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ORIGINAL ARTICLE

Autoimmunity and Clinical Immunology

Allergen-Specific IgE and IgG4 Signatures in IgG4-Related Disease Revealed by a Large-Scale PhIP-Seq Study

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Received: 13 January 2025 | **Revised:** 9 October 2025 | **Accepted:** 24 October 2025

Funding: This research was supported by grants from the National Key Research and Development Program of China (2024YFA1307604, 2022YFC2703104), the National Natural Science Foundation of China (82472348, 82471829, 82271848), Beijing Natural Science Foundation (M23008, 7232113), and the National High Level Hospital Clinical Research Funding (2022-PUMCH-B-124, 2022-PUMCH-C-006).

Keywords: allergen | IgE | IgG4 | IgG4-RD | pathogenesis

ABSTRACT

Objectives: Immunoglobulin G4-related disease (IgG4-RD) is frequently accompanied by allergic/atopic manifestations, yet its allergen-specific antibody landscape remains poorly defined. Therefore, we detected allergen-specific IgE and IgG4 levels in patients with IgG4-RD to clarify their contribution to IgG4-RD pathogenesis.

Methods: In total, 826 plasma samples from 370 patients with IgG4-RD and 354 healthy controls were used to perform Phage Immunoprecipitation Sequencing. Follow-up samples from 102 patients (median 3 years) captured temporal dynamics. Machine learning (XGBoost) was used to identify key allergen peptides. ELISA was used for validation in 51 IgG4-RD patients and 24 healthy controls, including an independent cohort of 20 treatment-naïve patients.

Results: Abnormal IgE and IgG4 allergen reactivity was observed in IgG4-RD patients. Enhanced responses to *Apis mellifera* and *Arachis hypogaea* were particularly prominent and correlated with lacrimal gland, parotid gland, paranasal, pulmonary, renal (*A. mellifera*) and pancreatic (*A. hypogaea*) involvement. XGBoost achieved an AUC of 0.91–0.96 and consistently ranked 10 peptides, mainly from bee, peanut, vespid-venom and fish allergens. ELISA confirmed significantly elevated Ara h 1-specific IgG4 in IgG4-RD. Follow-up sampling showed a global decline in reactivity to most top allergens and an IgE-to-IgG4 shift to *Staphylococcus aureus* peptides, suggesting therapy-associated modulation and emerging tolerance.

Conclusions: Comprehensive pan-allergen profiling suggests distinct IgE and IgG4 signatures in IgG4-RD and links specific environmental antigens to organ-selective disease patterns. These findings support a contributory role for aberrant allergen responses in IgG4-RD pathogenesis and provide a foundation for biomarker development and targeted allergen-based interventions, especially for future mechanistic investigations.

Zhan Li, Songxin Yan, and Jialei Zhang contributed equally to this work.

1 | Introduction

Immunoglobulin G4-related disease (IgG4-RD) is an autoimmune disease characterized by multi-organ involvement [1]. Elevated serum levels of IgG4 are evident in 55% to −97% of IgG4-RD patients, although this wide range is influenced by assay variations, population differences, and diagnostic cutoffs. However, serum IgG4 is a highly sensitive initial screening approach for IgG4-RD diagnosis [2].

Clinical observations indicate a strong atopic component in IgG4-RD. Up to 71% of patients report lifetime allergy symptoms including aero-allergy, food allergy, and skin allergy; rhinitis and asthma are the most frequent allergic diseases; approximately 35% to −89% of patients have high IgE levels, and 20% to −38% show peripheral eosinophilia [3–9]. In some individuals, strict allergen avoidance leads to symptomatic and serological improvement [10, 11]. Nonetheless, elevated IgE also occurs in non-atopic patients [6], initiating the question of whether IgE drives inflammation or is an epiphenomenon. Allergen-specific IgE could plausibly initiate or amplify disease activity. A study of 12 IgG4-RD patients and elevated IgE levels suggested positivity for at least one of four broad allergen panels (grass, mold, tree, and nut mixes) [5]. However, the breadth of allergen recognition and its association with the clinical phenotype remain undefined.

IgG4 biology is equally complex. Whereas IgG4 may function as a noninflammatory "blocking" antibody that competes with IgE or IgG1, possessing both pathogenic potential [12–14]. Experimental data from an adoptive-transfer model show that patient-derived IgG4 and IgG1 both may drive tissue injury, particularly in the pancreas and submandibular gland, both of which are the common sites of IgG4-RD, and this effect was attenuated but not abolished by co-administration of IgG1 and IgG4, while the translational relevance of this mouse adoptive transfer model to human pathophysiology requires further clarification [15]. Parallel human studies have identified IgG4-containing immune complexes and complement component 3 deposits in affected kidney and pancreatic tissues, indicating that IgG4 may act as an effector antibody [16–18]. Conversely, rising serum IgG4 might reflect a failed attempt to downregulate a primary immune response [19]. These seemingly conflicting observations highlight that IgG4 may exhibit pathogenic or beneficial roles in IgG4-RD based on the condition. Collectively, these apparently conflicting data imply—but do not yet prove—that qualitative differences in antigen specificity or antibody architecture (e.g., Fab-arm exchange, Fc glycosylation) could influence whether IgG4 responses are pathogenic or regulatory [16, 20]; however, definitive mechanisms remain to be elucidated.

Evidence from other immune-mediated disorders further supports an antigen-driven IgG4 response. In pemphigus and muscle-specific kinase-myasthenia gravis, pathogenic autoantibodies are predominantly IgG4, while in melanoma and cholangiocarcinoma, tumor-derived antigens may induce IgG4 responses that blunt cell-mediated cytotoxicity [14]. In eosinophilic esophagitis, food-specific IgG4 may accumulate within the inflamed site [21]. Similarly, elevated wheat- and rice-specific IgG/IgG4 titres have been reported in coeliac disease, *Helicobacter pylori* gastritis, and IgE-mediated wheat allergy [22]. A pilot study involving 24 IgG4-RD patients further documented broad polyclonal IgG4 reactivity to diverse food and animal allergens [23]. Taken together, these findings suggest that adaptive responses to environmental antigens may contribute to tissue-specific inflammation in IgG4-RD.

Despite this hypothesis, no large-scale investigation has simultaneously profiled allergen-specific IgE and IgG4 in IgG4-RD, necessitating a study on the precise allergen repertoire and its clinical relevance. Therefore, this large-scale phage immunoprecipitation sequencing (PhIP-Seq) analysis was conducted to characterize allergen-specific IgE and IgG4 antibody signatures, thereby clarifying disease-specific allergen responses and laying the groundwork for future mechanistic investigations.

2 | Materials and Methods

2.1 | Study Population

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Medical Ethics Committee of Peking Union Medical College Hospital (approval number: ZS-3193). The discovery cohort included 370 IgG4-RD patients and 354 age-, sex- matched healthy controls. The follow-up cohort included 102 IgG4-RD patients. The independent validation cohort for enzyme-linked immunosorbent assay (ELISA) included 20 treatment-naïve IgG4-RD patients. Plasma samples from the discovery cohort were collected during their initial course of disease. Sequential samples were collected from the follow-up cohort at a median duration of 3 years from the initial collection. All patients were diagnosed according to 2020 revised Comprehensive Diagnostic Criteria or the 2019 American College of Rheumatology/European League Against Rheumatism classification criteria [24, 25]. Patients with other autoimmune diseases or malignancies were excluded from this study. Healthy controls were from a population who self-reported good health, had no abnormal physical examination results, and did not take any medication for the last 6 months. Written informed consent was obtained from all participants.

2.2 | Comprehensive Allergen-Specific IgE and IgG4 Detection

High-throughput analyses of allergen-specific IgE and IgG4 antibodies in plasmas were conducted using PhIP-Seq [26–28]. The allergen related phage display library comprised 10,750 peptides from 2195 allergen proteins, downloaded from the AllergenOnline V21 [29]. The presence of peptide-specific antibodies was based on the following enrichment cutoffs: Z score >3, read count >100, and positive rate >0.1. Allergens with at least three distinct peptides enriched are supposed as reactive allergen. Differentially expressed peptides were identified using a $\log_2$ fold change ($\log_2$FC) threshold of $|\log_2 FC| > 0.263$ (corresponding to FC >1.2 or FC <0.833) and an adjusted p-value threshold of $-\log_{10}(\text{adj. } p) > 1.301$ (adj. $p < 0.05$). The flowchart outlining the study approach to data analysis is provided in the Data S1 (Figure S1).

2.2.1 | Library Construction

The allergen epitope (ALE) library was constructed following previously described protocols from PhIP-Seq studies [30]. Briefly, 10,750 peptides from 2195 allergen proteins were downloaded from the AllergenOnline V21 [29]. To generate polypeptide fragments covering all protein sequences, 40-mer polypeptide fragments with seven-amino acid overlaps were generated and clustered using CD-HIT at 95% sequence identity at the amino acid level. They were reverse translated to 120 nt DNA oligonucleotides. Adaptor sequences (15-mer) were added to both sides of the oligonucleotides to introduce *Eco*RI and *Hin*dIII restriction sites; the sequences were synthesized in TWIST Bioscience. Oligonucleotide pools were amplified, cloned and packaged according to the manufacturer's instructions (T7 Select 10-3 Cloning Kit, EMD Millipore). Briefly, Polymerase chain reaction (PCR) amplified pooled oligos were digested and cloned into the *Eco*RI/*Hin*dIII sites of the T7Select 10-3b vector (Novagen) with an average representation of at least 100 copies of each peptide maintained during each cloning step. The ligation reaction was incubated overnight at 16°C and added directly to T7 Packaging Extracts for in vitro packaging. The phage packaging reaction was incubated at room temperature (22°C) for 2 h. The amplified library was titrated and stored in a medium containing 8% glycerol at −80°C.

2.2.2 | Phage ImmunoPrecipitation and Binding Protocol

For IgE (1:1 mixture of Omalizumab and Ligelizumab, with variable regions grafted onto the murine IgG1 constant region) and IgG4 (MaI4-09, Nittobo Medical) PhIP-Seq, biotinylated IgE- or IgG4-specific antibodies were conjugated to Streptavidin-bound magnetic beads (Dynabeads MyOne Streptavidin T1; ThermoFisher-35602) at 5× binding capacity. Excess unbound antibody was washed away with PBST (0.01% (W/V) Tween in PBS, pH 7.5). The binding efficiency of anti-human IgE and anti-human IgG4 specific antibody coated magnetic beads was 40 μg of antibody per 1 mg of beads. For each immunoprecipitation, 20 μL of IgE- or IgG4-coated T1s was used. Before binding, the beads were blocked with 2% fraction V bovine serum albumin (BSA) in Phosphate-buffered saline (PBS) and washed with PBST. A total input of 200 ng IgG4 or 10 ng IgE was used to ensure sufficient reactivity based on the relationship between allergen-specific and total antibody levels, and as previously described [31]. Briefly, 20 μL of the coated magnetic bead slurry was added to each well of a 96-deep-well plate. Plasma samples were added to the wells, and adjusted to a final volume of 500 μL with PBST. The samples were incubated for 30 min at room temperature. The beads were washed five times with 500 μL immunoprecipitation (IP) wash buffer (150 mM NaCl, 50 mM Tris–HCl, 0.1% (vol/vol) NP-40, and adjust pH to 7.5) and once with PBS. The phage library was added to each well containing immunoglobulin-bound beads. At least 10^5 PFU plaque-forming units (PFU) per phage library were ensured. The final volume was adjusted to 500 μL with PBS, and the samples were incubated for 30 min at room temperature. The beads were washed six times with IP buffer. Finally, the beads were resuspended in 100 μL of double-distilled water. All procedures were automated using a 96-channel liquid handling workstation (PP96, SciProtech).

2.2.3 | Sequencing and Bioinformatics

PCR reactions were conducted in 50 μL samples containing 0.3 mM each deoxynucleotide triphosphate, 2 mM magnesium ion, 0.3 μM each primer, 1 U KAPA HiFi HotStart Polymerase (Roche), and one-tenth of the bead slurry per reaction, using different index primers. The thermal cycling profile was as follows: 95°C for 20 min of denaturing and DNA release, followed by 20 cycles of 98°C 20 s, 55°C for 15 s, and 72°C for 15 s. A final extension was conducted at 72°C for 1 min. Mock-immunoprecipitation (mock IP) reactions (with no plasma added) were included and sequenced in all experiments. PCR products were purified using MagMAX Pure Bind Beads (Thermo Fisher), and Illumina adaptors were added via PCR. Next-generation sequencing was conducted using the Illumina Novaseq platform to a read depth of approximately 2 million reads per sample.

FASTQ sequencing results were demultiplexed and aligned to the synthesized oligonucleotide pool using Bowtie 2 to obtain a count matrix [32]. A pseudocount of 1.0 was added to each peptide-encoding oligonucleotide to include zero-read peptides in statistical analysis. All reads were normalized to reads per million (RPM), and Z-scores for each peptide were calculated using a Python-based software package, phippery [33]. Z-scores were based on the read distribution of mock-IP samples (IgE- or IgG4-coated T1s). Peptides with similar read abundance in mock-IP samples were grouped into bins. The top and bottom 5% of peptides were disregarded. Z-scores were calculated using the following formula:

$$Z - \text{score} = (\text{counts} - \text{mean}) / \text{standard deviation}$$

where "counts" denote the sequencing read counts for a specific peptide within a bin, and "mean" and "standard deviation" denote the read distribution within that bin.

2.3 | XGBoost Classifier

The eXtreme Gradient Boosting (XGBoost) classifier was used for outcome classification and to highlight allergen peptide features that differentiate IgG4-RD patients from healthy individuals. The "sample.split()" function from the *caTools* package in R was used to divide the dataset into training (80%) and testing (20%) sets. A random seed (e.g., set.seed(123)) was applied to ensure reproducibility. A five-fold cross-validation strategy was applied with 20 repetitions using the "train()" function from *caret* package in R for hyperparameter tuning. The XGBoost model was retrained with the best parameters identified from xgb_model$bestTune (including eta, max_depth, gamma, colsample_bytree, min_child_weight, and subsample). Predictions were made on the independent testing set. The "model_parts()" function from the *DALEX* package was used to interpret the models and the effect of features on classification. The permutation importance of a feature was defined as the degradation in root mean square error after

permutation (ΔRMSE) caused by randomizing its values, reflecting its predictive contribution. Random seeds were varied five times to re-train and test the models, ensuring stability and confidence in results. Performance of these models was evaluated using receiver operating characteristic (ROC) and confusion matrices.

2.4 | Validation of Results

Plasma *Arachis hypogaea* allergen Arah 1 specific IgG4 levels were measured with ELISA in 51 IgG4-RD patients and 24 healthy controls, including an independent IgG4-RD cohort of 20 treatment-naïve patients. Ninety-six-well immunoplates (Corning) were coated with 200 ng/well of *A. hypogaea* allergen Arah 1.0101 (303-ARAH0200, Sino Biological) overnight. ELISA was conducted as described previously [34]. Briefly, the coated plates were blocked with 2% BSA in PBS at 37°C for 2 h. After washing, the plasma diluted 1:20 in 2% BSA/PBS was added and incubated at 37°C for 2 h. The plates were washed and incubated with anti-human IgG4 antibody (1 μg/mL) (AbM59499-29A30, Beijing Protein Innovation) for 1 h, followed by incubation in horseradish peroxidase conjugated goat anti-mouse IgG antibody (1:10,000) (BE0102, EASYBIO) for 1 h. After washing, tetramethylbenzidine was added to react and then stopped by 2 M H_2SO_4. Standard curves were generated by plotting optical densities (OD_{450nm}) against known concentrations of the human IgG4 standard (Beijing Protein Innovation) [35]. A blocking experiment was conducted by pre-incubating plasma samples with Arah 1.0101 at 1 μg/mL or unrelated poly-histidine (His)-tagged protein (Protein-RdRP) overnight, followed by ELISA. Each sample was assessed in duplicate.

2.5 | Statistical Analysis

2.5.1 | Data Collection

Demographic data (age, sex) and clinical data (organ involvement, self-reported allergy history) were extracted from the medical records. Laboratory findings included serum IgE and IgG4 levels and the relative number of eosinophils in the peripheral blood.

2.5.2 | Statistical Analysis

FASTQ sequencing results were processed according to previous studies [32, 33]. Data analysis was conducted using GraphPad Prism 9.5 software (San Diego, CA, USA), SPSS Version 27.0 software (SPSS Inc., Chicago, IL, USA), R (version 4.2.3), and the online BioLadder tool (bioladder.cn) [36]. Normality was assessed using the Kolmogorov–Smirnov test or Shapiro–Wilk test. Independent sample *t*-tests were used to compare differences between normally distributed data. The Mann–Whitney test compared differences between non-normally distributed data. Categorical variables were compared using the Chi-squared test. A two-tailed p-value < 0.05 was considered significant. For multiple comparisons, the Bonferroni correction was applied ($\alpha = 0.05$, adjusted p-values $= p$-values $\times n$, where n is the number of comparisons).

3 | Results

3.1 | Patient Characteristics

The clinical characteristics of the enrolled patients with IgG4-RD are shown in Table 1. Patients with IgG4-RD analyzed in this study included 228 men (58.5%) and 162 women (41.5%) with a mean age of 55.5 years at the time of plasma sampling. Of these patients, 141/232 (60.8%) had a self-reported history of allergies such as allergic asthma, allergic rhinitis, and allergic dermatitis. In addition, 381/390 (97.7%) of patients had complete medical record information regarding the involved organs. Overall, 364/390 (93.3%), 281/344 (81.7%), and 77/388 (19.8%) of

TABLE 1 | Clinical characteristics of IgG4-RD patients.

Characteristics	IgG4-RD ($n = 390$)
Age, mean (range), years	55.5 (17–88)
Sex, no. male/female	228:162 (1.4:1)
IgG4-RD RI, median (Q1, Q3)	6 (4, 10)
Allergy history, *n* (%)	141/232 (60.8)
Allergic rhinitis, *n* (%)	78/232 (33.6)
Allergic asthma, *n* (%)	32/232 (13.8)
Allergic dermatitis, *n* (%)	2/232 (0.9)
Involved organs	
Pancreas, *n* (%)	140/381 (36.7)
Bile duct, *n* (%)	47/381 (12.3)
Paranasal sinus, *n* (%)	61/381 (16.0)
Lacrimal gland, *n* (%)	166/381 (43.6)
Submandibular gland, *n* (%)	159/381 (41.7)
Parotid gland, *n* (%)	56/381 (14.7)
Retroperitoneum, *n* (%)	38/381 (10.0)
Kidney, *n* (%)	37/381 (9.7)
Lung, *n* (%)	59/381 (15.5)
Pituitary, *n* (%)	6/381 (1.6)
Lymph node, *n* (%)	105/381 (27.6)
Artery, *n* (%)	28/381 (7.3)
Liver, *n* (%)	8/381 (2.1)
Thyroid, *n* (%)	7/381 (1.8)
Skin, *n* (%)	2/381 (0.5)
Gastrointestinal tract, *n* (%)	1/381 (0.3)
Mediastinum, *n* (%)	9/381 (2.4)
Elevated serum IgG4 (> 1400 mg/L), *n* (%)	364/390 (93.3)
Elevated serum IgE (> 60KU/L), *n* (%)	281/344 (81.7)
Elevated eosinophils (> 5%), *n* (%)	77/388 (19.8)
Both elevated IgE and IgG4, *n*%	269/344 (78.2)

these patients respectively exhibited elevated IgG4 levels, IgE levels, and relative numbers of eosinophils. 269/344 (78.2%) of these patients exhibited both elevated IgE and IgG4 levels in their plasmas.

3.2 | Distinct Allergen-Specific IgE and IgG4 Profiles in IgG4-RD

Given that studies have reported that IgG4 competes with IgE for allergens, we sought to explore the patterns of immunodominance and reactivity that characterize allergic antibody responses in IgG4-RD. Accordingly, the PhIP-Seq approach to whole-human proteome seroreactivity analyses was utilized [37], analyzing the reactivity of both IgE and IgG4 immunocaptures in plasma samples from 370 IgG4-RD patients and 354 sex- and age- matched healthy controls. IgE and IgG4 reactivity to allergen peptides suggested heterogeneity between IgG4-RD patients and healthy controls (Figure 1A). Differences in the distribution of antibody responses against allergens and allergen reactive signatures are displayed in Figure 1B,C. The identification of the differences between enriched allergen-specific IgE and IgG4 antibody signatures in IgG4-RD represents a step toward understanding the potential pathogenesis of IgG4-RD.

3.3 | Identifying Disease-Specific Allergens in IgG4-RD

Furthermore, differences in the composition of overall enriched allergen-specific IgE and IgG4 antibody repertoires were explored in patients with IgG4-RD and healthy controls by analyzing the proportions of reactive allergens and the number of mapped peptides associated therewith (Figure 2A). The results identified a total of 42 allergens. Enriched IgE responses to 10 allergens were only observed in IgG4-RD patients, including *Equus caballus*, *Hevea brasiliensis*, *Lens culinaris*, *Olea europaea*, *Penicillium citrinum*, *Periplaneta americana*, *Polybia paulista*, *Prunus dulcis*, *Staphylococcus aureus*, and *Vespula vulgaris*; Similarly, IgG4 responses toward 10 allergens were only observed in patients, including *Anisakis simplex*, *A. hypogaea*, *Fagopyrum esculentum*, *Lates calcarifer*, *Lupinus angustifolius*, *Oryza sativa Japonica Group*, *Poa pratensis*, *P. paulista*, *Tyrophagus putrescentiae*, and *V. vulgaris*. Notably, both IgE and IgG4 allergic responses toward *P. paulista* and *V. vulgaris* were observed in patients but not in controls. Moreover, IgE-based allergic responses to *A. simplex*, *A. hypogaea*, *L. calcarifer*, and *O. sativa Japonica Group* were observed among controls whereas these responses were IgG4-associated among patients. Conversely, allergic responses to *S. aureus* were IgE- and IgG4-dominant in patients and controls, respectively.

Because the discrepancies in antibody responses toward the same allergens may account for varying allergic trajectories between patients and controls, differential analyses were conducted by calculating fold change (FC) and adjusted *p*-values based on the RPM. For each peptide, FC values were calculated by dividing the RPM value of each IgG4-RD sample by the mean RPM of all matched HC samples [27]. Volcano plots suggested significant differences in allergen peptides associated with IgE and IgG4 responses between patients and controls (FC >1.2, adjusted $p<0.05$) (Figure 2B). In total, IgE responses toward 15 peptides of 11 allergens were found to be enhanced in individuals with IgG4-RD compared to matched controls. Of these, eight allergens were consistent with the allergens associated with IgE responses only observed in patients in the above analyses. The remaining four peptides were derived from three allergens including *Apis mellifera*, *Dermatophagoides farina*, and *Triticum aestivum*. In addition, IgG4 responses toward one peptide from *Aspergillus fumigatus* was observed to be upregulated in patients compared to controls. IgG4 responses against 19 peptides mapped to 13 allergens including *Aedes aegypti*, *A. mellifera*, *Canis lupus familiaris*, *Corylus avellana*, *D. farinae*, *Dermatophagoides pteronyssinus*, *Dolichovespula maculata*, *Gallus gallus*, *Pangasianodon hypophthalmus*, *P. americana*, *Sarcoptes scabiei*, *S. aureus*, and *Zea mays* were overrepresented among healthy controls relative to patients. This could be associated with greater tolerogenicity against these allergens in controls but does not exclude the possibility that some IgG4-mediated allergic responses against certain allergens may be linked to the pathogenesis of IgG4-RD.

3.4 | Identification of the Top 10 Disease-Specific Allergen Peptides in IgG4-RD

A total of 40 peptides associated with 13 allergens were selected as candidate IgE-reactive peptides, while 45 peptides associated with 11 allergens were selected as candidate IgG4-reactive peptides. Subsequently, XGBoost-based machine learning was applied to predict IgG4-RD onset using these allergen peptides. XGBoost models were trained and tested repeatedly with 10/15/20 repetitions of five-fold cross-validation with five distinct random seed initializations for both training and testing phases (Figure 3A). ROC curves and confusion matrices evaluated the model's performance. Outputs encompassed feature importance plots for both training and independent test datasets, and ROC curves and confusion matrices for independent datasets. The models achieved an area under the ROC curve (AUC) ranging from 0.91 to 0.96 (Figure 3A,B). The frequency of allergen peptides and their representative proteins ranked in the top 10 for feature importance across repetitions. These peptides made up the core features differentiating patients with IgG4-RD from controls (Figure 3C). Principal component analysis (PCA) was conducted to visualize individual diversity in allergen repertoires based on the top 10 allergen-associated peptides (Figure 3D). Furthermore, allergic responses toward these peptides were compared based on RPM (Figure 3E). The prevalence and corresponding odds ratios (ORs) for these responses are shown in Figure 3F. Detailed description about top 10 and other main allergen peptides in this study can be found in Data S1 (Table S1) and Data S2.

3.5 | Identification of Allergens Related to IgG4-RD Clinical Characteristics

To further explore the association between allergen-specific antibody response and serum levels of total IgE and total IgG4, as well as peripheral blood eosinophils, patients were stratified by

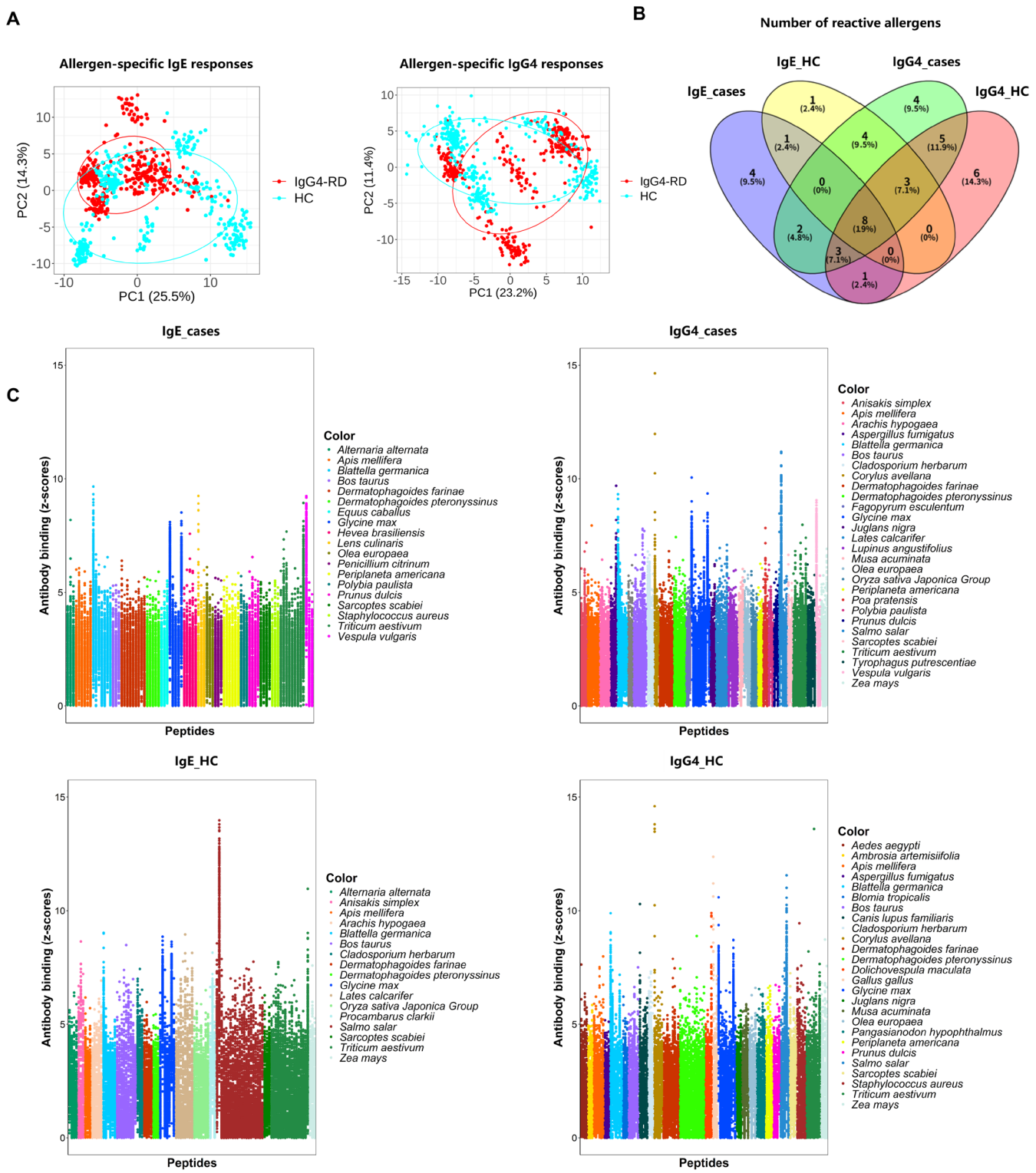

FIGURE 1 | Patterns of allergen-specific IgE and IgG4 reactivity. (A) Principal component analysis (PCA) of reactive allergen peptides suggests heterogeneity in allergen-specific IgE and IgG4 antibody responses between IgG4-RD patients and healthy controls. (B) Venn diagram showing the number of reactive allergens recognized by IgE and IgG4 antibodies in patients with IgG4-RD and matched controls. (C) Scatter diagrams for allergen-specific IgE and IgG4 responses in patients with IgG4-RD and matched controls.

these parameters, as well as confounding factors, such as age and sex. Heatmap showed enrichment patterns of IgE/IgG4 reactivity among the top 10 peptides. Cluster A showed stronger IgE responses to peptides ALE00808|330-370 from *Dermatophagiodes farinae*, ALE01754|0-40 from *P. paulista*, ALE00179|1386-1426 and ALE00172|363-403 from *A. mellifera*, while cluster B exhibited stronger IgG4 responses to peptides ALE00302|66-106 from *A. fumigatus*, ALE00197|231-271 from *A. hypogaea*,

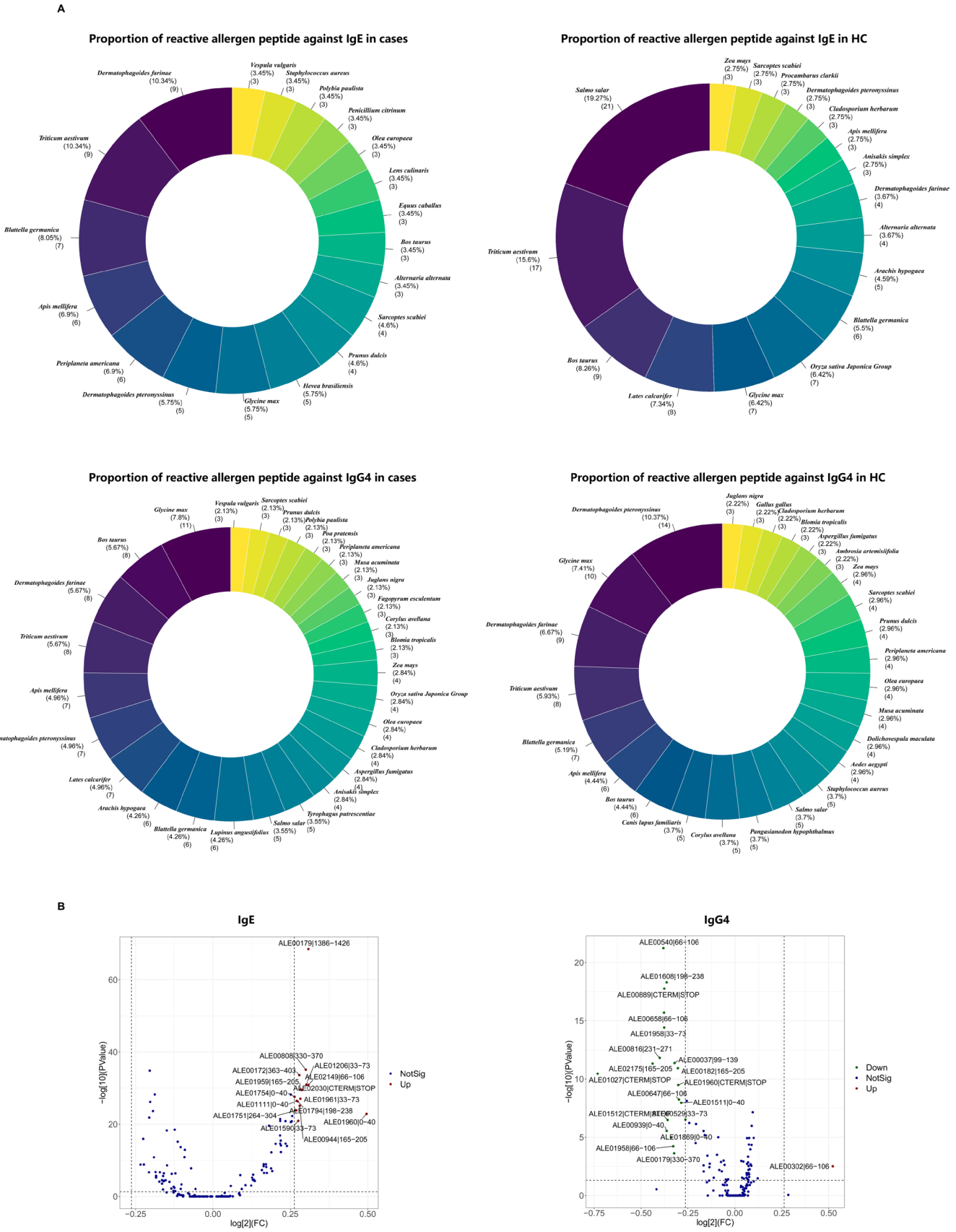

FIGURE 2 | The identification of disease-specific allergens in IgG4-RD. (A) Donut charts showing the proportions and numbers of mapped peptides for reactive allergens identified in the allergen-specific IgE and IgG4 antibody repertoires of patients and matched healthy controls. (B) Volcano plots of IgE- and IgG4-reactive allergen peptides that differed significantly between patients and matched controls. Differentially expressed peptides are identified using a $\log_2$ fold change ($\log_2$FC) threshold of $|\log_2 FC| > 0.263$ (corresponding to FC > 1.2 or FC < 0.833) and an adjusted p-value threshold of $-\log_{10}(\text{adj. } p) > 1.301$ (adj. $p < 0.05$). Corresponding allergen names and more peptide details are available in the Data S2.

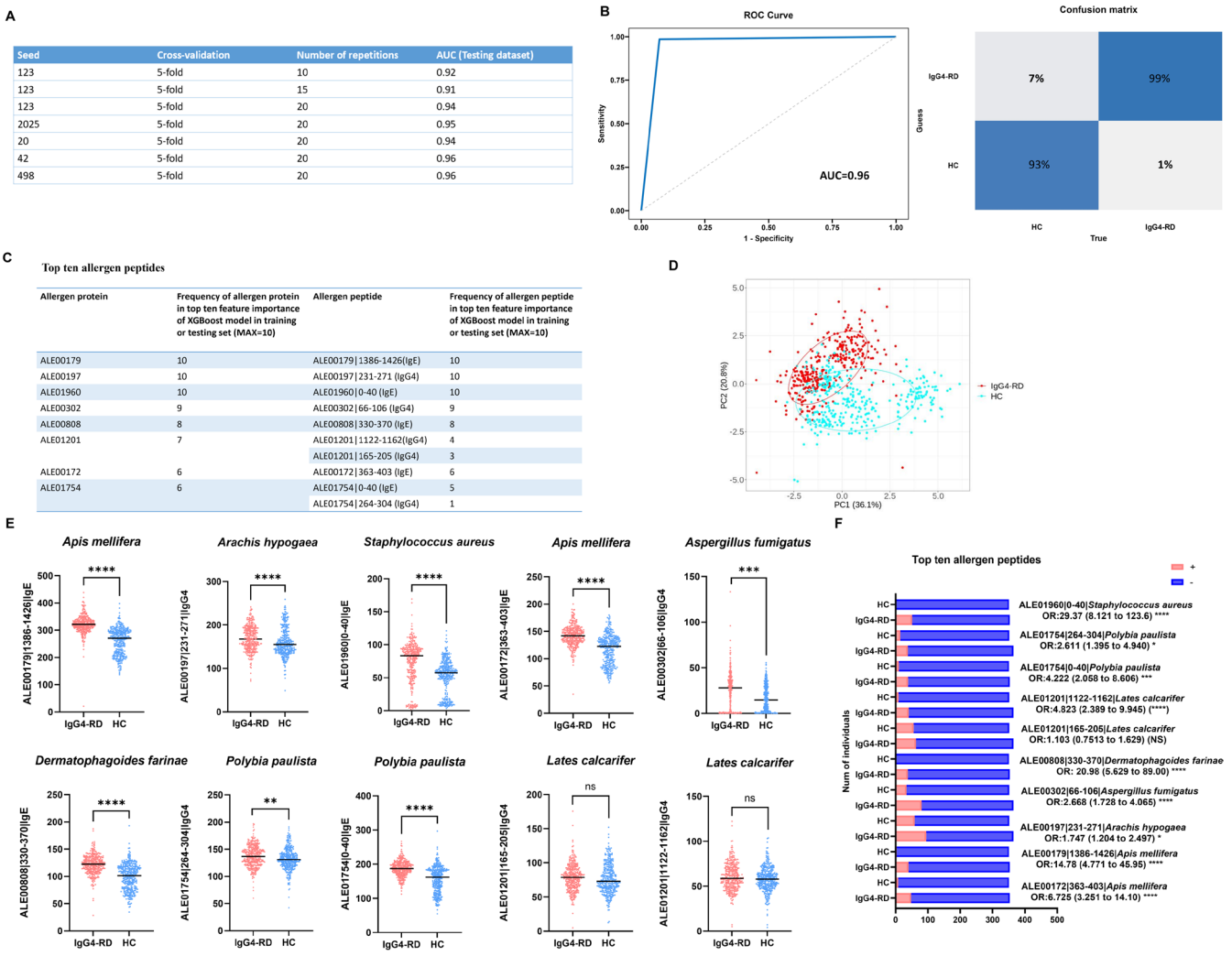

FIGURE 3 | Antibody binding to candidate peptides in patients and matched controls (A) Development and evaluation of an XGBoost-based machine learning model for IgG4-RD onset. (B) The ROC curve and confusion matrix of an XGBoost-based machine learning model (seed: 498, five-fold cross-validation, 20 repetitions) (C) Top 10 most frequently occurring allergen peptides in feature importance plots from training and testing datasets. (D) Principal component analysis (PCA) of the top 10 allergen-associated peptides. (E) Levels of antibody responses toward the top 10 allergen-associated peptides. (F) Odds ratios (ORs) and 95% confidence intervals (CIs) for these peptides. *adjusted $p < 0.05$; **adjusted $p < 0.01$; ***adjusted $p < 0.001$; ****adjusted $p < 0.0001$.

and ALE01754|264-304 from *P. paulista* (Figure 4A). We observed that IgE/IgG4 reactivity to top 10 allergen peptides are possibly different in IgG4-RD patients with different levels of EOS%, IgE, IgG4, and IgG4-RD responder index (RI) score, but have a weak capacity of differentiating patients with different urban residency and self-reported allergy history (Figure 4B, Figure S2A–E). Notably, patients with higher IgG4 response to ALE01201|1122-1162 showed a tendency toward higher IgG4, EOS%, and IgE levels (Figure 4A,C). Additionally, the association between organ susceptibility and the top 10 peptides was investigated, organ-specific subgroups containing fewer than 10 positive patients were omitted from analyses. For the most frequently involved organs in our cohort, higher responses to *P. paulista* and *A. mellifera* were associated with lacrimal gland and submandibular gland involvement, *A. hypogaea* and *L. calcarifer* with pancreas involvement. Additionally, higher responses to *A. mellifera* were associated with parotid gland, kidney, and lung involvement, and *L. calcarifer* with bile duct, paranasal sinus, and lymphnode. Hence, allergen-specific responses may be associated with organ involvement (Figure 4D). Pie charts display the relative contribution of each peptide to specific organ involvement, without implying causal associations.

3.6 | Verification of *A. hypogaea* Allergen Arah 1 in IgG4-RD Patients

A. hypogaea allergen Arah 1 was selected to validate these findings based on the feature importance of ALE00197 across XGBoost models, the importance of Arah 1 protein in *A. hypogaea*, and the other five candidate peptides from *A. hypogaea* in the initial selection (Table 2, Figure 5A).

ELISA was conducted to identify IgG4 levels against *A. hypogaea* allergen Arah 1 in plasma samples from IgG4-RD patients, compared with healthy controls. Samples from 51 IgG4-RD patients and 24 healthy controls were collected, including samples from 20 additional patients independent of the previously tested

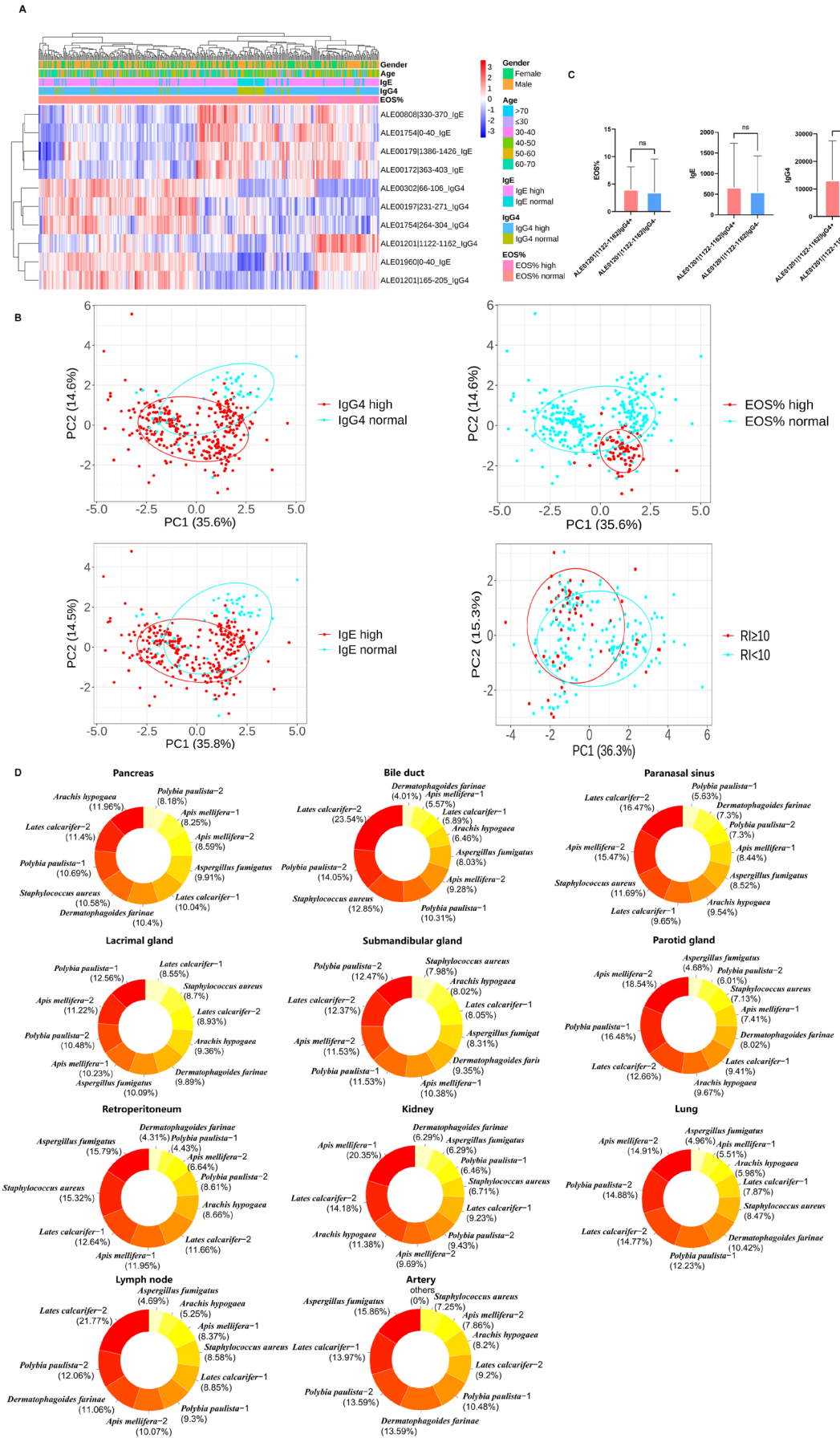

FIGURE 4 | Legend on next page.

FIGURE 4 | Correlations between identified allergens and patient characteristics. (A) Nonbiased hierarchical clustering of patients based on antibody responses to the top 10 peptides, stratified by age, sex, EOS%, IgG4, and IgE levels. (B) Principal component analysis of allergen peptide responses among different subgroups. (C) Comparison of EOS%, IgE, and IgG4 in patients with higher and lower IgG4 response to ALE01201|1122-1162. (D) Allergen response relative contribution in IgG4-RD patients with different organ involvement.

TABLE 2 | All candidate allergen peptides from *Arachis hypogaea*.

Allergen peptide	Antibody response	Allergen	IUIS	Sequence
ALE00197|231-271	IgG4	*A. hypogaea*	Ara h 1	NLREGEPDLSNNFGKLFEVKPDKKNPQLQDLDMMLTCVEI
ALE00231|396-436	IgG4	*A. hypogaea*	Ara h 1	EPDLSNNFGRLFEVKPDKKNPQLQDLDMMLTCVEIKEGAL
ALE00224|396-436	IgG4	*A. hypogaea*	Ara h 1	NLREGEPDLSDNFGRLFEVKPDKKNPQLQDLDMMLTCVEI
ALE00196|330-370	IgG4	*A. hypogaea*	Ara h 3	TICTATVKKNIGRNRSPDIYNPQAGSLKTANELNLLILRW
ALE00210|0-40	IgG4	*A. hypogaea*	Ara h 10.0101	MTDRTQPHTVQVHTTAGRFGDTAAGTNRYPDRGPSTSKVI
ALE00234|CTERM|STOP	IgG4	*A. hypogaea*	Ara h 12.0101	TNASCDDHCKNKEHFVSGTCMKMACWCAHNC

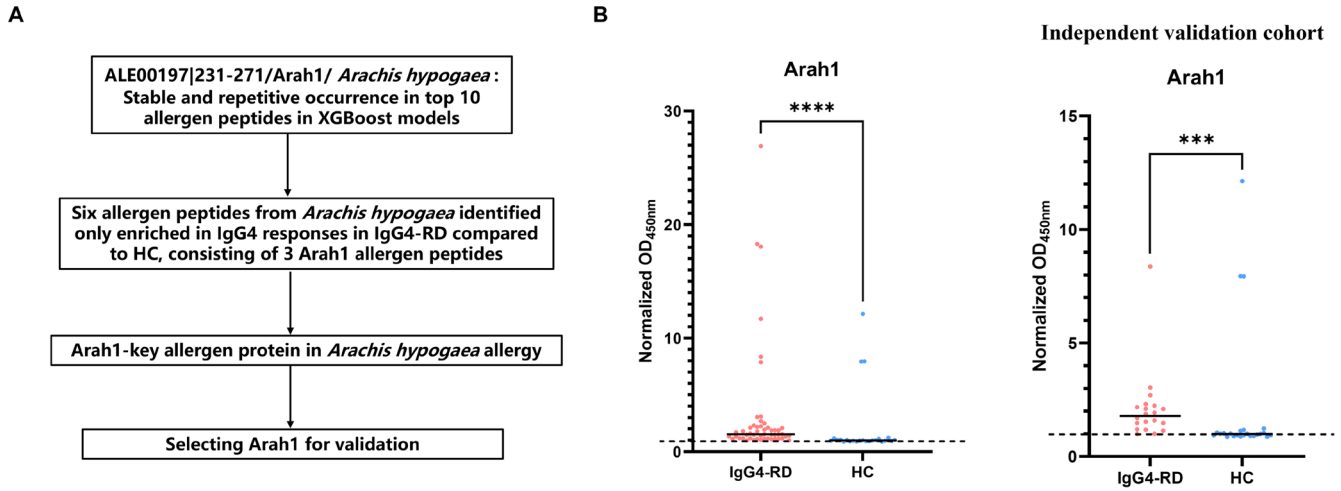

FIGURE 5 | Validation of Arah1-specific IgG4 levels assessed by ELISA. The results are normalized as the ratio of the OD value to median OD + 3MAD obtained in HCs per experiment. (A) Rationale of selecting Arah1 for validation. (B) ELISA results for 51 IgG4-RD patients and 24 HCs, including 20 additional patients independent of peptide-based high-throughput immunoprecipitation sequencing cohort. ***adjusted $p < 0.001$; ****adjusted $p < 0.0001$. OD, optical density, HC, healthy control, MAD, Median absolute deviation.

cohorts. Arah1-specific IgG4 levels were significantly higher in IgG4-RD patients than in healthy controls, with similar results observed in independent validation cohort (adjusted $p < 0.05$, Figure 5B). Results for Arah1 blocking experiment eliminated interference of nonspecific binding (Figure S3).

3.7 | Temporal Analysis of the Top 10 Allergen Peptides in IgG4-RD

Next, a follow-up analysis of 102 IgG4-RD patients was conducted after a median period of 3 years (median, Q1, Q3, 3.0, 2.1, 4.0), evaluating their IgE and IgG4 responses to allergens as above. Treatment details of follow-up cohort are shown in Table 3. The results showed that a single peptide among the top 10 peptides—ALE01960|0-40 belonging to *S. aureus*—remained reactive in IgG4-RD patients at this follow-up time point (Figure 6A,B). Significantly, the IgE-to-IgG4 transition of allergic responses to ALE01960|0-40 was observed in patients with IgG4-RD (Figure 6B), suggesting the potential establishment of allergic tolerance against *S. aureus*, in line with the stronger IgG4 responses against *S. aureus* observed in healthy

TABLE 3 | Treatment of follow-up patients with IgG4-RD.

Follow-up cohort	IgG4-RD
Treatment received, N (%)	
GC monotherapy	13/102 (12.7)
GC + IMs	69/102 (67.6)
IMs monotherapy	11/102 (10.8)
Watchful waiting	9/102 (8.9)

Note: IMs were primarily Mycophenolate mofetil ($n = 34$), Methotrexate ($n = 15$), Leflunomide ($n = 14$), Azathioprine ($n = 5$), and others.
Abbreviations: GC, glucocorticoids; IM, immunosuppressive therapy.

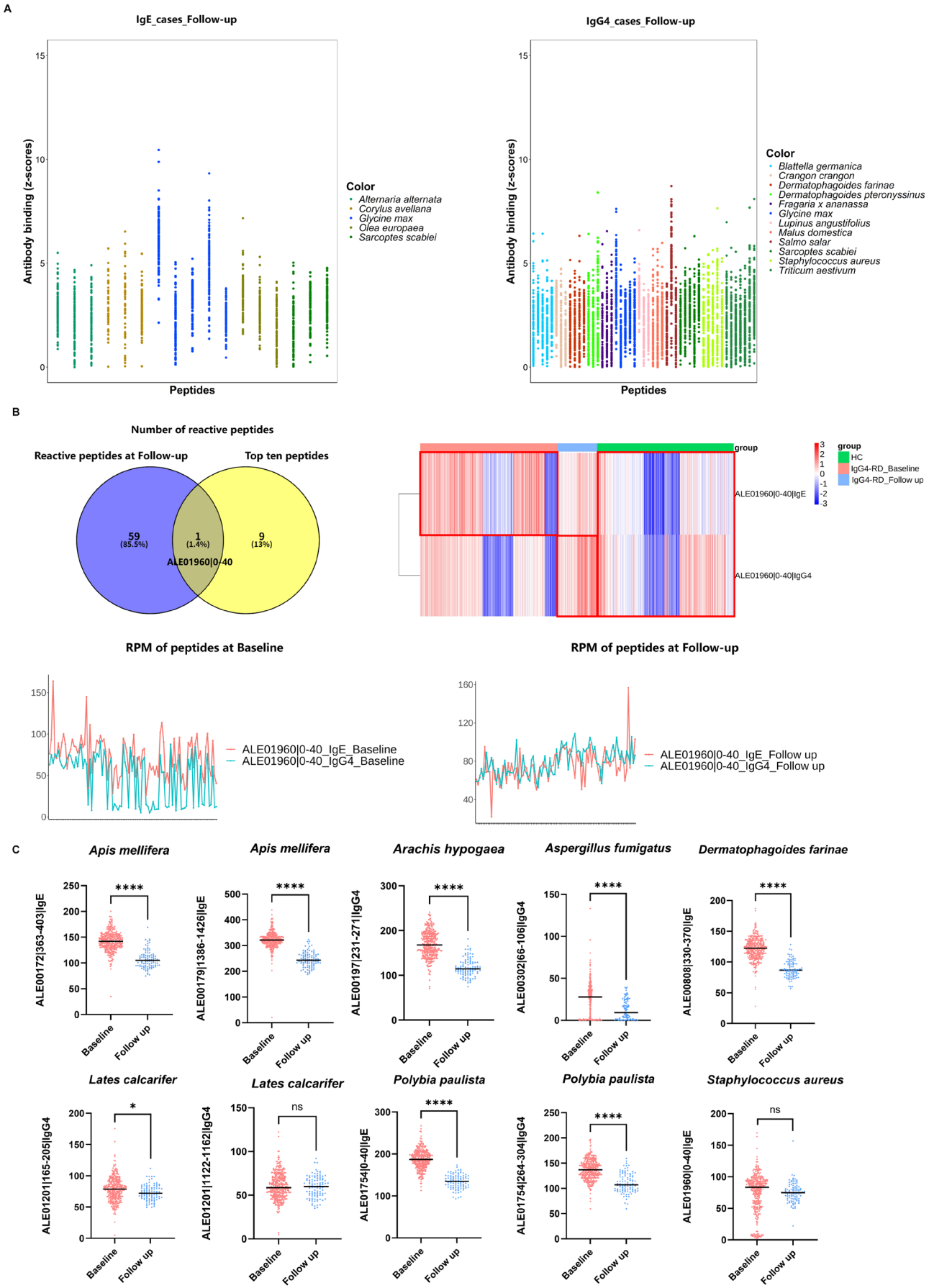

FIGURE 6 | Legend on next page.

FIGURE 6 | Temporal verification of candidate allergens and their peptides. (A) Scatter diagrams corresponding to allergen-specific IgE and IgG4 responses in IgG4-RD patients at the follow-up time point. (B) Identification of 1 of the top 10 peptides at both time points, and the overall transition of antibody responses. (C) Responses toward the top 10 allergens in sequential samples. *adjusted $p < 0.05$; ****adjusted $p < 0.0001$.

controls (Figure 2B). Furthermore, patient responses toward the top 10 allergens were characterized in the sequential samples, and 8 of them were significantly decreased, which may be attributable to the general inhibition of abnormal immune responses when undergoing treatment (Figure 6C).

4 | Discussion

This study involved a pan-allergen-specific IgE and IgG4 trajectory assessment and sequential analysis in a large cohort of IgG4-RD patients, compared with those from sex- and age-matched healthy controls via PhIP-Seq. This study offers novel insights into the distinct allergic responses that may differentiate individuals with IgG4-RD and healthy controls, while also exploring their association with clinical features. The top 10 allergen peptides linked to the pathogenesis of IgG4-RD were identified through XGBoost-based machine learning. The analysis suggested significant differences in IgE and IgG4 responses toward particular allergens, notably *A. mellifera* and *A. hypogaea*, implying their potential roles in IgG4-RD pathogenesis and associated organ involvement. Although experimental confirmation remains necessary, delineating these allergen specific antibody signatures furnishes a focused roadmap for future mechanistic and translational investigations in IgG4-RD.

The allergens detected in healthy controls in this study are consistent with frequently reported common allergens in other domestic and international studies published to date, emphasizing the strong credibility of our results. In 1981, the Codex Alimentarius Commission (CAC) defined the eight most important food allergens as gluten, crustaceans, eggs, fish, peanuts, soybeans, milk, and nuts, accounting for more than 90% of all allergic reactions to foods [38, 39]. In China, a 13-year multicenter retrospective study found that the top three most common aeroallergens among allergic rhinitis patients were *D. farinae*, *Dermatophagiodes pteronyssinus*, and *Blatella germanica* [40]. These reported allergens were also largely represented in our dataset.

To the best of our knowledge, our study presents the first detailed description of a predominant association between *A. mellifera* and allergen-induced IgE allergic responses in patients with IgG4-RD. Regarding bee allergies, research has shown that the frequency of IgG4-switched phospholipase A2 (PLA)-specific memory B cells were increased in allergic patients during allergen immunotherapy (AIT) and healthy beekeepers after venom exposure, while circulating IgE-switched PLA-specific B cells cannot be detected, which may be due to the very low frequencies of these circulating IgE-switched memory B cells [41]. Increased expression of IL-10 and IgG4 are observed in B cells specific for PLA isolated from nonallergic beekeepers [42]. In our results, we identified specific allergens uniquely recognized by either IgE or IgG4 antibodies in IgG4-RD patients, underscoring the complexity of the immune mechanisms underlying this disease. Notably, enhanced IgE reactivity against allergens such as *A. mellifera* was more frequently observed in patients with involvement of organs like the lacrimal glands, parotid glands, paranasal sinuses, lungs, and kidneys. Conversely, the stronger IgG4 reactivity observed among healthy controls suggests potential immunoregulatory mechanisms that are impaired or dysregulated in IgG4-RD, contributing to disease onset or progression.

Validation of increased IgG4 responses to *A. hypogaea* allergen Arah 1 by ELISA in independent cohorts further supports the hypothesis of allergen-driven immune dysregulation in IgG4-RD. Notably, this elevated response was linked to pancreatic involvement, suggesting a possible mechanistic connection between allergen exposure and organ-specific inflammation. While previous research has reported aberrant IgG4 responses to peanut allergens [23], our comprehensive analysis provides stronger empirical evidence, highlighting the importance of detailed allergen-specific profiling.

Another significant observation from our study was the shift from IgE to IgG4 antibody responses against *S. aureus* allergens during follow-up. Though *S. aureus* is not a typical allergen with common knowledge, several research has reported that it can induce IgE/IgG4 responses in allergic diseases [43, 44]. Additionally, as an infectious factor, *S. aureus* has been reported as a risk factor for relapses in granulomatosis with polyangiitis (GPA) [45–48]. Behind the GPA, the possible role of *S. aureus* carriage condition has been explored in other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus [49]. Prior studies have also reported a shift from IgE to IgG/IgG4 reactivity in response to AIT [26, 41, 50]. Our finding could indicate the emergence and establishment of allergic tolerance against this bacterial species in line with the stronger IgG4 responses against *S. aureus* we observed in healthy controls, but we cannot exclude alternative explanations such as the influence of natural half-lives of different antibody isotypes or the broad effects of immunosuppressive therapy. The shift from disease-associated to healthy-like antibody responses against *S. aureus* is possibly induced by reduced inflammation, therapy-mediated modulation, or epithelial barrier repair. This phenomenon, evident in our longitudinal data, suggests that therapeutic interventions may favorably modulate immune responses, reflecting the dynamic antibody-mediated immune regulation in IgG4-RD.

IgG4 antibodies uniquely undergo Fab-arm exchange, a stochastic and dynamic process involving swapping of a heavy-light chain pair (half-molecule) with another molecule, resulting in bispecific antibodies [51]. Additionally, IgG4 antibodies exhibit increased glycosylation within variable domains [13]. Conventionally, IgG4 is viewed as an anti-inflammatory or tolerogenic antibody due to its limited ability to cross-link antigens and form immune complexes [51, 52]. However, advances in analytical and structural methods have progressively unveiled potential pathogenic roles of IgG4 in allergic conditions such as allergic rhinitis, asthma, and atopic dermatitis [52].

Notably, in eosinophilic esophagitis, significant IgG4 deposition in esophageal tissue has been reported, implying that IgG4 molecules can accumulate and form immune complexes under certain conditions, such as high antibody concentrations, cross-linking among antibodies from a single source, and optimal allergen size and conformation [21, 53]. Therefore, the function of IgG4 is complex and requires further investigation. IgG4 biological function may depend on multiple factors including antibody isotype proportion and concentration, antigen concentration and physical properties, immune microenvironment, as well as genetic background of individuals. Our findings suggest heterogeneous IgG4 responses toward different allergens. Some responses appear consistent with the known anti-inflammatory properties of IgG4, exemplified by an IgE-to-IgG4 class switch against *S. aureus* peptides observed in IgG4-RD patients following treatment, and elevated IgG4 reactivity to various allergens even among healthy individuals. In contrast, other responses may reflect pathogenic contributions of IgG4 in IgG4-RD, exemplified by significantly increased IgG4 reactivity against *A. hypogaea* correlating with pancreatic involvement in patients relative to controls. The precise mechanisms underlying these diverse IgG4-mediated effects remain unclear, necessitating additional mechanistic studies to elucidate these relationships fully.

By integrating machine learning (XGBoost), our study robustly identified the most predictive allergen peptides, offering potential biomarkers for diagnosing and monitoring IgG4-RD. The high predictive accuracy (AUC: 0.91–0.96) suggests the clinical relevance of allergen-specific IgE and IgG4 signatures, which may facilitate personalized therapeutic approaches. However, additional research is required to elucidate the biological implications of these identified allergen-antibody interactions fully.

Our findings align with globally recognized allergens such as *D. farinae* and *D. pteronyssinus*, reinforcing the external validity of our allergen repertoire. Nevertheless, although extensive, our allergen set was not exhaustive, and inherent limitations of PhIP-Seq—including the inability to detect discontinuous epitopes and post-translational modifications—must be acknowledged. Moreover, the bispecific nature of IgG4 antibodies resulting from Fab-arm exchange was not explicitly addressed, highlighting another critical area for future investigation. Another limitation is that we did not ascertain the isotype purity of the immunoprecipitation eluates. The bead-bound state and low concentrations of bead-enriched IgE and IgG4 precluded precise isotype quantification of the eluates; accordingly, we restricted measurements to plasma IgE and IgG4 concentrations and cannot exclude low-level co-elution of IgG1/IgA that could confound downstream assays. Although we used highly specific capture antibodies and low-nonspecific-binding magnetic beads to minimize carryover, residual uncertainty remains. Future studies incorporating direct analysis of eluate composition will be essential to confirm and refine these findings. In addition, we did not perform stratified analyses by clinical phenotype (e.g., pancreas, lacrimal gland, submandibular gland) due to small subgroup sizes in our cohort; therefore, definitive conclusions regarding organ-specific antibody signatures in IgG4-RD will require larger, phenotype-enriched cohorts.

Despite these limitations, this study substantially enhances understanding of IgG4-RD pathogenesis by highlighting the importance of allergen-directed immune responses and providing a foundation for mechanistic work. Abnormal IgE and IgG4 reactivity to certain allergens was more prevalent in patients than in matched healthy controls and may be associated with organ involvement. These findings are compatible with a possible role for allergen-directed antibody responses in the inflammation and immune activation underlying IgG4-RD and may inform future mechanistic studies; nevertheless, confirmation and refinement through assays that directly quantify isotype carryover remain necessary. Our results also underscore the emerging association between allergy/atopy and IgG4-RD, although the precise roles of allergy/atopy in disease onset remain to be defined. Further research is warranted to clarify these mechanistic links and to evaluate whether allergen profiling can improve clinical monitoring or inform targeted therapeutic strategies for this complex disease.

Author Contributions

Conceptualization: Z.L., S.Y., W.Z., Yz.L.; Methodology: Z.L., Jl.Z., C.Y.; Investigation: Z.L., Jl.Z., H.L., Z.W., S.W., X.F., C.Y.; Visualization: Z.L., S.Y.; Funding acquisition: W.Z., Yz.L.; Project administration: Z.L., Z.W., H.X., F.F., Jd.Z.; Supervision: L.C., Ym.L., H.Z., L.P., Jx.Z., Y.F.; Writing – original draft: Z.L., S.Y., Jl.Z.; Writing – review and editing: W.Z., Yz.L. All authors provided final approval of the version of the article to be published.

Conflicts of Interest

The authors declare no conflicts 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. **Appendix S1:** all70162-sup-0001-AppendixS1.docx. **Appendix S2:** all70162-sup-0002-AppendixS2.xlsx.