

Amelioration of Muc5b mucin hypersecretion is enhanced by IL-33 after 2-APB administration in a murine model of allergic rhinitis

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Abstract

We attempted to clarify whether hypersecretion of Muc5b mucin from mouse nasal submucosal glands that is enhanced by interleukin (IL)-33 under allergic conditions can be ameliorated by administration of 2-APB. Immunohistochemistry was used to examine both the distribution of T cells in the nasal mucosa of an allergic rhinitis mouse model and expressions of IL-33 receptor ST2 and Muc5b protein in mouse submucosal gland cells. The amounts of protein and mRNA of Orai1, Muc5b, IL-4, IL-5, IL-13 and IL-33 in mouse nasal lavage fluid (NLF) and nasal mucosa were determined using enzyme-linked immunosorbent assay and real-time reverse transcription-polymerase chain reaction. Expressions of Orai1, Muc5b, IL-4, IL-5, IL-13 and IL-33 were up-regulated in the allergic state and IL-33 increased the levels of Muc5b, IL-4, IL-5 and IL-13, but did not influence proliferation of T cells; however, ST2 was diminished in nasal submucosal gland cells. 2-APB reduced proliferation of T cells and the Orai1 level in the nasal mucosa. It also reduced the concentrations of IL-4, IL-5 and IL-13 in NLF and nasal mucosa, and hypersecretion of Muc5b from glandular cells that was enhanced by IL-33, but did not affect IL-33 production. 2-APB decreased Muc5b mucin hypersecretion from submucosal gland that was enhanced by IL-33 in allergic mice by limiting Ca^{2+} release-activated Ca^{2+} channel activity in which Orai1 plays a crucial role in the gland cells and/or by controlling channel activation in T cells and proliferation of these cells.

Key words: allergic rhinitis, 2-APB, IL-33, interleukins, mouse, Muc5b, Orai1

Airway mucus maintains airway surface hydration and traps particulates, bacteria and viruses. The mucin protein backbones are encoded by genes called MUCs (mucins) in humans. Many Muc genes are expressed in the airway mucosa. The primary mucin glycoproteins present in human airway secretions include Muc5AC and Muc5B with Muc-specific antibodies (Thornton et al. 2008). Muc5B is the predominant mucin expressed in the mucous cells of the human submucosal gland, and Muc5b is the principal mucin released from the mouse

submucosal gland (Thai et al. 2008). Whether goblet cells or submucosal glands are the major source of in airway mucins in allergic airway diseases is not known. It is likely that the relative contribution of goblet cells and gland cells varies according to the location in the respiratory tree, disease severity and disease type.

It is known that mucus secretion can be mediated by cytokines during allergic inflammation (Schmitz et al. 2005). The relation between mucin and interleukin (IL)-33 in allergic rhinitis (AR), however, is unclear. IL-33 and its receptor, ST2, are part of the IL-1 family and their interactions promote a variety of responses in various cell types. Within the IL-1 family, only IL-33 is associated with the promotion of systemic T helper 2 (Th2) cell responses. The IL-33/ST2 axis is thought to be involved in the acceleration and maintenance

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of allergic inflammation by a number of cell types including Th2 cells, mast cells, basophils and structural cells including airway epithelium and smooth muscle cells (Lloyd et al. 2010). IL-33 is located in the nucleus of resting cells and it binds chromatin by the H2A–H2B histone complex, which exhibits a potential transcriptional suppressor effect (Rousset et al. 2008). Administration of IL-33 results in lymphocyte-independent airway hyper-reactivity, goblet cell hyperplasia and eosinophilic and monocytic infiltration (Schmitz et al. 2005). Hypertrophy and increased mucous secretion in the bronchi and bronchioles emerge after repeated applications in mice models (Schmitz et al. 2005).

In addition to cytokine promotion, other physiological processes depend on intracellular Ca^{2+} signals including secretion, muscle contraction, cell growth and gene expression. Changes in cytoplasmic Ca^{2+} levels are initiated by altered influx across plasma membranes and by release from intracellular compartments that have large cellular Ca^{2+} stores, such as the endoplasmic reticulum (ER). Store-operated Ca^{2+} entry (SOCE) is activated by depletion of ER Ca^{2+} stores, and it is a broadly investigated mechanism of plasma membrane Ca^{2+} influx (Parekh et al. 2005). The store-operated Ca^{2+} channel (SOCC) was first identified in 1992 by patch-clamp electrophysiology (Hoth et al. 1992) and was named the Ca^{2+} release-activated Ca^{2+} (CRAC) channel. This kind of SOCC is the most extensively characterized SOCE pathway (Hoth et al. 1992). An earlier study used linkage analysis and a *Drosophila* S2 cell genome-wide RNA interference (RNAi) screen to identify Orai1 as a component of the CRAC channel (Feske et al. 2006). Mammalian cells have two additional Orai1 isoforms, Orai2 and Orai3, but their initiating Ca^{2+} currents were 2–3 fold smaller than that of Orai1 (Mercer et al. 2006).

The combination of Orai1 and stromal interaction molecule 1 (STIM1), a molecule that resides in ER and which was identified by RNAi screening in *Drosophila* S2 cells, sharply increases SOCE and CRAC channel activity (Varnai et al. 2009). Excessive expression of Orai1 produces a great influx of extracellular Ca^{2+} and extensive up-regulation of cytoplasmic Ca^{2+} (Varnai et al. 2009), which may contribute to cellular activities including secretion, proliferation, gene transcription etc. Persistent Ca^{2+} influx activates nuclear factor of activated T cells (NFAT), a family of four transcription factors (NFAT1–4). NFAT proteins are heavily phosphorylated and reside in the cytoplasm of immune system cells. When these cells are stimulated, they are dephosphorylated by calcineurin, a calmodulin-dependent serine/threonine phosphatase, and trans-

located to the nucleus. These processes represent significant stages in the induction and development of allergic reactions (Rao et al. 1997).

2-Aminoethoxydiphenyl borate (2-APB) is a useful pharmacological tool for studying SOCE. This drug affects SOCE by adjusting CRAC channel activity (Bootman et al. 2002, Peinelt et al. 2008). Whether 2-APB could regulate mouse Muc5b secretion from submucosal glands and IL-33 production in nasal mucosa, however, is not clear. According to our previous work, Orai1 is expressed in mouse submucosal gland cells and is up-regulated in AR (Lin et al. 2010). We introduced IL-33 and 2-APB into the nostrils of mice with AR to assess the relations among Muc5b, 2-APB and IL-33.

Materials and methods

Animals

Female BALB/c mice, 6–8 weeks old, were purchased from the Chinese Academy of Sciences Shanghai Laboratory Animal Center. They were maintained in horizontal laminar flow cabinets and provided sterile food and water *ad libitum* in a pathogen-free facility. Animal studies were approved by the institutional Animal Care and Use Committee of Fudan University. The mice were assigned randomly to seven groups as follows: two nonsensitized (normal) groups, two control AR groups, a 2-APB-treated group, a IL-33-treated group and a IL-33 + 2-APB-treated group (n = 12 for each group).

Allergic model

According to published procedures (Ikeda et al. 2002), mice were administered 0.5 mg/ml ovalbumin (OVA) (Grade V, Sigma-Aldrich, St. Louis, MO) and 20 mg/ml aluminium hydroxide (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) in saline at a dosage of 0.2 ml/mouse by intraperitoneal (i.p.) injection. The sensitization was repeated three times at 7 day intervals (days 1, 8, and 15) followed by introduction of OVA solution drops (40 mg/ml in saline) into the nostrils (0.02 ml/mouse) using a micropipette daily from day 22 to 29 (challenge). Some groups of mice were administered 2-APB intranasally (i.n.; 225 ng/mouse, Sigma-Aldrich) (2-APB-treated group), IL-33 (20 ng/mouse) (IL-33-treated group) or IL-33 (i.n., 20 ng/mouse) + 2-APB (i.n., 225 ng/mouse) (IL-33 + 2-APB-treated group) on challenge days, whereas another group of mice was not treated (control group). The dosage of IL-33 was determined by a preliminary experiment and the dosage of 2-APB was based on our previous

investigations (Lin et al. 2011, 2012). As a negative control, a group of mice received neither sensitization nor challenge treatment, except for the last challenge on day 29 (nonsensitized group).

Tissue and nasal lavage fluid (NLF)

After sacrificing one group of mice, the head of each mouse was obtained for subsequent immunohistochemical staining. Another group of mice was sacrificed and the nasal mucosa of each mouse was excised and divided into two portions: one was dissected for protein isolation, the other was for mRNA extraction. All specimens were frozen at -70°C .

After the mice were sacrificed, 1000 μl phosphate-buffered saline (PBS) was injected into the nasal cavities through the external nares using a blunted 18 gauge needle and the NLF was collected with a tube under both nares. NLF was centrifuged for 10 min at $200 \times g$ at 4°C . T cells were counted using flow cytometry. The supernatants were stored at -70°C and concentrations of IL-4, IL-5, IL-13 and IL-33 were determined using ELISA.

Orai1 antibody

Orai1-L1 rabbit polyclonal antibody (NewEast Biosciences, Malvern, PA) was raised against a peptide from the extracellular loop 1 region of human Orai1 and was used for detecting mouse Orai1 by western blotting at 1:1000–1:2000, immunohistochemistry at 1:100–1:200 and ELISA at 1:1000–1:5000 dilutions according to the manufacturer's instructions. The antibody may also recognize Orai2 and Orai3 proteins. The broad reactivity of polyclonal antibodies may be advantageous in some cases, e.g., they can be used for both immunoblotting and immunostaining of chemically fixed cells and tissues (Drenckhahn et al. 1993). Monoclonal antibodies often are specific for isoforms and spliced variants, and their cross-reactivity is rare. Cross-reactivity, however, cannot be eliminated by absorption or affinity purification if it occurs (Drenckhahn et al. 1993). We did not perform pre-incubation experiments and affinity purification, because Orai1-L1 rabbit polyclonal antibody cross-reacted to a lesser extent with Orai2 and Orai3 owing to their lower levels in plasma membrane than Orai1 (Lis et al. 2007).

Immunohistochemical staining

Twelve hours after the final nasal challenge, the heads of some mice were immersed in 10% neutral buffered formalin. After fixation, these heads were

decalcified in 8.8% formic acid for 6 days. The specimens then were embedded in paraffin, sectioned at 4 μm , and mounted on glass slides. Slides were allowed to dry in a 37°C incubator overnight, then baked at 60°C for 30 min. The tissues were deparaffinized through xylenes, rehydrated through graded alcohols, and rinsed in distilled water before staining. Slides then were dehydrated through graded alcohols, cleared in xylene, embedded in resin and coverslipped.

For immunofluorescence staining of T cells, sections prepared as above first were incubated with goat serum for 30 min to block the Fc γ receptor. After washing in PBS, these sections were incubated with anti-CD3 antibody diluted 1:100 with the antibody diluent (Abcam, Cambridge, MA), then with biotin-conjugated rat anti-mouse IgG diluted 1:100 with the antibody diluent (eBiosciences, San Diego, CA), followed by streptavidin-rhodamine (Rockland, Gilbertsville, PA). Anti-CD4 antibody diluted 1:100 with the antibody diluent (Abcam) then was applied to the sections and binding was detected by incubation with fluorescein isothiocyanate (FITC)-conjugated goat or rabbit anti-mouse IgG (Southern Biotechnology Assoc., Inc., Birmingham, AL). These sections were mounted in fluorescent mounting medium to prevent fading and images were acquired immediately under UV illumination using a microscope with FITC (U-MWIBA2) and TRITC (U-MNG) filters, with a Sony 3CCD color video camera and analysis software. The T cell counts were determined microscopically in a blinded manner at $\times 400$ magnification. Pictures were processed and superimposed using Photoshop CS5 (Adobe, San Jose, CA).

For horseradish peroxidase staining of sub-mucosal glands, sections were deparaffinized and rehydrated to water. Endogenous peroxidase was blocked by 3% hydrogen peroxide for 10 min. Normal goat serum (Vector Lab, Burlingame, CA) was used to block nonspecific binding sites and an optimized concentration (5 $\mu\text{g}/\text{ml}$) of primary antibody, either anti-Muc5b polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-ST2 polyclonal antibody (Abcam), was applied immediately to the sections and incubated for 45 min. Unbound antibody was removed by three washes in PBS solution. Biotinylated anti-goat or anti-rabbit polyclonal IgG (Vector Labs), secondary antibody was applied to each section for 30 min followed by 20 min incubation with Horseradish peroxidase streptavidin (Vector Labs). Slides were washed in PBS, stained with 3,3'-diaminobenzidine (DAB) chromogen (Sigma-Aldrich) for 5 min and counterstained with hematoxylin. Sections were dehydrated through an alcohol series, cleared in

xylene, then mounted in neutral balsam mounting medium. Antibody localization was detected microscopically by brown staining (DAB) of Muc5b or ST2. Sections were examined at 200, 400 and 1,000 $\times$ by two independent observers who were blinded to the sample groups. Immunoreactivities of the proteins above were evaluated for intensity of staining according to the following four grades: no staining, light brown, brown, and dark brown, and distributions of the proteins were estimated on the basis of percentage of stained cells. The intensity and distribution of immunostaining were assessed by light microscopy.

Flow cytometry

The cell suspension was centrifuged for 10 min at 200 $\times$ g at 4 $^{\circ}$ C and cells were harvested for flow cytometry analysis. The cells were washed with 200 μ l flow cytometry medium (FCM, 1% BSA and 0.1% sodium azide in PBS) and incubated with 50 μ l rabbit serum for 5 min at room temperature to block the Fc γ receptor (Salmon et al. 2000). The cells then were washed three times with FCM and incubated with 50 μ l of primary antibody at 4 $^{\circ}$ C for 30 min with gentle stirring in the dark. They were washed again and incubated with 50 μ l secondary antibody (biotin-conjugated rabbit anti-mouse IgG; Dako Denmark A/S, Glostrup, Denmark) followed by 50 μ l R-phycoerythrin-conjugated streptavidin (RPE-streptavidin; Dako).

For double CD3/CD4 staining, cells were incubated first with anti-CD3 antibody (Abcam), then with biotinylated goat anti-mouse IgG followed by RPE-streptavidin. Cells were incubated with mouse serum to block sites not occupied by the anti-mouse antibody, then incubated with FITC-conjugated rabbit anti-mouse CD4 antibody.

Controls for single color analysis were carried out by incubating cells with a primary antibody of the same isotype, but of irrelevant specificity at matched concentrations followed by secondary reagents, as described above.

Finally, cells were re-suspended in 100 μ l FCM and analyzed using a Becton-Dickinson fluorescence-activated cell scanner (FACScan; Becton-Dickinson, Mountain View, CA) using the LYSYS II software analysis program. Set-up of the instrument was carried out visually and selection of the isotype control antibody was according to the primary antibodies' host species, isotype, and conjugation format. For double staining, compensation was adjusted using control samples stained with antibodies that reacted with mutually exclusive populations (anti-CD3-RPE

and anti-CD4-FITC). Data then were analyzed using WinMDI 2.8 software.

Enzyme-linked immunosorbent assay (ELISA)

Other frozen tissues were sliced into small pieces, homogenized, then lysed in RIPA buffer (1 $\times$ PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail for mammalian tissue (Sigma), 1 mM sodium orthovanadate). Tissue lysates were centrifuged at 12,000 $\times$ g for 15 min at 4 $^{\circ}$ C to clarify, then stored at -70 $^{\circ}$ C. Tissue Orai1 protein from nasal mucosa was measured using a 96-well microplate (Corning Inc., New York, NY) coated with 1 μ g/ml Orai1 rabbit polyclonal antibody (NewEast Biosciences, Malvern, PA) and incubated overnight at 4 $^{\circ}$ C. After washing, the microplate was incubated in 3% bovine serum albumin (Calbiochem, La Jolla, CA) at 37 $^{\circ}$ C for 1 h. Samples diluted 1:10 with PBS then were added followed by incubation at 37 $^{\circ}$ C for 1 h. After washing, Orai1 rabbit polyclonal antibody that had been biotinylated using a biotinylation kit (American Qualex International Inc., San Clemente, CA) was added at 1 μ g/ml and allowed to incubate at 25 $^{\circ}$ C for 1 h. After washing, 1.5 μ g/ml streptavidin peroxidase was added followed by incubation at 25 $^{\circ}$ C for 1 h. After washing, tetramethylbenzidine (TMB) substrate (12.5 ml citric-phosphate buffer, 200 μ l TMB stock solution (6 mg/ml in DMSO), 100 μ l 1% H₂O₂, Fluka Chemical Co., Ronkonkoma, NY) was added to produce a color reaction. The reaction was terminated by adding 6 N H₂SO₄. Optical density was determined at 450 nm using a microplate reader (Microplate Reader MTP-32, Corona Electric, Ibaraki, Japan).

Concentrations of Muc5b protein in nasal mucosa and IL-4, IL-5, IL-13 and IL-33 in NLF were evaluated using corresponding ELISA kits purchased from Antibodies-Online Inc. (Atlanta, GA) Ever Systems Biology Laboratory, Inc. (Sacramento, CA) and R&D Systems Inc. (Minneapolis, MN). The ELISAs were conducted according to the manufacturers' protocols.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA of some specimens was extracted using Trizol (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Ambion, Austin, TX) twice at 37 $^{\circ}$ C for 30 min each time to eliminate contaminating genomic DNA. Absorbances at 280 nm (A280) and 260 nm (A260) were measured using

a UNICO UV-2000 ultraviolet spectrophotometer (Unico Instruments Co. Ltd., Shanghai, China) for the diluted sample in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). To estimate the total RNA concentration of the sample, the reading was multiplied by the dilution factor of the sample and a constant of 40, assuming that 40 µg/ml sample has an absorbance of 1, i.e., if a sample containing pure RNA has an absorbance of 1 at 260 nm, then it contains approximately 40 µg/ml of RNA. The ratio of the A260 to A280 absorbance was used to assess the purity of the RNA. The ratios for all samples fell within the range 1.9–2.1. For reverse transcription, 2 µg of RNA was transcribed reversely with random hexamers (Invitrogen) and cDNA was amplified according to the manufacturer's instructions. Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA) from sequences available in Genbank (Geneland Biotech, Shanghai, China). RT-PCR was performed to determine mRNAs of Orai1, Muc5b, IL-4, IL-5, IL-13 and IL-33. Orai1 primers were: forward primer 5'-TTTGCCCTCATGATCAGCAC-3' and reverse primer 5'-TTGGCGACGATGACTGATTC-3'. Muc5b primers were: forward primer 5'-ACTTGAGGAGGGTTCCAGGT-3' and reverse primer 5'-ACAGTGCCAGGGTTTATGC-3'. IL-4 primers were: forward primer 5'-ATCGGCATTTTGAACGAGG-3' and reverse primer 5'-TCGAAAAGCCCGAAAGAGTC-3'. IL-5 primers were: forward primer TCAGCTGTGTCTGGGCCACT and reverse primer TTATGAGTAGGGACAGGAAGCCTCA. IL-13 primers were: forward primer 5'-TGCTTGCCTTGGTGGTCTC-3' and reverse primer 5'-CAGGTCCACACTCCATACC-3'. IL-33 primers were: forward primer 5'-GATGGGAAGAAGGTGATGGGTG-3' and reverse primer 5'-TTGTGAAGGACGAAGAAGGC-3'.

GAPDH mRNA also was assessed to control sample-to-sample variation in RNA isolation and integrity using a pair of primers: forward primer, 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer, 5'-TCCACCACCCTGTTGCTGTA-3'. After initial denaturation at 95° C for 10 min, the amplification profile was 15 sec of denaturation at 95° C, 1 min of annealing and extension at 60° C for 45 cycles. Negative control RT (reverse transcription) reaction mixtures contained no reverse transcriptase and no cDNA in the PCR amplification mixtures.

For measurement, 2 µl of diluted cDNA was amplified in a total reaction volume of 20 µl using a 7500 real time PCR System (Applied Biosystems, Foster City, CA) with 20× SYBR green mixture (Invitrogen). Specificity of PCR products was evaluated by melting curve analysis and by size in

agarose gels. The melting peak distinguished specific products from other products that melted at different temperatures and the bands in the different lanes in the gel confirmed the presence of different PCR products. Linearity of PCR amplification was controlled using three dilutions of cDNA. Evaluation of data was performed using the threshold cycle (CT) values (Δ CT method) with GAPDH as internal standard (Livak et al. 2001, Beekman et al. 2011, Narasimhan et al. 2012).

Statistical analysis

Statistical analysis was performed using Statistical Software Prism 5.0 (GraphPad Software Inc., San Diego, CA). Data were analyzed using the unpaired Student's t test $\pm$ Welch's correction and presented as mean $\pm$ SEM. Values for $p < 0.05$ were considered statistically significant.

Results

CD3⁺ CD4⁺ T cells in nasal mucosa in allergic state

It is widely accepted that induction of antigen-specific CD4⁺ T cells is a key event in many allergic diseases including AR. Increased numbers of activated CD4⁺ T cells are detectable in the nasal mucosa of AR patients (Horiguchi et al. 2005). To analyze probable functions of T cells in the AR mice, we examined their appearance and number in murine nasal mucosa. In our study, positive double immunofluorescence staining of CD3 and CD4 appeared yellow and was present in nasal mucosa of mice; furthermore, intense immunoreactivity was found in AR mice by contrast to normal mice (Fig. 1A,B). In addition, we demonstrated that the CD3⁺ CD4⁺ T cell counts in nasal mucosa were enhanced in the AR mouse models compared to the normal mice (Fig. 1C, $p < 0.001$). These results indicate the proliferation and activation of CD4⁺ T cells under allergic conditions.

2-APB effects on Orai1 expression without IL-33 production

Orai1 protein is present in mouse submucosal glands and immunostaining appeared stronger under allergic conditions than we observed under normal conditions in our previous work (Lin et al. 2010). The Orai1 protein and its mRNA that were extracted from the nasal mucosa of mice were evaluated by ELISA and RT-PCR in the study reported here. ELISA results showed that Orai1 protein was

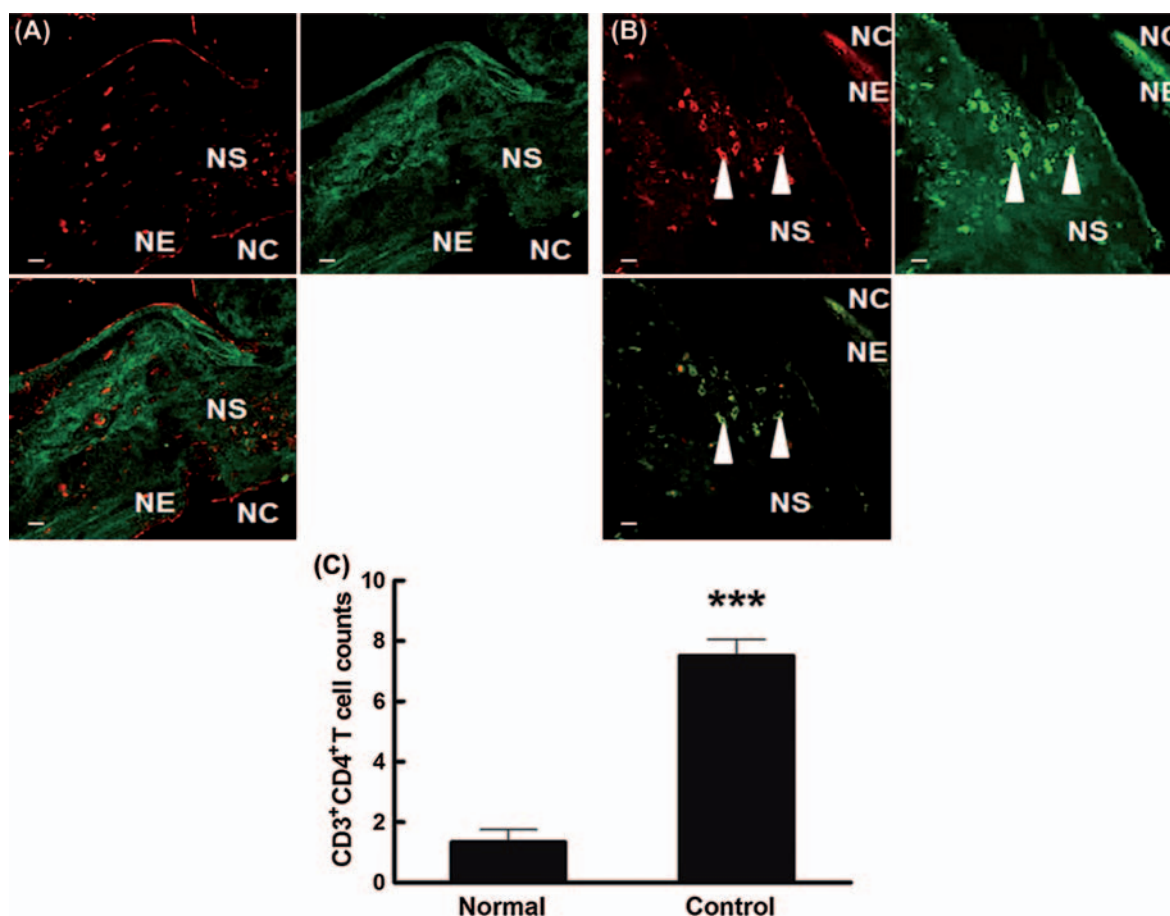


Fig. 1. CD3⁺CD4⁺ T cells in the nasal mucosa of allergic mice. Immunofluorescence staining of CD3 and CD4 proteins in the plasma membrane of T cells indicated increased numbers of Th2 cells in nasal mucosa of AR mice (B) compared to normal mice (A). CD3 staining was red, CD4 staining was green and the combined signal was yellow. A) Nonsensitized group. B) Control group. C) CD3⁺CD4⁺ T cell counts. Arrowheads indicate single and dual staining of CD3⁺ and CD4⁺ T cells. NC, nasal cavity; NE, nasal epithelium; NS, nasal submucosa; normal, nonsensitized group; control, control group. *** $p < 0.001$ vs. control group. Original magnification $\times 400$, Scale bar = 10 μ m.

increased in the control group compared to the normal group (Fig. 2A, $p < 0.001$). As shown in Fig. 2B, Orai1 mRNA of nasal mucosa was up-regulated in control mice compared to normal mice (Fig. 2B, $p < 0.001$). 2-APB administration reduced the production of Orai1 protein and its mRNA in the AR nasal mucosa (Fig. 2A, $p < 0.01$, 2-APB vs. control, Fig. 2B, $p < 0.001$, 2-APB vs. control). Similarly, IL-33 and its mRNA were increased in the AR group compared to the normal group (Fig. 2C, $p < 0.001$, normal vs. control, Fig. 2D, $p < 0.001$, normal vs. control). There were no statistical differences, however, in IL-33 and its mRNA content between control and 2-APB-treated mice (Fig. 2C and D, 2-APB vs. control). Our results indicated that Orai1 expression in nasal mucosa of mice was increased under allergic conditions and was decreased after administration of 2-APB. The IL-33 cytokine was up-regulated after sensitization and challenge with OVA allergen in our

model system and 2-APB intervention did not influence directly its expression in the allergic state.

Decreased secretion of Muc5b mucin and expression of Th2 cytokines after 2-APB treatment

Excess production of mucins is an important factor in the morbidity and mortality of chronic airway disease, e.g., asthma. To assess the regulation of Muc5b secretion by 2-APB, we performed immunohistochemical staining, ELISA, and RT-PCR of Muc5b. Immunohistochemical staining of Muc5b showed that this protein was localized primarily in cytoplasm of nasal submucosal gland cells. Immunostained cells appeared brown. Immunostaining was more intense in allergic mice (Figs. 3B, 5B) than in nonsensitized mice (Figs. 3A, 5A). The intensity of staining, however, was weaker in submucosal gland

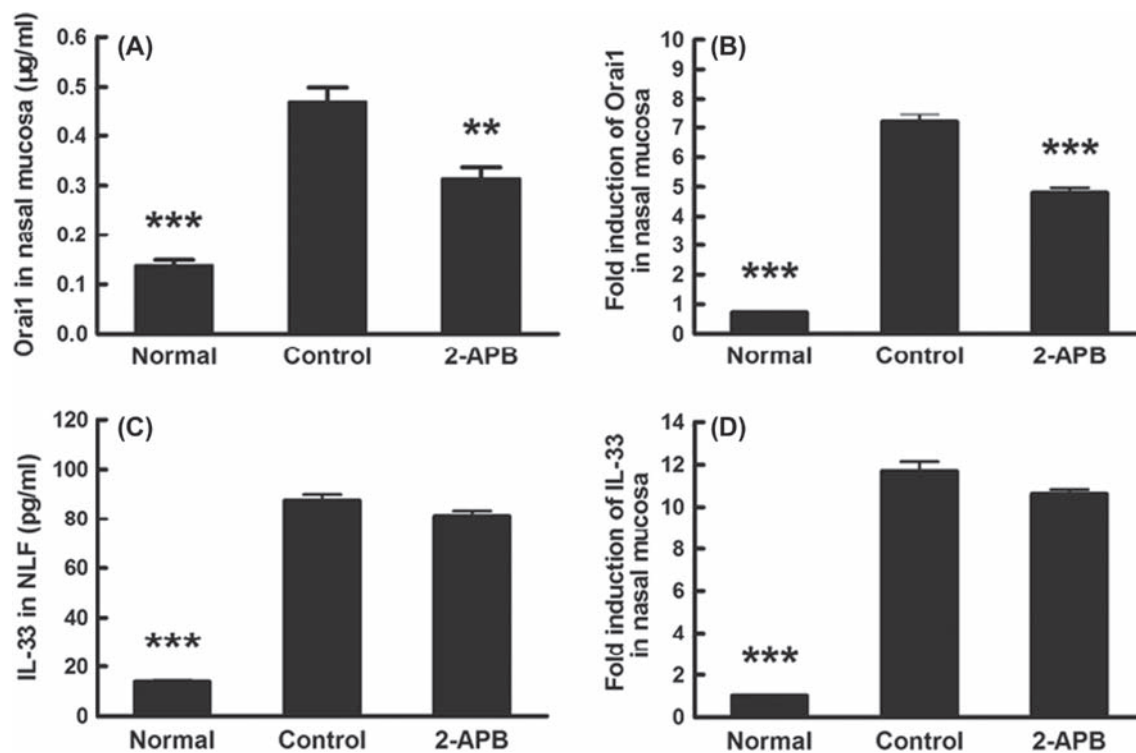


Fig. 2. 2-APB effects on Orai1 and IL-33 expression in AR mice. ELISA was used to evaluate the amounts of Orai1 and IL-33 proteins, and RT-PCR was used to assess mRNA expression of Orai1 and IL-33 in mouse models. A) Orai1 protein. B) Orai1 mRNA. C) IL-33 protein. D) IL-33 mRNA. Each value represents the mean $\pm$ SEM of six mice in each group. Normal, nonsensitized group; Control, control group; 2-APB, 2-APB-treated group; NLF, nasal lavage fluid; IL, interleukin; 2-APB, 2-aminoethoxydiphenyl borate. *** $p < 0.001$ vs. control group, ** $p < 0.01$ vs. control group.

epithelium (Fig. 3C) in 2-APB treated mice (Fig. 3B) than in allergic specimens. The ELISA and RT-PCR analysis showed that the expressions of Muc5b protein and mRNA were increased in control mice compared to nonsensitized mice (Fig. 4A, $p < 0.001$; Fig. 6A, $p < 0.001$; Fig. 4E, $p < 0.001$; Fig. 6E, $p < 0.001$), and also revealed that 2-APB treatment decreased production of both Muc5b and mRNA (Fig. 4A, $p < 0.001$; Fig. 4E, $p < 0.001$, respectively).

It is known that Th2 cytokines such as IL-4, IL-5 and IL-13 play important roles in the pathophysiology of allergic diseases including AR and asthma. Therefore, we investigated the effects of 2-APB on these inflammation mediators. The concentrations of IL-4, IL-5 and IL-13 in NLF of the mice were increased in allergic mice compared to normal mice (Fig. 4B, $p < 0.001$; Fig. 4C, $p < 0.001$; Fig. 4D, $p < 0.001$; Fig. 6B, $p < 0.001$; Fig. 6C, $p < 0.001$; Fig. 6D, $p < 0.001$); the corresponding mRNAs in the nasal mucosa also were elevated (Fig. 4F, $p < 0.001$; Fig. 4G, $p < 0.001$; Fig. 4H, $p < 0.001$; Fig. 6F, $p < 0.001$; Fig. 6G, $p < 0.001$; Fig. 6H, $p < 0.001$) in allergic inflammation. Nostril administration of 2-APB decreased the amounts of the cytokines, IL-4, IL-5 and IL-13, for both the production of protein (Fig. 4B, $p < 0.001$; Fig. 4C, $p < 0.001$;

Fig. 4D, $p < 0.001$) and production of mRNA (Fig. 4F, $p < 0.001$; Fig. 4G, $p < 0.001$; Fig. 4H, $p < 0.001$). These results suggest that 2-APB may improve symptoms of AR by decreasing Muc5b secretion from submucosal glands and down-regulating Th2 cytokines in nasal mucosa in the allergic state.

Decreased Muc5b mucin hypersecretion caused by IL-33 after 2-APB administration

IL-33 is involved in the development of allergic reaction by way of Th2 cells and is reported to aggravate inflammation (Lloyd et al. 2010). Therefore, we investigated the impact of IL-33 on mucin hypersecretion and the relation between IL-33 function and 2-APB drug treatment. Topical application of IL-33 in the mouse nasal cavity increased Muc5b mucin expression in the IL-33-treated group (Fig. 5C) compared to the control group (Fig. 5B) and 2-APB treatment lessened immunoreactivity (Fig. 5D). ELISA and RT-PCR demonstrated that the mucin protein and mRNA contents were increased in the IL-33-treated group compared to AR mice (Fig. 6A, $p < 0.001$; Fig. 6E, $p < 0.001$). To the contrary, the contents of Muc5b protein and its

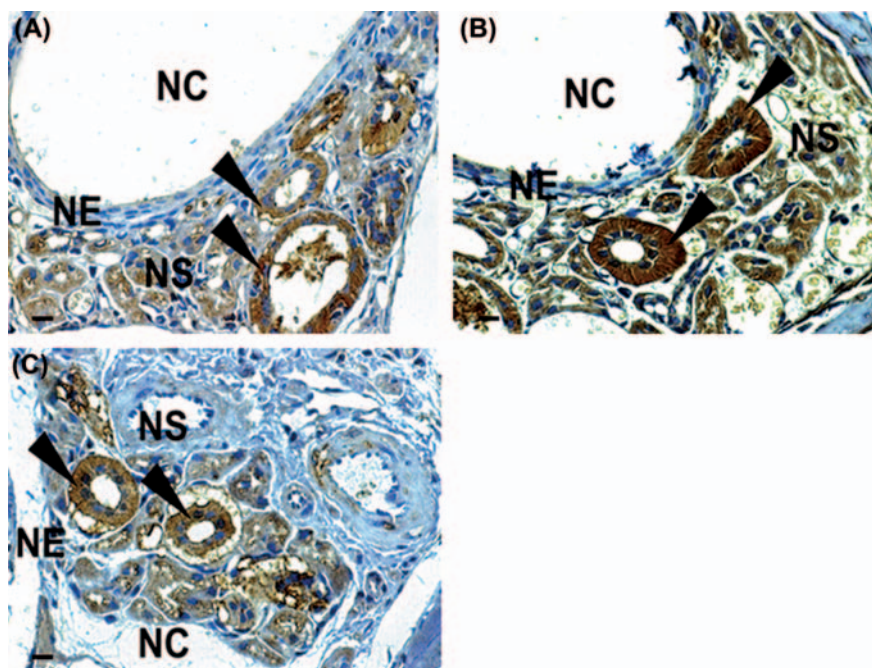


Fig. 3. Immunohistochemical analysis of Muc5b mucin expression in submucosal gland cells after administration of 2-APB. Immunohistochemical staining for Muc5b mucin. Muc5b was localized in the cytoplasm of submucosal gland acinus and appeared brown. The intensity of immunostaining indicated relative protein amounts. A) Nonsensitized group. B) Control group. C) 2APB-treated group. Arrowheads indicate staining of submucosal gland epithelium. NC, nasal cavity; NE, nasal epithelium; NS, nasal submucosa. Original magnification $\times 400$, Scale bar = 10 μm .

mRNA were decreased in the IL-33 + 2-APB-treated group (Fig. 6A, $p < 0.001$; Fig. 6E, $p < 0.001$).

IL-33 accelerates allergic inflammation by promoting Th2 cells (Lloyd et al. 2010) and these cells subsequently function by releasing IL-4, IL-5 and IL-13 cytokines (Horiguchi et al. 2005). We studied the influence of IL-33 on Th2 cytokines in a murine model of AR. We found that IL-33 encouraged the expression of IL-4, IL-5 and IL-13 cytokine proteins in NLF of AR mice (Fig. 6B, $p < 0.001$; Fig. 6C, $p < 0.001$; Fig. 6D, $p < 0.001$) and mRNA in AR mouse nasal mucosa (Fig. 6F, $p < 0.001$; Fig. 6G, $p < 0.001$; Fig. 6H, $p < 0.001$), whereas 2-APB decreased IL-4, IL-5 and IL-13 cytokine production of not only protein (Fig. 6B, $p < 0.001$; Fig. 6C, $p < 0.001$; Fig. 6D, $p < 0.001$), but also mRNA (Fig. 6F, $p < 0.001$; Fig. 6G, $p < 0.001$; Fig. 6H, $p < 0.001$). These data suggest that IL-33 may mediate the hypersecretion of Muc5b mucin and aggravate allergic inflammation by promoting Th2 cytokines, although 2-APB may counteract the action of IL-33.

Negative expression of ST2 in submucosal glands

Increased amount and activity of glandular tissue is characteristic of allergic nasal mucosa (Salib et al. 2003). Immunohistochemical staining was performed to analyze the expression of IL-33 receptor ST2 in submucosal

gland cells. No staining was observed in either normal (Fig. 7A) or allergic mice (Fig. 7B). Consequently, IL-33 does not affect submucosal glands directly.

Opposite effects of IL-33 and 2-APB on CD3⁺ CD4⁺ T cells

We divided our determination of CD3⁺ CD4⁺ T cells into two parts. One part was to assess the effect of 2-APB on the distribution of these cells; the other part was to determine the effects of 2-APB on Th2 cell response caused by IL-33. According to flow cytometry analysis, CD3⁺ CD4⁺ T cell counts were increased by allergic inflammation (Fig. 8B, E, H, $p < 0.001$) compared to normal cells (Fig. 8A, D, H, $p < 0.001$, normal vs. control) and 2-APB could decrease their population (Fig. 8C, H, $p < 0.001$). We found that IL-33 did not appear to effect the appearance of CD3⁺ CD4⁺ T cells (Fig. 8F, I) when these cells maintained their population under allergic conditions (Fig. 8E, I); the numbers of cells were controlled when IL-33 was applied together with 2-APB (Fig. 8G, I, $p < 0.001$). We found that IL-33 could increase allergic inflammation by stimulating Th2 cell activity, but it did not play a role in Th2 cell proliferation. 2-APB could reverse the allergic situation by inhibiting propagation of Th2 cells.

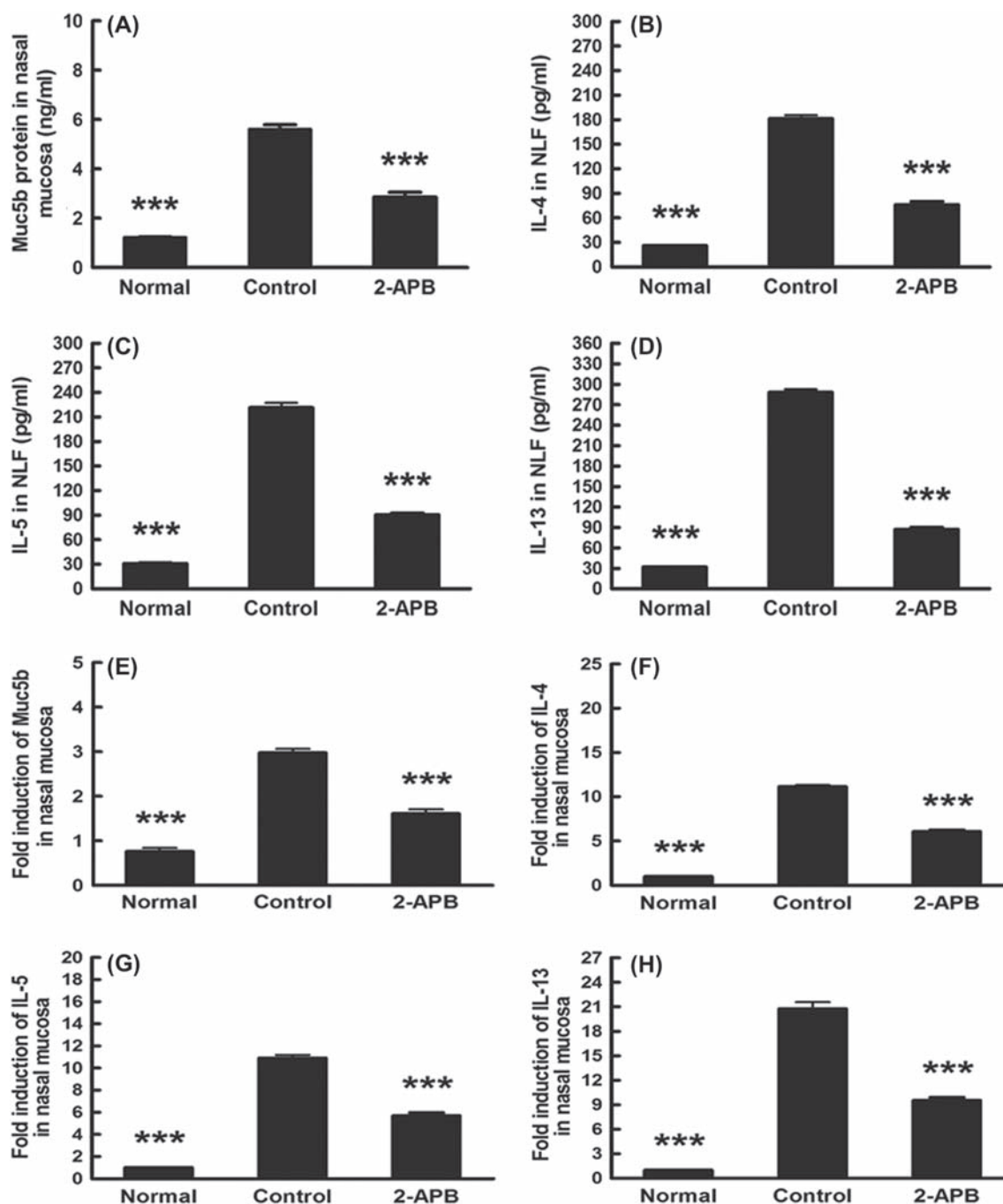


Fig. 4. Production of protein and mRNA of Muc5b and Th2 cytokines after 2-APB treatment. ELISA and RT-PCR were used to determine the concentrations of Muc5b mucin and its mRNA in nasal mucosa, and IL-4, IL-5 and IL-13 protein in NLF and their mRNAs in nasal mucosa among groups. A) Muc5b protein. B) IL-4 protein. C) IL-5 protein. D) IL-13 protein. E) Muc5b mRNA. F) IL-4 mRNA. G) IL-5 mRNA. H) IL-13 mRNA. Each value represents the mean $\pm$ SEM of six mice in each group. Normal, nonsensitized group; control, control group; 2-APB, 2-aminoethoxydiphenyl borate. *** $p < 0.001$ vs. control group.

Discussion

AR is an inflammatory disease of the nasal mucosa that results from IgE reaction to an allergen. It is characterized clinically by nasal symptoms including

rhinorrhea and obstruction (Salib et al. 2003). Hypersecretion of mucus and serum contributes to rhinorrhea. In other words, exocytosis from both mucous and serous cells of submucosal glands contributes to airway secretions. Mucous hypersecretion is a key

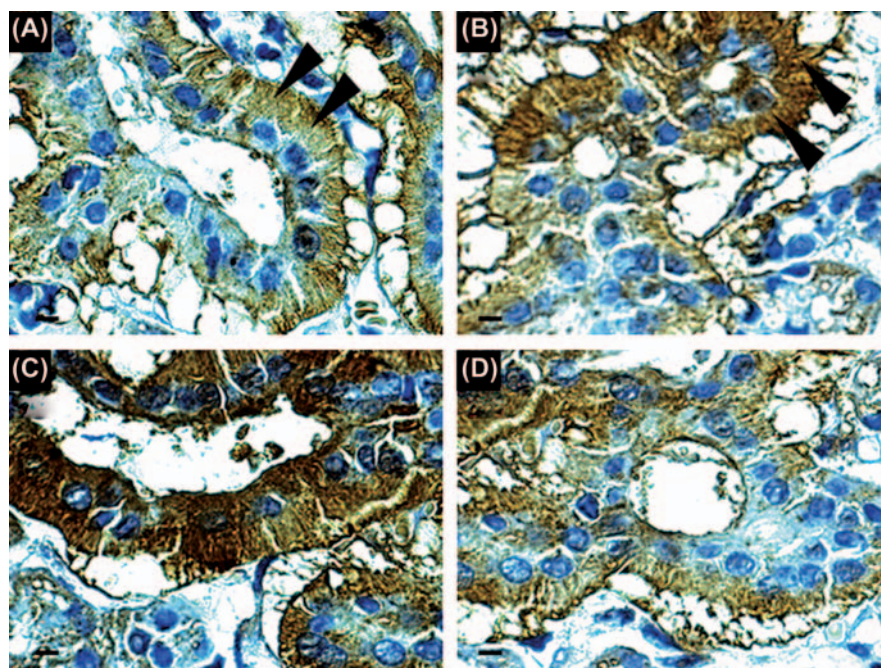


Fig. 5. Immunohistochemical staining of Muc5b mucin in submucosal gland cells after application of IL-33 and 2-APB. Muc5b was localized in the cytoplasm of submucosal gland acini and appeared brown. The intensity of immunostaining indicates relative protein amounts. A) Nonsensitized group. B) Control group. C) IL-33-treated group. D) IL-33 + 2-APB-treated group. Arrowheads indicate staining of submucosal gland epithelium. Original magnification $\times 1,000$, scale bar = 5 μm .

pathophysiological feature of the chronic inflammatory reaction of AR. The process involves increased generation of macromolecules, salts and water from the airway into the lumens. The major secreted airway mucins are Muc5b from submucosal glands and Muc5ac from goblet cells (Jonckheere et al. 2011).

High concentrations of Ca^{2+} are required for goblet cell degranulation; however, the mechanism for entrance of extracellular Ca^{2+} into the cytoplasm of goblet cells and nasal submucosal glandular cells is not clear (Fahy et al. 2002). We found Orai1 expression in submucosal gland cell membranes in our previous study (Lin et al. 2010), which suggested that the CRAC channel may regulate the cytoplasmic Ca^{2+} concentration of the acinus. We used ELISA and RT-PCR to evaluate Orai1 expression in nasal mucosa. We found that the protein and mRNA of Orai1 were up-regulated after OVA sensitization and challenge, and were down-regulated after 2-APB administration. We conclude that this drug may inhibit CRAC channel function by limiting Orai1 expression in submucosal glands and may result in decreased Muc5b secretion directly.

IL-33 can promote secretion of Th2 cytokines from Th2 cells (Lloyd et al. 2010). This cytokine resides in the nuclei of static cells. How they migrate from the nucleus to interact with the extracellular

receptor, ST2, remains to be clarified. It has been reported that IL-33 lacks classical secretion signals and displays cytokine activity independent of processing by ER-Golgi, and it is released by necrotic cells, but not apoptotic cells (Roussel et al. 2008). Allergens may trigger tissue damage, which causes IL-33 secretion after necrotic cell death in the allergic state; this cytokine thus may exacerbate the symptoms of asthma (Roussel et al. 2008).

It is essential that IL-33 be released extracellularly to bind to its ST2 receptor to play a role in allergic inflammation. We found no evidence of immunostaining of ST2 in gland cells, however. This finding suggests that extracellular IL-33 may not control the secretory activity of gland cells because of a scarcity of its receptor in this kind of cell. We did find that Muc5b protein and mRNA were increased after OVA immunization and they apparently were increased after IL-33 administration i.n. compared to controls. This finding suggests that IL-33 accelerates production of Muc5b indirectly by other mechanisms under allergic condition.

Mucus secretion is stimulated by cytokines such as IL-4 and IL-13 (Adler et al. 1987, Temann et al. 1997). Intratracheal administration of IL-13 to hyper-responsive BP2 mice initiates bronchopulmonary hyper-reactivity, eosinophilia, and mucus and

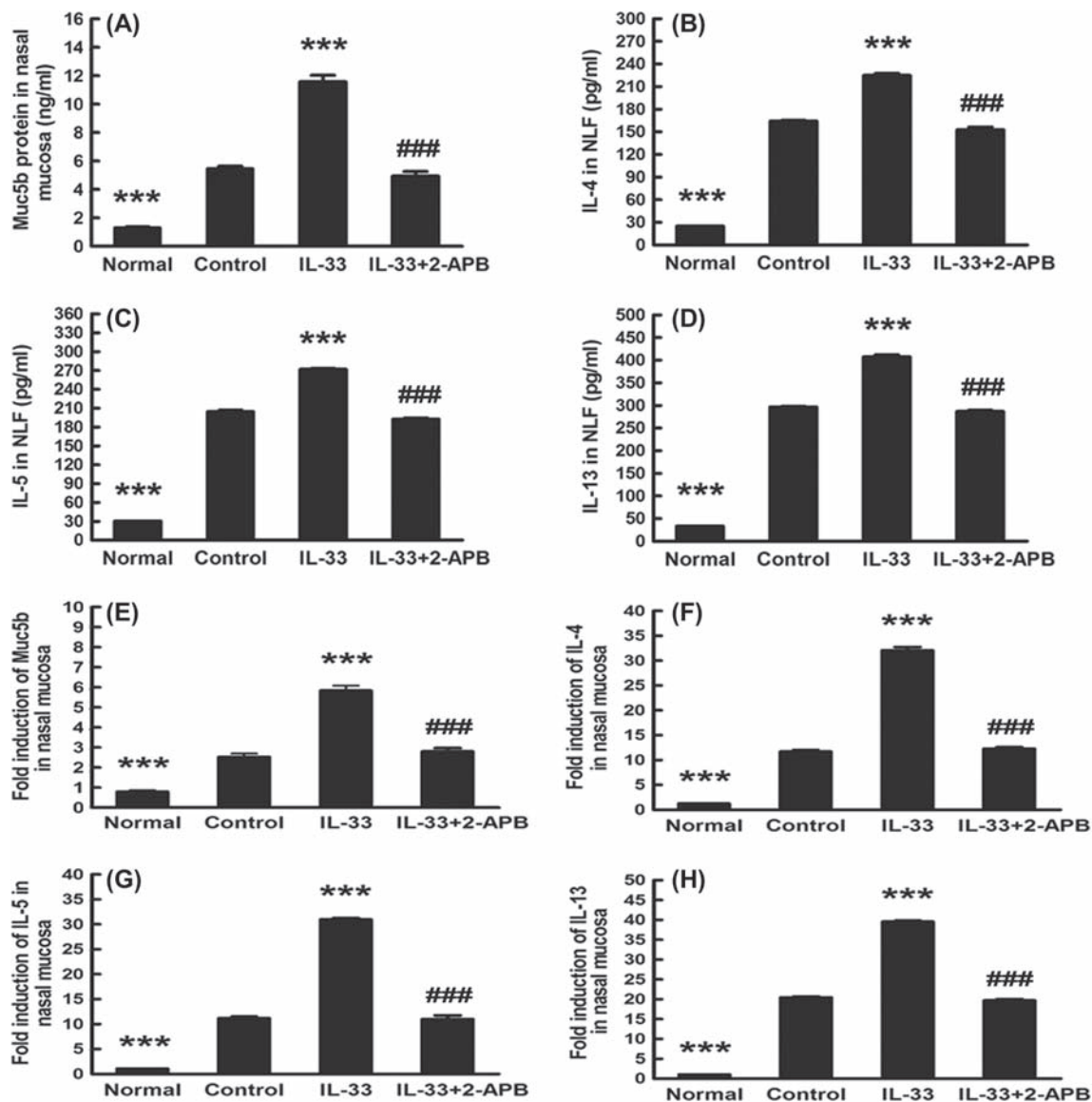


Fig. 6. Decreased protein and mRNA over-production of Muc5b and Th2 cytokines after IL-33 after 2-APB treatment. ELISA and RT-PCR were used to determine the concentrations of Muc5b mucin and its mRNA in nasal mucosa, and IL-4, IL-5 and IL-13 protein in NLF and their mRNAs in nasal mucosa among groups. A) Muc5b protein. B) IL-4 protein. C) IL-5 protein. D) IL-13 protein. E) Muc5b mRNA. F) IL-4 mRNA. G) IL-5 mRNA. H) IL-13 mRNA. Each value represents the mean $\pm$ SEM of six mice in each group. Normal, nonsensitized group; Control, control group; IL-33, IL-33-treated group; IL-33 + 2-APB, IL-33 + 2-APB-treated group; Muc, mucin; NLF, nasal lavage fluid; IL, interleukin; 2-APB, 2-aminoethoxydiphenyl borate. *** p < 0.001 vs. control group, ### p < 0.001 vs. IL-33-treated group.

Muc5ac aggregation similar to what is observed after OVA treatment when mice are sensitized (Eum et al. 1995, Hailé et al. 1999). Administration of IL-33 directly into the lung causes airway hyper-reactivity, goblet cell hyperplasia, lung IL-4, IL-5 and IL-13 hypersecretion; i.n. IL-33 application also produced this allergic pathology even in the absence of T and B cells (Kondo et al. 2008). IL-33 administration into mouse nostrils increased Th2 cytokine production as in our experiment. We conclude that

IL-33 may induce extensive IL-13 release from Th2 cells in mouse nasal mucosa and subsequently lead to over-expression of Muc5b in submucosal glands.

SOCE is the principal pathway for Ca^{2+} influx in mammalian immune system cells such as T cells. Stimulation of these cells causes depletion of Ca^{2+} from ER stores and triggers sustained Ca^{2+} entry through the CRAC channel, an important pathway for activation and proliferation of these cells (Lewis et al. 2001). Activation of Th2 cells contributes to

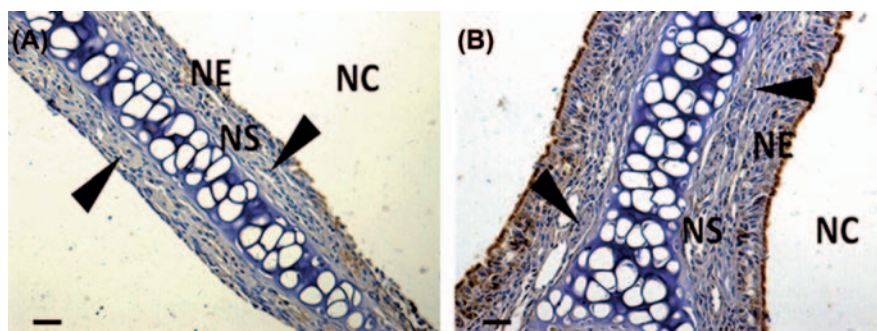


Fig. 7. ST2 expression in submucosal gland cells after immunohistochemical staining for the IL-33 receptor, ST2. There was no staining in either normal or allergic mouse submucosal gland acini. A) Nonsensitized group. B) Control group. Arrowheads indicate staining of submucosal gland epithelium. NC, nasal cavity; NE, nasal epithelium; NS, nasal submucosa. Original magnification $\times 200$, scale bar = 30 μm .

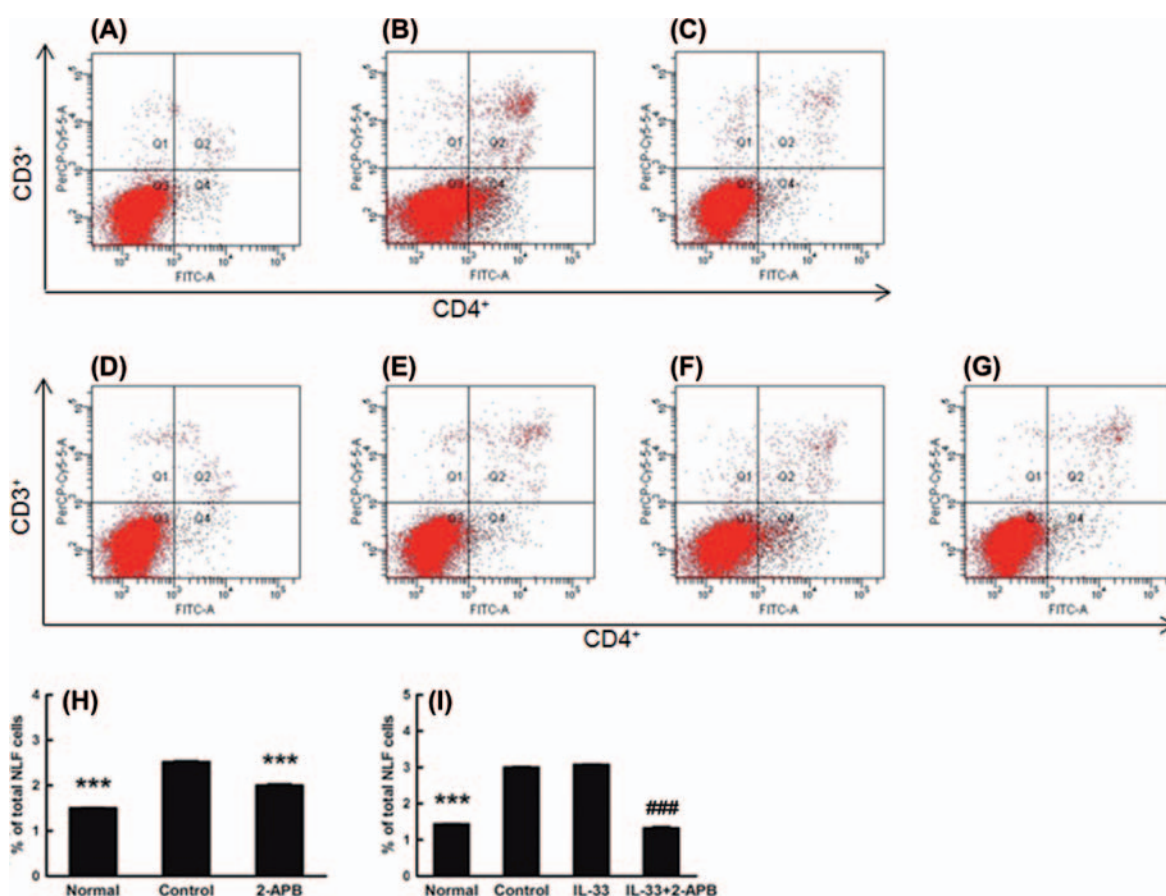


Fig. 8. Proliferation of $\text{CD3}^+ \text{CD4}^+$ T cells after IL-33 and 2-APB. Cells were stained with fluorochrome-conjugated antibodies and analyzed by flow cytometry. Dot plots shown were from representative experiments. Q1 quadrant, CD3^+ staining; Q2 quadrant, $\text{CD3}^+ \text{CD4}^+$ staining; Q3 quadrant, $\text{CD3}^- \text{CD4}^-$ staining and Q4 quadrant, CD4^+ staining. Cell counts represent mean percentage values of expression of six independent experiments (using pooled cells of six mice per sample). The values were calculated for the entire population within an NLF cell suspension and displayed as percent of total NLF cells. A, D) Nonsensitized group. B, E) Control group. C) 2-APB-treated group. F) IL-33-treated group. G) IL-33 + 2-APB-treated group. H, I) Relative $\text{CD3}^+ \text{CD4}^+$ T cell counts. Each value represents the mean $\pm$ SEM of six mice in each group. Normal, nonsensitized group; Control, control group; 2-APB, 2-aminoethoxydiphenyl borate. *** $p < 0.001$ vs. control group, ### $p < 0.001$ vs. IL-33-treated group.

increased Th2 cytokine expression and proliferation of Th2 cells produces an enlarged lymphocyte clone. 2-APB, a reliable blocker of SOCE by modulating CRAC channel activity, may interfere with the CRAC channel and cause the subsequent low Ca^{2+} concentration in the cytoplasm, which results in cellular inactivity. IL-4, IL-5 and IL-13 cytokines were decreased in the mouse nasal mucosa after 2-APB treatment and the number of $\text{CD3}^+ \text{CD4}^+$ T cells also was reduced after the drug administration. These results suggest that 2-APB may regulate cellular activity and proliferation of Th2 cells by regulation of the CRAC channel in their cytoplasmic membranes. Furthermore, our results suggest that 2-APB could counteract the promotion of IL-33 on Th2 cells, which was characterized by the reduced secretion of Th2 cytokines. 2-APB treatment, however, did not lower the expression of IL-33 protein and mRNA in mouse NLF and nasal mucosa. Therefore, 2-APB may block Th2 cytokine over-expression by limiting the activity and proliferation of Th2 lymphocytes rather than by the intervention of IL-33. Decreased Th2 cytokines may be responsible for decreased Muc5b mucin under allergic conditions.

IL-33 may increase release of Muc5b mucin from submucosal glands by way of Th2 cytokines, and 2-APB may control Muc5b hyper-secretion mediated by IL-33 in AR mouse models by controlling the activation of the CRAC channel in submucosal gland cells and/or by regulating the cellular activity and proliferation of $\text{CD3}^+ \text{CD4}^+$ T cells.

Conflict of interest: The authors have no financial conflicts of interest and have no financial support.

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