H2-EB1 Molecule Alleviates Allergic Rhinitis Symptoms of *H2-Eb1* Knockout Mice

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ABSTRACT

Background: H2-EB1 molecule which is the homolog of Human HLA-DRB1 is proposed to be associated with allergic rhinitis (AR). Construction of *H2-Eb1* knockout animal models provides a tool to elucidate the role of H2-EB1 and AR pathogenesis. **Objective:** To establish the *H2-Eb1* knockout model and investigate the H2-EB1 functions in *H2-Eb1* knockout mice as a model of AR. **Methods:** The Cre/LoxP system and ES gene knockout technology were applied to create heterozygous *H2-Eb1* (+/-) knockout mice and their offspring of knockout homozygous(-/-), heterozygous (+/-) and wild type (+/+) *H2-Eb1* mice. After identification, offspring of heterozygous (+/-) and homozygous (-/-) *H2-Eb1* knockout mice were randomly selected to establish AR models to demonstrate the role of *H2-Eb1* in AR pathogenesis. **Results:** The *H2-Eb1* knockout mice model was successfully established. The reproduction and feeding of the homozygous (-/-) *H2-Eb1* knockout mice were successful. Compared with the control group, the serum OVA-IgE and IL-4 levels significantly increased, while IFN-γ levels significantly dropped ($p<0.05$) in the experimental groups. For the two experimental groups, the homozygous (-/-) mice group had lower serum OVA-IgE and IL-4 levels, and higher IFN-γ levels than their heterozygous (+/-) counterparts ($p<0.05$), concomitant with slighter allergic symptoms (gentle behavior and less eosinophils in nasal mucosa). **Conclusion:** Our study demonstrated that knockout of *H2-Eb1* gene could alleviate mouse AR Symptoms, indicating *H2-Eb1* may play an important role in regulating Th1/Th2 balance during the pathogenesis of AR.

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Keywords: *H2-Eb1*, Allergic Rhinitis, Knockout,Th1, Th2

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INTRODUCTION

Belonging to the human lymphocyte antigen (HLA) class II DR beta chain paralogs, HLA-DRB1 is a classical polymorphic class II gene relating to antigen processing and presentation. As early as 1987, Winther *et al.* found HLA-DR antigen in the epithelial cells of nasal mucosa which indicated that the epithelial cells of nasal mucous can recognize and process antigens, and pointed out that HLA-DR antigen played an important role in immune adjustment (1). Scnchez Velasco P *et al.* found the IgE-producing regulatory genes located on the HLA-DR region of chromosome 2 and 5 (2). Therefore it was concluded that HLA-DR locus had a great contribution to the clinical manifestation and total IgE level of asthma patients who are allergic to the weeds pollens (3). Moreover, Kim *et al.* reported that HLA-DRB1*07 was related to the specific IgE (SIgE) of house dust mites (4). It was suspicious that HLA-DRB1*0406 and HLA-DRB1*0405 might be the candidate susceptibility alleles of people with Type I hypersensitivity allergies to Platanus acerifolia pollen allergens (5). It was also reported that HLA-DRB1*01 and HLA-DQB1*05 might be the susceptibility gene of Uyghur people in China suffering from AR, HLA-DRB1*11 would be the susceptibility gene of Han people with AR; while HLA-DRB1*0402 could be related to resistance (6,7). Current research shows that HLA-DRB1 is related to not only AR, but also autoimmune diseases like bronchial asthma (8), multiple sclerosis (9), keloid (10) and phthisis (11), and cervical cancer (12).

Construction of HLA-DRB1 knockout animal models provides a tool, not only to elucidate the pathogenesis of AR and related diseases. The difficulty of breeding and reproduction of *H2-Eb1* knockout mice has hampered the universal use of the model. In addition, finding out the pathogeneses of autoimmune diseases need the availability of certain numbers of *H2-Eb1* knockout mice. Our study is to report the successful establishment of *H2-Eb1* knockout animal models regarding the breeding, reproduction and genotypes of the animals that required for modeling. We also discusses the roles that *H2-Eb1* might play during the pathogenesis of AR.

MATERIALS AND METHODS

Animals. Our study was approved by the ethical review board of the first Affiliated Hospital, Xinjiang Medical University (Number: IACUC-20120705003).To knock out the target gene *H2-Eb1* in a conventional manner, *H2-Eb1* was targeted by H2Eb1-pBR233-KO, a homologous recombination targeting vector, which introduced a PGK-neo cassette and loxp into exons 1 and 2 of H2-Eb1 locus. Then, the Cre/LoxP system and ES gene knockout technology were jointly applied by our research group and Shanghai Biomodel Organism Science and Technology Development Co., Ltd. to create heterozygous *H2-Eb1* knockout 129/SV mice models (+/-).

All mice were raised in a laminar air flow isolation feeding room at 22℃ ~ 26℃ and 50 % ~ 65% humidity under a 12 h light:12 h complete darkness lighting schedule. The cages, bedding and drinking water were all sterilized with high pressure and temperature. The total nutrient feed treated with co-irradiation sterilization purchased from Guangdong Medical Laboratory Animal Center. The feeding room was visited

once a day for mice growth observation and food and water supplement; and the bedding was changed twice a week.

Heterozygous *H2-Eb1* mice. The 70-80-day-old heterozygous (*H2-Eb1*+/-) mice were inbred in the proportion of 1 to 1 in accordance with the rules of genetics for reproduction. After their offsprings were born, the whole genomic DNA was extracted from the tail tissues of the young mice whose genotypes were then identified using PCR and western blot.

Reagents. The 2X PCR Master Mix was purchased from Thermo Co., Ltd., the kits for extracting the genome DNA from the tail tissue of the mice were provided by Beijing Kangwei Biotechnology Co., Ltd., and the PCR primer was synthesized by Shanghai Invitrogen. The rabbit anti-mouse HLA-DRB1 antibody (ab98108) for western blotting was purchased from Abcamco., LTD (USA).

DNA Extraction from Mice Tissue. Young mice (offspring) of approximately 2 weeks old were taken out for toe numbering. The tail ends of 2-3 mm were cut and put into 1.5 mL centrifuge tubes to extract DNA from tissue samples in accordance with the instructions of the cell/tissue DNA extraction kit produced by Shanghai Generay Biotech Co., Ltd. The concentrations and quality of extracted genomic DNA were quantified using spectrophotometry.

PCR Analysis. PCR primers for the genotype identification were as follows:

P1: 5'-ACAACCCAAAGCAAAACCCTACTC-3';

P2: 5'-TGGCCCAAAACAACAGAAAACAT-3';

P3: 5'-GTTGGCGCCTACCGGTGGATGTG-3'.

The PCR reaction condition was denaturation at 94°C for 3 min, 94°C for 20s, 52°C for 30s and 72°C for 1 min in 34 cycles, and extension at 72°C for 5 min. PCR products were analyzed on 1% agarose gel.

Western Blotting. *H2-Eb1* protein expression in the tissues of three genotypes of mice was detected by western blot as reported (13). Briefly, tissue from the nasal mucosae, thymuses and lungs of 12-week-old *H2-Eb1*+/+, *H2-Eb1*+/- and *H2-Eb1*-/- mice were homogenized with cell lysis buffer (Cell Signaling Technology Co., Ltd.) and the debris were removed by centrifugation. Aliquots containing identical amounts of protein were resolved on 10% SDS-PAGE gels, and transferred to the methanol pre-activated polyvinylidene difluoride (PVDF) membranes, after which were blocked with 5% milk, incubated with primary antibodies, and subsequently with a secondary antibody for immunoblotting via enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, UK).

Allergic Rhinitis Model Establishment. Two groups (12 mice/group) of 8-week-old female wild type (*H2-Eb1*+/+) mice and one group (12 mice/group) of 8-week-old female homozygous (*H2-Eb1*-/-) mice were randomly selected. One group of wild type (*H2-Eb1*+/+) mice and the group of homozygous (*H2-Eb1*-/-) mice were used as the experimental groups, and the remaining group of wild type (*H2-Eb1*+/+) mice served as the control group. The mice of the experimental groups were sensitized and challenged with ovalbumin (OVA, sigma A5503, USA) to establish AR models. In the control group, OVA was substituted by phosphate buffer solution (PBS).

For Sensitization (14), the experimental groups received an intraperitoneal injection of 200 μL OVA and aluminium hydroxide (as immunologic adjuvant; Sigma 239186) once every two days for 7 times (13 days in total). Each injection contained 40 μg of OVA and 2 mg of aluminium hydroxide in 200 μL with PBS. In the control group, OVA was substituted by PBS.

One week after the intraperitoneal injection, namely, from the 21st day of the modeling, the models started to receive 5% OVA aerosol inhalation once a day for 7 days. The aerosol inhalation lasted for 30 min each time, after then 10% OVA solution was dropped into the nasal cavities, with 10 μL for each cavity. OVA was substituted by PBS for the control group.

Enzyme Linked Immunosorbent Assay. Serum OVA-specific IgE, IL-4 and IFN-γ levels were analyzed with ELISA (Enzyme Linked Immunosorbent Assay). Blood was obtained from the mice tail vein and IgE, IL-4 and IFN-γ levels were assayed by ELISA according to the instructions of mouse OVA specific IgE (BioLegend, USA), IL-4 and IFN-γ (Wuhan Boster Biological Engineering Co., LTD, China) ELISA kits.

Histopathological Analysis. Pathomorphology analysis of mouse nasal mucosa was carried out 24 hours after the last challenge. In brief, nasal septum mucosa tissues from each group were fixed with paraformaldehyde, then underwent paraffin embedding, slicing and then HE staining (Nanjing Jiancheng Bioengineering Institute, China), dehydration and mounting, pathomorphology of mouse nasal mucosa was observed with optical microscope (Leica DMI3000B, Germany). HE staining was used for eosinophil (EOS) analyzing. Eosinophils that infiltrated the nasal mucosa of mice were counted (5 scopes were randomly chosen to average the numbers under 400 × magnification).

Statistical Analysis. All values in the study are presented as mean ± SD from three independent experiments. The two groups of data were compared using one-way analysis of variance (one-way ANOVA) using the SPSS17.0 software to determine their significance of difference. Value of $p < 0.05$ was considered statistically significant.

RESULTS

Feeding, Reproduction and Genotype Identification of *H2-Eb1* Knockout Mice. *H2-Eb1*+/- mice was originally selected for *H2-Eb1*-/- mice reproduction. Then *H2-Eb1*+/- mice and *H2-Eb1*-/- mice were used to reproduce *H2-Eb1*-/- mice which can be bred healthily with common total nutrient feed.

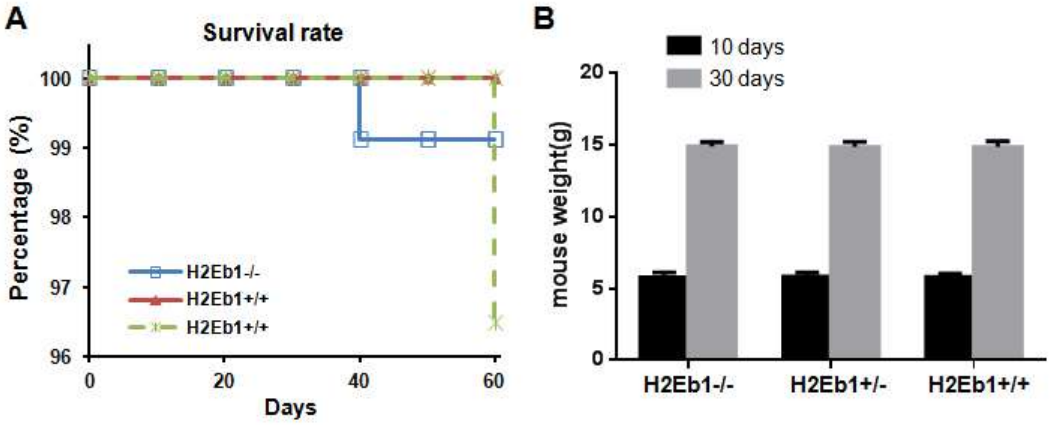

Figure 1.Breeding of the *H2-Eb1* gene knockout mice. A. The survival of three genotypes *H2-Eb1*+/+, *H2-Eb1*+/- and *H2-Eb1*-/- knockout mice; B. Weights of the three Genotypes of mice on 10d and 30d.

The gestation period of each female mouse was 19-21 days, the average litter size per labor was 6-8, the survival rates of *H2-Eb1*+/-, *H2-Eb1*-/- and *H2-Eb1*+/+ mice were very close and above 98%, and were relatively stable at each stage and did not show any significant difference between groups (Figure 1A).

In the first few days after birth, the three genotypes of mice of the same brood were almost the same in size; on 10d and 30d day, the *H2-Eb1*-/-, *H2-Eb1*+/+ and *H2-Eb1*+/- mice showed no significant difference in weight (Figure 1B). Adult *H2-Eb1*-/- mice were normal in weight, growth and activity relative to *H2-Eb1*+/+ and *H2-Eb1*+/- mice, and no phenotypic difference was observed. By as the increase of the number of generations, the mice of three genotypes didn't show such defects like growth retardation, low activity or poor physical state. These data suggest that the reproduction and feeding of *H2-Eb1* knockout mice were successful.

Genotype Identification of *H2-Eb1* Knockout Mice. Whole genomic DNA was extracted from the tail tissues of the young offspring mice and were then identified using PCR through three specific primers. As shown in Figure 2A, in lane 1, 946 bp and 593 bp bands appeared simultaneously, thus the genotype was determined to be *H2-Eb1*+/-; while in lane 2, 6 and 7, only 593 bp band appeared, therefore the genotype was *H2-Eb1*-/-; and in lane 4 and 5, only 946 bp band was seen, and the genotype was thus determined to be *H2-Eb1*+/+; the results indicated that among the offspring, there were wild type (*H2-Eb1*+/+), heterozygous (*H2-Eb1*+/-) and homozygous (*H2-Eb1*-/-) mice.

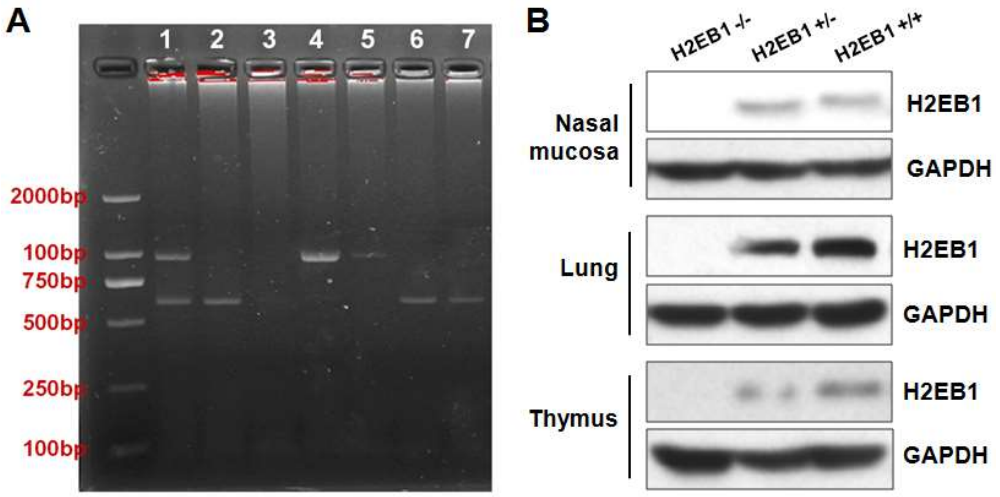

Figure 2. Identifications of the three genotypes of *H2-Eb1* knockout mice. A. Genome PCR of the three genotypes of *H2-Eb1*+/+, *H2-Eb1*+/- and *H2-Eb1*-/- knockout mice; B. Western Blot analysis of H2-EB1 expressions in nasal mucosa, lung and thymic tissues of mice.

H2-EB1 distribution in the different tissues of mice with the three genotypes were detected by western blot. As shown in Figure 2B, *H2-Eb1* expression was found in the nasal mucosa, thymus and lung tissues of the *H2-Eb1*+/- and *H2-Eb1*+/+ mice, but not in that of *H2-Eb1*-/- mice; again indicating the successful construction of *H2-Eb1* knockout mice model.

Behavior Observation of *H2-Eb1* Knockout AR Mice Model. Heterozygous (*H2-Eb1*+/-) knockout mice crossed offspring of female wild type (*H2-Eb1*+/+) and homozygous (*H2-Eb1*-/-) mice were randomly selected for AR modeling as mentioned. After being sensitized with OVA for 2-3 times, the mice of the experimental groups showed the symptoms of nose scratching. After being challenged, the symptoms of rhinocnesmus, sneezing and rhinorrhea were observed and scored significantly in both of the two AR modeling groups, but were less obvious in the homozygous knockout model group (Table 1 and Figure 3A, $p<0.01$), indicating the successful establishment of AR mouse model.

Table 1. Average and total scores of allergic rhinitis behavior in mice (Mean ± SD).

		Score			
Group	N	Rhinocnesmus	Sneezing	Rhinorrhea	Total
H2Eb1-/- (AR)	12	2.167 ± 0.577	1.917 ± 0.669	1.417 ± 0.515	5.500 ± 0.798
H2Eb1+/+ (AR)	12	2.583 ± 0.515	2.500 ± 0.522	2.000 ± 0.603	7.083 ± 0.793
H2Eb1+/+ (Control)	12	0.333 ± 0.492	0.25 ± 0.452	0.083 ± 0.289	0.667 ± 0.492

The symptoms appeared later and less obviously in the homozygous knockout (*H2-Eb1*-/-) model group than in the wild type knockout (*H2-Eb1*+/+) AR model group. The mice of wild type (*H2-Eb1*+/+) AR group were more excited and active. The mice of the control group (*H2-Eb1*+/+) were as quiet as usual and behaved normally. These data suggest that knockout of *H2-Eb1* gene could alleviate mouse AR Symptoms.

Histopathological Anaylysis of Eosinophils. The pathological analysis showed that eosinophils were mainly distributed in the lamina propria of nasal mucosa. In the homozygous knockout (*H2-Eb1*-/-) model group, nasal mucosae were incomplete, ciliated epithelial cells were irregular in formation, basilar membranes were uneven, submucosa serous gland hyperplasia was more obvious than that of control group (*H2-Eb1*+/+), and a few infiltrating eosinophils were seen (Figure 3B, left). For the wild type knockout (*H2-Eb1*+/+) model group, cilia laid flat, submucosal serous gland hyperplasia was evident, and infiltrating eosinophils and plasma cells could be seen (Figure 3B, middle). In the control group, nasal mucosae were complete, ciliated epithelial cells were regular in formation, basilar membranes were even, and submucosal serous and mucous glands were homogenously distributed (Figure 3B, right).

Table 2. Eosinophil count in nasal mucosa of AR animal models (Mean ± SD).

Group	Mice Number	Eosinophils
H2Eb1-/- (AR)	12	10.75 ± 1.57
H2Eb1+/+ (AR)	12	22.58 ± 2.62
H2Eb1+/+ (Control)	12	1.25 ± 0.26

Moreover, It can be seen from Table 2 that the eosinophil count in the nasal mucosae of the AR model groups [(*H2-Eb1*-/-) and (*H2-Eb1*+/+)] were significantly higher than that of the control group (Figure 3C and Table 2, p<0.01), and the eosinophil counts of AR modeling (*H2-Eb1*+/+) group were also significantly higher than that of *H2-Eb1*-/- group (Figure 3C and Table 2, p<0.01); indicating that knockout of *H2-Eb1* gene may play a role during the pathogenesis of AR.

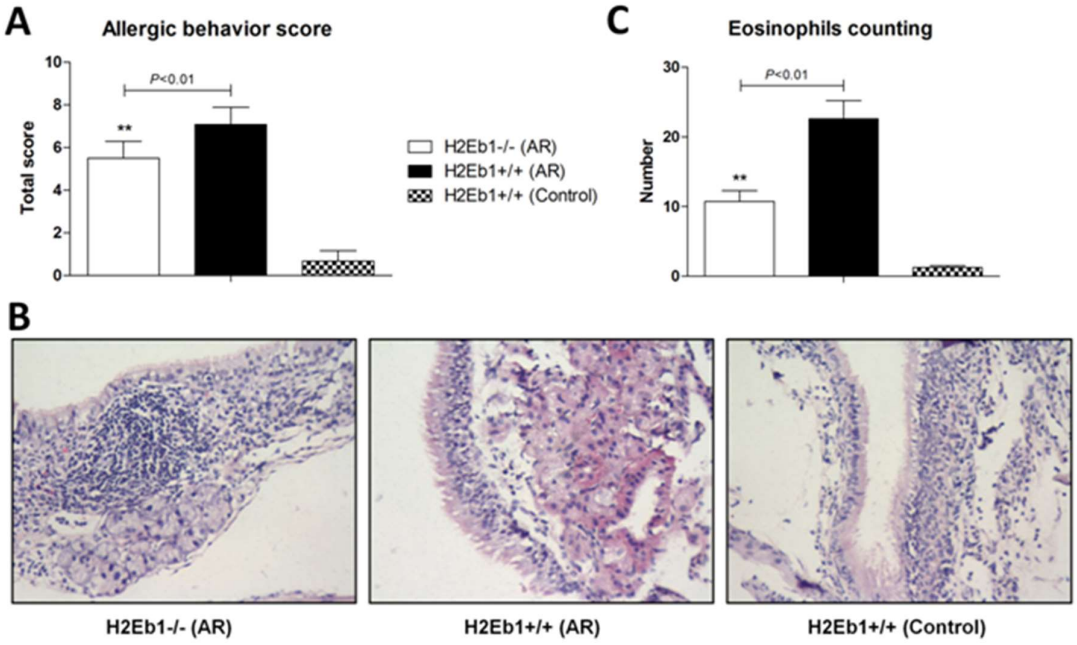

Figure 3. Behavior and histopathological analysis of the *H2-Eb1* knockout allergic rhinitis mice model. A. Behavior observation and score of *H2-Eb1* knockout allergic rhinitis and control mice; B. HE staining of eosinophils that in mouse nasal mucosa in the allergic rhinitis and control mice (400×magnification); C. Eosinophils counting of the HE staining analysis.

Specific IgE, IL-4 and IFN-γ Levels in Mouse Sera. According to our ELISA result, the specific IgE and IL-4 concentrations in the serum samples of AR model groups (*H2-*

Eb1-/-and *H2-Eb1*+/+) were significantly higher than that of the control non-AR group (*H2-Eb1*+/+), while their concentrations of *H2-Eb1*-/-group were lower than those of *H2-Eb1*+/+ group (Figure 4, p<0.01). Moreover, the IFN-γ concentrations in the serum samples of AR model groups were lower than that of the control group, and such concentration of *H2-Eb1*-/- group were higher than those of *H2-Eb1*+/+ group (Figure 4, p<0.01). These data indicate that knockout of *H2-Eb1* gene may result in the decreased serum Th2 immune response yet increased Th1 immune response, suggesting *H2-Eb1* may be involved in Th1/Th2 balance regulation.

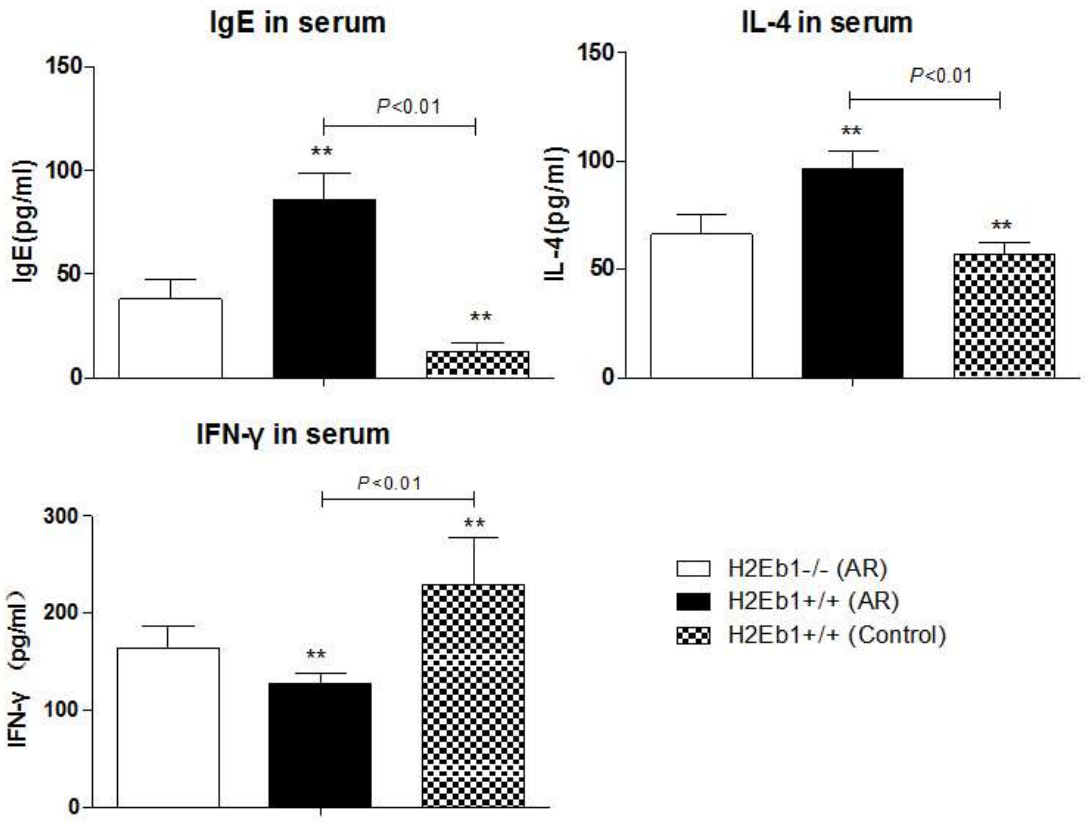

Figure 4. ELISA analysis of serum OVA specific IgE, IL-4 and IFN-γ.

DISCUSSION

To our knowledge, by using gene knockout technology, we for the first time successfully conducted *H2-Eb1* knockout mice model with successful breeding and reproduction. Moreover, based on the knockout Allergic rhinitis (AR) models, we demonstrated that knockout of *H2-Eb1* gene could alleviate mouse AR symptoms, indicating the important role *H2-Eb1* may play in regulating Th1/Th2 imbalance during the pathogenesis of AR.

It was reported that the IgE-producing regulatory genes localized in the HLA-DR region of chromosome 2 and 5 (2,15); and HLA-DR locus was reported to be associated with allergic asthma caused by the pollen of weeds (3). Allergic rhinitis is a Type I IgE-mediated hypersensitivity disease featured with Th1 and Th2 imbalance of T cells (16). In case of heritable variation or the change of environmental factors, the Th1/Th2 balance may be broken to cause allergic diseases(17). In 1991, Maggi *et al*. found human Th1/Th2 subgroups similar to those of mice, and thus started to study allergic diseases using mouse models (18). Therefore, to further understand the role played by *H2-Eb1* in the AR pathogenesis and in Th1/Th2 balance regulation, we started our *H2-Eb1* gene knockout mice construction and in turn the *H2-Eb1* gene knockout AR model establishment.

Through the Cre/LoxP system and ES gene knockout technology, we firstly obtained the knockout mice and indicated that *H2-Eb1*+/- mice (*H2-Eb1*+/- mice were inbred for *H2-Eb1*-/- and *H2-Eb1*+/- mice) were identical in phenotype with *H2-Eb1*+/+ mice, and were all fertile; found that the reproduction and feeding of *H2-Eb1* knockout mice were successful, the three genotypes of mice were of normal viability, had very similar survival rates, and showed no significant difference in size, weight, flexibility and activity (Figure 1). The PCR results, together with the western blot results of protein expression in the nasal mucosae, lungs and thymuses, showed that *H2-Eb1* was not expressed in the tissues of *H2-Eb1*-/-, but in *H2-Eb1*+/- and *H2-Eb1*+/+ mice, proving the successful establishment of *H2-Eb1* knockout mice model (Figure 2). After then, *H2-Eb1*+/+ and *H2-Eb1*-/- mice were selected randomly to establish AR animal models to explore the role of *H2-Eb1* in the AR pathogenesis. The typical AR responses like rhinocnesmus, sneezing and rhinorrhea were observed and scored (19). The AR symptoms of *H2-Eb1*+/- model mouse was more serious than the *H2-Eb1*-/- modeling mouse (Figure 3A). Together with the HE staining pathological results (Figure 3B and 3C), we can conclude that *H2-Eb1* knockout could restrain the hypersensitivity and eosinophils infiltration of the AR mice, which demonstrated that *H2-Eb1* played an important role in the pathogenesis of airway allergic diseases (6,8).

Mosmann *et al*. discovered in 1986 that the CD4+ T cell lines of mice can be divided into Th1 and Th2 cell lines according to the types and biological functions of the generated cytokines (20). IFN-γ and IL4 are respectively the characteristic cell factors of Th1 and Th2 cells (21). The increase of IL4 level or the decrease of IFN-γ level will cause Type I IgE reactions; on the contrary, increase in IFN-γ level or decrease in IL4 level can restrain the synthesis of IgE (22). To understand the mechanisms of *H2-Eb1* during the pathogenesis of AR, we further evaluated the Th1 and Th2 cytokines in the knockout AR model. The experimental results illustrated that the IgE and IL-4 levels of the experimental group (*H2-Eb1*-/- and *H2-Eb1*+/+) were significantly higher than those of the control group (*H2-Eb1*+/+), while the IFN-γ of the former was significantly lower than the latter (Figure 4). Moreover, the IgE and IL-4 levels of the homozygous (*H2-Eb1*-/-) knockout model were higher than those of the control (*H2-Eb1*+/+) but lower than those of the wild type (*H2-Eb1*+/+) knockout model groups; indicating that knockout of *H2-Eb1* gene could alleviate mouse AR Symptoms and *H2-Eb1* play role in Th1/Th2 balance regulation. Large numbers of studies have provided evidence that AR is regulated by multiple genes, and subjected to environmental and genetic factors such as IgE, IFN-γ, TAP, Treg cells, T cells, cell factors and corresponding receptors (23-29). In the present study, we showed that *H2-Eb1* (homolog for HLA-DRB1) is closely

related to AR, and may play the role of resisting against nasal inflammation during its occurrence and development.

In sum, *H2-Eb1* knockout animal models were established by our study and showed that *H2-Eb1* is related to the pathogenesis of AR and Th1/Th2 regulation, though more studies need to be conducted to find out its exact role in AR pathogenesis. Our study provides clues to provide the potential of H2Eb1 as a new target for AR diagnosis and therapy.

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