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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 Th17cells [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-mediatedTh2 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 (APExBIO 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) and1μl 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 evaluated 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 the FastStart Universal SYBR Green Master (Rox) (Roche, Germany) kit with primers specific for the target