Liu, Li, Yin World Allergy Organization Journal (2023) 16:100807
http://doi.org/10.1016/j.waojou.2023.100807

WORLD ALLERGY ORGANIZATION JOURNAL

Open Access

Changes of allergic inflammation and immunological parameters after Alt a 1 and *A. alternata* immunotherapy in mice

Juan Liu[a,b,1], Junda Li[a,b,1] and Jia Yin[a,b]*

ABSTRACT

Background: The efficacy of allergen-specific subcutaneousimmunotherapy (SCIT) with Alt a 1 of the fungus *A. alternata* is still unknown. Yet, few studies compare the therapeutic effects and immunological mechanisms of Alt a 1 and *A. alternata* extracts. We aim to explore and compare the changes in allergic inflammation and immunological mechanisms of Alt a 1 and *A. alternata* in mice.

Methods: Female BALB/c mice administrated recombinant Alt a 1 (rAlt a 1), native Alt a 1 (nAlt a 1), and *A. alternata*. Lung histology, airway hyper-reactivity (AHR), bronchoalveolar lavage fluid (BALF) cytokine levels, serum immunoglobulin responses, the expression of Bcl-6, the percentages of T follicular helper cells (Tfh), cytokine-related Tfh subtypes, regulatory B cells (Breg), and IL-10$^+$ Breg cells were detected.

Results: High-purity nAlt 1 protein was obtained. SCIT with Alt a 1 and *Alternaria* decreased airway and lung inflammation, including improvement of lung pathology, lower levels of AHR, reduction of total cell numbers, and IL-4 and IL-13 levels in BALF. Furthermore, Alt a 1-SCIT effectively suppressed the IgE responses, elevated IgG titers, and was superior in decreasing the expression of Bcl-6. Additionally, *Alternaria*-SCIT significantly decreased the expression of Tfh cells, L-4$^+$ Tfh, and IL-5$^+$ Tfh cells in the spleen, whereas Alt a 1 showed superior therapeutic effects in the lymph node. IL-13$^+$ Tfh cells in these two treatment groups not being significant. IL-17A$^+$ Tfh cells were alleviated most effectively after *A. alternata*-SCIT in both the spleen and lymph node. Intriguingly, IL-10$^+$ Breg cells decreased remarkably in response to SCIT with rAlt a 1.

Conclusions: Treatments with Alt a 1 and *A. alternata* extracts had beneficial effects on allergic inflammation. Alt a 1-SCIT resulted in prominent improvement in the immunoglobulin responses, Bcl-6, and IL-10$^+$ Breg cells. *Alternaria*-SCIT was more likely to suppress the expression of Tfh and cytokine-related Tfh subtypes.

Keywords: Allergic asthma, Subcutaneous allergen-specific immunotherapy, Alt a 1, *Alternaria alternata*, T follicular cells, Regulatory B cells

[a]Department of Allergy, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing Key Laboratory of Precision Medicine for Diagnosis and Treatment on Allergic Diseases, Beijing, China
*Corresponding author. Department of Allergy, Peking Union Medical College Hospital, No.1 Shuai Fu Yuan, Dong Cheng District, Beijing 100730, China. E-mail: doctoryinjia@163.com
[1] Juan Liu and Junda Li contributed equally to this study.
Full list of author information is available at the end of the article

http://doi.org/10.1016/j.waojou.2023.100807
Received 13 December 2022; Received in revised from 18 July 2023; Accepted 24 July 2023
Online publication date xxx

1939-4551/© 2023 The Authors. Published by Elsevier Inc. on behalf of World Allergy Organization. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

INTRODUCTION

Sensitization to fungi affects at least 3%–10% of the world's population,[1] and research performed in Beijing has revealed that fungi have gradually replaced dust mites as the most important inhalation allergens.[2] *Alternaria* is one of the most common allergic fungal genera and is considered to promote the development, persistence, and exacerbation of asthma in children.[3] Surveys conducted in several European countries have shown that the sensitization rate of *Alternaria* reaches 11.9%,[4] and a similar rate has been noted in the United States.[5]

Allergen-specific immunotherapy (AIT) has been viewed as a disease-modifying therapy for allergic diseases. Many authors highlighted the therapeutic effects of AIT for *Alternaria* extracts in patients with allergic asthma and/or rhinitis, which applied to adults and children.[6–8] Kuna et al emphasized AIT with standardized *Alternaria* extracts reduced symptoms of asthma in children.[9] Besides, studies also showed good tolerance and similar effectiveness of sublingual immunotherapy (SLIT) with *Alternaria* extracts compared with subcutaneous immunotherapy (SCIT) in patients.[8,10] However, the compliance of SCIT with extracts might be poor because of its long duration of treatment.[11] Alt a 1 is the major and most relevant allergen of *A. alternata* and is recognized by 80% of patients induced by this mold.[12] Although both native and chemically recombinant forms of Alt a 1 can be obtained, only three studies have performed SCIT with Alt a 1 in patients.[13–15] Our previous study showed that SCIT with recombinant Alt a 1 (rAlt a 1) suppressed allergic inflammation in a mouse model; however, we did not compare the changes in allergic inflammation after accepting treatment with different forms of Alt a 1 and *Alternaria* extracts in an established asthmatic model.

The immunological mechanisms of AIT are associated with the production of Breg cells, regulatory T cells (Tregs), and immune responses that deviate from Th2 to Th1.[16] Recently, a novel subset of T cells, Tfh cells, has been found in the germinal central (GC), which expresses the transcription factor B cell lymphoma 6 (Bcl-6) and produces IL-4, IL-13, and IL17A. Tfh cells and their cytokine-related subtypes participate in the progression of allergic diseases.[17] One study has demonstrated that SCIT with grass pollen influenced the role and function of circulating Tfh cells.[18] It is unknown whether Alt a 1-SCIT and *Alternaria*-SCIT have similar effects on Tfh and its subtypes, since differences exist between fungi and pollen. Similarly, it is worth exploring whether immune tolerance induced by Breg and IL-10^{+} Breg cells after SCIT with pollen also occurs after SCIT using Alt a 1 and *Alternaria* extracts in mouse models.

In this study, we aimed to investigate the laboratory parameters on allergic inflammation and immune responses induced by SCIT with Alt a 1 and *A. alternata* extracts in experimental SCIT mouse models, and further compare the similarities and differences between these two immunotherapy agents.

MATERIALS AND METHODS

Preparation of Alt a 1

rAlt a 1 was prepared by cloning, transforming, and purification, as previously described.[19] Native Alt a 1 (nAlt a 1) was purified using an NHS-activated HP column (17-0716-01, GE Healthcare, Sweden) incubated with rabbit anti-rAlt a 1 polyclonal antibody. In brief, a total of 17.52 mg (c = 1.46 mg/mL) of anti-rAlt a 1 antibodies were recovered from New Zealand rabbits immunized by the Biotyscience company (Beijing, China). Anti-rAlt a 1 antibody was coupled to NHS-activated HP columns following manufacturer's instructions. A culture filtrate extract of *Alternaria alternata* (364,216-1, Greer, USA) was applied to the column, then the nAlt a 1 protein was eluted using elution buffer (100 mM glycine, 0.5 M NaCl, pH 2.7). Finally, the nAlt a 1 protein was stored at −20 °C until further use.

Electrophoresis and immunoblotting

Samples of nAlt a 1 were subjected to 12% SDS-PAGE for Coomassie staining and immunoblotting under reducing and non-reducing conditions,

respectively. The protocols and analyses were according to a previous study.[19]

Testing of basophil activation

Basophil activation testing (BAT; FK-CCR, BÜHLMANN, Switzerland) was performed according to the manufacturer's protocol. Briefly, fresh whole blood was stimulated with 0.4 ng nAlt a 1 and rAlt a 1 for 15 min at 37 °C. The nAlt a 1 and rAlt a 1 were obtained as previously described, and the concentration used for stimulation was determined by a preliminary experiment. The stimulation control in the detection kit served as a positive control. After lysis of red blood cells, basophil activation was measured using the expression of CD63 and CCR3 as assessed by flow cytometry.

Mice and grouping

Animal experiments were conducted using 6-8-week-old female BALB/c mice (Charles River Laboratories, Beijing, China) and housed in a specific pathogen-free (SPF) environment. The mice were divided into 5 groups as follows: negative control group (NC), positive control group (PC), nAlt a 1-SCIT group (nAlt a 1), rAlt a 1- SCIT group (rAlt a 1), and *Alternaria*-SCIT group (*Alternaria*). All protocols were approved by the Institutional Animal Care and Use Committee of the Peking Union Medical University (XHDW-2020-046).

SCIT-treatment protocol

BALB/c mice in the PC, nAlt a 1, rAlt a1, and *Alternaria*-SCIT groups were anesthetized with isoflurane and intranasally sensitized with 10 μg A. *alternata* extract (362,083, Greer, USA) in total volume of 50 μL PBS on days 0, 7, and 14, and subsequently challenged with 25 μg A. *alternata* extract in total volume of 50 μL PBS by intranasal instillation on days 21, 22, and 23. On days 30, 32, 34, 36, 38, 40, and 42, each mice received 5 μg nAlt a 1, 5 μg rAlt a1, or 50 μg A. *alternata* extract that dissolved in total volume of 100 μL PBS via seven subcutaneous injections on the neck skin, as previously described.[19] Finally, mice were intranasally re-challenged with 25 μg A. *alternata* extract in 50 μL PBS or PBS only on days 49, 50, and 51. All mice were euthanized on day 52, and BAL, serum, lung, spleen, and lymph node (LN) tissues were collected (Supplementary Table 1).

Measurement of airway hyper-reactivity

Airway hyper-reactivity (AHR) was measured 24 h after the last re-challenge with a Buxco Fine Point System (Buxco, Wilmington, USA) according to a previously described method.[20] Briefly, mice were challenged for 10 s with PBS to set a baseline value and then exposed to increasing methacholine (Sigma-Aldrich, MO, USA) concentrations (1.5, 3, 6, 12, and 24 mg/mL). The airway resistance doses (cm H_2O.s/mL) were calculated after each methacholine intervention.

Assessment of airway and lung inflammation

BAL fluid total cellular counts and IL-4, IL-10, and IL-13 levels were measured 24 h after the final re-challenge. Lung inflammation was evaluated by histological analysis, and formalin-fixed lungs were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). All protocols were conducted according to a previous method.[19]

Measurement of immunoglobulins and mouse mast cell protease-1 in serum

The levels of T-IgE, *Alternaria*-sIgE, Alt a 1-sIgE, sIgG1, sIgG2a, and sIgG2b in the serum were measured by ELISA, as previously described.[19] The concentration of mouse mast cell protease-1 (MMCP-1, BioLegend, San Diego, CA, USA) was detected using a commercially available ELISA kit according to the manufacturer's instructions.

Immunohistochemical staining

Spleen tissues from mice in the 5 different groups were cut into formalin-fixed, paraffin-embedded samples (3 μm) for immunohistochemical staining. Slides were incubated with an anti-Bcl-6 antibody (GTX101338, GeneTex, 1:1000) and then stained with a secondary biotinylated goat anti-rabbit IgG antibody, followed by horseradish peroxidase-conjugated streptavidin (PV-6001, Zsbio, China). Phosphate-buffered saline (PBS) was used as the negative control to exclude non-specific binding. The semiquantitative scoring method referred to the previous standard and was divided into 2 parts: the scores of

percentage and the staining degree of positive cells, respectively.[21]

Flow cytometry

Tfh cells were collected from the LN and spleen of the SCIT-treated groups, whereas Breg cells were obtained from single-cell suspensions of the spleen. Dead cells were removed by incubating with live/dead fixable dye antibodies. To determine Tfh surface marker expression, live cells were stained with anti-mouse fluorochrome-conjugated antibodies against CD4 (FITC, GK1.5), CXCR5 (APC-Cy7, L138D7), and PD-1 (PE-Cy7, 29F.1A12), according to the manufacturer's protocol. The surface markers of Breg cells were identified as B220 (PE-Cy7, RA3-6B2), CD1d (BV421, 1B1), and CD5 (PE, 53-7.3). To detect intracellular factor expression, cells were stimulated with ionomycin, phorbol myristate acetate, and monensin (550,583, BD Biosciences) for 6 h. Stimulated cells were then fixed and permeabilized using a fixation/permeabilization kit (00-523-00, eBioscience). Antibodies against IL-4 (APC, 11B11), IL-5 (BV421, TRFK5), IL-17A (BV510, TC11-18H10.1), IL-13 (PE, W17010B), and IL-10 (APC, JES5-16E3) were used for intracellular staining. Finally, all samples were analyzed using LSRII (BD Biosciences) and FlowJo software. All antibodies were purchased from BioLegend (San Diego, CA).

Statistical analysis

All statistical analyses were performed using the GraphPad Prism software (version 9.0, San Diego, CA, USA). Data are presented as the mean ± S.E.M., and the Mann-Whitney *U* test was used to compare significant differences between groups. Statistical significance was set at $P < 0.05$.

RESULTS

The purity and allergenic potency of Alt a 1

rAlt a 1 was shown to have high purity and immunogenicity in our previous study.[19] We evaluated 3 major aspects of the purity and allergenic potency of nAlt a 1 (Fig. 1). SDS-PAGE for Coomassie staining analysis confirmed that nAlt a 1 displayed high a degree of purity, and molecular weights of approximately 15–16 kDa under reducing conditions and 30 kDa under non-reducing conditions (Fig. 1A). IgE immunoblotting using serum from an *Alternaria*-allergic patient reacting to nAlt a 1 showed positive binding but

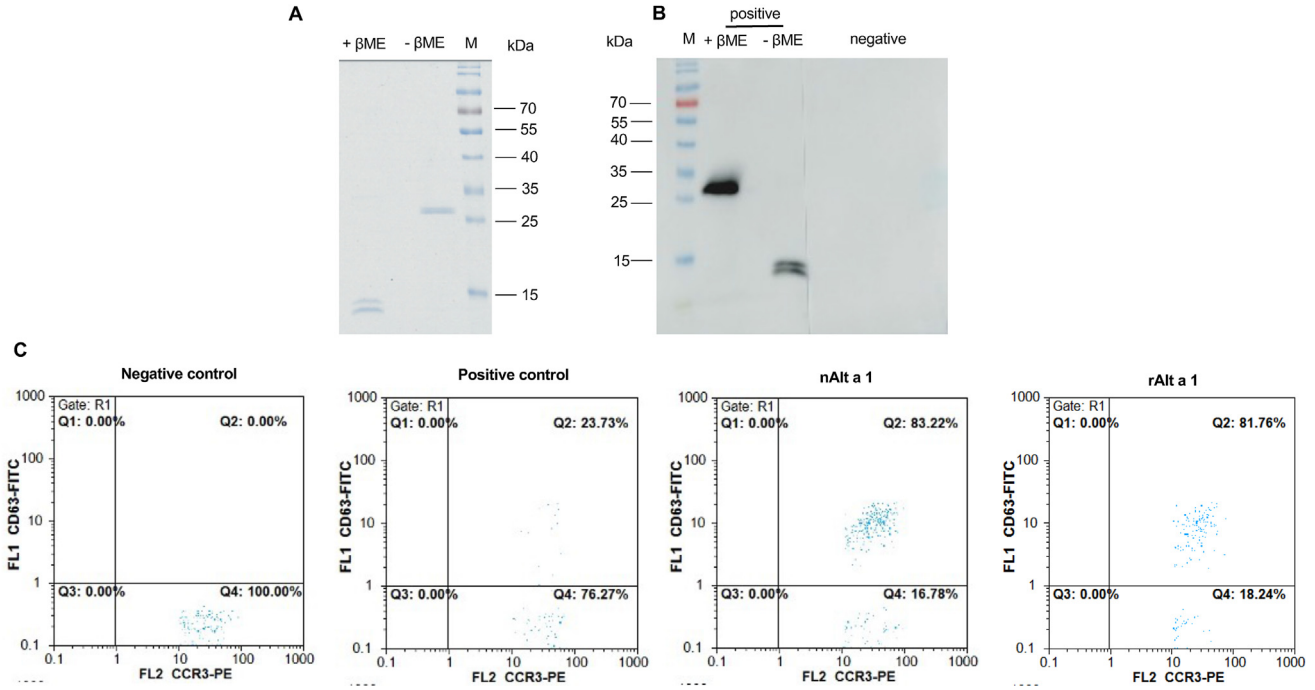

Fig. 1 nAlt a 1 showed high purity and allergenic potency. (A) SDS-PAGE and Coomassie staining of nAlt a 1. (B) Western blotting using serum from *Alternaria*-allergic and non-allergic patients. (C) nAlt a 1 and rAlt a 1 induced basophil reactivity in sera of *Alternaria*-positive patient showed surface activation marker CD63 on basophils.

did not react with healthy controls (Fig. 1B). BAT results demonstrated that nAlt a 1 significantly activated CCR3 and CD63 double-positive basophils in patients with *Alternaria* allergy, with an activation rate of up to 83.22%, which showed a similar basophil activity compared with rAlt a 1 (Fig. 1C). These results suggest that nAlt a 1 was isolated to a high degree of purity and was suitable for further immunological analyses.

Airway and lung inflammation are alleviated after SCIT with Alt a 1 and *A. alternata* extracts

To clarify the therapeutic effects of Alt a 1 and *A. alternata* extracts, female BALB/c mice were used as a well-established SCIT model (Fig. 2A). In the PC group, an increased AHR tendency was observed, but not in either Alt a 1 or *Alternaria*-SCIT mice (Fig. 2B), although these differences lacked statistical significance. Lung inflammation was assessed histopathologically using previously described methods. Compared with the control group, the PC group showed strong inflammatory cell infiltration around blood vessels and airways ($P = 0.0002$) as well as mucus production in lung tissues ($P = 0.0002$), as demonstrated by H&E (Fig. 2C, red arrow) and PAS staining (Fig. 2D, red arrow), respectively. Following treatment with an effective concentration of Alt a 1 and *A. alternata* extracts, lung inflammation showed significant improvements among the three SCIT groups, and the reduction of mucus secretion was most significant in the rAlt a 1-SCIT group ($P = 0.0023$). Hence, immunization with Alt a 1 and *A. alternata* extracts had great treatment efficacy in asthmatic mice, but rAlt a 1 was most efficacious in reducing the release of mucus.

BALF cytokines and MMCP-1 levels in Alt a 1 and *A. alternata*-SCIT mice

After treatment with Alt a 1 and *A. alternata* extracts, we measured the total cell numbers and Th2-related cytokines in the BALF (Fig. 3). Total cell counts decreased significantly in both the rAlt a 1 and nAlt a 1-SCIT groups when compared to the PC control ($P < 0.01$), while this change was not significant in the *A. alternata* extracts group (Fig. 3A). Furthermore, we analyzed cytokine release in the supernatant of BALF after the last re-challenge (Fig. 3B), and observed a remarkable suppression of IL-4 levels in the nAlt a 1-SCIT group ($P = 0.0229$) and IL-13 levels in the *Alternaria*-SCIT group ($P = 0.0411$), while the expression of IL-10 showed a tendency to be upregulated in the rAlt a 1 and *Alternaria* groups, but this was not statistically significant. In addition, we also measured MMCP-1 levels in the serum, and observed a slight suppression after Alt a 1-SCIT treatment (Fig. 3C). Overall, we observed

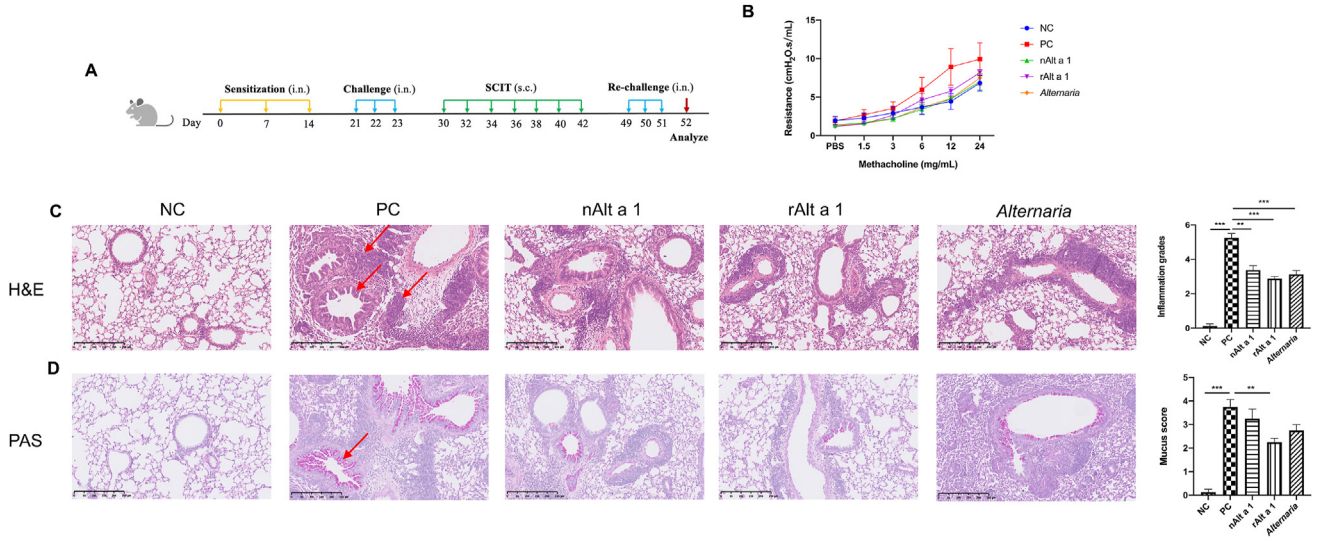

Fig. 2 SCIT with Alt a 1 and *A. Alternata* extracts alleviated lung inflammation and mucus production in mice. (A) Experimental protocol of immunization with Alt a 1 and *A. alternata* extracts in asthmatic mice. (B) AHR was measured and plotted as airway resistance. (C) H&E staining and semi-quantitative analysis of the lung inflammation. *Scale bar* = 250 μm. (D) PAS staining and mucus score in lung histology. *Scale bar* = 250 μm. Values are means ± SEMs (n = 8). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the PC group. NC: Negative group, PC: Positive group. i.p. intraperitoneally; i.n. intranasally. s.c. subcutaneous injection. H&E: hematoxylin and eosin; PAS: periodic acid-Schiff.

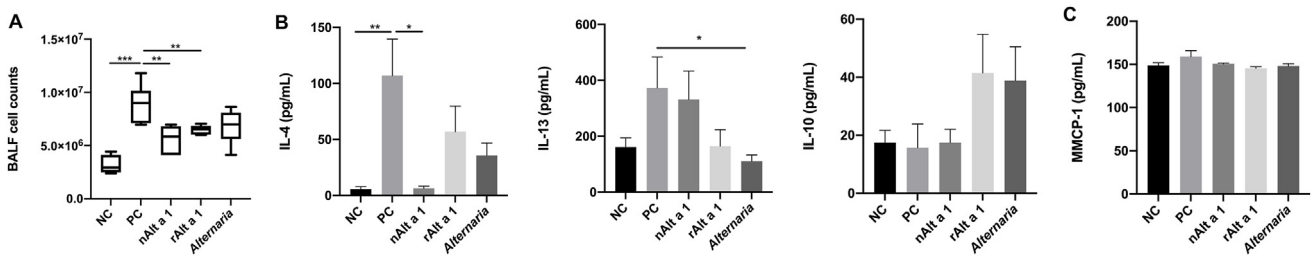

Fig. 3 Total cell counts, IL-4, IL-13, IL-10 levels in BALF, and MMCP-1 levels in the serum. (A) Total cell counts in BALF. (B) Levels of IL-4, IL-10, and IL-13 were measured in BALF by ELISA. (C) Serum MMCP-1 levels. Values are means ± SEMs (n = 6). *$P < 0.05$, **$P < 0.01$ compared to the PC group. NC: Negative group, PC: Positive group, BALF: Bronchoalveolar lavage fluid. MMCP-1: Mouse mast cell protease 1.

reduced inflammation in the BALF and decreased production of IL-4 and IL-13 in response to *Alternaria* stimulation in Alt a 1- and *Alternaria* -SCIT-treated mice.

Serum immunoglobulin responses are influenced by Alt a 1 and *A. Alternaria*-SCIT

In this section, we sought to examine the total, Alt a 1-, and *Alternaria*-specific immunoglobulin levels (Fig. 4). Mice in the PC group showed higher induction of T-IgE ($P = 0.0012$) and allergen-specific IgE titers than the NC mice, while a trend toward reduced T-IgE and *Alternaria*-sIgE was achieved by rAlt a 1-SCIT ($P < 0.05$), but Alt a 1-sIgE levels exhibited a strong response after SCIT (Fig. 4A). Moreover, treatment of mice with Alt a 1 and *A. alternata* extracts led to an increase in IgG antibodies compared to allergic mice. Significant increases in Alt a 1-sIgG1, sIgG2a, and sIgG2b levels were observed in the Alt a 1-SCIT group (Fig. 4B), and mice that received nAlt a 1 SCIT were more obviously affected (nAlt a 1-sIgG1: $P = 0.0004$; nAlt a 1-sIgG2a: $P = 0.0024$; nAlt a 1-sIgG2b: $P = 0.0006$). The neutralizing activity in the ratios of *Alternaria* and Alt a 1-sIgG1/sIgE, sIgG2a/sIgE, and sIgG2b/sIgE were also calculated in the SCIT groups (Fig. 4C), in which the ratios of Alt a 1-related neutralizing antibodies were higher than those of the PC control ($P < 0.05$), but only the levels of *Alternaria*-sIgG1/sIgE had an increasing ratio in the group treated with *A. alternata* extracts ($P = 0.0411$). Overall, SCIT with Alt a 1 resulted in a greater decrease in IgE titers and increased IgG responses in the serum of mice than in the group treated with *A. alternata* extracts.

SCIT with Alt a 1 and *A. alternata* extracts suppress the expression of Bcl-6

Bcl-6 is a key transcription factor in Tfh cells and always expresses in germinal central (GC). Among the best studied, Bcl-6 is important and central to GC differentiation.[22] Han et al [23] verified both the mRNA and protein expression of Bcl-6 in the spleen increased in a fine particular matter-induced asthma model, and we determined the location and protein expression level of Bcl-6 in the spleen using immunohistochemistry (Fig. 5). Our examination showed that cells expressing Bcl-6 were mainly localized in the nucleus of the red pulp of the spleen germinal central (red arrow), although Bcl-6 was occasionally expressed in the white pulp and marginal areas (Fig. 5A). Following Alt a 1-SCIT and *Alternaria*-SCIT, the expression scores of Bcl-6 were evaluated by a semi-quantitative assessment tool (Fig. 5B) and indicated more highly positive cells in the PC group than in the negative control ($P < 0.01$), while the strength of expression decreased in the SCIT groups, especially in the Alt a 1 groups (nAlt a 1: $P = 0.0022$; rAlt a 1: $P = 0.0043$), although Bcl-6 in the *Alternaria*-SCIT mice also displayed reduced expression. These data indicated that SCIT with Alt a 1 was more effective in alleviating expression of Bcl-6 in the spleen of mice.

Alt a 1 and *A. alternata* extracts influence the proportion of Tfh cells

We determined the number of Tfh ($CD4^+$ $CXCR5^+$ $PD\text{-}1^+$) cells in the spleen of asthmatic mice using a therapeutic concentration screening

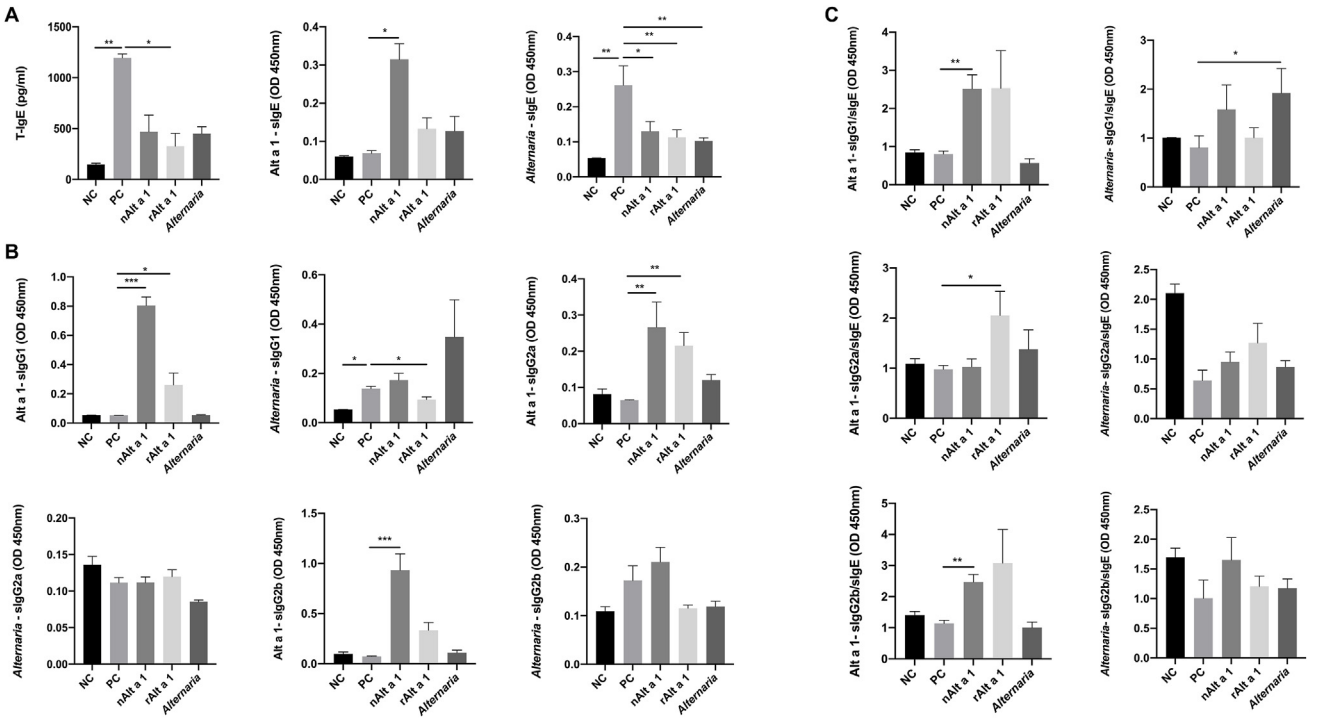

Fig. 4 Serum immunoglobulin responses in each group after SCIT. (A) T-IgE, Alt a 1-sIgE, and *Alternaria*-sIgE levels in the serum. (B) Serum *Alternaria*- and Alt a 1-sIgG1, sIgG2a, and sIgG2b levels. (C) The expressions of neutralizing antibodies in serum after SCIT. Values are means ± SEMs (n = 6–8). **P* < 0.05, ***P* < 0.01 and ****P* < .001compared to the PC group. NC: Negative group, PC: Positive group.

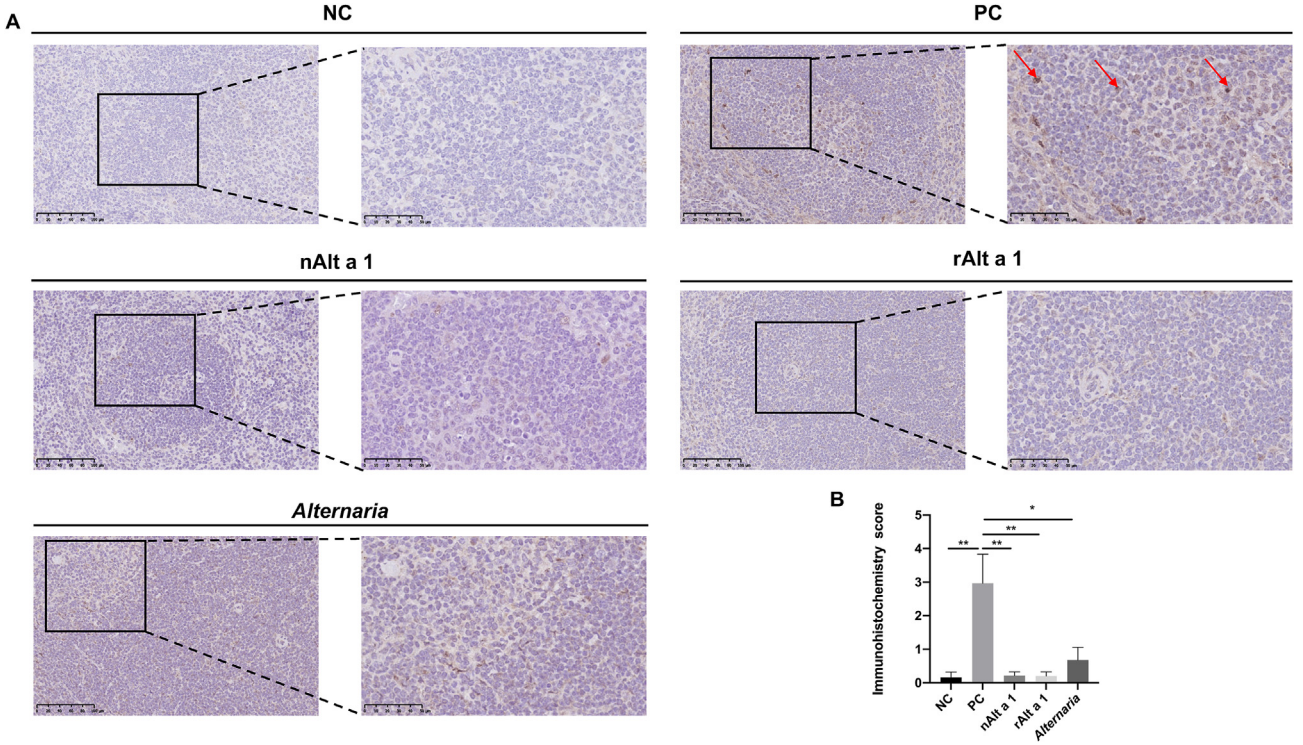

Fig. 5 The location and expression of Bcl-6 in the spleen from each group. (A) Bcl-6 was primally located in the germinal central and expressed in the cell nucleus. *Scale bar* = 100/50 μm. (B) Semiquantitative scores of Bcl-6 were analyzed via immunohistochemistry. Values are means ± SEMs (n = 6). **P* < 0.05, ***P* < 0.01 compared to the PC group. NC: Negative group, PC: Positive group. Bcl-6: B cell lymphoma 6.

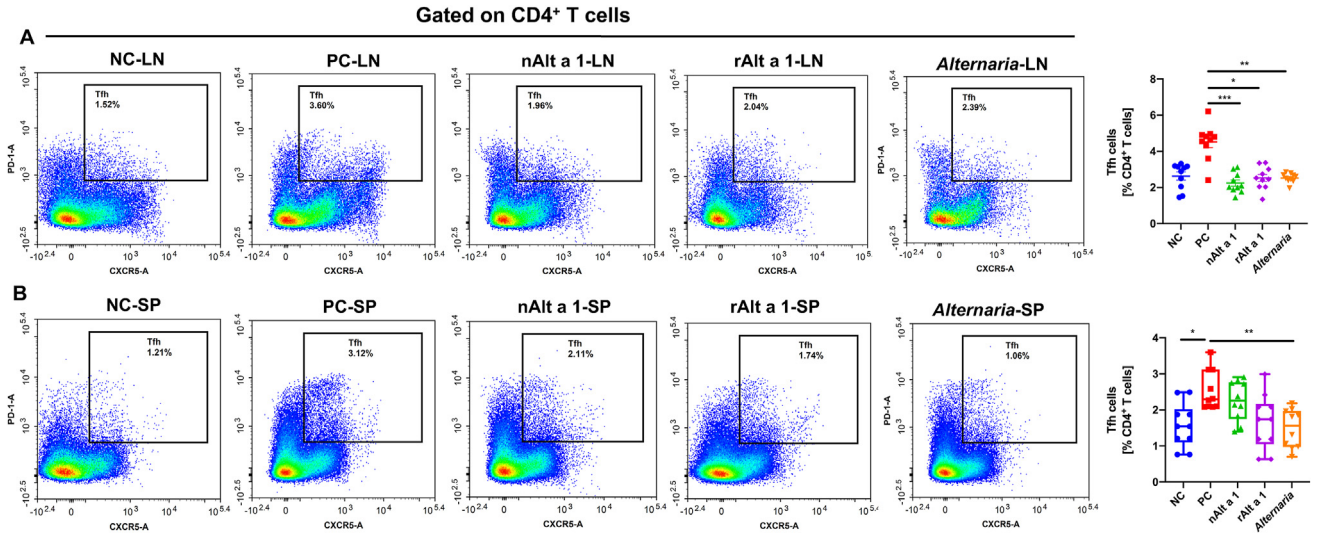

Fig. 6 The percentages of Tfh cells were decreased following Alt a 1 and *A. Alternata* subcutaneous treatment. (A) Analysis and quantification of Tfh cells in the lymph node. (B) Analysis and proportions of Tfh cells in the spleen. *Symbols* represent individual samples. Values are means ± SEMs (n = 10). **P* < 0.05, ***P* < 0.01, ****P* < .001compared to the PC group. NC: Negative group, PC: Positive group. LN: lymph node, SP: spleen.

stage of rAlt a 1.[19] In this study, we further compared the percentages of Tfh cells in the Alt a 1 and *Alternaria*-SCIT groups of the LN and spleen (Supplementary Figure 1). Notably, the PC group had a higher proportion of Tfh cells among the $CD4^+$ T cells in the LN and spleen than the control group (Fig. 6). Conversely, after Alt a 1-SCIT and *Alternaria*-SCIT, there was a sharply decreased expression of Tfh cells in these groups in the LN compared to the PC control, and all showed statistical significance, especially in the nAlt a 1-SCIT mice ($P = 0.0002$), followed by the *Alternaria* group ($P = 0.0074$) (Fig. 6A), whereas no significant difference was observed in the proportion of Tfh cells in spleen cells between the Alt a 1-SCIT and the PC control groups, although lower levels of Tfh cell expression were observed in the *Alternaria* group ($P = 0.0087$) (Fig. 6B). Therefore, our results suggested that SCIT with Alt a 1 attenuated the accumulation of Tfh cells in the LN, but moderate changes were observed in the spleen, and *Alternaria*-SCIT showed greater effects on Tfh cells in the spleen.

The effects of Alt a 1 and *A. alternata* extracts on the percentage of IL-4^+ Tfh, IL-5^+ Tfh, IL-13^+ Tfh, and IL-$17A^+$ Tfh cells

Since IL-4, IL-5, IL-13, and IL-17 participate in the regulation of Tfh cells, we determined the percentages of these cells in the LN and spleen (Supplementary Figure 1). Notably, the proportions of IL-4^+ Tfh, IL-5^+ Tfh, IL-13^+ Tfh, and IL-$17A^+$ Tfh cells were higher in the PC control than in the negative control in both the LN and spleen (Fig. 7). However, following Alt a 1-SCIT and *Alternaria*-SCIT, there was a significant reduction in IL-4^+ Tfh and IL-5^+ Tfh levels in the LN, while a moderate decrease in the spleen was not statistically significant (Fig. 7A and B). Additionally, the expression of IL-$17A^+$ Tfh cells in both the LN and spleen was significantly decreased after administration of the *Alternaria* extracts (LN: $P = 0.0018$; spleen: $P = 0.0103$). Alt 1-SCIT levels showed a mild reduction in this subtype (Fig. 7D). We also investigated the percentage of IL-13^+ Tfh cells (Fig. 7C), no distinct reduction was found in either the Alt a 1 or *Alternaria* groups. Taking these results together, Alt a 1-SCIT was more likely to suppress the expression of IL-4^+ Tfh and IL-5^+ Tfh cells in the LN, rather than the levels of IL-13^+ Tfh and IL-$17A^+$ Tfh cells; in contrast, *A. alternata* extracts presented stronger improvement of IL-4^+ Tfh and IL-5^+ Tfh in the spleen, and alleviated expression of IL-$17A^+$ Tfh in both the spleen and LN.

Alt a 1 and *Alternaria*-SCIT regulate the Breg and IL-10^+ Breg cells response in the spleen

To clarify the responses of Breg cells and their subsets in the spleen following Alt a 1-SCIT and

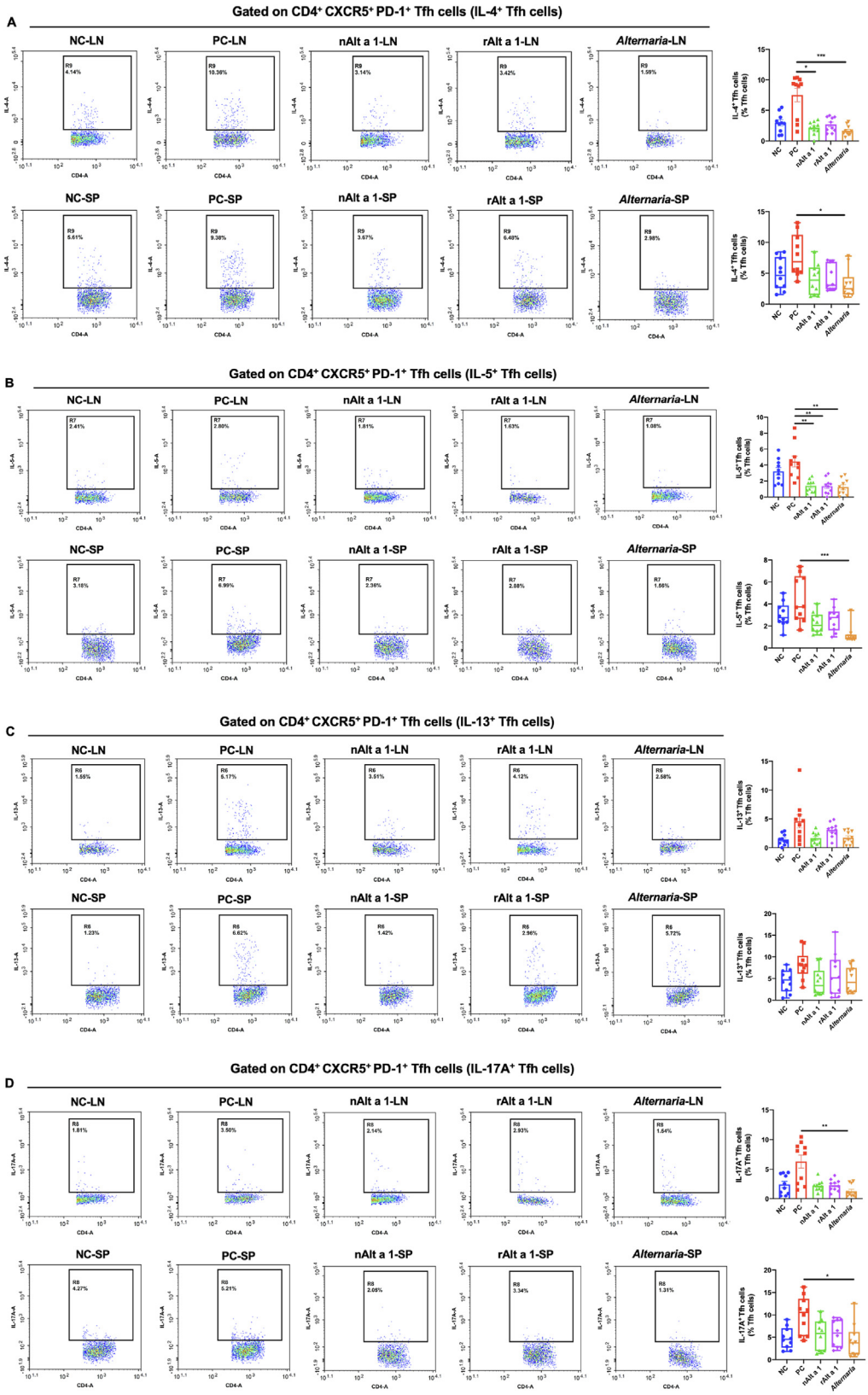

Fig. 7 The proportions of IL-4$^+$ Tfh, IL-5$^+$ Tfh, IL-13$^+$ Tfh, and IL-17A$^+$ Tfh cells in the LN and the spleen after accepted Alt a 1 and *A. alternata* treatment. (A) Flow cytometry analysis of IL-4$^+$ Tfh cells. (B) The levels of IL-5$^+$ Tfh cells in each group. (C) The expression of IL-13$^+$ Tfh cells among these groups. (D) The proportions of IL-17A$^+$ Tfh cells. *Symbols* represent individual samples. Values are means ± SEMs (n = 10). **P* < 0.05, ***P* < 0.01, ****P* < .001compared to the PC group. NC: Negative group, PC: Positive group. LN: lymph node, SP: spleen.

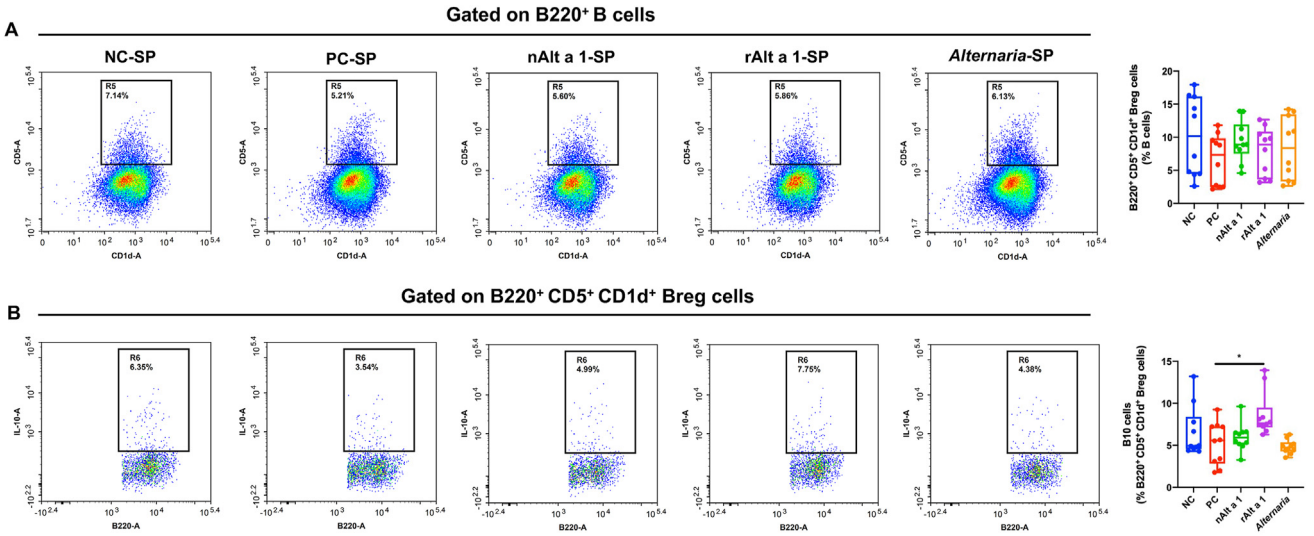

Fig. 8 The percentages of Breg and B10 cells in asthmatic mice of each group. (A) Flow cytometry analysis and quantification of Breg cells in spleen tissues. (B) Flow cytometry analysis of B10 cells in different groups. *Symbols* represent individual samples. Values are means ± SEMs (n = 10). *$P < 0.05$ compared to the PC group. NC: Negative group, PC: Positive group. SP: spleen.

Alternaria-SCIT, we defined Breg cells as $B220^+$ $CD1d^+$ $CD5^+$ and $IL\text{-}10^+$ Breg cells as $IL\text{-}10^+$ $B220^+$ $CD1d^+$ $CD5^+$ (Supplementary Figure 2, Fig. 8). Compared with the NC group, the levels of Breg cells were decreased in the PC mice, in contrast, following Alt a 1-SCIT and *Alternaria*-SCIT, there was a slight improvement in Breg cells, but without statistical significance (Fig. 8A). Next, we investigated the percentage of $IL\text{-}10^+$ Breg cells, since IL-10 is the main negative regulator of the immune response. The proportion of $IL\text{-}10^+$ Breg cells significantly increased in the rAlt a 1-SCIT group compared to the PC group ($P = 0.0374$), although there was almost no upward trend in the nAlt a 1 and *Alternaria* groups (Fig. 8B). Our data suggested upregulation of $IL\text{-}10^+$ Breg cells in the spleen in response to SCIT with rAlt a 1, but not with nAlt a 1 or *A. alternata* extracts.

DISCUSSION

Conventional AIT for fungi using whole *A. alternata* extracts is associated with adverse effects,[10,24-26] including anaphylaxis[8,9] and poor patient compliance. The use of isolated Alt a 1 in AIT represents an encouraging and novel therapeutic method for *Alternaria*-induced allergic diseases. However, few studies of fungal AIT with Alt a 1 and no direct comparison studies have focused on the efficacy and immune responses between both treatments in an experimental mouse model. The results observed in the present study suggest that both treatments share a similar therapeutic efficacy in suppressing airway and lung inflammation. While the Alt a 1 vaccine possessed stronger responses of immunoglobulins, lower levels of Bcl-6, and higher percentages of $IL\text{-}10^+$ Breg cells than *A. alternata* extracts, Tfh and cytokine-related Tfh cells were remarkably suppressed in the *Alternaria*-SCIT mice.

Inflammation of airway and lung tissues was decreased in both the Alt a 1 and *A. alternata* extracts therapeutic groups, which indicated that Alt a 1-SCIT was at least as effective in suppressing the *Alternaria*-induced allergic responses as whole crude extracts. This is in line with results of another study that compared the efficacy of purified Der p1/2 and house dust mite-SCIT.[27] Our initial study confirmed that different dosages of rAlt a 1-SCIT caused a slight decrease in MMCP-1 levels, which was also observed in therapy with *A. alternata* extracts, suggesting that neither crude extracts nor purified components could induce anaphylaxis in this mouse model, as MMCP-1 in serum acts as an indicator of severe anaphylaxis.[28]

The difference in reduction of IgE levels and induction of neutralizing antibodies might be due to a higher protein dose of Alt a 1 that was supplied by purified allergens compared to crude extracts since *A. alternata* extracts only contained 0.04 mg/mL Alt a 1 protein. We observed that Alt a 1-sIgE levels in the

nAlt a 1-SCIT group were even higher than those in the PC control, which could be explained by continuous stimulation with nAlt a 1 and variation in concentrations of drugs. Similar transient increases in sIgE levels have been recorded in other AIT studies.[29] Of note, we also only found a sIgG and sIgG/sIgE response to Alt a 1 rather than in therapy with *A. alternata* extracts, showing that increases in blocking IgGs were always linked with AIT efficacy.[30] However, in an experimental dust mite SCIT model, the IgG response was not induced by similarly crude extracts.[27] They speculated that another molecule named Der p23 in the mixture might influence the value of a vaccine. The same phenomenon may have occurred in our study, where Alt a 6 in *A. alternata* extracts has a highly relevant sIgE value.[31] Overall, subcutaneous treatment with Alt a 1 correlated with high levels of serum immunoglobulin responses.

Next, we explored the expression of Bcl-6 in the spleen. Bcl-6 plays a critical role in the production, differentiation, and migration of Tfh cells, and works by binding to DNA in the nucleus.[32] Bcl-6 likely has multiple functions in allergic diseases, since there was no IgE production in response to allergen sensitization in the absence of Tfh cells that lack Bcl-6.[33] Furthermore, the expression of Bcl-6 was significantly higher in the acute asthma children than in the healthy group.[34] Another study also suggested that Bcl-6 may be important for reducing Th2 immune responses.[35] However, there has been no research highlighting the levels of Bcl-6 in AIT models. The results of our study showed that expression of Bcl-6 was significantly decreased after SCIT, but therapy with Alt a 1 presented greater efficacy than with *A. alternata* extracts. One could postulate that this improved reduction in Alt a 1-SCIT is associated with structural features of the antigen. Alt a 1 is a dimeric protein rich in β-barrel regions that increases its stability and heat-resistance.[36] It is thought that dimerization of Alt a 1 might contribute to its allergenicity, although it is not essential,[37] and allows the immune system to recognize the location of T and B cell epitopes on Alt a 1. *A. alternata* contains other components and different molecular forms of these components may also interfere with immunogenicity. Ultimately, this could result in improved therapeutic efficacy of Alt a 1 compared to *Alternaria alternata* in reducing the expression of Bcl-6.

To address the factors that prompt AIT immunological responses, we investigated the percentage of Tfh, cytokine-related Tfh cells, and IL-10^+ Breg cells. Tfh cells participate in class switching, somatic mutation, and affinity maturation of antibodies, especially IgE.[38] Our clinical study demonstrated that the proportion of circulating Tfh cells greatly decreased after *Alternaria*-SCIT compared to that in non-AIT patients (data not shown). Meanwhile, Sharif et al[39] verified that IL-4- and IL-21-producing Tfh cells decreased in the *Lolium perenne* peptide-treated AIT group, while IFN-γ-producing Tfh cells increased in the same group. These findings suggest that Tfh cells and their subtypes are pathogenic in patients with allergies and AIT. However, this is the first study to detect the number of Tfh subtypes in Alt a 1 and *Alternaria*-SCIT animal models, and our observations are in agreement with the findings obtained by others, illustrating that either pollen or fungal AIT can reduce the levels of Tfh cells and cytokine-related Tfh cells, although several differences in the changes in Tfh and subtypes occurred in both treatments. A potential explanation might be attributed to the lack of nonprotein constituents in the purified proteins, including β-glucans, chitin, and endotoxins, which activate other pro-inflammatory innate responses during injection.[40] In addition, it has been reported that sensitization with Alt a 1 alone can induce allergic responses in mice.[41] Therefore, the whole extract was more likely to contribute to the reduction in Tfh cells and subtypes. Accumulating evidence suggests the induction of IL-10^+ Breg cells following AIT.[42] IL-10 can induce B cells to produce sIgG4, reduce IgE production, and mediate immune tolerance.[43] Our data also showed an upregulation of IL-10^+ Breg cells exclusively in the rAlt a 1-SCIT group, which might be explained by the efficacy of a purified natural allergen being equal to crude extracts.[44] Additionally, several studies have suggested that Toll-like receptors are required for the activation of B10 cells,[45] and these molecules can be activated by *A. alternata*. Hence, it is worth considering whether the inconsistent changes in the levels of IL-10^+ Breg cells in both treatments

were caused by modulation of B10 cells through receptor molecules. Unfortunately, we could not directly or in depth analyze this mechanism.

Taken together, we confirmed for the first time the effect of Alt a 1 and *A. alternata* extracts on inflammatory biomarkers in an experimental mouse model, and we made a direct comparison between these two different treatment methods and the changes in immune responses. There are some limitations associated with this analysis that will need to be addressed in future studies. First, the mechanisms by which Alt a 1 and *A. alternata* extracts regulate Tfh and Breg cells is still unknown. Gene knockout mice are likely to be a valuable tool for further exploration of this aspect. Second, inflammatory cells in BALF were not fully classified and counted, and we also did not test the percentages of Breg and IL-10^+ Breg cells in the LN. Third, antigen-specific Tfh and Breg cells might need to be sorted to clarify the mechanisms.

CONCLUSIONS

Immunotherapy using a novel preparation of Alt a 1, as well as using *A. alternata* extracts, reduced airway and lung inflammation in our established mouse model. Alt a 1-SCIT showed greater improvement in the responses of immunoglobulins, Bcl-6, and IL-10^+ Breg cells, whereas SCIT with *A. alternata* extracts was more inclined to decrease the levels of Tfh and their subtypes. These results lend further support to the view that SCIT with Alt a 1 is a promising and effective therapeutic approach for mice with *Alternaria* allergy, and may lay the foundation for Alt a 1 in the future research of clinical allergen immunotherapy.

Abbreviations

SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; AIT, allergen-specific immunotherapy; rAlt a 1, recombinant Alt a 1; nAlt a 1, native Alt a 1; Tfh, T follicular helper; Breg, regulatory B cells; GC, germinal central; MMCP-1, mouse mast cell protease-1; Bcl-6, B cell lymphoma 6.

Statement of financial support

This study was supported by the CAMS Initiative for Innovative Medicine (CAMS-I2M), Grant/Award Number: 2021-I2M-1-017; Major National Science and Technology Projects for "Significant New Drugs Creation", Grant/Award Number: 2019ZX09301131.

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

Juan Liu performed the experiments, analyzed the data, and drafted the manuscript. Junda Li participated in all the experiments. Jia Yin designed the experiments and provided funding. All authors have contributed to the manuscript and approved the submitted version.

Ethics approval

All protocols were performed and approved by the Institutional Animal Care and Use Committee of Peking Union Medical University (XHDW-2020-046).

Authors' consent for publication

All authors have approved the final manuscript and approved of the submission to World Allergy Organization Journal.

Declaration of competing interest

The authors have declared no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2023.100807.

Author details

[a]Department of Allergy, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing Key Laboratory of Precision Medicine for Diagnosis and Treatment on Allergic Diseases, Beijing, China. [b]National Clinical Research Center for Dermatologic and Immunologic Disease (NCRC-DID), Beijing, China.

REFERENCES

1. Pulimood TB, Corden JM, Bryden C, Sharples L, Nasser SM. Epidemic asthma and the role of the fungal mold Alternaria alternata. *J Allergy Clin Immunol*. 2007;120:610–617.
2. Song X, Liu CH, Wang W, Huang GM, Zhao J, Sha L. Characteristics and changes of sensitization patterns of major allergens in children from 2010 to 2020 in a hospital of pediatric in Beijing. *Zhonghua Yufang Yixue Zazhi*. 2022;56:763–773.
3. Bush RK, Prochnau JJ. Alternaria-induced asthma. *J Allergy Clin Immunol*. 2004;113:227–234.

4. Heinzerling L, Frew AJ, Bindslev-Jensen C, et al. Standard skin prick testing and sensitization to inhalant allergens across Europe-a survey from the GALEN network. *Allergy*. 2005;60: 1287–1300.
5. Arbes Jr SJ, Gergen PJ, Elliott L, Zeldin DC. Prevalences of positive skin test responses to 10 common allergens in the US population: results from the third National Health and Nutrition Examination Survey. *J Allergy Clin Immunol*. 2005;116:377–383.
6. Di Bona D, Frisenda F, Albanesi M, Di Lorenzo G, Caiaffa MF, Macchia L. Efficacy and safety of allergen immunotherapy in patients with allergy to molds: a systematic review. *Clin Exp Allergy*. 2018;48:1391–1401.
7. Kiliç M, Altintaş DU, Yilmaz M, Bingöl-Karakoç G, Burgut R, Güneşer-Kendirli S. Evaluation of efficacy of immunotherapy in children with asthma monosensitized to Alternaria. *Turk J Pediatr*. 2011;53:285–294.
8. Pozzan M, Milani M. Efficacy of sublingual specific immunotherapy in patients with respiratory allergy to Alternaria alternata: a randomised, assessor-blinded, patient-reported outcome, controlled 3-year trial. *Curr Med Res Opin*. 2010;26:2801–2806.
9. Kuna P, Kaczmarek J, Kupczyk M. Efficacy and safety of immunotherapy for allergies to Alternaria alternata in children. *J Allergy Clin Immunol*. 2011;127:502–508. e501-506.
10. Cortellini G, Spadolini I, Patella V, et al. Sublingual immunotherapy for Alternaria-induced allergic rhinitis: a randomized placebo-controlled trial. *Ann Allergy Asthma Immunol*. 2010;105:382–386.
11. Di Bona D, Albanesi M, Macchia L. Is immunotherapy with fungal vaccines effective? *Curr Opin Allergy Clin Immunol*. 2019;19:646–653.
12. Asturias JA, Ibarrola I, Ferrer A, et al. Diagnosis of Alternaria alternata sensitization with natural and recombinant Alt a 1 allergens. *J Allergy Clin Immunol*. 2005;115:1210–1217.
13. Ferrer Á. Clinical evaluation of subcutaneous immunotherapy with a polymerized molecular allergoid of Alt a 1 in patients with allergic rhinoconjunctivitis and/or allergic asthma caused by the mould Alternaria alternata. *Eur Ann Allergy Clin Immunol*. 2022. https://doi.org/10.23822/EurAnnACI.1764-1489.267.
14. Tabar AI, Prieto L, Alba P, et al. Double-blind, randomized, placebo-controlled trial of allergen-specific immunotherapy with the major allergen Alt a 1. *J Allergy Clin Immunol*. 2019;144:216–223.e213.
15. Prieto L, Palacios R, Aldana D, et al. Effect of allergen-specific immunotherapy with purified Alt a1 on AMP responsiveness, exhaled nitric oxide and exhaled breath condensate pH: a randomized double blind study. *Allergy Asthma Clin Immunol*. 2010;6:27.
16. Shamji MH, Sharif H, Layhadi JA, Zhu R, Kishore U, Renz H. Diverse immune mechanisms of allergen immunotherapy for allergic rhinitis with and without asthma. *J Allergy Clin Immunol*. 2022;149:791–801.
17. Olatunde AC, Hale JS, Lamb TJ. Cytokine-skewed Tfh cells: functional consequences for B cell help. *Trends Immunol*. 2021;42:536–550.
18. Sharif H, Acharya S, Dhondalay GKR, et al. Altered chromatin landscape in circulating T follicular helper and regulatory cells following grass pollen subcutaneous and sublingual immunotherapy. *J Allergy Clin Immunol*. 2021;147:663–676.
19. Liu J, Yin J. Immunotherapy with recombinant Alt a 1 suppresses allergic asthma and influences T follicular cells and regulatory B cells in mice. *Front Immunol*. 2021;12, 747730.
20. Xie Z, Yin J. Chinese birch pollen allergy and immunotherapy in mice. *Inflammation*. 2019;42:961–972.
21. Liu J, Liang R, Huang H, Zhang Y, Xie A, Zhong Y. Effect of an antagonistic peptide of CCR5 on the expression of autophagy-related genes and β-arrestin 2 in lung tissues of asthmatic mice. *Allergy Asthma Immunol Res*. 2021;13:106–121.
22. Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science*. 1997;276:589–592.
23. Han Y, Yu X, Lu Y, et al. Di-(2-ethylhexyl) phthalate aggravates fine particulate matter-induced asthma in weanling mice due to T follicular helper cell-dependent response. *Toxicology*. 2023;484, 153406.
24. Tabar AI, Lizaso MT, García BE, et al. Double-blind, placebo-controlled study of Alternaria alternata immunotherapy: clinical efficacy and safety. *Pediatr Allergy Immunol*. 2008;19:67–75.
25. Horst M, Hejjaoui A, Horst V, Michel FB, Bousquet J. Double-blind, placebo-controlled rush immunotherapy with a standardized Alternaria extract. *J Allergy Clin Immunol*. 1990;85:460–472.
26. Criado Molina A, Guerra Pasadas F, Daza Muñoz JC, et al. [Immunotherapy with an oral Alternaria extract in childhood asthma. Clinical safety and efficacy and effects on in vivo and in vitro parameters]. *Allergol Immunopathol*. 2002;30:319–330.
27. Hesse L, van Ieperen N, Habraken C, et al. Subcutaneous immunotherapy with purified Der p1 and 2 suppresses type 2 immunity in a murine asthma model. *Allergy*. 2018;73:862–874.
28. Finkelman FD. Anaphylaxis: lessons from mouse models. *J Allergy Clin Immunol*. 2007;120:506–515. quiz 516-507.
29. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol*. 2014;133:621–631.
30. Shamji MH, Layhadi JA, Sharif H, Penagos M, Durham SR. Immunological responses and biomarkers for allergen-specific immunotherapy against inhaled allergens. *J Allergy Clin Immunol Pract*. 2021;9:1769–1778.
31. Postigo I, Gutiérrez-Rodríguez A, Fernández J, Guisantes JA, Suñén E, Martínez J. Diagnostic value of Alt a 1, fungal enolase and manganese-dependent superoxide dismutase in the component-resolved diagnosis of allergy to Pleosporaceae. *Clin Exp Allergy*. 2011;41:443–451.
32. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity*. 2014;41:529–542.
33. Kobayashi T, Iijima K, Dent AL, Kita H. Follicular helper T cells mediate IgE antibody response to airborne allergens. *J Allergy Clin Immunol*. 2017;139:300–313.e307.

34. Zhang D, Wu Y, Sun G. miR-192 suppresses T follicular helper cell differentiation by targeting CXCR5 in childhood asthma. *Scand J Clin Lab Invest*. 2018;78:236–242.

35. Ogasawara T, Kohashi Y, Ikari J, et al. Allergic T(H)2 response governed by B-cell lymphoma 6 function in naturally occurring memory phenotype CD4(+) T cells. *Front Immunol*. 2018;9:750.

36. Sánchez P, Vélez-Del-Burgo A, Suñén E, Martínez J, Postigo I. Fungal allergen and mold allergy diagnosis: role and relevance of Alternaria alternata Alt a 1 protein family. *J Fungi (Basel)*. 2022;8.

37. Chruszcz M, Chapman MD, Osinski T, et al. Alternaria alternata allergen Alt a 1: a unique β-barrel protein dimer found exclusively in fungi. *J Allergy Clin Immunol*. 2012;130:241–247. e249.

38. Gowthaman U, Sikder S, Lee D, Fisher C. T follicular helper cells in IgE-mediated pathologies. *Curr Opin Immunol*. 2022;74:133–139.

39. Sharif H, Singh I, Kouser L, et al. Immunologic mechanisms of a short-course of Lolium perenne peptide immunotherapy: a randomized, double-blind, placebo-controlled trial. *J Allergy Clin Immunol*. 2019;144:738–749.

40. Hernandez-Ramirez G, Barber D, Tome-Amat J, Garrido-Arandia M, Diaz-Perales A. Alternaria as an inducer of allergic sensitization. *J Fungi (Basel)*. 2021;7.

41. Vélez-Del-Burgo A, Sánchez P, Suñen E, Martínez J, Postigo I. Purified native and recombinant major Alternaria alternata allergen (Alt a 1) induces allergic asthma in the murine model. *J Fungi (Basel)*. 2021;7:896.

42. Yamanaka K, Shah SA, Sakaida H, et al. Immunological parameters in prophylactic sublingual immunotherapy in asymptomatic subjects sensitized to Japanese cedar pollen. *Allergol Int*. 2015;64:54–59.

43. van de Veen W, Stanic B, Yaman G, et al. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol*. 2013;131:1204–1212.

44. Pauli G, Larsen TH, Rak S, et al. Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis. *J Allergy Clin Immunol*. 2008;122:951–960.

45. Baba Y, Matsumoto M, Kurosaki T. Signals controlling the development and activity of regulatory B-lineage cells. *Int Immunol*. 2015;27:487–493.