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Comparison of rArt v 1-based sublingual and subcutaneous immunotherapy in a murine model of asthma

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Mugwort-allergic patients frequently experience severe respiratory allergies due to sensitization to the major allergen Art v 1, with allergen-specific immunotherapy (ASIT) as the only causal treatment to halt disease progression. This study evaluated the effects of subcutaneous (SCIT) and sublingual (SLIT) ASIT with purified recombinant Art v 1 (rArt v 1) in a murine model of mugwort pollen of asthma. BALB/c mice were sensitized with *Artemisia vulgaris* pollen extract and treated with either rArt v 1-based SCIT adjuvanted with Montanide ISA-51, rArt v 1-based SLIT, an extract-based commercial SLIT vaccine, or PBS. Both rArt v 1-based SCIT and SLIT improved lung pathology and reduced airway reactivity following allergen challenge, with rArt v 1-based SCIT inducing Th1-polarized immune responses marked by increased IFN-γ production and rArt v 1-specific IgG_1/IgG_{2a}, while SLIT induced stronger mucosal IgA responses. These findings highlight the therapeutic potential of rArt v 1-based ASIT for mugwort allergy.

Mugwort (Artemisia) pollen is one of the most important sources of aeroallergens globally, contributing significantly to allergic rhinitis and bronchial asthma[1,2]. The major allergen of mugwort, Art v 1, is a glycoprotein consisting of a highly stable N-terminal defensin-like domain and a C-terminal hydroxyproline-rich domain[3]. Besides Art v 1 also other mugwort pollen allergens have been described[4,5] but Art v 1 is the most important allergenic component in mugwort pollen. Interestingly, IgE recognition of Art v 1 is strongly MHC II-restricted and immunodominant Art v 1 T cell epitopes have been reported[6,7]. Since mugwort pollen allergy is a seasonal allergy and dominated by one major allergen (i.e., Art v 1) it is an excellent model system to investigate allergen-specific immunotherapy (ASIT).

ASIT is the only treatment for IgE-mediated allergies that not only alleviates symptoms but also alters the disease course and has sustained effects after discontinuation[8,9]. Unlike symptomatic treatments with antihistamines and corticosteroids, ASIT induces the production of allergen-specific blocking antibodies and affects cellular immune responses[10]. This is accomplished by gradually administering increasing doses of the allergen, leading to desensitization and symptom relief upon future allergen exposure[11].

Subcutaneous allergen-specific immunotherapy (SCIT) with natural allergen extracts or allergoids with or without adjuvants (e.g., aluminum hydroxide or aluminum phosphate) is the most frequently used form of ASIT[12]. SCIT offers long-lasting therapeutic benefits[12], but it may cause severe adverse local and/or systemic reactions[13]. Accordingly, this method is administered in specialized medical facilities under physician supervision[14].

More recently sublingual immunotherapy (SLIT) has gained considerable attention, and numerous clinical studies have been performed to evaluate its efficacy[15]. Although numerous studies suggest that SLIT is clinically effective, relatively few have focused on investigating its underlying immunological mechanisms or directly comparing SLIT with SCIT. One human study compared the efficacy and immunological effects of both SCIT and SLIT in a double-blind, double-dummy, placebo-controlled study in patients allergic to grass pollen[16] and showed differential induction of allergen-specific IgA with SLIT versus SCIT, suggesting key differences in the

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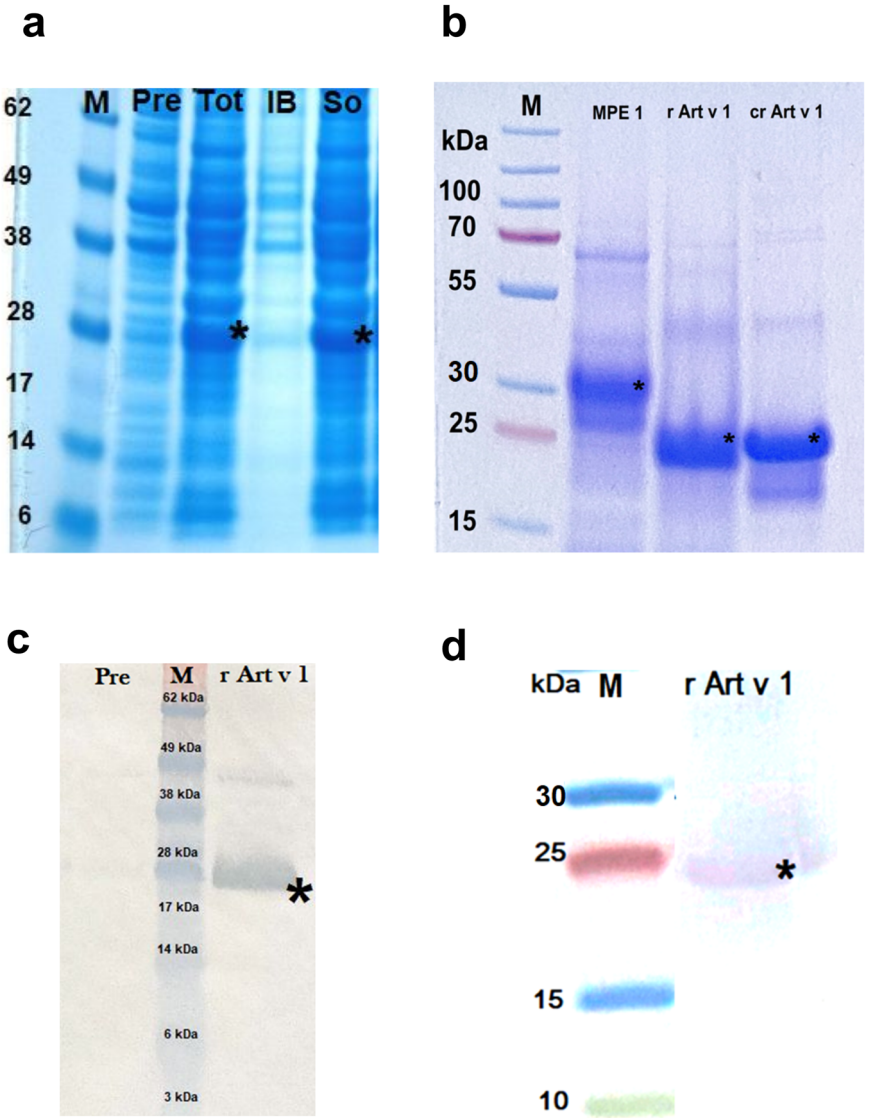

Fig. 1 | Cloning, expression, purification, and analysis of rArt v 1 protein. a Electrophoretic expression profile of the rArt v 1 gene. M – SeeBlue Plus2 Pre-Stained Protein Standard (Thermo Fisher Scientific); Pre – cell lysate before induction; Tot – after IPTG induction; IB – insoluble protein fraction; So – soluble protein fraction; * - indicates the location of the target protein. **b** SDS-PAGE analysis of protein expressed by rArt v 1 in *E. coli* and mugwort pollen extracts (MPE 1 – *Artemisia vulgaris*, Burly, Almaty, Kazakhstan). The protein gel was loaded with 10 μg of purified bacterially expressed rArt v 1 protein, commercial recombinant major mugwort pollen protein (crArt v 1; AtaGenix laboratories, China), and 1000 PNU MPE 1. **c** Western blot analysis of rArt v 1 protein expressed in *E. coli* using His Tag Monoclonal antibody. **d** Western blot analysis of rArt v 1 protein expressed in *E. coli* using *Artemisia vulgaris* major pollen allergen Art v 1 polyclonal antibodies.

mechanisms of action of the two forms of ASIT. Several studies have investigated SLIT in experimental animal models of allergy. One study investigated SLIT with house dust mite (HDM) extract in a murine model of HDM allergy and found an induction of allergen-specific IgA[17]. Another study performed SCIT and SLIT in a murine model of birch pollen allergy with birch pollen extract and found that both treatments reduced airway symptoms[18]. So far, no study has compared SCIT and SLIT with a purified recombinant Art v 1 allergen in mice who had been previously sensitized with an allergen extract.

In our study we report for the first time the comparison of SLIT and SCIT performed with purified recombinant major mugwort pollen allergen, rArt v 1, in mice who had been sensitized with mugwort pollen allergen extract. We found that both approaches work through reducing allergen extract-induced airway responses. Furthermore, we were able to show that SCIT induced higher levels of allergen-specific IgG than SLIT whereas SLIT induced more allergen-specific IgA. This suggests both approaches would through different mechanisms and supports additional studies into the utility of rArt v 1 SLIT for treatment of mugwort allergy.

Results

Cloning, expression, purification, and analysis of rArt v 1 protein

The rArt v 1 protein was successfully expressed in *E. coli* C41. After induction and cell lysis, SDS-PAGE analysis confirmed that the protein was present in the soluble fraction (Fig. 1a). Figure 1b shows a major band at approximately 23 kDa. Electrophoretic analysis demonstrated that the obtained rArt v 1 looks similar to a commercial rArt v 1 protein but shows a lower molecular mass than the natural glycosylated protein found in *Artemisia vulgaris* pollen extract. Importantly, 10 μg of purified bacterially expressed rArt v 1 protein corresponded well to the 1000 PNU of mugwort pollen extract used in the commercial vaccine. Purification of the clarified lysate by metal-affinity chromatography, followed by desalting and endotoxin removal, yielded 22 mg of rArt v 1 protein per liter of bacterial culture with a purity greater than ~95% and an endotoxin content of less than 5 EU/mL (Fig. 1b). Immunoblotting confirmed the specific binding of purified rArt v 1 with both anti-His Tag monoclonal antibodies (Fig. 1c) and polyclonal antibodies raised against the natural Art v 1 allergen (Fig. 1d). Uncropped and unprocessed scans of gels and blots shown in Fig. 1 Supplementary (Supplementary Data).

SLIT and SCIT with rArt v 1 prevent increases of total IgE levels induced by allergen challenge

Sensitization with *Artemisia vulgaris* pollen extract successfully induced relevant levels of total IgE antibodies in all experimental groups whereas no induction of IgE antibodies was noted for the negative control group (Fig. 2a, b, day 0). Of note, total IgE levels were well balanced among the groups before they received different forms of ASIT or no treatment

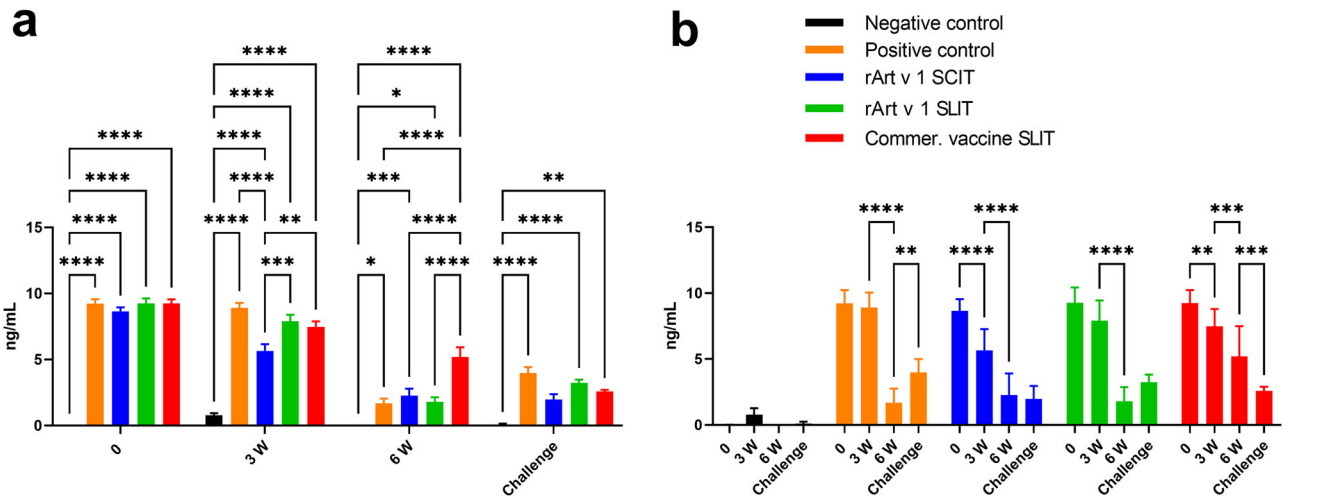

Fig. 2 | Inter- and intra-group- as well as time-wise comparison of total IgE levels. **a** Comparison of total IgE levels (y-axes) at different time points (week 0, 3, 6 ASIT and after challenge) (x-axes) between the different treatment groups. **b** Comparison of total IgE levels (y-axes) at different time points (week 0, 3, 6 ASIT and after challenge) (x-axes) within each of the treatment groups. Differences in IgE antibody levels between groups were assessed using Tukey's multiple comparisons test. A *P*-value of <0.05 was considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

(Positive control) (Fig. 2a, b, day 0) indicating proper randomization of mice. Neither SCIT nor SLIT with rArt v 1-based vaccines increased the levels of total IgE antibodies throughout the observation period (Fig. 2b).

Already by the 3rd week of ASIT, there was a distinct decrease of total IgE antibodies as compared to the positive control group which had not received ASIT. In fact, a significant reduction in total IgE antibodies compared to the positive control group was observed in the rArt v 1 SCIT group, which was also significantly lower than in the rArt v 1 SLIT and commercial vaccine groups (Fig. 2a).

By the sixth week of ASIT total IgE antibody levels had strongly decreased and there were no significant differences observed between the rArt v 1-based SCIT and SLIT groups and the positive control group, except for the commercial vaccine group which showed significantly higher total IgE levels as compared to the positive control group and the other ASIT groups (Fig. 2a). After three rounds of allergen provocation, total IgE levels significantly increased in the positive control group but were markedly reduced in the group treated with the commercial vaccine compared to baseline levels at 6 weeks (Fig. 2b). Importantly, only the rArt v 1-based SCIT group achieved total IgE levels comparable to the negative control (Fig. 2a).

Significant induction of Art v 1-specific IgG_1 and IgG_{2a} by SCIT and SLIT with rArt v 1-based vaccines

By week 6 of ASIT we observed a significant induction of Art v 1-specific IgG_1 and IgG_{2a} antibody levels in the mice treated with rArt v 1-based SCIT and SLIT as well as in the commercial vaccine SLIT-treated group (Fig. 3a, b). The Art v 1-specific IgG_1 levels were higher in the rArt v 1 SCIT group than in the SLIT groups, but this difference was not significant. The rArt v 1 SCIT group exhibited the highest titers of Art v 1-specific IgG_{2a} antibodies, significantly surpassing those in the sublingual vaccine groups (Fig. 3b). The assessment of the IgG_{2a}/IgG_1 ratios suggested a predominant Th1 immune response in the rArt v 1 SCIT group, whereas it indicated a Th2 response for the SLIT groups and the unvaccinated sensitized mouse group (Fig. 3c).

Following allergen challenge (Fig. 3a), Art v 1-specific IgG_1 titers significantly increased only in the positive control group (compared to week 6 of ASIT), reaching levels where no significant difference from the ASIT groups was observed. Art v 1-specific IgG_{2a} titers remained significantly elevated across all ASIT groups, with the highest levels observed in mice treated with rArt v 1-based SCIT (Fig. 3b). The IgG_{2a}/IgG_1 ratio trend remained consistent, with a polarization toward a Th1 immune response observed in the rArt v 1-based SCIT group (Fig. 3c).

SLIT vaccines induce higher levels of Art v 1-specific IgA in the lungs as compared to SCIT

It has been reported that SLIT induces higher levels of allergen-specific IgA than SCIT in allergic patients[16]. Accordingly, we were interested to study Art v 1-specific IgA levels in the lungs and serum samples of the different treatment groups. Results obtained showed that only the rArt v 1-based SLIT vaccine and commercial vaccine SLIT led to a significant induction of allergen-specific IgA antibodies in the lungs (Fig. 3d). A similar trend was observed for Art v 1-specific IgA in the serum samples of mice; however, the levels were not significantly different in any of the ASIT groups compared to the negative control (data not shown).

rArt v 1-based SCIT Induces a Th1-polarized IFN-γ response

To assess the Th1-polarized cellular immune response, the level of IFN-γ production was measured in splenocyte suspensions from all groups at week 6 post-ASIT following restimulation with rArt v 1 protein (Fig. 3e). All ASIT groups produced Art v 1-induced IFN-γ; however, only the rArt v 1-based SCIT group exhibited a Th1 response, characterized by this cytokine, that was significantly higher compared to the positive control.

rArt v 1-based SCIT and SLIT vaccines reduce lung inflammation but only SCIT suppresses late phase allergic responses

The effects of ASIT in the mouse groups on late phase allergic symptoms were assessed through ear swelling testing whereas effects on airway inflammation/response was investigated by allergen inhalation followed by methacholine provocation, and histological analysis of lung pathology.

The ear swelling test was conducted at week 0 and at week 6 after ASIT as well as after respiratory allergen challenge. At baseline (day 0), the ear swelling test confirmed successful sensitization to *Artemisia vulgaris* pollen in the experimental groups which had been sensitized. Auricle thickening in response to allergen injection was significantly higher compared to the negative control group and there were no significant differences among the sensitized groups indicating proper randomization of groups (Fig. 4a). After completion of ASIT, ear swelling in the experimental groups, including the positive control, remained significantly higher than in the negative control group (Fig. 4a, 6 W), except for the mice treated by rArt v 1-based SCIT.

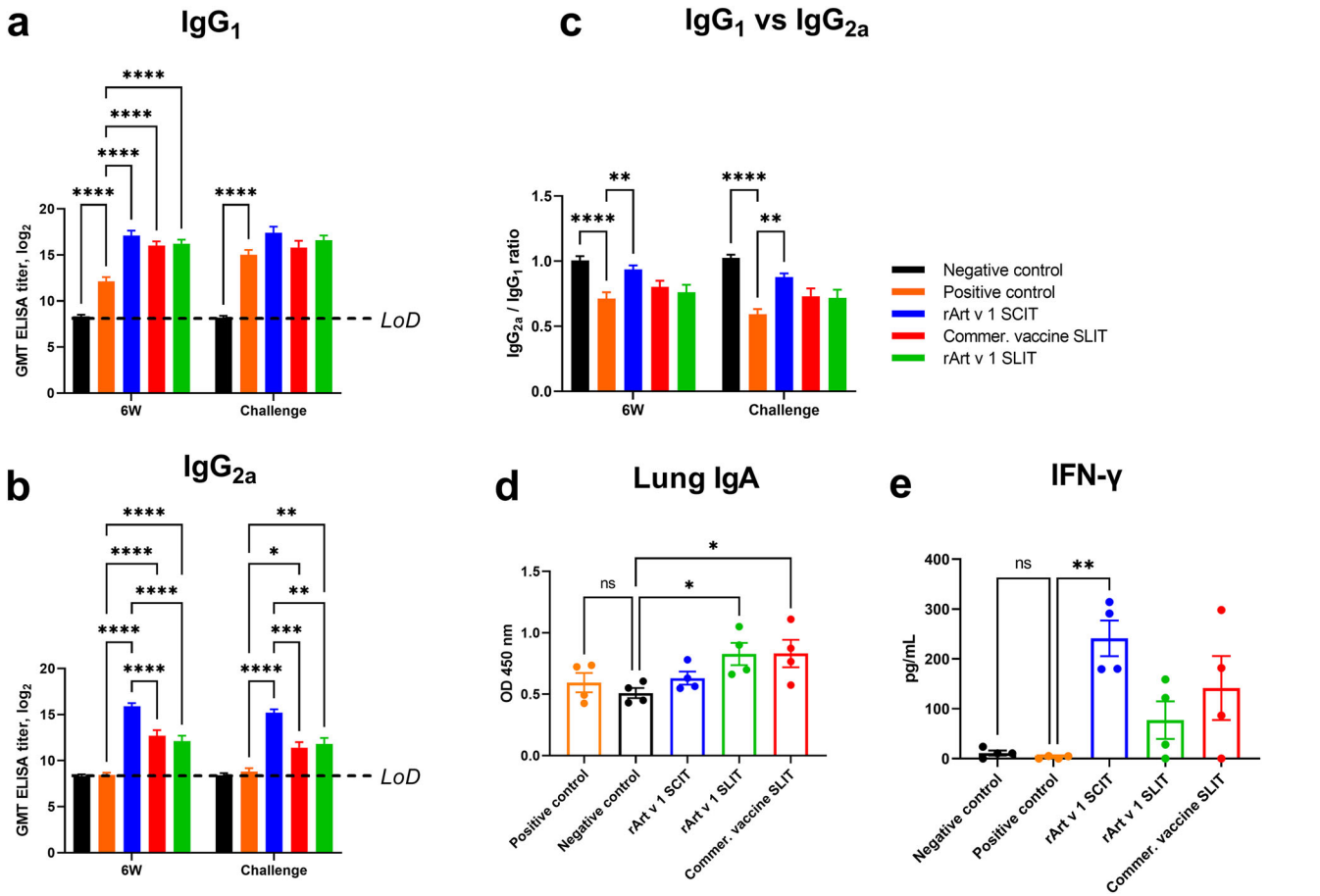

Fig. 3 | Comparison of allergen-specific IgG_1, IgG_{2a} and IgA and IFN-γ production levels in the different mouse groups. Antibody response following ASIT. rArt v 1-specific IgG_1 (**a**) and IgG_{2a} (**b**) log_2 titers (y-axes) in the different treatment groups assessed at week 6 of ASIT. **c** IgG_1/IgG_{2a} ratios (y-axis) in the different treatment groups (x-axis). **d** rArt v 1-specific lung IgA levels (y-axis) in the different treatment groups (x-axis). **e** rArt v 1-induced IFN-γ production levels (y-axis) in the different treatment groups (x-axis). LoD: Level of detection. Differences in antibody titers between groups were assessed using Tukey's multiple comparisons test. A *P*-value of <0.05 was considered statistically significant. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns not significant.

Notably, unlike the rArt v 1-based SLIT group, the commercial SLIT vaccine group exhibited a significant increase in ear swelling after completing the full ASIT course, exceeding both baseline (day 0) levels and the responses observed in the positive control and rArt v 1-based SCIT groups. After respiratory allergen challenge, the greatest increase in ear swelling was observed in the positive control group. While the SLIT groups exhibited slightly lower ear swelling compared to the positive control, it remained significantly higher than that of the negative control group. Notably, only the rArt v 1-based SCIT group showed no significant difference when compared to the negative control group (Fig. 4a). Interestingly, a trend toward a noticeable reduction in ear swelling after allergen challenge compared to post-ASIT levels was observed exclusively in the group treated with the commercial SLIT vaccine.

One day after the respiratory allergen provocation mice from all groups were exposed to methacholine inhalation to assess airway responsiveness using a whole-body plethysmograph. ASIT with both rArt v 1-based vaccines and the commercial vaccine significantly reduced airway responsiveness compared to the positive control group, with levels comparable to those of the negative control group (Fig. 4b).

Histological analysis of lung samples after allergic provocation was conducted using a scoring scale based on perivascular and peribronchial inflammation, the presence of eosinophils in inflammatory foci, and goblet cell metaplasia in the bronchi. The highest level of pathological changes was observed in the lungs of the positive control group (Fig. 4c), where 4 out of 5 mice exhibited marked lymphocytic peribronchial inflammation with numerous eosinophils, and one mouse showed moderate peribronchial inflammation with occasional eosinophils (Fig. 4d). Goblet cell metaplasia was widespread in 4 out of 5 mice, with single goblet cells observed in 1 mouse. The lung inflammation score for the positive control group averaged 6.4 out of 7 points.

In the ASIT groups, lung inflammation varied slightly depending on the vaccine used, with foci of mild lymphocytic peribronchial inflammation and occasional moderate inflammation (Fig. 4d). Single eosinophils were present in inflammatory foci, and goblet cells were rare in the bronchi. The average lung inflammation scores in the ASIT groups ranged from 1.6 to 3.8 points and thus were significantly lower than that in the positive control group. The rArt v 1-based SCIT group demonstrated the highest therapeutic efficacy, with minimal lung inflammation levels comparable to those of the negative control group (Fig. 4c, d).

Discussion

According to extensive meta-analysis data[15] SCIT and SLIT are effective in reducing respiratory symptoms of allergy. However, there is only one clinical study which has compared SCIT and SLIT and investigated immunological parameters underlying the two forms of ASIT[16]. To the best of our knowledge, our study is the first to compare in an experimental mouse model the effects of SCIT and SLIT on respiratory symptoms and to investigate underlying immune responses. Our study is also unique because it compares SCIT and SLIT-based on a single recombinant allergen, the major mugwort allergen, rArt v 1, with SLIT based on a mugwort pollen extract which is assumed to resemble the majority of the natural allergen repertoire of mugwort pollen. In this context, it should be mentioned that

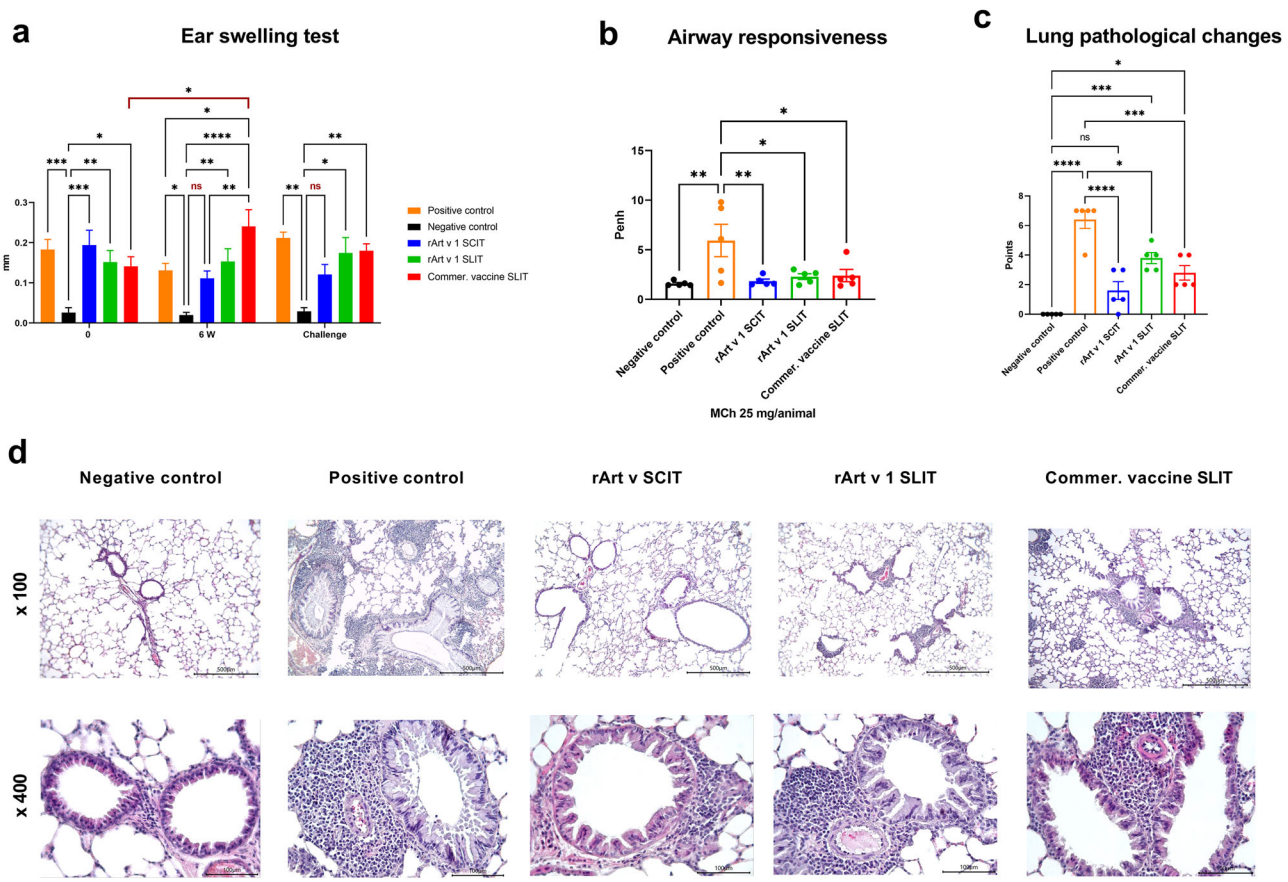

Fig. 4 | Effects of different forms of ASIT on allergen-induced ear swelling, airway responsiveness and allergen-induced lung inflammation. a Allergen-induced ear swelling (y-axes: mm) assessed for mice having received different forms of ASIT, positive and negative control at different time points (week 0, 6 and after allergen challenge) (x-axis). **b** Airway responsiveness measured as Penh (y-axis) in mice having received different forms of ASIT, positive and negative control at different time points (week 0, 6 and after allergen challenge) (x-axis). **c** Allergen-induced lung inflammation (y-axis: Points) scored in mice having received different forms of ASIT, positive and negative control at different time points (week 0, 6 and after allergen challenge) (x-axis). Panel (**d**) shows representative lung histology images from each of the mouse groups at 100x and 400x magnification. Differences between groups were evaluated using Tukey's multiple comparisons test. NS not significant. A *P*-value of <0.05 was considered statistically significant. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

our mouse model is clinically relevant because mice were sensitized with natural mugwort pollen extract and the effects of treatment on ear swelling, airway responses and airway inflammation induced by natural pollen extract were investigated. Several important results were obtained. First, it could be shown that rArt v 1-based SCIT and SLIT reduced airway responses and airway inflammation induced by natural allergen extracts indicating that rArt v 1-based vaccines comprised the relevant epitopes of the natural mugwort pollen extract.

The findings demonstrate that rArt v 1-based SLIT, as evaluated by efficacy parameters such as airway responsiveness and pathological changes in the lungs, provided a therapeutic effect comparable to that of a commercial vaccine derived from natural *Artemisia vulgaris* pollen extract containing an equivalent amount of nArt v 1. This is notable given that the commercial vaccine includes a full complement of major and minor proteins. Both vaccines showed similar profiles for rArt v 1-specific IgG_1, IgG_{2a}, and secretory IgA antibodies in the lungs after the full 6-week SLIT course. The most notable difference between the rArt v 1-based SLIT and the commercial SLIT vaccine was that the rArt v 1-based vaccine did not significantly increase total IgE antibody levels, nor did it significantly enhance sensitization, as measured by the ear swelling test, in mugwort pollen-sensitized mice following ASIT. These findings align with another study[19], which demonstrated reduced allergenicity of the recombinant full-length rArt v 1 protein expressed in *E. coli* in skin and nasal provocation tests on *Artemisia* pollen-sensitized patients, compared to nArt v 1 and *Artemisia* pollen extracts.

Another important finding of our study was that SCIT and SLIT differently induced allergen-specific IgG and IgA responses. As reported in clinical trial on allergen-specific IgG_{2a}/IgG_1 ratios in grass pollen allergic patients[16], we found that SCIT induced more allergen-specific IgG than SLIT whereas SLIT induced more allergen-specific IgA. In addition, SCIT performed with rArt v 1 combined with ISA-51 adjuvant induced high allergen-specific IgG levels and a more Th1-prone immune response whereas SLIT seemed to induce a more Th2 immune responses based on allergen-specific IgG_1/IgG_{2a} ratios of vaccine induced antibodies and IFN-γ production levels. This may be due to the ISA-51 adjuvant in the vaccine formulation, which creates a water-in-oil emulsion (with antigen droplets up to 2 µm in size encapsulated within the oil phase). The selection of the ISA-51 adjuvant for our allergy vaccine was supported by in vivo studies involving Art v 1-based formulations, where the vaccine formulated with ISA-51 appeared more effective in the immunotherapy of *Artemisia* pollen-induced bronchial asthma when compared to formulation with other adjuvants including aluminum hydroxide, squalene-water emulsion [SWE]) adjuvants, and some novel adjuvants[20]. The ISA-51 formulated vaccine (PollenVax) polarized the immune response towards a Th1 profile, as evidenced by an increased ratio of allergen-specific IgG_{2a}/IgG_1 and an increased IFN-γ/IL-4 ratio[21]. ISA-51 emulsion ensures a slow release of the rArt v 1 protein at the subcutaneous injection site, and at least in theory this may reduce the risk of immediate-type hypersensitivity reactions to the injected allergen. During necropsy of mice from the rArt v 1 SCIT group at the 6th week of ASIT, undissolved remnants of the ISA-51 emulsified vaccine (approximately 50–60% of the

total injected volume) were observed at the subcutaneous injection site in all mice of this group. However, no macroscopic pathological changes were detected in the subcutaneous tissue surrounding the vaccine residues (data not shown). ISA-51 has previously been tested in vaccines for cancer, HIV/AIDS, and malaria[22]. Additionally, a therapeutic lung cancer vaccine containing ISA-51, administered in a four-dose regimen at one-week intervals, has been registered in seven countries, including Kazakhstan[23]. ISA-51 has been evaluated in influenza vaccine trials[24,25] but this is the first instance of ISA-51 being used as an adjuvant in ASIT.

Of note, rArt v 1-based SCIT as well as rArt v 1- and allergen extract-based SLIT reduced IgE induction following respiratory allergen exposure as it was found in earlier human studies[26,27]. Unlike tablet formulation used for grass pollen SLIT[28], the SCIT and SLIT vaccines did not induce increases of total IgE production.

The rArt v 1-based vaccine formulated with ISA-51 adjuvant demonstrated efficacy against bronchial asthma in our mouse model with an ultrashort SCIT regimen of just four weekly subcutaneous injections[20], which used 6 times less rArt v 1 protein compared to the SLIT regimen (147.7 μg over 42 daily doses).

The superior efficacy of the subcutaneous rArt v 1-based vaccine compared to the sublingual version aligns with findings from comparative clinical studies of these two ASIT methods[16]. Specifically, the level of pathological changes in the lungs of mice in the rArt v 1 SCIT group was 57.8% and 42.8% lower than those in the rArt v 1-based SLIT and commercial vaccine SLIT groups, respectively. Previously, the rArt v 1-based vaccine formulated with ISA-51 adjuvant (PollenVax) also demonstrated a 43% greater efficacy in treating bronchial asthma caused by *Artemisia vulgaris* pollen compared to the commercial preparation CLUSTOID®, which contains a processed *Artemisia vulgaris* extract with an aluminum hydroxide adjuvant (ROXALL Medizin GmbH, Hamburg, Germany)[21].

The limitations of this study include the small group sizes, the performance of study in inbred mice and the lack of replication, which constrain the generalizability of the findings. Additionally, it remains uncertain whether the mouse model accurately mirrors *Artemisia* pollen allergy in humans, given the significant differences between murine and human immune responses, which could impact the study's outcomes. A further limitation of this study is the lack of significant differences between the active groups which could have been influenced by variations in antigen doses, timing of administrations, and use of the ISA-51 adjuvant. Another limitation is that the cellular response was assessed solely based on IFN-γ production, with no data being able to be generated on other relevant Th1, Th2, or Treg cytokine responses to rArt v 1, which might have provided further mechanistic insights. Future studies should include a broader cytokine profiling to better understand the immune mechanisms underlying the observed therapeutic effects. Yet another limitation was the inability to perform SLIT in mice using a commercial vaccine produced in tablet form. Unlike the liquid formulation used in this study (containing the active ingredient), the tablet form allows for prolonged retention and resorption in the sublingual region which may result in greater efficacy. A further limitation is the lack of direct evidence demonstrating that recombinant Art v 1 produced in *E. coli* shares identical protein folding and IgE reactivity with the glycosylated natural Art v 1 which will need to be looked at in the future. Yet another limitation is the use of a single dose of methacholine (25 mg/mL) in the airway hyperresponsiveness (AHR) assay, chosen for its established ability to reliably induce measurable airway responses in sensitized murine models of asthma. While this approach allowed for the assessment of treatment efficacy under conditions of significant bronchoconstriction and minimized animal usage and stress, it meant a dose-response curve for methacholine could not be generated. Future studies will incorporate a range of methacholine concentrations to construct dose-response curves, although we don't believe this would affect the overall study conclusions. Another limitation is the absence of bronchoalveolar lavage fluid (BALF) analysis to quantify inflammatory cells, such as eosinophils, neutrophils, and lymphocytes, which could otherwise have provided valuable insights into airway inflammation and immune cell recruitment. Instead, lung inflammation was assessed primarily through histological scoring of peribronchial and perivascular inflammatory infiltrates, eosinophil presence, and goblet cell metaplasia, focusing on structural and cellular changes associated with allergen-induced airway remodeling and pathology. While these methods effectively evaluated lung tissue changes, the inclusion of BALF analysis in future studies would complement the histological findings and offer a more comprehensive understanding of the inflammatory response. While this study quantified IgG_1, IgG_{2a}, and IgA antibody levels to assess their induction by ASIT and their correlation with observed therapeutic effects, it was unable to directly evaluate the functional capacities of these antibodies. Specifically, the ability of antibodies to block IgE-allergen interactions using competitive ELISA or basophil degranulation tests, was unable to be assessed. Future studies will aim to address this limitation by incorporating functional evaluations. Yet another limitation is the absence of Periodic Acid–Schiff (PAS) staining to assess mucus production in the lung histopathology analysis. While hematoxylin and eosin (H&E) staining effectively demonstrated structural changes and inflammation, PAS staining could have provided additional insights into mucus production, which is a critical feature of airway remodeling in allergic responses. Incorporating PAS staining in future studies would strengthen the findings by confirming the impact of SLIT and SCIT on mucus production. Another limitation is the absence of a detailed quantification of eosinophil numbers in the H&E-stained lung sections. While a qualitative scoring approach was used to approximate the presence of eosinophils and assess inflammation, a more precise count and statistical comparison between groups would provide a more detailed and quantitative assessment of the inflammatory response. Incorporating specialized staining or alternative methods such as flow cytometry in future studies would address this limitation. Another limitation is the potential for transient IgE responses following bronchial provocation, as highlighted by the results in Fig. 2. Although the anti-Art v 1 IgE did not increase in the immunotherapy groups following the final respiratory boost, it is possible that the IgE response observed was part of a transient reaction. Without a time-course analysis, it is challenging to determine whether the assay measured an accelerated or delayed response, as well as the actual size and duration of the IgE response. This limitation underscores the need for future studies incorporating multiple time points to fully characterize the kinetics of IgE responses after allergen provocation. A final limitation is the lack of irrelevant allergen-alone controls to distinguish allergen-specific effects from allergen non-specific effects induced by repeated allergen administration in sensitized mice. Including an irrelevant allergen controls, such as birch extract, could have simplified the interpretation of allergen specificity in the observed results.

In conclusion, the vaccine based on the recombinant major protein rArt v 1, administered through a 6-week SLIT regimen, did not lead to an increase in total IgE levels in *Artemisia vulgaris* pollen-sensitized mice, unlike the commercial vaccine based on natural *Artemisia vulgaris* pollen extracts. At the same time, it provided a comparable and significant reduction in the symptoms of bronchial asthma after provocation with *Artemisia vulgaris* pollen extract. These results support further development of a SLIT vaccine based on the recombinant rArt v 1 protein. However, the highest efficacy in the immunotherapy of bronchial asthma in the mouse model was achieved with the rArt v 1-based vaccine formulated with the ISA-51 adjuvant, delivered through an ultrashort subcutaneous SCIT regimen.

Methods

Cloning and expression of recombinant Art v 1

The recombinant plasmid containing the codon-optimized gene for the *Artemisia vulgaris* major pollen allergen Art v 1 was constructed at AtaGenix laboratories, China. The synthetic Art v 1 gene (GenBank ID PQ223694.1) was inserted into the pET28b vector using NdeI and XhoI restriction sites. The nucleotide sequence was verified following the Dye Terminator Cycle Sequencing protocol (Applied Biosystems). The plasmid was transformed into electrocompetent *Escherichia coli* C41 (DE3) cells (Sigma, St. Louis, MO, USA). For rArt v 1 expression, a single transformant was cultured in 10 mL LB medium containing 50 μg/mL kanamycin at 37 °C with shaking at

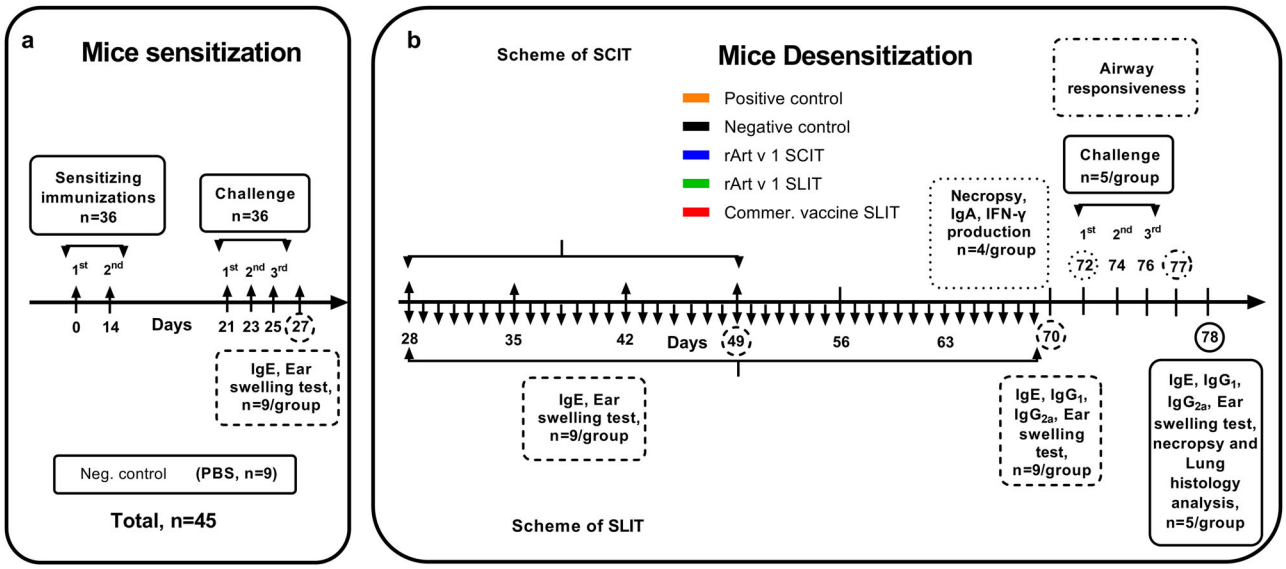

Fig. 5 | Study design. The schematic illustration shows the sensitization (**a**) of mice with *Artemisia vulgaris* pollen extract and their subsequent desensitization (**b**) using rArt v 1-based vaccine formulations administered via sublingual or subcutaneous routes. SCIT subcutaneous immunotherapy, SLIT sublingual immunotherapy, Positive and Negative control (Table 1).

250 rpm overnight. This culture was then used to inoculate 1 L of LB medium containing 50 μg/mL kanamycin and incubated at 37 °C with shaking at 250 rpm for 4 h. When the OD600 reached 0.8, IPTG was added to a final concentration of 0.125 mM, and the culture was grown at 16 °C with shaking at 250 rpm overnight. After protein induction, cells were centrifuged, resuspended in 60 mL buffer (20 mM sodium phosphate, 500 mM NaCl, 2% Triton X-100, 5% glycerol, 30 mM imidazole, pH 7.4), and lysed by sonication (three cycles of 2 min with 2-min intervals). The lysate was then cleared by centrifugation at 16,000 × *g* for 30 min at 4 °C.

Purification of recombinant protein

The recombinant protein was purified using the Akta Start system (Cytiva, Uppsala, Sweden) with Immobilized Metal Ion Affinity Chromatography. The cleared lysate was applied to a HisTrap FF 1 mL column (Cytiva), washed with 20 mM sodium phosphate, 500 mM NaCl, and 30 mM imidazole (pH 7.4), and eluted with a linear gradient up to 500 mM imidazole. Fractions were analyzed by SDS-PAGE, and those fractions containing pure rArt v 1 were pooled and desalted using a HiTrap 5 mL column (Cytiva) against PBS (pH 7.4). Bacterial endotoxins were removed using Pierce™ High-Capacity Endotoxin Removal Spin Columns, 0.5 mL (Thermo Fisher Scientific, Carlsbad, CA, USA), following the manufacturer's instructions. The purified protein was aliquoted into 1 mL portions and stored at −80 °C. Protein concentration was determined using the Bradford assay (Sigma-Aldrich, SL, USA), and endotoxin levels were quantified with the Chromogenic Endotoxin Quant kit (Invitrogen, Rockford, IL, USA).

SDS-PAGE and immunoblotting

Bacterial lysates and purified rArt v 1 protein were analyzed using SDS-PAGE, with 4–12% Bis-Tris acrylamide gels and MES SDS (Thermo Fisher Scientific) running buffer. Proteins were stained with Coomassie Brilliant Blue R-250 for visualization. For immunoblotting, proteins were separated on acrylamide gels and transferred onto PVDF membranes using the iBlot2 system (Invitrogen). After protein transfer, the PVDF membrane was briefly incubated for 1 h at room temperature (RT) in blocking buffer (1× PBS, containing 0.1% (v/v) Tween 20 and 3% BSA). Then, the membrane was probed with 6×His Tag Monoclonal antibody (Invitrogen) or *Artemisia vulgaris* major pollen allergen Art v 1 Polyclonal antibody (Invitrogen) using a dilution of 1:5000 in blocking buffer for 16 h at 4 °C, washed three times with PBS-Tween 20 (1× PBS supplemented with 0.1% (v/v) Tween 20) and incubated in HRP-conjugated goat anti-Rabbit IgG secondary antibody (Invitrogen) for 2 h at 4 °C. After three washes with PBS-Tween 20, the membrane was developed by utilizing the 1-Step TMB-Blotting substrate (Invitrogen) according to the manufacturer's instructions.

Vaccine preparation

To prepare the sublingual rArt v 1-based vaccine (rArt v 1 SLIT), a stock solution of rArt v 1 protein at 5 mg/mL was diluted with PBS to final concentrations of 0.001, 0.01, 0.1, 1, and 10 μg/0.1 mL. The diluted rArt v 1 solutions were sterilely aliquoted into 1 mL cryovials, stored at −70 °C, and thawed on the day of administration.

The commercial vaccine (Burly, Almaty, Kazakhstan) is typically produced in tablet form and packaged in blisters. However, for this study, the manufacturer provided a bulk form of the vaccine as a native *Artemisia vulgaris* pollen extract, with a concentration of 10,000 PNU/mL (nArt v 1—100 μg/mL) (batch 190823, valid until 08.2025). This extract was diluted with PBS to final concentrations of 0.1, 1, 10, 100, and 1000 PNU/0.1 mL (commercial vaccine SLIT). The preparations were stored at 2–8 °C until use.

To prepare the PollenVax vaccine, the stock solution of rArt v 1 protein was diluted with PBS to a concentration of 44 μg/mL (rArt v 1 SCIT) and then emulsified with Montanide ISA-51 VG oil adjuvant (a water-in-oil emulsion, Seppic, France) in a 50:50 ratio (by weight), following the manufacturer's guidelines. The emulsification process was carried out using IKA® ULTRA-TURRAX® disperser tubes with a DT-50-M-gamma rotor-stator element (IKA®-Werke GmbH & Co. KG, Germany), as previously described[21]. The final vaccine was aliquoted into vials and stored at 2–8 °C until use.

Sensitization and ASIT of mice

Sensitization of mice was performed as previously described[20,21]. Briefly, 8–12-week-old specific pathogen-free (SPF) male BALB/c mice (36 mice in total) were intraperitoneally injected twice at 14-day intervals with a dose of 1000 PNU/200 μL (Burly) *Artemisia vulgaris* pollen extract sorbed on aluminum hydroxide (InvivoGen, San Diego, CA, USA; 1 mg/mouse). Negative control mice ($n = 9$) were similarly injected with PBS (200 μL). On day 21, all mice were subjected to three times provocation at daily intervals (on days 21, 23, 25) by inhalation of *Artemisia vulgaris* pollen extract (1000 PNU/group) according to the previously described method[19], as well as intranasal injection under ketamine-xylazine anesthesia of allergen at a dose of 200 PNU/20 μl or the same volume of PBS (negative control) (Fig. 5a). On day 27, an ear swelling test was conducted as previously described[20], and blood samples were collected to measure total IgE levels (Fig. 5a). To

Table 1 | Allergen-specific immunotherapy regimens

Group	Route and frequency of administration	Conditional ASIT phase	Vaccine administration sequence number (days by study design)	Vaccine dose, mL (rArt v 1 dose) per mouse per administration
rArt v 1 SLIT	Sublingually, daily for 42 days	Primary	1–7 (28–34)	0.1 mL (0.001 μg)
			8–14 (35–41)	0.1 mL (0.01 μg)
		Main	15–21 (42–48)	0.1 mL (0.1 μg)
			22–28 (49–55)	0.1 mL (1 μg)
			29–35 (56–62)	0.1 mL (10 μg)
		Maintenance	35–42 (63–69)	0.1 mL (10 μg)
Commercial vaccine SLIT	Sublingually, daily for 42 days	Primary	1–7 (28–34)	0.1 mL (0.1 PNU)
			8–14 (35–41)	0.1 mL (1 PNU)
		Main	15–21 (42–48)	0.1 mL (10 PNU)
			22–28 (49–55)	0.1 mL (100 PNU)
			29–35 (56–62)	0.1 mL (1000 PNU)
		Maintenance	35–42 (63–69)	0.1 mL (1000 PNU)
rArt v 1 SCIT	Subcutaneously, 4 times at 7-day intervals	Primary	1 (28)	0.1 mL (2 μg)
			2 (35)	0.2 mL (4 μg)
		Main	3 (42)	0.4 mL (8 μg)
		Maintenance	4 (49)	0.4 mL (8 μg)
Positive control	Sublingually, daily for 42 days	PBS	1–42 (28–69)	0.1 mL
Negative control	Sublingually, daily for 42 days	PBS	1–42 (28–69)	0.1 mL

The cumulative doses of a full course of ASIT for the vaccines studied were: rArt v 1 SCIT—1.1 mL (22 μg rArt v 1)/mouse; rArt v 1 SLIT—4.2 mL (147.777 μg rArt v 1)/mouse; Commercial vaccine SLIT—4.2 mL (14777.7 PNU)/mouse. PBS - phosphate-buffered saline

perform the ear swelling test, 10 μl (100 PNU) of *Artemisia vulgaris* pollen extract or PBS (negative control) was injected into the right auricle. After 1.5–2 h, auricle thickness was measured with an electronic micrometer (MCC-25 DSWQ0-100II, China). Results are expressed as the thickness difference (mm) between the right (injected) and left (non-injected) auricles.

On day 28, following randomization by weight, the sensitized mice (Fig. 5b) were divided into five groups, with each group consisting of 9 animals. Three groups received SLIT or SCIT according to the vaccines and regimens outlined in Table 1. To increase the contact time of the vaccine with the sublingual area and to reduce swallowing, mice were held in a supine position for 60 s after the vaccine was administered sublingually. The positive and negative control groups were administered PBS following the sublingual vaccine regimen. On days 49 (corresponds to 3 weeks of ASIT) and 71 (corresponds to 6 weeks of ASIT), all mice underwent an ear swelling test, and blood samples were collected to measure total IgE, Art v 1-specific IgG_1, IgG_{2a}, IgA levels were measured at 6 weeks of ASIT. On day 72, four mice per group were necropsied to collect lungs for preparing 20% lung suspensions to test for Art v 1-specific IgA antibodies, and spleen samples were collected to assess cellular immune response factors, including Art v 1-specific IFN-γ production. On the same day (day 72) and days 74 and 76 the remaining mice ($n = 5$/group) underwent allergen provocation. Mice in the negative control group ($n = 5$) received PBS instead of allergen. On day 77, all mice were assessed for airway responsiveness in a WBP-M Whole-body Plethysmography System chamber (Shanghai TOW Intelligent Technology Co. Ltd., Shanghai, China) after methacholine (25 mg/mouse) or PBS (negative control) inhalation. Airway resistance was measured and expressed as an enhanced pause (Penh) during a 5-min chamber stay. On the final day of the experiment (day 78), all mice underwent an ear swelling test, had blood samples taken to determine total IgE level, Art v 1-specific IgG_1 and IgG_{2a} titers, and were necropsied for histological lung analysis to assess inflammatory reactions.

Antibody response evaluation

Total IgE antibodies were measured using the ELISA MAX™ Standard Set Mouse IgE (BioLegend, USA) following the manufacturer's instructions, with results expressed in μg/mL.

The ELISA for IgG_1, IgG_{2a}, and IgA was conducted as previously described[20]. Briefly, 96-well microplates were coated with 5 μg/10 mL of rArt v 1 protein in ELISA Coating Buffer (BioLegend) and incubated overnight at 4 °C. On the following day, ELISA Assay Diluent (BioLegend) in PBS was added to the plates at 200 μL/well and incubated on a PST-60HL thermal shaker (BIOSAN, Latvia) for 1 h at RT. The plates were then washed four times with ELISA Wash Buffer (BioLegend). Mouse serum samples were serially diluted two-fold in ELISA Assay Diluent, beginning with a 1:250 dilution and continuing up to 1:8,192,000. From each dilution, 100 μL was added to the wells and incubated with continuous shaking for 1.5–2 h at RT. The secondary antibodies used were anti-mouse biotinylated detection antibodies for IgG_1 (1:1000, BioLegend) and IgG_{2a} (1:1000, BioLegend), each at a volume of 100 μL/well. To determine the presence of IgA antibodies, serum samples were diluted 1:5 with ELISA Assay Diluent, and lung suspensions were used undiluted. Anti-mouse biotinylated detection antibody for IgA (1:1000, BioLegend) was used for detection. Plates were washed four times and then incubated with horseradish peroxidase (HRP) streptavidin (BioLegend, 1:1000, 100 μL/well) for 30 min at RT with shaking. After washing five times, TMB substrate (BioLegend, 100 μL/well) was added. The color reaction was stopped by adding 2.5 M H_2SO_4 (100 μL/well), and the optical density (OD) was measured at a wavelength of 450 nm with a reference wavelength of 630 nm on a Stat Fax 2100 analyzer (Awareness Technology). The cut-off values for IgG_1, IgG_{2a} titers were determined by calculating the average OD of the wells containing only Assay Diluent (blank) plus three standard deviations.

Assessment of cellular immune response by IFN-γ production

Harvested spleens were mechanically processed into single-cell suspensions using Falcon® 70-μm cell strainers (Corning, USA) in disposable sterile Petri dishes (Piove di Sacco, Italy) with 10 mL of 3% fetal bovine serum (FBS; US Origin, Millipore Corp., Germany) in PBS. Erythrocytes in the suspensions were lysed using RBC lysis buffer (BioLegend). The resulting splenocytes were washed and cultured in a 5% CO_2 incubator (INCO 153, Memmert, Germany) at 37 °C in 24-well flat-bottom plates (Sigma-Aldrich, USA) at a density of 1×10^6 cells/well in 1 mL of RPMI-1640 + GlutaMax™ medium (Gibco) supplemented with 20 mM HEPES (Gibco), 10% heat-inactivated

Table 2 | Scoring system for evaluating pathological changes in mouse lungs

Evaluated trait	Points for the evaluated trait
Perivascular/peribronchial inflammation	0 – no changes; 1 – moderate inflammation; 2 – pronounced inflammation; 3 – severe inflammation
Presence of eosinophils in foci of perivascular/peribronchial inflammation	0 – absent; 1 – single eosinophils in the field with magnification (x1000); 2 – multiple eosinophils in the field with magnification (x1000)
Metaplasia of the Goblet cells in the bronchi	0 – absent; 1 – several Goblet cells are present in one or two bronchiolar profiles; 2 – numerous Goblet cells are present in bronchioles
Maximum score	**7**

FBS, and 1% Antibiotic-Antimycotic solution (Gibco). Cells were stimulated with 10 μg of purified rArt v 1 protein per well or left unstimulated (control). After 48 h of incubation, supernatants were collected and analyzed for interferon-gamma (IFN-γ) production using the ELISA MAX™ Deluxe Set Mouse IFN-γ (BioLegend) according to the manufacturer's instructions. The results were expressed as the difference (Δ) in cytokine concentrations (pg/mL) between wells stimulated with rArt v 1 and the unstimulated controls.

Lung histology analysis

Histologic analysis of mouse lungs was performed as previously described[21]. Briefly, mouse lungs were fixed in 10% formaldehyde, processed through graded isopropyl alcohol and xylene, and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin (H&E), then deparaffinized and cleared in ascending alcohol concentrations and xylene. The slides were examined under an Mshot MF52-N microscope at 100x, 400x, and 1000x magnifications, with 100 μm and 500 μm scale bars included in the photographs. Pathological changes in the lungs were scored using a point-based scale (Table 2).

Animal housing and ethical considerations

All work with laboratory animals was conducted at the vivarium of the M. Aikimbayev National Scientific Center for Especially Dangerous Infections (NSCEDI), Ministry of Health of the Republic of Kazakhstan. Care, housing, and feeding of SPF BALB/c mice were carried out as described previously[20,21]. The study was conducted in accordance with Protocol #16, dated 31.10.2022, approved by the Institutional Animal Care and Use Committee (IACUC) at NSCEDI. This study was conducted in full compliance with both national and international laws and guidelines pertaining to the handling of laboratory animals.

Mice were anesthetized via intraperitoneal (IP) injection of ketamine (50 mg/kg) and xylazine (10 mg/kg) in sterile PBS. Humane endpoint criteria, in accordance with IACUC-approved scoring parameters, were applied to determine when animals should be humanely euthanized. For terminal anesthesia prior to lung sample collection, mice were euthanized using an IP injection of ketamine (100 mg/kg) and xylazine (40 mg/kg), followed by cervical dislocation.

Statistical analysis

GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) was used for graphing and statistical analysis of the experimental data. Differences in antibody and IFN-γ levels, ear swelling test results, airway responsiveness, and lung pathology between animal groups were assessed using Tukey's multiple comparisons test. Geometric mean titers were calculated for IgG_1 and IgG_{2a} antibodies and expressed as $\log_2$. The detection limit for IgG_1 and IgG_{2a} titers was set at 8.0 $\log_2$. A P-value of <0.05 was considered statistically significant. All bars in the graphs represent the standard error of the mean (SEM).

Data availability

The nucleotide sequence of the synthetic gene encoding the major pollen allergen Art v 1 from *Artemisia vulgaris* has been deposited in the GenBank database with the accession number PQ223694.1. All relevant data are available upon reasonable request.

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Author contributions

Kr.T. and Kt.T. conceptualized the study. Data curation was performed by Kr.T., E.T., and G.R. Formal analysis was conducted by Kr.T., Kt.T., E.T., and G.R. Kr.T. secured the funding. Investigation was carried out by Kt.T., E.T., G.R., T.B., Y.H.L., G.F., and M.B. Methodology was developed by Kt.T., E.T., G.R., G.F., and M.B. Kr.T. managed the project. Resources were provided by Kr.T. Software support was given by Kt.T. and G.F. Supervision was overseen by Kr.T. and Kt.T. Validation was performed by Kt.T. Visualization was carried out by Kr.T., Kt.T., E.T., G.R., T.B., Y.H.L., G.F., and M.B. Kr.T. wrote the original draft. Kr.T., Kt.T., E.T., T.B., Y.H.L., G.F., M.B., and R.V. reviewed and revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

Competing interests

Kaissar Tabynov and Kairat Tabynov are affiliated with T&TvaX. Rudolf Valenta has received research grants from HVD Life-Sciences, Vienna, Austria and from WORG Pharmaceuticals, Hangzhou, China. He serves as consultant for HVD and WORG Pharmaceuticals. The authors with a Russian affiliation declare that they have prepared the article in their “personal capacity” and/or that they are employed at an academic/research institution where research or education is the primary function of the entity. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Additional information

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