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Oral immunotherapy using polymeric nanoparticles loaded with peanut proteins in a murine model of fatal anaphylaxis

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Background: Peanut allergy is the most common cause of anaphylaxis and food-related death. However, there is currently no approved immunotherapy treatment. Hence, this warrants the need for relevant and convenient animal models to test for adequate immunotherapies. **Materials & methods:** In this study, we compared three mouse strains: CD1, BALB/c and C57, to select a model of peanut allergy. After that, we conducted then a therapeutic study using an immunogenic peanut extract encapsulated in nanoparticles made with polymer Gantrez[®] following the solvent displacement method. **Results & conclusion:** After implementing a dosing schedule with oral commercial peanut butter, the antibody responses, cytokine profiles and, above all, the anaphylaxis induced after a challenge with peanut proteins, showed that the outbred CD1 strain was the most susceptible to peanut sensitization. CD1 sensitized mice were orally immunized with three doses of the nanoparticle formulation capable of protecting them against the severe anaphylactic symptoms induced by the peanut challenge.

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Allergies represent the most prevalent of all diseases in developed countries and, specifically, the rate of food allergies is increasing alarmingly across the world, with peanut (*Arachis hypogaea*, Ara h) being the food most frequently involved in serious anaphylactic reactions [1]. However, there is still no peanut-specific immunotherapy available, although several forms are being researched [2–4]. Still, current approaches show limitations in terms of long-term efficacy and safety, due to their related local side effects and risk of anaphylaxis [5]. Therefore, safe and effective vaccines are needed to reduce the risks related to the presence of the allergens, as well as the inclusion of new adjuvants that can aid in achieving allergen tolerance. Traditional adjuvants such as Alum have several disadvantages due to their toxicity and inability to generate Th1 immune responses [6]. We propose the use of polymeric nanoparticles for the encapsulation of allergens and their subsequent use in immunotherapy [7,8]. Administered orally, polymeric nanoparticles may avoid direct contact between the cargo allergen and the sensitized mast cells. Moreover, due to their ability to interact with antigen-presenting cells, they facilitate the induction of the adequate protective immune response [9,10]. Additionally, the encapsulation of allergens in these nanocarriers protects them from premature chemical, enzymatic or immunological degradation, thus facilitating their arrival to the target effector cells [7]. In short, this strategy may allow for a more rational and adapted therapeutic approach by increasing the efficacy and safety of the immunotherapy.

However, there is a need for relevant and convenient animal models to test for new forms of immunotherapy against food allergies. Allergic diseases are mediated by Th2 cytokine responses, as evidenced by the high levels of allergen-specific IgE and the reduced number of Th1 IFN- γ -producing cells. Therefore, effective immunotherapies are likely to shift the immune responses from the proallergic Th2 to the Th1, Th17 and Treg subsets. However,

researchers have focused on certain inbred mice strains showing Th1 (e.g., C57BL/6) or Th2 (e.g., BALB/c) responses and, consequently, on researching their different innate abilities to produce IgE anaphylactic antibodies [11–14]. Based on our results, we recommend the use of CD1 outbred mice over the immune-oriented inbred strain. To our knowledge, this is the first article available in the published literature describing conditions that facilitate the oral sensitization of outbred mice to peanut. In this study, we used nanoparticles formed by the synthetic copolymer between methyl vinyl ether and maleic anhydride (commercialized as Gantrez[®] AN). One of the most important properties of the resulting poly(anhydride) nanoparticles is their ability to reach the gut mucosa (following their oral administration), including Peyer's patches, and to develop strong adhesive interactions with components of the intestinal epithelium [15]. Gantrez AN also has an affordable price. Moreover, the resulting nanoparticles can be considered a safe oral delivery system, even with chronic administration regimens [16]. Considering our findings, we believe that Gantrez AN nanoparticles are good candidates for oral immunotherapy treatments. We recently optimized the formulation and preparation of Gantrez AN nanoparticles containing a peanut extract (PE) [17,18]. In these studies, we demonstrated that complete roasted extracts (containing lipids) showed higher immunogenicity than other types of extracts [17]. Similarly, the manufacturing process of these nanoparticles (particularly the technique used to dry the resulting nanoparticles) may also affect the activity level of the resulting formulation [18].

In our study, we used these optimized nanoparticles to define a relevant and convenient animal model of peanut allergy. For this purpose, we compared the effect of these nanoparticles in three different mouse strains: CD1, BALB/c and C57, to determine which would be the most suitable as a model of peanut allergy.

Materials & methods

Chemicals

PE was obtained from Diater[®] Laboratories (Madrid, Spain). Poly(methyl vinyl ether-co-maleic anhydride) (PVM/MA), Gantrez AN 119 (MW 200.000), was kindly provided by Ashland (Barcelona, Spain). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and a protease inhibitor cocktail, were purchased from Sigma (Spain). The peroxidase immunoconjugates (GAM/IgG1/PO, GAM/IgG2a/PO, GAM/IgE/PO and GAM/IgA/PO) were obtained from Nordic Immunology (Tilburg, The Netherlands). The ELISA Mouse IgE Set and the cytometric bead array mouse TH1/TH2/TH17 Cytokine Kit were purchased from BD[®] Biosciences (Belgium). The protein assay reagent kit was obtained from Pierce[®] (IL, USA); RPMI-1640 medium with L-glutamine from eBioscience[®], Affymetrix; fetal bovine serum and penicillin-streptomycin from GIBCO, Invitrogen[™]; and protein A-conjugated magnetic beads from Dynabeads[®], Life Technologies. All other chemicals used were of reagent grade and obtained from Merck (Spain).

In vivo studies

Animals

All experiments were conducted in compliance with the regulations of the Ethics Committee of the University of Navarra and in line with the European legislation on animal experiments (approved protocol numbers 016/13 and 006/15).

Anaphylaxis study (oral sensitization)

CD1, BALB/c and C57BL/6 mice (Harlan Interfauna Ibérica, Spain) (n = 5) were sensitized intragastrically once a week, for a total of 4 weeks, through the administration of 4.35 mg of peanut butter per mouse (equivalent to 1 mg of peanut proteins) with 5 µg of adjuvant cholera toxin (Sigma-Aldrich, Germany) in a final volume of 200 µl of PBS. Once sensitized, mice were challenged intraperitoneally with 2 mg of the hydrophilic fraction of roasted PE either 2 or 4 weeks after administering the last sensitization dose. Naive mice were challenged in the same way. Anaphylactic symptoms were monitored for 45 min and evaluated based on the rectal temperature of the mice and a scoring system ranging from 0 to 5 [0: no symptoms; 1: scratching; 2: reduced activity and increased respiratory rate; 3: labored breathing and cyanosis around the mouth and tail; 4: no activity on prodding; 5: death]. Blood and fecal samples were taken throughout the course of the experiment to determine the levels of total IgE and peanut-specific IgG1, IgG2a, IgE and IgA. Following the challenge, spleens from dead and live mice were extracted to determine the cytokine profile produced by their splenocytes.

Immunotherapy schedule

Four of the five groups ($n = 6$) of CD1 mice (Harlan Interfauna Ibérica) were sensitized following the method explained above. One week after the last sensitization dose was administered, three of the sensitized groups received the PE formulation intragastrically, consisting of three weekly doses of whole roasted PE (1 mg), either free, encapsulated in lyophilized nanoparticles (PE-LF) or loaded into spray-dried nanoparticles (PE-SD). Then, 1 week after the last dose of PE was administered, mice were challenged as described above. Blood and fecal samples were taken throughout the course of the experiment to determine the content of total IgE and peanut-specific IgG1, IgG2a, IgE and IgA. Following the challenge, spleens from dead and live mice were extracted to determine the cytokine profile produced by their splenocytes.

Preparation of poly(anhydride) nanoparticles

A complete roasted PE was used (Diater Laboratories, Madrid, Spain). In order to improve the encapsulation process, we removed salts contained in the extract by dialysis. Poly(methyl vinyl ether-co-maleic anhydride) nanoparticles (NP) were prepared following a solvent displacement method. After purifying them, the nanoparticles were dried by lyophilization or spray-drying [18].

To prepare the PE-loaded lyophilized nanoparticles (PE-NP-LF), 10 ml of a 1.5 mg/ml PE solution was dispersed by ultrasonication (MCD10son™) for 1 min. The PE dispersion was added to 10 ml of acetone containing 200 mg of PVM/MA, and the mixture was stirred for 45 min at room temperature (RT). The organic solvents were eliminated under reduced pressure (Büchi® R-144, Switzerland). Finally, the suspensions of nanoparticles were purified twice by centrifugation $12,000 \times g$ for 20 min and lyophilized using an aqueous solution containing sucrose (5%, p/v) as a cryoprotector (Genesis 12EL, Virtis®, USA). Control empty lyophilized nanoparticles (NP-LF) were prepared in the same way, without PE.

To obtain the PE-loaded spray-dried nanoparticles (PE-NP-SD), 500 mg of PVM/MA were dissolved in 15 ml of acetone. At the same time, 15 ml of a 3 mg/ml PE solution was dispersed by ultrasonication (MCD10son) and added to the previous mixture. Following incubation with a magnetic stirrer (45 min at RT), acetone was eliminated under reduced pressure. The nanoparticles were then purified by centrifugal filtration. Next, a mixture of a solution containing 1 g of mannitol in 15 ml of H₂O was added and combined with a magnetic stirrer (5 min at RT). Finally, nanoparticles were dried in a Mini Spray-dryer Büchi B191 (Büchi Labortechnik AG, Switzerland). Control empty spray-dried nanoparticles (NP-SD) were prepared in the same way, without PE.

Characterization of nanoparticles

Size & ζ potential

The size and ζ potential of the empty and PE-loaded nanoparticles were determined by photon correlation spectroscopy and electrophoretic laser Doppler anemometry, respectively, using a Zetamaster analyzer system (Malvern® Instruments, UK). Samples were diluted with 0.05 mM PBS or KCl 0.1 mM to measure the size and ζ potential, respectively. All analyses were performed in triplicate, at 25°C and with a scattering angle of 90°. The average particle size was expressed as a volume mean diameter (Vmd) in nanometers (nm), and the average surface charge was given in mV.

Protein loading & encapsulation efficiency

In order to quantify the amount of peanut protein contained in the nanoparticles, 10 mg of either lyophilized or spray-dried nanoparticles were digested in NaOH 0.1 M by shaking the mixture at 37°C for 1 h. Next, their protein content was measured following the bicinchoninic acid method. Each sample was analyzed in triplicate and PE loads were expressed as an amount of protein (in μ g) per milligram of NP. Similarly, the encapsulation efficiency (EE) was calculated with the following formula:

$EE (\%) = (Q_{\text{associated}}/Q_{\text{initial}}) \times 100$, where Q_{initial} was the initial amount of PE added per mg of polymer forming the nanoparticles and $Q_{\text{associated}}$ was the amount of loaded PE per milligram of nanoparticle, which was calculated following the bicinchoninic acid method. Main AraH allergens were contained in the free PE and loaded into the nanoparticles, as described previously [12,13].

Peanut-specific antibodies & total IgE (ELISA)

The presence of PE-specific antibodies in the mice sera (IgG1, IgG2a and IgE) and feces (IgA) was analyzed by indirect ELISA. Measurements were taken in triplicate, including negative controls.

Specific serum IgG1 & IgG2a

Microplate wells (Immuno-Maxisorp, Nunc®, Roskilde, Denmark) were coated with 100 µg/ml of peanut proteins diluted in a sodium carbonate–bicarbonate buffer (0.05 M, pH 9.6) in a moist chamber at 4°C for 12 h. The plates were blocked with PBS containing 3% bovine serum albumin (BSA) for 1 h at room temperature (RT). The wells were then incubated with serum samples diluted at 1:100 (1 h at RT) and subsequently with goat horseradish peroxidase (PO)-conjugated secondary antibody (antimouse IgG1 or IgG2a) diluted at 1:1000 in PBS containing 1% BSA for 1 h at RT. Finally, the plates were developed using a chromogenic substrate (H₂O₂-ABTS solution, Sigma, MI, USA) and a mixer. Optical density (OD) was measured at 405 nm (iEMS Reader MF, Labsystems) after 15 min of incubation at RT.

Specific serum IgE

Plates were coated with peanut proteins (100 µg/ml) and blocked with PBS plus 10% fetal bovine serum (FBS) (1 h at RT). After washing them, pooled serum samples which had previously been incubated with protein A-conjugated magnetic beads (Dynabeads) were added to a buffer at a 1:10 dilution (0.085% BSA in PBS-T) (2 h at RT). Following their incubation, the plates were washed and PO-conjugated secondary antibody (antimouse IgE) was added at a 1:1000 dilution (in PBS with 10% FBS, pH 7.0). The plates were then washed, developed and measured as described above.

Specific fecal IgA

To measure the levels of PE-specific IgA, plates were coated and then blocked with PBS plus 1% skim milk (1 h at RT). The wells were incubated with serum samples diluted at 1:20 (4 h at 37°C) and treated with peroxidase (PO)-conjugated secondary antibody (antimouse IgA) diluted at 1:1000 in PBS containing 1% BSA (1 h, RT). The plates were then washed, developed and measured as described above.

Total serum IgE

Total IgE was quantified with the ELISA Mouse Set kit purchased in BD Biosciences, following the manufacturer's instructions. MCD10plate wells (BD Falcon™) were coated with capture antibodies diluted in coating buffer (0.1 M Sodium Carbonate, pH 9.6) for 12 h in a moist chamber at 4°C. The plates were blocked with assay-diluent (PBS with 10 % FBS) for 1 h at RT. After washing them, the wells were incubated with serum samples (diluted at 1:10 in assay diluent) or standards (purified mouse IgE) for 2 h at RT, and then with working detector (biotinylated detection antibody and streptavidin-horseradish peroxidase conjugate diluted at 1:250 in assay diluent) for 1 h at RT. Finally, the plates were washed, developed and measured as described above.

Splenocyte isolation & culture for cytokine production (PE stimulation)

To prepare single cell suspensions of the mice splenocytes, their spleens were isolated following an aseptic technique and placed in sterile Petri dishes containing RPMI-1640 medium with 4 mM L-glutamine. Splenocyte suspensions were prepared by passing the minced spleens through a 40-µm cell strainer and hemolyzing them with a 1X RBC Lysis Buffer (eBioscience). The resulting cell suspensions were then washed twice and resuspended in culture medium (RPMI-1640 medium with 4 mM L-glutamine) supplemented with 10% FBS and 1% penicillin-streptomycin. After conducting the cell count and the viability study following the trypan blue dye exclusion method, 100 µl of the splenocyte suspensions at a dilution of 1×10^6 cells/ml were plated in the flat bottom wells of a 96-well plate. The cells were then stimulated with concanavalin A (10 µg/ml) or PE (150 µg/ml in the sensitization study and 45 µg/ml in the vaccination study). After 48 h of incubation at 37°C in an atmosphere containing 5% CO₂, the culture media was cleared of cells and debris by centrifugation at $18,000 \times g$ for 3 min. The cleared media was transferred to sterile tubes and stored at -80°C until it was analyzed by cytometry for cytokine levels.

Determination of cytokine levels

The BD cytometric bead array Mouse TH1/TH2/TH17 Cytokine Kit uses bead array technology to simultaneously detect multiple cytokine proteins (IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A and IL-10) in samples. Seven bead populations with distinct fluorescence intensities were coated with capture antibodies specific for each cytokine. During the assay procedure, the seven beads were mixed together with samples and incubated with phycoerythrin-conjugated detection antibodies. The intensity of the phycoerythrin fluorescence of each sandwich complex reveals the concentration of each cytokine.

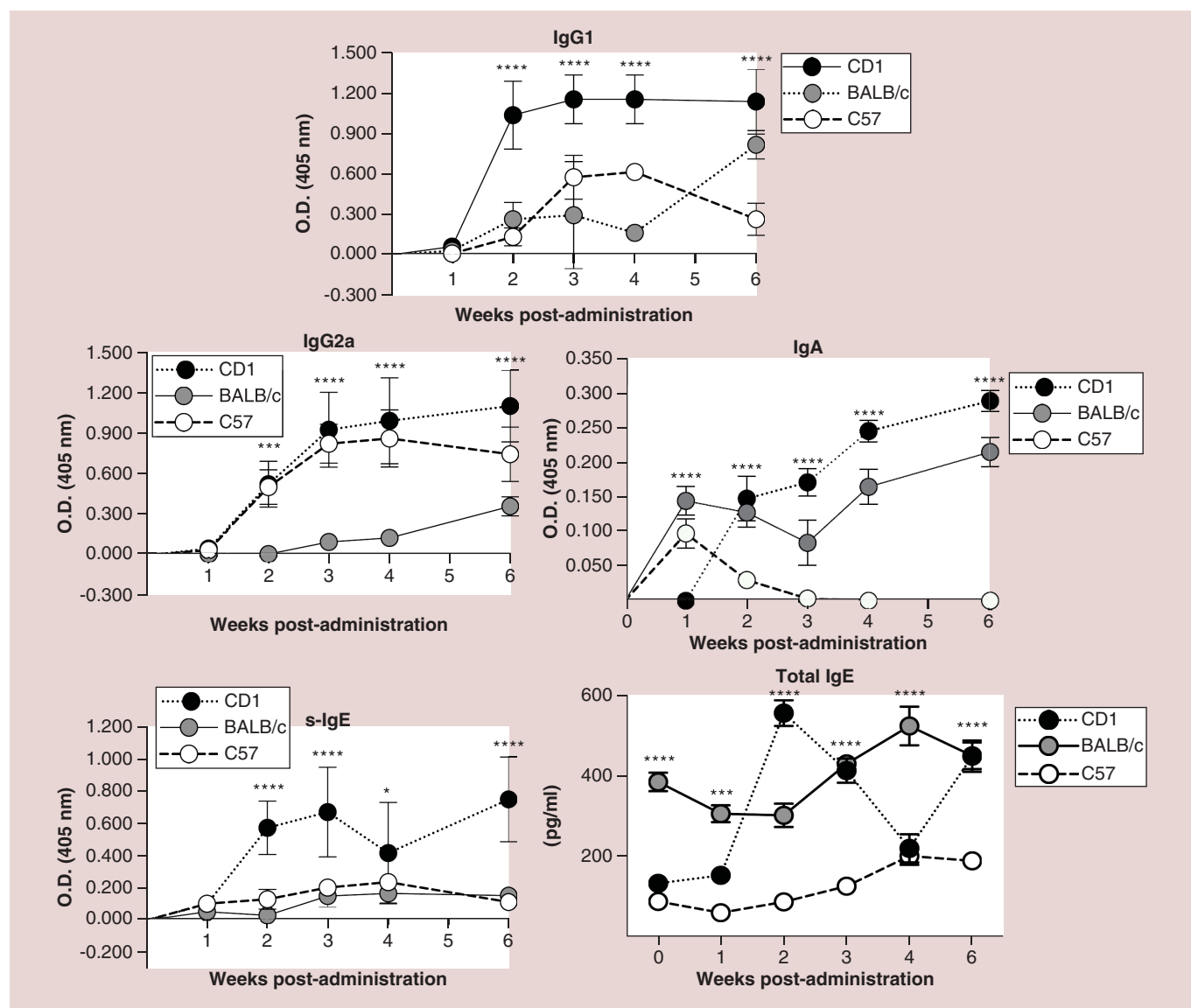


Figure 1. Serum and fecal levels of peanut-specific antibodies obtained in CD1, BALB/c or C57BL/6 mice during oral sensitization. Mice ($n = 5$) received four doses of 1 mg of roasted peanuts adjuvanted with cholera toxin. Specific antibodies were determined by indirect ELISA and results were expressed as optical densities. Total IgE was determined by a sandwich ELISA kit and results were expressed as ng/ml of IgE in serum. Data are given as means $\pm$ SD.

Statistics

Statistical comparisons were performed using the analysis of variance test (ANOVA), followed by Bonferonni's *post hoc* test. $p < 0.05$ was considered as a statistically significant difference. All calculations were performed using GraphPad Prism® 6.0 statistical software program (Microsoft, USA). Data are expressed as the mean $\pm$ SD from independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

Oral sensitization: anaphylactic murine model

Since the purpose of our study was to find a suitable model to study immunotherapy protection, it was crucial to determine which mouse strain would outperform peanut sensitization and challenge. The C57BL/6, BALB/c and CD1 strains were compared for peanut sensitization. During intragastric sensitization, CD1 mice produced higher levels of peanut-specific IgE than the other two mice strains (Figure 1), indicating a greater sensitization capacity.

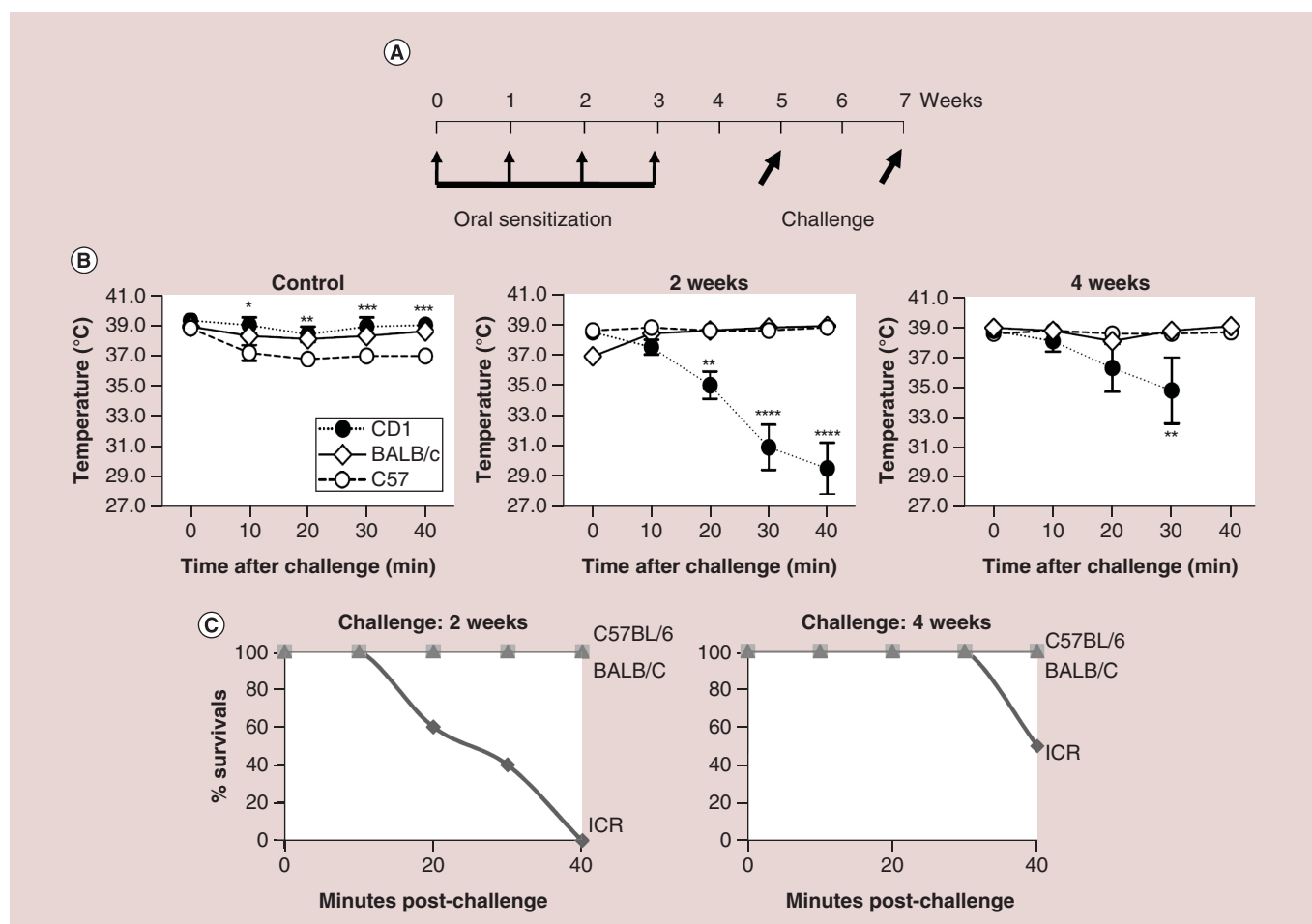


Figure 2. Peanut sensitization and challenge in mouse strains. CD1, BALB/c or C57BL/6 mice were sensitized by means of intragastric gavage with whole roasted peanut plus cholera toxin. **(A)** Time schedule. Mice were intraperitoneally challenged with a hydrophilic fraction of peanut proteins at week 2 or 4 post-sensitization: **(B)** rectal temperature post-challenge; **(C)** mortality rates post-challenge.

Peanut-specific IgE levels showed two peaks on weeks 2 and 6 following the first administration in CD1 mice, which were also correlated with the total IgE levels (Figure 1). High basal levels of total IgE was characteristic of the BALB/c mice strain. Oral sensitization also resulted in significantly higher levels of IgG1 in the CD1 and BALB/c strains. The CD1 and C57BL/6 strains had high levels of IgG2a, whereas these levels were very low in the BALB/c strain. These data are consistent with the pro-Th2 and pro-Th1 tendencies of the BALB/c and C57BL/6 strains, respectively. Peanut protein administration also induced an important mucosal antibody response characterized by increased levels of fecal IgA in the CD1 strain.

Temperature drop & mortality rate following the challenge

Sensitized animals (see section Materials & methods) were challenged intraperitoneally with 2 mg of a hydrophilic fraction of roasted PE either 2 or 4 weeks after the administration of the last sensitization dose (Figure 2). Systemic anaphylactic symptoms were only evidenced in the sensitized CD1 mice. Puffiness around the eyes, followed by wheezing and labored breathing, was manifested within the first 20 min postchallenge. Only CD1 mice experienced a drop in their body temperature, measured rectally, at two point challenge-times. This drop was particularly significant during the challenge applied at week 2 postsensitization, with a mortality rate of up to 100%. When CD1 mice were challenged at week 4 postsensitization, the mortality rate was 50%; one survivor experienced severe anaphylactic symptoms and a rectal temperature drop of up to 4.8°C, and one animal showed no anaphylactic symptoms whatsoever. Neither the BALB/c nor the C57BL/6 mice experienced a drop in their body temperature (Figure 2).

Table 1. Physico-chemical characterization of empty or peanut-loaded lyophilized and spray-dried poly(anhydride) nanoparticles.

Nanoparticle formulation	Size(nm)	ζ potential (mV)	PE content(μg/mg NP)	Encapsulation efficiency (%)	Yield (%)
NP-L	201 ± 3	-49 ± 2	–	–	80 ± 2
NP-SD	172 ± 4	-42 ± 4	–	–	63 ± 1
NP-PE-L	155 ± 2	-45 ± 5	30 ± 3	72 ± 6	76 ± 4
NP-PE-SD	143 ± 6	-48 ± 3	32 ± 3	60 ± 4	61 ± 3

NP-L: Empty lyophilized nanoparticle; NP-SD: Empty spray-dried nanoparticle; NP-PE-L: Peanut-loaded lyophilized nanoparticle; NP-PE-S: Peanut-loaded spray-dried nanoparticle.

Cytokine production after splenocyte stimulation with PE

Cytokine-specific responses on spleen cells were analyzed and compared in the three mouse strains following their *in vitro* stimulation with PE. Once again, after the challenge, significant differences were found between the CD1 strain and the other two mice strains, as well as between live and dead CD1 mice (Figure 3). Splenocytes from the three strains responded similarly in control mice, but sensitized BALB/c and C57BL/6 mice showed an increased cytokine production capacity, especially of Th1 and Th17 cytokines. However, sensitized CD1 mice experienced a greater increase in their levels of Th2-associated cytokines. In the CD1 strain, live mice had high levels of IFN-γ (signature Th1 cytokine) and TNF-α (produced by IFN-γ activated antigen-presenting cells). Spleen cells from mice who had died from an anaphylactic shock expressed high levels of IL-10 and IL-17 (Figure 3).

Oral immunotherapy with peanut-nanoparticles in sensitized CD1 mice

Characterization of NPs

Nanoparticles loaded with roasted PE were prepared following a solvent displacement method and subsequently dried, either by lyophilization or spray-drying. Table 1 summarizes the main physico-chemical characteristics of the NPs. Their mean size ranged between 180 and 380 nm with polydispersity values lower than 0.3, corresponding to homogeneous batches of nanoparticles. As expected, it was noted that the loading of PE had an impact on their size. Moreover, in PE-loaded nanoparticles, the ζ potential of these nanocarriers had a lower net negative charge than empty nanoparticles, suggesting the presence of proteins on the surface of the nanoparticles. It is also interesting to note that both the yield of the manufacturing process and the EE) were higher in the lyophilized NPs than in the spray-dried ones.

IgE, cytokine profile & anaphylaxis during the challenge

CD1 mice were selected from the previous study as being able to get sensitized with orally administered peanut. Sensitized CD1 mice were then treated with the formulations containing PE and challenged a week later (Figure 4). Total IgE levels decreased following the immunization, with the largest drop being recorded in the group treated with SD-nanoparticles (Figure 4). As expected, control mice (unsensitized) did not experience symptoms of anaphylaxis. Sensitized mice, either untreated or treated with free PE, had a similar mortality rate (67%), whereas the mortality dropped to 33% in the PE-NP-SD nanoparticle-group (Figure 4). Live mice had high levels of IFN-γ, IL-17 and IL-10. In contrast, dead mice produced more TNF-α (Figure 5).

Discussion

Relevant animal models are of interest in allergy research to test the efficacy of immunotherapies [11–13]. In this study, we compared three mice strains in which peanut-specific IgE was induced by oral sensitization. The most severe anaphylactic reactions induced by the peanut challenge were observed in CD1 mice. To our knowledge, this is the first paper in the available literature describing the conditions capable of orally sensitizing outbred mice to peanut. The same administration schedule failed at sensitizing inbred BALB/c and C57BL/6 mice, which are widely used by other authors [19]. The genetic background of inbred mice strains is important in terms of the Th balance toward a Th1, Th2 or Th17 phenotype, conditioning a high (Th2) or low (Th1, Th17) IgE response. In mice, IgE and IgG1 are induced by IL-4 and IL-13 secreted by Th2 cells. Th1 cells produce IFN-γ, which, in turn, downregulates Th2 and mediates IgG2a production. Th17-derived cytokines have been described as good biomarkers for tolerance to food allergens [20]. In this context, T cells from C57BL/6 mice (H-2b genotype) preferably produce Th1 cytokines with high IFN-γ and low IL-4, whereas those from BALB/c (H-2d genotype)

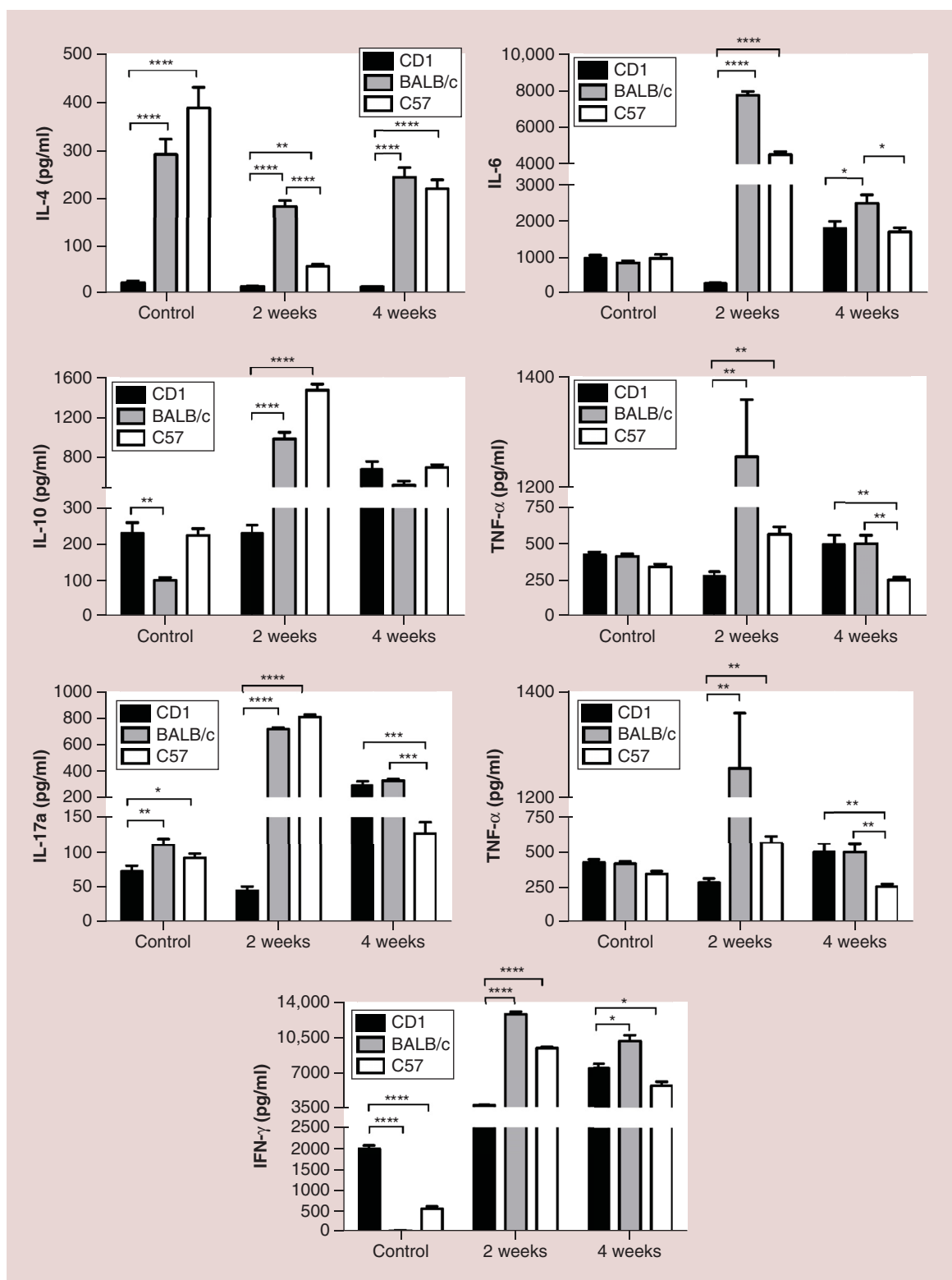


Figure 3. Cytokine response to peanut in sensitized mice. (A) Induced cytokine secretion profiles in control and sensitized CD1 (open box), BALB/c (gray box) and C57BL/6 (black box) mice post-challenge (2 -w2- or 4 -w4- weeks after administering the last sensitizing dose) and subsequent splenocyte stimulation with PE (150 µg/ml). Splenocyte culture supernatants were quantified by cytometry and cytokine levels are shown in picograms per milliliter. **(B)** When CD1 mice were challenged at 4 weeks post-sensitization, spleens from live and dead mice were collected. The figure shows the cytokine levels in both groups: dead (dark boxes) versus live (gray boxes).

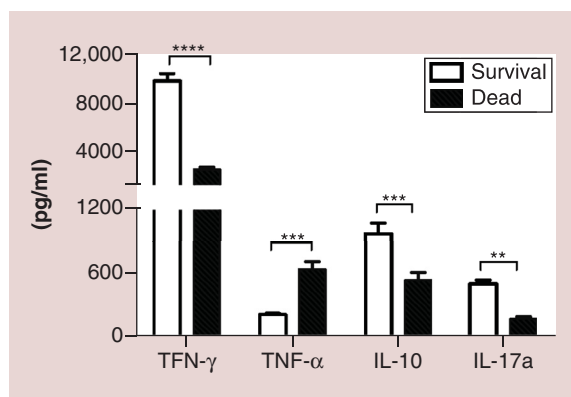


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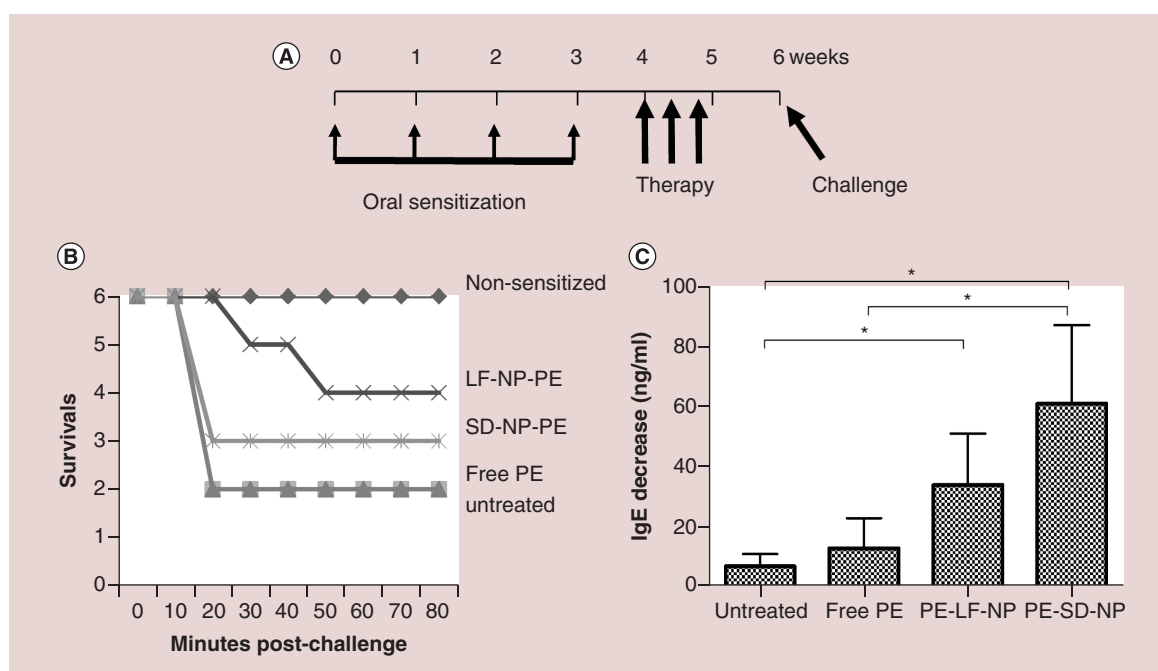


Figure 4. Peanut allergy oral immunotherapy study in sensitized CD1 mice. (A) Experimental setting for the sensitization and therapeutic approaches used. (B) Mortality rates secondary to the peanut challenge in sensitized CD1 mice ($n = 6$): untreated (control animals); Free-PE (mice treated with free peanut extract); SD-NP-PE (mice treated with peanut-loaded spray-dried nanoparticles); LF-NP-PE (mice treated with peanut-loaded lyophilized nanoparticles); non-sensitized challenged mice are also included. (C) Total IgE decrease levels after oral immunotherapy in peanut-sensitized CD1 mice before the challenge.

mice favor Th2 cytokine production with low IFN- γ and high IL-4 [21]. The strains also differ in terms of the ability of their macrophages to be activated and differentiated into M1/M2 phenotypes. Thus, macrophages from C57BL mice preferably produce an M1 phenotype, whereas those from BALB/c mice are typically M2 responders [22,23].

As a result, the C57BL/6 genotype will predispose mice to have a lower susceptibility to allergens, which, in contrast, tends to be higher in BALB/c mice. However, in our experimental conditions, both strains were equally resistant to allergen sensitization. The cholera toxin is widely used in sensitizing animal models as a potent mucosal

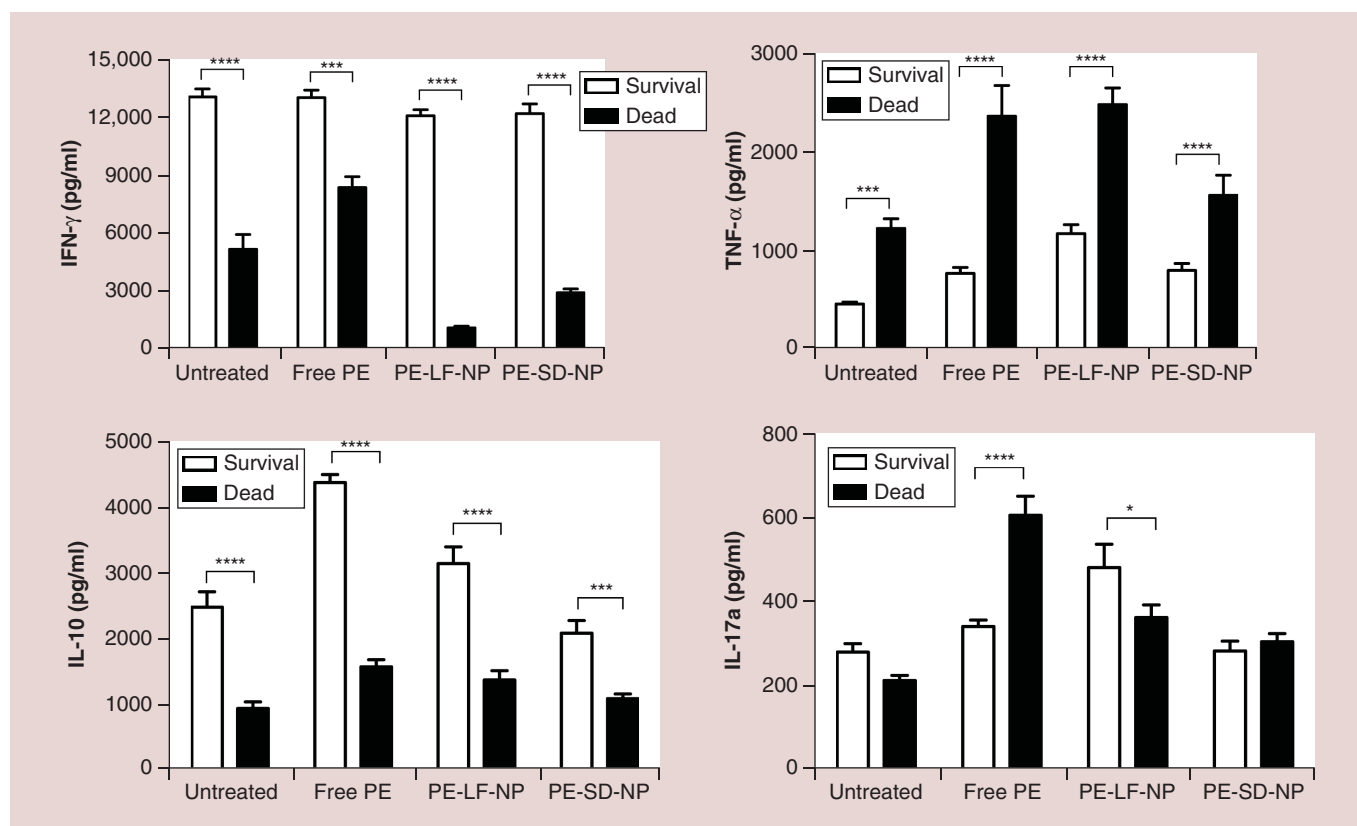


Figure 5. Cytokine profile in peanut-sensitized CD1 mice following oral immunotherapy. Cytokine secretion induced in sensitized CD1 mice following a challenge and subsequent splenocyte stimulation with PE (45 μ g/ml). Spleens of dead (gray boxes) and live mice (dark boxes) were analyzed after the challenge. Splenocyte culture supernatants were quantified by cytometry and cytokine levels are shown in picograms per milliliter.

adjuvant to activate Th2 cells and promote production of IL-4 and IgE antibodies by overcoming the innate immunologic tolerance to ingested antigens [24–26]. The cholera toxin mediates the release of IL-33 by enterocytes, increases mucosal permeability to proteins that have not been destroyed by enzymatic digestion, bile salts or low gastric pH, and also induces a Th2 proallergic promotion by DCs [27]. However, the adjuvant capacity of this cholera toxin also depends on certain particularities of the animal host with respect to its ability to process, recognize and respond to a particular antigen. For instance, the adjuvant effect of the cholera toxin has proven to be greater in C57BL/6 mice than in BALB/c mice [28]. Nevertheless, in our sensitization study it was inefficient at promoting sensitization to peanut proteins. Contrarily, high levels of peanut-specific and nonspecific IgE were linked to a successful sensitization to peanut in CD1 mice, which was also accompanied by a production of high levels of IgG1, IgG2a and IgA. We also observed that fatal anaphylaxis only occurred in sensitized CD1 mice following a challenge with peanut proteins. Furthermore, splenocytes of mice that had died after such challenge showed higher levels of IL-10 and IL-17 than those of live mice, producing increased levels of pro-Th1 cytokines (IFN- γ and TNF- α). In short, all these findings support the use of CD1 outbred mice over the immune-oriented inbred ones. Moreover, the genetically diverse response of outbred mice is expected to generate results that could be better extrapolated to the human population. In contrast, the homogeneous genetic background of inbred mice strains offers a limited repertoire of responses. Thus, despite the current opinion that outbred mice are too variable for their use in most research settings due to being detrimental to the study's statistical power [29], some publications actually support their use [30]. In fact, intragroup variability among individual responses in inbred mice is not an uncommon finding in allergy models [31].

The focus of our study was to analyze oral immunotherapy and, therefore, it was crucial to determine if the sensitization window status in CD1 mice allows for the administration of peanut immunotherapy. Our results showed that the mice became sensitive to peanuts for 2–4 weeks. The immunotherapy administered consisted

in three oral doses of PE contained in polymeric nanoparticles. This platform offered a safety profile, as the allergens were encapsulated within the nanoparticles, thus limiting their interaction with mast cells and reducing the risk of anaphylaxis during the therapy. In addition, the nanoparticles used in this study proved to be safe in previous toxicity studies [16,32]. Moreover, in the particular case of Gantrez AN-based nanoparticles, the use of these devices as adjuvants allowed us to enhance the duration of the mucosal contact and to specifically target innate receptors [17,18,33–35]. In fact, these poly(anhydride) nanoparticles have an innate ability to activate TLR-2 and TLR-4 and induce DC maturation [36], thus demonstrating their efficacy to induce Th1 immune responses [10]. When using ovalbumin as an allergen model, a single oral dose of these nanoparticles given to mice induced high serum levels of specific IgG2a and IgA, as well as high levels of IL-10 [10]. Moreover, these nanoparticles were also able to decrease the anaphylactic symptoms of sensitized BALB/c mice and protect them from death after they had received an intraperitoneal challenge with the native protein [37]. In another study, similar nanoparticles were used to encapsulate peanut proteins and the BALB/c mice immunized with peanut protein-loaded NPs experienced a drop in their levels of peanut-specific IgE, enhanced class switching to IgG2a, and had higher levels of IFN- γ and IL-10 than the control animals [17,18]. Based on our results, we believe that oral immunotherapy based on Gantrez nanoparticles offers protection against an induced anaphylactic shock. There are several publications using nanoparticles and focusing on allergy immunotherapy strategies, but, to our knowledge, this is the first one reporting that protection can be achieved against an oral peanut challenge in a treatment model with established severe peanut hypersensitivity [14,38].

Conclusion & future perspective

Despite the fact that the underlying immune mechanisms of peanut allergy are unclear and controversial [39,40], the results of our study provide information on the peanut-driven sensitization in an outbred murine model that may be considered analogues to clinical peanut-allergic immune pathways. We support CD1 mice as a suitable animal model for future studies. Furthermore, we describe a nanoparticle-based oral immunotherapy capable of partially conferring protection against an induced anaphylactic shock. Our findings suggest that this approach merits further research before application in human subjects.

Executive summary

- With the aim to develop a vaccine formulation against peanut allergy, the majority of studies are conducted in inbred mouse models giving a range of differences in the genetic profile and consequently in the immune system. The studies presented here offer preliminary proof of concept on outbred CD1 as a suitable animal model for immunotherapeutic studies.
- After a dosing schedule with oral commercial peanut butter, the antibody responses and cytokines profile and, what is more, the anaphylaxis induced after a challenge with peanut proteins, indicated that the outbred CD1 was susceptible to sensitization to peanut.
- There is considerable interest in the oral route in immunotherapy because of its convenience, safety and cost. An immunogenic peanut extract encapsulated in nanoparticles made with the polymer Gantrez[®] using the solvent displacement method. Nanoparticles protect the encapsulated antigens, facilitate transport through intestinal mucus, controls its uptake by intestinal mucosae and triggers an immune response after oral delivery.
- Peanut sensitized CD1 mice were protected against severe anaphylactic symptoms induced by peanut challenge by oral immunization with three doses of the nanoparticle formulation.
- CD1 mice may thus provide a suitable model for the peanut sensitization and polymeric nanoparticles show to be a good choice for immunotherapy against peanut allergy.

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