Received: 14 December 2023 | Revised: 29 April 2024 | Accepted: 20 May 2024

DOI: 10.1111/all.16181

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI WILEY

ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

Mould allergen Alt a 1 spiked with the micronutrient retinoic acid reduces Th2 response and ameliorates Alternaria allergy in BALB/c mice

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Funding information
Danube Allergy Research Cluster DARC #8 of the Karl Landsteiner University Krems, Austria

Abstract

Background: We investigated the biological function of the mould allergen Alt a 1 as a carrier of micronutrients, such as the vitamin A metabolite retinoic acid (RA) and the influence of RA binding on its allergenicity in vitro and in vivo.

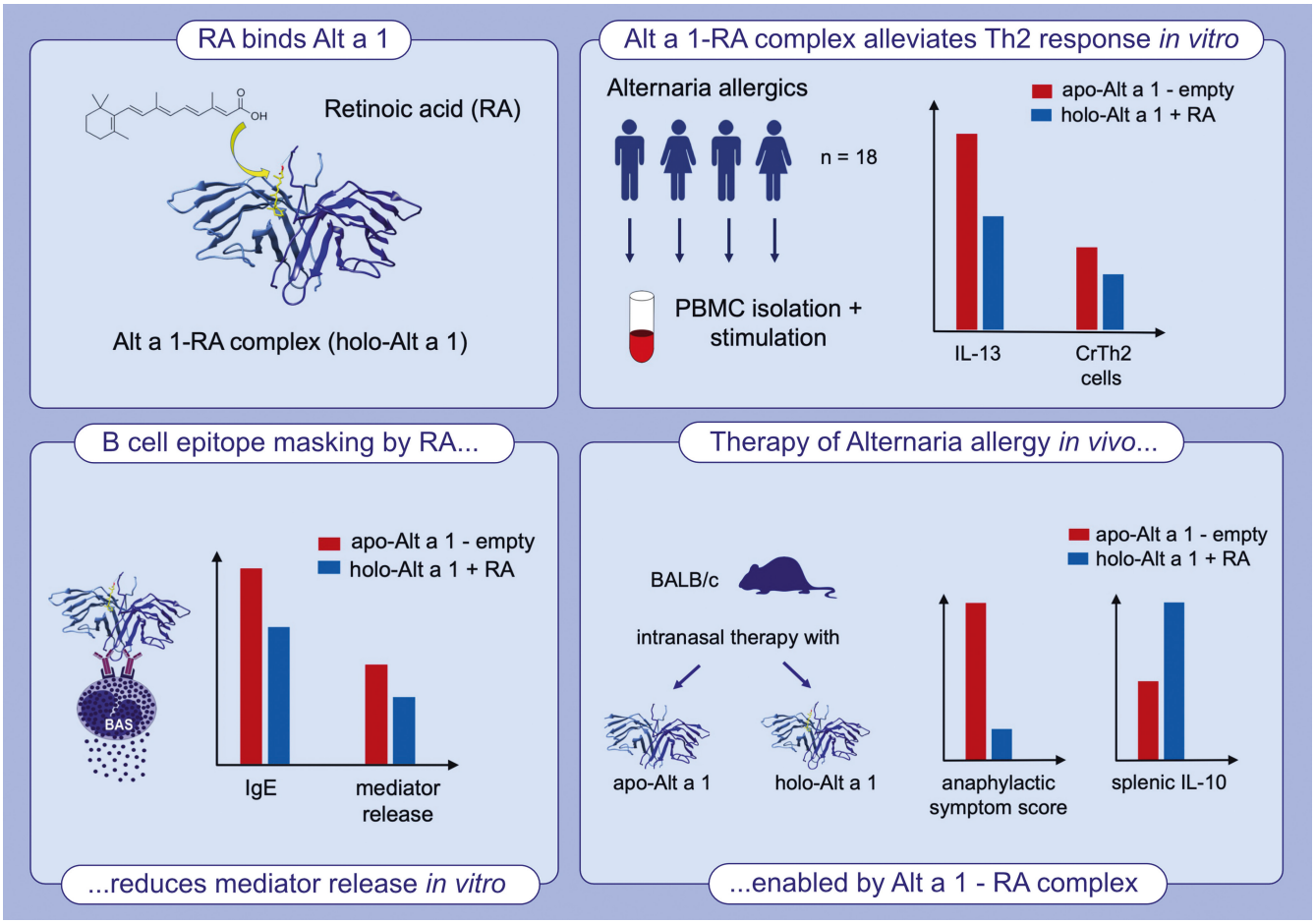
Methods: Alt a 1-RA complex formation was analyzed in silico and in vitro. PBMCs from Alternaria-allergic donors were stimulated with Alt a 1 complexed with RA (*holo*-Alt a 1) or empty *apo*-Alt a 1 and analyzed for cytokine production and CD marker expression. Serum IgE-binding and crosslinking assays to *apo*- and *holo*-protein were correlated to B-cell epitope analysis. Female BALB/c mice already sensitized to Alt a 1 were intranasally treated with *apo*-Alt a 1, *holo*-Alt a 1 or RA alone before measuring anaphylactic response, serum antibody levels, splenic cytokines and CD marker expression.

Results: In silico docking calculations and in vitro assays showed that the extent of RA binding depended on the higher quaternary state of Alt a 1. *Holo*-Alt a 1 loaded with RA reduced IL-13 released from PBMCs and CD3+CD4+CRTh2 cells. Complexing Alt a 1 to RA masked its IgE B-cell epitopes and reduced its IgE-binding capacity. In a therapeutic mouse model of Alternaria allergy nasal application of *holo*-Alt a 1, but not of *apo*-Alt a 1, significantly impeded the anaphylactic response, impaired splenic antigen-presenting cells and induced IL-10 production.

Conclusion: *Holo*-Alt a 1 binding to RA was able to alleviate Th2 immunity in vitro, modulate an ongoing Th2 response and prevent anaphylactic symptoms in vivo, presenting a novel option for improving allergen-specific immunotherapy in Alternaria allergy.

Abbreviations: ANS, 1-anilino-8-naphthalene sulfonate; BCA, bicinchoninic acid; E_{aff}, affinity energy; FCS, fetal calf serum; IL, interleukin; kDa, kilodalton; K_D, binding constant; MFI, mean fluorescence intensity; OD, optical density; PBMCs, peripheral blood mononuclear cells; PDB, protein data bank; RA, retinoic acid; RFUs, relative fluorescence units.

KEYWORDS
Alternaria alternata, fungal allergy, immunomodulation, retinoic acid, vitamin A



GRAPHICAL ABSTRACT

Complexing Alt a 1 from *Alternaria alternata* to RA masks IgE B-cell epitopes and reduces the IgE-binding capacity of the allergen. Alt a 1 complexed to RA leads to reduced Th2 response in vitro. Alt a 1 complexed to RA is able to modulate an ongoing Th2 immune response and to prevent anaphylactic symptoms in vivo.

Abbreviations: *holo*-Alt a 1, Alt a 1 complexed with RA; IgE, immunoglobulin E;IL, interleukin; PBMC, peripheral blood mononuclear cells; RA, retinoic acid.

1 | INTRODUCTION

Alternaria alternata belongs to the fungal phylum Ascomycota and is the most common saprophytic mould found worldwide.[1] As a plant pathogen it infests fruits and vegetables and is associated with agricultural landscapes.[2] In addition to its role as a plant pathogen, Alternaria represents an important source of fungal allergens, triggering allergic sensitization and in consequence the development of respiratory allergic diseases such as asthma.[3] High fungal allergen exposure causes a higher usage of asthma medication and is often associated with hospitalization of asthmatic patients.[4] In a clinical setting fungal allergy seems to be underdiagnosed, as sporulation of fungi in warm and humid weather overlaps with the major pollen season.[5] This was documented in a grass pollen immunotherapy trial in North America, where 22% of all enrolled patients had additional sensitization to fungi/mould.[6] Overall, worldwide estimations on prevalence of fungal allergy are up to 10% and positive skin prick tests for Alternaria and Cladosporium can be found in up to 14% of the adult European population.[3,7]

Alternaria alternata contains a single major allergen Alt a 1, which is responsible for IgE-binding in 90% of sensitized patients.[8,9] Alt a 1 is a 30 kDa homodimer protein found in mould spores that dissociates into 15 kDa subunits under reducing conditions or acidic pH.[10] Studies on high-resolution structure revealed a unique β-barrel comprised of 11 β-strands, forming a butterfly-like structure in the homodimer which is stabilized by an intermolecular disulphide bridge between Cys30 from both monomers and by hydrophobic and polar interactions. The

biological function of Alt a 1 has not yet been identified, although its localization in the cell wall of Alternaria spores together with functional and structural features indicate a role as ligand transport protein.[8,10]

We previously found a striking functional homology of the fungal Alt a 1 to mammalian and plant innate defence molecules, such as lipocalin 2 (LCN2) expressed at the respiratory barrier,[11] bovine lipocalin β-lactoglobulin (BLG), and pathogenesis-related plant protein Bet v 1, otherwise known as major birch pollen allergen. In all examples we were able to demonstrate that the proteins attain immunomodulatory capacity when complexed with micronutrient ligands, such as vitamins A and D or iron-siderophore complexes.[12,13] Ligand binding even transformed the generally allergenic proteins BLG and Bet v 1 into tolerogens.[14–19] This principle may open up novel dietary management strategies for allergy,[16] as in humans, micronutrient deficient conditions and anaemia are associated with atopy and development of allergic diseases especially in the paediatric population.[20–25]

In our previous studies with other allergenic proteins we concentrated on vitamin A and its main metabolite retinoic acid (RA) as ligand, which, next to vitamin D, is a key component for the development and homeostasis of the immune system. RA has a high counter-regulatory impact on inflammation and oxidative stress, two key factors in the clinical manifestation of allergic diseases.[20,26,27] Consequently, we here investigated whether the fungal protein Alt a 1 may act as a micronutrient carrier, and which influence this has on its immunogenicity in vitro and in a therapeutic mouse model of Alternaria allergy. Our results indicate that Alt a 1 has a high affinity to form complexes with RA. Thereby RA masks major IgE epitopes of the allergenic molecule, which leads to reduced IgE binding and reduced Th2 response in vitro. In fact, binding of Alt a 1 to RA was able to modulate an ongoing Th2 immune response and prevent anaphylactic symptoms in vivo. Spiking of Alt a 1 with RA may thus be a novel option for improving allergen-specific immunotherapy in Alternaria allergy.

2 | MATERIALS AND METHODS

2.1 | Alt a 1 expression in *Pichia pastoris*

Escherichia coli DH5α strain was grown in low-salt Luria Bertani broth and transfected with the pPICZα A vector (Invitrogen, Waltham, MA, USA) with the synthetic Alt a 1 gene (General Biosystems, Morrisville, NC, USA), then selected with Zeocin™ antibiotic (Invitrogen, USA).[28] The plasmid DNA was isolated and checked with PCR using AOX1 gene forward and reverse primers. Next, the plasmid DNA was transferred to the *Pichia pastoris* X33 strain using electroporation and cultured on yeast extract peptone dextrose medium (YPD) and +Zeocin™ plates. A positive Mut+ colony was selected using colony PCR and cultivated in expression medium. Finally, supernatant was collected and purified with anion exchange chromatography using 10 mM Na-phosphate buffer (pH 7.5). SDS-PAGE and circular dichroism (CD) spectroscopy were used to verify Alt a 1 protein purity, identity and secondary structure. For detailed information see online supplement.[14,28,29]

2.2 | Generation of *apo*- and *holo*-Alt a 1

Preparation of *apo*- and *holo*-Alt a 1, ANS binding assay and spectral analysis were done as previously described,[14] and detailed information of all methods can be found in the online supplement.

2.3 | In silico docking analysis

Protein-ligand docking calculations were carried out with the standalone version of AutoDock Vina v1.2.3-mod.[30–32] Binding modes with the lowest affinity energies (E_{aff}) were considered as best docking solutions and used to estimate the dissociation constant K_D for the protein-ligand complexes. Figures were prepared and rendered with UCSF Chimera.[33] For detailed information, also on B-cell epitope selection, see online supplement.[8,34,35]

2.4 | Alternaria-allergic patients and non-allergic participants

Experiments with blood samples and PBMCs from Alternaria-allergic patients and non-allergic study participants were approved by the institutional ethics committee of the Medical University of Vienna and conducted in accordance with the Helsinki Declaration of 1975 (EK number 1318/2021). All subjects gave their full written informed consent. PBMCs from Alternaria-allergic donors and non-allergic participants were stimulated with Alt a 1 complexed with RA (*holo*-Alt a 1) or empty *apo*-Alt a 1 and analyzed for cytokine production and CD marker expression with detailed description of all methods provided in the online supplement (Table S1).

2.5 | Animals

Female inbred 7-week-old BALB/c mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice were kept under conventional housing and treated according to European Union rules of animal care with the permission of the Austrian Ministry of Science (BMBWF 2021–0.550.211). Intraperitoneal Alt a 1-sensitized mice were intranasally treated with *apo*-Alt a 1, *holo*-Alt a 1 or RA alone, followed by a final intraperitoneal Alt a 1 challenge (Figure S1, S2). Anaphylactic response, serum antibody levels, splenic cytokines and CD marker expression were measured. For methodological details see online supplement.[14,36,37]

2.6 | Statistical analysis

Comparison of more than two groups was done by one-way ANOVA following Tukey multiple comparison test or Kruskal–Wallis test using GraphPad Prism 9 software (GraphPad, San Diego, CA, USA). Differences between two groups were analyzed using paired *t*-test.

Data are shown as mean +/− SEM. A value of $p < 0.05$ was considered significant.

3 | RESULTS

3.1 | Alt a 1 complexes with RA in silico and in vitro

In a first step we investigated RA binding to Alt a 1 by in silico docking analysis and in vitro binding assays (Figure 1). In silico docking calculations predicted that the strength of RA binding is dependent on the quaternary state of Alt a 1 with an affinity energy of −6.62 kcal/mol in monomeric Alt a 1, of −6.78 kcal/mol in dimeric Alt a 1 up to −8.53 kcal/mol in tetrameric Alt a 1, corresponding to dissociation constants K_D of 13.88, 10.56 and 0.548 μM, respectively (Figure 1A,B). These data were corroborated by in vitro ANS binding assay, showing a dose-dependent increase of fluorescence signal with increasing concentrations of RA (Figure 1C). Similarly, in spectral analysis addition of RA led to an increase in the absorption curve between 380 and 400 nm (Figure 1D), indicating complex formation of the mould allergen with the vitamin A metabolite. Both assays were conducted at pH 7.4, in which Alt a 1 is mainly present in dimeric or tetrameric form,[10] thereby providing the basis to use the Alt a 1-RA complex in the following in vitro and in vivo experiments in physiological conditions.

3.2 | *Holo*-Alt a 1 specifically alleviates the allergic Th2 immune response in vitro

Next, we investigated the influence of the Alt a 1-RA complex on the cellular immune response. For this purpose, we stimulated PBMCs from Alternaria-allergic donors with empty *apo*- or RA-loaded *holo*-Alt a 1 or RA alone and measured cytokine levels in supernatants as well as surface marker expression on immune cells (Figure 2; Figure S4; Table S1).

Compared to medium control levels, *apo*-Alt a 1 induced pro-inflammatory cytokine IL-6 and even reduced IL-13 levels (Figure 2A,D). On the other hand, *holo*-Alt a 1 like RA-treatment of cells significantly reduced the cytokines IL-6, TNFα and IL-10 compared to *apo*-Alt a 1 and medium controls, indicating direct RA-specific effects (Figure 2A,B, C). The Th2 cytokine IL-13 was only reduced in *holo*-Alt a 1 treated PBMCs (Figure 2D). Comparison to medium control revealed significantly higher IL-6, TNFα, IL-10 and

(A)

Ligand RA	Alt a 1 monomer	Alt a 1 dimer	Alt a 1 tetramer
Energy (kcal/mol)	- 6.62	- 6.78	- 8.53
K_d (μM)	13.88	10.56	0.548

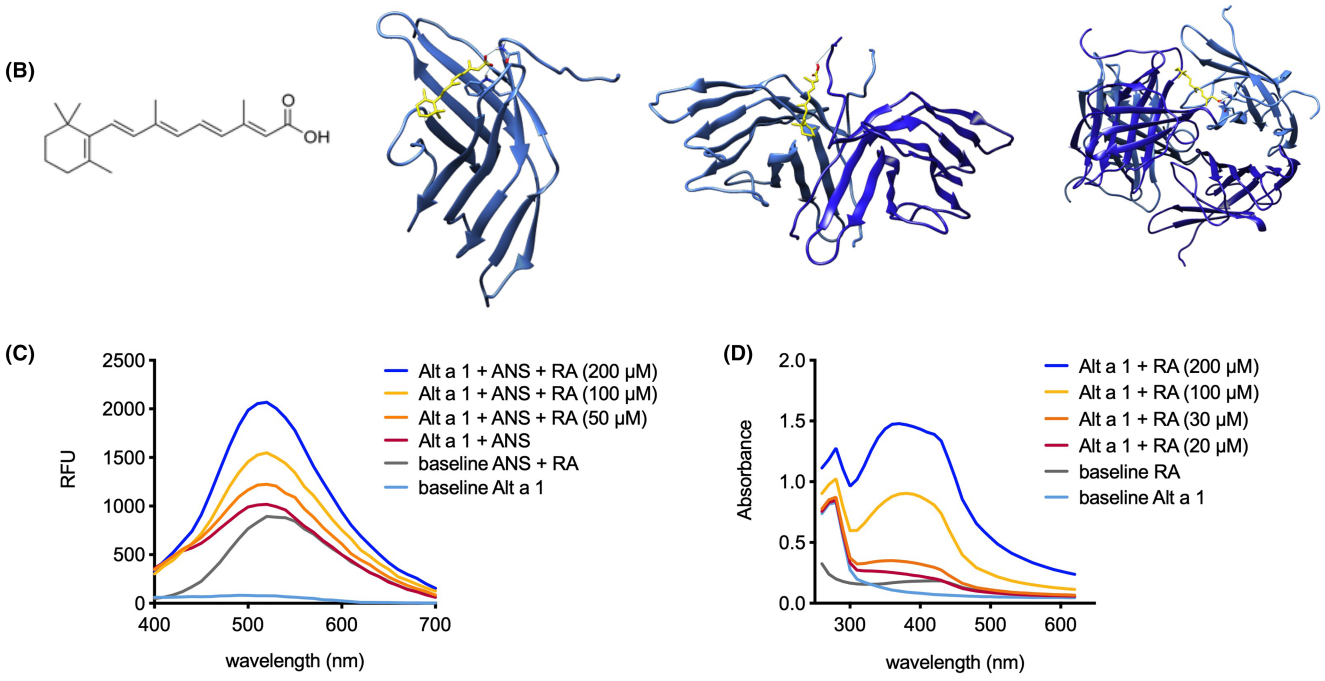


FIGURE 1 RA is a ligand of Alt a 1. In silico docking analysis with (A) affinity energy (kcal/mol) and dissociation constant (K_d, μM) of Alt a 1 monomer, dimer and tetramer with its ligand RA; (B) molecular structure of RA (left) and crystal structure of monomeric, dimeric and tetrameric Alt a 1 (middle to right) with docked ligand RA (sticks in yellow); (C) ANS binding assay (RFU relative fluorescence units) and (D) optical spectrum of Alt a 1 with increasing concentrations of RA.

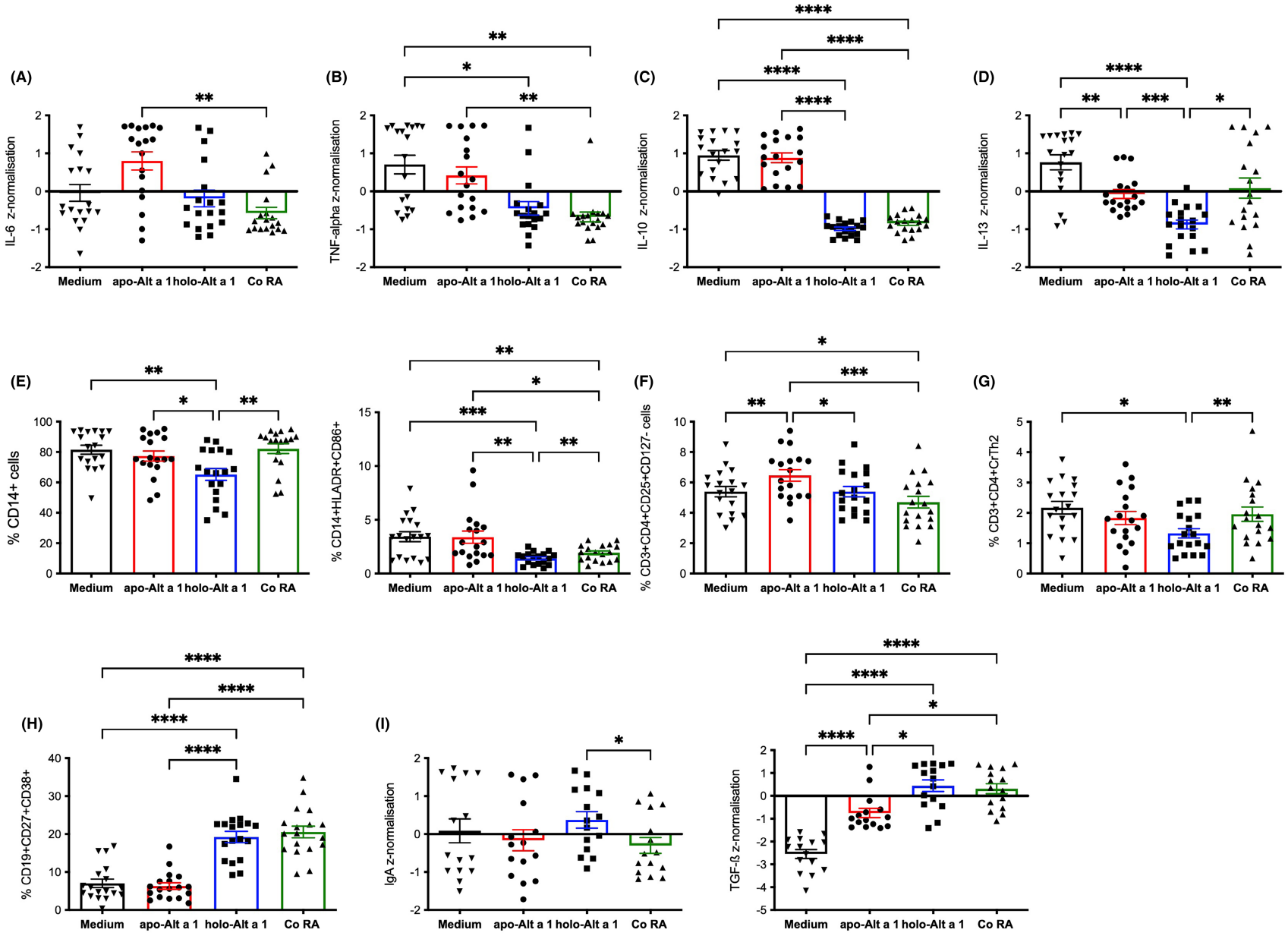


FIGURE 2 Binding of RA to Alt a 1 alleviates the allergic immune response in vitro. (A) IL-6, (B) TNFα, (C) IL-10 and (D) IL-13 production in PBMCs from 18 Alternaria-allergic donors stimulated in vitro with medium only, *apo*- or *holo*-Alt a 1 or RA alone (Co RA); (E) relative number of CD14+ monocytic cells (left graph) and of CD14+HLADR+CD86+ monocytic cells (right graph); (F) relative number of regulatory CD3+CD4+CD25+CD127- T-cells; (G) relative number of CD3+CD4+CRTh2 cells; (H) relative number CD19+CD27+CD38+ B-cells; (I) human total IgA and TGF-β production in supernatants of stimulated PBMCs; z-normalization of pg/mL for each cytokine and total IgA (OD_{450nm}) was performed as described in material and methods; statistical analysis was done with ANOVA followed by Tukey's multiple comparison test; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

IL-13 production in Alternaria allergic donors, but not in healthy non-allergic donors (Figure S3).

Analysis of immune cell markers in PBMCS from Alternaria allergics corroborated these effects. Only when RA was complexed to Alt a 1 (*holo*-Alt a 1), stimulation resulted in significantly reduced CD3+CD4+ cells expressing the Th2 marker CRTh2, compared to medium or RA only treated immune cells (Figure 2G).

In the *holo*-Alt a 1 treated group, the relative numbers of monocytic CD14+ cells and monocytic cells expressing activation markers HLADR+ and CD86+ (Figure 2E), as well as CD3+CD4+CD25+CD127- T-regulatory cells (Figure 2F) were significantly decreased, suggesting resilience to immune activation by impaired antigen-presentation.[16,28]

When analysing B-cells expressing activation/plasma cell markers CD38+ and CD27+, both RA alone and RA complexed in *holo*-Alt a 1 significantly enhanced its expression (Figure 2H). This is in line with the observation that RA mediates differentiation of human peripheral B lymphocytes into plasma cells with enhanced CD38+ and CD27+ expression.[38,39] These studies also proposed that the cytokine TGF-β may synergize with RA in the IgA class switching of B-cells,[38,39] with a role in immune homeostasis and peripheral tolerance induction.[40] Indeed, in our samples (Figure 2I) we detected enhanced total IgA levels rather when PBMCs were treated with RA complexed to *holo*-Alt a 1, than with RA or Alt a 1 alone. The TGF-β production was also significantly elevated compared to the *apo*-Alt a 1 group, independent of whether RA was in a complex with Alt a 1 or not (Figure 2I).

Taken together, our results indicate that sensitization to Alternaria and manifestation of Alternaria-allergy seems to upregulate intrinsic inflammatory/Th2 cytokine production, making PBMCs from Alternaria-allergic donors susceptible to immunomodulatory effects induced by Alt a 1 loaded with RA or RA alone. Therefore we hypothesize that *holo*-Alt a 1 complexed with RA is able to alleviate Th2 immunity in vitro, with a trend to foster a type of immunomodulation which originally was termed Th3 immunity.[41]

3.3 | Reduced IgE binding and cross-linking in vitro via B-cell epitope masking by *holo*-Alt a

We then investigated if IgE-binding to Alt a 1 was affected by RA complexing, as we had observed in previous studies with BLG or Bet v 1.[14,15] In ELISA, *holo*-Alt a 1 showed significantly lower serum IgE-binding compared to the *apo* form in some, but not all donors resulting in an overall 28% reduction of IgE binding in pooled serum (Figure 3A,B). Additionally, *holo*-Alt a 1 induced significantly less mediator release from humanized RBL-SX38 cells sensitized with serum IgE from Alternaria allergics (Figure 3C).

We aimed next to verify a potential effect of RA-binding on Alt a 1´s IgE binding capacity. Further investigations with mouse monoclonal 2C10 antibody revealed a much lower dose–response curve for RA loaded *holo*-Alt a in ELISA (Figure 3D). This antibody was selected as its N-terminal sequence covers an IgE binding region and part of a major B-cell epitope of Alt a 1.[35,42] Alt a 1 has four described B-cell epitopes to which Alt a 1-specific IgE can bind, two high IgE-binding epitopes spanning from P31 to P50 (epitope 1) and from Y54 to K63 (epitope 2), as well as two low-IgE binding epitopes spanning from Y87 to D96 (epitope 3) and from V119 to C128 (epitope 4) (Figure 4A–D).[8,34,35] Our in silico analysis revealed that at least three of the epitopes were influenced by binding of RA, affecting mainly epitope 1 at the N-terminus of Alt a 1 (VAL32, THR33, ASP37, TYR38, VAL39, TRP40), epitope 3 (PHE94) and epitope 4 (THR123, PRO125) in dimeric or tetrameric Alt a 1 (Figure 4A–D). Accordingly, 2C10 monoclonal antibody competed and reduced the IgE binding from Alternaria patients to *apo*-Alta 1 dose-dependently, while IgE binding to *holo*-Alt a1 remained unaltered by preincubation with the monoclonal antibody (Figure 3E). Based on these data we propose that in *holo*-Alt a 1, B-cell epitope masking by RA leads to decreased IgE-reactivity.

3.4 | Therapeutic treatment of Alternaria allergy in vivo is enabled by *holo*-Alt a 1

Consequently, we aimed to explore in a mouse model of Alternaria allergy if an already established allergic immune response could be influenced differently by *holo*-Alt a 1 than by *apo*-Alt a 1. For this purpose, we sensitized female BALB/c mice with two intraperitoneal injections of recombinant Alt a 1 adjuvanted with aluminium hydroxide (Figure S1, S2). Subsequently, mice were subjected to three treatment groups and treated intranasally with *apo*-Alt a 1, *holo*-Alt a 1 or RA alone. After a final i.p challenge with Alt a 1 the anaphylactic symptoms and immunological changes were analyzed (Figures 5, 6).

In the mice treated with *holo*-Alt a 1 a significantly reduced symptom score and a significantly lower body temperature drop upon allergen challenge could be recorded in the non-invasive anaphylaxis imaging cage,[36] compared to *apo*-Alt a 1 or control RA treatments (Figure 5A, left and middle graph). In the *holo*-Alt a 1 treated group the level of physical activity over 20 min was not impaired by the allergen challenge (Figure 5A, right graph; Figure 5B). The alleviated allergic symptoms in *holo-Alt a* 1 treated mice were accompanied by significantly enhanced allergen-specific IgG2a, IgG2b and IgA serum levels compared to control mice, while *apo-Alt a* 1 treated mice showed no significant (Figure 5C). Allergen-specific IgE levels were not affected by any of the treatments (Figure 5C).

The systemic immune response was analyzed by cytokine expression in supernatants of splenocytes in vitro stimulated with Alt a 1. *Holo*-Alt a 1, but not *apo*-Alt a 1 treated mice displayed significantly higher IL-10 production from splenocytes compared to the control group (Figure 6A, left graph), whereas Th1 (IFN γ) and Th2 (IL-4, IL-13) responses were unaltered (Figure 6A). Analysis of splenic surface markers revealed unaltered regulatory CD4+CD25+Foxp3+ expression between groups (Figure 6D). Notably, from *holo*-Alt 1 treated mice the percentage of antigen presenting CD19+CD138+ plasma cells (Figure 6E) and dendritic CD11c+ CD86+ classII+ cells (Figure 6B) was significantly reduced among splenocytes, the latter

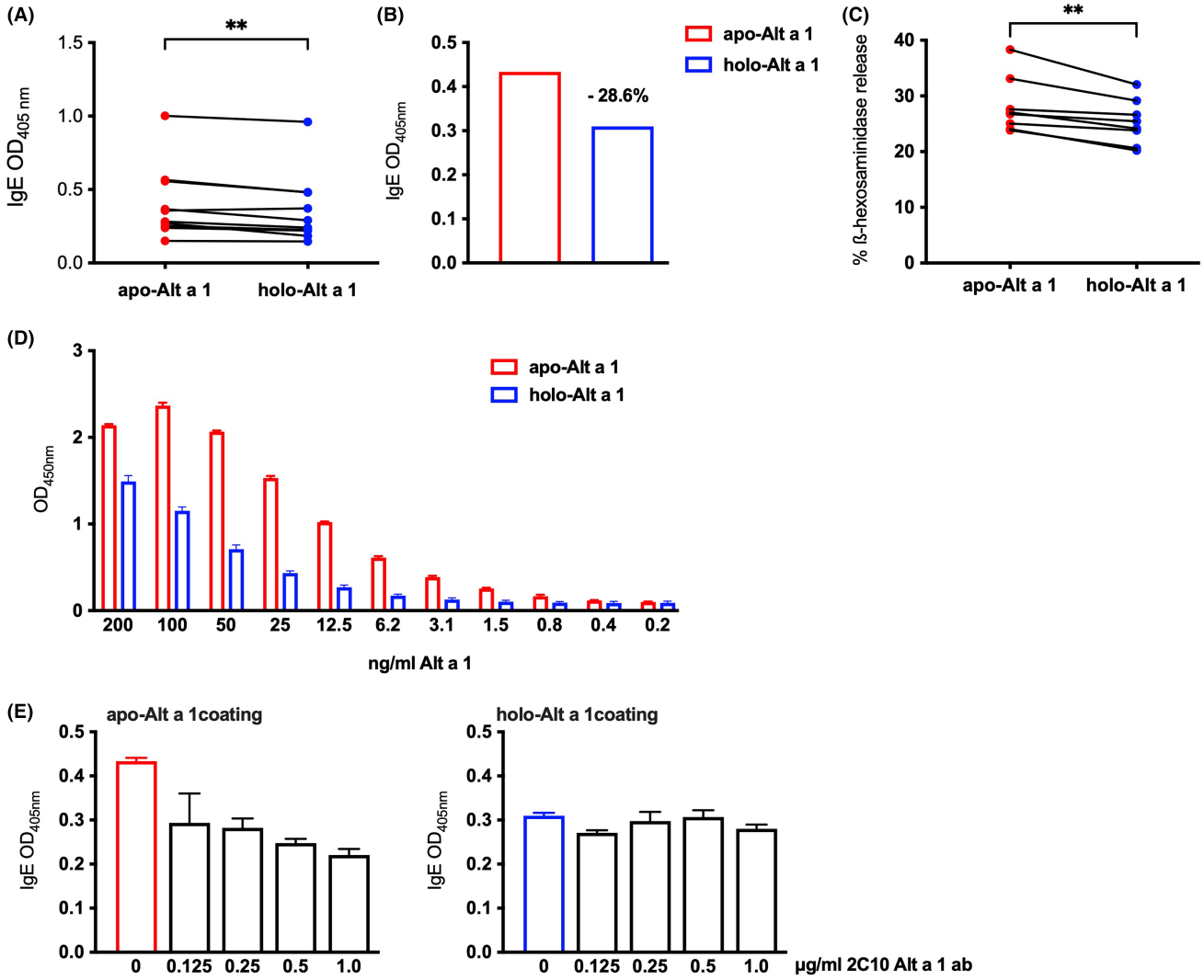


FIGURE 3 *Holo*-Alt a 1 displays reduced IgE binding and cross-linking abilities due to B-cell epitope masking. (A) serum IgE binding of 10 Alternaria-allergic donors to *apo*-Alt a 1 (red) and *holo*-Alt a 1 complexed with RA (blue) (paired samples *t*-test); (B) serum IgE binding of a serum pool of 15 Alternaria-allergic donors to *apo*-Alt a 1 (red) and *holo*-Alt a 1 complexed with RA (blue); (C) β-hexosaminidase release from humanized RBL-cells sensitized with serum IgE from eight Alternaria-allergic donors against *apo*-Alt a 1 (red) or *holo*-Alt a 1 (blue) (paired samples *t*-test); (D) Alt a 1 monoclonal antibody ELISA for detection of *apo*- Alt a 1 (red) and *holo*-Alt a 1 (blue) in a concentration range of 0.2–200 ng/mL; (E) human serum IgE competition ELISA using 2C10 monoclonal Alt a 1 antibody (2C10 Alt a 1 ab) in increasing concentrations (µg/mL) on *apo*-Alt a 1 (left graph) and *holo*-Alt a 1 (right graph) coated plates; $^{**}p < 0.01$; OD, optical density.

indicating interruption of pro-inflammatory antigen presentation of the allergen.

Thus, in this preclinical setting *holo*-Alt a 1 complexed to RA was superior to *apo*-Alt a 1 or unbound RA in modulating an ongoing Th2 immune response in vivo and protecting from anaphylactic symptoms.

4 | DISCUSSION

The functionality of the major *Alternaria alternata* allergen Alt a 1 as a carrier molecule of small hydrophilic ligands has so far been investigated in few studies only.[11,28,43] In the present work we add to this by showing for the first time that (i) RA, the main vitamin A metabolite, is able to bind to Alt a 1, and (ii) that *holo*-Alt a 1 when complexed to RA is superior in immunomodulating Th2 immunity in vitro and protecting from anaphylactic symptoms in vivo.[14,15]

Alternaria alternata is a mould in need of micronutrients to support its growth and sporulation. Therefore, retaining and transporting micronutrients via Alt a 1 would be a survival benefit for this fungus.[44] In fact, an intrinsic carotenoid synthesis has been reported in *Alternaria alternata* and fungi benefit from their antioxidative capacity. In this context, fungi are often utilized for large-scale carotenoid production.[45,46]

In this study we aimed first to in-depth study the interaction capacity of Alt a 1 with RA. Our in silico investigations on RA binding to Alt a 1 calculated similar affinity energies as previously reported for BLG and RA.[15] Binding affinities of β-carotene to

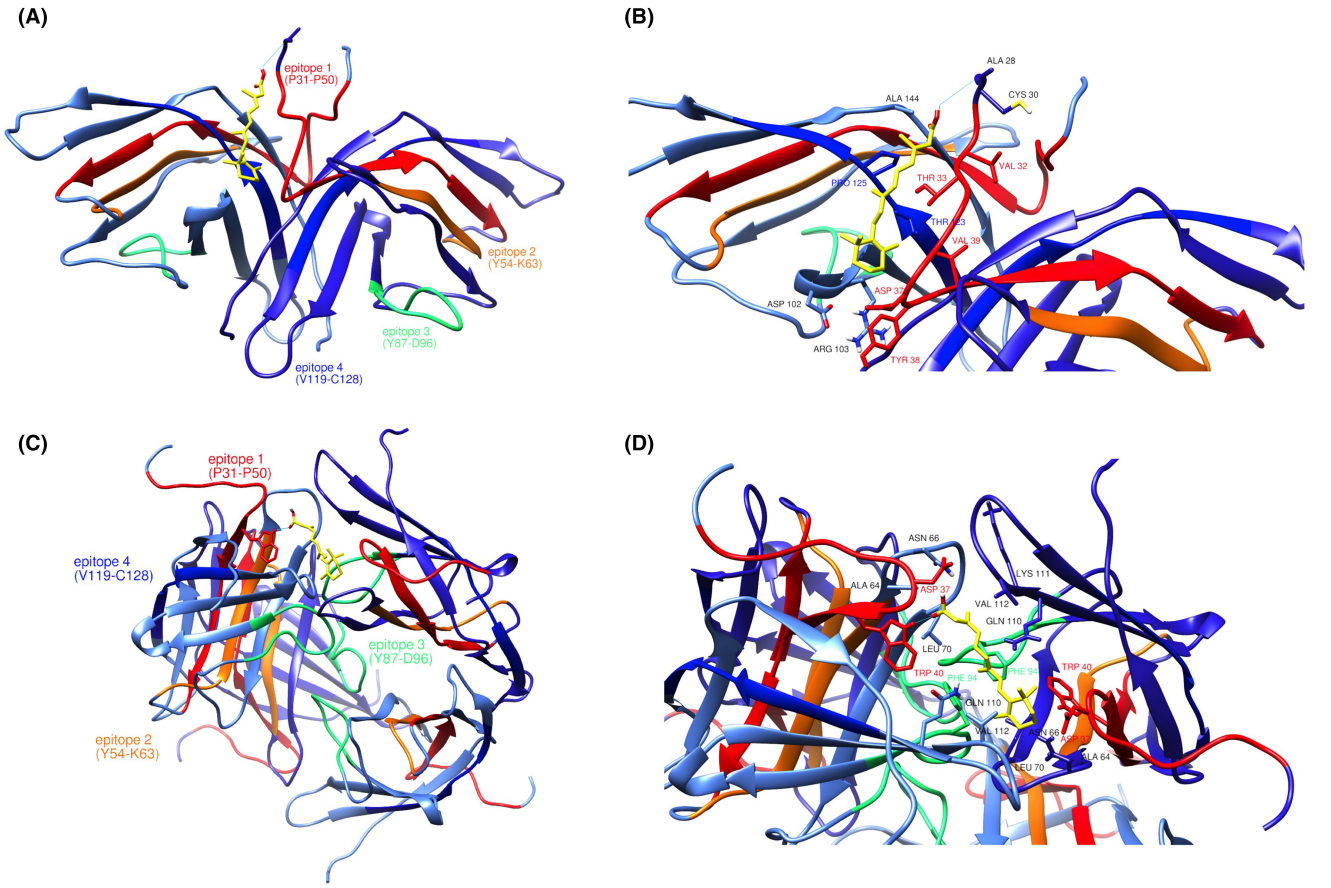


FIGURE 4 In silico docking analysis of Alt a 1 and ligand RA. (A) dimeric Alt a 1-RA complex (ligand RA as sticks with carbons in yellow) and localization of B-cell epitopes (carbons in red: epitope 1 P31-P50; carbons in orange: epitope 2 Y54-K63; carbons in green: epitope 3 Y87-D96; carbons in blue: epitope 4 V119-C128), (B) close-up view of dimeric Alt a 1 with ligand RA as yellow sticks; colored labels indicate all epitope residues that are within 4 Å of the ligand (epitope 1 with carbons in red: VAL32, THR33, ASP37, TYR38, VAL39; epitope 4 with carbons in blue: THR123, PRO125); (C) geometry of tetrameric Alt a 1-RA complex and (D) close-up view of tetrameric Alt a 1 with ligand RA as described for Figure A (epitope 1 with carbons in red: ASP37, TRP40; epitope 3 with carbons in green: PHE94).

Alt a 1 were in the range of a dissociation constant of 2.97 μM for dimeric, and of 0.173 μM for tetrameric Alt a 1, respectively (Figure S5). In vitro binding assays confirmed Alt a 1-RA binding leading to increased fluorescence (ANS assay) and absorbance (optical spectrum) signals upon RA addition. Interestingly, we found an increased ANS signal upon RA addition, while generally other ligands quench the ANS fluorescence signal.[15,47] These phenomena could be explained by further in silico analysis that revealed that one ANS molecule in combination with one RA molecule enhanced Alt a 1 binding to these ligands, with calculated affinities reaching 0.01 μM for the dimer and 0.0001 μM in the tetramer (Figure S6). Thus, combined binding of the two molecules seems to enhance binding sites accessible for the ANS molecule, instead of impairing those. This phenomenon has also been described for ligand kinetin to Bet v 1 and in our group with zinc-binding to BLG.[47,48] As its binding pocket is smaller compared to other carrier proteins,[10] Alt a 1 seems to rely on oligomerization for ligand binding and transport.[49] Importantly, our spectral analysis indicates that RA binding does not require any other molecule to assist, as also by itself the absorption increases.

When we used the Alt a 1-RA complex or RA alone for stimulation of PBMCs from Alternaria-allergic donors we anticipated to find RA-specific effects, as RA itself is an immunomodulatory metabolite.[20] RA-induced effects after treatment—independent of protein binding—were evident in a significant lower expression of costimulatory molecules on CD14+ monocytic cells and lower number of T-regulatory cells. In addition, IL-6 and TNFα were significantly suppressed in *holo*-Alt 1 or RA stimulated cells, completely opposite to *apo*-Alt a 1, which might be due to counter-regulatory effects in human peripheral immune cells as recently described.[50,51] In *holo*-Alt 1 stimulated PBMCs, both impaired T-regulatory cell activation as well as impaired antigen presentation, shown by significant lower maturation state of CD14+HLADR+CD86+ cells, indicate higher resilience to immune activation due to ligand binding, as previously observed with iron-quercetin and BLG or Bet v 1.[16,18]

Another important factor in peripheral tolerance and immune homeostasis is IgA, which has been described as counter-regulatory for IgE antibodies in allergy and immunotherapy.[52] Since RA is known to contribute to IgA class switch of B-cells,[40] we analyzed IgA levels in our cultured PBMCs. Indeed, *holo*-Alt a 1 incubation

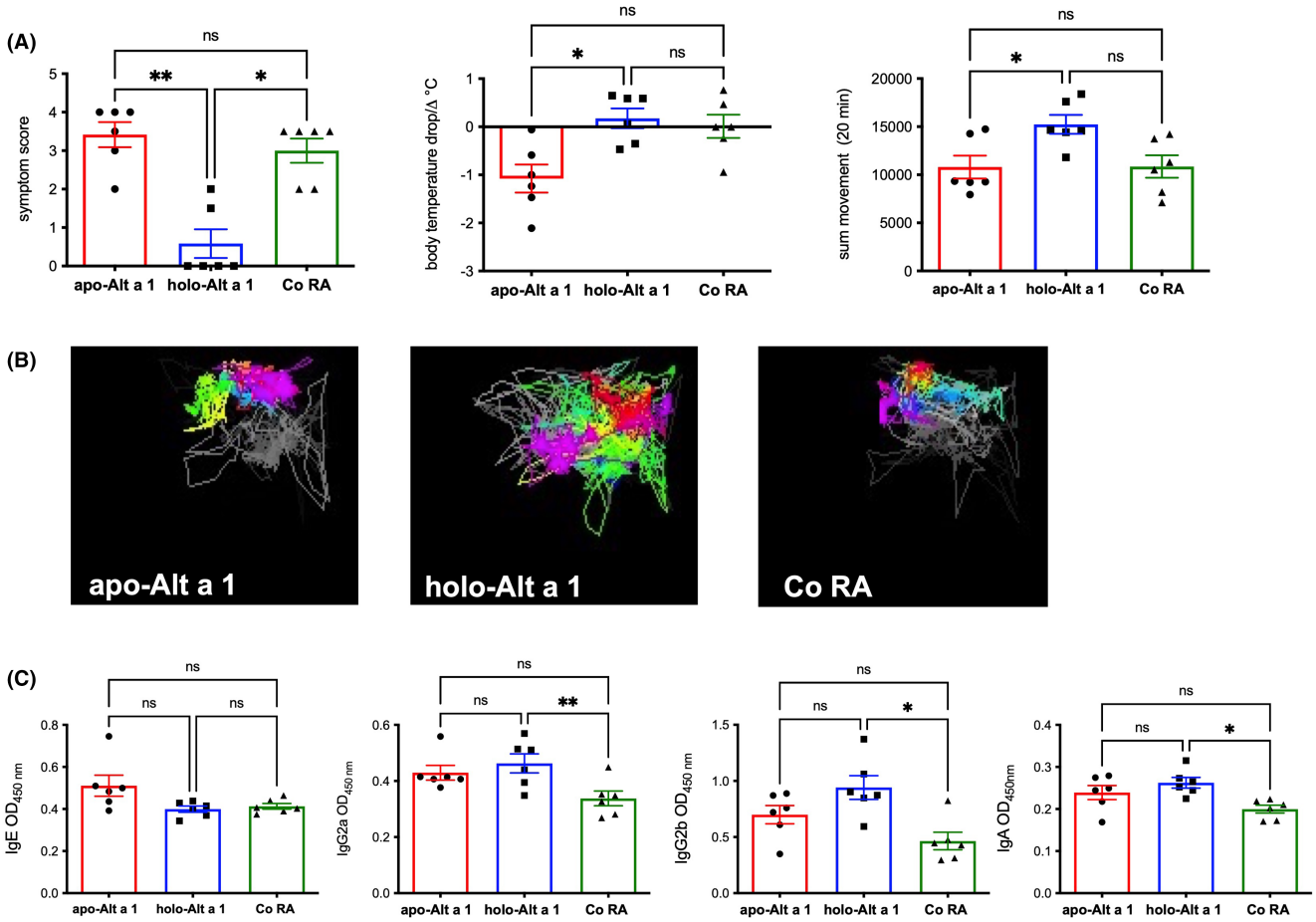


FIGURE 5 *Holo*-Alt a 1 improves specific allergen immunotherapy in a mouse model of Alternaria allergy. After sensitization to Alt a 1, mice were therapeutically treated intranasal with *apo*-Alt a 1, *holo*-Alt a 1 or RA alone (Co RA) and then subjected to a specific allergen challenge with Alt a 1 (Figure S1). (A) anaphylactic symptom score (left), body temperature drop (°C, middle) and mouse movement (right) recorded 20 min after i. p. Alt a 1 challenge; (B) representative images of body temperature (blue to red indicates low to high temperature) and movements (lines) recorded by the imaging cage; (C) Alt a 1-specific serum IgE, IgG2a, IgG2b and IgA levels from sensitized mice treated with *apo*-Alt a 1, *holo*-Alt a 1 or RA alone; groups ($n = 6$) were compared with ANOVA followed by Tukey's multiple comparison test or Kruskal–Wallis test; $^{*}p < 0.05$, $^{**}p < 0.01$; OD, optical density.

resulted in increased total IgA levels and enhanced TGF-β-levels, a pleiotropic cytokine described to be critical for B-cell class switching to IgA.[53] Importantly, RA stimulation alone was not sufficient to induce TGF-β or IgA production in our experimental settings. Along with its IgA switching abilities, RA is naturally involved in B cell differentiation into plasma cells in human PBMCs. As such, RA activated and matured B-cells, leading to enhanced CD38+ and CD27+ expression.[39]

Thus, binding of RA to Alt a 1 may promote B cell maturation and IgA class switching, thereby influencing the already imprinted Th2 immune response in PBMCs from Alternaria allergics, as evidenced by significant reduction of Th2 cytokine IL-13 and the percentage of Th2 cells expressing prostaglandin 2 receptor CRTh2. This receptor found mainly on Th2 cells, basophils and eosinophils is involved in amplification of the allergic immune response[54] and CRTH2/PGD_2 receptor antagonists are under investigation in asthma and allergic diseases.[55] Moreover, we already reported similar results with ligands RA and vitamin D3 on the Th2 response inflicted by birch pollen allergen Bet v 1 in immune cells from BP-allergics.[14,19]

Binding of RA significantly reduced IgE-binding in ELISA and IgE-cross-linking abilities of the mould allergen in effector cells. Based on our in silico data and results from human serum IgE competition ELISA using a monoclonal Alt a 1 antibody 2C10, we propose that this could be due to masking of IgE epitopes by RA and, accordingly, affecting one major and two minor IgE epitopes in the molecular structure of dimeric and tetrameric Alt a 1.[10,14]

Taken together, our in silico and in vitro results so far indicated favourable properties of *holo*-Alt a 1 in Alternaria allergy. Consequently, we tested the RA-loaded allergen in a therapeutic mouse model of Alternaria allergy. Mice therapeutically treated with *holo*-Alt a 1 showed significant reduction of anaphylactic symptoms compared to *apo*-Alt a 1 treatment, accompanied by enhanced antigen-specific IgA, IgG2a and IgG2b serum levels as well as splenic IL-10 production compared to the control group. The data strongly mirror our previous data when birch pollen allergic mice

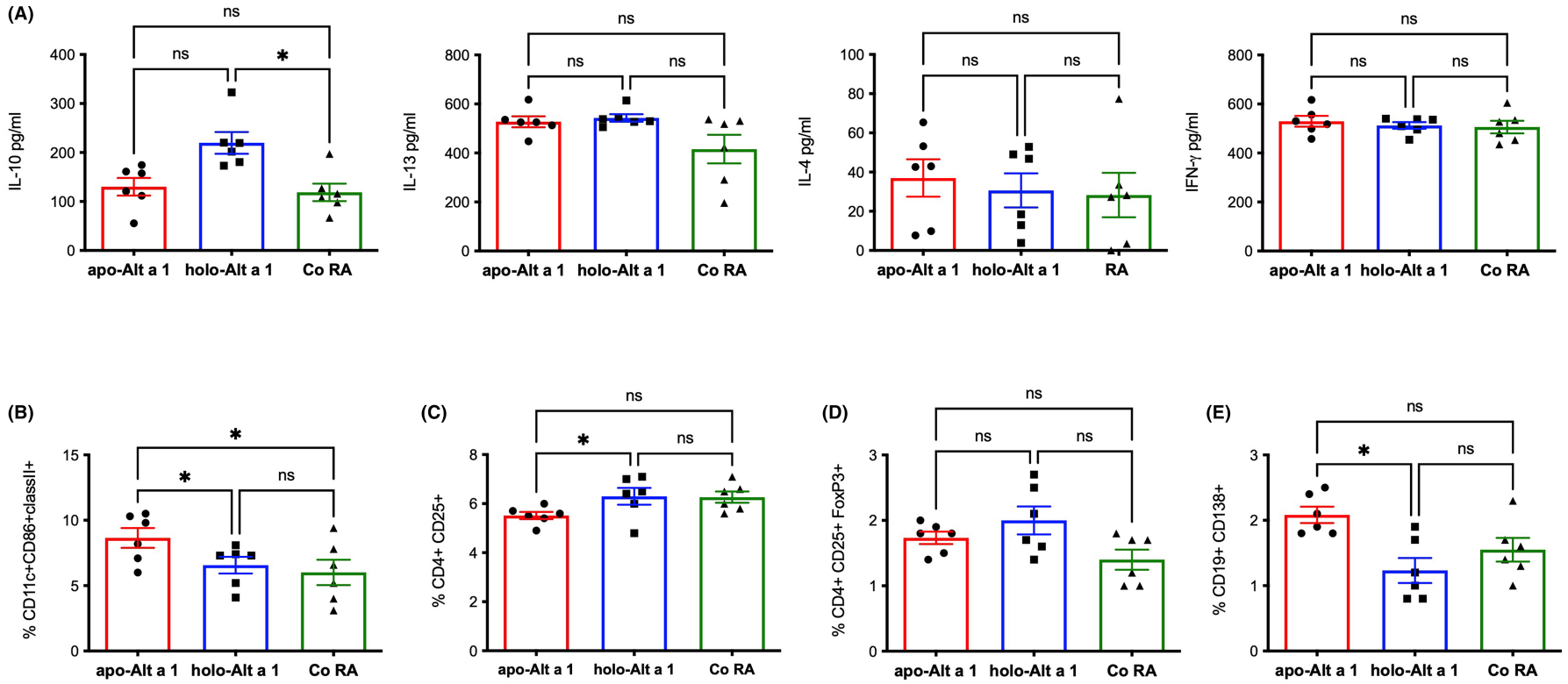


FIGURE 6 Reduced antigen presentation and elevated IL-10 production after *holo*-Alt a 1 treatment in vivo. (A) IL-10, IL13, IL-4 and IFN-γ production (pg/mL) in mouse splenocytes after in vitro stimulation with Alt a 1 in respective treatment groups; relative number of (B) CD11c+CD86+classII+ antigen presenting cells (C) CD4+CD25+ T-cells (D) CD4+CD25+Foxp3 T-cells and (E) CD19+CD138+ B-cells in splenocytes from mice intranasally treated with *apo*-Alt a 1, *holo*-Alt a 1 or RA alone. Statistical analysis was done with ANOVA followed by Tukey's multiple comparison test; $^{*}p < 0.05$; ns, non-significant.

were therapeutically treated with RA-loaded Bet v 1.[14] Along these lines, therapeutic oral administration of ovalbumin and RA in the diet reduced anaphylactic response together with induction of splenic IL-10 levels in a mouse model of food allergy.[56] In this example, however, the possible complex formation of ovalbumin and RA was not addressed, therefore RA might exert an adjuvant effect only.

It was also recently shown that IgA antibodies are able to inhibit IgE-mediated mast cell and basophil degranulation in vivo in an allergen-specific manner,[57] which could in part account for the reduced anaphylactic reaction in our *holo*-Alt a 1 treated mice. In *holo*-Alt a 1 treated mice we observed significant lower numbers of CD19+CD138+ differentiated B-cells in splenocytes, although allergen-specific serum IgE levels were unaltered, the latter being also reported during human allergen-specific immunotherapy.[52] It is known that plasma cells expressing the differentiation marker CD138+ increase the number of mast cells at mucosal barriers in mice, correlating with the severity of anaphylactic symptoms.[58,59] In addition, we found impaired antigen presentation after *holo*-Alt a 1 treatment, similar as in human PBMCs, indicated by a decreased expression of costimulatory markers on dendritic cells, which may account for the attenuation of an already ongoing Th2 immune response.[37]

Taken together, our data provide first evidence that RA is a potent ligand to the major mould allergen and that complexing of Alt a 1 with RA is superior to *apo*-Alt a 1 or RA alone in alleviating an ongoing Th2 response in vitro and in vivo. We have demonstrated the underlying principle of resilience to immune activation induced by micronutrient-spiked allergenic proteins in several studies,[14,16,18,19,28,37] highlighting that this principle could be further utilized to improve management strategies in allergic disease.

AUTHOR CONTRIBUTIONS

AF performed mouse experiments, stimulation and flow cytometry of PBMCs, analyzed data and contributed to writing; FRW co-designed the study, conducted experiments, analyzed data and contributed to manuscript writing; GH performed protein expression and provided support; MW provided in silico data and analysis; SAJ and MB conducted the clinical study with Alternaria allergics and provided support; NS performed in vitro experiments; RB contributed to flow cytometric experiments and provided support; IPS contributed to mouse experiments, provided support and contributed to manuscript writing; EJJ co-directed the study, analyzed data, contributed to manuscript writing and provided support; KH co-designed and directed the study, participated in all experiments, analyzed and interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We thank Andrea Vlasaty for technical assistance.

FUNDING INFORMATION

This work was supported by the Danube Allergy Research Cluster DARC #08 of the Karl Landsteiner University, Krems, Austria, to EJJ; KH was also supported by AllergyCare, Vienna, Austria.

CONFLICT OF INTEREST STATEMENT

EJJ and FRW declare inventorship on "LCN2 as a tool for allergy diagnostic and therapy, by Roth-Walter F, Gomez-Casado C, Jensen-Jarolim E, Pacios LF, Singer J." EP 14150965.3, Year: 01/2014; US 14/204,570; EJJ is shareholder of Biomedical Int. R+D GmbH, Vienna, Austria. The other authors declare they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Fakhimahmadi A, Roth-Walter F, Hofstetter G, et al. Mould allergen Alt a 1 spiked with the micronutrient retinoic acid reduces Th2 response and ameliorates Alternaria allergy in BALB/c mice. *Allergy*. 2024;79:2144-2156. doi:10.1111/all.16181