ORIGINAL ARTICLE

Immunological Parameters for Assessing the In Vitro Safety and Efficacy of Allergoid Mixtures for Immunotherapy

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Abstract

Background: Most patients with respiratory allergy are polyallergic. Combining different allergen extracts in the same allergen-specific immunotherapy is a common practice. However, it should be justified.
Objective: To analyze the stability, safety, and immune response of allergen extract mixtures from nonhomologous groups.
Methods: We analyzed 2 depigmented-polymerized mixture extracts (DPmixEs): cat dander–grass pollen and *Alternaria alternata*–grass pollen. The stability of the mixtures was investigated by studying proteolysis and degradation effects. The allergenicity and humoral and cellular immune responses of DPmixEs were also evaluated using various technical approaches, including the Bradford assay, enzyme-linked immunosorbent assay, rabbit immunization, peripheral blood mononuclear cell culture, and flow cytometry. The results were compared with those of individual depigmented-polymerized extracts (DPEs) and native mixture extracts (NmixEs).
Results: The proteolytic activity of DPmixEs was lower than that of NmixEs. The protein content of DPmixEs remained stable for 18 months, whereas that of NmixEs decreased significantly during the first month. The allergenicity of DPmixEs was similar to that of DPEs and lower than that of NmixEs. Regarding the immune response, DPmixEs induced functional specific IgG antibodies in rabbits and blocked sIgE-allergen binding. Moreover, DPmixEs induced IL-10 secretion in peripheral blood mononuclear cells from polyallergic patients, improving the Treg/T_H2 cell balance.
Conclusion: These findings support the use of DPmixE as a promising formulation for allergen immunotherapy, combining stability, reduced enzymatic activity, and enhanced immunological stimulation, while preserving in vitro safety and efficacy comparable to separated depigmented-polymerized extracts.

Key words: Allergen-specific immunotherapy. Polyallergy. Tolerogenic response. Multiallergen treatment. Allergoid. Immunomodulation. Mechanisms of action.

Resumen

Antecedentes: La mayoría de los pacientes respiratorios alérgicos son polialérgicos. La combinación de alérgenos en una misma vacuna de AIT es una comúnmente utilizada para tratarlos, pero su uso debe justificarse.
Objetivo: Analizar la estabilidad, seguridad y respuesta inmunológica de mezclas de extractos alergénicos de grupos no homólogos.
Métodos: Se analizaron dos mezclas de extractos despigmentadas-polimerizadas (DPmixEs): caspa de gato-polen de gramíneas y *Alternaria alternata*-polen de gramíneas. Se evaluó la estabilidad del contenido proteico y las actividades proteolíticas. Para los estudios de alergenicidad y la respuesta inmunitaria, se utilizaron diferentes enfoques técnicos: ELISA, inmunización en conejos, cultivos de PBMC y citometría de flujo. Los resultados de las DPmixEs se compararon con extractos polimerizados despigmentados individuales (DPEs) y mezclas nativas (NmixEs).
Resultados: El contenido proteico de DPmixEs se mantuvo estable durante 18 meses de estudio, mientras que el contenido de NmixEs disminuyó significativamente en el primer mes. La alergenicidad de las DPmixEs fue similar a la de los DPEs. En cuanto a la respuesta inmunitaria, las DPmixEs indujeron anticuerpos IgG en conejos con capacidad de bloquear la unión de sIgE-alérgenos. Los DPmixEs indujeron la secreción de IL-10 en PBMC de pacientes alérgicos, mejorando el balance de las poblaciones Treg/T_H2.
Conclusión: La mezcla de DPEs no afectó a la estabilidad. Además, los DPmixE no incrementaron su alergenicidad y activaron la respuesta inmunotolerogénica a nivel humoral y celular. Estos resultados refuerzan la adecuación de DPmixEs para el tratamiento de pacientes polialérgicos.

Palabras clave: Inmunoterapia específica de alérgeno. Polialergia. Respuesta tolerogénica. Tratamiento basado en mezclas de alérgenos. Alergoide. Inmunomodulación. Mecanismos de acción.

Summary box

- **What do we know about this topic?**
  Treating polyallergic patients with various allergens eliminates the need for multiple injections and potentially reduces both cost and time required.
- **How does this study impact our current understanding and/or clinical management of this topic?**
  The current study on depigmented-polymerized mixture extracts offers evidence of potential clinical efficacy, which should be considered when treating polyallergic patients.

Introduction

Allergic respiratory diseases constitute a global health problem that seriously impacts the daily lives of affected individuals [1]. Most patients are sensitized to various allergenic sources simultaneously (polysensitization). This phenomenon is attributed to sensitization to panallergens, different molecular allergens from the same allergenic source, and/or multiple allergens with no common structural features from different environmental sources [2,3]. The last of these 3 factors is especially relevant in countries with multiple types of aeroallergens and high allergen pressure [1,4]. From a clinical perspective, polysensitization is generally considered a risk factor for the subsequent development of severe allergic symptoms and more severely impaired quality of life [5].

Despite the clinical relevance of polysensitization, no consensus regarding the most appropriate approach to treating affected patients has been reached [2,6]. Allergen-specific immunotherapy (AIT) is the only treatment that can correct the course of allergic diseases, and the strategy of using allergenic mixtures in the same vaccine has been commonly adopted by physicians, with favorable clinical outcomes [7]. AIT is well tolerated, eliminates the need for multiple injections, and reduces costs and time. However, the approach is controversial, and its use varies from country to country [1,8]. European recommendations released some years ago by the European Medicines Agency [9] are based on the principle of homologous groups generally related to taxonomic families and imply minor variations in the composition, physicochemical properties, and biological properties of allergen extracts. In this sense, these properties cannot be extrapolated to other, nonhomologous groups, and mixtures containing allergens that do not belong to the same group must be justified [1,9,10]. Mixtures likely present several drawbacks owing to the potential risk of allergen degradation. Moreover, some studies have suggested that combining nonhomologous allergens could increase allergenic potency and overstimulate the immune response [11]. Therefore, key considerations for formulating mixed AIT vaccines include the following: (*1*) selecting adequate proportions and concentrations of each allergen to guarantee the safety and efficacy of the final products; and (*2*) ensuring the absence of activated proteolytic enzymes that could degrade proteins and compromise the stability of the final formulation [8].

The development of allergoids for AIT provided an important competitive advantage over native extracts. In Europe, the allergoid approach has been widely adopted with the aim of minimizing the risk of adverse effects [12]. Allergoids are allergen extracts that have been chemically modified to reduce their allergenic activity while maintaining their immunogenicity [13,14]. Moreover, several studies have demonstrated the strong positive impact of this AIT strategy in terms of stability, safety, tolerability, and efficacy and clarified the mechanisms of action underlying these types of products [2,14-18].

The primary objective of the study was to evaluate the stability, safety, and immunological efficacy profiles of mixtures of modified (depigmented and polymerized) allergen extracts, specifically cat dander–grass pollen and *Alternaria alternata*–grass pollen, which encompass diverse allergenic sources (pollen, animal epithelia, and molds).

Methods

Allergen Extracts

Allergen extract preparations

Lyophilized native extracts (NEs) of cat dander, *A alternata*, 5 grass pollens (*Phleum pratense, Dactylis glomerata, Lolium perenne, Poa pratensis*, and *Festuca elatior*) and their corresponding depigmented-polymerized extracts (DPEs) were manufactured based on Good Manufacturing Practices standards following in-house procedures (LETI Pharma S.L.). The grass mixture was prepared with equal concentrations of each of the 5 grass extracts in purified water or phenolated saline solution. Cat dander and *A alternata* extracts were also resuspended in the same solvents.

Solutions of native mixture extracts (NmixEs) and depigmented-polymerized mixture extracts (DPmixEs) were prepared by mixing cat dander–grass pollen and *A alternata*–grass pollen extracts. Both mixed products contained the same amount of each individual component.

Characterization

The molecular weight distribution of each extract was determined using high-performance size exclusion chromatography with a Bio SEC-3 column (Agilent Technologies) on a 1260 series high-performance liquid chromatography system (Agilent Technologies). Additionally, SDS-PAGE (2.67%C-15%T acrylamide) was performed under reducing conditions, and staining was with Coomassie-Blue

R-250 (Bio-Rad Laboratories). The allergenic properties of the extracts were studied using Western blotting with pools of sera from patients allergic to cat dander, grass pollen, or *A alternata* (Plasmalab International and SerApply International) and verified using monoclonal α-human-IgE-PO (Southern Biotech). Major allergens (Fel d 1 for cat dander, Alt a 1 for *A alternata*, and Phl p 5 for grass pollen) in the NEs, NmixEs, DPEs, and DPmixEs were quantified using specific enzyme-linked immunosorbent assay (ELISA) kits (EL-FD1, EL-AA1, and EL-PP5; Inbio Biotech) following the manufacturer's instructions.

Stability Studies

Enzymatic activity

Enzymatic activity was evaluated using an Api-Zym kit (BioMérieux) according to the manufacturer's instructions. Each reaction was scored between 0 and 5 (0 corresponds to a negative reaction and 5 to maximum intensity). Total proteolytic activity was obtained from the sum of the individual scores for the activity of leucine-arylamidase, valine-arylamidase, cysteine-arylamidase, trypsin, and α-chymotrypsin. Enzymatic activities were also evaluated using gelatin zymography SDS-PAGE.

Protein content

Protein content was measured at 0, 1, 3, 6, 12, and 18 months using the Bradford method with Coomassie protein assay reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Extracts were resuspended in a phenolated-saline solution and stored at 4ºC. Acceptance criteria were established according to the European Pharmacopeia [19] at 80%-120% of the total protein value of each mixture obtained at time 0.

Biological potency

The allergenicity of each DPmixE was measured at 0, 2, 4, 8, and 16 weeks using ELISA competition assays following the criteria recommended in the European guidelines of allergen products [9] and using pools of sera from patients allergic to grass pollen, *A alternata*, and cat dander (Plasmalab International and SerApply International). The 50% inhibition point (IP) values for DPmixEs were compared with the corresponding fresh individual DPE values. Acceptance criteria were established at 50%-200% of the 50% IPs of each individual DPE obtained at time 0.

This assay also enables the in vitro safety profile of DPmixEs to be compared with that of DPEs.

Immunological Studies

Humoral response

Specific IgG production was induced in 2 New Zealand white rabbits after 3 immunizations administered every 3 weeks. The immunizations were with 200 mg of DPmixE in the presence of complete Freund adjuvant for the first immunization and incomplete Freund adjuvant for the second and third immunizations. All procedures were approved by the Biolab Institutional Review Board (Madrid, Spain; reference number, PROEX 075/17) and adhered to local ethics requirements for animal experimentation.

The capacity of DPmixEs to induce allergen-specific polyclonal IgG antibodies that block human IgE binding to each allergen was evaluated using inhibition ELISA, as previously described [20]. The percentage of inhibition was calculated by comparing IgE binding after incubation with preimmune or final bleeding polyclonal antibodies as follows: $100 - (ODf/ODP) \times 100$, where ODf and ODP are the optical densities of serum after preincubation with the rabbit's final sera and the corresponding preimmune sera, respectively.

Cellular response

Different immune cell populations and cytokines were characterized in response to allergen extract stimuli using peripheral blood mononuclear cells (PBMCs) from patients with allergies and healthy controls.

Recruitment of allergic patients and healthy controls

Participants were recruited at the Allergy Services of Hospital Universitario Ramón y Cajal (Madrid, Spain), Hospital Universitario La Paz (Madrid, Spain), and Hospital Universitario de Guadalajara (Guadalajara, Spain) after ethics committee approvals (Ref. HULP: PI-4583, Ref. 438/20, and Ref. 2021.01.EO NO EPA, respectively). The inclusion criteria for patients with allergy were age ≥18 years and a history of rhinitis/rhinoconjunctivitis with or without allergic asthma for at least 2 previous allergen seasons. All participants signed informed consent forms. Twenty-four and 12 participants were included in the allergic and healthy groups, respectively, according to their clinical symptoms/history and related skin prick test results. Skin prick tests were performed according to European guidelines using a commercialized battery of extracts (LETI Pharma), including those from *A alternata*, cat epithelia, and *P pratense* (see Supplementary material). The response was considered positive when the wheal diameter was >3 mm greater than that induced by a negative saline control.

Finally, approximately 40 mL of peripheral blood was obtained from each participant.

Specific immunoglobulin determination

Serum sIgE levels against *A alternata*, cat epithelia, and *P pratense* were determined using ImmunoCAP (Thermo Fisher Scientific).

Basophil activation test

The basophil activation test (BAT) was performed to study the capacity of the NEs, DPEs, NmixE, and DPmixEs to activate effector cells, serving as a functional study of the in vitro safety profile. Peripheral blood was stimulated with the extracts at 4 different concentrations (0.025, 0.25, 2.5, and 25 μg/mL), and the expressions of the specific markers CCR3 and CD63 were evaluated using flow cytometry (Flow CAST Basophil Activation Test Flow Cytometry; BÜHLMANN

Diagnostics Corp.) and FlowJo 10.9.0 software (FlowJo, LLC). The number of activated cells was reported as a percentage (cells expressing CD63 among all basophils gated; a minimum of 500 basophils was analyzed in each case). Data were analyzed using FACSDiva 8.0 software (Becton Dickinson) and Flowjo 10.9.

Cell cultures

PBMCs from peripheral blood were isolated by Ficoll gradient centrifugation and cultured in the presence of the stimuli (NEs, DPEs, or DPmixEs). PBMCs incubated with only culture media and those stimulated with concanavalin A (5 µg/mL) were used as negative and positive controls, respectively. PBMCs were cultured in RPMI media supplemented with fetal calf serum at 20% for 16 and 48 hours at 37°C and in 5% CO_2. Finally, the supernatant and cells were isolated for further analysis.

Cytokine determination

The production of IFN-γ, IL-2, IL-5, IL-4, and IL-10 in the supernatants of cell cultures was determined using a CBA kit (Thermo Fisher Scientific) and flow cytometry (FACs Canto A Cytometer and FCAP™ Array Software, BD Biosciences) following the manufacturer instructions.

Cell population phenotype

The phenotypes of different lymphocyte subpopulations were analyzed using flow cytometry. Cell subpopulation phenotypes were defined as follows: $CD4^+$ $CCR6^-CXCR3^-$ as T_H2, $CD1^+CD5^+CD19^+$ as $Breg^+$ cells, and $CD4^+CD25^+FOXP3^+$ as Tregs. Detailed data are provided in Table S1. First, for each participant, the proportion of $CD4^+CCR6^-CXCR3^-$ T cells was calculated with respect to the total T-cell population, as were that of $CD1^+CD5^+CD19^+$ B cells with respect to total B cells and that of $CD4^+CD25^+FOXP3^+$ T cells with respect to the total T-cell population. Second, stimulation indexes were calculated as the ratio between the proportion of each cell subtype for each stimulus and the proportion obtained in cell cultures in the absence of stimulation.

Statistical Analysis

Statistical analyses were performed using Prism 10 (GraphPad Software Inc.). Descriptive statistics were used to characterize the study population. Quantitative variables are

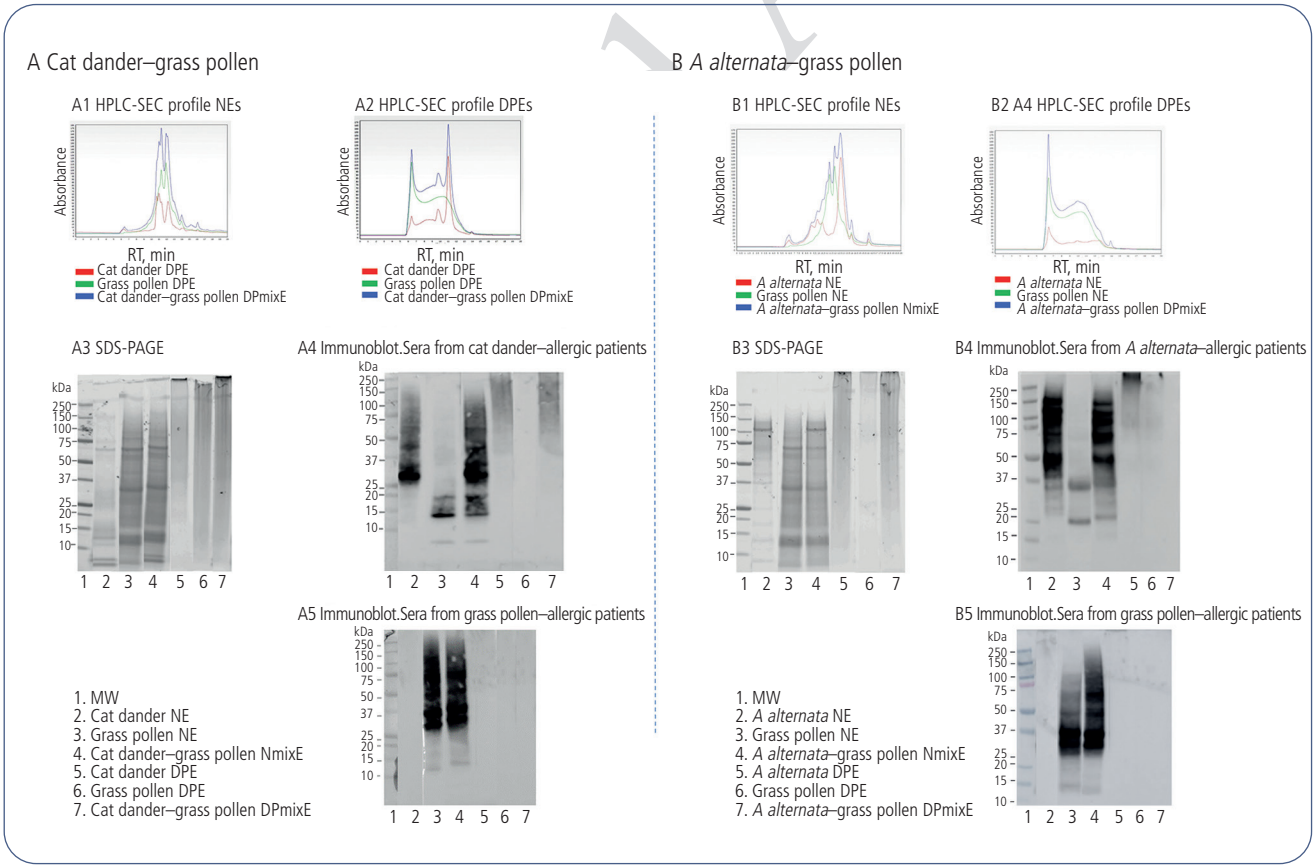

Figure 1. Extract characterization. A, Cat dander and grass pollen allergens. A1-A3, Protein profile analyzed using size-exclusion high-performance liquid chromatography and SDS-PAGE. A4-A5, Allergenic profile of cat dander and grass pollen extracts using a pool of sera from polyallergic patients. B, *Alternaria alternata*-grass pollen. B1-B3, Protein profile analyzed using size-exclusion high-performance liquid chromatography and SDS-PAGE. A4-A5, Allergenic profile of A alternata and grass pollen extracts using a pool of sera from allergic patients. MW indicates molecular weight; NE, native extract; DPE, depigmented and polymerized extract; NmixE, native mixture extract; DPmixE, depigmented-polymerized mixture extract.

presented as mean (SD) or median (IQR). Categorical variables are presented as frequencies and percentages. The normality of distributions was calculated using the Shapiro–Wilk test. Nonnormal distributions were compared using the Friedman and Dunn multiple comparisons tests.

Results

Allergen Extract Characterization

All individual allergen extracts fulfilled the internal acceptance criteria. Protein profiles are shown in Figure 1. Cat dander–grass pollen and *A alternata*–grass pollen NmixEs and DPmixEs were characterized by prominent peaks owing to the sum of allergenic sources rather than to the individual allergens (Figure 1, A1-B2). SDS-PAGE showed that individual NEs presented a profile of protein bands in the entire range of molecular weights analyzed, whereas NmixEs presented a more complex profile (Figure 1, A3 and B3). As expected, DPEs and DPmixEs did not show any defined protein bands below the 250-kDa protein marker.

Sera from allergic patients recognized the same protein bands in individual NEs and NmixEs. The absence of recognition in DPEs and DPmixEs indicated that the polymerization process was appropriate owing to the reduced access for allergen-IgE antibody binding (Figure 1, A4-A5 and B4-B5).

Regarding allergen quantification, the mean (SD) Phl p 5 levels in the grass pollen extract were 19.91 (3.25) µg/mg for the lyophilized extract and 18.24 (2.90) µg/mg in the grass pollen–cat dander NmixE. Fel d 1 levels were 18.12 (3.44) µg/mg in the cat dander NE and 17.39 (2.84) µg/mg in NmixE. In the *A alternata*–grass pollen NE, the mean values were 0.91 (0.01) µg/mg for Alt a 1 and 24.27 (5.66) µg/mg for Phl p 5, similar to values obtained for individual NEs (Alt a 1, 1.00 [0.041] µg/mg; Phl p 5, 24.27 [5.66] µg/mg). As expected, nonallergens could be quantified in DPEs and DPmixEs.

Stability Studies

Api-Zym results showed that the proteolytic activity score was between 3.5- and 5-fold lower in DPmixEs than in NmixEs (Figure 2A, Figure S1). The presence of clear bands in the zymography gel indicates enzymatic activity. These bands were observed in cat dander NE (2 bands of around 17-18 kDa), *A alternata* NE (1 band at 22 kDa), and in both NmixEs but not in the corresponding DPEs and DPmixEs (Figure 2B). Regarding total protein content, the 2 DPmixEs (cat dander–grass pollen and *A alternata*–grass) were stable for 18 months. However, their corresponding NmixEs exhibited progressively reduced protein levels, and after a month of study, levels were under the specification limit (Figure 2C). Moreover, the biological potency of both DPmixEs was stable for the 16 weeks of the study and fulfilled the established acceptance criteria (Figure 3A).

In Vitro Safety Profile

The biological potency of each DPmixE was compared with that of individual DPEs to determine whether the combination of different types of allergens increased biological potency. In both DPmixEs, the 50% IP value was similar to that of individual DPEs (P>.05) (see Figure 3A, mean values).

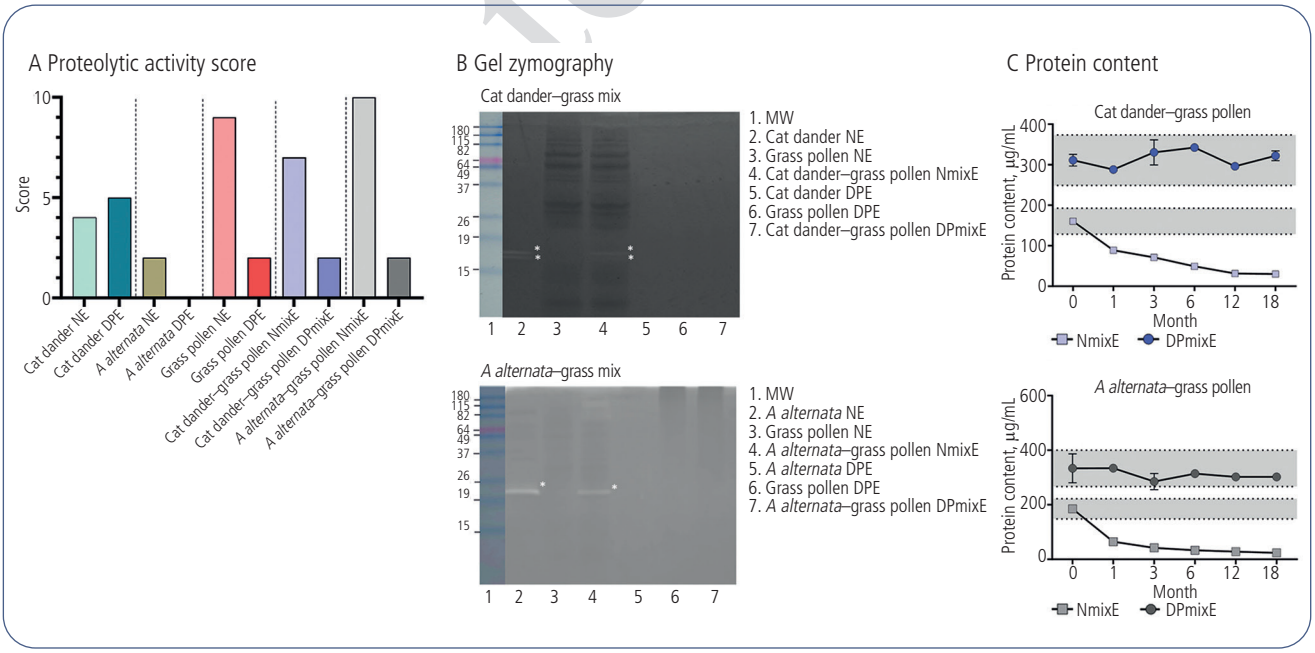

Figure 2. Protein stability. A, Proteolytic activity scores obtained by summing the individual scores for activity of leucine-arylamidase, valine-arylamidase, cysteine-arylamidase, trypsine, and α-chymotrypsine according to Api-Zym. B, Gel zymography. Asterisk-marked white bands indicate proteins with enzymatic activity. C, Protein content of allergen extract mixtures with time. NE indicates native extracts; DPE, depigmented and polymerized extracts; NmixE, native mixture extract; DPmixE, depigmented-polymerized mixture extract.

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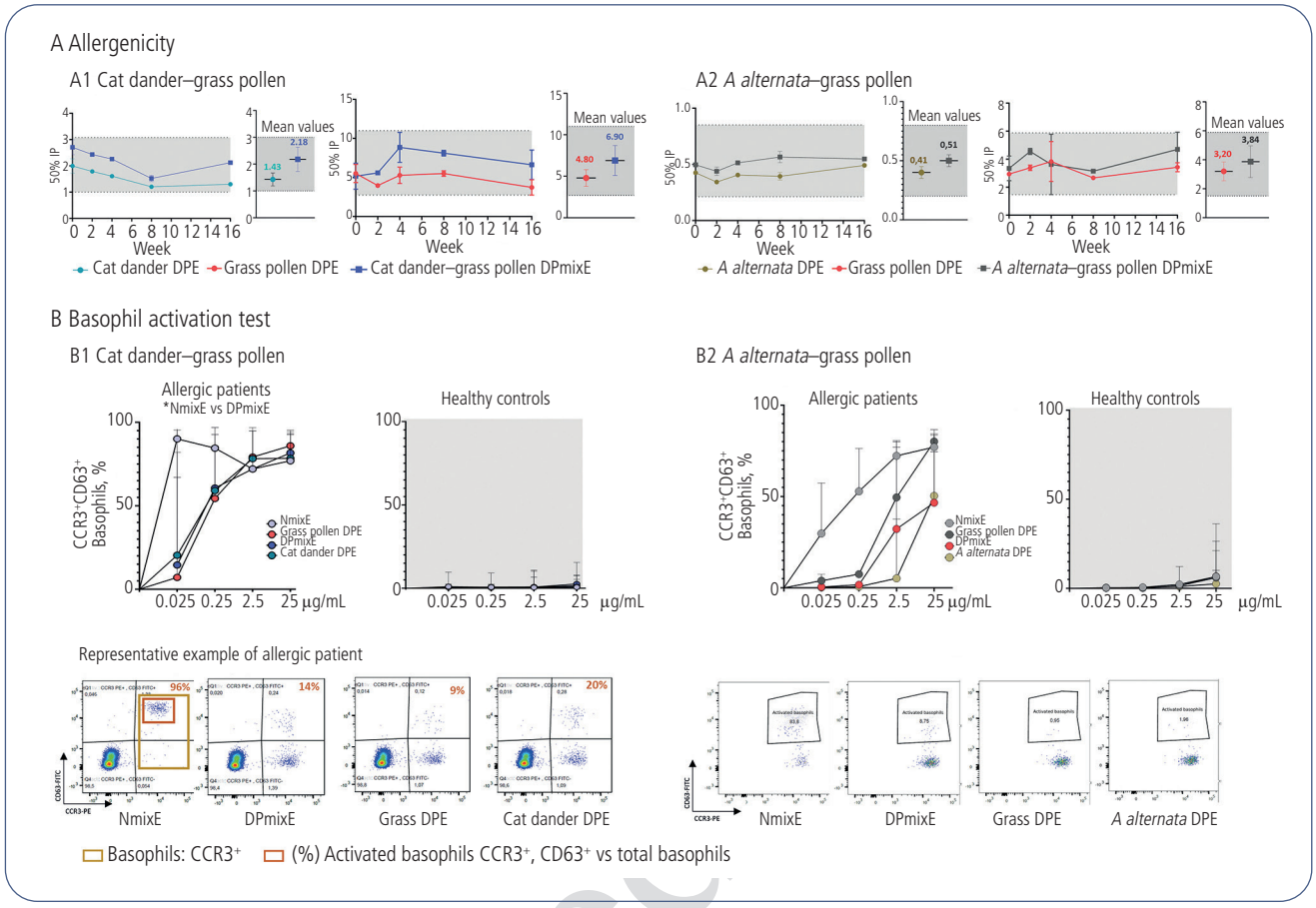

Figure 3. In vitro safety profile. A, Biological potency of DPmixEs compared with that of individual DPEs. The figure shows the 50% inhibition parameters obtained during the 16 weeks of study. Mean levels obtained in the global study. B, Results of basophil activation test in allergic patients and healthy controls using different concentrations of individual DPEs, NmixEs, and DPmixEs. Below: a representative example of an allergic patient. NmixEs indicates native mixture extracts; DPE, depigmented and polymerized extract; DPmixEs, depigmented-polymerized mixture extracts. The median and range are shown. Cat dander–grass pollen studies, N=10 polyallergic patients. *Alternaria alternata*–grass pollen, N=4 polyallergic patients. **P*<.05

An additional potency analysis was conducted based on a BAT in allergic patients and healthy controls (clinical and demographic data are summarized in the Table). A dose-dependent increase in the proportion of activated basophils was observed in response to all extracts in allergic patients but not in healthy controls (Figure 3B). NmixEs produced a dose-response curve that shifted towards the left, with a higher maximal response than that of DPEs and DPmixEs and a statistically significant difference in the cat dander–grass pollen mixture (*P*=.001; Figure 3B1). Moreover, the percentage of activated basophils in DPmixEs was similar to that of individual DPEs. All data obtained are shown in Figure S2.

Table. Demographic and Clinical Parameters of the Population Used in the Basophil Activation Test and Cellular Response.

Mixture studied	Population, No.	Mean (SD) age, y	Sex, female/male	Clinical symptoms	*Mean (SD) sIgE, kU_A/L		
					P pratense	Cat epithelia	*A alternata*
Cat dander–Grass pollen	Healthy controls, 8	37 (11)	75%/25%	___	<0.35	<0.35	___
	Polyallergic patients, 12	41 (12)	84%/16%	100% rhinoconjunctivitis, 92% asthma	25 (30)	25 (30)	___
A alternata–Grass pollen	Healthy controls, 4	30 (5)	100%/0%	____	<0.35	___	<0.35
	Polyallergic patients,12	27 (10)	75%/25%	100% rhinoconjunctivitis, 83% asthma	15 (14)	___	8 (8)

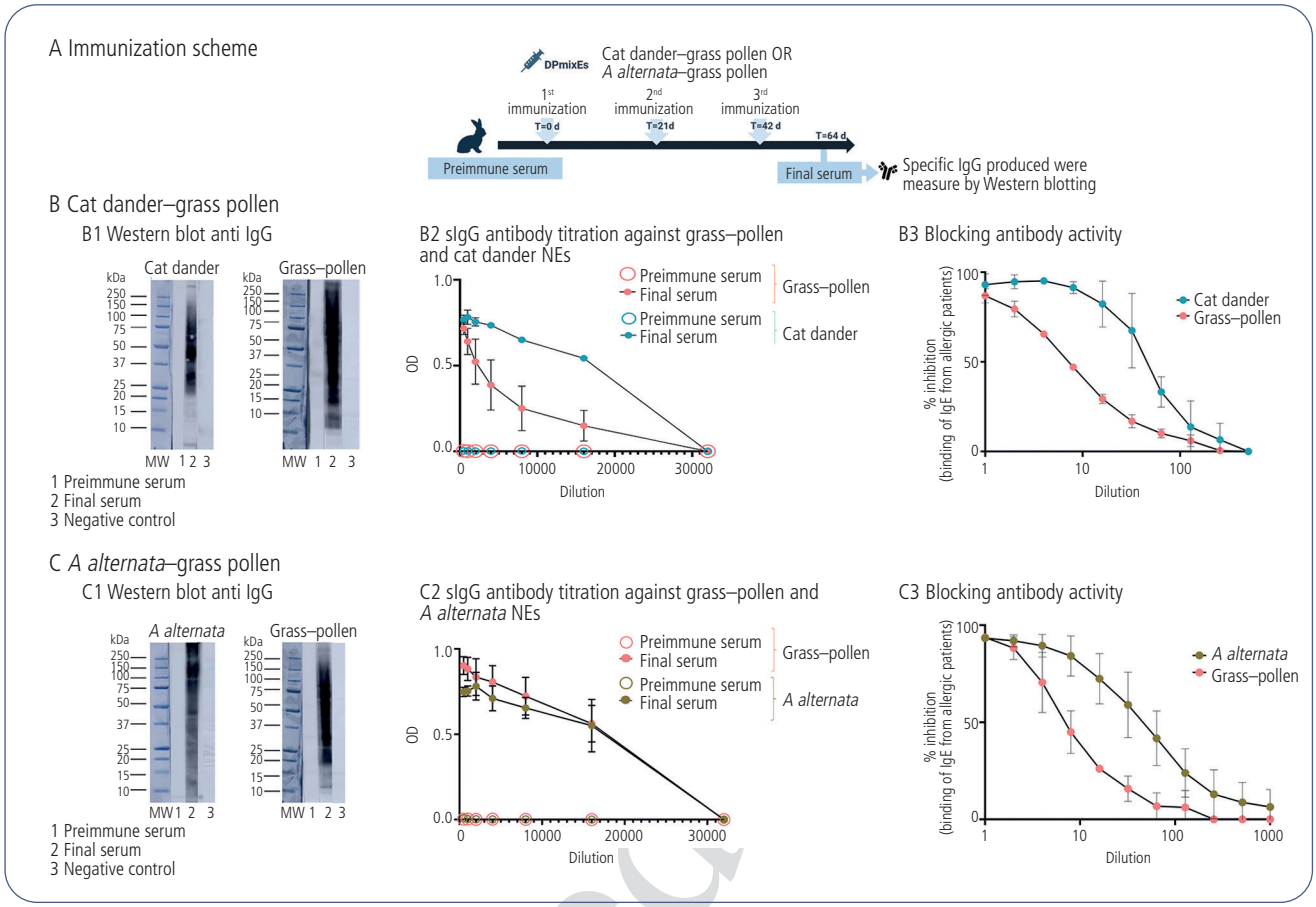

Figure 4. Humoral response. A, Rabbit immunization model with DPmixEs. B, Results of grass pollen-cat dander DPmixE. C, Results of grass pollen-*A alternata* DPmixE. Specific IgG antibody detection against allergens using sera before and after immunization. B1, C1, Immunoblot studies. B2, C2, IgG antibody titration through ELISA assays at different dilutions. The mean (SD) is shown. C3, Percentage of inhibition of IgE binding of allergen by IgG antibodies according to serum dilution. The mean (SD) is shown. NE indicates native extracts; DPmixE, depigmented-polymerized mixture extract.

Immunological Efficacy Studies

Humoral response

The immunization of rabbits with DPmixEs induced specific polyclonal IgG antibodies against each individual extract, as demonstrated by Western blotting (Figure 4, B1 and C1) and ELISA (Figure 4, B2 and C2). The antibodies produced were able to block approximately 95% of the human IgE-binding sites (Figure 4, B3 and C3).

Cellular response

Focusing on cytokine production in PBMC cultures, the most relevant results were obtained after 48 hours of culture in allergic patients. However, INF-γ and IL-4 values were lower than the detection limit (data not shown).

In the cat dander–grass pollen studies, IL-2 and IL-5 levels remained unchanged. IL-10 secretion was higher in DPEs than in NEs, although the difference was not statistically significant. Stimulation with cat dander–grass pollen DPmixE maintained high IL-10 levels, similar to those elicited by grass pollen DPE (Figure 5, A3). Although IL-5 production was not significantly reduced, DPEs presented IL-10/IL-5 ratio values that were 1.7- and 3-fold higher than those of cat dander and grass pollen NEs, respectively. Notably, the ratio of DPmixE was similar to that of cat dander DPE (Figure 5A). These data also correlated with those of cell populations. The cat dander–grass pollen DPmixE induced significantly higher regulatory B-- and T-cell indices than those of cat dander NE (P=.04 and .034, respectively) (Figure 6A).

Numerous comparisons in the *A alternata*–grass pollen cultures revealed statistically significant differences in IL-10 secretions. The highest levels were observed for grass pollen DPE stimulation, followed by DPmixE. Both stimuli produced a high IL-10/IL-5 balance (Figure 5B). Nevertheless, flow cytometry did not reveal any differences in regulatory B- and T-cell populations (Figure 6B).

As expected, statistically nonsignificant differences were observed in the PBMC cultures from healthy controls (Figure S3).

Discussion

Several studies have demonstrated that polyallergy can affect clinical disease presentation and is a risk factor for more severe symptoms and diminished quality of life [3,21,22]. From

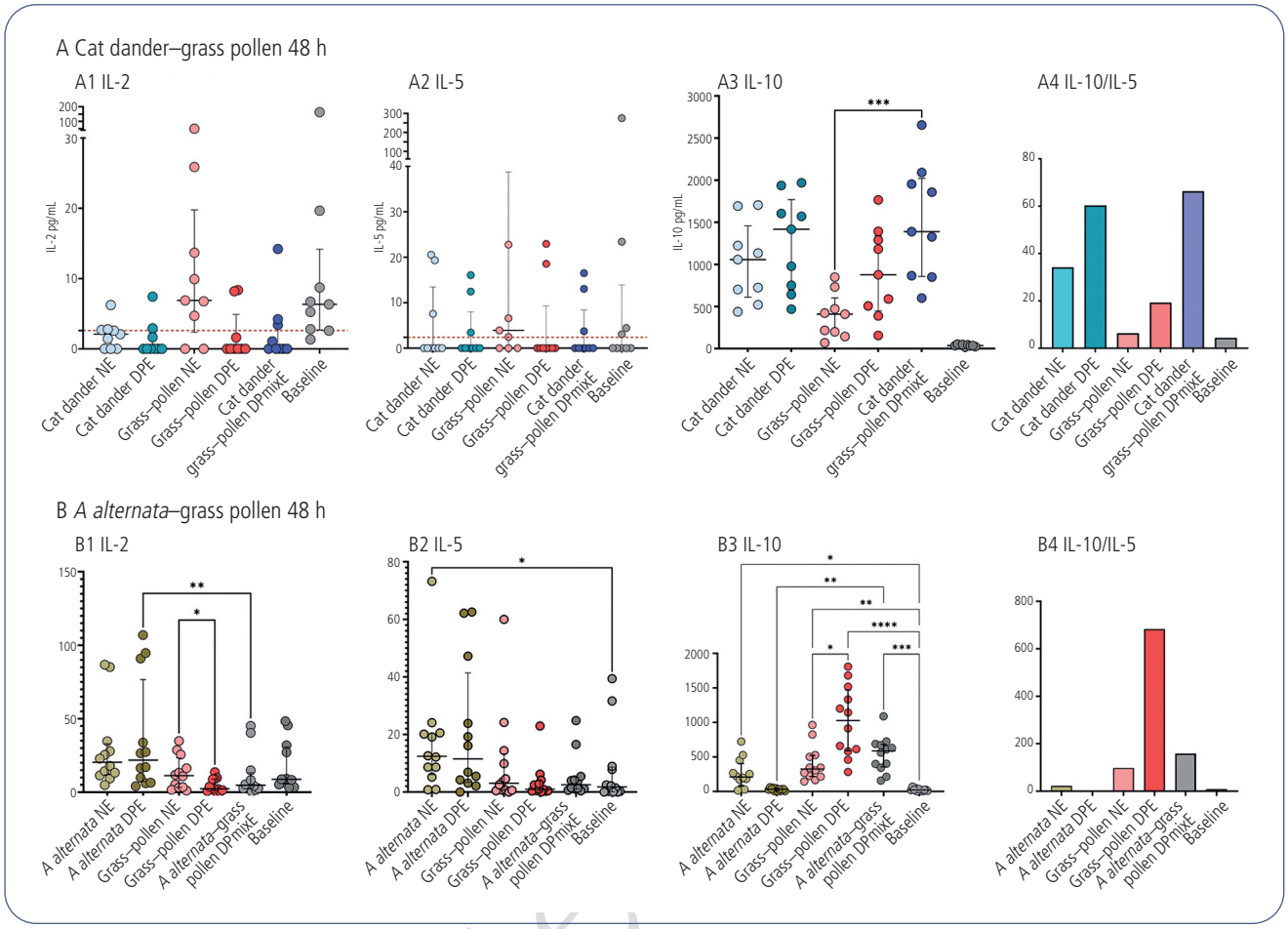

Figure 5. Cytokine levels in peripheral blood mononuclear cell culture supernatant after 48 hours of stimulation. The median (IQR) is shown. A, Patients allergic to cat dander and grass pollen. B, Patients allergic to *Alternaria alternata* and grass pollen. **P*<.05, ***P*<.01; ****P*<.001. NE indicates native extracts; DPE, depigmented and polymerized extracts; DPmixE, depigmented-polymerized mixture extract. A4 and B4, Ratio of IL-10/IL-5.

this perspective, understanding the spectrum of sensitivities that drive patient symptoms could optimize treatment strategies toward more personalized AIT products [23]. In this context, epidemiological studies demonstrated that 42% and 13% of allergic patients sensitized to grass pollen were also sensitized to animal epithelia and molds, respectively [3]. Moreover, according to unpublished data from our group, over 50% of patients sensitized to cat dander and 75% of those sensitized to *A alternata* were also sensitized to *P pratense*. Therefore, combining these mixtures in the same vaccine seems reasonable as long as the requirements established by regulatory agencies are met. These requirements include demonstrating the absence of allergen degradation, safety, and vaccine efficacy [8,11]. Under these premises, the present study compared the compatibility, stability, and in vitro safety and efficacy between cat dander–grass pollen and *A alternata*–grass DPmixEs and their corresponding NmixEs and individual DPEs.

Regarding compatibility and stability, the protein patterns fulfilled all internal criteria and were consistent with our previous findings [18,24,25]. What is more relevant is that DPmixEs conserved the low proteolytic content of individual DPEs and, interestingly, DPmixEs had lower enzymatic activity than that of NmixEs. This reduced activity explains the higher stability of DPmixEs, with protein content remaining stable throughout the 18 months of the study. In summary, mixing different DPEs did not affect allergen stability, which is a key concern and a requirement for assessing allergen compatibility in the same AIT prescription [8,10,11]. This was attributed to the reduced proteolytic activity.

The in vitro safety studies revealed a clear dose-response relationship between the percentage of activated basophils and increasing allergen concentrations. Although NEs and DPEs could activate the same percentage of basophils at the highest concentrations, NE curves shifted leftward, indicating more allergenic activity than that of DPEs, as previously demonstrated for dog dander and grass pollen extract [15,18]. This reduction in the allergenicity of allergoids is caused by the modification of IgE epitopes during the polymerization process [14]. Notably, DPmixEs did not increase the activation of these effector cells more than individual DPEs, as also demonstrated using competitive ELISA. Therefore, the combination of different allergen extracts did not change the in vitro biological potency, which is crucial for assessing the

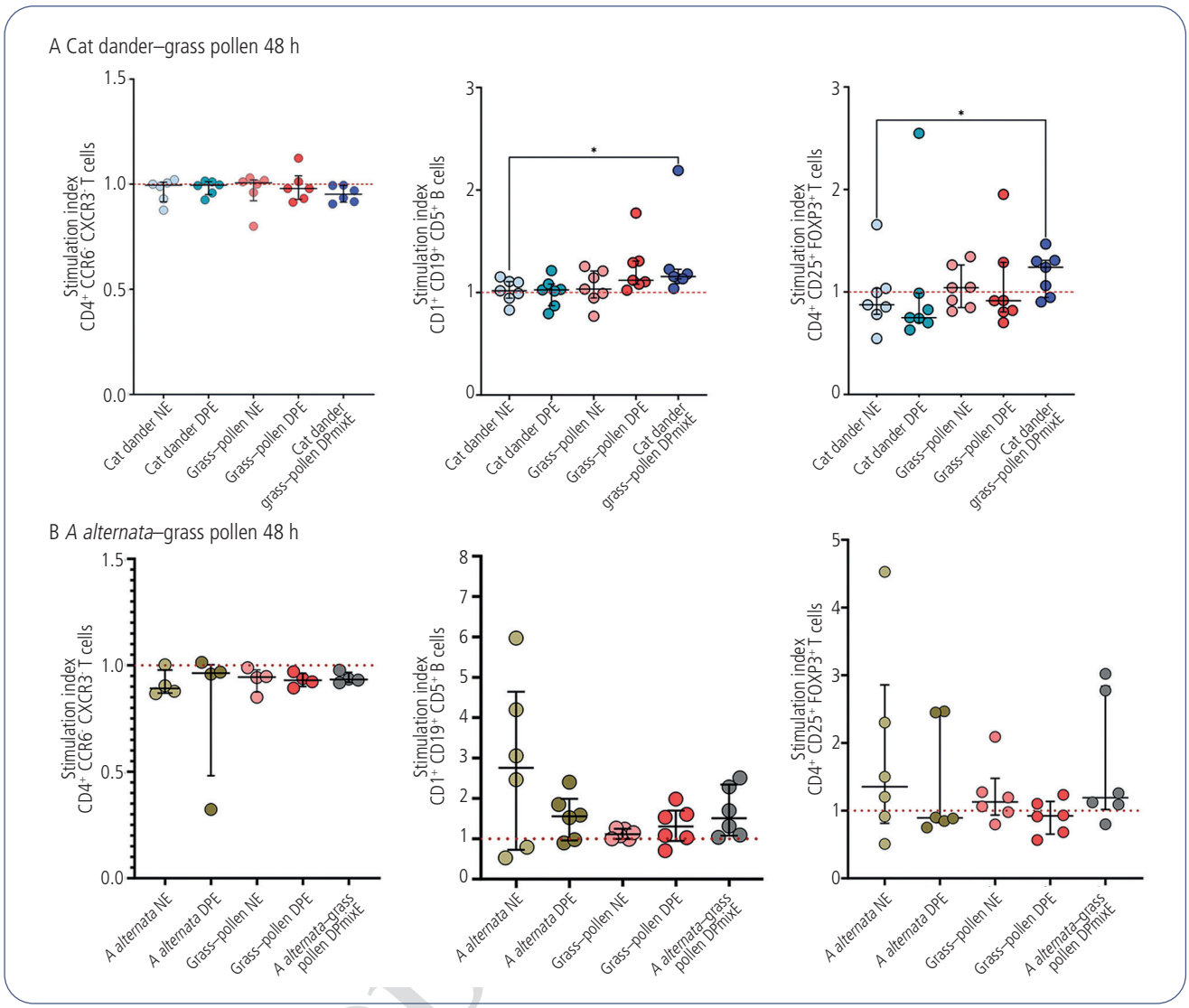

Figure 6. Cell population response. Stimulation index of $CD4^+CCR6^-CXCR3^-$T, $CD1^+CD19^+CD5^+$ B, and $CD4^+CD25^+FOXP3^+$ T cells in peripheral blood mononuclear cells from polyallergic patients after 48 hours of stimulation with native extracts (NEs) and depigmented and polymerized extracts (DPEs) alone or in combination (DPmixE). The median (IQR) is shown. A, Patients allergic to cat dander and grass pollen. B, Patients allergic to *Alternaria alternata* and grass pollen. Stimulation indexes were calculated as the ratios between the proportion of each cell subtypes found in each stimulus and the proportion obtained in cell cultures in the absence of stimulation. * $P<.05$

safety profile of allergen mixtures in immunotherapy and is a major requirement of regulatory authorities [1,8].

Several clinical trials have demonstrated the efficacy of DPEs [26-28]. Moreover, in vitro studies have clearly confirmed that the immunological changes associated with effective AIT involve both humoral and cellular responses [29].

The humoral tolerogenic response is associated with an increase in specific IgG4. Several intrinsic features of IgG4 may play a noninflammatory role [30]. Moreover, IgG antibodies can bind allergens directly and compete with binding of allergens by IgE, thus inhibiting basophil FcεRI-mediated responses [29,31,32]. This aspect was demonstrated in the individual DPEs of grass pollen, *A alternata*, and cat dander [18,24,25]. The present study demonstrated that combining these allergens in the same AIT produced specific IgGs against each allergen in rabbits. Furthermore, these IgGs could block allergen binding to IgE from patients. This capacity is related to the potential ability to suppress the allergic response in a specific manner.

Regarding the cellular response, AIT results in changes in memory-type allergen-specific T- and B-cell responses [31], with IL-10 being a key player. IL-10 is secreted by various cell populations, including dendritic cells, macrophages, innate lymphoid cells, and, especially, regulatory B and T cells [33,34]. Incidentally, grass pollen AIT has been demonstrated to be able to restore induction of IL-10 [35]. In the present study, IL-10 values were higher following DPE-based stimulation of cat dander and grass pollen than with NE-based stimulation, consistent with previous studies [18,24]. This response was not observed with

stimulation of *A alternata*, probably because of the different culture incubation times in the current study compared with those in previous studies [25]. Nevertheless, and more importantly, IL-10 production was higher in DPmixEs than in NEs, indicating that immune tolerance is maintained in the combination of allergen extracts from different environmental sources and could explain the molecular mechanisms underlying the efficacy of AIT formulations. In the cat dander–grass pollen experiments, the stimulus did not induce IL-5 in most patients. However, in those with IL-5 levels above the detection limits, DPEs and DPmixEs produced lower levels than NEs, suggesting that these allergoids down-regulated the T_H2 response. During the treatment of allergic diseases, multiple proinflammatory molecules are balanced with those exhibiting anti-inflammatory effects. Therefore, using a ratio covering several analytes between T_H2 and regulatory responses could better represent the balance of the molecules contributing to tolerance [36]. In the current study, DPEs and DPmixEs restored the IL-10/IL-5 balance, indicating that IL-10 could inhibit the T_H2 response and promote proregulatory phenotypes [37]. This finding is associated with the regulatory balance of regulatory B- and T-cell/T_H2 populations observed in stimulation of DPEs and DPmixEs. Cat dander–grass pollen DPmixE exhibited the highest stimulation index for both $CD1^+CD19^+CD5^+$ regulatory B cells and $CD4^+CD25^+FOXP3^+$ T cells. However, no statistically significant differences were found in the *A alternata* and grass pollen experiment. *A alternata* NE induced the highest levels of $CD1^+CD19^+CD5^+$ regulatory B cells followed by *A alternata* DPE and DPmixE. As for $CD4^+CD25^+FOXP3^+$ T cells, the stimulation index was higher for DPmixE than for *A alternata* DPE and grass pollen DPE alone. Both populations are considered especially relevant during AIT in patients with allergies, where high-dose allergen exposures restore these regulatory responses, promoting tolerance [37,38].

Considering the humoral response by IgG secretion in rabbits and the cytokine response in the PBMCs of patients with allergies, we demonstrated an efficient immunoregulatory mechanism involving the use of DPmixEs, inducing a shift from an allergic towards a regulatory pathway.

In summary, we fully characterized 2 allergoid mixtures from different nonhomologous allergen sources. Both DPmixEs, cat dander–grass pollen and *A alternata*–grass pollen, remained stable with no significant modifications in protein composition. Moreover, although it has been tested in vitro, the safety of the mixtures was confirmed by the absence of increased allergenicity, and induction of tolerance was demonstrated. These results suggest a promising alternative for AIT in patients with multiple allergies. However, further clinical studies are needed to complement the in vitro results.

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Conflicts of Interest

Calzada D, Parody N, Osuna C, Moya R, and Carnés J are employees of LETI Pharma. The remaining authors declare that they have no conflicts of interest.

Previous Presentation

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