Predicting probability of tolerating discrete amounts of peanut protein in allergic children using epitope-specific IgE antibody profiling

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ARTICLE SUMMARY

- Existing diagnostic testing is not predictive of severity or the threshold dose of clinical reactivity, and many patients still require an Oral Food Challenge (OFC). While OFCs are very useful for making an allergy diagnosis and determining clinical reactivity, they often cause anaphylaxis, which can increase patient anxiety. and are time and resource intensive.[1]
- An extensive validation was performed across 5 cohorts (all with confirmed oral food challenge results) across six different countries. Cohorts used: BOPI, OPIA, CAFETERIA, CoFAR6, and PEPITES with specimens from Australia, UK, US, Ireland, and Germany.
- This paper reports the first validated algorithm using two key peanut specific IgE epitopes to predict probabilities of reaction to different amounts of peanut in allergic subjects and may provide a useful clinical substitute for peanut oral food challenges.
- Using the algorithm, subjects were assigned into "high", "moderate", or "low" dose reactivity groups. On average, subjects in the "high" group were 4 times more likely to tolerate a specific dose, compared to the "low" group.[1] For example, 88% of patients in the high dose reactivity group were able to tolerate ≥ 144 mg of peanut protein whereas only 29% were able to tolerate the same amount in the low dose reactivity group.[1-2]

CLINICAL CONSIDERATIONS

- The new epitope test offers more granular information to help clinicians stratify treatment and peanut avoidance plans for their patients.
- See below for summary of clinical considerations based on threshold reactivity level.[1]

allergenis peanut diagnostic result	clinical considerations[1]
likely allergic – low dose reactor	• inform or avoid oral food challenge to reduce risk of anaphlyaxis • confirm strict avoidance of peanut • consider immunotherapy to reduce risk of reaction
likely allergic – moderate dose reactor	• consider a single oral food challenge (30 to 100 mg) to reduce anxiety and improve quality of life • less stringent avoidance of peanut regime • consider inclusions of precautionary labeled foods such as 'May contain peanut' • consider immunotherapy to reduce risk of reaction
likely allergic – high dose reactor	• consider a single oral food challenge (100 to 300 mg) to reduce anxiety and improve quality of life • less stringent avoidance of peanut regime • consider inclusions of precautionary labeled foods such as 'May contain peanut' • consider starting immunotherapy at higher doses to shorten time to maintenance dose
unlikely allergic	• oral food challenge to rule out the diagnosis of peanut allergy

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Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY

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ORIGINAL ARTICLE EXPERIMENTAL ALLERGY AND IMMUNOLOGY

The lipid interaction capacity of Sin a 2 and Ara h 1, major mustard and peanut allergens of the cupin superfamily, endorses allergenicity

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To cite this article: Angelina A, Sirvent S, Palladino C, Vereda A, Cuesta-Herranz J, Eiwegger T, Rodríguez R, Breiteneder H, Villalba M, Palomares O. The lipid interaction capacity of Sin a 2 and Ara h 1, major mustard and peanut allergens of the cupin superfamily, endorses allergenicity. *Allergy* 2016; **71**: 1284–1294.

Keywords
11S and 7S globulins; allergens; dendritic cells; food allergy; lipid-binding capacity.

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Accepted for publication 12 March 2016

DOI:10.1111/all.12887

Edited by: Reto Crameri

Abstract

Background: Sin a 2 (11S globulin) and Ara h 1 (7S globulin) are major allergens from yellow mustard seeds and peanut, respectively. The ability of these two allergens to interact with lipid components remains unknown.
Objective: To study the capacity of Sin a 2 and Ara h 1 to interact with lipid components and the potential effects of such interaction in their allergenic capacity.
Methods: Spectroscopic and SDS-PAGE binding assays of Sin a 2 and Ara h 1 with different phospholipid vesicles and gastrointestinal and endolysosomal digestions in the presence or absence of lipids were performed. The capacity of human monocyte-derived dendritic cells (hmoDCs) to capture food allergens in the presence or absence of lipids, the induced cytokine signature, and the effect of allergens and lipids to regulate TLR2-L-induced NF-kB/AP-1 activation in THP1 cells were analyzed.
Results: Sin a 2 and Ara h 1 bind phosphatidylglycerol (PG) acid but not phosphatidylcholine (PC) vesicles in a pH-dependent manner. The interaction of these two allergens with lipid components confers resistance to gastrointestinal digestion, reduces their uptake by hmoDCs, and enhances their stability to microsomal degradation. Mustard and peanut lipids favor a proinflammatory environment by increasing the IL-4/IL-10 ratio and IL-1β production by hmoDCs. The presence of mustard lipids and PG vesicles inhibits TLR2-L-induced NF-kB/AP-1 activation in THP1 cells.
Conclusion: Sin a 2 and Ara h 1 interact with lipid components, which might well contribute to explain the potent allergenic capacity of these two clinically relevant allergens belonging to the cupin superfamily.

Abbreviations
3D, three-dimensional; CBS, coomassie blue staining; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; hmoDCs, human monocyte-derived DCs; LTP, lipid transfer proteins; ML, mustard lipids; PC, phosphatidylcholine; PG, phosphatidylglycerol; PL, peanut lipids; PMSF, phenylmethanesulfonyl fluoride; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; TCS, theoretical cleavage sites; TLR2-L, toll-like receptor 2-ligand.

IgE-mediated food allergy is a worldwide health problem of increasing incidence affecting around 6–8% of children and 4–6% of adults (1, 2). Peanut, tree nuts, and some seeds such as yellow mustard seeds are among the most potent allergenic foods of plant origin (3, 4). Although the prevalence of mustard allergy is not very high, it is frequently associated with severe symptoms (4). Mustard is consumed in homemade meals and added as hidden condiment in sauces, salad dressing,

and processed foodstuffs. Four clinically relevant allergens have been characterized from yellow mustard seeds (www.allergen.org). Sin a 1 (2S albumin) is a diagnostic marker for sensitization to mustard (4, 5), Sin a 2 (11S globulin) predicts severity of symptoms and is involved in cross-reactivity among mustard, tree nuts, and peanut (4, 6, 7), and Sin a 3 (LTP) and Sin a 4 (profilin) are associated with sensitization to other plant-derived foods (4, 8). Sin a 2 is a basic 51-kDa protein composed of 2 polypeptide chains of 36 and 23 kDa bound by 1 disulfide bridge that forms hexameric structures (6, 9). Peanut allergy affects around 1–2% of the world population (1). Peanuts are used for the preparations of a wide variety of foods, and small doses can trigger severe reactions (1). Seventeen peanut allergens (Ara h 1 to Ara h 17) have been characterized (www.allergen.org). Ara h 1 (7S globulin) is a major peanut allergen recognized by more than 90% of peanut-allergic patients showing cross-reactivity with other food allergens (10). Ara h 1 consists of a polypeptide chain of 63 kDa (11) that forms trimers, may oligomerize, and may bind some small molecular compounds (12–15).

What makes a protein to become an allergen is still an open question of intensive debate (16). Food allergens that are able to sensitize via the gastrointestinal tract share common features such as relatively low molecular weight (10–70 kDa), high solubility in biologic fluids, thermal stability, and digestion resistance (17, 18). Allergic sensitization is a multifactorial process that depends not only on the allergens but also on other molecules contained within the allergen source (19, 20). Food allergens can bind ligands such as sugars or lipids modifying their structural, immunogenic, and allergenic properties. Lipid binding has been reported for food allergens that belong to different protein families (20). The interaction between food allergens and lipids can alter their degradation within the gastrointestinal tract, their transport across the intestinal epithelial cells, the induced immune responses, and their potential allergenicity (20). Mouse models of allergy and human data have firmly demonstrated that dendritic cells (DCs) play a critical role in all the phases of allergy as well as in the generation of tolerance to allergens (21–23).

The capacity of Sin a 2 (11S globulin) and Ara h 1 (7S globulin) to interact with lipids and the effects of such interaction in their allergenic capacity have not been investigated so far. In this study, we demonstrated for the first time that Sin a 2 and Ara h 1 are able to interact with phospholipid acid vesicles. The interaction of these two allergens with lipid components confers resistance to gastrointestinal digestion, reduces their uptake by human DCs, enhances their stability to microsomal degradation, and favors the production of pro-inflammatory cytokines by human DCs, which might well contribute to explain the potent allergenic capacity of these two clinically relevant allergens of the cupin superfamily.

Material and methods

Human sera, allergens, and lipid extracts

Serum samples were obtained from a well-defined cohort of patients allergic to mustard and peanut from the Allergy Service of Hospital Fundación Jiménez Díaz, Madrid, Spain (4). The study was approved by the Ethic Committee of Hospital Fundación Jiménez Díaz, and written informed consent was obtained from all subjects. Two pools of sera from patients specifically sensitized to Sin a 2 or Ara h 1 were used. Ara h 1 was purified from peanut seed extract as described with minor modifications (12). Sin a 1 and Sin a 2 were purified from a yellow mustard seed extract (5, 6), and Sin a 3 was produced as a recombinant allergen in *Pichia pastoris* (8) as previously described. Peanut and mustard lipids were obtained following the protocol described in (24) with minor modifications. Pam3CSK (InvivoGen) was used as TLR2-ligand (TLR2-L).

Detailed protocols are fully described in the supporting information of this article.

Results

Sin a 2 and Ara h 1 interact with phosphatidylglycerol vesicles

To assess the capacity of Sin a 2 and Ara h 1 to interact with lipid membranes, increasing concentrations of these allergens were incubated with different kinds of phospholipid vesicles at several pHs and the absorbance at 360 nm was continuously monitored. If the protein–lipid interaction induces vesicle aggregation, the generated larger particles would scatter the visible light, which can be determined as an absorbance variation at 360 nm. The maximum absorbance variation ($\Delta A_{360\ nm}$) after 10 min was assessed. Sin a 2 induced $\Delta A_{360\ nm}$ after incubation with phosphatidylglycerol (PG) but not phosphatidylcholine (PC) vesicles at pH 2.0 and 5.0 but not at pH 7.0 in a dose-dependent manner. This indicates that Sin a 2 interacts with PG acidic vesicles at these conditions (Fig. 1A). Supporting these data, SDS-PAGE analysis after centrifugation demonstrated the presence of Sin a 2 in the pellet fraction (protein bound to vesicles) when the allergen was incubated with PG at pH 2.0 and 5.0, but not at pH 7.0, or after incubation with PC vesicles at each of the three pHs (Fig. 1B). Ara h 1 induced most aggregation of PG but not PC vesicles at pH 5.0 in a dose-dependent manner (Fig. 1A). This was also confirmed by SDS-PAGE after centrifugation and analysis of the proteins contained in the pellet and supernatants (Fig. 1B). Marginal or no variations in $A_{360\ nm}$ were observed after incubation of Ara h 1 with PG or PC vesicles at pH 2.0 and 7.0 (Fig. 1A). At pH 2.0 and 7.0, most of Ara h 1 remained soluble in the supernatants, but a small fraction of the allergen was also identified within the pellet in the presence of PG vesicles. Sin a 2 and Ara h 1 were in the soluble fraction without vesicles (control, C) at all the assayed pHs (Fig. 1B).

PG vesicles protect Sin a 2 and Ara h 1 from gastric and intestinal digestion

Sin a 2 was almost completely degraded after 10 min of gastric digestion in the absence (control) or presence of mustard lipids or PC vesicles (Fig. 2A). The IgE reactivity to Sin a 2 was abolished after 1 min of digestion in the control, and

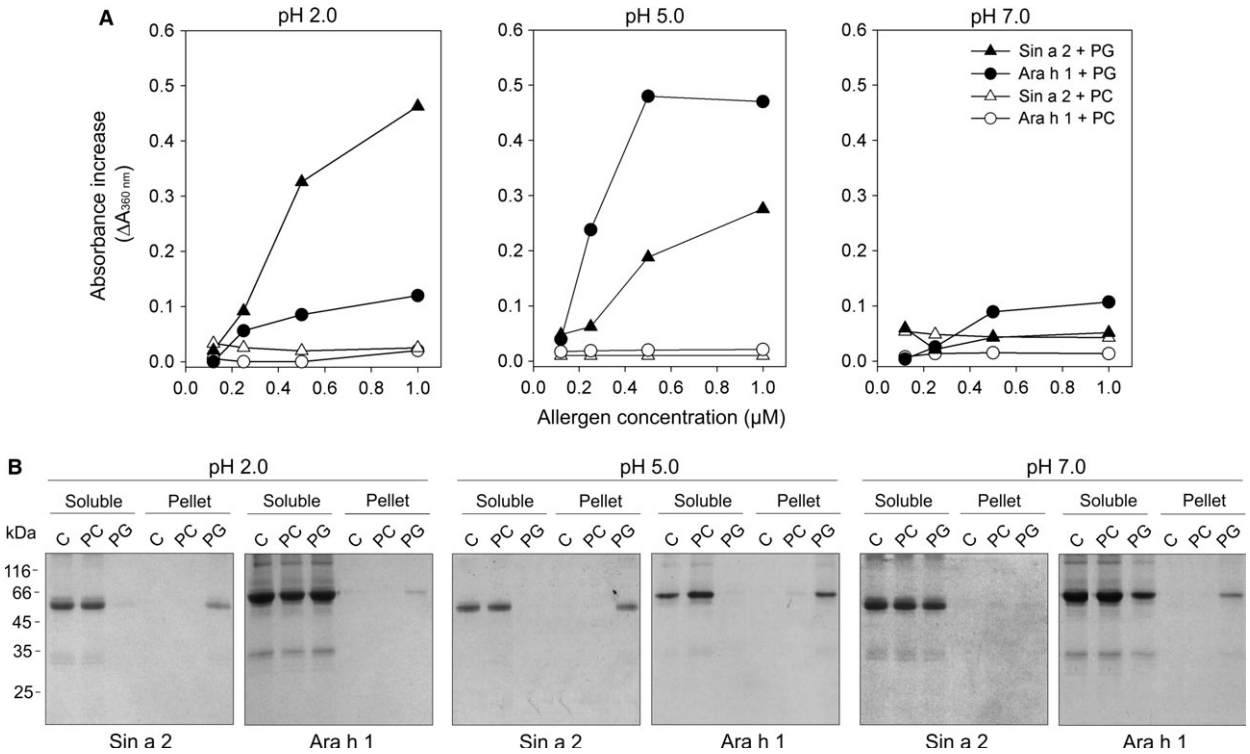

Figure 1 Interaction of Sin a 2 and Ara h 1 with PC or PG vesicles at different pHs. (A) Maximum absorbance increase ($\Delta A_{360\ nm}$) after 10-min incubation of vesicles with allergens *vs* allergen concentration at pH 2.0, 5.0, and 7.0. (B) SDS-PAGE and Coomassie blue staining of the pellet and soluble fractions after centrifugation of the allergen–vesicle mixture (10-min incubation) at the maximum allergen concentration. Molecular mass markers are indicated in kDa. The controls (C) show the corresponding allergen at the same protein concentration and pH without phospholipid vesicles.

after 10 min in the presence of mustard lipids or PC vesicles. PG vesicles significantly enhanced the resistance of Sin a 2 to gastric digestion. After 2 h, around 17% of Sin a 2 remained intact and the allergen retained its IgE reactivity after 30 min of digestion (Fig. 2A). Ara h 1 was quickly processed in the absence or presence of peanut lipids or PC vesicles, and proteolytic fragments of around 44, 32, 20, and 11 kDa were visualized (Fig. 2B). The IgE-binding capability of intact Ara h 1 was abolished within the first 5 min, and only the proteolytic fragments showed IgE reactivity, which disappeared after 30 min of treatment (Fig. 2B). PG vesicles significantly increased the resistance of Ara h 1 to gastric digestion, and around 40% of the allergen remained intact with IgE binding still detectable after 2 h (Fig. 2B). The theoretical cleavage sites (TCS) for pepsin were located in the three-dimensional (3D) structure of Sin a 2 and Ara h 1 (Fig. 2C,D). Although from academic point of view TCS were mainly located in the inner parts of the molecules, Sin a 2 and Ara h 1 were rapidly digested by pepsin in all the tested conditions except in the presence of PG vesicles. Circular dichroism (CD) analysis at pH 2.0 showed that PC vesicles did not considerably alter the secondary structure content of the allergens (Fig. S1). In contrast, PG vesicles slightly increased the percentage of α-helix in Sin a 2 or β-sheet in Ara h 1, indicating that at pH 2.0, the presence of PG vesicles enhances the content of ordered secondary structures in both allergens.

Sin a 2 was very resistant to intestinal digestion, but after 2 h of incubation around 55% of the protein was degraded. In the presence of PG vesicles, Sin a 2 remained undigested after 2 h of treatment (Fig. 3A). The IgE reactivity of Sin a 2 was significantly reduced after 5 min of digestion and almost completely abolished after 2 h, but in the presence of PG vesicles Sin a 2 retained the IgE reactivity (Fig. 3A). Intestinal digestion of Ara h 1 resulted in the rapid appearance of different proteolytic fragments. Neither peanut lipids nor PC or PG vesicles modified this process (Fig. 3B). The resulting fragments of around 39 and 20–30 kDa retained IgE-binding capability, which was abolished after 2 h of digestion (Fig. 3B). The academic analysis of the TCS for trypsin and chymotrypsin in the 3D structure of these allergens revealed that the number of the TCS for trypsin in Ara h 1 ($n = 73$) was significantly higher than in Sin a 2 ($n = 35$), which might justify the observed differences to intestinal digestion. Half of the TCS were located on the surface of both molecules (Fig. 3C,D). CD analysis at pH 7.0 demonstrated that the presence of PC or PG vesicles did not significantly alter the secondary structure content of Sin a 2 (Fig. S2). In contrast, PG but not PC vesicles slightly increased the percentage of random coil secondary structure in Ara h 1 at pH 7.0, suggesting that PG vesicles might favor the unfolding of Ara h 1 at this pH.

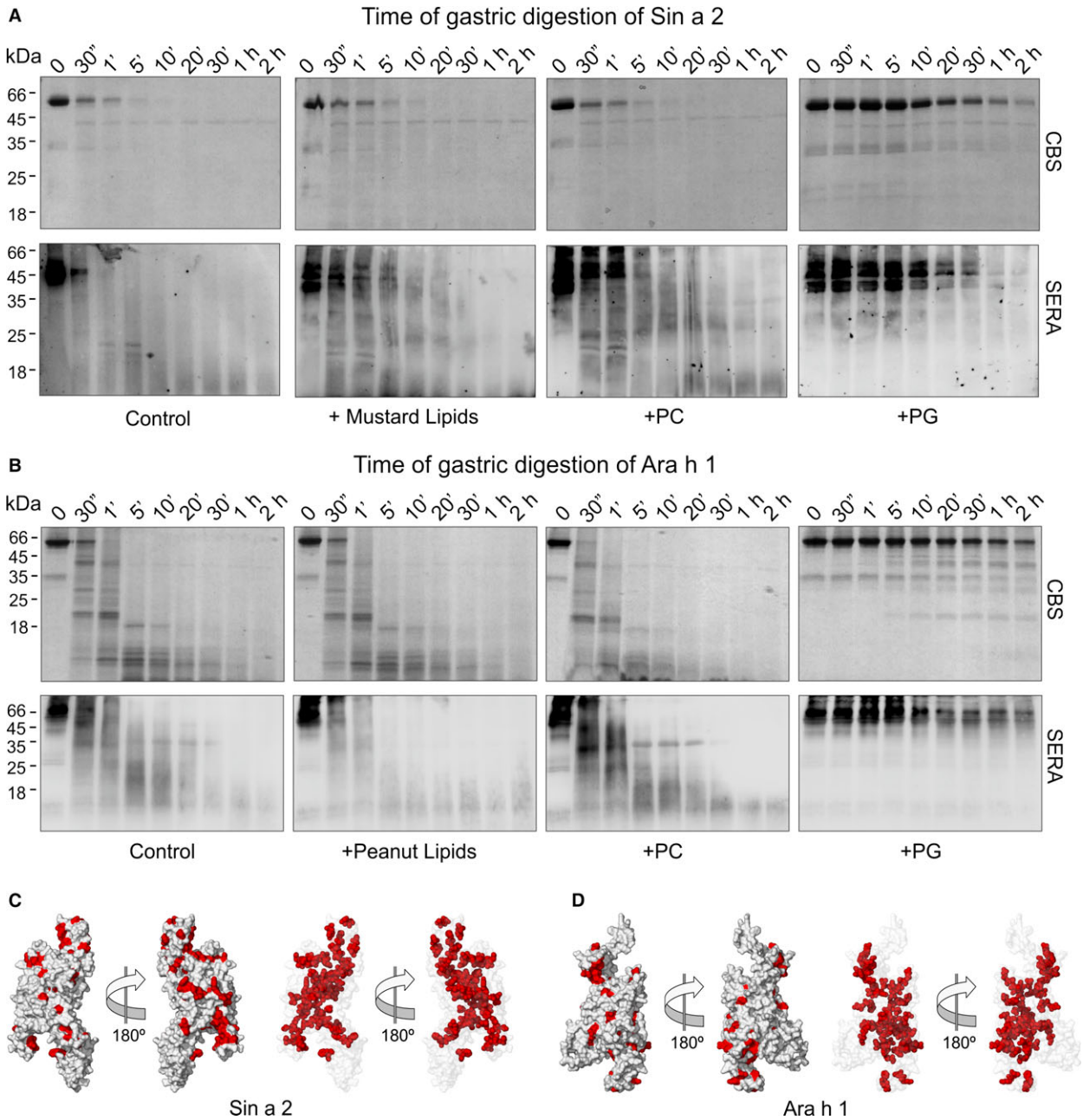

Figure 2 Simulated gastric digestion of purified Sin a 2 (A) and Ara h 1 (B). (A–B) Coomassie blue staining (CBS) and Western blot of the digestion products with a pool of sera from patients sensitized to Sin a 2 or Ara h 1 in the absence of lipids (control) or presence of mustard or peanut lipids, respectively, and PC or PG vesicles. Molecular mass markers are indicated in kDa. (C–D) Theoretical cleavage sites (TCS) for pepsin at pH < 2 in the 3D structure of Sin a 2 (modeled based on the 11S globulin from *B. napus*, PDB accession number 3KGL) and Ara h 1 (PDB accession number 3S7I) are shown in red: on the surface (left) or in the inner parts of the molecule (right).

Lipid components modify the uptake of food allergens by human dendritic cells

Human monocyte-derived DCs (hmoDCs) from healthy donors were cultured with different fluorescence-labeled plant-derived food allergens, and the capture was analyzed by flow cytometry and confocal microscopy (Fig. 4A,B). Sin a 2 and Ara h 1 were efficiently captured by around 80–90% of hmoDCs after 1 h of incubation (Fig. 4A). Sin a 1 (2S albumin) and Sin a 3 (LTP), two well-defined allergens from yellow mustard seeds able to induce primary sensitization and trigger systemic reactions, were also efficiently captured by hmoDCs (Fig. 4A). These data were confirmed by confocal microscopy (Fig. 4B). Next, hmoDCs were cultured with

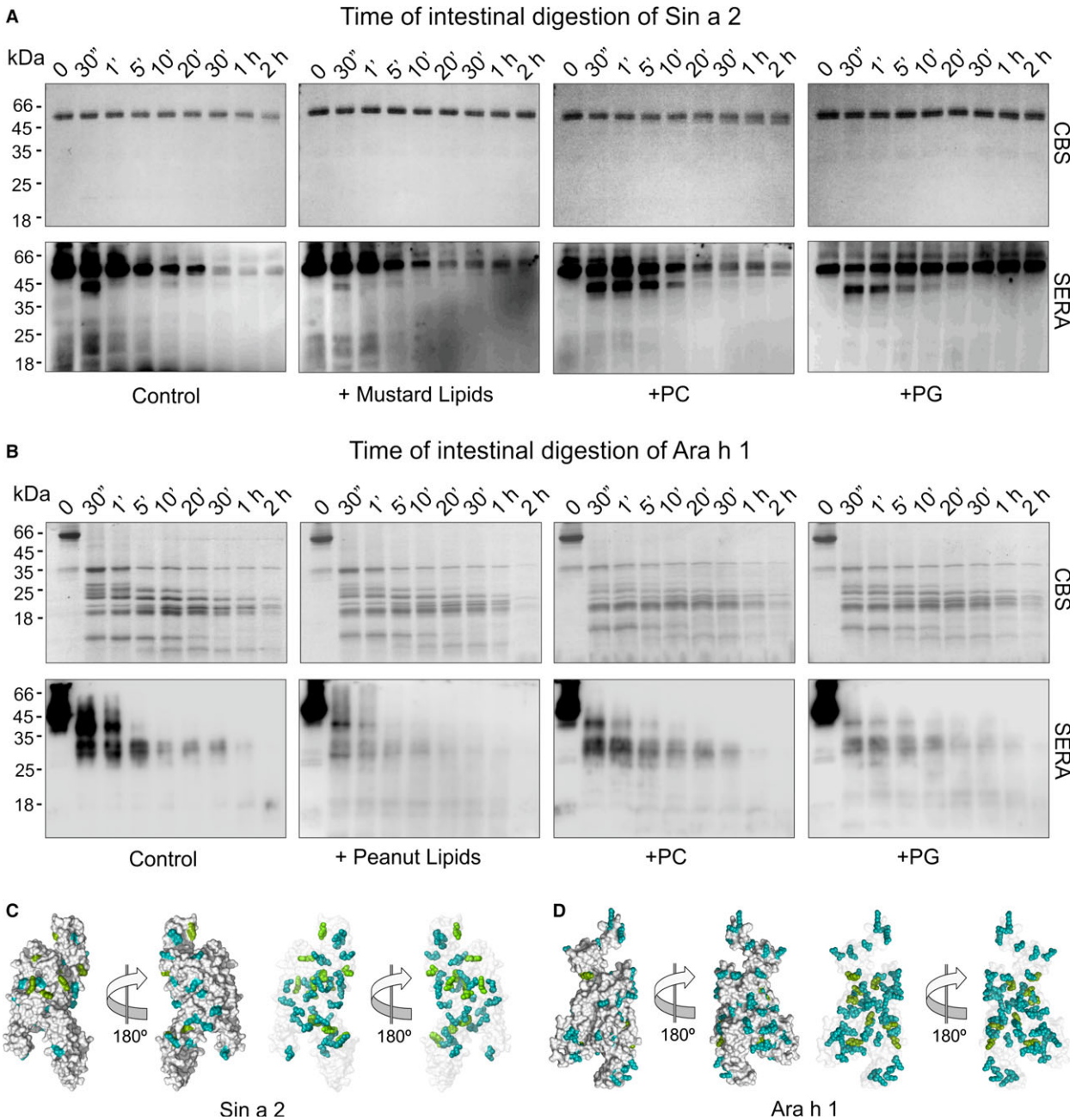

Figure 3 Simulated intestinal digestion of purified Sin a 2 (A) and Ara h 1 (B). (A–B) CBS and Western blot with a pool of sera from patients sensitized to Sin a 2 or Ara h 1 of the digestion products in the absence of lipids (control) or presence of mustard or peanut lipids, or PC or PG vesicles. Molecular mass markers are indicated in kDa. (C–D) TCS for trypsin and chymotrypsin are shown in blue and green, respectively, in the 3D structure of Sin a 2 and Ara h 1 (as described in Fig. 2): on the surface (left) or in the inner parts of the molecule (right).

the different food allergens in the presence of mustard or peanut lipids, and PC or PG vesicles. The presence of mustard lipids or PG but not PC vesicles impaired the capacity of hmoDCs to capture Sin a 2 after 1 h of incubation (Fig. 4C). Although none of the lipid components altered the capture of Sin a 3, the presence of PG vesicles for Ara h 1, or mustard lipids or PG vesicles for Sin a 1 slightly reduced the capture of these allergens after 1 h of incubation (Fig. 4C). We tested whether longer incubation times in the presence of lipids contained in the food matrix would affect allergen uptake. The presence of mustard or peanut lipids significantly reduced the capture of Sin a 2 and Ara h 1, respectively, by hmoDCs after 24, 48, and 72 h of incubation (Fig. 4D). As shown in this figure, mustard lipids signifi-

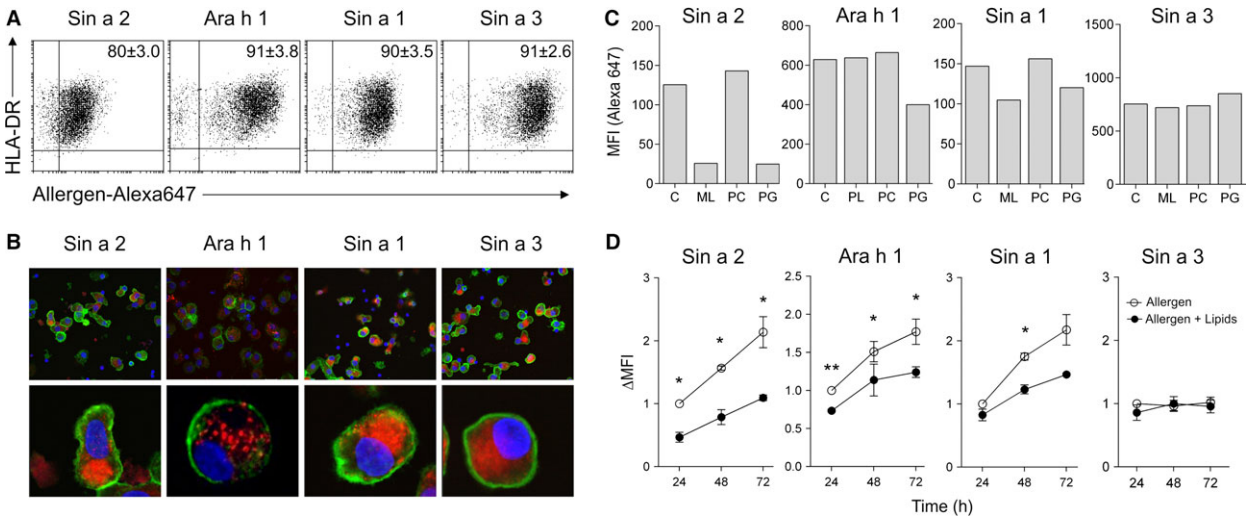

Figure 4 Capture of allergens by hmoDCs. (A) Representative dot plots for the capture of the indicated fluorescence-labeled allergens by hmoDCs after 1 h of incubation. The percentage of HLA-DR$^+$/allergen-Alexa 647$^+$ cells is indicated (mean ± SEM of 4 independent experiments). (B) Confocal microscopy images of hmoDCs after 20 h of incubation with the indicated allergens: HLA-DR (green), nucleus with DAPI (blue), and Alexa 647-labeled allergens (red). (C) Mean fluorescence intensity (MFI) of Alexa 647-labeled allergens captured by HLA-DR$^+$ hmoDCs after 1 h of incubation with the indicated allergens alone or in the presence of mustard lipids (ML) or peanut lipids (PL), and PC or PG vesicles. One of three representative experiments with the same results is displayed. (D) Increment of MFI (ΔMFI) after 24, 48, and 72 h of incubation of hmoDCs with the indicated allergens alone (open circles) or in the presence of mustard lipids (ML) or peanut lipids (PL) (closed circles). ΔMFI was calculated as the ratio between the MFI values of each condition respect to the MFI value at 24 h in the absence of lipids for each allergen. The data show the mean ± SEM of four independent experiments. Paired *t*-test, $*P < 0.05$; $**P < 0.01$.

cantly reduced the capture of Sin a 1 after 48 h without affecting Sin a 3 uptake at any of the assayed times.

Lipid components protect food allergens from endolysosomal degradation

The allergens captured by DCs are processed in endolysosomal compartments to generate MHC-II–peptide complexes that are presented to T cells. Antigens that are rather stable to endolysosomal proteolysis ensure efficient presentation to T cells and high immunogenicity (25). To assess the potential effect of lipids in the resistance to endolysosomal degradation, the purified food allergens were incubated alone or in the presence of mustard or peanut lipids with microsomal enzymes under reducing conditions. After 48 h of digestion, around 40% of Sin a 2 was degraded, but in the presence of mustard lipids around 90% of the allergen remained undigested as determined by scanning densitometry of the subunits linked by a disulfide bond (36 and 23 kDa in the SDS-PAGE) that constitute the allergen (Fig. 5). The endolysosomal digestion of Ara h 1 rendered a main proteolytic fragment of around 55 kDa after 48 h of digestion in both the absence and the presence of peanut lipids. The presence of peanut lipids slightly enhanced the resistance of this Ara h 1-derived fragment (50% *vs* 65% after 48 h digestion). Mustard lipids did not significantly modify the high stability of Sin a 1 to endolysosomal degradation. In contrast, the stability of Sin a 3 was also enhanced and around 48% of the allergen remained undigested in the presence of mustard lipids compared with around 20% of Sin a 3 alone. The high susceptibility of peroxidase to microsomal degradation was not modified by mustard or peanut lipids (Fig. 5).

Lipid components condition the capacity of human dendritic cells to produce cytokines and inhibit TLR2-L-induced NF-kB/AP-1 activation in THP1 cells

We assessed the capacity of hmoDCs to mount cytokine responses against allergens in the presence of lipid components. The IL-4/IL-10 ratio was significantly higher when hmoDCs were stimulated with Sin a 2 in the presence of mustard lipids than with Sin a 2 alone, which was also accompanied by a significant increment in the production of IL-1β (Fig. 6A). As shown in this figure, the same profile for IL-4/IL-10 ratio was observed for the mustard allergens Sin a 1 and Sin a 3 but without significant enhanced production of IL-1β. Although the IL-4/IL-10 ratio was not significantly altered when hmoDCs were stimulated with Ara h 1 in the presence of peanut lipids compared with Ara h 1 alone, the levels of IL-1β were significantly higher in the presence of peanut lipids (Fig. 6A). Mustard or peanut lipids in combination with Sin a 2 or Ara h 1, respectively, increased the mRNA expression of IL-1β compared with the allergens alone without modifying other inflammasome-associated genes such as caspase 1, ASC, or NLRP3 (Fig. 6B). Western blot experiments showed that the combination of the allergens and lipids also slightly increased the activation of caspase 1 compared with the allergens alone (Fig. 6C).

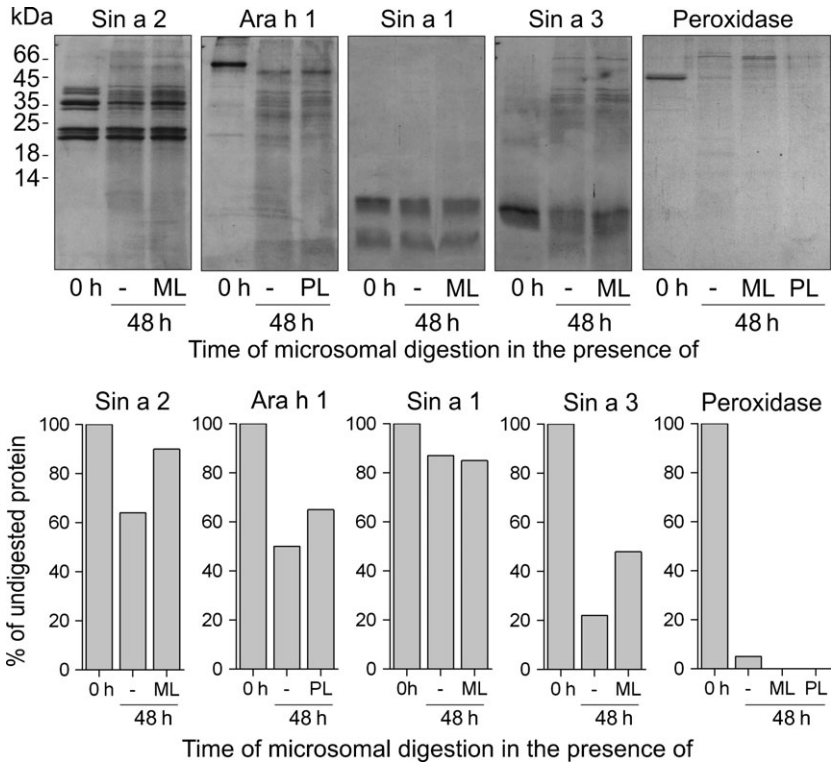

Figure 5 Endolysosomal degradation of allergens. SDS-PAGE and Coomassie blue staining of the digestion products after 0 (control without enzymes) and 48 h of endolysosomal degradation of Sin a 2, Ara h 1, Sin a 1, Sin a 3, and peroxidase in the absence (−) or presence of mustard lipids (ML) or peanut lipids (PL). The percentage of degradation as determined by scanning densitometry for each condition is also displayed.

We studied the capacity of the purified allergens, lipid-free mustard or peanut protein extracts, and different lipid components to induce NF-κB/AP-1 activation in THP1 cells as a suitable commercially available reporter cell line. None of the assayed components directly induced NF-kB/AP-1 activation in THP1 cells (Fig. S3A,B). In the same way, the combination of the purified allergens with the different lipids did not induce NF-kB/AP-1 activation (Fig. S3C). Next, we analyzed the capacity of purified allergens, protein extracts, or lipids to interfere with TLR2-L-induced NF-kB/AP-1 activation in THP1 cells. As shown in Fig. 6D, Sin a 2, Ara h 1, Sin a 1, or Sin a 3 did not modify TLR2-L-induced NF-kB/AP-1 activation. In contrast, mustard and peanut protein extracts inhibited TLR2-L-induced NF-kB/AP-1 activation in THP1 cells in a dose-dependent manner. Interestingly, mustard lipids and PG vesicles but not peanut lipids or PC vesicles significantly inhibited TLR2-L-induced NF-kB/AP-1 activation in THP1 cells in a dose-dependent manner (Fig. 6D). Cell viability was not modified in any case (Fig. S4).

Discussion

We demonstrate for the first time that Sin a 2 (11S globulin) and Ara h 1 (7S globulin), two clinically relevant allergens from yellow mustard seeds and peanut, respectively, are able to interact with phospholipid acid vesicles. The capacity of Sin a 2 and Ara h 1 to interact with membranes confers protection to gastrointestinal degradation, which might well allow them to cross the gastrointestinal tract in an immunological active form. Mustard and peanut lipids significantly reduced the capacity of human DCs to capture Sin a 2 and Ara h 1 and increased the resistance of Sin a 2- or Ara h 1-derived proteolytic fragment to endolysosomal degradation. The IL-4/IL-10 ratio was significantly higher when human DCs were simultaneously stimulated with mustard allergens and lipids than allergens alone, which was also accompanied by significant higher levels of IL-1β for Sin a 2. Stimulation of human DCs with peanut lipids and Ara h 1 did not alter the IL-4/IL-10 ratio compared with Ara h 1 alone, but it significantly increased the production of IL-1β. In conclusion, our results indicate that the capacity of Sin a 2 and Ara h 1 to interact with lipids is a relevant feature of allergens belonging to the cupin superfamily that might well contribute to their potent allergenicity.

The allergic sensitization depends not only on the allergens but also on the presence of other biological components contained in the allergenic sources that are concomitantly ingested or inhaled (19, 20). In the case of food allergy, different studies showed that lipid components are of paramount importance to induce allergic sensitization (20). For example, the major allergen from Brazil nut, Ber e 1 (2S albumin), required the lipid fraction obtained from the seeds

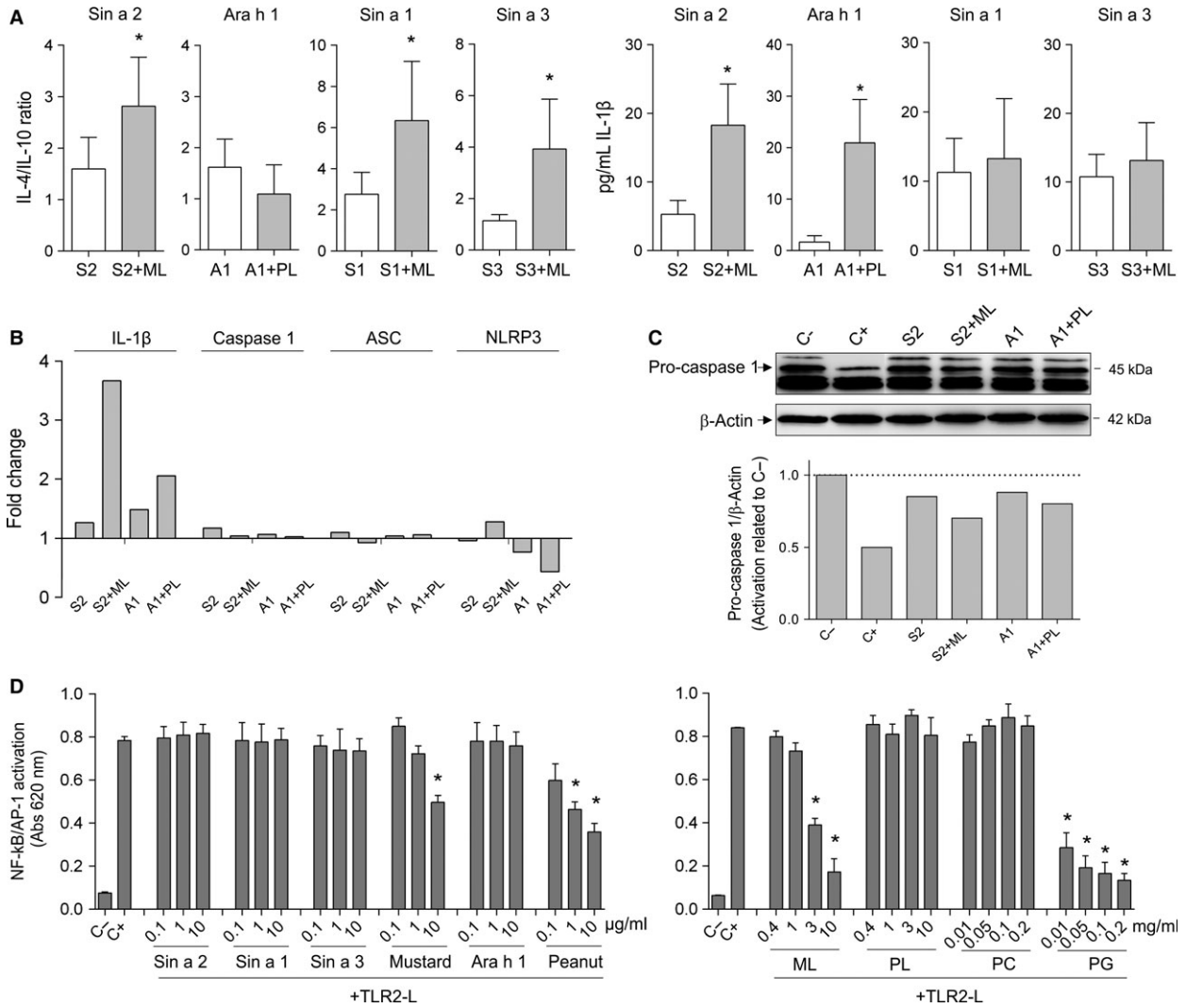

Figure 6 Capacity of purified allergens and lipid components to stimulate the production of cytokines by hmoDCs and modulate NF-κB/AP-1 activation in THP1 cells. (A) IL-4/IL-10 ratio and IL-1β production by hmoDCs treated with purified Sin a 2, Ara h 1, Sin a 1, or Sin a 3 in the absence or presence of mustard lipids (ML) or peanut lipids (PL). The data are the mean ± SEM of five independent experiments. Wilcoxon test, $*P < 0.05$. (B) Analysis of IL-1β, caspase 1, APC, and NLRP3 mRNA expression in hmoDCs stimulated with purified Sin a 2 or Ara h 1 in the absence or presence of ML or PL. One of three representative experiments with same results is displayed. (C) Western blot for pro-caspase 1 and quantitative analysis of pro-caspase 1 processing respect to β-actin and relative to C- in hmoDCs stimulated with Sin a 2 or Ara h 1 in the presence or absence of ML or PL for 6 h. C-, cells alone; C+, cell stimulated with aluminum hydroxide 100 μg/ml plus LPS 100 ng/ml. (D) Modulation of TLR2-L-induced NF-κB/AP-1 activation in THP1 cells by purified allergens, peanut or mustard protein extract, ML, PL, and PC or PG vesicles. C−, cells alone; C+, TLR2-L-induced NF-κB/AP-1 activation. The data show the mean ± SEM of four independent experiments. Wilcoxon test, $*P < 0.05$.

for eliciting IgE responses (26). Similarly, peanut lipids were shown necessary to induce potent allergic sensitization (27). Lipid-binding capacity has been reported for food allergens belonging to different protein families such as Bet v 1-like proteins (28), LTPs (29), 2S albumins (26, 30, 31), lipocalins (32), or oleosins (33). The potential lipid interaction capacity of clinically relevant allergens belonging to the cupin superfamily, such as the 11S globulin Sin a 2 from yellow mustard seeds or the 7S globulin Ara h 1 from peanut, was unknown prior to this study. Herein, we showed that Sin a 2 and Ara h 1 are able to interact with PG but not PC vesicles in a pH-dependent manner, indicating that both allergens are able to physically interact with biological membranes.

A large number of dietary proteins come in contact with the gastrointestinal tract daily, but only a few of them reach the gut immune system in an immunological active form able to induce allergic sensitization (22). Resistance to gastrointestinal digestion has been proposed as a useful tool for evaluating potential food allergenicity, but it cannot be considered as definitive criterion to define a given protein as

a food allergen (34). Mustard allergens Sin a 1 and Sin a 3 are considered as genuine food allergens able to induce primary sensitization and trigger systemic reactions (18), but their capacity to interact with human DCs has not been previously studied. Herein, we showed that Sin a 2 is highly susceptible to gastric digestion but resistant to intestinal degradation as shown for its homologous Act d 12 from kiwi seeds (24, 35). Ara h 1 was rapidly degraded by gastric and intestinal enzymes generating fragments with IgE-binding capacity as previously reported (36, 37). Interestingly, the presence of PG acid vesicles protected both allergens from gastrointestinal digestion, suggesting that their capacity to interact with phospholipid vesicles may enhance their resistance to degradation. Although we employed PC and PG from chicken egg as a suitable model to generate vesicles (38), similar lipid components are contained in allergenic sources from plant origin such as mustard and peanut (30). Ingestion of egg or other lipid sources at the same time as eating mustard or peanut might well also lead to the reported effects.

DCs play a key role in the sensitization phase of allergic diseases; however, how the interaction of food allergens with lipid components could condition the capacity of DCs to promote allergic sensitization and the underlying molecular mechanism are not yet completely understood (20). We questioned whether the capacity of food allergens to interact with lipid components could influence the ability of human DCs to uptake them. All the tested food allergens were efficiently captured by human DCs, and the presence of mustard or peanut lipids significantly reduced the uptake of Sin a 2 or Sin a 1 and Ara h 1, respectively. The capture of LTP Sin a 3 was not affected by the presence of mustard lipids, indicating that only the uptake of those allergens able to interact with membranes (2S albumin, 7S or 11S globulins) was reduced in the presence of lipids. The effective dose of allergen captured by DCs is essential in the generation of tolerance (high doses) or allergic sensitization (low doses) (22, 39, 40). Our data support that the capacity of lipids to interact with food allergens could reduce the doses of allergen captured by DC, thus favoring the generation of allergic Th2 responses. Sin a 2- and Ara h 1-derived proteolytic fragment displayed moderate resistance to endolysosomal degradation, but the presence of mustard or peanut lipids, respectively, increased their stability. Although Sin a 1 showed a high intrinsic resistance to microsomal degradation, the presence of mustard lipids also increased the resistance of Sin a 3. Antigen stability to lysosomal proteolysis leads to low-density MHC-II–peptide complexes on DC surface, which favors Th2 responses in contrast to high-density expression (40, 41).

Different phospholipids can interact with immune cells and modulate immune responses (20). Our data showed that the presence of mustard lipids in combination with allergens significantly increased the IL-4/IL-10 ratio produced by DCs, suggesting that lipids might influence the capacity of human DCs to promote allergic Th2 response. For Sin a 2, mustard lipids also increased the production of IL-1β, a proinflammatory cytokine able to break tolerance to allergens (23). Although peanut lipids did not modify the IL-4/IL-10 ratio induced by Ara h 1 alone, they also enhanced IL-1β production by human DCs. Acidic phospholipids have been previously shown as antagonist of LPS-induced inflammation by interacting with CD14/MD-2 components (42). Phospholipids from helminthes or bacteria are also able to generate Th2 responses by interfering with TLR4 signaling pathways (43). Recently, it has been shown that the probiotic *Lactobacillus rhamnosus* is able to promote tolerance by generating IL-10-producing Treg cells through mechanisms involving TLR2 and DC-SIGN (44). We used the human THP-1 XBlue reporter cell line to assess the capacity of allergens and lipids to induce NF-κB/AP-1 activation or to cross talk with TLR signaling pathways as a suitable commercially available system. We showed that whole mustard and peanut protein extracts, mustard lipids, and acidic PG vesicles inhibited TLR2-L-induced NF-κB/AP-1 activation in THP1 cells, suggesting that the capacity of lipids interacting with food allergens to cross talk with TLR-mediated signaling pathways might well contribute to break tolerance to allergens. The increment of IL-1β induced by Sin a 2 and Ara h 1 in the presence of mustard and peanut lipids, respectively, was accompanied by a slight increase in IL-1β mRNA expression and activation of caspase 1, suggesting inflammasome induction. Whether the capacity of these two allergens to interact lipids might lead to lysosome disruption and subsequent release of inflammatory components as it has been previously described by other adjuvants such as aluminum hydroxide needs to be further investigated (45).

In summary, our results demonstrated for the first time that Sin a 2 and Ara h 1 bind acidic phospholipid vesicles and interact with lipid components, which might well contribute to explain the potent allergenic capacity of these two clinically relevant allergens of the cupin superfamily.

Acknowledgments

The authors' laboratories are supported by the Spanish Ministerio de Economía y Competitividad (MINECO) SAF2011-26716 to MV and SAF2014-52706-R to OP. CP is supported by the Austrian Science Fund (FWF) Doctoral Program MCCA W1248-B13. AA is supported by SAF2014-52706-R. OP is a Ramon y Cajal Scholar funded by MINECO and the European Social Fund.

Author contributions

OP conceived and designed the experiments. AA and SS performed the experiments. AV and JCH performed clinical characterization of the patients. OP, AA, and SS analyzed and discussed the data. CP, HB, TE, RR, MV, and OP contributed reagents/materials/analysis tools. OP, AA, and SS wrote the manuscript. All the authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest in relation to this manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. CD spectra of Sin a 2 (left) and Ara h 1 (right) in the far-UV at pH 2.0 in the absence or presence of PC or PG vesicles.

Figure S2. CD spectra of Sin a 2 (left) and Ara h 1 (right) in the far-UV at pH 7.0 in the absence or presence of PC or PG vesicles.

Figure S3. Capacity of purified allergens, lipids and combinations to activate NF-κB/AP-1 in THP1 cells.

Figure S4. Viability of THP1 cells stimulated with TLR2-L in combination with purified allergens, peanut or mustard protein extracts, mustard lipids (ML) or peanut lipids (PL), PC or PG vesicles.

Appendix S1. Methods.

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