Title:

Immunologic responses to the major allergen of *Olea Europaea* in local and systemic allergic rhinitis subjects

Short title: Immunologic responses to nOle e 1 in local and systemic allergic rhinitis

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ABSTRACT

Background: Evaluate the *in vivo* and *in vitro* responses to nOle e 1 in allergic rhinitis (AR) and local allergic rhinitis (LAR) patients sensitized to olive tree pollen (OL) confirmed by nasal allergen provocation test (NAPT).

Methods: Twelve subjects with AR, 12 with LAR, and 12 subjects as control group (CG) were selected. Skin testing and NAPT with nOle e 1 were performed. ECP and tryptase were measured in nasal lavages before and after NAPT. Serum IgE to OL allergens were measured by ELISA. Basophil activation tests (BAT) with OL and nOle e 1 and dendritic cell maturation/proliferation studies were carried out.

Results: All AR (12/12) and 10/12 (83%) of LAR had a +NAPT to nOle e 1. ECP levels in nasal lavages were significantly increased after NAPT in both AR and LAR compared with CG ($p<0.05$). Serum IgE was positive only in AR. All AR had +BAT responses to OL and 10/12 to nOle e 1 (83%); 8/12 LAR (66.6%) had a +BAT with OL and 4/12 (33%) to nOle e 1, with only one subject of the control group with a +BAT to both OL and nOle e 1 (8%). Dendritic cell proliferation to nOle e 1 was increased in AR compared to LAR and CG ($p=0.019$ and $p=0.001$ respectively).

Conclusion: Both AR and LAR had a similar *in vivo* response to nOle e 1 with release of inflammatory mediators. Specific basophil activation with OL and nOle e 1 was observed in LAR confirming previous data obtained with dust mites.

Keywords:

Basophil activation test, local allergic rhinitis, nasal allergen provocation test, purified allergen

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Abbreviations

AR: allergic rhinitis

BAT: basophil activation test

CG: control group

DC: dendritic cell

ID: intradermal skin testing

IU/mL: international units per milliliter

kU/L: kilounits per liter

LAR: local allergic rhinitis

LTT: lymphocyte transformation test

nOle e 1: natural Ole e 1

NAPT: nasal allergen provocation test

OL: olive tree pollen

SI: stimulation index

sIgE: specific IgE

SPT: skin prick testing

VAS: visual analogue scale

INTRODUCTION

Allergic rhinitis (AR) affects 20-40% of population (1), and constitutes a health and economic burden with important comorbidities such as asthma. Local allergic rhinitis (LAR) is a phenotype of AR characterized by a positive response to nasal allergen provocation test (NAPT) and local production of specific IgE (sIgE) (2,3). In LAR subjects, specific nasal responses to allergens have been demonstrated with dust mites, pollens and molds (4-6). Inflammatory mediators´ release and a cellular Th2 pattern in nasal lavages are present after challenge with all these allergens (4,7).

Olive tree (*Olea europaea*) pollen is a major cause of respiratory allergy in Mediterranean regions and some areas of North America (8-12). Olive tree is closely related to other plants from the Oleaceae family such as ash tree *(Fraxinus excelsior),* jasmine *(Jasminum)*, lilac *(Syringa)*, privet *(Ligustrum)* and forsythia *(Forsythia)* (13). Ole e 1, the major allergen, is recognized by almost 80% of patients with olive pollinosis (13-16) and shows 88% sequence identity to Fra e 1. The structure of Ole e 1 has been thoroughly studied and constitutes an interesting model of purified allergen (15, 17-20).

LAR shows many similarities with AR in terms of clinical presentation, comorbidities and inflammatory patterns (21). However, LAR seems to constitute a unique entity that does not evolve to AR (22). In LAR subjects, NAPT have been performed with whole commercial extracts, which contain a known amount of major allergens. Some studies have evaluated nasal responses to purified allergens in classic AR patients (23,24), but up to date there are not such studies in LAR subjects. Also, each allergen may produce a different response in the nasal mucosa according to their properties (25). If the response in the target organ is the same in AR and LAR subjects still remains unknown.

Moreover, a recent study by Gómez et al. demonstrated a basophil response in 50% of

subjects with LAR to *D. pteronyssinus* (26), and this response was IgE specific demonstrated by wortmannin pre-treatment. Since these studies were performed only with dust mites, it seemed interesting to confirm these results with other relevant allergens such as pollens.

The aim of this study was to thoroughly evaluate the *in vivo* and *in vitro* responses to natural Ole e 1 purified from olive pollen (nOle e 1) as a model of purified allergen from a relevant pollen in many areas around the world. The study was performed in AR and LAR patients with known sensitization to OL demonstrated by NAPT. The AR group represents the classic model of rhinitis, and the LAR group represents a novel phenotype whose responses to purified allergens have not been investigated yet.

METHODS

Study subjects

The study included 36 subjects classified in 3 groups: 12 subjects with AR, 12 with LAR and 12 healthy subjects as control group (CG). Subjects were recruited in the Allergy Department, Regional Hospital of Málaga. The study was performed outside the pollen season when patients were symptom-free.

AR inclusion criteria: history of seasonal rhinitis symptoms ≥ 2 years, positive NAPT with olive tree pollen (NAPT-OL), positive skin prick test (SPT) and serum specific IgE (sIgE) to OL.

LAR inclusion criteria: history of seasonal rhinitis symptoms≥ 2 years, positive NAPT-OL, negative skin testing to OL (intradermal (ID)/SPT), and negative sIgE to OL and a battery of common aeroallergens.

CG inclusion criteria: healthy subjects with negative NAPT-OL, negative SPT/ID and serum sIgE to aeroallergens.

Exclusion criteria: subjects with chronic rhinosinusitis evaluated by CT-scan; massive polyposis, septal perforation, total nasal blockage, upper respiratory infection or any condition that prevents performing nasal challenge; pregnancy/breastfeeding; autoimmune or any severe disease; psychosomatic disorders or unable to follow the instructions.

The study was conducted according to the principles of the Declaration of Helsinki and approved by the Ethic Committee of Málaga. All participants and parents of patients below 16 years old signed the corresponding informed consent.

The complete description about n Ole e 1 purification, *in vivo* evaluation (skin testing, NAPT with OL extract and nOle e1, ECP/tryptase measurement in nasal lavages), *in vitro evaluation* (total/specific IgE measurement in serum, basophil activation test with whole OL extract and nOle e 1, studies in dendritic cells with generation of monocyte

derived DC, DC maturation and lymphocyte transformation tests (LTT) by CFSE dilution-proliferation assay) and complete statistical analysis are thoroughly explained in the **Online Repository**.

RESULTS

Clinical characteristics of participants

Clinical and demographical data are summarized in **Table 1**. Subjects were mostly non-smoker young women (AR mean age: 33.1 yr, LAR: 38.2 yr, CG: 37.6 yr), with more active smokers in both LAR and CG ($p<0.05$). The mean time of rhinitis symptoms was 8.5 years (range 2-30) for AR subjects and 9.7 years (3-20) for LAR. Sixty six percent of AR subject had also asthma symptoms *vs.* to 58% of LAR patients.

In vivo responses to OL and nOle e 1

Skin testing

SPTs with nOle e 1 at 0.5, 1, 5 and 10 µg/mL were performed in all subjects. All AR subjects recognized nOle e 1 in SPT and none of the LAR or CG subjects (**Table 1**).

NAPT responses to nOle e 1 and mediators´ release in nasal lavages

The responses to NAPT are shown in **Figures 1A and B**. All AR subjects (12/12) had a positive response to nOle e 1, and 10/12 (83%) of LAR subjects had a positive NAPT. Healthy controls did not react to nOle e 1 (**Figure 1A**) at the highest concentration. AR and LAR show differences in the nOle e 1 concentration to obtain a positive NAPT with 60% of AR and 38% of LAR patients reacted at 0.5 µg/mL, whereas 62% of LAR required 1 µg/mL or higher nOle e 1 concentration in order to obtain a positive NAPT response compared to 40% of AR subjects (**Figure 1B**).

The median VOL 2-6 cm decreased significantly from 100% (after saline lavage) to 50% at 15 min in AR subjects and to 55% in LAR after NAPT with nOle e 1 (**Figure 2A**). The maximum reduction was observed at 15 min in both groups. Patients from AR and LAR groups also showed significant increases in total VAS scores compared to controls **(Figure 2B**).

ECP levels (Figure **3A)** in nasal lavages were significantly increased at baseline, 15 and 60 min after NAPT in AR subjects compared with CG ($p<0.05$). Comparisons for

related sample showed a significant increase of ECP at 15 min after challenge in LAR subjects ($p<0.05$, Figure **3A**). Tryptase levels in nasal lavages were increased at 15 and 60 minutes after NAPT for AR ($p<0.05$, **Figure 3B**), and no significant increase was observed in LAR or CG subjects.

In vitro responses to nOle e 1

Serum sIgE to nOle e 1 and other OL allergens

In AR subjects, total IgE was significantly higher compared to those determined in LAR and CG ($p<0.05$). Specific IgE to OL was also significantly higher in AR group as expected ($p<0.05$) (**Table 2**). Serum specific IgE antibodies against nOle e 1, rOle e 2, rOle e 3, nOle e 7, rOle e 9 and rOle e 11 were also measured by ELISA in all subjects. Both LAR and CG subjects had undetectable serum levels of sIgE to all allergens tested. AR subjects showed positive values to allergens, being nOle e 1 and rOle e 2 the most commonly detected (60%), followed by nOle e 7, rOle e 9 and rOle e 11 (20%) and rOle e 3 (10%).

Basophil activation test (BAT) with OL and nOle e 1

BAT was performed with OL whole commercial extract and nOle e 1 in all subjects at final concentrations 0.1, 0.5, 1 and 5 µg/mL. **Figure 4 A** represents the SI of all subjects after basophil stimulation with both OL and nOle e 1. All AR subjects had positive responses to OL stimulation, and 10/12 AR subjects (83.3%) had a positive response to nOle e 1 (**Figure 4B**). In the LAR group, 8/12 subjects (66.6%) had a positive BAT with OL, and 4/12 with nOle e 1 (33%). In the control group, only one subject (8.3%) had a positive BAT with both OL and nOle e 1 stimulation. Specificity of the test was 91.7% for OL and n Ole e 1 in both groups.

Dendritic cell maturation and T cell proliferation studies

Changes in DC maturation after stimulation with nOle e 1 were assessed. The percentage of cases with positive DC maturation was 66.6% for SAR, 57.4% for LAR

and 40% for CG. There were no significant differences in the percentage of positive maturation among the groups for any of the markers evaluated. The analysis of the proliferative response of T lymphocytes after stimulation with nOle e 1 was also assessed by flow cytometry. The percentage of cases with positive proliferative responses was again higher in SAR (90%) than in LAR (37.5%) and CG (11.1%). Chi-square analysis demonstrated that in SAR showed a significant increase in the percentage of proliferation compared to LAR and CG ($p=0.019$ and $p=0.001$ respectively). Although results were higher in LAR compared to CG, the differences were not significant. Results are summarized in **Figure 5**.

DISCUSSION

Olive tree pollen (OL) is one of the most important causes of allergy in Mediterranean countries (12, 16, 27). Several allergens have been characterized, which show sequence similarity to proteins from different vegetable tissues (8,28,29). Ole e 1 is the most abundant protein (up to 20% of the total protein content). It is a polymorphic protein of 145 amino acids with a glycosylated (80-85% of total allergen) and a non-glycosylated forms (14,15), and constitutes a good model of purified allergen (30,31). Several studies have tested its reactivity *in vivo* and *in vitro*, (measurement of sIgE (32), SPT (33)) using natural and recombinant forms of Ole e 1. However, to the best of our knowledge there are no studies assesing the response after nasal challenge with nOle e 1 in AR patients, since all challenges were performed with whole OL for diagnostic purposes (34,35). Moreover, the cellular responses after nOle e 1 stimulation have not been studied in depth. A recent study evaluated the Th1, Th2 and T regulatory responses in nasal biopsies of subjects with AR due to OL (36), and there is only one study performing histamine release in 5 patients after incubation with Ole e 1 (37).

In this study, the aim was to investigate the in *vivo* and *in vitro* responses to nOle e 1 as a model of purified allergen in a group of AR and LAR patients sensitized to OL where the phenotype had been confirmed by NAPT. Moreover, the basophil response of LAR subjects to pollens was also unknown, since the prior study performed in LAR was only done with dust mites (26).

All AR subjects reacted to nOle e 1 after NAPT. Interestingly, most LAR subjects (10/12, 83.3%) had a positive response to nasal challenge with the purified allergen. This is the first time where *in vivo* positive responses to a purified structure of known allergenicity are demonstrated in LAR subjects. Also, in both AR and LAR subjects a significant decrease in acoustic rhinometry measures with an increase in VAS scores

were detected with no response in healthy controls. These results correlated with ECP release that increased at the different time points in nasal lavages in both AR and LAR group. Tryptase leves were elevated only in AR subjects with no activity in LAR or the controls. These data could indicate that mast cells may not be involved in these responses in LAR, or could be also due to technical limitations of the technique (e.g: dilution effect), since increases of tryptase levels had been previously detected in other challenge studies using non-purified extracts in LAR patients (4,6,7), and in patients with AR using Art v 3 (38). This is the first study performing nasal provocation test with a natural purified allergen in LAR subjects, so more studies are necessary in order to better understand the mechanism of the response.

Regarding the cellular response, BAT was positive to OL stimulation in 100% of AR subjects and in 10/12 with nOle e 1 (83.3%). In the LAR group 8/12 subjects (66.6%) had a positive BAT with OL. The BAT responses with purified nOle e 1 were lower in this group, with 4/12 (33%) allergen stimulation. In the control group, only one subject (8%) had a positive BAT with both OL and nOle e 1 stimulation indicating a good specificity (91.7%) for both OL and nOle e 1. These results are in agreement with the ones obtained in a previous study (26), where subjects with LAR to DP showed a 50% of positive results in the BAT, with 7% of controls having a positive response in BAT similar to the results found with OL and nOle e 1. This study confirms the presence of allergen-specific IgE in the surface of basophils since this cell may be the target of the specific IgE leaked from the nose or other organs (39,40). It has also been demonstrated that basophils circulate to inflamation sites in allergic individuals (41), and have been found in nasal secretions after allergen challenge (42). The demostration of the reactivity of basophils with no presence of specific IgE antibodies could be explained by the fact that basophils are highly responsive to IgE-mediated activation, being twice more sensitive than mast cells (43). However, the exact

mechanism that may cause this phenomenon and how the exchange of IgE antibodies between the nasal mucosa and the general circulation is produced still remains unclear (40).

Also, this work represents the first study that evaluates the cellular responses to allergens in LAR patients. These results demonstrated that although nOle e 1 induced DC maturation in a similar way in all groups analyzed, both AR and LAR showed a higher specific T cell proliferation compared to CG, although with a very low sensitivity in the latter. The significant differences obtained in AR are in agreement with those obtained previously with other purified allergens as Pru p 3, Bet v 1 and Phl p 5 (44,45,46).

In conclusion, this study has described the *in vivo* and *in vitro* responses to a purified allergen, nOle e 1, in 3 different groups of subjects, demonstrating *in vivo* a nasal response with obstruction and mediators´ release in both AR and LAR patients. The *in vitro* studies confirmed the absence of serum specific IgE to OL and OL allergens in both LAR and controls. The BAT was positive in AR and LAR subjects with both OL and nOle e 1, confirming a BAT response with pollens in a similar percentage as described with dust mites, and also responses to nOle 1 from the basophils but in a lower percentage. Cellular studies revealed that nOle e 1 induced mild maturation and proliferation of dendritic cells in AR ald LAR with no significant differences. Further studies with higher number of subjects and other whole and purified allergens will be performed in the near future to gain insights into the pathogenesis of this novel form of AR.

Conflict of interest statement

The authors: Paloma Campo, Mayte Villalba, Esther Barrionuevo, , Carmen Rondón, Luisa Galindo, Maria José Rodríguez, Juan Carlos López-Rodríguez, María José Torres MD, Miguel Blanca MD, Cristobalina Mayorga declare that they have no conflicts of interest for this manuscript.

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Table 1.

	Age (y) Median (range)	Sex (F/M)	Smoking (%) (yes/no/ex)	Diagnosis of rhinitis(yr) Median(range)	Asthma Symptoms (%)	Positive SPT/IDOL (%)	Positive SPT nOle e1
AR n=12	33.09 (15-64)	9/3	0/83/17	8.5 (2-30)	66%	100%¥	100%¥
LAR n=12	38.15 (27.52)	6/6	17*/75/8	9.75 (3-20)	58%	0%	0%
CG n=12	37.6 (27-58)	9/3	17*/83/0	n/a	n/a	0%	0%

TABLES AND FIGURE LEGENDS

Table 1.

Clinical characteristics and SPT responses to olive tree (OL) pollen extract and purified nOle e 1 in allergic rhinitis (AR), local allergic rhinitis (LAR) and control subjects (CG).

*p<0.05 (LAR and CG *vs.* AR)

¥p<0.05 (AR *vs.* LAR and CG)

Table 2.

Serum total IgE and specific IgE determination to olive tree pollen (OL) and purified OL allergens nOle e 1, rOle e 2, rOle e 3, nOle e 7, rOle e 9 and rOle e 11

IU/mL: international units per milliliter kU/L: kilounits per liter

Figure 1A.

Percentage of positive nasal allergen provocation test (NAPT) with olive tree pollen (OL) extract and purified nOle e 1 in allergic rhinitis (AR), local allergic rhinitis (LAR) and control subjects (CG).

Figure 1B.

Percentage of nasal allergen provocation test (NAPT) obtained with the different doses of nOle e 1 in allergic rhinitis (AR) and local allergic rhinitis (LAR) subjects.

Figure 2A.

Nasal allergen provocation test (NAPT) with nOle e 1 in allergic rhinitis (AR), local allergic rhinitis (LAR) and control subjects (CG). Figure shows decrease of VOL 2-6 cm values at different timepoints. The solid horizontal bar represents the cut-off point for a positive NAPT response (decrease of 30% of vol 2-6 cm).

Figure 2B.

Mean visual analogue scale (VAS) values after nOle e 1 NAPT in allergic rhinitis (AR), local allergic rhinitis (LAR) and control subjects (CG). Values are represented at different time points after NAPT.

Figure 3A.

Levels of ECP in nasal lavages at 0, 15 and 60 minutes after nOle e 1 NAPT in AR, LAR and CG.* p<0.05

Figure 3B.

Levels of tryptase in nasal lavages at 0, 15 and 60 minutes after nOle e 1 NAPT in AR, LAR and CG.* p<0.05

Figure 4A.

Basophil activation test results after adding OL and nOle e 1 at different concentrations. The horizontal line indicates the cut-off point of the assay.

SI: stimulation index; OL: olive tree pollen extract; IgE C+: positive IgE control.

Figure 4B.

Positive responses of basophil activation test (BAT) in response to olive tree (OL) pollen extract and purified nOle e 1 in allergic rhinitis (AR), local allergic rhinitis (LAR) and control subjects (CG).

Figure 5.

Percentage of positive responses in terms of maturation and proliferation of dendritic cells after OL and n Ole e1 incubation in allergic rhinitis (AR), local allergic rhinitis (LAR) and control subjects (CG).

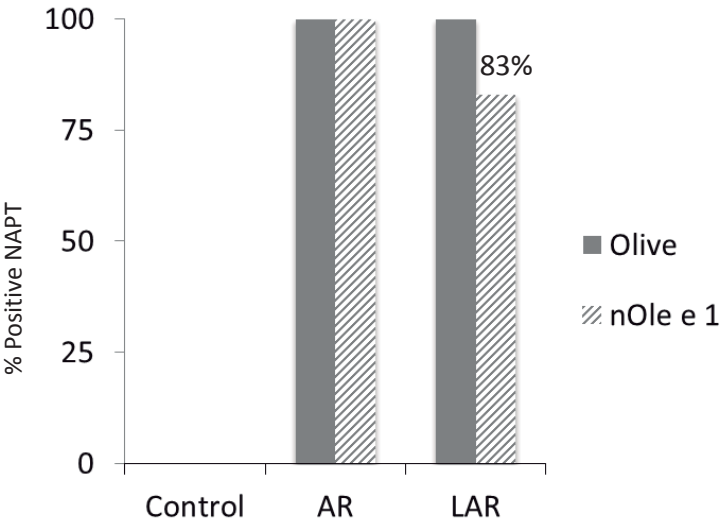
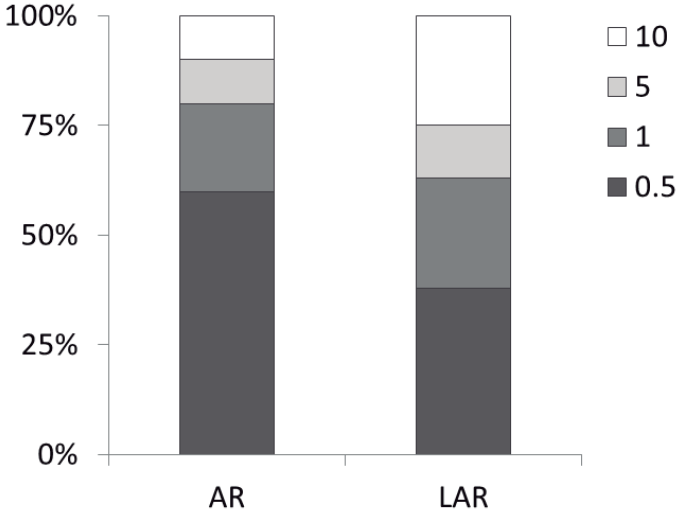


Figure 1A.



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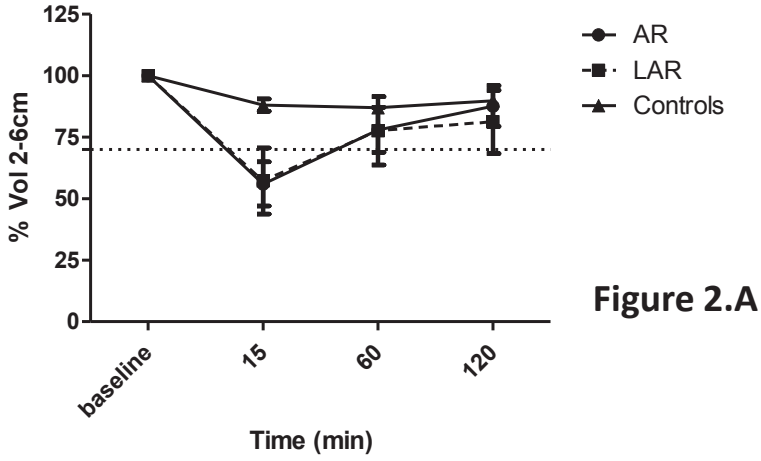

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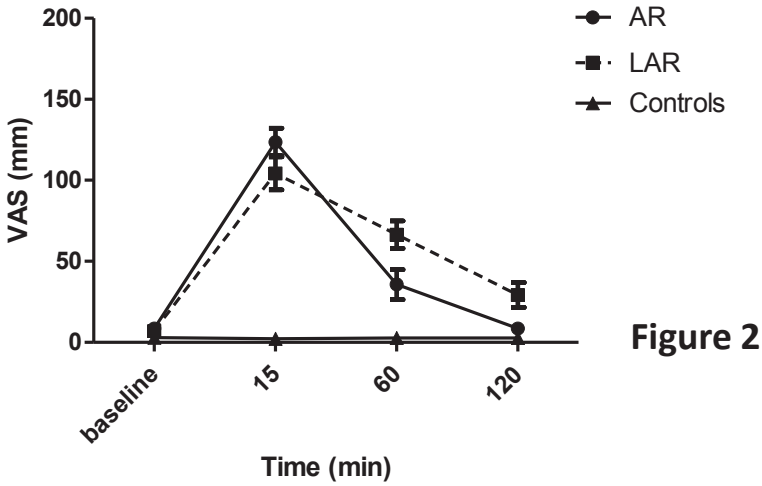


Figure 2.B

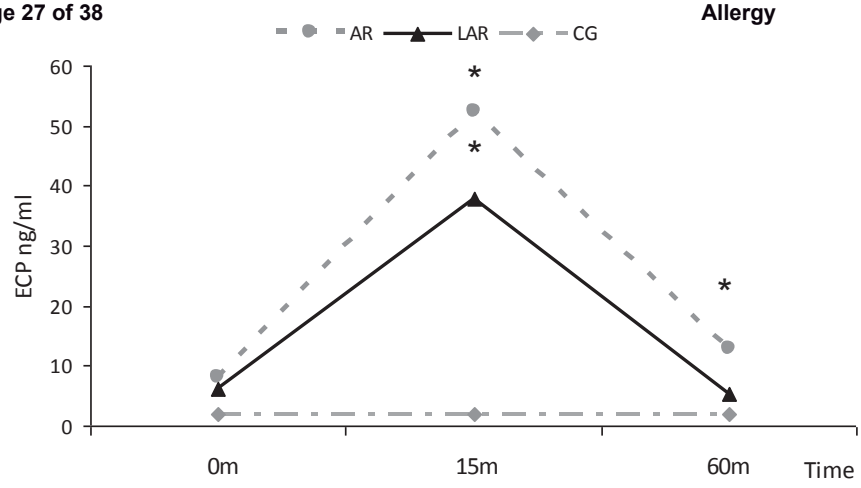


Figure 3.A.

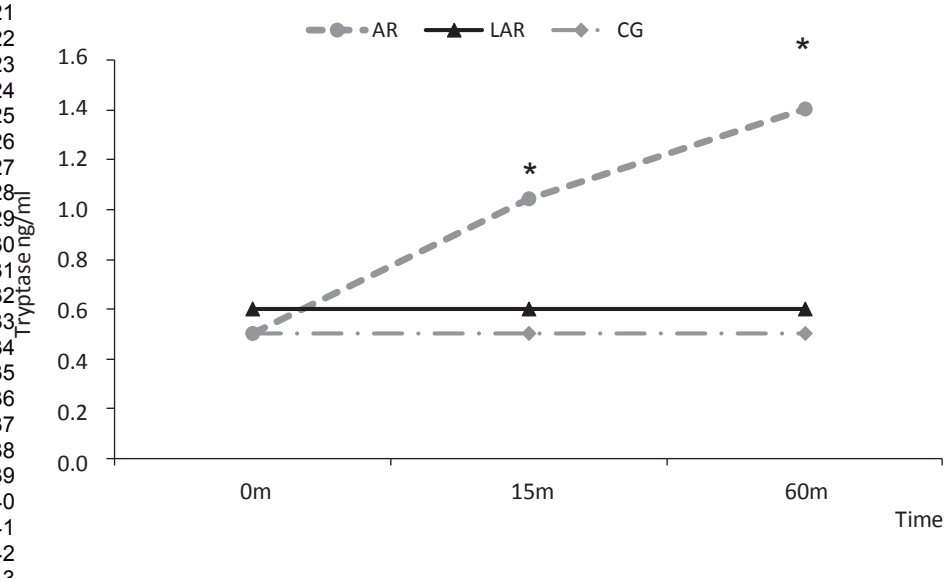
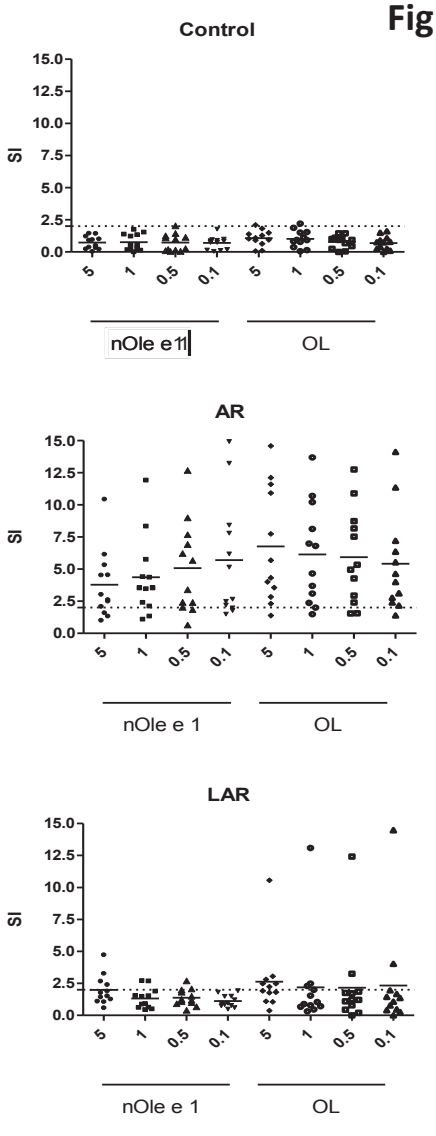


Figure 3.B.

Figure 4A.



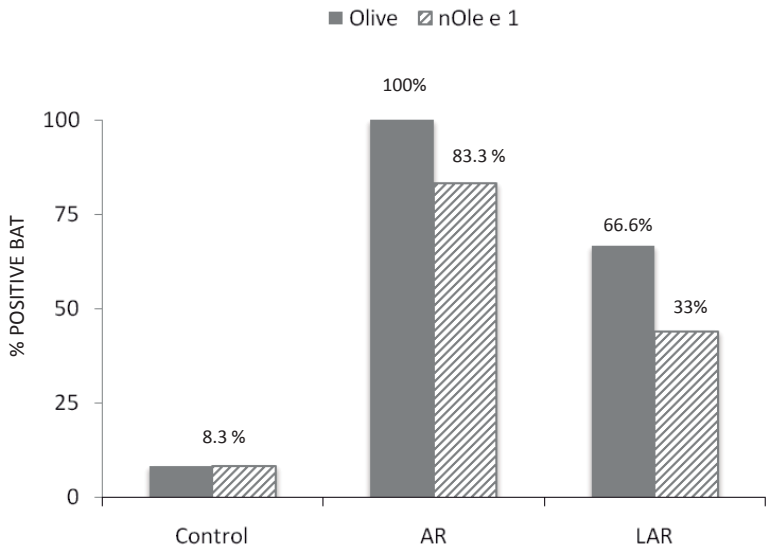


Figure 4B.

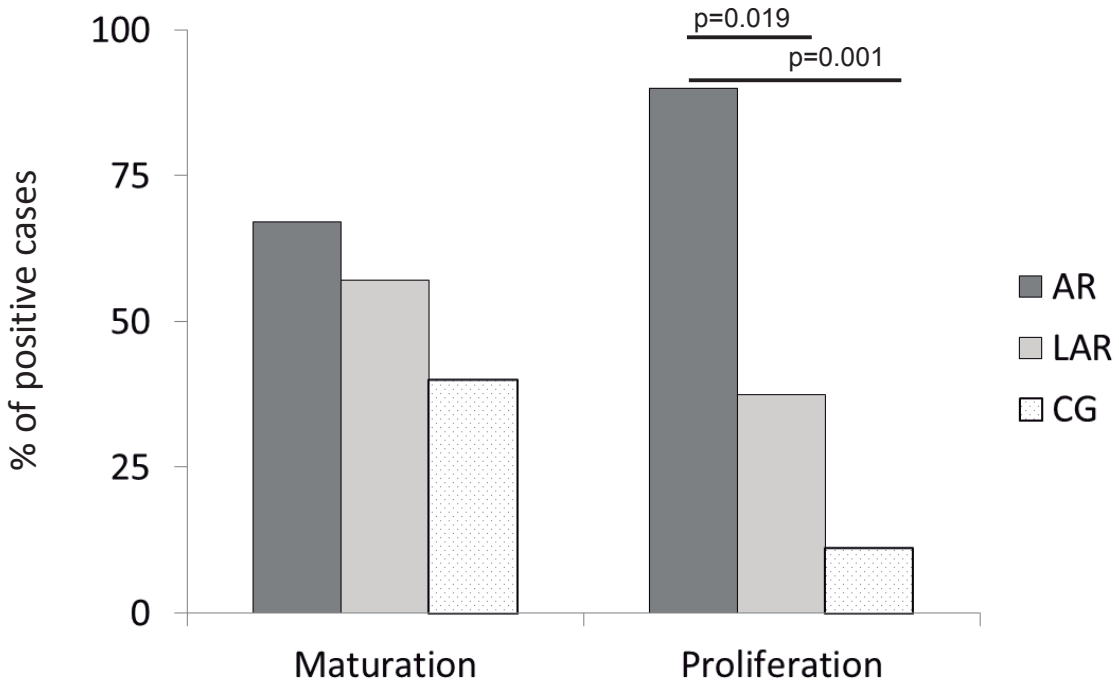

100
75
50
25
0
% of positive cases
p=0.019
p=0.001
AR
LAR
CG
Maturation
Proliferation

Figure 5.

REPOSITORY

Title:

Immunologic responses to the major allergen of *Olea Europaea* in local and systemic allergic rhinitis subjects

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METHODS

Purification and characterization of nOle e 1

nOle e 1 allergen was purified from ethyl-ether-defatted olive tree (*Olea europaea*) pollen (Allergon AB) extracted in 50mM ammonium bicarbonate pH 8.0, containing 1 mM PMSF (1 g pollen/30 mL) for 1 h as described (15). After centrifugation at 20.000g for 30 min at 4°C supernatant was collected. The procedure was repeated three times, supernatants were pooled and stored at –20°C. Olive pollen extract was loaded into a Sephadex G-75 *medium* column and afterwards in a Sephadex G-75 *superfine*, both in 0.2M ammonium bicarbonate, pH 8.0. Fractions containing nOle e 1 were finally loaded onto a Nucleosil C_{18} column with an acetonitrile gradient (0-60%) in 0.1% trifluoroacetic acid. The elution profile was continuously monitored at 214 nm and 280 nm.

Skin testing

SPTs were performed with a wide panel of prevalent aeroallergenic sources in the area: *D. pteronyssinus, D.farinae, Phleum pratense, Lolium perenne, Cupressus arizonica, Platanus acerifolia, Olea europaea, Chenopodium album, Artemisia vulgaris, Parietaria judaica, Salsola kali, Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, Penicillium,* dog and cat epithelia (ALK-Abelló, Madrid, Spain) (E1). Also, SPTs with nOle e 1 at 0.5, 1, 5 and 10 µg/mL were performed in all subjects. ID skin test was performed with freshly reconstituted freeze-dried OL extract (0.6 µg/mL) (ALK-Abelló) as described in all LAR and CG subjects (E2).

Nasal provocation test with whole olive extract and nOle e 1

NAPT with OL extract and nOle e 1 were performed according to published methods (7,6). All subjects underwent a NAPT with OL as an inclusion criterion. Two weeks later, challenge with n Ole e 1 was performed using serial dilutions at 0.5, 1, 5 and 10 µg/mL of nOle e 1. For all allergen challenges, a nasal challenge with 0.9% saline was performed prior to the application of the allergen in the nose in order to rule out nasal

hyperresponsiveness. Two puffs (100µL) of freshly reconstituted OL extract (Ole e 1 at 6 µg/mL, ALK-Abelló, Denmark) were applied using a metered pump. For the nasal challenge with the purified allergen, 100 µL of nOle e 1 at serial dilutions were applied per nostril by means of a micropipette. Responses were monitored by symptoms score (rhinorrhea, itching, nasal obstruction and sneezing) placing a vertical mark on a horizontal visual analog scale (VAS) of 100 mm. The volume of the nasal cavity that corresponds to the lower turbinate (VOL 2–6 cm) in each nostril was measured after challenge at different timepoints (baseline, 15, 60 and 120 minutes) by acoustic rhinometry (SRE 2000 Rhinometer, Rhinometrics, Lynge, Denmark) following the current guidelines (E4,E5). A nasal lavage was performed at baseline, 15 and 60 minutes following the Naclerio method (E6). A positive NAPT was considered to be an increase ≥30% in the total nasal symptoms and a decrease ≥30% in the total VOL 2-6 cm of both nasal cavities compared with the baseline test.

ECP and tryptase measurement in nasal lavages

Nasal lavages were obtained from both nostrils at baseline, 15 and 60 minutes after NAPT as described (E6). Measurement of tryptase and ECP was performed by UNICAP method (Thermofisher, USA).

Total and specific IgE measurement in serum

Serum total and sIgE were measured to the same aeroallergens of the SPT panel including OL by fluoroenzyme immunosorbent assay (UNICAP, Thermofisher, USA). Also, specific IgE antibodies against Ole e 1, Ole e 2, Ole e 3, Ole e 7, Ole e 9 and Ole e 11 were measured by ELISA in the sera of all participants. Briefly, ELISA was performed in microtiter plates coated with 100 µg/well of each aforementioned purified allergen. Plates were incubated with sera (diluted 1:10). The binding of human IgE was detected by mouse anti-human IgE antibodies (diluted 1:5000) donated by ALK-Abelló (Madrid, Spain) followed by horseradish peroxidase-labeled goat anti-mouse IgG

(diluted 1:5000; Pierce Chemical Co, Rockford, Ill). The reaction was developed with o-phenylendiamine and the optical density (OD) measured at 492 nm. Each value was calculated as mean of two determinations (E7).

Basophil activation test with whole olive extract and nOle e 1

A hundred microliters of heparinized whole blood was aliquoted per test and 20 µL of stimulation buffer was added and incubated for 10 min with agitation at 37ºC in a water bath as described (E8). After this, 100 µL of the washing solution was added to the negative control tube, 100 µL of anti-human IgE antibodies (BD Pharmingen San Diego, CA, USA) to the positive control tubes and 100 µL of the allergen (OL extract, ALK-Abello, Denmark and purified nOle e 1), were added at final concentrations of 5, 1, 0.5 and 0,1 µg/mL. These concentrations were chosen based on a dose-response curve (data not shown). The samples were incubated for 30 minutes at 37ºC in a water bath in agitation. The degranulation was stopped by incubating the samples on ice for 5 minutes and then, 3 µL of monoclonal antibodies, anti-CD63-FITC, CD203c-PE, CD193-APC (Caltag Laboratories, Burlingame, CA) were added to each tube. After 20 minutes at 4ºC in dark, 2 mL of pre-warmed lysis solution was added and centrifuged 5 min at 4ºC. Cells were washed and analyzed in a FACSCalibur flow cytometer (Becton-Dickinson Bioscience, San Jose, CA) by acquiring at least 500-1000 basophils per sample. Results were considered as positive when the stimulation index (SI), calculated as the ratio between the percentage of allergen-degranulated basophils and the negative control, was ≥2 in at least one of the allergen concentrations mentioned above. When the percentage of spontaneously activated basophils was lower than 2.5%, an additional condition was required, i.e. that the percentage of basophils activated after contact with the antigen should be ≥5%, as previously described (E9).

Generation of monocyte derived DC

From each subject, 40 mL of peripheral blood was obtained. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density gradient (GE Healthcare UK Ltd, Buckinghamshire, England). Monocytes were purified from PBMC by positive selection using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's protocol. A purity of 90-95% was assessed by flow cytometry. Immature dendritic cells (imDC) were derived from monocytes by culturing the $CD14^+$ fraction in complete medium R10 (RPMI 1640, 10% foetal bovine serum (BioWhittaker, Pittsburgh PA), 2 mM L-glutamine (BioWhittaker), and 5 mg/mL gentamicin (Normon, Madrid, Spain)) with 200 ng/mL rhGM-CSF and 100 ng/mL rhIL-4 (both from R&D Systems Inc, Minneapolis, MN), for 5-6 days at 5% CO_2 and 37°C. The $CD14^-$ fraction was frozen in a culture medium containing 10% dimethylsulphoxide (DMSO) (Sigma, St Louis, Mo) for further experiments of lymphocyte transformation test (LTT) (E10).

DC maturation

imDC were incubated in complete medium at $5x10^5$ cells/mL in 48-well plates (Nunc AS, Roskilde, Denmark) with Ole e 1 at 10, 1 and 0.1 µg/mL. including LPS at 1 mg/mL (Sigma, St Louis, MO) and TNF-α (R&D Systems) at 10 ng/mL as positive controls. After 72h of stimulation at 37°C in 5% CO_2, treated or untreated imDC were harvested and the maturation state was assessed by upregulation of CD80, CD86, and CD83 co-stimulatory molecules (all three from Immunotech, Marseilles, France) and HLA-DR (BD Pharmigen, San Diego, CA), in a FACSCanto II Cytometer (BD Biosciences, Milpitas, CA). Data were processed with FACSDiva (BD Biosciences). Results were expressed both as a percentage of positive cells compare to non-stimulated cells and as maturation index (MI), calculated as the ratio between stimulated DC and non-stimulated cells, considered positive when greater than 2.

Lymphocyte transformation tests (LTT) by CFSE dilution-proliferation assay

The LTT were performed by using imDC as APC, the $CD14^-$ fraction (that included autologous lymphocytes (lymphs)) and nOle e 1 at different concentrations (10, 1 and 0.1 µg/mL). Autologous lymphs at 0.5-1x10^7/mL were labelled with CFSE (5(6)-Carboxyfluorescein diacetate N-succinimidyl ester, Molecular probes) following the manufacturer's instructions. One hundred µL of CFSE-labelled lymphs at 1.5×10^6cells/mL were cultured with imDC at 1.5×10^5cells/mL (ratio 10:1) at a final volume of 250 µL of complete medium, in 96-well plates in triplicate, with or without allergen, for 7 days at 37°C and 5% CO_2. Tetanus toxoid (TT) at 5µg/mL (Calbiochem, San Diego, CA) and phytohaemagglutinin (PHA) at 10µg/mL (Sigma) were used as positive proliferative controls. The proliferation of different lymphs subsets, either T cells, NK cells, B cells or T regulatory cells, was assessed by flow cytometry, analyzing the percentage of $CD3^+$, $CD56^+$, $CD19^+$ or $CD4^+CD25^{high}CD127^-$ cells that expressed $CFSE^{low}$, respectively. The results were considered positive when the proliferation index (PI), calculated for each subset as the ratio between [%$CFSE^{low}$ stimulated-(Lymphs + DC)-%$CFSE^{low}$ unstimulated-(Lymphs + DC)] / %$CFSE^{low}$ (Lymphs), was greater than 3.

Statistical Analysis

Data were expressed as median and range. Clinical and demographic data were compared between groups by chi-square analysis and the Mann–Whitney U-test. Friedman's test was used to examine the overall differences. If significant differences occurred, the Wilcoxon's signed-ranks test was used to identify them within groups. For cellular studies, comparisons of quantitative variables were carried out by non-parametric Kruskall-Wallis test and Mann-Whitney U test. All p-values of <0.05 were considered statistically significant.

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Table 2.

	Total IgE OL (IU/ml) Median (range)	sIgE OL (kU/L) Median (range)	Serum sIgE nOle e 1 (% positive)	Serum sIgE rOle e 2 (% positive)	Serum sIgE rOle e 3 (% positive)	Serum sIgE nOle e 7 (% positive)	Serum sIgE rOle e 9 (% positive)	Serum sIgE rOle e 11 (% positive)
AR n=12	322.89 (12-909)*	12.9 (0.7-53.7)*	60%*	60%*	10%*	20%*	20%*	20%*
LAR n=12	26.17 (9-68)	<0.35	0%	0%	0%	0%	0%	0%
CG n=12	13 (8-18)	<0.35	0%	0%	0%	0%	0%	0%