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EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells

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Abstract

Background: Lipids are required for mice sensitization to Ber e 1, Brazil nut major allergen. Here, we characterized different lipid fractions extracted from Brazil nuts and the lipid-binding ability of Ber e 1. Further, we determined their *in vivo* ability to induce Ber-specific anaphylactic antibodies and the role of invariant natural killer T (iNKT) cells in this process.

Methods: Wild-type (WT) and iNKT cell-deficient mice were sensitized with Ber e 1 and specific lipid fractions, and anaphylactic antibodies were measured by enzyme-linked immunosorbent assay (ELISA) and passive cutaneous anaphylaxis (PCA). The lipid-binding characteristic of Ber e 1 (Ber) was established by using fluorescent probes and ¹⁵N-labeled NMR. *In vitro* production of IL-4 was determined in Ber/lipid C-stimulated mouse iNKT cells and human T-cell lines containing NKTs primed with CD1d+C1R transfectants by flow cytometry and ELISA, respectively.

Results: Only one specific lipid fraction (lipid C), containing neutral and common phospholipids, induced Ber anaphylactic antibodies in mice. Ber e 1 has a lipid-binding site, and our results indicated an interaction between Ber e 1 and lipid C. iNKT-deficient mice produced lower levels of anaphylactic antibodies than WT mice. *In vitro*, Ber/lipid C-stimulated murine iNKT cells produced IL-4 but not IFN-gamma. Human T-cell lines derived from nut-allergic patients produced IL-4 to Ber/lipid C in a CD1d- and dose-dependent manner.

Conclusion: Lipid fraction C from Brazil nut presents an essential adjuvant activity to Ber e 1 sensitization, and iNKT cells play a critical role in the development of Brazil nut-allergic response.

Food allergy is on the rise worldwide, and it is estimated that 6% of young children and 4% of adults are affected in Western societies (1). Despite the heterogeneous protein intake in normal diets, only few protein families are commonly implicated as food allergens (2). Nuts are among the commonest food allergens, and allergy to Brazil nut is now well documented (3). The protein Ber e 1, a seed storage 2S albumin, was identified as the major allergen in Brazil nuts (4).

The mechanisms that lead to the development of food allergy (sensitization) are still elusive; however, once established, the cascade of events leading to elicitation of symptoms is well documented [for review, (5)]. The range of signs

and symptoms observed in food-allergic patients include urticaria, abdominal discomfort, nausea, diarrhea, and, in some cases, anaphylactic shock (6).

Ber e 1 possesses a compact, alpha-helical, disulfide-bridged rigid structure that may confer on it resistance to gastro-intestinal conditions (7, 8). However, 2S albumin proteins with similar biophysical characteristics present in highly consumed foods, such as sunflower seeds, are not frequently associated with allergy. In addition to the properties of allergenic proteins, extrinsic factors present in the food matrix may also participate in the development of an allergic response (9). Previously we have shown that sensitization of

mice with recombinant Ber e 1 alone did not induce the production of anaphylactic antibodies, which, however, were produced when Brazil nut lipids were added to the purified protein (10). This characteristic is not particular to Ber e 1, and the food matrix of peanuts was also necessary to the immunostimulatory activity of purified peanut allergens (11).

Lipid binding is a common characteristic of major allergens (12). Lipid molecules have also been implicated in allergic disorders (13), and pollen-allergic patients, but not nonallergic subjects, displayed specific IgE and cutaneous reactions to phospholipids present in pollen. There is evidence that the recognition of pollen lipids as antigens by human T cells involves CD1 (13), a MHC class-I-like molecule. CD1 binds distinct lipid-based antigens, which are recognized by invariant natural killer T (iNKT) cells (14). Specifically in allergy, it was shown that iNKT cells participate in Th2 responses through a CD1d-dependent mechanism. OVA-immunized *Jalpha18^{-/-}* mice, deficient in the invariant Valpha14(+) and CD1d-restricted NKT cells, exhibited a reduced OVA-specific IgE, airway hyper-reactivity, and inflammation compared with wild-type (WT) mice (15).

In the present study, we confirmed that Ber e 1, the major allergen of Brazil nut, requires lipid association to trigger allergic Th2 responses in mice. One specific active lipid fraction (lipid C) was identified, and its ligation to Ber e 1 was carefully characterized. In addition, we showed that the Th2-type responses induced by Ber e 1 and lipid C were enhanced by iNKT cells on an *in vivo* model. Finally, we showed that Ber/lipid C-activated NKT cells from mice and nut-allergic patients produced IL-4, a key Th2 cytokine.

Material and methods

Proteins

Uniformly, $^{13}\text{C}/^{15}\text{N}$ -labeled, ^{15}N -labeled, and nonlabeled recombinant Ber e 1 were expressed as secreted proteins by the methylotrophic yeast *Pichia pastoris* and purified by FPLC using a heparin–Sepharose column as previously described (7).

Lipid fractionation and analyses

Total lipid fraction (fraction T) was extracted from Brazil nut seeds (70 g) with chloroform–methanol (2 : 1, 150 ml) and then with chloroform alone (100 ml) and filtered through filter paper (n.1; Whatman, Maidstone, UK). Fraction T (250 ml) was added to a glass silica gel column (230–400 mesh; Fluka, Darmstadt, Germany; 3 × 20 cm), and the flow through fraction was named fraction L. Then, the following solvents were added, and the respective fractions were collected: chloroform/methanol (150 ml; fraction C) and methanol (500 ml; fraction M). Lipid samples were concentrated in a vacuum rota-evaporator and stored in chloroform–methanol (2 : 1) and BHT (0.01%, m/V) at -20°C protected from light.

Neutral lipid content of lipid fraction C was determined by HPLC essentially as described by Christie et al. (16); the

polar lipid content of lipid fraction C was determined by TLC (17) and confirmed by NMR as described by Petzold et al. (18); and the phosphorus concentration of lipid fraction C was determined by perchloric acid digestion followed by molybdenum blue method, as previously described (19).

ANS and bis-ANS fluorescence titrations

The ability of the fluorescent probes ANS (1-anilinonaphthalene-8-sulfonic acid; Sigma-Aldrich, St. Louis, MO, USA) and bis-ANS (4,4-dianilino-1,1-bisnaphthyl-5,5 disulfonic acid; Sigma-Aldrich) to bind recombinant Ber e 1 was measured as previously described (20). Fluorescent probes were dissolved in 20% methanol (V/V), and the concentration of the stock solution was determined spectrophotometrically using an extinction coefficient of 4990/M/cm at 350 nm for ANS and 16 790/M/cm at 383 nm for bis-ANS (21). All fluorescence measurements were carried out on Jasco/FP-6500 spectrofluorometer, using an excitation/emission wavelength of 383/498 nm for bis-ANS and 350/495 nm for ANS. A reversed binding experiment was performed in a quartz cuvette, using 12 μM ANS or bis-ANS in PBS buffer containing methanol (2%, V/V). Ber e 1 in PBS (0–50 μM) was titrated, and the fluorescence was measured at 25°C . The dissociation constant of Ber e 1–bis-ANS complex was estimated from a modified Hill equation: $F_{\text{obs}} = F_{\text{max}} [\text{Ber e 1}]^n / (K_d + [\text{Ber e 1}]^n)$. F_{obs} : observed fluorescence; F_{max} : maximal fluorescence when all bis-ANS is bound, [Ber e 1]: protein concentration; K_d : apparent dissociation constant; n : Hill coefficient. Fitting of the binding curves was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

NMR lipid titration

NMR titration experiments were conducted at a temperature of 303 K on Bruker/DRX-600 spectrometer equipped with a cryo triple-resonance ($^1\text{H}/^{13}\text{C}/^{15}\text{N}$) probe with Z-gradient capability. The ^{13}C and ^{15}N chemical shifts were referenced indirectly using the gyromagnetic ratio of $^{13}\text{C}/^1\text{H}$ and $^{15}\text{N}/^1\text{H}$ (22). All NMR spectra were processed in NMRPipe (23) and analyzed with CCPN analysis (24). Fraction C was titrated into a ^{15}N -labeled sample of Ber e 1. The spectral change was followed in a ^1H – ^{15}N -correlated heteronuclear single-quantum coherence (HSQC) spectrum, and the chemical shift changes for each residue were scaled as $0.15 \Delta\omega^{15}\text{N} + \Delta\omega^1\text{H}$. A control experiment was performed with fraction C solvent without lipids, and no spectral changes were observed.

Animals

Young adult (8–12 weeks old) female mice were used. BALB/c mice were obtained from Harlan-Seralab (Oxford, UK), Charles River (Margate, UK), or ICB-IV, USP (Brazil). *CD1d^{-/-}* and C57BL/6 WT mice were obtained from ICB-IV. *Jalpha18^{-/-}* and C57BL/6 WT mice were bred in CNRS UMR8147 facilities (Paris, France) or purchased from

Janvier (Le Genet St Isle, France). Food (containing no nuts) and water were available *ad libitum*. The ambient temperature was maintained at $21 \pm 2^\circ\text{C}$, and relative humidity was $55 \pm 10\%$ with a 12-h light/dark cycle. All experiments were carried out following the provision of each country/institution. UK: Home Office Licence, following UK Animals (Scientific Procedures) Act 1986; Brazil: in accordance with the Ethical Principles of the Brazilian College of Animal Experimentation; and France: guidelines of the French Institutional Committee.

Sensitization protocol

Animals received two intraperitoneal (i.p.) injections of recombinant Ber e 1 (2.5 mg) alone or in the presence of different lipid fractions (600 μg) (stoichiometry protein lipid 1 : 1), in 0.2 ml of PBS, on days 0 and 7. Animals were bled on day 14, by intracardiac route, after lethal anesthetic doses (pentobarbital, 200 mg/kg).

Enzyme-linked immunosorbent assay

Total mouse sera IgE was determined by sandwich ELISA and specific IgG1 or IgG2a by indirect ELISA, using commercial kits (Bethyl Laboratories®, Montgomery, TX, USA or BD Biosciences®, San Diego, CA, USA). For specific immunoglobulins, 384- or 96-well Maxisorp plates (Nunc®, Copenhagen, Denmark) were coated with Ber e 1, at 400 $\mu\text{g}/\text{ml}$. The absorbance was measured at 450 nm using FLUOstar Galaxy microplate reader (BMG Labtech, Ortenberg, Germany).

Passive cutaneous anaphylaxis

Pooled serum samples (25 μl), neat or diluted in PBS, were injected into the dermis of naïve BALB/c mice's ears. Forty-eight hours later, Ber e 1 (0.25 mg) and Evans blue dye (1.25 mg) in physiological saline solution (0.25 ml) were intravenously injected in the recipient mice. Animals were kept at 39°C and culled after 30 min with lethal doses of anesthesia (Pentobarbital, 200 mg/kg). Cutaneous reactions' diameters >3 mm were considered positive reactions. Results express PCA titers that represent the reciprocal of the highest dilution of serum that gave a lesion of >3 mm in diameter.

Activation and cytokine production by mouse iNKT cells

15×10^6 splenocytes from C57BL/6 WT mice were stimulated *in vitro* with Ber e 1 and lipid C (50 μg and 1.2 mg, respectively) or α -galactosylceramide (α -GalCer, 200 ng) in the presence of 2 μg of brefeldin A (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 4 h, final volume of 1 ml. Cells were stained with CD1d tetramer and anti-CD3 (BD Pharmingen, Le pont de Claix, France). For intracellular staining, cells were fixed in 1% PFA, washed, and permeabilized with 0.5% saponin (Sigma-Aldrich, Saint-Quentin Fallavier, France) and incubated with anti-IL-4 and anti-IFN γ or isotype controls

(eBioscience, Paris, France). Cells were analyzed in a FACSCanto II (Becton Dickinson, Le pont de Claix, France) using FlowJo software.

Human samples

Food-allergic subjects were selected based on their symptoms (abdominal discomfort and diarrhea, urticaria, and/or angioedema related to food ingestion), positive skin prick tests with one or more of the selected food extracts (walnuts, Brazil nuts, peanuts), and nut-specific IgE above 1.75 IU/dl (ImmunoCap, Pharmacia, Sweden). Informed consent approved by the local Ethical Committee was previously obtained from each participant. PBMCs from normal subjects (NC, $n = 5$) and from patients with food allergy (FA, $n = 4$) were incubated with autologous dendritic cells previously induced by incubation with rhGM-CSF and rhIL-4 for 4 days, as previously described (13).

Generation of Brazil nut-specific T-cell lines

Bulk cultures were exposed to Ber e 1 (10 $\mu\text{g}/\text{ml}$), Ber e 1 and lipid C (10 $\mu\text{g}/\text{ml}$ and 2.5 $\mu\text{g}/\text{ml}$, respectively), or vehicle re-stimulated every 2 weeks with CD1+ autologous dendritic cells plus previously added extracts. Proliferating lines were further expanded with recombinant human interleukin-2 (rhIL-2, 50–100 U/ml, EuroClone, Pero, Italy) and tested *in vitro* for antigen specificity, as previously described (13). Growing microcultures were expanded at weekly intervals with 10^5 irradiated feeder cells and rIL-2. Immunophenotype of emerging lines was detected by flow cytometry using monoclonal anti-CD4-Cy-chrome (clone/RPA-T4; Pharmingen, San Diego, CA, USA), anti-V β 11-PE (clone/21, Serotec, Oxford, UK), anti-CD161-FITC (NKR-P1A Ag, clone DX12; Pharmingen, San Diego, CA, USA), anti-CD8 (clone/LT8, Serotec), anti- $\alpha\beta$ (clone BMA031, Serotec), anti-V α 24 (clone C15, Serotec), anti-pan TCR $\gamma\delta$ (clone 5A6.E9; T Cell Diagnostics, Woburn, MA, USA) or -V γ 9 (clone 7A5; T Cell Diagnostics), -V δ 1, and -V δ 2 (clones TS-1 and 15D, all from T Cell Diagnostics).

Functional characterization of Brazil nut-specific T-cell lines

For evaluation of CD1 restriction, CD1 transfectants of cervical carcinoma cell line HeLa or C1R lymphoblastoid B-cell line expressing CD1a, CD1b, CD1c, or CD1d, and CD1-negative mock transfectants were employed. Irradiated (50 Gy) CD1 transfectants (10^5 per well) were seeded with T cells (2×10^4 per well) in a 96-well round-bottom plate (Costar) in 0.2 ml medium alone or containing either Ber e 1 or Ber e 1 and lipid C (from 0.1 to 10 $\mu\text{g}/\text{ml}$). Specific response and CD1 restrictions were evaluated, after 5 days of culture, by measurement of IL-4 production (AID-DiagnostikaGmbH, Strassberg, Germany) using ELISPOT commercial Kits. The antigens used for the assay were as follows: Ber e 1 alone (Ber), Ber e 1 and lipid C (Ber/lipid C), cypress-derived phosphatidylglycerol (PG), and α -GalCer (GalCer).

Results

Production of Th2-related antibodies is dependent on a specific Brazil nut lipid fraction

We have previously shown that Ber e 1, the major allergen from Brazil nuts, in its purified form is not able to induce significant antibody responses in an animal model of sensitization (10). In the present study, we assessed the effect of various lipid fractions extracted from Brazil nuts (fractions T, L, C, or M) in the sensitization to Ber e 1 in a near 1 : 1 stoichiometry. Figure 1 shows that Ber e 1-sensitized (Ber/PBS group) or naive mice presented similar levels of antibodies, confirming previous results (10). In contrast, the association of Ber e 1 and lipid C (Ber/lipid C group) promptly induced high levels of Ber-specific IgG1, total IgE, and anaphylactic antibodies in the serum of sensitized mice. The presence of

lipid fraction M induced increased levels of Ber-specific IgG2a, whereas the presence of fractions T and L did not induce significant immune response, showing no alteration in the titers of antibodies tested. The endotoxin content in the lipid C fraction was undetectable by LAL assay. It was further confirmed in an *in vitro* HEK-293 cell screening of TLR activation that lipid C did not activate TLR-2, 3, 4, 5, 7, 8, or 9, and in the *in vivo* model of sensitization with Ber/lipid C, C57BL/6 MyD88 KO animals produced similar titers of anaphylactic antibodies as WT animals (data not shown).

Altogether, these results corroborate our previous findings (10); Ber e 1 is not able to sensitize animals to produce protein-specific antibodies. Even at the small stoichiometric concentrations, a specific fraction of Brazil nut lipids (lipid C) was necessary to induce an *in vivo* Th2-type humoral response.

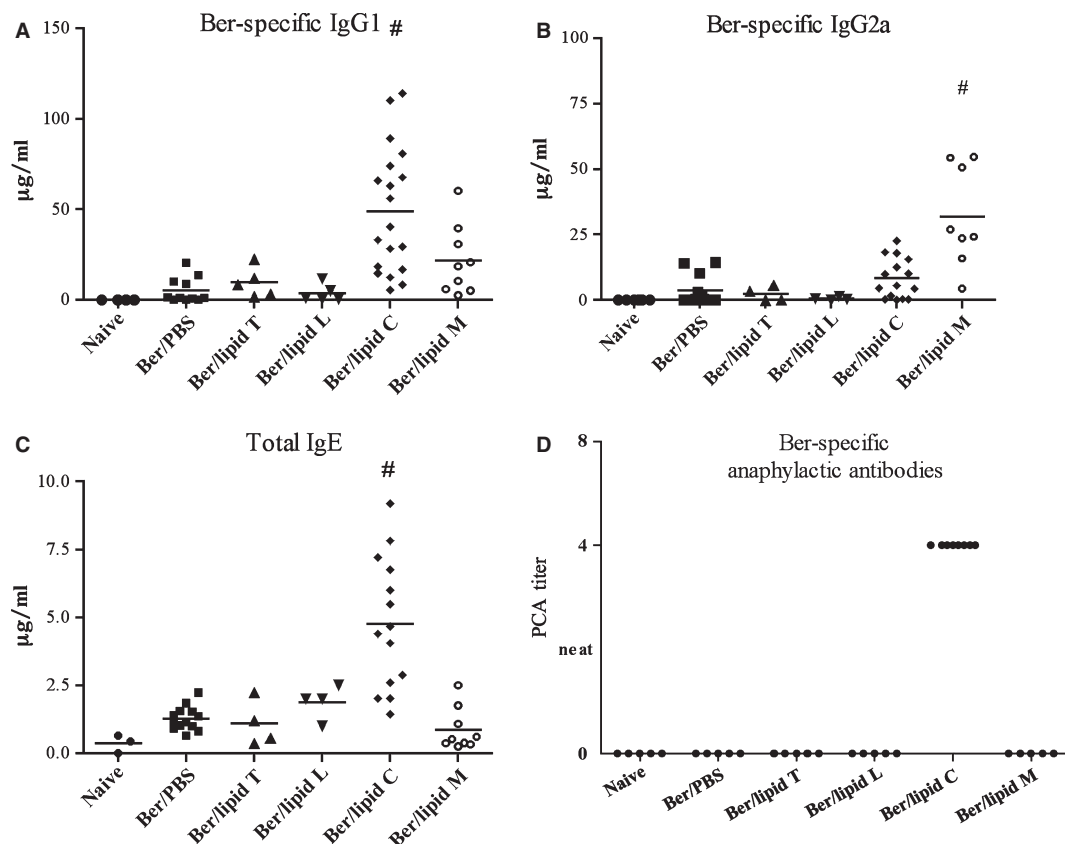


Figure 1 Antibody response induced by Ber e 1 and different Brazil nut lipid fractions. Antibodies in the serum of BALB/c mice sensitized with PBS (Naive), Ber e 1 alone (Ber/PBS), or in the presence of different lipid fractions (Ber/lipid T, Ber/lipid L, Ber/lipid C, and Ber/lipid M) on days 0 and 7, intraperitoneally. Antibody levels were measured on day 14. (A–C) Antibody concentration measured by enzyme-linked immunosorbent assay (ELISA); (D) Ber-specific anaphylactic antibodies measured by passive cutaneous anaphylaxis (PCA). PCA titers represent the reciprocal

of the highest dilution of serum that gave a lesion of >3 mm in diameter. Symbols represent individual animals. Statistical analysis: one-way ANOVA and Tukey's post test. #significant difference: (A) $P < 0.05$ (compared with Ber/lipid T), $P < 0.01$ (compared with naive, and Ber/lipid L), and $P < 0.001$ (compared with Ber/PBS); (B) $P < 0.001$ (compared with all groups); (C) $P < 0.05$ (compared with Ber/lipid L), $P < 0.001$ (compared with naive, Ber/PBS, Ber/lipid T, Ber/lipid M); (D) $P < 0.05$ (compared with all groups).

Ber e 1 has a hydrophobic binding site: interaction with lipid fraction C

The ability of Ber e 1 to bind hydrophobic molecules was investigated using ANS and bis-ANS. These molecular probes dramatically increase their fluorescence when transferred from a polar environment (water) to a more hydrophobic environment such the hydrophobic pockets in proteins (25). Bis-ANS, in particular, consists of two ANS molecules and has been shown to possess structural similarity to lipopolysaccharide (LPS) (26).

Ber e 1 is capable of interacting with both ANS and bis-ANS (Fig. 2A), but the ANS–Ber e 1 complex shows a Hill coefficient below 1, indicating unspecific binding. The bis-ANS–Ber e 1, however, shows a 1 : 1 binding stoichiometry with an apparent K_d of $5.6 \pm 0.1 \mu\text{M}$. The Hill coefficient for ANS is estimated to be 1.7, which suggests a slightly cooperative binding mechanism that involves a two-step binding event. These interactions provide two possible binding scenarios: (i) the Hill coefficient reflects the cooperativity between the hydrophobic interaction in one part of the bis-ANS molecule, and the other part makes electrostatic interactions; (ii) one of the two identical halves of the bis-ANS molecule makes the initial interaction, placing the other half of the molecule into position, thus yielding a stronger interaction.

The hydrophobic binding sites were further investigated using NMR spectroscopy. Lipid fraction C was titrated into a ^{15}N -labeled Ber e 1 sample, and the spectral changes for each residue were followed. The chemical shift changes upon addition of fraction C are visualized in Fig. 2B, C. As shown, many residues throughout the protein are affected, which is indicative of an interaction between Ber e 1 and fraction C components.

These results indicate that Ber e 1 possesses one hydrophobic site able to accommodate one bis-ANS or a lipid molecule of approximately the size, electrostatic and hydrophobic properties of an LPS molecule. This is also a strong indication that Ber e 1 interacts with lipid fraction C. We recently showed structural evidence that Ber e 1 possesses a hydrophobic pocket of the same size as the lipid-binding area in wheat ns-lipid transfer proteins (LTP). It is, therefore, most likely that Ber e 1 interacts with lipids in the same manner as ns-LTP's, which have lipid affinity similar to the affinity between Ber e 1 and bis-ANS (27).

Murine invariant natural killer T cells are critically involved in anaphylactic antibody production

In mice, invariant NKT cells, expressing V α 14J α 18 TCR rearrangement, recognize lipids presented by CD1d, a MHC class-I-like antigen-presenting molecule. When activated, iNKT cells rapidly produce large amounts of cytokines, such as IL-4 and IFN- γ , and are implicated in allergic responses (28). Because the lipid fraction C proved to be required for Th2-type antibody production, we investigated the participation of iNKT cells in our model. To evaluate cytokine production by iNKT cells, we stimulated spleen cells from naïve

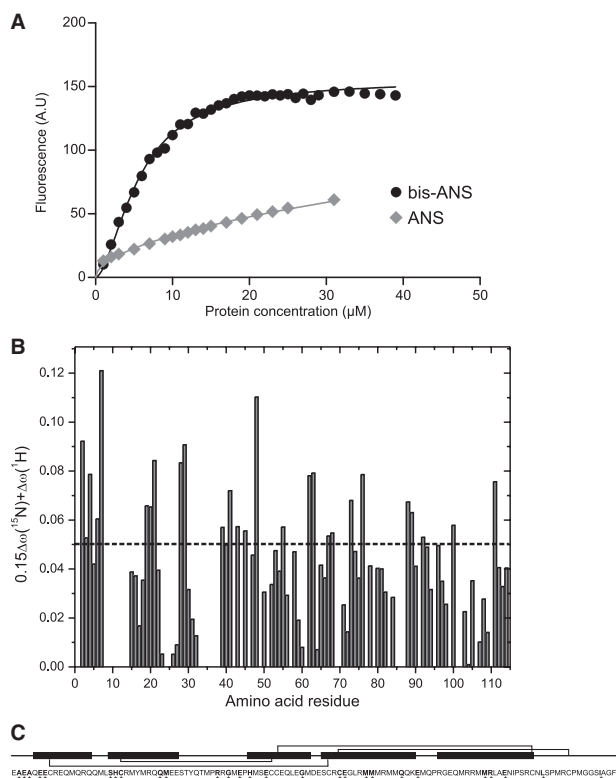


Figure 2 Lipid-binding properties of Ber e 1 (A) Ber e 1 was titrated into a reaction mixture containing $12 \mu\text{M}$ of bis-ANS or ANS, and the emission fluorescence were followed at 498 and 495 nm, respectively. The bis-ANS binding shows a specific interaction with Ber e 1 and apparent K_d of $5.6 \pm 0.1 \mu\text{M}$. ANS shows unspecific interaction with Ber e 1 as seen by the lack of a plateau; (B) HN, ^{15}N NMR chemical shift perturbations upon addition of lipid fraction C. A threshold value of 0.05 was used to identify residues that show significant chemical shift perturbation upon addition of lipid fraction C; and (C) distribution of significant accumulated chemical shift perturbations mapped on the primary structure of Ber e 1, along with a schematic representation of the secondary structure. Boxes correspond to helices, lines are random coil regions, and the pattern of disulfide bridges are connected with lines. (*) indicates that the chemical environment around the amide group in the protein backbone for those residues has changed. Black boxes indicate alpha-helical regions of the protein. ANS, 1-anilinonaphthalene-8-sulfonic acid.

C57BL/6 mice with Ber e 1 and lipid fraction C (Ber/lipid C) for 4 h followed by intracellular staining. For positive control, we used αGalCer , a derivative of a marine sponge, which specifically binds CD1d and strongly stimulates NKT cells to secrete large amounts of cytokines including IL-4 and IFN- γ . Figure 3A indicates that splenic iNKT cells produced IL-4 in response to *in vitro* Ber/lipid C stimulation. IL-4 production was low but significant ($2.7 \pm 0.2\%$ vs $1.6 \pm 0.3\%$ of IL-4+ cells among gated iNKT lymphocytes stimulated with Ber/lipid C or PBS, respectively; $P < 0.05$). Neither IFN- γ (Fig. 3A) nor IL-17 (data not shown) production were observed. As expected, αGalCer induced large

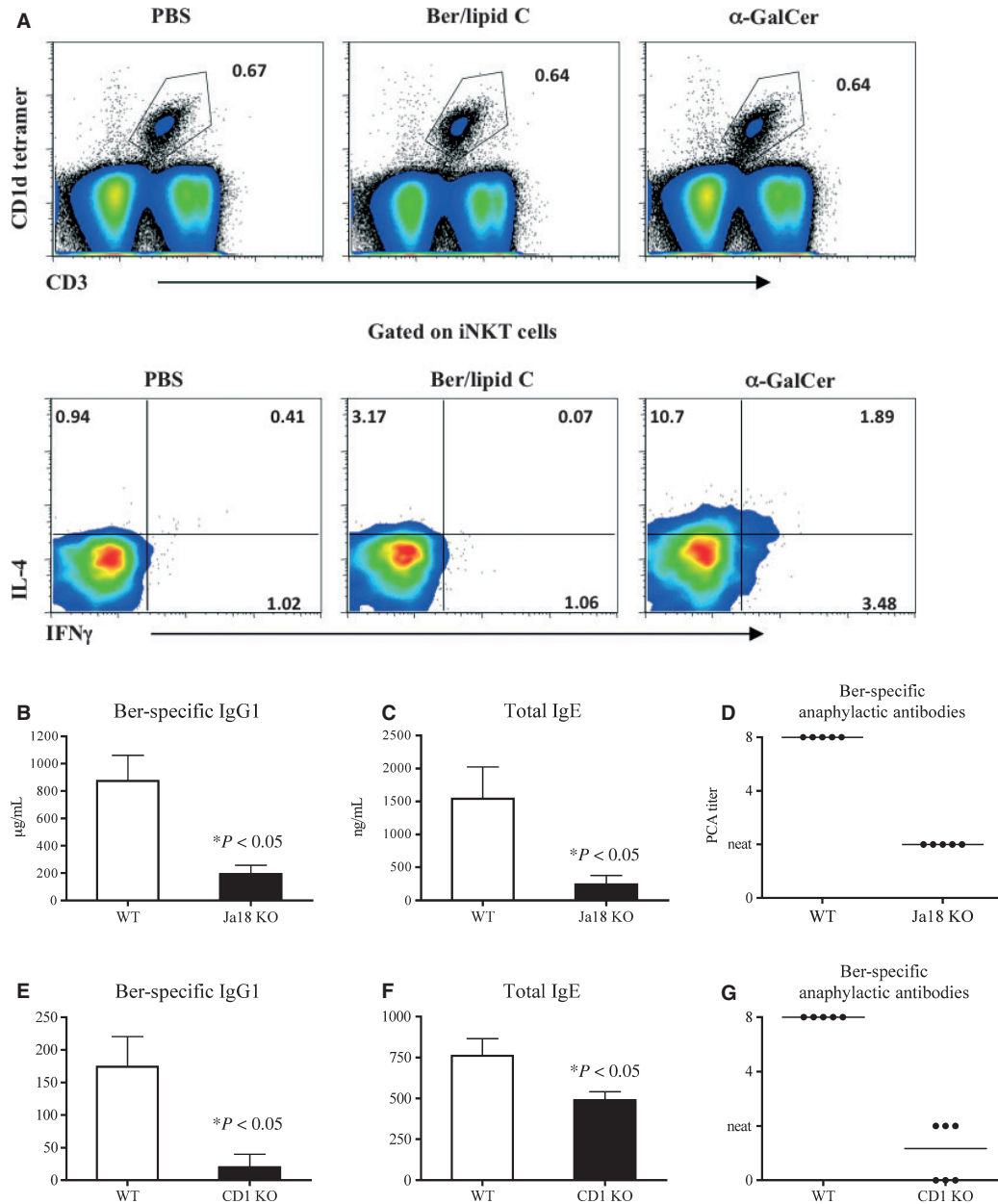


Figure 3 *In vitro* cytokine production by splenic invariant natural killer T (iNKT) cells and *in vivo* antibody response in Ber/lipid C-sensitized wild-type (WT) and iNKT-deficient C57BL/6 mice. (A) Splenocytes from naïve WT C57BL/6 mice were stimulated *in vitro* with medium (PBS) Ber e 1 (50 μg) and lipid C (1.2 mg) (Ber/lipid C) or α-GalCer (200 ng), for 4 h. Representative FACS profile showing the gate used to specifically detect iNKT cells (CD3^{pos} CD1d tetramer^{pos}) (top). IL-4 and IFNγ production by these gated iNKT cells (bottom). Data are representative of at least three independent experiments with 2–3 mice per experiment. Percentages of each subset are indi-

cated in quadrants. *T*-test, $P < 0.05$ (Ber/lipid C- vs PBS-stimulated cells) (B) Antibodies in the serum of WT C57BL/6, Jalpha18 knockout (Jalpha18 KO), or CD1 knockout (CD1 KO) mice sensitized with Ber e 1 (2.5 mg) and lipid fraction C (600 mg), on days 0 and 7, intraperitoneally. Parameters evaluated on day 14. (B, C, E, and F). Antibodies measured by enzyme-linked immunosorbent assay (ELISA); (D and G) Ber-specific anaphylactic antibodies measured by passive cutaneous anaphylaxis (PCA). PCA titers represent the reciprocal of the highest dilution of serum that gave a lesion of >3 mm in diameter. Symbols represent individual animals. Statistical analysis: *T*-test.

secretion of IL-4 and IFN-γ. To further test the role of iNKT cells in our model, animals deficient in iNKT cells, namely Jα18^{-/-} (lacking the TCR receptor Vα14Jα18) or

CD1d^{-/-} (lacking all CD1d-restricted T cells including iNKT cells), and their WT counterparts were sensitized with Ber e 1 and lipid fraction C, and the antibody production was

Table 1 Clinical characteristics of study subjects and frequency of lipid-specific T-cell lines

Subject	Age	Sex	Clinical symptoms	Nut-specific IgE (IU/dl)	Prick tests (main reactivity)	Lipid-specific T-cell lines	CD161 + T cells (%)
FA Patient 1	22	Male	Abdominal discomfort, diarrhea, asthma	2.35	Nuts	FA-1 FA-2 FA-3	48* 13 21
FA Patient 2	15	Male	Urticaria	15.5	Nuts	FA-4 FA-5	22 41
FA Patient 3	16	Female	Urticaria/angioedema	20.0	Brazil nuts, nuts	FA-6 FA-7 FA-8 FA-9	24 43 31 67
FA Patient 4	18	Female	Abdominal discomfort	6.35	Nuts	FA-10	35
NC-1	15	Female	None	<0.35	Negative		0.2
NC-2	22	Male	None	<0.35	Negative	NC-1	1
NC-3	18	Female	None	<0.35	Negative		0.2
NC-4	17	Male	None	<0.35	Negative		0.4
NC-5	20	Female	Diarrhea	<0.35	Milk	NC-2	5

NC, normal subjects; FA, food allergy.

*Percentage of T cells co-expressing the CD161 in proliferating T-cell lines after priming with Ber/lipid C.

determined. As shown in Fig. 3, $J\alpha 18^{-/-}$ mice produced significantly lower titers of anti-Ber IgG1, total IgE, and Ber-specific anaphylactic antibodies compared with WT animals (Fig. 3B–B–B–D). $CD1d^{-/-}$ animals also produced less Ber-specific IgG1, total IgE, and Ber-specific anaphylactic antibodies than WT C57BL/6 mice (Fig. 3E–E–E–G). Collectively, these results indicate that iNKT cells are involved in the production of Th2-type antibodies induced by Ber e 1 and lipid fraction C.

Human iNKT cells are also activated by lipid fraction C

Stimulation of PBMCs from nut-allergic patients and healthy controls generally gives rise to multiple T-cell lines; however, only two vs ten proliferating lines were recovered from plates containing control (NC, $n = 5$) and nut-allergic patients' T lymphocytes (FA, $n = 4$), respectively (Table 1). The phenotypes of FA and NC T-cell lines were quite different, with a clear prevalence of NKT cells in cultures primed with Ber e 1 and lipid fraction C (Ber/lipid C) extract from the FA group. As all emerged T-cell lines, from both NC and FA patients, were derived from wells primed with Ber/lipid C, their CD1-restriction and cytokine production after re-stimulation with relevant and irrelevant lipid substances in the presence of C1R transfectants were determined. Figure 4 illustrates the results obtained from all experiments, performed in triplicate. NC-derived T-cell lines, which contained few iNKT cell proportions (CD161+ cells ranging from 1% to 5% of all T cells) produced very low amounts of IL-4 when stimulated with Ber/lipid C or α GalCer, comparable with that of the vehicle. On the contrary, they produced appreciable amounts of IFN- γ when stimulated with α GalCer (data not shown) in a CD1d-restricted manner. The patient T-cell lines with a

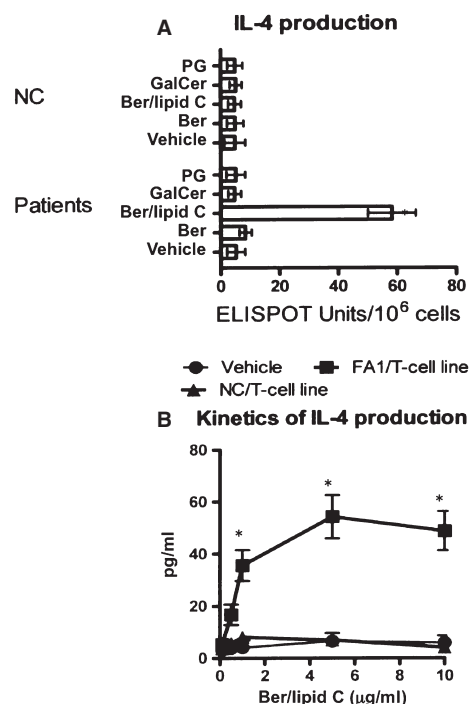


Figure 4 IL-4 responses of human T-cell lines. (A) Average (means of triplicates) IL-4 results (ELISPOT) from all T-cell lines comparing health controls (NC) and nut-allergic patients (FA) stimulated with cypress-derived phosphatidylglycerol (PG), α -GalCer (GalCer), Ber e 1 and lipid C (Ber/lipid C), Ber e 1 alone (Ber), and vehicle; (B) Ber e 1 and lipid fraction C (Ber/lipid C) dose-response curve of two T-cell lines, one from a nut-allergic patient (FA1) and one from a normal control (NC) primed with CD1d+C1R transfectants as APC. Statistical analysis: (A) one-way ANOVA and Tukey's post test, (B) T-test. *Significant difference ($P < 0.001$).

Table 2 Descriptive table of lipid fraction C components. Neutral lipids, polar lipids, and phosphorus concentration. Analyses performed as described in Material and Methods

Lipid classes	Components	%
Neutral lipids	Triglycerides	37.8
	Sterol	39.9
	Acylsterylglucosides	3.3
	Sterylglucosides	15.5
	Unknown	3.3
	Total	100
Polar lipids (184 mg/l of phosphorus)	Phosphatidylcholine	10.0
	Phosphatidylethanolamine	45.0
	Phosphatidylinositol	27.0
	Phosphatidic acid	9.0
	Sulfo-digalactosyldiacylglycerol	9.0
	Total	100

higher frequency of CD161⁺ T cells, comprising iNKT cells (i.e., FA5, FA6, FA9: TCR $\alpha\beta$ ⁺ (85–90%), FA1: 55% CD4⁺CD8⁺ V α 24/V β 11 + iNKT; FA7 and FA10: 45 and 78% TCR $\gamma\delta$ ⁺ T cells, respectively), were all responsive to the stimulation with Ber/lipid C in a CD1d-restricted manner to produce IL-4. In particular, the FA1 iNKT cell line, with a higher frequency of iNKT V α 24/V β 11 cells, produced very large amounts of IL-4 when primed with CD1d+C1R transfectants, demonstrating a clear specific dose–response curve, with a maximal cytokine production upon stimulation with a mixture of the Ber/lipid C in a constant stoichiometry ratio of 1 : 1 (5 μ g/ml of protein and 1.25 μ g/ml of lipid) (Fig. 4B). These findings underscore the ability of human iNKT cells issued from allergic patients to produce IL-4 in response to Ber e 1 and lipid fraction C stimulation.

Discussion

The intrinsic immunogenicity of Ber e 1, the major allergen from Brazil nuts, has been investigated. Early experiments in animals showed that Ber e 1, when injected intradermally, did not induce specific IgG1 or IgE (29). More recently, we confirmed that the purified native or recombinant Ber e 1 when injected in mice did not elicit antibody production, indicating that components other than the proteins might influence the intrinsic sensitization potential of this protein. Indeed, lipids from Brazil nuts were shown to be required for the sensitization of mice and for the induction of an anti-Ber e 1 anaphylactic antibody response (10).

In the present work, we used various lipid fractions extracted from Brazil nuts and found that a specific fraction (lipid fraction C) was essential for the induction of a Th2 response. Fraction C is composed of complex lipids, mainly triglycerides, sterylglucosides, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid, and a sulfonated di-galacto lipid with c.a. 184 mg/l of phosphorous (Table 2). The specific active component(s) present in fraction C were not identified, as a further fractionation of this fraction did not elicit immune responses (data not shown).

However, the presence of PE and PC alone could explain the activity observed (18).

The amount of the putative lipid necessary to trigger the observed response is low as the animal experiments were designed with a stoichiometry of protein/lipid of 1 : 1. Due to its structural similarity to plant LTP, Ber e 1 may also be able to bind lipids or other hydrophobic compounds from Brazil nuts. Indeed, the results of the ANS binding and NMR experiments showed that Ber e 1 possesses a hydrophobic site able to accommodate a lipid molecule with a stoichiometry close to 1 : 1. The chemical shift differences upon addition of fraction C were distributed throughout the whole protein, similarly to what is observed in LTPs, showing that components in lipid fraction C interact with Ber e 1 (30). The fact that lipid C is still a complex mixture certainly has a strong influence on the large number of AA affected. In LTPs, the long fatty acid chain of a lipid is buried in a hydrophobic pocket within the protein, hence interacting with many residues. In lipid fraction C, the vast majority of the fatty acid side chain contains either 16 or 18 carbon atoms as determined by gas chromatography (data not shown). In addition, the size of a bis-ANS molecule is comparable with the length of a fatty acid from fraction C, thus is likely that the hydrophobic pocket of Ber e 1 is large enough to accommodate a lipid.

Some allergens possess structural similarity to the Toll-like receptor accessory protein MD-2, and in this case, the high-affinity bis-ANS binding site overlaps with the LPS binding site (26). However, this might not be the case of Ber e 1, because in our previous work, we did not find differences in DC-T-cell polarization between Myd88^{−/−} compared with WT animals (31). Further, lipid fraction C had no detectable endotoxin levels, it was not able to activate human TLRs *in vitro*, and C57BL/6 Myd88 KO animals sensitized with Ber/lipid C produced titers of anaphylactic antibodies similar to WT mice (data not shown).

Lipid antigens can stimulate CD1d-restricted T cells. Five CD1 isoforms (CD1a–e) are present in humans, while CD1d-restricted T cells (iNKT) are the only subset in mice (32). Invariant natural killer T cells are involved in Th2 pathologies, such as respiratory allergy (15, 33). Consequently, iNKT cells were putative candidates to be implicated in the lipid-dependent specific immunoglobulin production in response to Ber e 1. First, we observed that Ber e 1 associated with lipid C induced IL-4 production by murine splenic iNKT cells in culture. We further used two different iNKT cell-deficient mice: animals lacking the invariant V α 14 + J α 18 + of iNKT cells (J α 18^{−/−}) and animals lacking the CD1d molecule (CD1d^{−/−}). Both iNKT cell-deficient groups of mice exhibited significant reduced Ber-specific Th2-type antibodies when compared with WT mice. These results clearly demonstrate the role of iNKT cells in the allergic response induced by Ber e 1 and lipid C.

We extended our investigation to human patients allergic to nuts. Nut-allergic patients exhibited a discrete array of Ber/lipid C-specific T-cell responses that give rise to both iNKT (V α 24 + V β 11+, FA1 cell line) and other CD1d-restricted NKT cells (CD161+), some of which belong to the $\gamma\delta$ TCR type, capable of recognizing CD1d-restricted aller-

gens as previously reported by us (34). A set of human CD1d-restricted T cells can recognize pollen lipids (13), but the possible implication of human iNKT CD1d-restricted cells on the Th2 responses to Brazil nut allergies was not analyzed before. Here, we demonstrated that CD1d-restricted human T-cell lines, including the iNKT cell FA1 line, expand in response to Ber/lipid C complex and produce IL-4. This type of response has been described in humans allergic to pollens both in the peripheral blood and at mucosal surfaces (13, 34), mostly sustained by pollen-derived PE. Like the humoral reaction to T-dependent antigens, such observed specific immune response to Ber/lipid C appears to develop from the synergistic action of innate-like T cells and DCs (35).

In summary, our results indicated that the immunogenicity of the Ber e 1 protein was dependent on a specific lipid fraction natural to Brazil nuts. Ber e 1 was able to bind this lipid fraction in a 1 : 1 stoichiometry. *In vivo*, iNKT cells were required for the production of Th2-type antibodies. Moreover, both murine iNKT and human CD1d-restricted NKT cell lines produced IL-4 in response to Ber e 1 and lipid C, suggesting that IL-4-producing iNKT cells could represent the missed link to explain the enhanced production of anaphylactic antibodies in subjects allergic to Brazil nuts. Therefore, our findings contribute to explain why a large number of experiments based solely on proteins failed to identify the intrinsic factor(s) that confer allergenic properties on common proteins. We also identified CD1d-restricted NKT cells as regulators of nut-allergic immune responses opening new potential targets for immunotherapy.

References

1. Sicherer SH, Sampson HA. Food allergy: recent advances in pathophysiology and treatment. *Annu Rev Med* 2009;**60**:261–277.
2. Radauer C, Breiteneder H. Evolutionary biology of plant food allergens. *J Allergy Clin Immunol* 2007;**120**:518–525.
3. Ridout S, Matthews S, Gant C, Twiselton R, Dean T, Arshad SH. The diagnosis of Brazil nut allergy using history, skin prick tests, serum-specific immunoglobulin E and food challenges. *Clin Exp Allergy* 2006;**36**:226–232.
4. Pastorello EA, Farioli L, Pravettoni V, Ispano M, Conti A, Ansaloni R et al. Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy. *J Allergy Clin Immunol* 1998;**102**:1021–1027.
5. Maddox L, Schwartz DA. The pathophysiology of asthma. *Annu Rev Med* 2002;**53**:477–498.
6. Cianferoni A, Spergel JM. Food allergy: review, classification and diagnosis. *Allergol Int* 2009;**58**:457–466.
7. Alcocer MJ, Murtagh GJ, Bailey K, Dumoulin M, Meseguer AS, Parker MJ et al. The disulphide mapping, folding and characterisation of recombinant Ber e 1, an allergenic protein, and SFA8, two sulphur-rich 2S plant albumins. *J Mol Biol* 2002;**324**:165–175.
8. Tengel T, Alcocer MJ, Schleucher J, Larsson G. Complete assignment and secondary structure of the Brazil nut allergen Ber e 1. *J Biomol NMR* 2005;**32**:336.
9. Brix S, Kjaer TM, Barkholt V, Frokiaer H. Lipopolysaccharide contamination of beta-lactoglobulin affects the immune response against intraperitoneally and orally administered antigen. *Int Arch Allergy Immunol* 2004;**135**:216–220.
10. Dearman RJ, Alcocer MJ, Kimber I. Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1. *Clin Exp Allergy* 2007;**37**:582–591.
11. van Wijk F, Nierkens S, Hassing I, Feijen M, Koppelman SJ, de Jong GA et al. The effect of the food matrix on in vivo immune responses to purified peanut allergens. *Toxicol Sci* 2005;**86**:333–341.
12. Thomas WR, Hales BJ, Smith WA. Structural biology of allergens. *Curr Allergy Asthma Rep* 2005;**5**:388–393.
13. Agea E, Russano A, Bistoni O, Mannucci R, Nicoletti I, Corazzi L et al. Human CD1d-restricted T cell recognition of lipids from pollens. *J Exp Med* 2005;**202**:295–308.
14. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1 + T lymphocytes. *Science* 1995;**268**:863–865.
15. Lisbonne M, Diem S, de Castro Keller A, Lefort J, Araujo LM, Hachem P et al. Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. *J Immunol* 2003;**171**:1637–1641.
16. Christie WW, Gill S, Nordbäck J, Itabashi Y, Sanda S, Slabas AR. New procedures for rapid screening of leaf lipid components from *Arabidopsis*. *Phytochem Anal* 1998;**9**:53–57.
17. Kupke IR, Zeugner S. Quantitative high-performance thin-layer chromatography of lipids in plasma and liver homogenates after direct application of 0.5-microliter samples to the silica-gel layer. *J Chromatogr* 1978;**146**:261–271.
18. Petzold K, Olofsson A, Arnqvist A, Gröbner G, Schleucher J. Semiconstant-time P-H-COSY NMR: analysis of complex mixtures of phospholipids originating from *Helicobacter pylori*. *J Am Chem Soc* 2009;**131**:14150–14151.

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Conflict of interests

The authors have no conflict of interests to declare.

Author contributions

All authors contributed to the manuscript. Luciana Mirotti performed all experiments of Figs 1 and 3 and wrote the article. Esther Florsheim performed the *in vitro* experiments with NKT cells and contributed with the writing. Louise Rundqvist and Göran Larsson performed the experiments of Fig. 2. Fabrizio Spinozzi performed the experiments with human samples (Fig. 4) and contributed with the manuscript preparation. Maria Leite-de-Moraes supervised all animal NKT experiments and contributed with manuscript writing. Momtchilo Russo and Marcos Alcocer thoroughly supervised the work and participated in the manuscript writing.

19. Harris WD, Popat P. Determination of the phosphorus content of lipids. *J Am Oil Chem Soc* 1954;**31**:124–127.
20. Rosen CG, Weber G. Dimer formation from 1-amino-8-naphthalenesulfonate catalyzed by bovine serum albumin. A new fluorescent molecule with exceptional binding properties. *Biochemistry* 1969;**8**:3915–3920.
21. Wassenberg JJ, Reed RC, Nicchitta CV. Ligand interactions in the adenosine nucleotide-binding domain of the Hsp90 chaperone, GRP94. II. Ligand-mediated activation of GRP94 molecular chaperone and peptide binding activity. *J Biol Chem* 2000;**275**:22806–22814.
22. Wishart DS, Bigam CG, Yao J, Abildgaard F, Dyson HJ, Oldfield E et al. 1H, 13C and 15N chemical shift referencing in biomolecular NMR. *J Biomol NMR* 1995;**6**:135–140.
23. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 1995;**6**:277–293.
24. Johnson BA, Blevins RA. NMR view: a computer program for the visualization and analysis of NMR data. *J Biomol NMR* 1994;**4**:603–614.
25. Pastukhov AV, Ropson IJ. Fluorescent dyes as probes to study lipid-binding proteins. *Proteins* 2003;**53**:607–615.
26. Mancek-Keber M, Jerala R. Structural similarity between the hydrophobic fluorescent probe and lipid A as a ligand of MD-2. *FASEB J* 2006;**20**:1836–1842.
27. Rundqvist L, Tengel T, Zdunek J, Björn E, Schleucher J, Alcocer MJC et al. Solution structure, copper binding and backbone dynamics of Ber e 1 – the major allergen from Brazil nut. *PLoS ONE* 2012;**7**:e46435.
28. Scanlon ST, Thomas SY, Ferreira CM, Bai L, Krausz T, Savage PB et al. Airborne lipid antigens mobilize resident intravascular NKT cells to induce allergic airway inflammation. *J Exp Med* 2011;**208**:2113–2124.
29. Melo VM, Xavier-Filho J, Lima MS, Prouvost-Danon A. Allergenicity and tolerance to proteins from Brazil nut (*Bertholletia excelsa* H. B. K.). *Food Agric Immunol* 1994;**6**:10.
30. Lerche MH, Poulsen FM. Solution structure of barley lipid transfer protein complexed with palmitate. Two different binding modes of palmitate in the homologous maize and barley nonspecific lipid transfer proteins. *Protein Sci* 1998;**7**:2490–2498.
31. Kean DE, Goodridge HS, McGuinness S, Harnett MM, Alcocer MJ, Harnett W. Differential polarization of immune responses by plant 2S seed albumins, Ber e 1, and SFA8. *J Immunol* 2006;**177**:1561–1566.
32. Brigl M, Brenner MB. CD1: antigen presentation and T cell function. *Annu Rev Immunol* 2004;**22**:817–890.
33. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med* 2003;**9**:582–588.
34. Russano AM, Agea E, Corazzi L, Postle AD, De Libero G, Porcelli S et al. Recognition of pollen-derived phosphatidyl-ethanolamine by human CD1d-restricted gamma delta T cells. *J Allergy Clin Immunol* 2006;**117**:1178–1184.
35. Munz C, Steinman RM, Fujii S. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *J Exp Med* 2005;**202**:203–207.