

## ***H2-Eb1* expression is upregulated in the nasal mucosa of allergic rhinitis**

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### Summary

**Background:** Allergic rhinitis (AR) is one of the most common allergic diseases. The results of recent studies of HLA-DRB1 suggest that HLA-DRB1 plays an important role in allergic disease.

**Objective:** The aim of the present study was to investigate the relationship between the *H2-Eb1* (orthologous gene of human HLA-DRB1 in mice) gene and AR pathogenesis in AR mice.

**Methods:** Female 129/sv mice were sensitized with ovalbumin (OVA) to establish an AR mouse model. After successful induction, the nasal mucosa was fixed and stained for pathologic analysis, such as eosinophil (EOS) evaluation and mast cell infiltration etc. The Th1 and Th2 cytokines IFN- $\gamma$ , IL-2, IL-4, IL-10 and the OVA-specific IgE levels were detected using the ELISA method. The *H2-Eb1* and GATA-3 (GATA-binding protein-3) and T-bet (T-box expressed in T-cells) protein expression in the nasal mucosa were detected by the western blot or immunohistochemistry, immunofluorescence technique. The expression of *H2-Eb1* mRNA in nasal mucosa was analyzed by quantitative real time PCR.

**Results:** Compared with the control group, the cilia layer in nasal mucosa of the experimental group was found to be partially desquamated; eosinophil (EOS) and mast cell infiltration was found in submucosal layer; serum OVA-IgE and IL-4, IL-10 levels were found to be significantly increased, together with a decrease of IFN- $\gamma$  and

IL-2 levels. The expression of *H2-Eb1* mRNA and *H2-Eb1* protein, as well as the ratio of GATA-3/T-bet expression, were upregulated in the nasal mucosa of the experimental group as compared to controls.

**Conclusion:** *H2-Eb1* expression, accompanied by an increased GATA-3/T-bet ratio were significantly upregulated in the nasal mucosa of our successfully established AR mouse model, suggesting that HLA-DRB1 may play an important role in the pathogenesis of AR and in Th1/Th2 balance regulation. (*Asian Pac J Allergy Immunol* 2014;32:308-15)

**Keywords:** allergic rhinitis, ovalbumin, nasal mucosa, HLA-DRB1, Th1/Th2 balance

### Introduction

Allergic rhinitis (AR) is one of the most common allergic diseases, whose pathogenesis is considered to be the result of the interplay between environmental, immunological and genetic factors.<sup>1</sup> A large number of scientists have focused on AR susceptibility gene studies in recent years and have reported that the MHC-II gene is one of the important genes that is involved in exogenous antigen presentation during AR pathogenesis.<sup>2,3</sup>

The human major histocompatibility complex (MHC), which is also called human leucocyte antigen (HLA), of which HLA-DRB1 is one of the functional MHC-II genes that is involved in encoding the  $\beta$  chain of the HLA-DR structure in the antigen binding slot.<sup>2,4,5</sup> The *H2-Eb1* (Histocompatibility 2, class II antigen E beta) gene of mouse major histocompatibility-2 (MHC) is the orthologous gene of the human HLA-DRB1 gene.<sup>3,6</sup>

People have placed great emphasis on the study of the relevance study of HLA-DRB1 to allergic disease in recent years.<sup>7</sup> Winther et al. discovered HLA-DR antigen in the epithelial cells of nasal mucosa and pointed out that HLA-DR is the important factor in immune regulation.<sup>8</sup> Knutsen et al. speculated that HLA-DRB1\*13 may be the susceptibility gene for asthma associated with sensitivity to alternaria mold, as the frequency of

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HLA-DRB1\*13 genes is obviously increased in children with severe asthma who are sensitive to alternaria mold.<sup>9</sup> Andiappan et al. showed that HLA-DRB1 is significantly associated with AR in a GWAS study of 4461 Singapore Chinese with allergic rhinitis.<sup>10</sup> Li et al. also proved that HLA-DRB1 is closely associated with asthma by a GWAS study ( $p < 0.001$ ).<sup>11</sup> Luckey et al. discovered that HLA-DR3 (DRB1\*0301) can regulate immune responses and increase IFN- $\gamma$  levels using an HLA-II transgenic mouse model.<sup>12</sup> These studies all suggest that HLA-DRB1 might play an important role in allergic disease.

In this study, we investigated the possible role of HLA-DRB1 and its relationship with AR, through the establishment of an AR mouse model, to explore whether HLA-DRB1 plays a role in the pathogenesis of AR and in Th1/Th2 balance regulation.

## Methods

### Animals

Thirty-six 8-week old mature female 129/sv mice (weigh 20 to 25g) were bought from the Shanghai Research Center for Model Organisms, and raised in a SPF (Specific Pathogen Free) grade environment by the section of laboratory animal research (SLAR) of the first affiliated hospital of Xinjiang medical university. Mice were randomly divided into two groups and underwent 3 independent experiments (18 mice for each group, and 36 in total). The experimental group were treated with Ovalbumin (OVA) for sensitization, while the control group received phosphate buffer solution (PBS) as a control. Our study was approved by the ethical review board of the First Affiliated Hospital, Xinjiang Medical University (Approval Number: IACUC-20120705003).

### Antibodies

Rabbit anti-mouse HLA-DRB1 antibody (ab98108) for western blotting and rat anti-mouse HLA-DRB1 antibody (ab134038) for immunohistochemistry and immunofluorescence were bought from Abcam co., LTD (USA); primary antibodies T-bet (4B10) and GATA-3 (B-10), and secondary antibody goat anti-mouse IgG-HRP (sc-2005) for western blotting were bought from Santa Cruz Biotechnology, Inc.(USA); goat anti-rabbit IgG H&L (HRP) (ab97051) secondary antibody for western blotting was bought from Abcam co., LTD (USA); goat anti-rat secondary antibodies for immunohistochemistry (PV-6004, Polink HRP

Detection System for Rat Primary Antibody) and immunofluorescence (ZF-0516, Alexa Fluor®594-Conjugated AffiniPure Goat Anti-Rat IgG) were bought from ZSGB biological engineering co., LTD (Beijing, China).

### Allergic rhinitis mouse model establishment

The AR mouse model was established as reported.<sup>13</sup> For Sensitization, each mouse in the experimental group received 200  $\mu$ L OVA [using aluminium hydroxide as an immune adjuvant; containing 40  $\mu$ g OVA (A5503, sigma, USA), 2 mg aluminium hydroxide (239186, Sigma, USA) in PBS] by intraperitoneal injection. Mice were sensitized once every other day, 7 times in total (from the 1st to 13th day). As for the control group, PBS was used as a control. For challenge after sensitization via intraperitoneal injection, the experimental group was treated with 5% OVA aerosol inhalation every other week until the 21st day. In brief, the mice were numbered and put in transparent PVC box, with the nozzle of a multi-functional compressed air atomizer inside. The atomizer was turned on and the mice received continuous aerosol inhalation for 30 minutes. After that, 10% OVA solution was dropped into the mouse nasal cavity, 10  $\mu$ L for each side. Mice underwent challenge once a day for 7 continuous days. PBS was used for challenge in the control group.

Each group (Control and Treated groups) had 6 randomly divided mice, and 3 independent experiments were undertaken (12 mice for each independent experiment). Nasal septal mucosa tissue was taken for the tissue pathomorphology study, tissue RNA analysis and Western blotting detection for proteins expression.

### Pathomorphology analysis

The pathomorphology of the mouse nasal mucosa was analyzed in the 24 hours after the last challenge. Briefly, nasal septum mucosa tissue from 6 mice in each group was firstly fixed with paraformaldehyde, then underwent paraffin embedding, slicing and HE staining, toluidine blue staining (Nanjing Jiancheng Bioengineering Institute, China), dehydration and mounting. The pathomorphology of the mouse nasal mucosa was observed with an optical microscope (Leica DMI3000B, Germany). The eosinophils and other inflammatory cells that infiltrated the nasal mucosa of the mice were analyzed. HE staining was used for eosinophil (EOS) counting and the toluidine blue staining (TBS) was used for mast cell counting (5



scopes were randomly chosen to average the numbers under 400× magnification).

### **ELISA Assay**

Serum OVA-specific IgE, IL-4, IL-10, IFN- $\gamma$  and IL-2 levels were analyzed with ELISA (Enzyme Linked Immunosorbent Assay). Blood was obtained from the tail vein of the mice and IgE and IL-4, IL-10, IFN- $\gamma$  and IL-2 levels were assayed by ELISA according to the instructions of mouse OVA specific IgE (BioLegend, USA), IL-4, IL-10, IFN- $\gamma$  and IL-2 (Wuhan Boster biological engineering co., LTD, China) ELISA kits.

### **Realtime PCR Analysis**

Realtime PCR was used to detect the expression of *H2-Eb1* gene. The nasal septum mucosa tissue of the mice was milled in mortar in liquid nitrogen and the total RNA of the nasal septum mucosa tissue was extracted using Trizol reagent (Invitrogen, USA) according to the RNA isolation kit instructions (Qiagen, Germany). The total quality and concentration of RNA were detected by 1% agarose gel electrophoresis and ultraviolet spectrophotometry. The RNA content was calculated.

The total RNA was reverse transcribed using a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA). Real-time PCR was performed using CFX96TM Real-Time System fluorescent quantitative PCR (Bio-Rad, USA) with SYBR green fluorescent. Samples were run in triplicate and the cycling parameters were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10s, 60°C for 30s, and a detection step at 72°C for 30 s. Human  $\beta$ -actin transcript was chosen as an internal control to eliminate variations in mRNA quantity and standardize the results ( $2^{-\Delta\Delta CT}$ ). *H2-Eb1* and  $\beta$ -actin genes primers were synthesized by Takara co. LTD (Dalian, China) and are as follows:

$\beta$ -actin: forward primer: 5'-AATCGTGCGTGA CATCAAAG-3'; reverse primer: 5'-CAAGAAGG AAGGCTTGAAAA-3';

*H2-Eb1*: forward primer: 5'-ACGGTGTGCA GACACAATGAG-3'; reverse primers: 5'-CTTGCCATTCCGGAACCATC-3';

### **Immunohistochemistry**

Nasal septum mucosal tissue slices were dewaxed and placed in water and hydrogen peroxide, each for 10 minutes. After that samples were blocked in citric acid for 12 minutes and incubated with rat anti-mouse HLA-DRB1

(ab134038) antibody (1:50 dilution) at 4°C overnight. Then slices were incubated with goat anti rat IgG antibody (PV-6004) (1:500 dilution) at 37°C for 0.5h. After DAB (3,3N-Diaminobenzidine Tetrahydrochloride) (DAB Substrate Kit, ab64238, USA) color development, haematoxylin re-staining, dehydration and mounting, slices were observed under a microscope (Leica DMI3000B, Germany). The positive cells may be a little bit granular, with a pale brown color of the cytoplasm and cell membrane. Unless more than 5% cells in vision were positively stained, the cells should be considered to be negative cells.

### **Immunofluorescence**

Nasal septum mucosal tissue slices were dewaxed and placed in water and hydrogen peroxide each for 10 minutes, blocked in citric acid for 12 minutes, and incubated with rat anti-mouse HLA-DRB1 (ab134038) antibody (1:50 dilution) at 4°C overnight. Then slices were incubated with goat anti rabbit IgG (ZF-0516) antibody (1:500 dilution) at 37°C for 0.5h. The nucleus was restained with DAPI (AR1176, Boshide biological engineering co., LTD, Wuhan, China) before mounting. A fluorescence microscope (Leica confocal microscopy TCS SP8, Germany) was used for photographing the slides to observe the expression file of *H2-Eb1*.

### **Western blotting**

*H2-Eb1* expression in nasal mucosal tissue was further detected via western blotting as reported.<sup>14,15</sup> Mice nasal mucosal tissue was collected and homogenized with a cell lysis buffer (10 mg tissue sample with 100  $\mu$ L) (Thermo Scientific, USA). Homogenate was placed on ice for 15 minutes and then centrifuged at 4°C (12000g/min) for 5 minutes; the supernatant then was collected for blotting. The protein concentration was quantified with a BCA kit (Thermo Scientific, USA), according to the instructions provided. Aliquots with 60  $\mu$ g amounts of protein were fractionated with 10% SDS-PAGE electrophoresis and then transferred to NC (Nitrocellulose) membranes (Millipore, Bedford, MA, USA). The NC membranes were in turn blocked and incubated with the primary (anti-mouse HLA-DRB1, ab98108) and secondary goat anti-mouse IgG-HRP (sc-2005) antibodies. Bands of the proteins were then visualized by the ECL (Enhanced chemiluminescence) plus system bought from Amersham Pharmacia Biotech (Buckinghamshire UK).



**Table 1.** The average score of allergic rhinitis symptoms in mice ( $\bar{x} \pm SD$ )

Group	N	rhinocnesmus	sneezing	rhinorrhea	total points
Allergic rhinitis group	6	2.500±0.548	2.333±0.516	1.667±0.516	6.500±0.548
Control group	6	0.667±0.516	0.33±0.516	0.167±0.408	1.00±0.632
<i>t</i> Value		5.966	6.708	5.582	16.102
<i>p</i> Value		<0.01	<0.01	<0.01	<0.01

### Statistical analysis

All values in the study were presented as mean±SD from the three independent experiments. Data were analyzed with SPSS 15.0. Statistical analysis was carried out using the Student's *t* test or non-parametric tests. A value of  $P < 0.05$  was considered to be statistically significant.

### Results

#### Behavior observation

After 2 to 3 times of OVA sensitization, the mice in the experimental group developed nose scratching; at the challenge phase, mice in the experimental group developed nose swelling, nose scratching, continuous sneezing and other symptoms, indicating our successful establishment of an AR mouse model (Table 1,  $P < 0.01$ ). Mice in the control group were as quiet as before and no obvious abnormalities of behavior were observed (Table 1,  $P < 0.01$ ).

#### Histopathological observation of Mast cells and eosinophil cells

Histopathological observation showed eosinophil granulocytes and mast cells mainly distributed in lamina propria of the nasal mucosa. In the control group complete nasal mucosa, aligned ciliated epithelial cells, goblet cells and supporting cell distribution, uniformed basement membrane were observed and the serous and mucous gland in submucosa were well-distributed (Figure 1a and 1c). In the experimental group, cilium binding, obvious serous fluid in the submucosa, visible eosinophil granulocytes and mast cell infiltration were observed (Figure 1b and 1d). Moreover, the amount of mast cells as well as eosinophils in the nasal mucosa in the experimental group were significantly higher than that in the control group (Table 2,  $P < 0.01$ ). These data again suggested our successful establishment of an AR model.

#### Serum OVA-specific IgE and IL-4, IL-10, IFN- $\gamma$ , IL-2 levels evaluation

From the ELISA results, we can see that the content of OVA-specific IgE ( $P < 0.01$ ), IL-4 ( $P < 0.01$ ) and IL-10 ( $P < 0.05$ ) of mice serum in the experimental group were significantly increased (Figure 2a, 2b and 2c), and the content of IFN- $\gamma$  ( $P < 0.01$ ) and IL-2 ( $P < 0.05$ ) were obviously lower compared with the control group ( $P < 0.01$ ) (Figure 2d and 2e). These data suggested that mice in the experimental group were successful induced with AR, and with an elevated serum Th2 immune response and a decreased Th1 immune response.

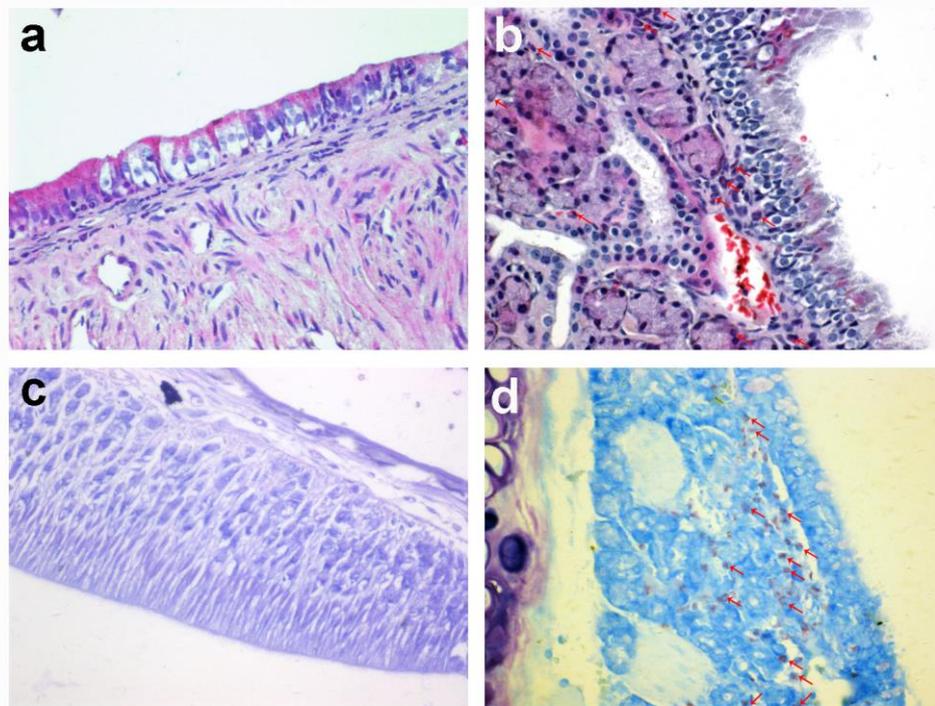
#### Immunohistochemistry and immunofluorescent analysis of H2-Eb1 protein content in nasal mucosal tissues

We firstly analyzed the H2-Eb1 protein content in the nasal mucosal tissue using immunohistochemistry methods. Cells expressing H2-Eb1 were stained to pale yellow. According to figure 3, the rate of cells positively stained pale yellow in the experimental group was higher than in the control group (Figure 3a and 3b). Moreover, consistent with immunohistochemistry results, fluorescent microscopy also showed increased H2-Eb1 expression (strong specific red fluorescence) in nasal mucosal tissue in the experimental group as compared to the controls (Figure 3c and 3d) suggesting the close correlation between H2-Eb1 expression in nasal mucosa and AR.

#### H2-Eb1 gene and H2-EB1, T-bet, GATA-3 protein, expressional profile Changed in allergic rhinitis mice

We used Realtime-PCR to analyze the H2-Eb1 gene expression in the nasal mucosal tissues. We found that H2-Eb1 mRNA levels in the experimental group were significantly higher than those in the control group (Figure 4a) ( $p < 0.01$ ). We further analyzed the H2-Eb1 protein content in





**Figure 1.** Histopathological analysis of eosinophils and mast cells in mouse nasal mucosal tissues. a. No obvious eosinophils were found in the lamina propria of mouse nasal mucosa in the control group (HE staining, 400×magnification); b. Eosinophil cells in the lamina propria of mouse nasal mucosa appear more in the AR group (see red arrow) (HE staining, 400×magnification); c. No obvious mast cells were found in lamina propria of mouse nasal mucosa in the control group (Toluidine blue staining, 400×magnification); d. More mast cells were found in the lamina propria of mouse nasal mucosa in the AR group (see red arrow) (Toluidine blue staining, 400×magnification).

**Table 2.** Mast and eosinophil cell counts in the nasal mucosa of animal models ( $\bar{x} \pm SD$ )

Group	Mice Number	Mast Cell	Eosinophils
Allergic rhinitis group	6	19.17±5.98	22.67±2.88
Control group	6	2.83±1.722	1.33±1.038
<i>t</i> Value	-	6.428	17.105
<i>p</i> Value	-	<0.01	<0.01

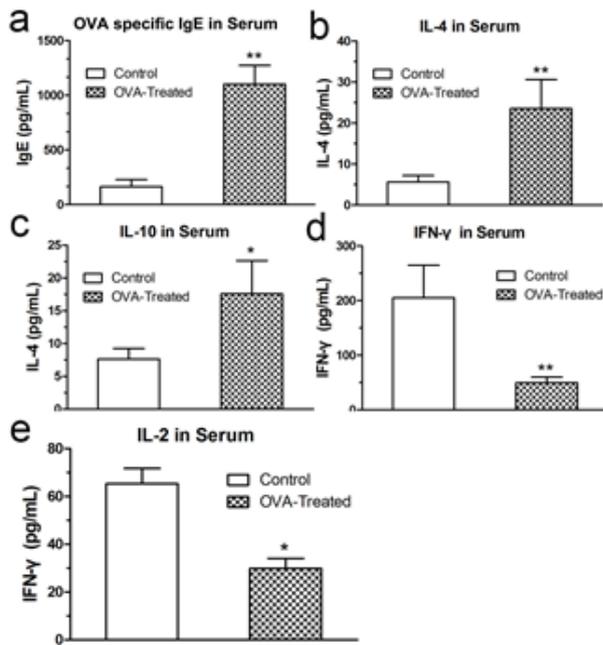
mouse serum by ELISA. As seen in Figure 4b, *H2-Eb1* protein levels in the experimental group were also significantly higher than those in the control group ( $p < 0.05$ ). Moreover, *H2-Eb1* protein was also found unregulated in the nasal mucosal tissue in the AR group compared with the control group (Figure 4c) and the expression of key transcription factor GATA-3 for differentiation Th2 cells was also significantly unregulated in the nasal mucosa of the OVA-induced allergic rhinitis group, while the

key factor T-bet for differentiation Th1 cells were also significantly down-regulated (Figure 4d). Altogether, these data suggested that *H2-Eb1* protein and *H2-Eb1* gene are associated with allergic rhinitis, indicating that they may play a role in allergic rhinitis pathogenesis and in Th1/Th2 regulation.

## Discussion

AR is generally considered to be an IgE mediated type I immune response with imbalance of Th1 and Th2.<sup>16-18</sup> After specific allergens have acted on a specific individual and induced sensitization, secondary allergen contact will stimulate the inflammatory response mediated by IgE.<sup>18</sup>

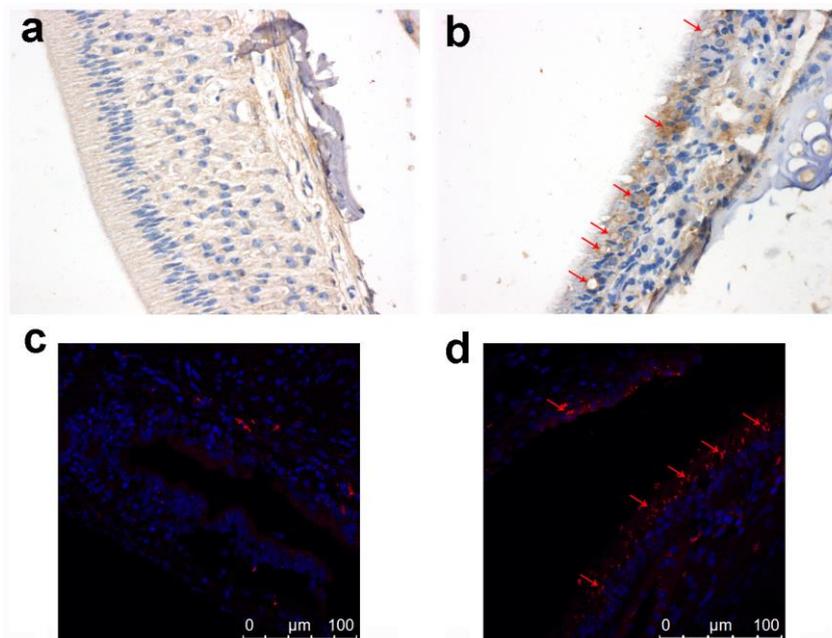
Our study chose 129/sv mice to establish allergic rhinitis mouse models. We evaluated eosinophil (EOS) and mast cell infiltration, *H2-Eb1* gene expression in allergic mouse nasal mucosa, serum cytokine IL-4, IL-10, IFN- $\gamma$ , IL-2 and the OVA-specific IgE levels. We found that *H2-Eb1*



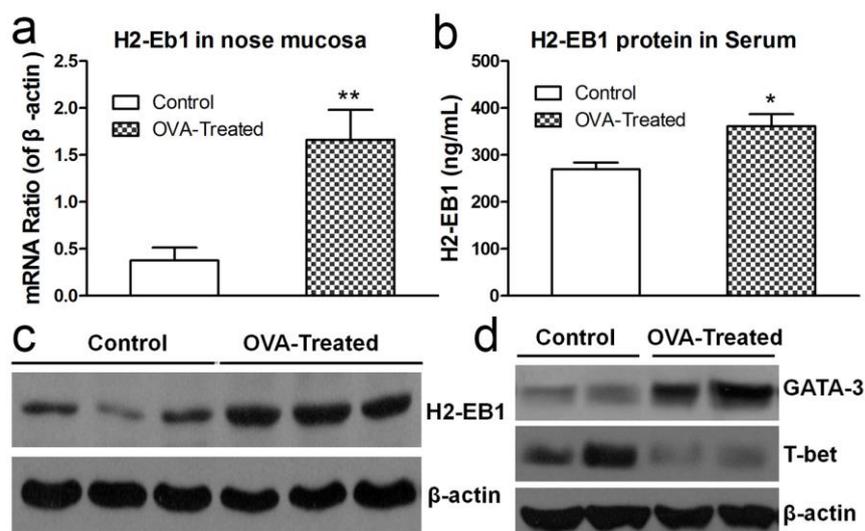
**Figure 2.** ELISA analysis of OVA specific IgE, IL-4, IL10, IFN- $\gamma$  and IL-2 in serum.

mRNA and *H2-Eb1* protein expression, as well as the key regulators GATA-3/T-bet for Th1 and Th2 differentiation in nasal mucosa were significantly upregulated (or downregulated) in our successfully established AR mouse model, suggesting that HLA-DRB1 may play a role in the pathogenesis of AR as well as in Th1/Th2 balance regulation.

In this study, we firstly found that at the challenge stage, the mice in the experimental group all began nose scratching, nasal swelling, continuous sneezing and other symptoms, indicating that allergic rhinitis had been successfully induced in the experimental group. Lodging cilia in nasal mucosa tissue and a typical increase of glands in the submucosa were also observed in the experimental group (Figure 1). Further observation of eosinophil accumulation and mast cells infiltration supported our successful establishment of an AR mouse model (Figure 1).<sup>19</sup> Moreover, an increase of OVA specific IgE, as well as Th2 cytokines IL-4 and IL-10 levels, and decreased Th1 IFN- $\gamma$  and IL-2 levels (Figure 2), together proved that a Th2 allergic immune response had been successfully induced in the experimental mice.<sup>18,20</sup>



**Figure 3.** Immunohistochemistry and Immunofluorescent analysis of H2-Eb1 expression in mice nasal mucosal tissues. a. In the control group, H2-Eb1 in nasal mucosa cells is expressed in the cytoplasm and membrane stained with a light yellow colour as fine particles (Immunohistochemistry of DAB color development, 400 $\times$ magnification); b. In the AR group, H2-Eb1 in nasal mucosa cells expressed in the cytoplasm and membrane stained with a brown color in a sediment-like distribution (see red arrow; Immunohistochemistry of DAB color development, 400 $\times$ magnification); c. In the control group, H2-Eb1 in nasal mucosa does not have significant expression, with weak specific fluorescence (Immunofluorescence, 200 $\times$ magnification); d. In the AR group, H2-Eb1 in nasal mucosa is expressed in mucosa epithelium and the lamina propria, with a strong specific red fluorescent signal (see red arrow; Immunofluorescence, 200 $\times$ magnification).



**Figure 4.** *H2-Eb1* gene and H2-EB1, GATA-3 and T-bet protein expression in serum or nasal mucosa.

a. Realtime PCR analysis of *H2-Eb1* gene expression in nasal mucosal tissues; b. ELISA analysis of H2-Eb1 protein expression in mouse serum; c. Western Blot Result of H2-Eb1 in nasal mucosal tissues of mice; d. Western Blot Result of GATA-3 and T-bet in nasal mucosal tissues of mice.

The human histocompatibility complex (MHC) codes the HLA genes. The classic HLA-II genes are HLA-DR, HLA-DP and HLA-DQ, whose coding products are all double-stranded ( $\alpha$ ,  $\beta$ ) molecules.<sup>2,5,21</sup> The  $\beta$ 1 functional domain of the HLA-II protein's light chain encoded by HLA-DRB1 gene comprises the antigen slot.<sup>21,22</sup> HLA-DRB1 genes therefore might play an important role in the regulation of the individual's immune response.

Recent studies have reported that the MHC II genes had a close relationship with Th0 cell differentiation.<sup>2,23,24</sup> After antigen-presenting by APC cells, MHC II molecules will combine with exogenous antigen to form an 'MHC II-antigen peptide' complex on the APC surface, which will be recognized by CD4+T cells and will drive the Th0 cells toward Th2 cells differentiation, producing Th2 type cytokines (IL-4, IL-10, IL-13, etc.) to stimulate B lymphocyte transform into IgE secreting plasma cells, accompanied with a decrease in Th1 type cytokines (IFN- $\gamma$ , IL-2, IL-12).<sup>6,25-27</sup> In our study, we confirmed that, accompanied with the AR symptoms, OVA induced a Th1/Th2 imbalance and led to a Th2 dominant immune response with increased cytokine IL-4 levels, and a decrease of IFN- $\gamma$  and IL-2 (Figure 2). The differentiation of naive T-helper (Th) cells towards Th2 or Th1 is decided by the transcription factors GATA-3/T-bet.<sup>28</sup> We found that the ratio of GATA-3/T-bet

significantly increased, indicating a successfully induced Th2 response (Figure 4). Moreover, the expression of *H2-Eb1* genes and *H2-Eb1* protein in the nasal mucosal tissues was found to be significantly increased (Figure 4) ( $P < 0.05$ ), which taken together suggests that H2-MHC Eb1 genes may play an important role during the process of Th0 cell differentiation and the pathogenesis of allergic rhinitis.

In summary, our data showed that HLA-DRB1 has a close correlation with Th1/Th2 regulation and the pathogenesis of AR diseases. Though the exact underlying mechanism(s) of its action still needs exploring, our study has added new knowledge and provides a clue as to the relationship between the HLA-DRB1 gene and AR. We believe that further study of HLA-DRB1 will offer us new strategies for the diagnosis and treatment of AR.

#### Conflict of interest

The authors confirm that there are no conflicts of interest.

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