



The Attune™ NxT Flow Cytometer system

Let's get to the science

Find out more

invitrogen
by Thermo Fisher Scientific



***Alternaria* B Cell Mimotope Immunotherapy Alleviates Allergic Responses in a Mouse Model**

This information is current as of May 18, 2019.

Jie Li, Lin Yang, Jingru Wang, Yongshi Yang, Yuying Wu, Qing Jiang, Yaqi Yang, Dongxia Ma, Rui Zhang, Nan Huang, Wenjing Li, Guanghui Liu and Rongfei Zhu

J Immunol published online 15 May 2019

<http://www.jimmunol.org/content/early/2019/05/14/jimmunol.1801182>

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



Alternaria B Cell Mimotope Immunotherapy Alleviates Allergic Responses in a Mouse Model

Jie Li, Lin Yang, Jingru Wang, Yongshi Yang, Yuying Wu, Qing Jiang, Yaqi Yang, Dongxia Ma, Rui Zhang, Nan Huang, Wenjing Li, Guanghui Liu, and Rongfei Zhu

Alternaria is a major outdoor allergen. Immunotherapy with *Alternaria* extracts has been documented to be effective in the sensitized patients. However, *Alternaria* extracts are notoriously difficult to standardize. Our aim is to screen the B cell mimotopes of *Alternaria* and to evaluate the therapeutic effects of B cell mimotope peptides on a BALB/c mouse model of *Alternaria* allergy. After a human sera pool from *Alternaria* monosensitized patients was established, B cell mimotopes were screened by a phage-displayed random heptamer peptide library that was identified via mixed *Alternaria*-specific IgE in the sera pool. B cell mimotopes with phage as a carrier were used to perform immunotherapy in an *Alternaria* allergy mouse model. Serological Ab levels, lung histology, and cytokine profiles were compared in the mimotope immunotherapy group, natural extract immunotherapy group, irrelevant phage control group, *Alternaria*-sensitized model group, and saline-blank group. Two mimotopes (MISTSRK and QKRNTIT) presented high binding ability with the sera of the *Alternaria*-allergic patients and mice and, therefore, were selected for immunotherapy in the mouse model. Compared with irrelevant phage control, model, and natural extract immunotherapy group, mimotope immunotherapy group significantly reduced serum IgE levels, inflammatory cells infiltration in the lung tissue, and IL-4 levels in bronchoalveolar lavage fluid, whereas serum IgG1 and IFN- γ levels in bronchoalveolar lavage fluid were increased. Our results indicate that B cell mimotopes of *Alternaria* alleviates allergic response in a mouse model and have potential as novel therapeutic agents for IgE-mediated *Alternaria*-allergic diseases. *The Journal of Immunology*, 2019, 203: 000–000.

A *lternaria* is one of the most common molds associated with allergic diseases. Exposure to high levels of atmospheric *Alternaria* spores is a risk factor for asthma attacks. Sensitization to *Alternaria* in early childhood is an independent risk factor for asthma in a longitudinal birth cohort study (1). There was substantial geographical variation in the prevalence of sensitization to *Alternaria* (lowest 0.2%, median 3.3%, and highest 14.4%) (2). Our study showed 4.6% of allergic rhinitis patients in central China were sensitized to *Alternaria* (3). The prevalence is gradually increasing, with children being especially affected (4).

Allergen immunotherapy (AIT) is still the only disease-modifying therapy for allergic diseases (5). However, AIT of mold is a relatively uncommon method of treatment as a result of the difficulty in acquiring standardized allergenic molds. There is still a lack of sufficient prospective studies that support the effectiveness of AIT with mold allergies (6). Although several studies presented the effectiveness of AIT for *Alternaria* in patients with allergic rhinitis and/or bronchial asthma, immunotherapy with natural *Alternaria* extracts

may lead to potentially severe adverse reactions, such as anaphylaxis and developing new sensitization to other components of the extracts (7). Recently, recombinant allergen, T cell epitope, and B cell epitope vaccine have been developed to overcome the drawback of natural allergen vaccines. B cell epitope vaccine immunotherapy has been shown to be effective and safe in grass allergy patients (8, 9).

B cell epitopes include linear epitopes and conformational epitopes. For inhalant allergens, conformational epitopes appear to be the primary targets of IgE response (10). B cell epitope information cannot be effectively obtained from the primary structure of amino acid sequence analysis. Phage-displayed libraries allow selection of mimotope peptides regardless of whether the epitope is linear or conformational (11, 12), which have been applied in Ag B cell epitopes identification (13). In our study, we established a patients' sera pool of *Alternaria* specific IgE (s-IgE) to screen mimotopes matching B cell epitopes on the surface of *Alternaria* allergens by phage display technology and performed B cell mimotope immunotherapy (MIT) in a mouse model.

Materials and Methods

Alternaria sera pool establishment

Thirty patients monosensitized to *Alternaria* and suffering from allergic rhinitis and/or asthma (s-IgEs to *Alternaria* ≥ 3.5 KUA/L, ImmunoCAP 250; Thermo Fisher Scientific/Phadia) were selected. Those with positive s-IgEs to other allergens (s-IgE ≥ 0.35 KUA/L), such as pollen, house dust mites, pets, and foods, were excluded from our study. Blood samples were obtained, and then serum was isolated and mixed to establish an *Alternaria* sera pool. The s-IgE level was measured. Meanwhile, a mixed sera pool served as normal control was also established from 30 healthy subjects (no allergic diseases and negative s-IgE). The study was approved by the Ethical Committee of Tongji Hospital (TJ-C20141227).

Mimotope selection, sequencing, and mapping of peptides with Alternaria alternata allergen Alt a 1

B cell mimotopes were screened with phage-displayed random peptide library technique. Biopanning, colonies picking, isolating, and amplifying

Department of Allergy, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 430030 Wuhan, China

ORCID: 0000-0003-2103-3381 (R. Zhu).

Received for publication August 24, 2018. Accepted for publication April 25, 2019.

This work was supported by the National Natural Science Foundation of China (81401326) and the Clinical Research Physician Program of Tongji Medical College, Huazhong University of Science and Technology.

Address correspondence and reprint requests to Dr. Rongfei Zhu, Department of Allergy, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 430030 Wuhan, China. E-mail address: zrf13092@163.com

Abbreviations used in this article: AIT, allergen immunotherapy; BALF, bronchoalveolar lavage fluid; i.n., intranasal, intranasally; MIT, mimotope immunotherapy; NIT, natural extract immunotherapy; PAS, periodic acid–Schiff; s-IgE, specific IgE; T-IgE, total IgE.

Copyright © 2019 by The American Association of Immunologists, Inc. 0022-1767/19/\$37.50

of the phages were performed as described by the manufacturer. First, two plates coated with anti-human IgE mAbs (NeoBioscience) were prepared, one incubated with the healthy sera pool (plate A) and the other incubated with sera from *Alternaria* pool (plate B) overnight to capture IgE, then they were blocked by 1% BSA (Sigma-Aldrich) for 2 h. Second, phages from the library of phage (Ph.D.-7 phage Display Peptide Library Kit; New England BioLabs) were screened by non-*Alternaria* s-IgE (plate A) for three-round selections (each time for a new plate A). The nonbinding phages were harvested and then screened by *Alternaria* s-IgE (plate B) three times (each time for a new plate B). The bound phages (targeted phages) were washed off from plate B (Fig. 1A). Third, the targeted phages in a monoclonal colony were picked and amplified respectively, and then the amplified phages were purified by polyethylene glycol/NaCl-precipitation and titrated. Endotoxin-pyrogenic LPS from the cell walls of Gram-negative bacteria was also detected (end point chromogenic tachyples amebocyte lysate; Bioendo). The measurement was repeated three times. Finally, DNA sequencing of the displayed heptamer peptides was performed using M13 phage-specific primers (Sangon Biotech). The nucleotide sequence was transformed into an amino acid sequence by Primer Premier 5 software (PREMIER Biosoft). We aligned mimotope peptides to the *A. alternata* allergen *Alt a 1* (National Center for Biotechnology Information GenInfo identifier number 390980892) linear sequence with Basic Local Alignment Search Tool. Selected mimotope peptides were mapped on the three-dimensional structure surface of *Alt a 1* (Protein Data Bank identification number 3V0R_A) using the Pepitope server (<http://pepitope.tau.ac.il/>).

Phage-capture ELISA for determination of IgE-binding ability, mimotope inhibition test, and basophil activation assays

The binding ability of the phage (mimotopes and irrelevant phages) to patients' IgE was measured by phage-capture ELISA. Two plates were prepared as described above (plate A and plate B). Both plates were incubated with monoclonal of amplified mimotope phages (1×10^9 virions/100 μ l) for 90 min and then reincubated with peroxidase-conjugated mouse mAb to M13 phages (PROGEN Biotechnik) and 3,3',5,5'-tetramethylbenzidine as substrate, consecutively. The OD value was obtained at

450 nm. The selected phages and irrelevant phages with IgE-binding ability were further tested with mice tail blood sera (mice for MIT group, irrelevant phage group, and healthy blank group), with the procedure being similar to above. The plates coated with anti-mouse IgE mAbs were also provided by NeoBioscience.

A dosage-dependent inhibition test was carried out to determine the IgE-binding potential with patients and mice. Irrelevant phage was used as control. The patients' sera were incubated with graded amounts of the mimotope phage with 1×10^8 , 1×10^9 , 1×10^{10} , 1×10^{11} , and 1×10^{12} as inhibitor. The s-IgE was tested by ImmunoCAP 250. For mice sera inhibition test, the s-IgE was tested by ELISA kit provided by eBioscience. Inhibition was presented as the percentage decrease in absorbance of samples with inhibitor to that of samples without inhibitor [(without inhibitor – with inhibitor)/without inhibitor $\times 100\%$].

Basophil activation assays were performed in nine *Alternaria*-associated allergic rhinitis patients. Whole blood was stimulated for 15 min (37°C) with stimulation buffer containing an equimolar mixture of the two mimotope phages (1×10^{12}) with *Alternaria* (2 μ g), irrelevant phage (1×10^{12}) with *Alternaria* (2 μ g), and an equimolar mixture of the two mimotope phages (1×10^{12}) with PBS. Buffer with anti-Fc ϵ RI mAb was as positive control and PBS as negative control. Samples were stained for the surface activation markers CD63 and basophils marker CCR3. Flow cytometry was performed with a CytoFLEX Flow Cytometer (Beckman).

Immunotherapy with mimotopes

Female 6-wk-old BALB/c mice, which were cohoused, were randomized into five groups of six mice each. Mice were sensitized intranasally (i.n.) with *Alternaria* extracts (2.5 μ g/100 μ l) (Beijing Macro-Union Pharmaceutical) on days 4, 5, and 6. The blank group was given 0.9% saline on days 4, 5, and 6. Mice were challenged i.n. with 5 μ g/100 μ l *Alternaria* extracts on days 18, 19, and 20 and were rechallenged with *Alternaria* extracts on days 36, 37, and 38, whereas the blank group was with 100 μ l 0.9% saline. Between days 20 and 23, 10 *Alternaria*-sensitized mice were randomly selected, and the tail blood was taken for phage-capture ELISA, of which, six mice had well-bound s-IgE with mimotope. The six mice were served as MIT group in the subsequent experiments. On days 23, 24, 25, and

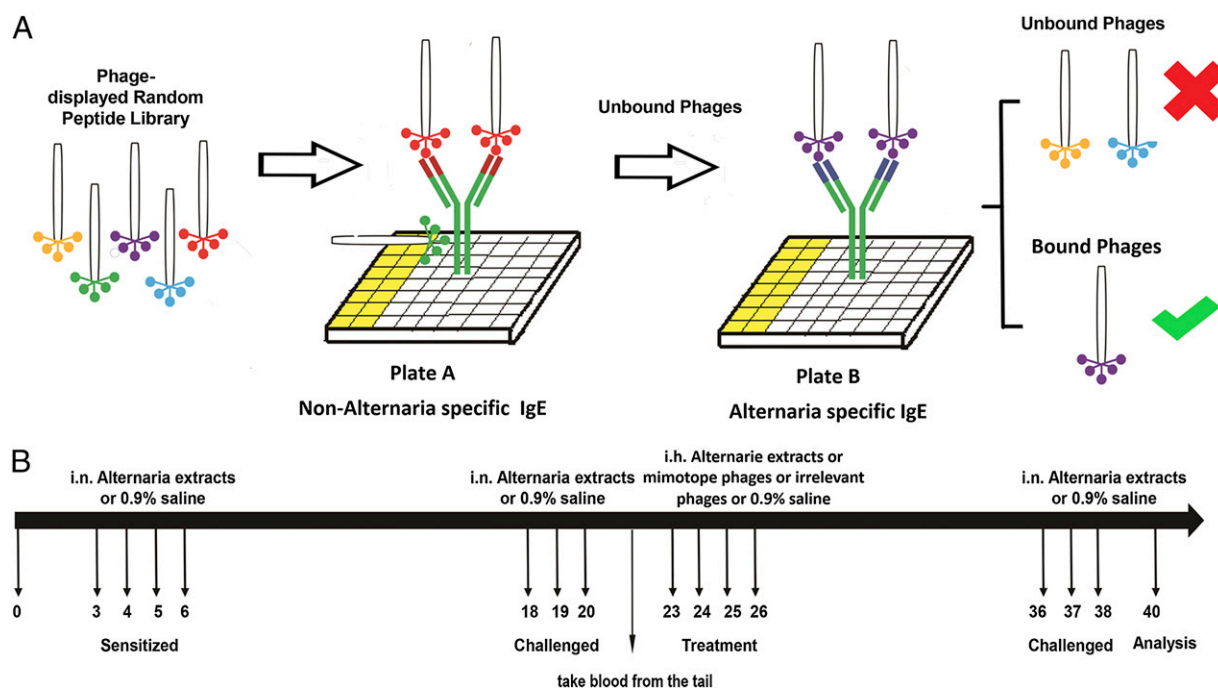


FIGURE 1. (A) The selection of phages: phages from the library of phage were screened by non-*Alternaria* s-IgE (plate A) for three-round selections (each time for a new plate A). The nonbinding phages were then screened by *Alternaria* s-IgE (plate B) three times (each time for a new plate B). The bound phages were targeted phages. (B) The procedure of mice model establishment and treatment: female 6-wk-old BALB/c mice were randomized into five groups of six mice each. Mice were sensitized i.n. with *Alternaria* extracts on days 4, 5, and 6, whereas the blank group was given 0.9% saline. Mice were challenged i.n. with *Alternaria* extracts on days 18, 19, and 20 and rechallenged on days 36, 37, and 38, and the blank group was with 0.9% saline. Between days 20 and 23, 10 *Alternaria*-sensitized mice were randomly selected, and the tail blood was taken for phage-capture ELISA, of which, six mice had well-bound s-IgE with mimotope. The six mice were served as MIT group in the subsequent experiments. On days 23, 24, 25, and 26, the mimotopes treatment group was performed: hypodermic injection with mimotope phages and 0.9% saline, the NIT group with natural *Alternaria* extracts and 0.9% saline, the control group with irrelevant phage and 0.9% saline, and the model and blank group with 0.9% saline. The mice were killed within 48 h after the last challenge.

26, the MIT group was performed by hypodermic injection with 200 μ l of 1×10^{11} virions (100 μ l) of MISTSRK phage and 1×10^{11} virions (100 μ l) of QKRNTIT phage and 0.9% saline, the natural extract immunotherapy (NIT) group with 10 μ g/200 μ l natural *Alternaria* extracts and 0.9% saline, the control group with 200 μ l irrelevant phage (1×10^{11} virions/100 μ l) and 0.9% saline, and the model and blank group using 200 μ l 0.9% saline. The mice were euthanized using sodium pentobarbital i.p. (100 mg/kg) within 48 h after the last challenge. Blood, bronchoalveolar lavage fluid (BALF), and lung were collected for further analysis (Fig. 1B). The animal study was approved by the Ethical Committee of Tongji Hospital.

Blood and BALF

Mice blood samples were collected after euthanasia. The blood was clotted for 1 h at room temperature, and serum was separated and stored at -80°C . With ligation of one side trachea, the lung was lavaged three times with 300 μ l PBS to collect BALF. The ligated pulmonary lobe was stored in 4% polyformaldehyde solution for histopathology test. BALF was centrifuged at 4000 rpm for 10 min at 4°C . The cell pellet was used for cell counting. The supernatant was decanted and stored at -80°C for cytokine analysis.

IgE and IgG1 Abs measurement. The serum s-IgE, s-IgG1 to *Alternaria*, and total IgE (T-IgE) and IgG1 were measured by ELISA following the manufacturer's protocol. For s-IgE and specific IgG1 test, *Alternaria* extracts (1 μ g/100 μ l/well) were incubated in carbonate buffer in a microtiter plate following the manufacturer's protocol. The plates and commercial test kits were provided by eBioscience.

Inflammatory cell counting. The total inflammatory cells were counted using trypan blue (Sigma-Aldrich) and hemocytometer (Neubauer chamber) under a light microscope. The proportion of cells was calculated with H&E staining. The percentage of neutrophils and eosinophils was determined by counting a minimum of 200 cells. Absolute numbers of neutrophils and eosinophil counts in per milliliter of BALF were calculated by total cell counts and percentage value.

Cytokine measurement. Expression of IL-4, IL-10, IL-33, and IFN- γ in BALF was measured by ELISA following the manufacturer's protocol (eBioscience).

Th1/Th2 ratio array. BALF cells were first stained with FITC anti-mouse CD4 (eBioscience). For detection of cytokines, cells were fixed and permeabilized using fixation and permeabilization buffer (eBioscience) according to the manufacturer's protocol. BALF cells were incubated with a mAb to CD16/CD32 (BD-Biosciences) for 10 min to block Fc receptors and then stained with PE anti-mouse IL-4, APC anti-mouse IFN- γ (eBioscience). Th1 cells were identified as IFN- γ -positive CD4 $^{+}$ cells, and Th2 cells were identified as IL-4-positive CD4 $^{+}$ cells. Flow cytometry was performed with a CytoFLEX Flow Cytometer (Beckman), and sample data were further analyzed with CytExpert (Beckman). The number of cells was used for calculating the ratio.

Histopathology. The lungs were embedded in paraffin, and 4- μ m thick sections were cut using microtome. Then tissue sections were stained with H&E and periodic acid-Schiff (PAS) and were examined with a microscope. Inflammation scoring was made from 1 to 5 as follows: 1, inflammatory reaction affecting 20% of the lung; 2, 20–40% of the lung affected; 3, 40–60%; 4, 60–80%; and 5, more than 80% of the lung affected (14).

Statistical analysis

Data were presented as mean $\pm$ SD. Data were assessed for normality, and statistical comparison was determined by one-way ANOVA, followed by *t* test using SPSS. Basophil activation assays data were assessed by paired *t* test. A *p* value <0.05 was considered significant.

Results

Mimotope selection, sequencing, and mapping with *A. alternata* allergen Alt a 1

The s-IgE concentration to *Alternaria* was 30.25 kU/l in the patients' sera pool. A total of 92 phage clones were selected. Two sequences were identified multiple times in different screening tests (Fig. 1). They were MISTSRK (5'-ATGATCAGCACGAGTCGCAAA-3', 40 times) and QKRNTIT (5'-CAAAAACGGAATACGATTACT-3', two times) in screening. The result of aligning mimotope peptides to the *A. alternata* allergen *Alt a 1* linear sequence was not found. However, MISTSRK and QKRNTIT were matched with the *Alt a 1* surface well. Those of MISTSRK were the following: 1) Ile-leu31, 2) Ser-Ser48, 3) Thr-Ala49, 4) Ser-Ser30, 5) Arg-Arg21, and 6) Lys-Lys22 (alignment score: 9.05354, $p < 0.001$; Fig. 2A). Those of QKRNTIT were the following: 1) Gln-Glu24, 2) Lys-lys22, 3) Arg-Arg21, 4) Asn-Asn29, 5) Thr-Ser30, 6) Ile-Tyr28, and 7) Thr-Thr26 (alignment score: 11.2645, $p < 0.001$; Fig. 2B).

Mimotopes IgE-binding ability, inhibition ability in human, and basophil activation assay

The binding ability to patients' sera *Alternaria* s-IgE with phage MISTSRK and QKRNTIT was significantly higher than the control group. There was no difference between *Alternaria* monosensitized patients' sera and the control group sera in IgE binding to irrelevant phage (Fig. 3A). In the mimotopes inhibition test, both these two mimotopes showed an inhibitory effect in a series of diluted allergen concentrations, compared with the irrelevant phage, which showed no inhibitory effect in patients (Fig. 3B). Basophil activation assays were performed in nine *Alternaria*-associated allergic rhinitis patients. There was no difference of the basophils activation induced by mimotope phages compared with the PBS. The basophils activation induced by *Alternaria* was significantly suppressed by mimotope phages ($p < 0.01$), whereas the inhibition effect was not found in irrelevant peptide phages (Fig. 3C). The two phages (MISTSRK and QKRNTIT) were tested for IgE-binding ability to *Alternaria*-allergic mice sera, and the binding ability of phage MISTSRK and QKRNTIT was significantly higher than the blank group. The mimotope phage also showed an inhibitory

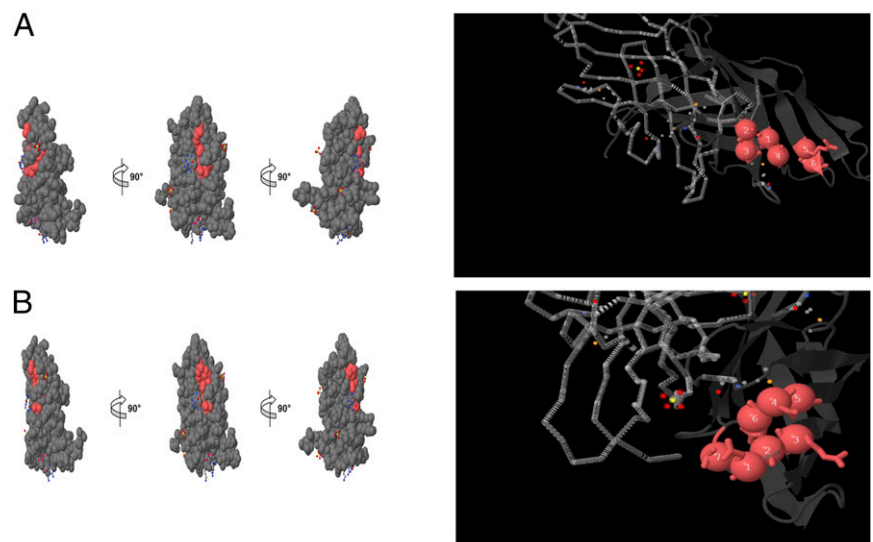


FIGURE 2. The two selected mimotope peptides MISTSRK and QKRNTIT were mapped on the surface of *Alt a 1*. (A) MISTSRK: 1) Ile-leu31, 2) Ser-Ser48, 3) Thr-Ala49, 4) Ser-Ser30, 5) Arg-Arg21, and 6) Lys-Lys22 (alignment score: 9.05354, $p < 0.001$). (B) QKRNTIT: 1) Gln-Glu24, 2) Lys-lys22, 3) Arg-Arg21, 4) Asn-Asn29, 5) Thr-Ser, 6) Ile-Tyr, and 7) Thr-Thr26 (alignment score: 11.2645, $p < 0.001$).

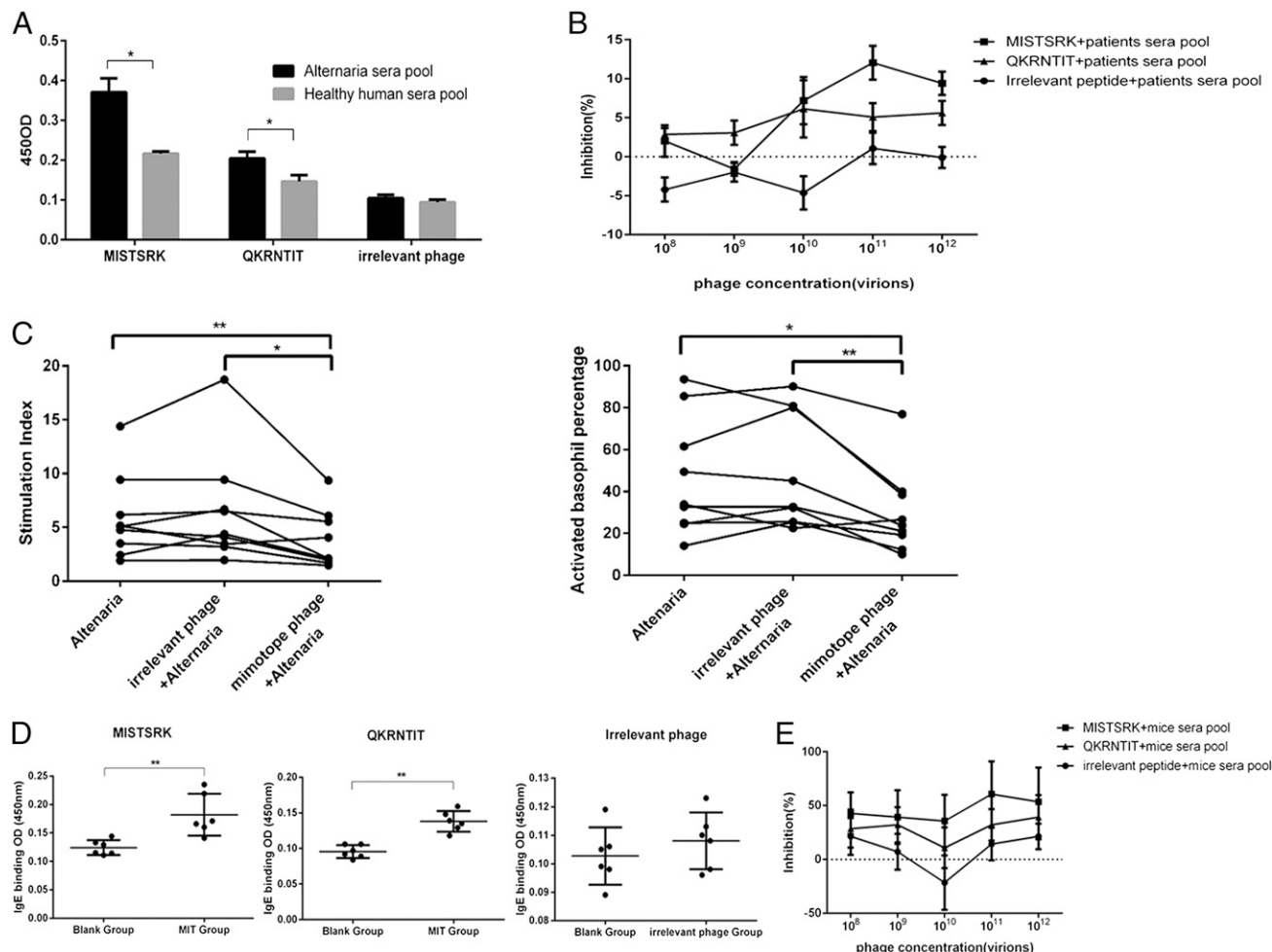


FIGURE 3. IgE binding ability of mimotopes phage to human and mice sera by ELISA. **(A)** IgE binding ability of phage MISTSRK, QKRNTIT, and irrelevant phage to *Alternaria* patients' sera pool (consists of 30 patients), whereas healthy human sera pool was as control. The measurement was repeated three times. **(B)** The patients' sera pool (consists of 30 patients) were incubated with graded amounts of the mimotope phage 1×10^8 , 1×10^9 , 1×10^{10} , 1×10^{11} , and 1×10^{12} as inhibitor for inhibition test. Irrelevant phage was used as control. The value of s-IgE of patients was tested by ImmunoCAP 250. The measurement was repeated three times. **(C)** Basophil activation assays were performed by flow cytometry with buffer containing an equimolar mixture of the two mimotope phages (1×10^{12}) with *Alternaria* (2 μ g), irrelevant phage (1×10^{12}) with *Alternaria* (2 μ g), and an equimolar mixture of the two mimotope phages (1×10^{12}) with PBS. Buffer with anti-Fc ϵ RI mAb was as positive controls and PBS as negative controls. Stimulation index is the ratio of basophils count in each group to that in PBS group. Activated basophil percentage is the percentage of activated basophils count in each experimental group. There were nine patients' samples for one-time measurement. Data are assessed by paired *t* test. **(D)** Mimotope phage (MISTSRK and QKRNTIT) and irrelevant phage were performed, and phage-capture ELISA with individual blood serum of mice ($n = 6$) from MIT group, irrelevant phage group, and healthy blank group (blank group). **(E)** Mimotope phage (MISTSRK and QKRNTIT) and irrelevant phage were performed inhibition test with the sera pool of mice ($n = 24$) after immunotherapy (the measurement was repeated three times). All data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

effect in mice sera. (Fig. 3D). The LPS content in mimotope phage vaccine (13.98 ± 1.88 endotoxin units per milliliter) and in irrelevant phage vaccine (14.26 ± 1.93 endotoxin units per milliliter) were lower than that in the *Alternaria* extracts (40.22 ± 2.8 endotoxin units per milliliter) ($p < 0.01$).

IgE and IgG1 analysis. In MIT group, the levels of T-IgE (114.83 ng/ml) and s-IgE (OD value, 0.05) were significantly reduced ($p < 0.05$), whereas IgG1 (857.17 ng/ml) and specific IgG1 (OD value 1.29) were increased ($p < 0.01$) compared with the control group (T-IgE 665.04 ng/ml, s-IgE OD value 0.18, T-IgG1 481.05 ng/ml, and s-IgG1 OD value 0.34) and the model group (T-IgE 687.53 ng/ml, s-IgE OD value 0.21, T-IgG1 505.80 ng/ml, s-IgG1 OD value 0.34). The MIT group had lower T-IgE (114.83 ng/ml) and s-IgE (OD value 0.05) than the NIT group (T-IgE 426.95 ng/ml, s-IgE OD value 0.16, $p < 0.01$). The NIT group showed no difference of IgE and IgG1 with the blank, model, and control group, but higher specific IgG1 (OD value 0.94) than the model group (OD value 0.34, $p < 0.05$) (Fig. 4A).

Inflammatory cells of BALF. The MIT group showed a reduction in total cell count (58.54) and eosinophil count (12.38) compared with control group (total cell, 172.50; eosinophil, 26.19) and model group (total cell, 173.10; eosinophil, 26.66) ($p < 0.05$). MIT group showed a lower total cell count (58.54) than NIT group (total cell 158.10, $p < 0.05$) (Fig. 4B).

Cytokine analysis in BALF. MIT group showed a higher IFN- γ level (167.02 pg/ml) and a lower IL-4 level (32.40 pg/ml) in BALF compared with the control group (IFN- γ , 120.77 pg/ml; IL-4, 102.14 pg/ml) and the model group (IFN- γ , 120.12 pg/ml; IL-4, 108.84 pg/ml) ($p < 0.05$). And IL-4 level in the MIT group (32.40 pg/ml) was decreased compared with the NIT group (68.53 pg/ml, $p < 0.05$). However, the level of IL-10 and IL-33 showed no difference among the four groups ($p > 0.05$) (Fig. 4C).

Th1/Th2 ratio array. The ratio of Th1/Th2 in the NIT and MIT group was significantly increased than that in the control group and the model group ($p < 0.05$). There was no difference between MIT

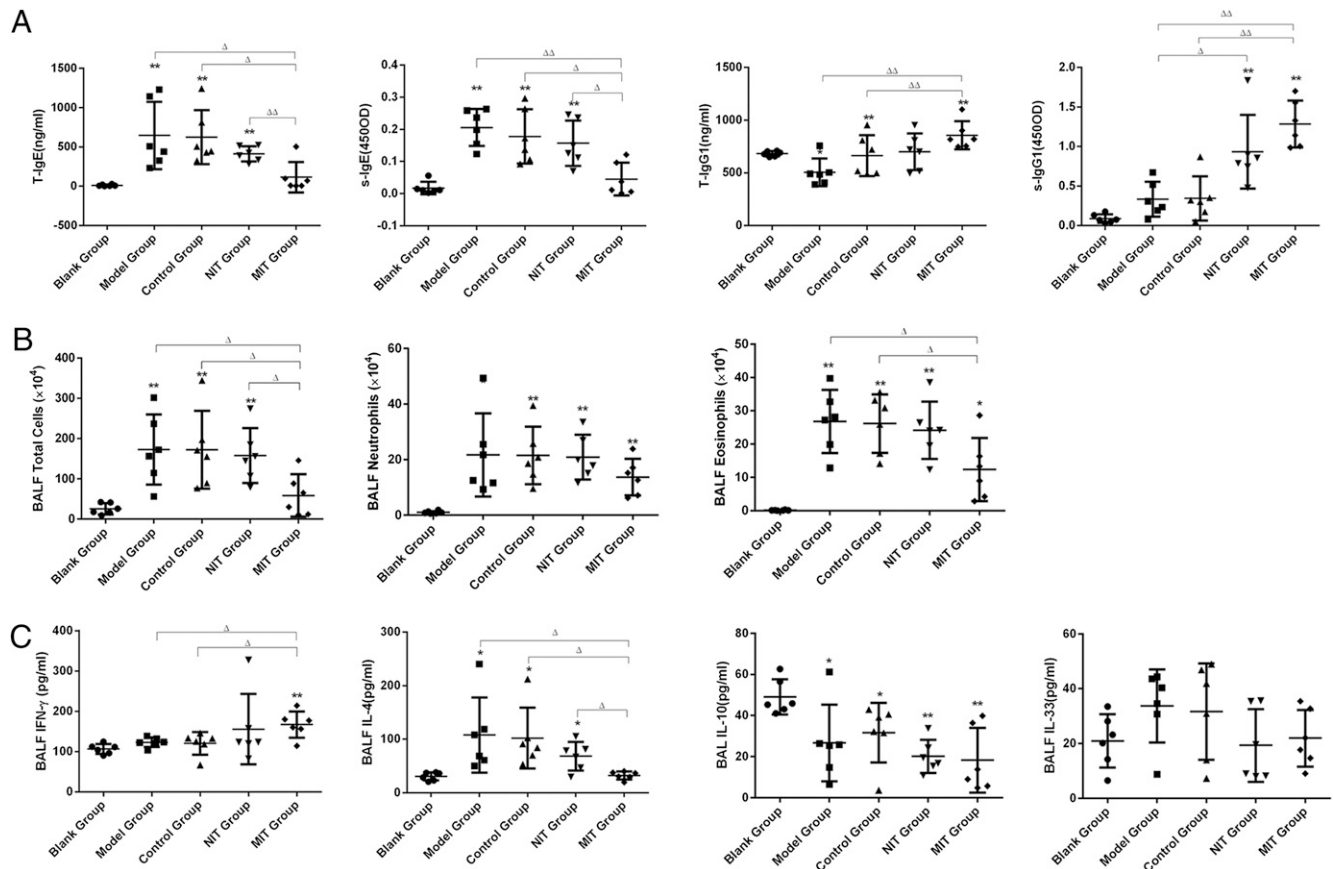


FIGURE 4. Ig in sera and cellular count and cytokine level in BALF after immunotherapy: **(A)** T-IgE, s-IgE, total-IgG1 (T-IgG1), and specific IgG1 (s-IgG1), **(B)** total cell count and total number of neutrophils, eosinophils, **(C)** IFN- γ , IL-4, IL-10, and IL-33. Ig and cytokine levels were determined by sandwich ELISA. Capture Ab (for T-IgE, T-IgG, and cytokine) or *Alternaria* extracts (for s-IgE and s-IgG1) were coated separately on microtiter plates and incubated overnight. After blocking, sera or BALF samples were added to the wells. Biotinylated detector Ab labeled with avidin-HRP anti-IgE, anti-IgG1, IFN- γ , IL-4, anti-IL-10, or anti-IL-33 were used to detect specific levels. BALF cells were fixed and stained with trypan blue to determine total cell counts and H&E staining for neutrophils and eosinophil counts. At least 200 cells were counted and analyzed with a hemocytometer under a microscope. Values are presented as mean $\pm$ SD; $n = 6$ per group. * $p < 0.05$, ** $p < 0.01$, compared with control group; $\Delta p < 0.05$, $\Delta\Delta p < 0.01$, between the two groups.

and NIT, and also no difference between MIT and blank group. The ratio of Th1/Th2 in the model and control group was significantly reduced than that in the blank group ($p < 0.01$) (Fig. 5).

Lung histology. Lung sections of blank, model, control, NIT, and MIT group mice were stained for histopathological analysis by H&E and PAS staining to evaluate the reduction in allergic inflammation upon MIT. Lung sections of mice from the model group showed perivascular and peribronchial infiltration of eosinophilic inflammatory cells, with loss of normal lung structure including alveolar and airway wall and narrowing of the airway lumen with goblet cell hyperplasia, tracheal epithelial cell proliferation, and mucous secretion. The inflammatory cells, especially eosinophilic infiltration, were also observed in the airways of mice in the MIT group; however, these were much fewer than in the control, model, and NIT groups (Fig. 6).

Discussion

As is known, AIT is the only curative treatment for allergic diseases. However, conventional s.c. injection with crude allergen extracts may lead to severe side effects, such as anaphylaxis (15). Allergen epitopes have been considered as an approach to improving AIT (16, 17). B cell epitopes, as short linear peptides lacking the structure to cross-link IgE, could prevent the allergen/IgE interaction without sensitizing effector cells, such as mast cells and basophils, via Fc ϵ RI, which made it a promising vaccine for AIT.

B cell epitopes of some allergen had been screened by phage display libraries, such as the peanut allergen Ara h 1 (18), and a major allergen of timothy grass pollen Phl p 5 (19). However, there were always unrelated phages to be screened in traditional ways, such as phages binding with plastic, albumin, and even IgE Fc regions and so on (20). In our study, we performed three-round selections with *Alternaria* non-s-IgE and *Alternaria* s-IgE. Most of the peptides binding to the C region of IgE were discarded in the selections with *Alternaria* non-s-IgE, which could enhance the *Alternaria*-epitope specificity. *Alternaria* mimotopes of MISTSRK and QKRNTIT were selected after the screening and confirmed by the binding and inhibition ability with *Alternaria* s-IgE. MISTSRK and QKRNTIT had a sequence alignment and the result showed no similarity to the sequences of *Alternaria* major components such as *Alt a 1*. However, they were well matched with the three-dimensional structure of *Alt a 1*. We propose that the two mimotopes mimic the conformational epitope of *Alternaria*, and *Alt a 1* might have a major area for IgE binding as the mapping position for MISTSRK and QKRNTIT on the three-dimensional structure was very close. As an evidence, the two mimotopes were confirmed to preserve high binding ability with *Alternaria* IgE in the phage-capture and -inhibition tests.

We investigated the therapeutic effect of the two mimotope by a modified *Alternaria* allergy mouse model. *Alternaria*-induced asthmatic mouse model has been successfully established in recent years (21). Compared with surrogate allergens (such as OVA),

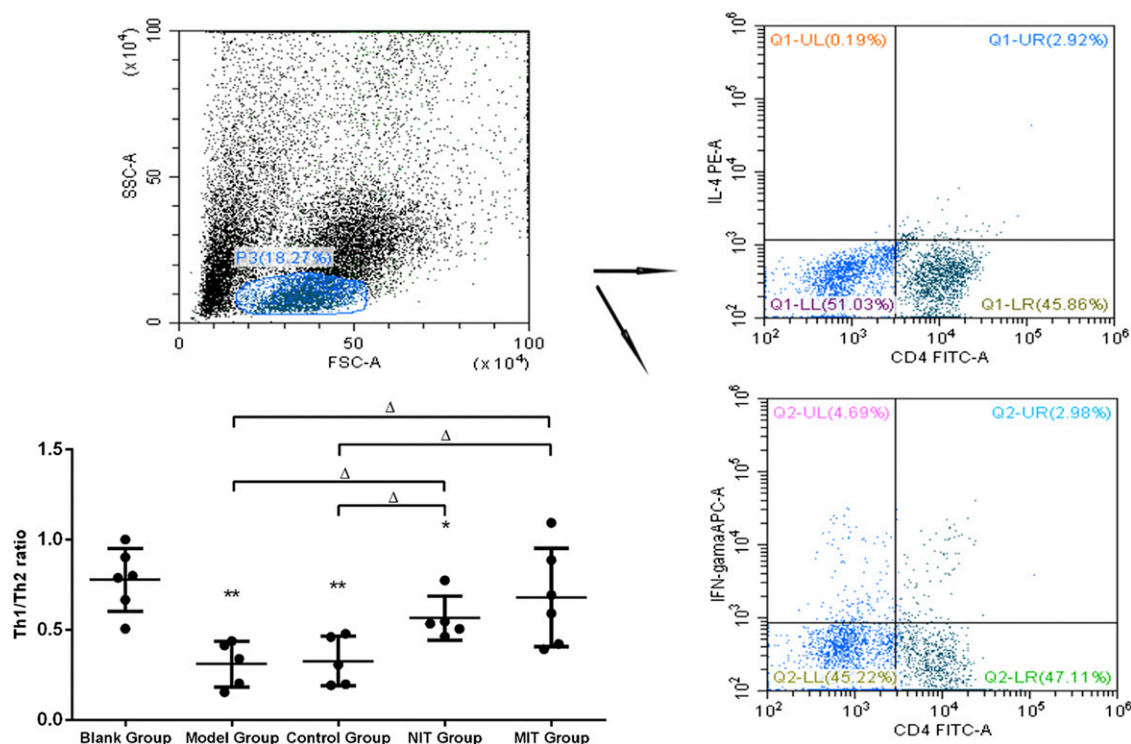


FIGURE 5. Analysis of Th1/Th2 ratio by flow cytometry. BALF cells were first stained with FITC anti-mouse CD4. For detection of cytokines, cells were fixed and permeabilized using fixation and permeabilization buffer according to the manufacturer's protocol. BALF cells were incubated with a mAb to CD16/CD32 for 10 min to block Fc receptors and then stained with PE anti-mouse IL-4, APC anti-mouse IFN- γ . Th1 cells were identified as IFN- γ -positive CD4⁺ cells, and Th2 cells were identified as IL-4-positive CD4⁺ cells. The gating strategies: first plot gating for lymphocyte cells, then the second plot for CD4⁺ cells, and then only for IL-4-positive CD4⁺ cells and IFN- γ -positive CD4⁺ cells. Values are presented as mean $\pm$ SD; $n = 6$ per group. * $p < 0.05$, ** $p < 0.01$, compared with control group; $\Delta p < 0.05$, between the two groups.

sensitized asthma mouse model, using natural allergens such as dust mite, pollen, and cockroach to sensitize mice could elicit chronic airway inflammation and structural remodeling, which mechanistically recapitulates some of the processes of asthma pathogenesis in human (22). In our study, modified mouse model of *Alternaria*-allergic asthma was used (23). The sensitization and challenge routes were carried out through i.n. drop rather than through i.p. injection, as this approach led to similar pathophysiologic changes in mice and yet bore more resemblance to natural allergen exposure route in humans (24). In our study, the levels of IgE, IL-4, and eosinophil count in the model group increased significantly and allergic inflammation in pulmonary tissue developed, whereas Th1/Th2 ratio decreased significantly compared with the blank group, which indicated the *Alternaria* allergy mouse model was successfully established.

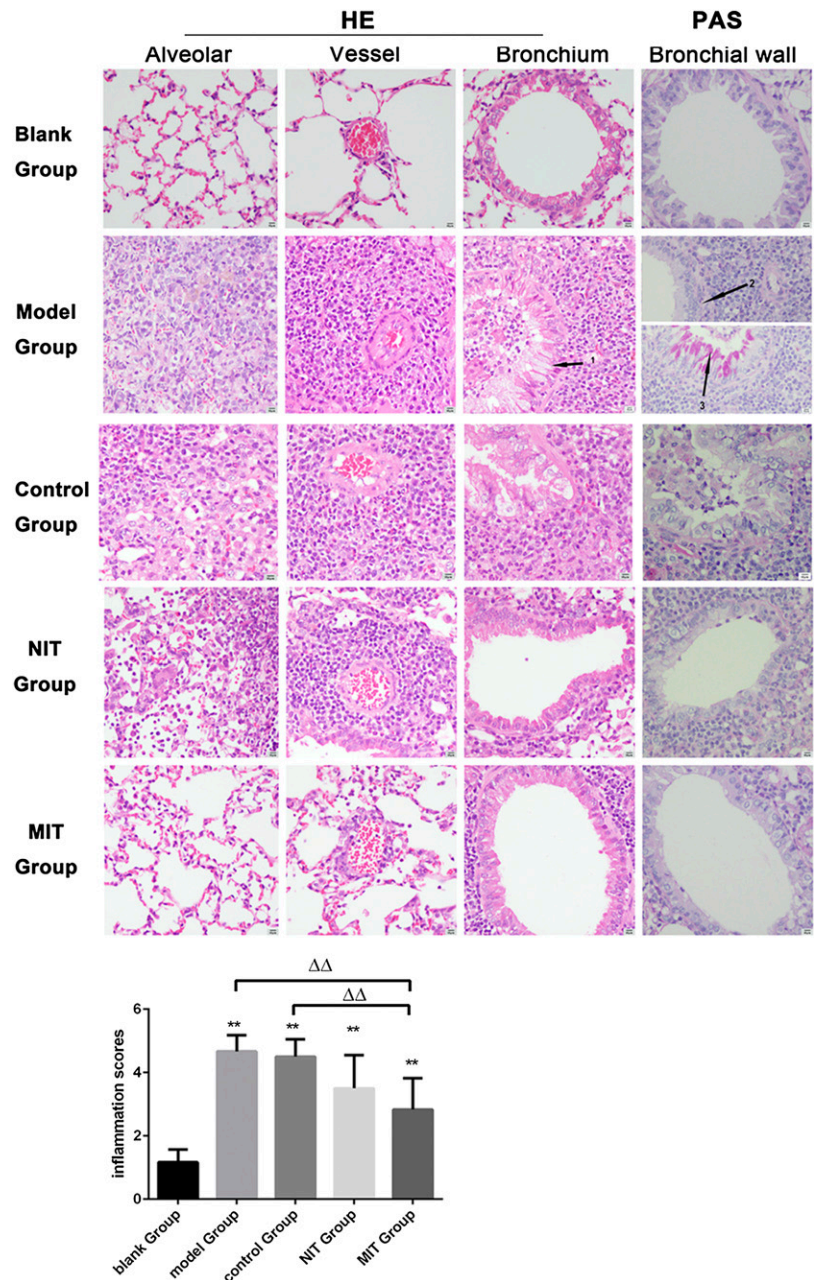
In the mice model, the binding ability of the two mimotopes was also tested to ensure they were suitable for immunotherapy. Several studies reported that crude allergen extracts containing all components might not necessarily produce better therapeutic effects than major components or epitope vaccines (9, 25, 26). We found that *Alternaria* B cell MIT had similar or even stronger efficacy than natural extracts in the mice model, which might be explained that the B cell vaccines preserved good immunogenicity and that they consist of major *Alternaria* epitopes with great exclusion of irrelevant component.

In our study, mimotope treatment reduced IgE reaction, enhanced IgG1 reaction, and led to Th1-skewed responses (the levels of IL-4 were reduced, whereas IFN- γ and Th1/Th2 ratio were increased), which helped to alleviate *Alternaria*-induced allergic reactions. For the reduced IgE reaction, we found that mimotope phages could not activate basophils in the patients, which implied

MIT could avoid severe allergic reactions, such as anaphylaxis induced by natural *Alternaria* extracts. In addition, the mimotopes significantly inhibit *Alternaria* extracts-induced basophil activation than irrelevant peptides. We presume that the mimotope phages could inhibit the allergic reactions by directly binding IgE either on the mast/basophil surface or free IgE in serum, thus preventing the effect cells from activation and resulting in alleviating the allergic symptoms. Two mimotope phages were found to have inhibitory effects in mice, but not as significant as in humans, which might be related to the low concentration of *Alternaria* s-IgE and the inhibition of *Alternaria* s-IgG (after mimotope and *Alternaria* AIT) in the mice sera pool.

The change of T cells and IgG in MIT might be related to the carrier phage. The filamentous bacteriophage (M13 phage was one kind of filamentous phage) consists of highly immunogenic particles that can be used as carrier proteins for peptides and the role of bacteriophages is to improve immunogenicity and focus the Ab response against the peptide (27–29). The constructed phages elicited T cell-mediated immune response, which was predominated by the type 1 T cell response in other research (30). According to previous research, peptide mimotopes may induce a protective IgG response, preventing IgE-mediated allergic reactions (31). The regulation of Th1/Th2 might be that allergen-specific T cell epitopes were devoid, and allergen s-IgE were inhibited by protected Abs and mimotope phage directly. It was reported that being devoid of allergen-specific T cell epitopes was able to downregulate inflammation in acute asthma (32). These conclusions were consistent with our results. However, we found that the irrelevant peptide phages had almost no regulatory effect on T cells. It may be due to the lack of memory B cells targeting irrelevant peptides. B cells were reported to be the dominant APCs that activate naive

FIGURE 6. Lung histopathology of the mice given immunotherapy with mimotope phages/*Alternaria* extracts/irrelevant phages/0.9% saline: H&E and PAS staining sections of the lungs showed inflammation, especially eosinophil infiltration and PAS staining showed destruction of bronchial wall structure. Lungs were fixed with formalin, embedded in paraffin, cut sections, and fixed on the slide for staining (original magnification, $\times 400$). Inflammation scoring was made from “1” to “5,” as follows: 1, inflammatory reaction affecting 20% of the lung; 2, 20–40% of the lung affected; 3, 40–60%; 4, 60–80%; and 5, more than 80% of the lung affected. Values are presented as mean $\pm$ SD, $n = 6$ per group. $**p < 0.01$, compared with control group; $\Delta\Delta p < 0.01$, between the two groups.



CD4⁺ T cells upon immunization with a virus-derived nanoparticle Ag (33). However, the B cells could not present *Alternaria* Ag to T cells in the irrelevant peptides group as what happened in the MIT group.

One of the concerns of natural allergen-specific immunotherapy is the possible boost of inflammatory allergen-specific T lymphocytes and subsequent amplification of allergic inflammation (32). In our study, lung histology sections from the MIT group showed a reduction in allergic inflammation, such as eosinophil infiltration, hypereosinophilia around bronchi, goblet cell hyperplasia, and mucus production, compared with control and model group. Analysis of the BALF indicates that the inflammatory cells, including eosinophils, were significantly decreased in MIT group compared with irrelevant phage control group and allergen sensitization model group. The inhibited activation of basophil/mast cell by MIT could be an explanation. In addition, the absence of allergen-specific T cell epitopes might contribute to this. Research had shown that anti-inflammatory effects of the mimotope vaccine were observed in the memory

mouse model of acute asthma (32). They presumed vaccination with a mimotope peptide representing a single IgE epitope of the allergen and being devoid of allergen-specific T cell epitopes was able to downregulate inflammation in acute asthma (32). We cannot be sure which way of IgE/mast cells and T cells is more effective, but we believe that the two ways both have an impact on suppressing infiltration of inflammatory cells. In addition, regulatory T cells play an important role in AIT, IL-10 was mainly secreted by regulatory T cells and had a potential to inhibit Th2 responses in allergic diseases. Regulatory T cells crucially contributed to the effectiveness of immunotherapy by promoting IL-10 production (34). It was also reported that AIT showed a trend toward suppression of IL-33 (26). In our study, however, we did not observe significant changes of IL-10 and IL-33 in MIT, and more data are needed to elucidate the role of the two cytokines in further B cell MIT studies.

Some limitations have hampered our study to some extent. First, we used the heptapeptide phage display library to screen mimotopes;

consequently, the screened mimotopes are all heptapeptide, which may not match the natural B cell epitope by 100%. In our experiments of mice, some sera and BALF results of the MIT group were not significantly different from those of the control group, which might be attributed to the use of only two mimotopes of *Alternaria* allergen for immunotherapy. However, the mimotope was effective in reducing s-IgE level and improving the lesion in the tissue, which might have resulted from that the selected mimotopes were from the major area for IgE binding in *Alternaria*. Second, the LPS could not be completely removed from the mimotope vaccines. The possible effect of LPS on immunotherapy is still controversial (35–37). Although we found that the LPS in the mimotope vaccines was lower than that in the natural extracts and set an irrelevant phage group as control, we could not exclude the possible effect of LPS on immunotherapy. Finally, the two mimotopes screened from the sera pool were highly matched with the *Alt a 1* molecular. The sensitization profile of IgE against *Alt a 1* for individual patient and the sera pool will support the study. Although previous study showed that 93% of asthma/rhinitis patients with *Alternaria* s-IgE > 3.5 kU/l had IgE to *Alt a 1* (38). IgE against *Alt a 1* in the sera pool was not measured because of the fact that ImmunoCAP to *Alt a 1* was unavailable in China.

In summary, our current study presents a novel B cell MIT for *Alternaria* allergy that effectively alleviates the allergic responses. We modified the screening procedure of phage display peptide to make it much simpler and more effective for mimotopes selection compared with traditional methods. This B cell peptide screening and treatment regimen also had a potential for other allergen-induced allergic diseases.

Acknowledgments

We thank Dr. Xiubo Zhu and Prof. Xiufang Weng for invaluable assistance throughout the preparation of the original paper.

Disclosures

The authors have no financial conflicts of interest.

References

- Stern, D. A., W. J. Morgan, M. Halonen, A. L. Wright, and F. D. Martinez. 2008. Wheezing and bronchial hyper-responsiveness in early childhood as predictors of newly diagnosed asthma in early adulthood: a longitudinal birth-cohort study. *Lancet* 372: 1058–1064.
- Bousquet, P. J., S. Chinn, C. Janson, M. Kogevinas, P. Burney, and D. Jarvis. European Community Respiratory Health Survey I. 2007. Geographical variation in the prevalence of positive skin tests to environmental aeroallergens in the European Community Respiratory Health Survey I. *Allergy* 62: 301–309.
- Wang, J., Y. Wu, J. Li, X. Huang, and R. Zhu. 2017. Eight aeroallergen skin extracts may be the optimal panel for allergic rhinitis patients in Central China. *Int. Arch. Allergy Immunol.* 173: 193–198.
- Perzanowski, M. S., R. Sporik, S. P. Squillace, L. E. Gelber, R. Call, M. Carter, and T. A. Platts-Mills. 1998. Association of sensitization to *Alternaria* allergens with asthma among school-age children. *J. Allergy Clin. Immunol.* 101: 626–632.
- Dhami, S., U. Nurmatov, I. Agache, S. Lau, A. Muraro, M. Jutel, G. Roberts, C. Akdis, M. Bonini, M. Calderon, et al. 2016. Allergen immunotherapy for allergic asthma: protocol for a systematic review. [Published erratum appears in 2017 *Clin. Transl. Allergy* 7: 32.] *Clin. Transl. Allergy* 6: 5.
- Bozek, A., and K. Pyrkosz. 2017. Immunotherapy of mold allergy: a review. *Hum. Vaccin. Immunother.* 13: 2397–2401.
- Coop, C. A. 2014. Immunotherapy for mold allergy. *Clin. Rev. Allergy Immunol.* 47: 289–298.
- Valenta, R., R. Campana, and V. Niederberger. 2017. Recombinant allergy vaccines based on allergen-derived B cell epitopes. *Immunol. Lett.* 189: 19–26.
- Niederberger, V., A. Neubauer, P. Gevaert, M. Zidarn, M. Worm, W. Aberer, H. J. Mallinger, O. Pfaar, L. Klimek, W. Pfützner, et al. 2018. Safety and efficacy of immunotherapy with the recombinant B-cell epitope-based grass pollen vaccine BM32. *J. Allergy Clin. Immunol.* 142: 497–509.e9.
- Pomés, A. 2010. Relevant B cell epitopes in allergic disease. *Int. Arch. Allergy Immunol.* 152: 1–11.
- Luzar, J., B. Štrukelj, and M. Lunder. 2016. Phage display peptide libraries in molecular allergology: from epitope mapping to mimotope-based immunotherapy. *Allergy* 71: 1526–1532.
- Davies, J. M., R. E. O'hehir, and C. Suphioglu. 2000. Use of phage display technology to investigate allergen-antibody interactions. *J. Allergy Clin. Immunol.* 105: 1085–1092.
- Aghebati-Maleki, L., B. Bakhshinejad, B. Baradaran, M. Motallebnezhad, A. Aghebati-Maleki, H. Nickho, M. Yousefi, and J. Majidi. 2016. Phage display as a promising approach for vaccine development. *J. Biomed. Sci.* 23: 66.
- Sharma, P., S. N. Gaur, and N. Arora. 2015. Immunotherapy with B cell epitopes ameliorates inflammatory responses in Balb/c mice. *Clin. Exp. Immunol.* 179: 128–136.
- Dong, X., N. Huang, W. Li, L. Hu, X. Wang, Y. Wang, N. Xiang, G. Liu, and R. Zhu. 2016. Systemic reactions to dust mite subcutaneous immunotherapy: a 3-year follow-up study. *Allergy Asthma Immunol. Res.* 8: 421–427.
- Oldfield, W. L., M. Larché, and A. B. Kay. 2002. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. *Lancet* 360: 47–53.
- O'Hehir, R. E., S. R. Prickett, and J. M. Rolland. 2016. T cell epitope peptide therapy for allergic diseases. *Curr. Allergy Asthma Rep.* 16: 14.
- Bøgh, K. L., H. Nielsen, T. Eiwegger, C. B. Madsen, E. N. Mills, N. M. Rigby, Z. Szépfalusi, and E. L. Roggen. 2014. IgE versus IgG4 epitopes of the peanut allergen Ara h 1 in patients with severe allergy. *Mol. Immunol.* 58: 169–176.
- Hantusch, B., S. Krieger, E. Untermayr, I. Schöll, R. Knittelfelder, S. Flicker, S. Spitzauer, R. Valenta, G. Boltz-Nitulescu, O. Scheiner, and E. Jensen-Jarolim. 2004. Mapping of conformational IgE epitopes on Phl p 5a by using mimotopes from a phage display library. *J. Allergy Clin. Immunol.* 114: 1294–1300.
- Vodnik, M., U. Zager, B. Strukelj, and M. Lunder. 2011. Phage display: selecting straws instead of a needle from a haystack. *Molecules* 16: 790–817.
- Valladao, A. C., C. W. Frevert, L. K. Koch, D. J. Campbell, and S. F. Ziegler. 2016. STAT6 regulates the development of eosinophilic versus neutrophilic asthma in response to *Alternaria alternata*. *J. Immunol.* 197: 4541–4551.
- Johnson, J. R., R. E. Wiley, R. Fattouh, F. K. Swirski, B. U. Gajewska, A. J. Coyle, J. C. Gutierrez-Ramos, R. Ellis, M. D. Inman, and M. Jordana. 2004. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am. J. Respir. Crit. Care Med.* 169: 378–385.
- Li, J., J. Wang, H. Chen, L. Yang, and R. Zhu. 2017. Establishment of a mouse model of *alternaria*-allergic asthma. *Chin. J. Allergy Clin. Immunol.* 4: 344–350.
- Matsumoto, A., K. Hiramatsu, Y. Li, A. Azuma, S. Kudoh, H. Takizawa, and I. Sugawara. 2006. Repeated exposure to low-dose diesel exhaust after allergen challenge exacerbates asthmatic responses in mice. *Clin. Immunol.* 121: 227–235.
- Weber, M., K. Niespodziana, B. Linhart, A. Neubauer, H. Huber, R. Henning, R. Valenta, and M. Focke-Tejkl. 2017. Comparison of the immunogenicity of BM32, a recombinant hypoallergenic B cell epitope-based grass pollen allergy vaccine with allergen extract-based vaccines. *J. Allergy Clin. Immunol.* 140: 1433–1436.e6.
- Hesse, L., N. van Ieperen, C. Habraken, A. H. Petersen, S. Korn, T. Smilda, B. Goedewaagen, M. H. Ruiters, A. C. van der Graaf, and M. C. Nawijn. 2018. Subcutaneous immunotherapy with purified Der p1 and 2 suppresses type 2 immunity in a murine asthma model. *Allergy* 73: 862–874.
- Willis, A. E., R. N. Perham, and D. Wraith. 1993. Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage. *Gene* 128: 79–83.
- Samoylova, T. I., M. D. Norris, A. M. Samoylov, A. M. Cochran, K. G. Wolfe, V. A. Petrenko, and N. R. Cox. 2012. Infective and inactivated filamentous phage as carriers for immunogenic peptides. *J. Virol. Methods* 183: 63–68.
- van Houten, N. E., K. A. Henry, G. P. Smith, and J. K. Scott. 2010. Engineering filamentous phage carriers to improve focusing of antibody responses against peptides. *Vaccine* 28: 2174–2185.
- Luzar, J., P. Molek, M. Šilar, P. Korošec, M. Košnik, B. Štrukelj, and M. Lunder. 2016. Identification and characterization of major cat allergen Fel d 1 mimotopes on filamentous phage carriers. *Mol. Immunol.* 71: 176–183.
- Jensen-Jarolim, E., A. Leitner, H. Kalchauer, A. Zürcher, E. Ganglbauer, B. Bohle, O. Scheiner, G. Boltz-Nitulescu, and H. Breiteneder. 1998. Peptide mimotopes displayed by phage inhibit antibody binding to bet v 1, the major birch pollen allergen, and induce specific IgG response in mice. *FASEB J.* 12: 1635–1642.
- Wallmann, J., M. M. Epstein, P. Singh, R. Brunner, K. Szalai, L. El-Housseiny, I. Pali-Schöll, and E. Jensen-Jarolim. 2010. Mimotope vaccination for therapy of allergic asthma: anti-inflammatory effects in a mouse model. *Clin. Exp. Allergy* 40: 650–658.
- Hong, S., Z. Zhang, H. Liu, M. Tian, X. Zhu, Z. Zhang, W. Wang, X. Zhou, F. Zhang, Q. Ge, et al. 2018. B cells are the dominant antigen-presenting cells that activate naive CD4⁺ T cells upon immunization with a virus-derived nanoparticle antigen. *Immunity* 49: 695–708.e4.
- Böhm, L., J. Maxeiner, H. Meyer-Martin, S. Reuter, S. Finotto, M. Klein, H. Schild, E. Schmitt, T. Bopp, and C. Taube. 2015. IL-10 and regulatory T cells cooperate in allergen-specific immunotherapy to ameliorate allergic asthma. *J. Immunol.* 194: 887–897.
- Dong, L., H. Li, S. Wang, and Y. Li. 2009. Different doses of lipopolysaccharides regulate the lung inflammation of asthmatic mice via TLR4 pathway in alveolar macrophages. *J. Asthma* 46: 229–233.
- Gyimesi, E., F. Gönczi, M. Szilasi, G. Pál, S. Baráth, and S. Sipka. 2013. The effects of various doses of bacterial lipopolysaccharide on the expression of CD63 and the release of histamine by basophils of atopic and non-atopic patients. *Inflamm. Res.* 62: 213–218.
- Post, S., M. C. Nawijn, T. L. Hackett, M. Baranowska, R. Gras, A. J. van Oosterhout, and I. H. Heijink. 2012. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax* 67: 488–495.
- Vailes, L. D., M. S. Perzanowski, L. M. Wheatley, T. A. Platts-Mills, and M. D. Chapman. 2001. IgE and IgG antibody responses to recombinant *Alt a 1* as a marker of sensitization to *Alternaria* in asthma and atopic dermatitis. *Clin. Exp. Allergy* 31: 1891–1895.