



The effects of resiquimod in an ovalbumin-induced allergic rhinitis model

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ABSTRACT

Growing evidence indicates that the Toll-like receptor7/8(TLR7/8) agonist resiquimod (R848) is a potential inhibitor of type-2 immunity. However, the mechanisms mediating its therapeutic effects are not fully understood. This study investigated the effects of R848 on OVA-induced allergic rhinitis(AR) mice and the expression of IL-25, IL-33, TSLP, T-cell immunoglobulin mucin1 (TIM1) and T-cell immunoglobulin mucin3 (TIM3). BALB/c mice were intranasally sensitized and challenged with ovalbumin (OVA), and R848 was intraperitoneally injected into AR mice. Histological changes in the nasal mucosa were evaluated by hematoxylin and eosin (H & E) and Periodic Acid-Schiff (PAS) staining; cytokine levels in serum were measured with enzyme-linked immunosorbent assays (ELISAs); the mRNA expression levels of IFN- γ , IL-17 and Foxp3 in the spleen determined by quantitative real-time RT-PCR (qRT-PCR); the proportions of Th1, Th2, Th17, Treg and TIM3 + IFN- γ + Th1 cells in the spleen were assessed with flow cytometry; TIM1, TIM3 and IL-33 expression levels in the nasal mucosa were evaluated with immunofluorescence staining(IF).R848 alleviated the nasal allergic symptoms; reduced eosinophil cell infiltration, goblet cell hyperplasia in the nasal mucosa; reduced IL-13, IL-17, IL-25 and IL-33 levels in serum; upregulated the relative mRNA expression of IFN- γ and Foxp3, and downregulated the relative mRNA expression of IL-17 in the spleen; decreased Th2, Th17 and TIM3 + IFN- γ + Th1 cells ratios, increased the proportion of Th1 and Treg cells in the spleen; suppressed TIM1 and TIM3, but increased IL-33 expression in the nasal mucosa in OVA-induced AR mice. R848 suppresses IL-25, IL-33 released and TIM1, TIM3 expression, which may contribute to its anti-allergic effects.

1. Introduction

Allergic rhinitis (AR) is an IgE-mediated allergic nasal disease characterized by nasal obstruction, rhinorrhea, sneezing, and itching, and it affects 10% to 40% of the population [1]. Th1/Th2 imbalance has been identified as an important immunological mechanism of AR. In addition, upregulation of Th17 cells [2] and downregulation of Treg cells [3] also increase the risk of developing AR.

Innate immunity represents the first line of defense against invading pathogens and a variety of environmental insults [4]. Toll-like receptors (TLRs) constitute a family of transmembrane receptors and are recognized as critical components of the innate immune system. TLRs consist of the following three physical domains: extracellular domains, transmembrane domains, and intracellular domains. Toll-interleukin 1 (IL-1) receptor (TIR) domains can recognize pathogen-associated molecular patterns (PAMPs), such as LPS, lipoteichoic, glycolipids, peptidoglycans, flagellin, unmethylated CpG DNA viruses and endogenous ligands, and then subsequently activate either the MyD88- or TRIF-

dependent pathway. To date, 10 and 12 functional TLRs have been identified in humans and mice, respectively. TLR1–TLR9, which are conserved in both species, act as critical proteins linking innate and acquired immunity [5,6]. TLR7 recognizes viral single-stranded RNA. Recent studies show that activation of Toll-like receptor 7 has therapeutic potency in the inhibition of allergic diseases [7]. R848, a TLR7/8 ligand, attenuates type 2 immune disorders by enhancing the Th1 immune response, downregulating Th2 adaptive responses to allergens [8], and inhibiting IgE secretion from B cells [9]. R848 also induces Treg cells that contributed to suppression of asthma [10], and activation of TLR7 signaling in T cells could inhibit Th17 cell differentiation from naive T cells and IL-17 production in experimental autoimmune encephalomyelitis [11].

The nasal epithelium, which is the first site of exposure to inhaled antigens, plays an essential role in innate immunity. Group 2 innate lymphoid cells (ILC2), which have recently been described in the nasal epithelium of both mice and humans, are associated with innate immunity and are specialized in the induction and maintenance of type 2

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inflammation [12]. Epithelial cell-derived cytokines, including thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, which are required for ILC2 activity, may directly or indirectly induce Th2 immune responses in nasal mucosal tissues [13,14]. Therefore, adaptive Th2 cells are no longer considered the only source of Th2-related cytokines. Recent studies have shown the associations between TLRs and epithelial cell-derived cytokine expression levels, such as IL-25, IL-33, TSLP. A wide range of TLR ligands, including poly(I:C), flagellin, R848, and PGN, can induce IL33 and long-form TSLP expression in nasal epithelial cells [15]. Toll-like receptor 7 gene deficiency contributes to the induction of IL33 and TSLP expression, recruitment of type 2 innate lymphoid cells and increased Th2-type cytokine production in the airway epithelium [16]. R848 inhibits the DC-mediated Th2 response, which is activated by TSLP (TSLP-DCs) [17]. These results suggest that R848 may attenuate AR by modulating the expression levels of IL-25, IL-33, and TSLP. Therefore, the effect of R848 on the expression levels of IL-25, IL-33, and TSLP in AR warrants further exploration.

TIM1 is preferentially expressed on T-helper 2 (Th2) cells and functions as a potent costimulatory molecule for T-cells, which promote the Th2 cell response and T cell proliferation [18,19]. Antagonists of TIM1 or TIM1 deficiency blocked airway inflammation in mouse models of asthma [20,21]. In contrast, TIM3 is preferentially expressed on Th1 cells [22], and negatively regulates Th1 responses [23]. TLR7/8 and TIM1/3 are implicated in Th2-mediated allergic inflammation, but the correlation between TLR7/8 and TIM1/3 is unknown. A recent study indicated that TIM4 expression in human DCs depends on the Toll-like receptor TLR2 and TLR4 pathways [24,25], suggesting that the expression of T cell immunoglobulin and mucin domain (TIM) proteins may be modulated by Toll-like receptors (TLRs) and TLR7/8 may influence the expression of TIM1 and TIM3 on CD4⁺ T cells in AR.

In this study, we investigated the effect of the TLR7/8 agonist R848 in ovalbumin-induced allergic rhinitis model and the expression of IL-25, IL-33 and TSLP, and TIM1 and TIM3.

2. Materials and methods

2.1. Mice

Specific pathogen-free (SPF) female BALB/c mice (6–8 weeks old and weighing 18–22 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd., and housed in the Guangxi Medical University Laboratory Animal Centre under SPF conditions. The experimental procedures were approved by the Animal Care and Use Committee of Guangxi Medical University.

2.2. Induction of allergic rhinitis in mice

Mice were sensitized and challenged via OVA administration referred to previous described [26]. Thirty-six mice were randomly divided into three groups, including the Control group, the AR group and the R848 group. The AR and R848 groups were sensitized by intraperitoneal 200 µl OVA mixing solution injection (25 µg of OVA (Grade V; Sigma) and 2 mg of an aqueous solution of aluminum hydroxide and magnesium hydroxide (Imject Alum; Perbio Science, Bonn, Germany) dissolved in 200 µl of phosphate buffer saline (PBS)) on days 0 and 7, and challenged intranasally with 5 µl of filter-sterilized 1.0% OVA in each side of the nares for 5 consecutive days, on days 21, 22, 23, 24, 25. The control group received the same amount of PBS solution as the other two groups. In addition, the R848 group received 50 nmol of R848 (APEX BIO Technology, Houston, USA) in 200 µl PBS by intraperitoneal injection for 6 consecutive days, on days 20, 21, 22, 23, 24, 25. The Control and AR groups were injected with the same dose of PBS solution instead of R848. Twenty-four hours after the final challenge and injection, the mice were sacrificed. The procedure is shown in Fig. 1.

2.3. Animal behaviour observation

To determine the change in nasal symptoms, the frequency of sneezing and nasal rubbing motions were counted for 15 min after the final nasal challenge with OVA or PBS. The final nasal challenge was performed by one operator. Other three observers blinded to counting.

2.4. Histological assessment

Twenty-four hours after the final challenge and injection, the mice were anesthetized with 1% pentobarbital sodium and then sacrificed after blood was collected. The skin, muscles and eyes were removed from the heads, and the decapitated heads were then immersed in 4% paraformaldehyde for 24 h to complete fixation, followed by decalcification for 6 days in 10% EDTA. After decalcification, the noses were dehydrated, embedded in paraffin, and sectioned into 4-µm-thick sections. After deparaffinization, the sections were stained with hematoxylin and eosin (H & E) to analyze eosinophil cell infiltration and Periodic Acid-Schiff (PAS) to measure goblet cell hyperplasia. Immunofluorescence staining was performed to detect the TIM1/TIM3/IL-33 positive cells in nasal mucosa.

2.5. Enzyme-linked immunosorbent assay

Blood samples were collected from the inner canthus vein, centrifuged to isolate serum, and then stored at −80 °C. The expression levels of IL-25 (CUSABIO, China), IL-33 (CUSABIO, China), IL-13 (CUSABIO, China), IL-17 (CUSABIO, China), and TSLP (Bioss, China) in serum were measured by ELISA kits according to the manufacturer's instructions.

2.6. Flow cytometry

Spleen tissues were ground and separated into single cells using 70-µm cell strainers, followed by cracking of the red blood cells with Red Cell Lysis Buffer (TIANGEN, China). The cells were then cultured in RPMI 1640 with 10% FBS at a concentration of 1.0×10^6 cells/ml. Then, the cells were stimulated with 100 ng/ml of PMA (Sigma-Aldrich), 1000 ng/ml of ionomycin (Sigma-Aldrich) and 1 µl/ml of BD Golgi Plug™ for 6 h. The cells were washed and resuspended in 100 µl of staining buffer (3% FBS in PBS), and 1 µl/ml of anti-CD16/32 (2.4G2) was added to block Fcγ receptors. Surface markers were stained with 1.5 µl of Alexa Flour 700-conjugated anti-mouse CD4(RM4-5) and/or 1 µl of Per-Cy5.5-conjugated anti-mouse CD25(PC61) and/or 1 µl of PE-Cy7-conjugated anti-mouse TIM3(RMT3-23). After the cells were washed and permeabilized (eBioscience, USA), they were stained intracellularly with 1 µl of APC-conjugated anti-mouse IFN-γ(XMG1.2) and 1 µl of Per-Cy5.5-conjugated anti-mouse IL-4 (11B11) and 1 µl of Alexa Flour 488-conjugated anti-mouse IL-17(TC11-18H10), or 1 µl of Alexa Flour 647-conjugated anti-mouse Foxp3(MF23). The cells were stained with Alexa Flour 700-conjugated anti-mouse CD4(RM4-5) and Per-Cy5.5-conjugated anti-mouse CD25(PC61) and Alexa Flour 647-conjugated anti-mouse Foxp3(MF23) in other test tube to evaluate the Treg cells. Then the samples were washed, resuspended in staining buffer and analyzed with a FACSCalibur flow cytometer (BD FACSCanto II). The data were analyzed with FlowJo 10.0.7 (Tree Star).

2.7. Quantitative real-time RT-PCR (qRT-PCR)

Spleen cells without red blood cells were treated as above, and total RNA in the spleen cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, California, USA). Reverse transcription of RNA into cDNA was carried out with the Transcriptor First Strand cDNA synthesis Kit (Roche, Germany) according to the manufacturer's protocol. Real-time PCR was performed according to the FastStart Universal SYBR Green Master (Rox) (Roche, Germany) kit with primers specific for the target

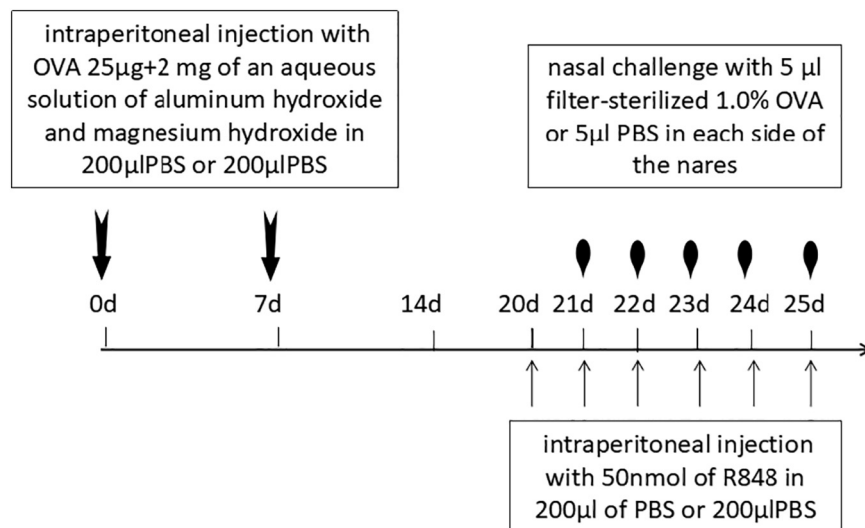


Fig. 1. The experimental protocol. AR mice were sensitized with intraperitoneal OVA mixing solution injection on days 0 and 7, and challenged intranasally with 5 µl of filter-sterilized 1.0% OVA in each side of the nares on days 21, 22, 23, 24, 25. R848 groups were injected intraperitoneally with R848 (50 nmol of R848 in 200 µl of PBS) on days 20, 21, 22, 23, 24, 25. Control groups were received PBS.

genes of IFN- γ , IL-17 and Foxp3 on the Biosystems 7500 Fast Real Time PCR System (Applied Biosystems). Two-step PCR reaction procedure were performed for all reactions: the PCR conditions for 1 cycles were 95 °C for 10 min for pre-denaturation, 40 cycles were 95 °C for 10 s for denaturation, 60 °C for 32 s for annealing/extension. The primer pair sequences as follows: IFN- γ : forward 5'-TGAACGCTACACACTGCATC-3', reverse 5'-CACTCGGATGAAGTCTCATTGA-3'; IL-17: forward 5'-TCAGCGTGTCCAAACACTGAG-3', reverse 5'-CGCCAAGGGATTAAAGACTT-3'; Foxp3: forward 5'-CACCTATGCCACCCTTATCCG-3', reverse 5'-CATGCGAGTAAACCAATGGTGA-3'; GAPDH: forward 5'-TGGCCTTCGGTTCCTAC-3', reverse 5'-GAGTTGCTGTTGAAGTCGCA-3'. Each sample was run in triplicate in separate tubes. Gene expression levels were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and relative expression levels were determined using the Livak method (the $2^{-\Delta\Delta C_t}$ method for real-time PCR) relative to the expression of GAPDH [27].

2.8. Immunofluorescence staining

Paraffin-embedded nasal tissues were cut into 4-µm-thick sections. After baking at 60 °C for 1 h, the sections were deparaffinized and rehydrated in density gradients of dimethylbenzene and ethanol, respectively, and then bathed in boiling citrate buffer (0.01 M, pH 6.0) for 10 min, followed by incubation in 20% goat serum for 45 min to block the positive and negative charges of tissues. Next, the sections were incubated with specific rabbit anti-mouse TIM1 (BIOSS, China, 1:150 dilution) / rabbit anti-mouse TIM3 (BIOSS, China, 1:150 dilution) / rabbit anti-mouse IL-33 (Bioss, China; 1:200 dilution) / nonspecific purified rabbit IgG (BIOSS, China; 1:150/200 dilution) at 4 °C overnight. After washing in PBS 5 times, the sections stained with TIM3 and IL-33 were incubated with Alexa Fluor 594-labeled goat anti-rabbit secondary antibody (Abcam; 1:500 dilution), and the slides stained with TIM1 were incubated with Alexa Fluor 488-labeled goat anti-rat (Abcam; 1:400 dilution) for 45 min at 37 °C. Nuclei were stained with DAPI. Rabbit IgG (BIOSS, China) was used for control staining. IL-33/TIM1/TIM3-positive cells in the nasal mucosa sections were viewed with a fluorescence microscope (Olympus) and analyzed using Image-Pro Plus 6.0. Two sections per mouse and six mice per group were assessed.

2.9. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0. The results are expressed as the mean $\pm$ standard deviation (SD). The comparison of variables between three groups was performed by analysis of variance (ANOVA), followed by Tukey's post hoc test. The

comparison of variables between two groups was performed with Student's *t*-test, followed by Homogeneity of variance test. *P* values < 0.05 were considered statistically significant.

3. Results

3.1. R848 treatment alleviates nasal symptoms in OVA-induced AR mice

The animal behaviour observation including sneezing and nasal rubbing motions were performed to assess the change of nasal symptoms. AR mice showed drastic sneezing and nasal rubbing motions in 15 min after the final OVA challenge. Treatment with R848 significantly alleviated the sneezing and rubbing motions (Fig. 2).

3.2. R848 treatment reduced eosinophil cell infiltration, goblet cell hyperplasia in OVA-induced AR mice

Compared with the control group, histological changes in the AR group showed significant upregulation of eosinophil cell infiltration, goblet cell hyperplasia in the nasal mucosa. Treatment with R848 significantly reduced the number of eosinophil cell and goblet cell in OVA-induced AR mice (Fig. 3).

3.3. R848 reduced the IL-13, IL-17, IL-25 and IL-33 levels relative to that in OVA-induced AR mice

IL-13 and IL-17 are the major cytokines of Th2 and Th17 cells, and IL-25, IL-33 and TSLP are epithelial cell-derived cytokines involved to type 2 inflammation. To explore the effect of R848 in AR, we analyzed IL-13, IL-17, IL-25, IL-33 and TSLP levels in serum. The concentrations of IL-13, IL-17, IL-25 and IL-33 were increased in the AR mice relative to that in the control mice, and they were decreased significantly after R848 treatment. TSLP levels in R848 group was lower than that in the control group, while there was no significant differences compared to AR group, and there was not significant differences between control group and AR group (Fig. 4). Therefore, the effect of R848 in the TSLP expression was not determined.

3.4. The mRNA expression levels of IFN- γ , IL-17 and Foxp3 after R848 treatment

The relative mRNA expression levels of IFN- γ , IL-17 and Foxp3 in spleen cells were assessed by qRT-PCR. The relative quantification of the AR group and the R848 group were normalized with the control group using the Livak method (the $2^{-\Delta\Delta C_t}$ method for real-time PCR).

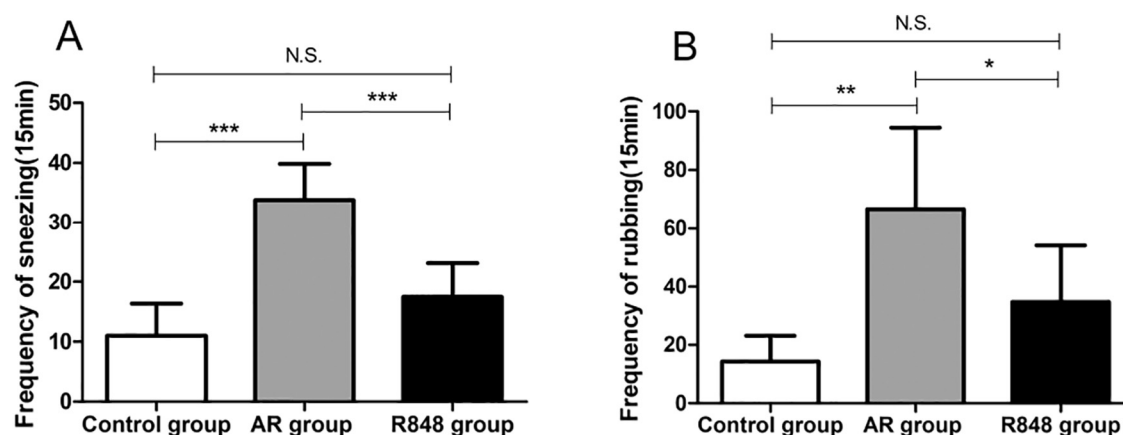


Fig. 2. Animal behaviour observation including sneezing and nasal rubbing motions. (A) The frequency of sneezing, (B) the frequency of nasal rubbing motions. The data are shown as the mean $\pm$ SD, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, N.S.: no significance.

As shown in Fig. 5, the mRNA expression level of IL-17 was higher in the AR group compared with the control group, while the IFN- γ and Foxp3 levels were decreased. In the R848 group, the mRNA expression of IFN- γ and Foxp3 were upregulated and IL-17 expression was down-regulated relative to that in the AR group. These results indicated that

R848 increased the mRNA expression of IFN- γ and Foxp3, and reduced the mRNA expression of IL-17 in spleen.

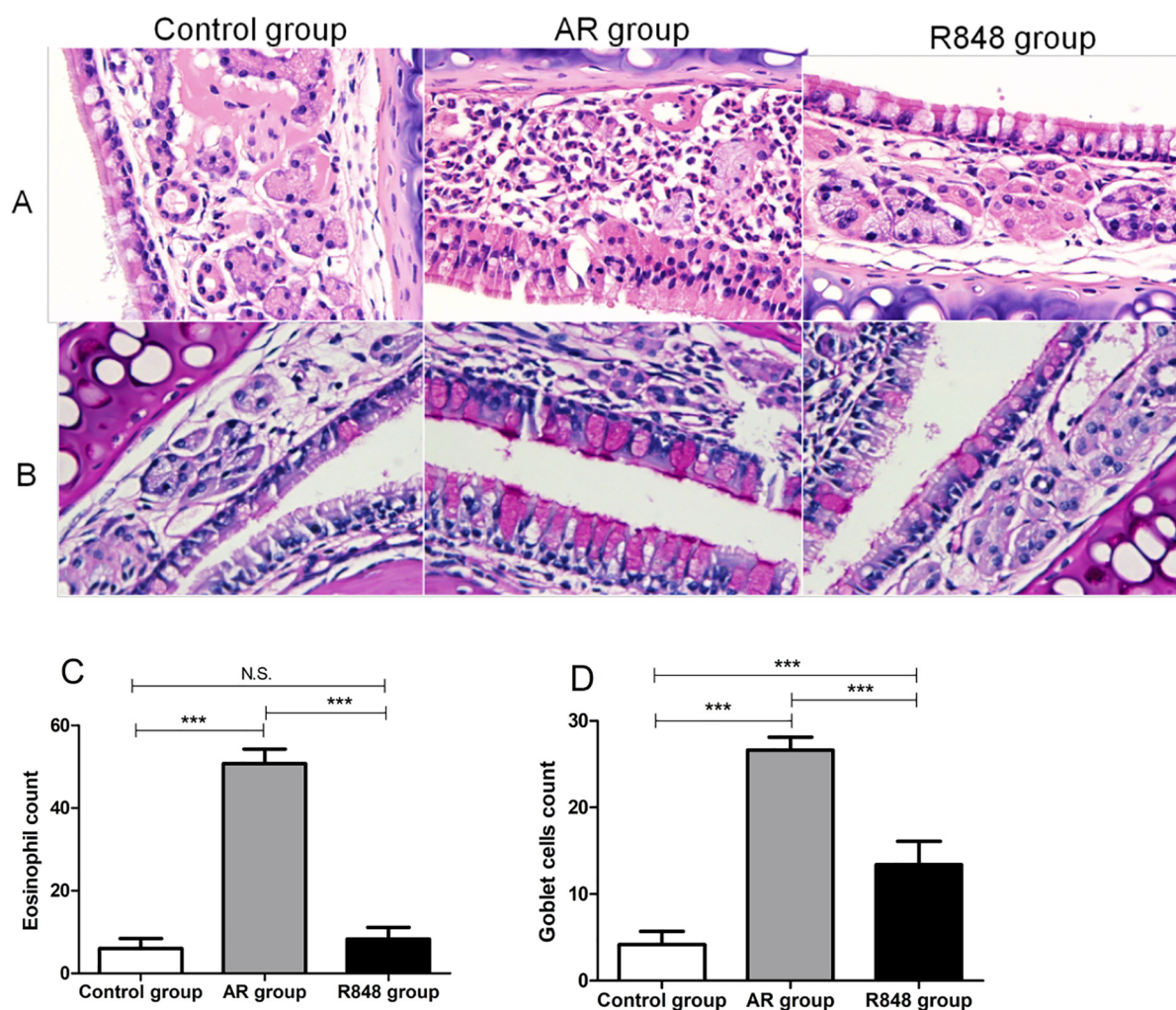


Fig. 3. Representative photomicrographs (original magnification $\times 400$) of H & E- and PAS-stained sections. (A) The nasal mucosa were evaluated by H & E to analyze eosinophil cell infiltration. (B) The nasal mucosa were evaluated by PAS to measure goblet cell hyperplasia. (C) The total number of infiltrated eosinophils in each microscopic field, (D) goblet cell hyperplasia in the nasal mucosa in each microscopic field. The data are shown as the mean $\pm$ SD, $n = 6$. $^{***}P < 0.001$, N.S.: no significance.

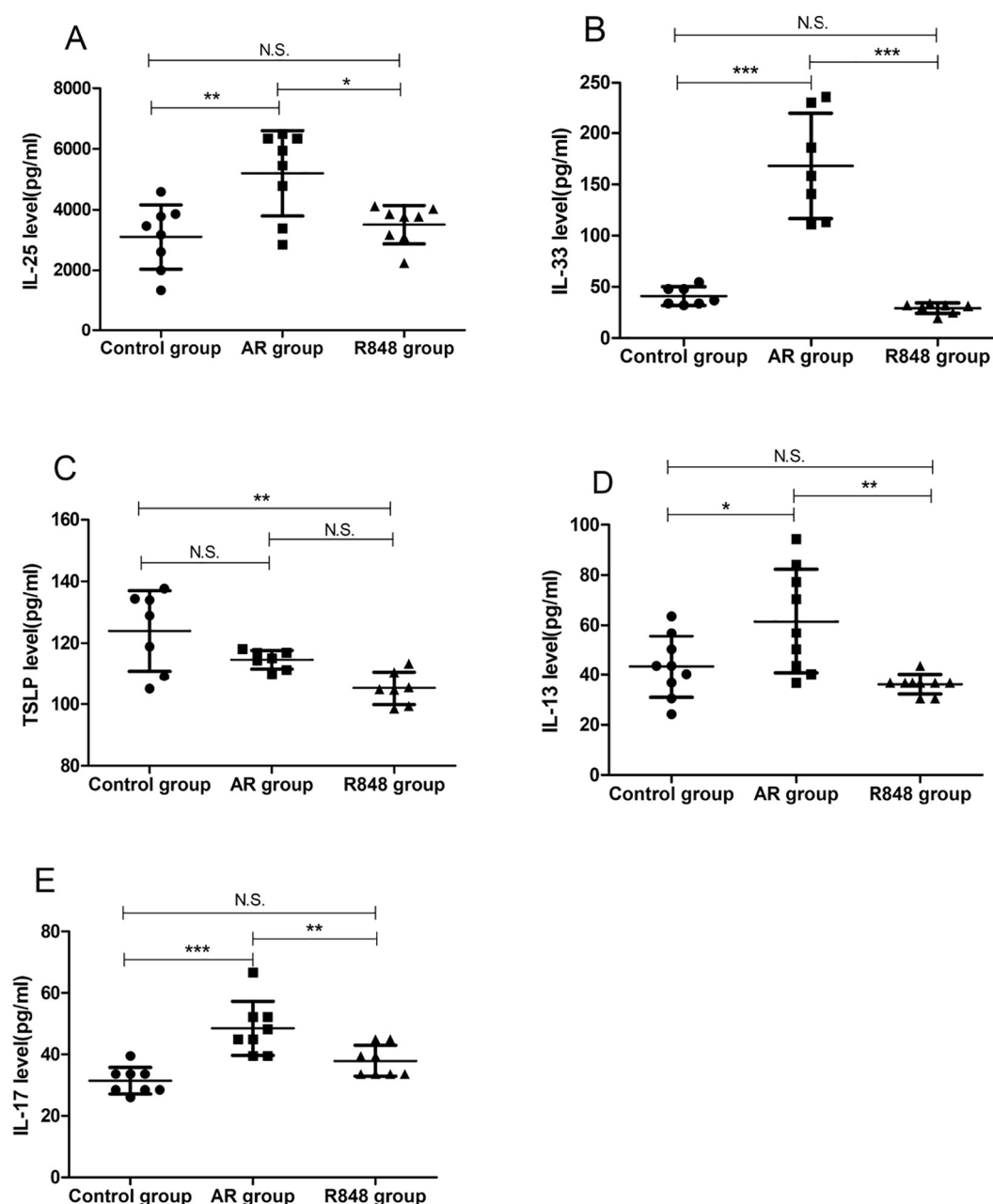


Fig. 4. R848 reduced the expression levels of IL-13, IL-17, IL-25, and IL-33 in serum in AR mice. The serum levels of IL-25, $n = 8$ (A); IL-33, $n = 7$ (B); TSLP, $n = 7$ (C); IL-13, $n = 9$ (D); and IL-17, $n = 8$ (E). The values represent the mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, N.S.: no significance.

3.5. R848 treatment decreased Th2, Th17 and TIM3 + IFN- γ + Th1 cell ratios and increased Th1, Treg cell ratios

As shown in Fig. 6, a higher proportion of Th2, Th17, TIM3 + IFN- γ + Th1 cells and lower percentages of Th1, Treg cells were observed in the AR group relative to that in the control group. The proportion of Th2, Th17, TIM3 + IFN- γ + Th1 cells in the R848 group were lower than that in the AR group, while the percentages of Th1, Treg cells in the R848 group were higher. The results indicated that R848 significantly reduced the Th2, Th17 and enhanced the percentages of Th1 and Treg cells. In addition, R848 inhibited the expression of TIM3 in Th1 cells.

3.6. R848 increased IL-33 and decreased TIM1 and TIM3 expression in the nasal mucosa in AR mice

Nasal sections (original magnification, $\times 400$) were stained with IF

to examine the expression of IL-33, TIM1, TIM3 in nasal mucosa. The data are representative of two independent experiments with four mice in each group, and similar results were obtained in both experiments. We found that IL-33-positive cells were significantly decreased in the AR mice, but they were increased after R848 treatment (Fig. 7A). TIM1 and TIM3 expression levels in the nasal mucosa showed the same trends following OVA and R848 treatment. Higher TIM1 and TIM3 levels were observed in the AR mice compared with those in the control mice, which significantly decreased after R848 treatment (Fig. 7BC). The results indicated that R848 increased IL-33 expression and suppresses TIM1 and TIM3 expression in the nasal mucosa.

4. Discussion

R848, a TLR7/8 ligand and imidazoquinolinone compound, is a potential inhibitor of Th2 cell-driven inflammatory responses and has

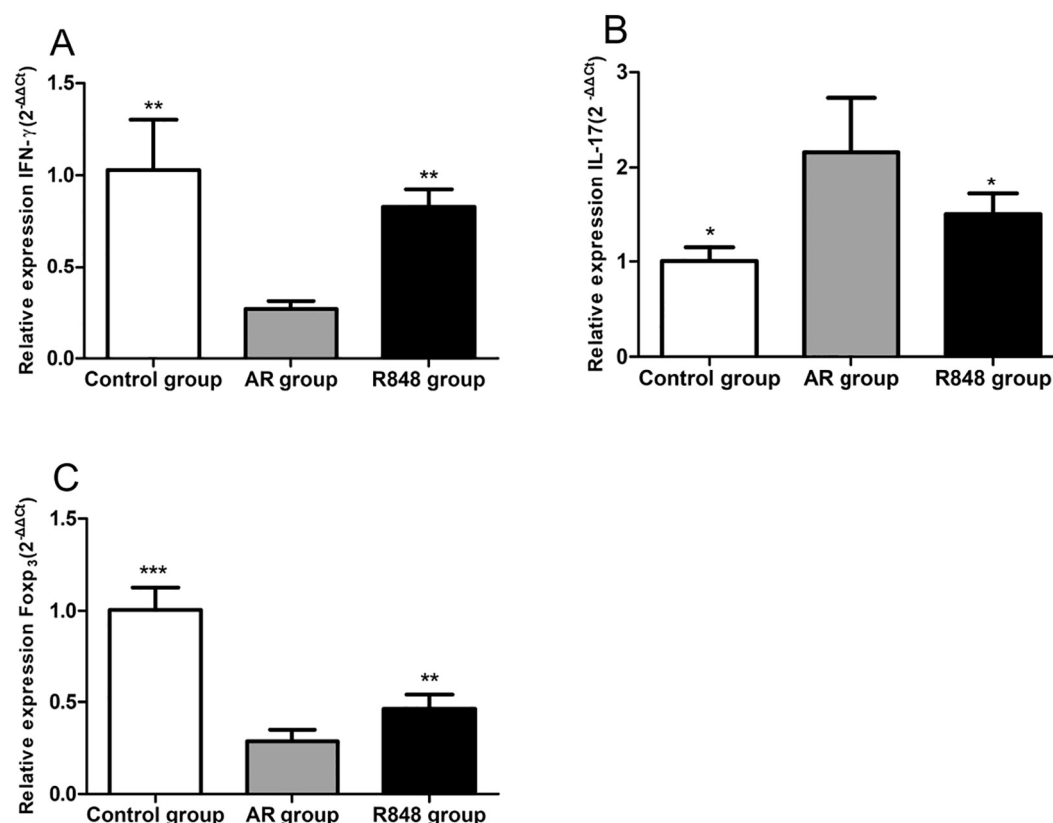


Fig. 5. The mRNA expression of IFN-γ, IL-17 and Foxp3 were detected with qRT-PCR. R848 increased the mRNA expression of IFN-γ and Foxp3, and reduced the mRNA expression of IL-17. (A) The relative mRNA expression of IFN-γ, (B) the relative mRNA expression of IL-17, (C) the relative mRNA expression of Foxp3. The bars represent the mean $\pm$ SD, $n = 4$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

been applied in experimental research in allergic diseases. R848 has been identified as a promising adjuvant in the treatment of allergic diseases. Epicutaneous immunization with rBet v 1 plus R848 promoted Bet v 1-specific Th1 responses and efficiently suppressed asthmatic features elicited by birch pollen [28]. Additionally, the TLR7a-Der f 1 vaccine exhibits interesting therapeutic potency in suppressing allergic asthma [29]. R848 suppressed experimental asthma by significantly reducing IL-5, IL-13, and IL-17 levels and inducing IFN-γ and IL-27 production [30]. However, the therapeutic effects of R848 in AR have received little attention. Imbalanced lymphocyte subsets, including reduced Th1 and Treg cell ratios and upregulated Th2 and Th17 cell ratios, have major roles in the immunological pathogenesis of allergic rhinitis. R848 is known to alleviate allergic inflammation by enhancing Th1 and Treg cell responses and inhibiting Th2- and Th17-mediated inflammation [8,10,11]. Recent study indicated that R848 can induce IL-10 and IFN-γ in peripheral blood mononuclear cells (PBMCs) in atopic donors with AR [31]. Collectively, these studies suggest that R848 also exhibits interesting therapeutic potency in the suppression of allergic rhinitis.

In this study, we found that R848 significantly reduced eosinophil infiltration and goblet cell hyperplasia, and alleviates nasal symptoms in OVA-induced AR mice. IL-13 and IL-17 levels in serum and mRNA expression of IL-17 in nasal mucosa in OVA-induced AR mice were also downregulated after R848 treatment. In addition, R848 increased Th1 and Treg ratios and decreased Th2 and Th17 ratios compared with the corresponding levels in OVA-induced AR mice. Our study provides evidence for the therapeutic potency of R848 in allergic rhinitis. The results demonstrated that R848 is a promising agent in the treatment of type 2 immune disorders. However, the mechanisms mediating its therapeutic effects are not fully understood.

IL-25, IL-33 and TSLP are involved in the development of AR. Anti-IL-33 treatment ameliorated allergic inflammation in the AR mice, as

shown by a significantly reduced number of nose-scratching events, total serum and OVA-specific IgE, and the numbers of eosinophils and Th2-type cytokines, including IL-4, IL-5, and IL-13, in BAL fluid [32]. Additionally, the IL-33/ST2 pathway plays a critical role in nasal Th2 activation during acute allergic insults in mice with AR, but both TSLPR and ST2 contribute to Th2 responses in chronically allergen-exposed mice with AR [33]. IL-25 is also involved in the development of AR. IL-25 expression is increased in human nasal epithelial cells (HNECs) in response to high-dose HDM stimulation and has been found to be significantly correlated with Th2 markers, such as ECP and GATA3 [14]. TSLP modulated the IgE-mast-cell/basophil pathway that played a critical role in the early phase response of AR [34]. Other studies have reported the correlation between TLRs and the epithelial cell-derived cytokine expression levels: IL-25, IL-33, and TSLP. A wide range of TLR ligands, including poly(I:C), flagellin, R848, and PGN, can induce IL-33 and long-form TSLP expression in nasal epithelial cells [15]. R848 repressed OX40 ligand expression on DCs and induced IL-12 production from DCs, then decreased the Th2-inducing potency of human DCs activated by TSLP (TSLP-DCs) [17]. Toll-like receptor 7 gene deficiency contributes to inducing IL-33 and IL-25 expression, recruiting type 2 innate lymphoid cells and increasing Th2-type cytokine production in the airway epithelium [16]. These studies suggest that R848 may modulate the expression of IL-25, TSLP and IL-33. In this study, we show that R848 treatment reduced IL-25 and IL-33 levels in serum relative to those in AR mice, and increased the expression of IL-33 in nasal mucosa, but the effect of R848 in the TSLP expression was not determined.

IL-33 is a dual function protein that may function as both a proinflammatory cytokine and an intracellular nuclear factor with transcriptional regulatory properties. In a resting state, Full-length IL-33 is located in the cell nucleus and functions as intracellular nuclear factor with transcriptional regulatory properties. Full-length IL-33 binds to the

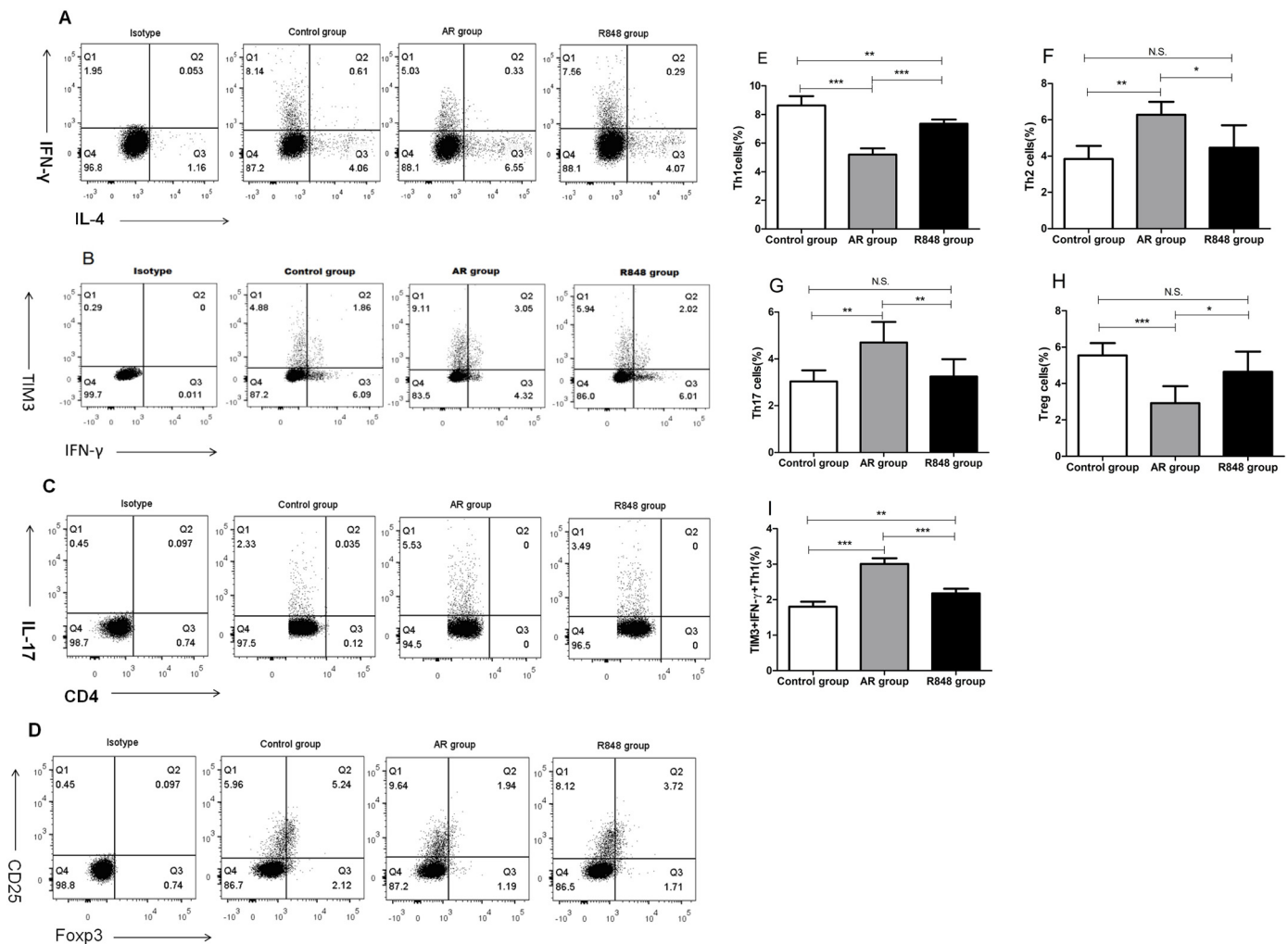


Fig. 6. (A–D) Phenotypic analysis of Th1, Th2, Th17, Treg and TIM3 + IFN- γ + Th1 cells in the spleen by FCM; IFN- γ + CD4 + represents Th1 cells, IL-4 + CD4 + represents Th2 cells, IL-17 + CD4 + represents Th17 cells, CD25 + Foxp3 + CD4 + represents Treg cells, TIM3 + IFN- γ + CD4 + represents TIM3 + IFN- γ + Th1 cells. (E) The frequency of Th1 cells. (F) The frequency of Th2 cells. (G) The frequency of Th17 cells. (H) The frequency of Treg cells. (I) The frequency of TIM3 + IFN- γ + Th1 cells. The data are shown as the mean $\pm$ SD, $n = 6$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, N.S.: no significance.

chromatin mediated by in homeodomain-like helix–turn–helix motif within the IL-33 N-terminal part, then modulates gene expression [35]. It has been demonstrated that nuclear IL-33 sequesters nuclear NF- κ B and reduces NF- κ B-triggered gene expression such as IkBa, TNF- α , and C-REL [36]. When cells experience mechanical stress, injury or death by necrosis, mature IL-33 is released extracellularly to trigger an inflammatory reaction [37,38]. Extracellular IL-33 binds to IL-1 receptor-related protein ST2, activates NF- κ B and MAP kinases and drives production of Th2-associated cytokines from polarized Th2 cells [39], mast cells, basophils [40], eosinophils [41], and innate immune cells [42]. In our previous study, we detected that IL-33 mainly located in the nucleus of epithelial cells, which significantly decreased in the nasal epithelial cells of OVA-induced AR mice and increased after 1,25-dihydroxyvitamin D3 treatment [43]. Other group study also shown that IL-33 protein continuously expressed in the nasal epithelial cells of healthy control subjects and downregulated in inflamed nasal epithelial cells of patients with AR [44]. In current study, we show that R848 treatment reduced IL-33 levels in serum in OVA-induced AR mice, and increased the expression of IL-33 in nasal mucosa. Which suggested that IL-33 may release from epithelial cells to extracellular to trigger allergic inflammation in the AR mice, and treatment with R848 contributed to maintaining IL-33 at a steady state and translocating to the nucleus. The results indicated that R848 is beneficial in alleviation of AR by inhibiting IL-25 and IL-33 released.

ILC2s, a subset of lymphoid cells involved in innate type 2 immunity, are strong responder cells to IL-25/IL-33/TSLP stimulation and rapidly induce robust production of type 2 cytokines, such as IL-4, IL-5 and IL-13, and airway eosinophils [15,42]. ILC2 involvement in allergic rhinitis has been confirmed. Peripheral blood type 2 innate lymphoid cells increased rapidly in allergic rhinitis subjects in response to nasal allergen exposure [45]. ILC2 also increased in patients with seasonal allergic rhinitis (SAR) during pollen season and was inhibited by subcutaneous grass pollen immunotherapy [46]. Considering that R848 effectively reduced the IL-25 and IL-33 levels in serum that are required for ILC2 activity, we speculate that R848 may suppress ILC2 response through inhibiting the IL-25 and IL-33 released, which contribute to the alleviation of innate immune responses related to type 2 immune disorders. Therefore, further research is needed to determine the effect of TLR7/8 in ILC2 response and innate immunity.

TIM1 is preferentially expressed on T-helper 2 (Th2) cells and positively regulates Th2-type immune responses. Ectopic retroviral expression of TIM1 in developing effector T cells elevated the production of IL-4 [47]. Antagonists of TIM1 or TIM1 deficiency blocked airway inflammation in mouse models of asthma [19,21]. In contrast, TIM3 is preferentially expressed on T helper type 1 (T(H)1) cells and negatively regulates Th1 responses [22,23]. Recent test results showed that TIM3 was highly expressed in allergic asthma patients and was related to Th1/Th2 imbalance. Inhibition of TIM3 favored Th1 induction,

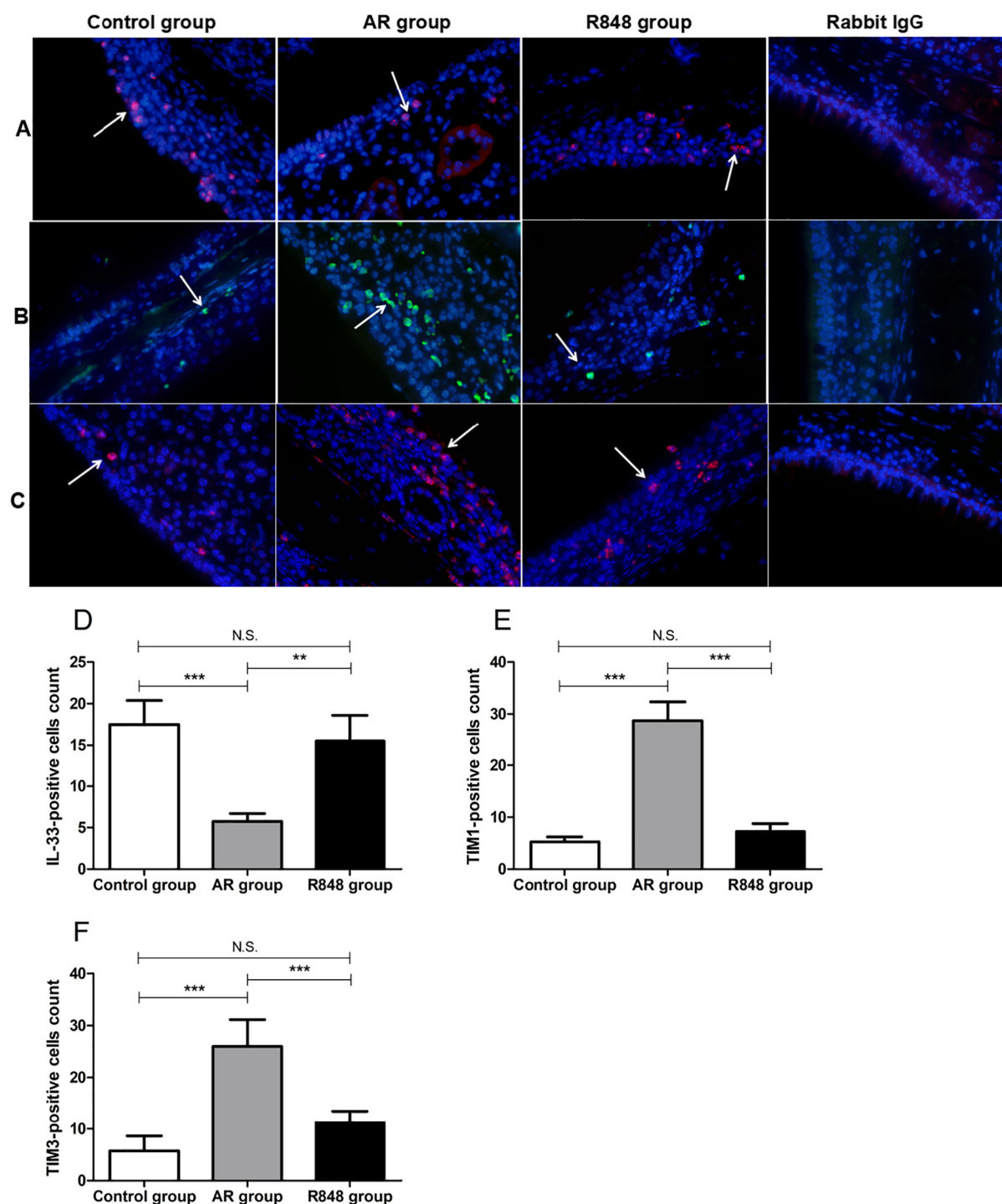


Fig. 7. (A) Nasal sections were stained with rabbit anti-mouse IL-33 and Alexa Fluor 594-conjugated goat anti-rabbit (red) antibodies, and nuclei were stained with DAPI (blue). IL-33-positive cells are indicated by the arrow. (B) Nasal sections were stained with rabbit anti-mouse TIM1 and Alexa Fluor 488-conjugated goat anti-rabbit (green) antibodies, and nuclei were stained with DAPI (blue). TIM1-positive cells are indicated by the arrow. (C) Nasal sections were stained with rabbit anti-mouse TIM3 and Alexa Fluor 594-conjugated goat anti-rabbit (red) antibodies, and nuclei were stained with DAPI (blue). TIM3-positive cells are indicated by the arrow. (D) The numbers of IL-33-positive cells, (E) TIM1-positive cells, and (F) TIM3-positive cells in each microscopic field. The data are shown as the mean $\pm$ SD, $n = 4$. ** $P < 0.01$, *** $P < 0.001$, N.S.: no significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inhibited Th2 cell responses, and decreased IgG/IgE production [48]. These study suggest that TIM1 and TIM3 increases in CD4+ T cells may induce the Th1/Th2 imbalance and play critical role in the immunopathology mechanism of AR. A recent study indicated that TIM4 expression in human DCs depends on the Toll-like receptor TLR2 and TLR4 pathways [24,25], which suggests that the expression of T cell

immunoglobulin and mucin domain (TIM) proteins may be modulated by Toll-like receptors (TLRs) and TLR7/8 may influence the expression of TIM1 and TIM3 on CD4+ T cells in AR.

To verify the speculations, we assessed the expression levels of TIM1 and TIM3 in the nasal mucosa by IF and the expression of TIM3 on Th1 cells by flow cytometry. In our study, we found that both TIM1-

and TIM3- positive cells were increased in the nasal mucosa in AR mice, and the TIM3 + IFN- γ + Th1 cells ratios were upregulated in the spleen. While these cells were downregulated in response to R848 treatment. The results suggest that TIM1 may actively regulate Th2 cells and TIM3 may negatively regulate Th1 cells, and R848-induced downregulation of TIM1 and TIM3 expression may favor AR alleviation.

In the current study, we showed that TIM3 was downregulated in Th1 cells in response to R848, but we did not analyze the effect of R848 on the expression of TIM1 in Th2 cells. Therefore, further exploration is warranted, and we will carry out in our future research.

In summary, R848 was beneficial for AR alleviation and inhibited IL-25 and IL-33 released and TIM1 and TIM3 expression. It is suggested that R848 inhibiting the IL-25 and IL-33 released from nasal epithelial cell may contribute to the alleviation of adaptive and innate immune responses related to type 2 immune disorders; and inhibiting the expression of TIM1 and TIM3 may contribute to the alleviation of adaptive immune responses related to type 2 immune disorders.

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Conflict of interest

The authors have no financial interests to disclose.

References

- J.L. Brozek, J. Bousquet, I. Agache, A. Agarwal, C. Bachert, S. Bosnic-Anticevich, et al., Allergic rhinitis and its impact on asthma (ARIA) guidelines-2016 revision, *J. Allergy Clin. Immunol.* 140 (2017) 950–958.
- G. Ciprandi, G. Filaci, F. Battaglia, D. Fenoglio, Peripheral Th-17 cells in allergic rhinitis: new evidence, *Int. Immunopharmacol.* 10 (2010) 226–229.
- K. Saad, A.M. Zahran, K.I. Elsayh, A. Abdelmoghny, M.D. Aboul-Khair, Variation of regulatory T lymphocytes in the peripheral blood of children with allergic rhinitis, *Arch. Immunol. Ther. Exp.* (2017).
- K.R. Bartemes, K. Iijima, T. Kobayashi, G.M. Kephart, A.N. McKenzie, H. Kita, IL-33-responsive lineage-CD25⁺ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs, *J. Immunol.* 188 (2012) 1503–1513.
- S. Akira, K. Takeda, T. Kaisho, Toll-like receptors: critical proteins linking innate and acquired immunity, *Nat. Immunol.* 2 (2001) 675–680.
- T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, *Nat. Immunol.* 11 (2010) 373–384.
- K.M. Lebold, D.B. Jacoby, M.G. Drake, Toll-like receptor 7-targeted therapy in respiratory disease, *Transfus. Med. Hemother.* 43 (2016) 114–119.
- F. Grela, A. Aumeunier, E. Bardel, L.P. Van, E. Bourgeois, J. Vanoirbeek, et al., The TLR7 agonist R848 alleviates allergic inflammation by targeting invariant NKT cells to produce IFN- γ , *J. Immunol.* 186 (2011) 284–290.
- E. Shen, L. Lu, C. Wu, TLR7/8 ligand, R-848, inhibits IgE synthesis by acting directly on B lymphocytes, *Scand. J. Immunol.* 67 (2008) 560–568.
- L.P. Van, E. Bardel, S. Gregoire, J. Vanoirbeek, E. Schneider, M. Dy, et al., Treatment with the TLR7 agonist R848 induces regulatory T-cell-mediated suppression of established asthma symptoms, *Eur. J. Immunol.* 41 (2011) 1992–1999.
- J. Ye, Y. Wang, X. Liu, L. Li, A. Opejin, E.C. Hsueh, et al., TLR7 signaling regulates Th17 cells and autoimmunity: novel potential for autoimmune therapy, *J. Immunol.* 199 (2017) 941–954.
- L. Cosmi, F. Liotta, L. Maggi, F. Annunziato, Role of type 2 innate lymphoid cells in allergic diseases, *Curr. Allergy Asthma Rep.* 17 (2017) 66.
- C. Butler, L.G. Heaney, Risk factors of frequent exacerbations in difficult-to-treat asthma, *Eur. Respir. J.* 27 (2006) 1324–1325 (1325–6).
- D.W. Kim, D.K. Kim, K.M. Eun, J.S. Bae, Y.J. Chung, J. Xu, et al., IL-25 could be involved in the development of allergic rhinitis sensitized to house dust mite, *Mediat. Inflamm.* 2017 (2017) 3908049.
- J. Mjosberg, J. Bernink, K. Golebski, J.J. Karrich, C.P. Peters, B. Blom, et al., The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells, *Immunity* 37 (2012) 649–659.
- G.E. Kaiko, Z. Loh, K. Spann, J.P. Lynch, A. Lalwani, Z. Zheng, et al., Toll-like receptor 7 gene deficiency and early-life Pneumovirus infection interact to predispose toward the development of asthma-like pathology in mice, *J. Allergy Clin. Immunol.* 131 (2013) 1331–1339.
- Y. Torii, T. Ito, R. Amakawa, H. Sugimoto, H. Amuro, T. Tanijiri, et al., Imidazoquinoline acts as immune adjuvant for functional alteration of thymic stromal lymphopoietin-mediated allergic T cell response, *J. Immunol.* 181 (2008) 5340–5349.
- S.E. Umetsu, W.L. Lee, J.J. McIntire, L. Downey, B. Sanjanwala, O. Akbari, et al., TIM-1 induces T cell activation and inhibits the development of peripheral tolerance, *Nat. Immunol.* 6 (2005) 447–454.
- J.A. Encinas, E.M. Janssen, D.B. Weiner, S.A. Calarota, D. Nieto, T. Moll, et al., Anti-T-cell Ig and mucin domain-containing protein 1 antibody decreases TH2 airway inflammation in a mouse model of asthma, *J. Allergy Clin. Immunol.* 116 (2005) 1343–1349.
- H.Y. Kim, Y.J. Chang, Y.T. Chuang, H.H. Lee, D.I. Kasahara, T. Martin, et al., T-cell immunoglobulin and mucin domain 1 deficiency eliminates airway hyperactivity triggered by the recognition of airway cell death, *J. Allergy Clin. Immunol.* 132 (2013) 414–425.
- S.S. Sonar, Y.M. Hsu, M.L. Conrad, G.R. Majeau, A. Kilic, E. Garber, et al., Antagonism of TIM-1 blocks the development of disease in a humanized mouse model of allergic asthma, *J. Clin. Invest.* 120 (2010) 2767–2781.
- W.D. Hastings, D.E. Anderson, N. Kassam, K. Koguchi, E.A. Greenfield, S.C. Kent, et al., TIM-3 is expressed on activated human CD4⁺ T cells and regulates Th1 and Th17 cytokines, *Eur. J. Immunol.* 39 (2009) 2492–2501.
- C. Zhu, A.C. Anderson, A. Schubart, H. Xiong, J. Imitola, S.J. Khoury, et al., The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity, *Nat. Immunol.* 6 (2005) 1245–1252.
- T. Liu, S.H. He, P.Y. Zheng, T.Y. Zhang, B.Q. Wang, P.C. Yang, Staphylococcal enterotoxin B increases TIM4 expression in human dendritic cells that drives naive CD4 T cells to differentiate into Th2 cells, *Mol. Immunol.* 44 (2007) 3580–3587.
- B.S. Feng, X. Chen, S.H. He, P.Y. Zheng, J. Foster, Z. Xing, et al., Disruption of T-cell immunoglobulin and mucin domain molecule (TIM)-1/TIM4 interaction as a therapeutic strategy in a dendritic cell-induced peanut allergy model, *J. Allergy Clin. Immunol.* 122 (2008) 55–61 (61.).
- K. Miyashita, J. Ohori, H. Nagano, S. Fukuyama, Y. Kuroono, Intranasal immunization with phosphorylcholine suppresses allergic rhinitis in mice, *Laryngoscope* (2017).
- K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2[−](Delta Delta C(T)) Method, *Methods* 25 (2001) 402–408.
- S. Siebeneicher, S. Reuter, M. Krause, A. Wangorsch, J. Maxeiner, S. Wolfheimer, et al., Epicutaneous immune modulation with Bet v 1 plus R848 suppresses allergic asthma in a murine model, *Allergy* 69 (2014) 328–337.
- L. Ma, X. Xiao, Y. Ma, H. Wu, S. Qiu, J. Li, et al., Effect of activation of Toll-like receptor 7 in the inhibition of allergic asthma on a mouse model, *Am. J. Transl. Res.* 9 (2017) 2143–2152.
- A.C. Jirno, K. Daluege, C. Happle, M. Albrecht, A.M. Dittrich, M. Busse, et al., IL-27 is essential for suppression of experimental allergic asthma by the TLR7/8 agonist R848 (Resiquimod), *J. Immunol.* 197 (2016) 4219–4227.
- G. Boghdadi, N. Hammad, A. Amer, S. Sammour, S. Sorour, R848, a Toll-like receptors 7 and 8 agonist, a potential therapy for allergic rhinitis patients, *Inflamm. Allergy Drug Targets* 13 (2014) 144–149.
- Y.H. Kim, T.Y. Yang, C.S. Park, S.H. Ahn, B.K. Son, J.H. Kim, et al., Anti-IL-33 antibody has a therapeutic effect in a murine model of allergic rhinitis, *Allergy* 67 (2012) 183–190.
- S. Akasaki, K. Matsushita, Y. Kato, A. Fukuoka, N. Iwasaki, M. Nakahira, et al., Murine allergic rhinitis and nasal Th2 activation are mediated via TSLP- and IL-33-signaling pathways, *Int. Immunol.* 28 (2016) 65–76.
- S. Akasaki, K. Matsushita, Y. Kato, A. Fukuoka, N. Iwasaki, M. Nakahira, et al., Murine allergic rhinitis and nasal Th2 activation are mediated via TSLP- and IL-33-signaling pathways, *Int. Immunol.* 28 (2016) 65–76.
- V. Carriere, L. Roussel, N. Ortega, D.A. Lacorre, L. Americh, L. Aguilar, et al., IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 282–287.
- S. Ali, A. Mohs, M. Thomas, J. Klare, R. Ross, M.L. Schmitz, et al., The dual function cytokine IL-33 interacts with the transcription factor NF- κ B to dampen NF- κ B-stimulated gene transcription, *J. Immunol.* 187 (2011) 1609–1616.
- R. Kakkar, H. Hei, S. Dobner, R.T. Lee, Interleukin 33 as a mechanically responsive cytokine secreted by living cells, *J. Biol. Chem.* 287 (2012) 6941–6948.
- C. Cayrol, J.P. Girard, The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 9021–9026.
- J. Schmitz, A. Owyang, E. Oldham, Y. Song, E. Murphy, T.K. McElanahan, et al., IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines, *Immunity* 23 (2005) 479–490.
- A. Otsuka, Y. Nonomura, K. Kabashima, Roles of basophils and mast cells in cutaneous inflammation, *Semin. Immunopathol.* 38 (2016) 563–570.
- B. Stolarzski, M. Kurowska-Stolarzka, P. Kewin, D. Xu, F.Y. Liew, IL-33 exacerbates eosinophil-mediated airway inflammation, *J. Immunol.* 185 (2010) 3472–3480.
- J.M. Mjosberg, S. Trifari, N.K. Crellin, C.P. Peters, C.M. van Drunen, B. Piet, et al., Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CCR2 and CD161, *Nat. Immunol.* 12 (2011) 1055–1062.
- B. Chen, S. Qu, M. Li, L. Ye, S. Zhang, T. Qin, et al., Effects of 1,25-dihydroxyvitamin D3 in an ovalbumin-induced allergic rhinitis model, *Int. Immunopharmacol.* 47 (2017) 182–189.
- Y. Haenueki, K. Matsushita, S. Futatsugi-Yumikura, K.J. Ishii, T. Kawagoe, Y. Imoto, et al., A critical role of IL-33 in experimental allergic rhinitis, *J. Allergy Clin. Immunol.* 130 (2012) 184–194.

- [45] T.A. Doherty, D. Scott, H.H. Walford, N. Khorram, S. Lund, R. Baum, et al., Allergen challenge in allergic rhinitis rapidly induces increased peripheral blood type 2 innate lymphoid cells that express CD84, *J. Allergy Clin. Immunol.* 133 (2014) 1203–1205.
- [46] M. Lao-Araya, E. Steveling, G.W. Scadding, S.R. Durham, M.H. Shamji, Seasonal increases in peripheral innate lymphoid type 2 cells are inhibited by subcutaneous grass pollen immunotherapy, *J. Allergy Clin. Immunol.* 134 (5) (2014) 1193.
- [47] A.J. de Souza, T.B. Oriss, K.J. O'Malley, A. Ray, L.P. Kane, T cell Ig and mucin 1 (TIM-1) is expressed on in vivo-activated T cells and provides a costimulatory signal for T cell activation, *Proc. Natl. Acad. Sci. U. S. A.* 102 (2005) 17113–17118.
- [48] F. Tang, F. Wang, L. An, X. Wang, Upregulation of Tim-3 on CD4(+) T cells is associated with Th1/Th2 imbalance in patients with allergic asthma, *Int. J. Clin. Exp. Med.* 8 (2015) 3809–3816.