

The role of major olive pollen allergens Ole e 1, Ole e 9, and Ole e 10 on mice sensitization

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Background: Olive pollen is an important cause of allergy in Mediterranean countries. To date, 10 allergens (Ole e 1 to Ole e 10) have been isolated and characterized. Animal models of olive pollen allergy are suitable tools for testing the efficacy and safety of new forms of immunotherapy.

Objectives: To characterize the immune response in mice sensitized with olive pollen extract and to compare it with that of allergic patients.

Methods: BALB/c mice were sensitized by 4 intraperitoneal injections of olive pollen extract in aluminum hydroxide. The allergic state was proved by measuring serum specific IgG1 and total IgE antibody levels. The IgG1 responses to olive pollen allergens were assayed by immunoblotting and enzyme-linked immunosorbent assay. Competition experiments between human IgE and mouse IgG1 binding to olive pollen allergens were performed.

Results: Sensitization with olive pollen extract induced high levels of specific IgG1 and total IgE in all tested animals. Immunoblotting experiments showed that the mouse IgG1 binding pattern to pollen extract was complex and heterogeneous, as occurs with human IgE. High IgG1 antibody levels to the major olive pollen allergens described for humans were detected in serum samples from sensitized mice, whereas minor olive pollen allergens induced no significant IgG1 response. Coincubation of mouse serum samples with a cocktail of Ole e 1, Ole e 9, and Ole e 10 resulted in a significant decrease (60%) in IgG1 binding to olive pollen extract. Specific mouse IgG1 strongly inhibited human IgE binding to olive pollen allergens.

Conclusions: This mouse model of olive pollen sensitization mimics immunologic features of human pollinosis and could be a useful tool for designing novel forms of immunotherapy for olive pollen allergy based on allergen cocktails.

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INTRODUCTION

Type I allergy is an important health problem that affects at least 25% of the population in industrialized countries.¹ Pollens are one of the most common sources of aeroallergens. Olive (*Olea europaea*) pollen is considered one of the main causes of allergy in the Mediterranean countries where this tree is extensively cultivated.² To date, 10 major and minor allergens (Ole e 1 to Ole e 10) have been identified and characterized from olive pollen.^{3–11} Ole e 1 has been recognized as the most prevalent allergen of the olive pollen extract, affecting 55% to 90% of patients depending on the population studied. However, other proteins, such as Ole e 9 and Ole e 10, have also been shown to be major allergens. Table 1 summarizes the main properties of these allergens.

Mouse models have been widely used in studies of various allergic diseases.^{14–18} Previously, we have established 2 murine models of allergic sensitization to Ole e 1¹⁷ and Ole e 9.¹⁸ However, there is no suitable mouse model of olive pollen allergy for performing preclinical studies with novel therapeutic approaches for allergy treatment. The simplest protocol for such models is based on sensitization against the

allergen with alum as adjuvant, which induces IgE synthesis in mice, thus reproducing typical immunologic features of human type I allergy.

In the present study, BALB/c mice were immunized with olive pollen extract, and the IgE and IgG1 responses against pollen allergens were analyzed and compared with the allergenic pattern described for human patients allergic to olive pollen. The contribution to the mouse allergic response of the major allergens described in humans was evaluated. Finally, we studied the capacity of mouse IgG to inhibit human IgE binding to olive pollen extract and the presence of cross-reactivity to mouse antibodies among different pollen species.

METHODS

Pollen Extracts, Purified Allergens, and Human Serum Samples

Olive (*O. europaea*), ash (*Fraxinus excelsior*), privet (*Ligustrum vulgare*), lilac (*Syringa vulgaris*), rye grass (*Lolium perenne*), birch (*Betula verrucosa*), ragweed (*Ambrosia trifida*), timothy grass (*Phleum pratense*), pellitory (*Parietaria judaica*), and cypress (*Cupressus sempervirens*) pollens were purchased from Allergon AB (Ängelholm, Sweden). Pollen protein extracts were prepared by homogenizing pollen (2% wt/vol) in 20-mmol/L ammonium bicarbonate (pH 8) containing 1-mmol/L phenylmethylsulfonyl fluoride using a potter and extracting for 3 hours at room temperature as previously described.⁵ Protein extracts were analyzed using

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Table 1. Olive Pollen Allergens and Their Main Features

Allergen	Molecular weight, kDa	Prevalence, %	Cross-reactivity	Function	Reference
Ole e 1	16.3/18.5*†	55–90	Yes	Unknown	3, 12
Ole e 2	14–16‡	>40	Yes	Profilin	4, 12
Ole e 3	9.2†	20	Yes	Polcalcin	5
Ole e 4	32‡	80	ND	Unknown	6
Ole e 5	16‡	35	ND	Superoxide dismutase	6
Ole e 6	5.8†	5–20	No	Unknown	7
Ole e 7	9.9–10†	47	ND	Lipid transfer protein	8
Ole e 8	18.8†	3–4	ND	Polcalcin	9
Ole e 9	46.4†	65	Yes	1, 3- β -glucanase	10, 13
Ole e 10	10.8†	55	Yes	Unknown	11

Abbreviation: ND, not determined.

* Glycosylated/nonglycosylated forms.

† Determined by mass spectrometry.

‡ Determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

sodium dodecyl sulfate–polyacrylamide gel electrophoresis¹⁹ and were stained using Coomassie brilliant blue (Serva, Heidelberg, Germany); after lyophilizing, the extracts were stored at -20°C until use. Protein concentrations were determined using the method of Lowry et al,²⁰ with bovine serum albumin (Sigma-Aldrich Corp, St Louis, MO) as standard. Olive pollen allergens (Ole e 1, Ole e 2, Ole e 3, Ole e 6, Ole e 7, Ole e 9, and Ole e 10) were purified from olive pollen as previously reported.^{3–5,7,8,10,11} Serum samples from 13 allergic patients with well-documented clinical histories, positive skin prick test results, and positive CAP-RAST (Amersham Pharmacia Biotech, Uppsala, Sweden) reactions to olive pollen were used.

Immunization of Animals

Female 6-week-old BALB/c mice were obtained from Harlan Interfauna Ibérica SA (Barcelona, Spain). Animals were maintained at the Animal Care Services of the Faculty of Biology, Universidad Complutense, according to the local guidelines for animal care. BALB/c mice ($n = 22$) were immunized by means of intraperitoneal injection with 50 μg of olive pollen extract adsorbed to 2 mg of aluminum hydroxide (Sigma-Aldrich Corp) in 150 μL of phosphate-buffered saline solution (PBS) on days 0, 7, 14, and 21, as previously described.¹⁷ Control animals received pollen extract, PBS, or adjuvant alone on those same days. Blood samples were collected from the retro-orbital plexus on day 28, and serum samples were stored at -20°C until analysis.

Enzyme-Linked Immunosorbent Assay

The IgG1 responses were measured using enzyme-linked immunosorbent assay (ELISA) as described elsewhere.¹⁷ Briefly, microtiter plates (Costar, Cambridge, MA) were coated with olive pollen extract (200 $\mu\text{g}/\text{mL}$) or purified proteins (1 $\mu\text{g}/\text{mL}$) in PBS (pH 7.2) overnight at 4°C . Non-specific binding sites were blocked with PBS containing 1% (wt/vol) bovine serum albumin (blocking buffer). Mouse serum samples (diluted in a ratio of 1:5,000) were incubated

in blocking buffer for 2 hours at room temperature. Bound mouse IgG1 was detected using peroxidase-conjugated rabbit anti-mouse IgG1 (diluted in a ratio of 1:5,000) antibody (Rockland, Gilbertsville, PA) and *o*-phenylenediamine chloride (Merck & Co Inc, Whitehouse Station, NJ) as substrate. Optical density (OD) was measured in an ELISA reader at 492 nm.

In the competition ELISA procedure,¹⁷ olive pollen extract-coated plates were incubated simultaneously with pooled serum samples from olive pollen-sensitive mice (diluted in a ratio of 1:5,000) and nondiluted individual serum samples from olive-allergic and nonallergic individuals, the latter for control purpose. Binding of IgG1 was detected as described previously herein. The inhibition percentage of IgG1 binding to olive pollen extract after coincubation with human allergic vs nonallergic serum samples was determined according to the following formula: percentage of inhibition of IgG1 binding = $(1^{\circ} \text{OD}_A / \text{OD}_N) \times 100$, where OD_A and OD_N represent OD after incubation with human allergic and nonallergic serum samples, respectively.

For ELISA inhibition experiments, mice olive pollen-sensitive serum samples (diluted in a ratio of 1:5,000) were preadsorbed for 2 hours at room temperature with extracts of different pollens (500 or 50 $\mu\text{g}/\text{mL}$) or with a mixture of Ole e 1 (100 $\mu\text{g}/\text{mL}$), Ole e 9 (50 $\mu\text{g}/\text{mL}$), and Ole e 10 (50 $\mu\text{g}/\text{mL}$) allergens as inhibitors. Total IgE (diluted in a ratio of 1:40) antibodies were measured using a sandwich ELISA and the OptEIA mouse IgE set (PharMingen, San Diego, CA) according to the manufacturer's instructions.

Western Blot Analysis

Pollen extracts (40 μg per lane) were separated by means of 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis¹⁹ and then were blotted onto nitrocellulose membranes (Hybond ECL; Amersham, Buckinghamshire, England).²¹ After blocking with 3% nonfat milk in PBS/0.1% Tween 20 (blocking buffer), membranes were incubated with

individual serum samples from sensitized mice (diluted in a ratio of 1:1,000 in blocking buffer) at room temperature for 2 hours.¹⁷ Bound antibodies were detected using peroxidase-labeled rabbit anti-mouse IgG1 (diluted in a ratio of 1:5,000) and visualized using the ECL Western blotting reagent and ECL-hyperfilm (Amersham). Immunodetection of blotted proteins with IgE (diluted in a ratio of 1:10) from patients allergic to olive pollen was performed as previously described.³

RESULTS

IgE and IgG1 Responses to Olive Pollen Proteins in Mice

The IgE and IgG1 responses to olive pollen proteins in serum samples from sensitized mice were analyzed by means of ELISA (Table 2). As previously reported,¹⁷ allergen specific IgG1 and total IgE levels peaked on day 28 after the first immunization; therefore, specific antibody responses to olive pollen extract were analyzed at this time. Sensitization of animals with olive pollen extract in aluminum hydroxide induced a dramatic increase in total IgE levels compared with control mice. High levels of olive pollen specific IgG1 were detected in serum samples from every sensitized animal. No specific antibody production was observed in control mice. Previous studies¹⁷ have shown the correlation between total IgE and specific IgE levels in sensitized mice, suggesting that total IgE consists primarily of olive pollen specific IgE. These data indicate that immunization of mice with olive pollen extract induced specific IgE that can be estimated by measuring total IgE levels in serum. The significantly increased production of IgE and IgG1 indicated the induction of a predominantly T_H2 response in the mouse model after immunization with olive pollen extract.

Table 2. Total IgE and Specific IgG1 Levels in Individual Mouse Serum Samples on Day 28 After First Immunization With Olive Pollen Extract in Aluminium Hydroxide*

Sample No.	IgE, ng/mL	IgG1, OD at 492 nm
1	3072 ± 36	1.612 ± 0.011
2	909 ± 29	0.522 ± 0.006
3	805 ± 163	1.395 ± 0.104
4	1577 ± 143	2.499 ± 0.002
5	1438 ± 11	1.332 ± 0.088
6	3007 ± 331	1.014 ± 0.032
7	1113 ± 29	0.587 ± 0.009
8	2672 ± 157	1.086 ± 0.068
9	3291 ± 487	1.533 ± 0.031
10	2007 ± 46	2.492 ± 0.008
Control†	147 ± 44	0.008 ± 0.001

Abbreviation: OD, optical density.

* Data are given as mean ± SD.

† Values are expressed as mean ± SD of the response of 4 control mice. Results are shown as mean ± SD of duplicate wells. Data are representative of 2 independent experiments.

Mouse IgG1 and Human IgE Binding Patterns

Human IgE and mouse IgG1 responses to olive pollen allergens were compared by means of immunoblotting assays (Fig 1). Results obtained with 10 representative mouse serum samples showed a complex and heterogeneous binding pattern, which is similar to that recognized by IgE from patients allergic to olive pollen, with many positive bands ranging from 10 to 70 kDa. All the serum samples showed specific reactivity against a 45-kDa protein band that is also recognized by human IgE. A high prevalence (9 of 10 mice) was detected for a 20-kDa band. According to their molecular weight, the 45- and 20-kDa bands seem likely to be the major allergens Ole e 9 and Ole e 1, respectively. However, the 10-kDa band also recognized by mouse IgG1 could not be assigned to a single allergen because Ole e 3, Ole e 6, Ole e 7, and Ole e 10 allergens exhibit similar electrophoretic mobility. No IgG1 reactivity was detected in control mice. These results suggest that IgG1 responses to olive pollen allergens in the mouse model are similar to those in humans.

To confirm this observation, specific IgG1 responses to 7 purified olive pollen allergens available for this study (Ole e 1, Ole e 2, Ole e 3, Ole e 6, Ole e 7, Ole e 9, and Ole e 10) were tested by means of ELISA (Fig 2A). The IgG1 reactivity was detected first against Ole e 9 (87%), followed by Ole e 10 (79%) and Ole e 1 (75%), which represent major olive pollen allergens in humans.^{3,10,11} None of the mouse serum samples presented specific IgG1 to Ole e 2, Ole e 3, Ole e 6, or Ole e 7 allergens, which were described as minor allergens from human allergic patients^{4,5,7,8} and whose sensitization depends on the pollen levels. These results suggest that major allergens in humans induce the highest IgG1 levels in mice and, in contrast, minor allergens elicit low or nondetectable IgG1 antibody responses.

To evaluate the combined contribution of Ole e 1, Ole e 9, and Ole e 10 allergens in the total immune response to the olive pollen extract, inhibition ELISAs were performed. A

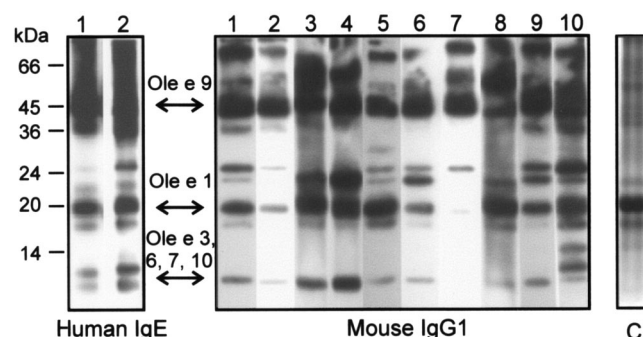


Figure 1. The IgG1 binding patterns of serum samples from mice sensitized to olive pollen extract. Ten serum samples were selected as being representative of the variability and complexity of the responses in individual sensitized animals (mouse IgG1). Also shown are the IgE binding patterns of 2 representative serum samples from patients allergic to this pollen (human IgE). Coomassie blue staining (C) of olive pollen proteins on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis is also shown.

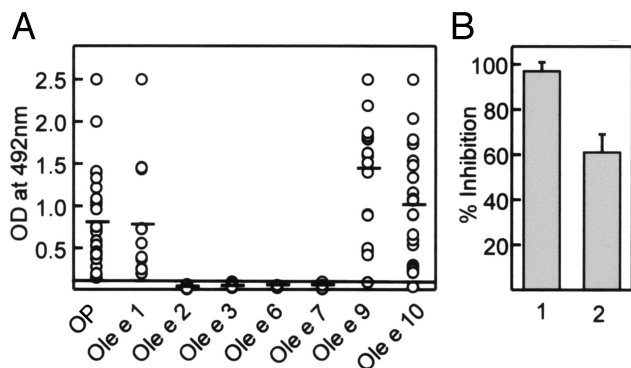


Figure 2. A, Specific IgG1 levels of purified olive pollen allergens for 22 serum samples from sensitized mice evaluated by means of direct enzyme-linked immunosorbent assay. Short bars indicate mean values; OP, olive pollen extract; OD, optical density. The baseline for specific IgG1 is represented by a horizontal black line. B, Inhibition enzyme-linked immunosorbent assay of mouse IgG1 binding to olive pollen extract-coated plates using olive pollen extract (1) or a cocktail of purified Ole e 1, Ole e 9, and Ole e 10 allergens (2) as inhibitors. Error bars represent SD.

significant percentage of mouse specific IgG1 antibodies can be preadsorbed with a cocktail of Ole e 1, Ole e 9, and Ole e 10 allergens (Fig 2B). Concentrations of 100 $\mu\text{g/mL}$ of Ole e 1, 50 $\mu\text{g/mL}$ of Ole e 9, and 50 $\mu\text{g/mL}$ of Ole e 10 were required to reach the maximum inhibition value of 60%. Complete inhibition was obtained with olive pollen extract as inhibitor (500 μg).

Specific IgE From Allergic Patients Blocks Binding of Mouse IgG1 to Olive Pollen Extract

To investigate whether human IgE antibodies inhibit the binding of mouse IgG1 to olive pollen proteins, competition experiments were performed. Figure 3 displays the percentage of inhibition of IgG1 binding to olive pollen extract determined for a pooled serum sample from sensitized mice in 13 serum samples from patients allergic to olive pollen as inhibitors. Human IgE inhibited mouse IgG1 binding to olive

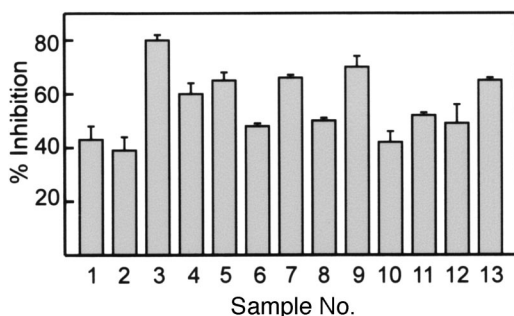


Figure 3. Competition enzyme-linked immunosorbent assay of mouse IgG1 binding to olive pollen extract with human IgE from allergic patients. The mean percentage of inhibition of IgG1 binding is displayed for 13 serum samples from patients allergic to olive pollen. Error bars represent SD. Results shown are from duplicate experiments.

pollen extract by 40% to 80%. These results indicated that mouse IgG1 recognized similar epitopes as human IgE antibodies.

Olive Pollen Extract Induces IgG1 Antibodies in Mouse That Cross-react With Homologous Pollen Proteins

The cross-reactivity of mouse IgG1 antibodies was tested with pollen extracts from botanically related (*F excelsior*, *L vulgare*, and *S vulgaris*) and unrelated (*L perenne*, *B verrucosa*, *A trifida*, *P pratense*, *P judaica*, and *C sempervirens*) species to olive tree (*O europaea*) (Fig 4). At the highest inhibitor value (500 μg), all the pollen extracts inhibited IgG1 binding to the olive pollen extract-coated plate (30%–58%). This finding indicates that mouse IgG1 antibodies react with homologous pollen proteins from different pollen sources.

DISCUSSION

Olive pollen contains many proteins, and a few of them bind IgE and elicit allergic reactions in humans. To examine the role and contribution of olive pollen proteins on IgE responses in vivo, a mouse model of olive pollen sensitization has been established. Specific IgE and IgG1 antibodies to olive pollen proteins were elicited in sensitized mice. This humoral response is considered to be the consequence of T_H2 cell activation in mice.²² The role of these 2 isotypes as homocytotropic antibodies is well characterized in allergic reactions in mice.²³ The antibody isotype responses in the model were comparable to the findings in olive pollen allergic patients.²⁴ The IgG1 binding pattern was similar in complexity and heterogeneity to that found in allergic patients. Complexity and heterogeneity were expressed as the number of IgG1 binding bands and differences in antibody response among sensitized animals, respectively.

The ELISA experiments with purified allergens showed that mice mainly elicited IgG1 responses against 3 major allergens of *O europaea* pollen described in human patients: Ole e 1, Ole e 9, and Ole e 10.^{3,10,11} The IgG1 binding

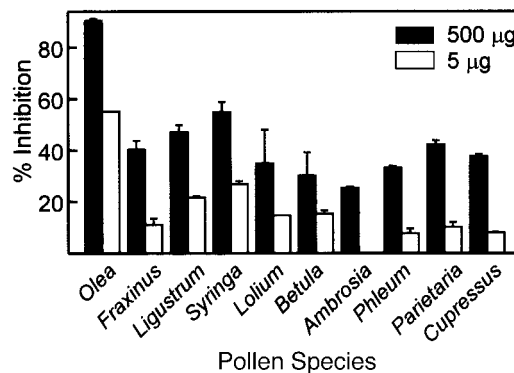


Figure 4. Cross-reactivity of mouse IgG1 with homologous proteins from different pollen species. Enzyme-linked immunosorbent assay inhibition of IgG1 binding to olive pollen-coated wells using extracts from pollens as inhibitors. Error bars represent SD.

frequencies of the sensitized mouse serum samples to the major allergens Ole e 1 (75%), Ole e 9 (87%), and Ole e 10 (79%) were comparable in the order of magnitude with those found in allergic patients (55%–90%,^{3,25} 65%,¹⁰ and 55%,¹¹ respectively). In contrast, we did not detect IgG1 antibodies against Ole e 2, Ole e 3, Ole e 6, or Ole e 7, which represent minor but clinically relevant allergens in humans.^{4,5,7,8} Nevertheless, a cocktail of major allergens did not completely inhibit IgG1 binding to the olive pollen extract, suggesting the presence in the pollen of allergens not yet characterized. This is an in vivo example of the fact that some components seem to produce sensitization in the mouse immune system and give IgE-mediated allergic reactions much more readily than others in a way that cannot be explained by the degree of exposure alone or the protein content in the extract because Ole e 3 levels, which did not mount an antibody response, are similar to Ole e 10 levels (~80 µg/g).¹¹ On the other hand, Ole e 1, the most abundant protein (10–12 mg/g of dried pollen) in the olive pollen extract,²⁵ and Ole e 10 induced similar IgG1 responses. These results suggest that the intrinsic properties of the individual allergens play an important role in the development of an allergic reaction. Besides the general features of the allergens, other factors inherent to the host, including genetics, immunologic and nonimmunologic, and type, degree, and time of exposition to the allergenic source could be the cause of the discrepancy between mouse and human responses. In any case, these findings cannot be attributed to a lack of immunogenicity of minor allergens from olive pollen per se because the immunization of rabbits and mice with purified proteins resulted in the production of polyclonal and monoclonal antibody, respectively (Rosalía Rodríguez, PhD, and Mayte Villalba, PhD, unpublished data, 2004).^{4,5,7} Finally, these data support the idea that proteins that represent major allergens in humans induce IgE and IgG1 responses in mice. In contrast, proteins with low or no allergenic potential in humans induce lower or no detectable antibody responses in mice.¹⁴

To further evaluate the suitability of this model as a tool for testing new therapeutic approaches based on the use of allergen cocktails, the capacity of the Ole e 1, Ole e 9, and Ole e 10 mixture to inhibit IgG1 binding to olive pollen extract was assayed. The cocktail strongly inhibited the binding of mouse IgG1 to olive pollen extract. Furthermore, it has been demonstrated that IgG1 antibodies of sensitized mice recognized epitopes in olive allergens defined by human IgE. These data support the idea that a cocktail of a few allergens (natural or recombinant molecules) can reproduce the complexity of natural allergenic extracts required for the diagnosis and treatment of allergy and that these allergens contain most of the IgE epitopes present in the corresponding allergen sources.^{26,27}

In this study, a strong cross-reactivity of olive pollen specific IgG1 antibodies with homologous proteins present in different pollen species has also been shown. The presence of Ole e 1-like proteins has been demonstrated in several Oleaceae pollens, which could explain the strong allergenic cross-reactivity observed among them. However, Ole e 1-like

proteins from taxonomically unrelated pollens do not cross-react to Ole e 1 at a significant level.^{12,25,26} Ole e 10 has been described as a new panallergen involved in pollen-latex-fruit syndrome.¹¹ Recently, Ole e 9 has been identified as a new candidate responsible for the cross-reactivity among pollens, latex, and plant-derived foods.¹³ Thus, Ole e 1, Ole e 9, and Ole e 10 would be responsible for the notable cross-reactivity detected among Oleaceae pollens, and at least Ole e 9 and Ole e 10 would be involved in the cross-reactivity process found among unrelated pollens. The identification of cross-reactive molecules responsible for cosensitizing allergic patients against different allergen sources may help develop simplified protocols based on cocktails of representative cross-reactive allergens, which carry most IgE epitopes, for immunotherapy (component-resolved immunotherapy) and diagnosis (component-resolved diagnosis) of pollen allergy.^{26,27} The fact that the olive pollen allergens induced IgG1 antibodies in mouse that cross-react with immunologically related proteins present in other pollens strongly supports the use of this model to study a novel immunotherapy approach (component-resolved immunotherapy).

Because mice sensitized to olive pollen extract reproduced the immunologic state of allergic patients, this model could be a potentially useful tool to study the IgE-mediated immune response elicited by major allergens of olive pollen and to evaluate new therapeutic approaches for olive pollen allergy based on cocktails of a few cross-reactive allergens, matching the IgE epitope complexity present in the natural pollen extract.

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