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Use of a Liver-targeting Nanoparticle Platform to Intervene in Peanut-induced anaphylaxis through delivery of an Ara h2 T-cell Epitope

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Abstract

To address the urgent need for safe food allergen immunotherapy, we have developed a liver-targeting nanoparticle platform, capable of intervening in allergic inflammation, mast cell release and anaphylaxis through the generation of regulatory T-cells (Treg). In this communication, we demonstrate the use of a poly (lactide-co-glycolide acid) (PLGA) nanoparticle platform for intervening in peanut anaphylaxis through the encapsulation and delivery of a dominant protein allergen, Ara h 2 and representative T-cell epitopes, to liver sinusoidal endothelial cells (LSECs). These cells have the capacity to act as natural tolerogenic antigen-presenting cells (APC), capable of Treg generation by T-cell epitope presentation by histocompatibility (MHC) type II complexes on the LSEC surface. This allowed us to address the hypothesis that the tolerogenic nanoparticles platform could be used as an effective, safe, and scalable intervention for suppressing anaphylaxis to crude peanut allergen extract. Following the analysis of purified Ara h 2 and representative MHC-II epitopes Treg generation *in vivo*, a study was carried out to compare the best-performing

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Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andre E. Nel is co-founder and equity holder in Westwood Biosciences Inc and Nammi Therapeutics. The remaining authors declare no conflict of interest.

CRediT authorship contribution statement

Qi Liu: Conceptualization, Experimentation, Data Curation and Analysis, Writing Original Draft. Xiang Wang: Experimentation, Data Curation and Analysis. Yu-Pei Liao: Animal Experimentation. Chong Hyun Chang: Nanoparticle Characterization. Jiulong Li: Animal Experimentation. Tian Xia: Conceptualization, Supervision, Writing - review & editing. Andre E. Nel: Conceptualization, Supervision, Writing - review & editing.

Supplementary material: this article's Supplementary material is available at https://www.journals.elsevier.com/nano-today. Additional figures, tables, and results, *e.g.*, outline of the establishment of a tolerogenic nanoparticle platform, additional details of the anaphylaxis, serological and late-phase cytokine responses in the animal model as described in the text.

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Ara h 2 T-cell epitope with a purified Ara h 2 allergen, a crude peanut protein extract (CPPE) and a control peptide in an oral sensitization model. Prophylactic as well as post-sensitization administration of the dominant encapsulated Ara h 2 T-cell epitope was more effective than the purified Ara h2 in eliminating anaphylactic manifestations, hypothermia, and mast cell protease release in a frequently used peanut anaphylaxis model. This was accompanied by decreased peanut-specific IgE blood levels and increased TGF-β release in the abdominal cavity. The duration of the prophylactic effect was sustained for two months. These results demonstrate that targeted delivery of carefully selected T-cell epitopes to natural tolerogenic liver APC could serve as an effective platform for the treatment of peanut allergen anaphylaxis.

Keywords

Peanut allergy; anaphylaxis; immunotherapy; tolerogenic nanoparticle; liver-targeting; Ara h2 T-cell epitope

INTRODUCTION

Food allergy affects 15 million people in the United States, including 8% of children. Of particular concern is the steady rise in the prevalence of peanut allergy. Over the last three decades, the prevalence of peanut allergy in children in the United States has more than tripled. The reasons behind this dramatic increase are unclear, but lifestyles, diet choices, and genetics all play a role. Different from other types of food allergy including cow's milk and egg, peanut allergy tends to be lifelong, with only 20% of affected children outgrowing peanut allergy by their teenage years. In addition, peanut allergy is responsible for most food-induced severe and fatal allergic reactions, including triggering anaphylaxis [1–6]. While avoidance of peanuts can alleviate the problem, the ubiquitous presence of trace amounts of peanuts in food makes complete avoidance difficult, in addition to severely impacting the quality of life for afflicted families. There is an urgent need, therefore, for an approved approach to peanut immunotherapy, with particular emphasis on interventions that can lead to sustained tolerance and prevention of life-threatening reactions to peanut allergens [7, 8].

Despite promising results from clinical trials that use oral or sublingual immunotherapy involving crude peanut protein extracts (CPPE), these interventions have many shortfalls [9–12]. Currently, there is only one FDA-approved oral immunotherapy treatment platform for peanut allergy, Palforzia, which is comprised of a powdered CPPE for oral desensitization therapy [13–16]. However, its shortcomings include the need for daily maintenance therapy, the occurrence of allergic side effects (including life-threatening anaphylaxis), and the lack of evidence that long-term tolerance can be accomplished [14, 16–18]. Thus, there is an urgent need for improved approaches to peanut immunotherapy that provides sustained tolerance, with a view of preventing life-threatening anaphylaxis [7, 8].

An important current trend for lessening the side effects of allergen immunotherapy is to use hypoallergenic proteins that eliminate the presence of IgE binding domains or to select peptide domains that can generate protective immune responses while avoiding IgE-binding epitopes. In particular, there is growing interest in so-called T-cell epitopes, which represent

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antigenic peptides that can be presented to $CD4^+$ T-cells through interaction with type II major histocompatibility (MHC) molecules on antigen-presenting cells (APC) [2, 8]. This includes epitope presentation to naïve $CD4^+$ T-cells, which can be induced to differentiate into $CD4^+/FoxP3^+$ regulatory T-cells (Tregs) upon engagement of T-cell antigen receptors (TCR) with the MHC-II/epitope complex [19]. Regulatory T-cells (Tregs) can suppress allergy and anaphylaxis through a variety of direct and indirect pathways [19]. Not only have O'Heir, Sampson and others identified dominant human T-cell epitopes for the major peanut antigens (Ara h 1, Ara h 2, and Ara h 6), but Aravax is a new startup company looking at the potential of T-cell epitopes being administered epicutaneously to develop a treatment for peanut anaphylaxis [20–25]. The advantage of using peptide antigens is the ease of production, scalability and safety compared to the delivery of whole protein or crude extracts.

One challenge for the PLGA platform is the different physicochemical properties (e.g., molecular weight, size, charge, hydrophobicity vs. hydrophilicity) of the cargo ingredients, which can impact loading capacity33. We will address this by: (i) adjusting the concentrations of the polymer components, antigens, and drugs; (ii) fine-tuning the ratio of aqueous vs. organic components; and (iii) adjusting the emulsifying parameters (e.g., duration of sonication and mechanical stirring).

We have recently demonstrated that biocompatible and biodegradable ~200 nm poly (lactide-co-glycolide acid) (PLGA) nanoparticles can be used for liver targeting and induction of systemic tolerance to the egg white protein, ovalbumin (OVA), prophylactically and therapeutically (Fig. 1 and Fig. S1A) [26, 27]. PLGA was selected due to its (i) excellent biocompatibility and lack of toxicity; (ii) ability to tune cargo loading and biodegradation rate by adjusting the molecular weight, concentration and ratio of the co-polymers; (iii) potential to modify surface properties for improved interactions at the nano/bio interface, and with (iv) adjusting the emulsifying parameters (e.g., duration of sonication and mechanical stirring) [26, 27]. As such, PLGA has been approved by the US Food and Drug Administration (FDA), as well as the European Medicines Agency (EMA), for orthopedics fixation, surgical sutures, and parenteral sustained-release drug delivery systems [28, 29]. The liver is an immune-privileged organ, capable of generating tolerogenic responses to food allergens [30, 31]. While a variety of liver cell types (Kupffer cells, sinusoidal endothelial cells, hepatocytes, etc.) contribute to establishing a natural tolerogenic environment, liver sinusoidal endothelial cells (LSECs) are endowed with the specialized ability to act as Treg-inducing antigen-presenting cells (APC) [32–34]. This includes a role for LSEC scavenger receptors, which are engaged in the endocytosis of foreign/damaged proteins and particulate matter [32, 34–36]. This also includes antigenic proteins that can be presented by the MHC-II complex on naïve T-cells, allowing for their activation and differentiation into antigen-specific $Foxp3^+$ Tregs [32, 34–36]. Thus, by using a covalently attached scavenger receptor Apo B100 peptide (ApoBP) to the PLGA surface, we have successfully constructed LSEC-targeting nanoparticles, as demonstrated by *in vivo* IVIS imaging studies, as well as the use of confocal microscopy, which demonstrates co-localization of the labeled particles with LSECs (Fig. S1B) [26, 27]. Moreover, we have demonstrated that the delivery of intact ovalbumin (OVA) OVA or a MHC-II binding OVA T-cell epitope (amino acids 323–339) to LSECs are capable of generating antigen-specific

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Foxp3$^+$ T-cells, which could successfully intervene in allergic airway inflammation and anaphylaxis, including through intraperitoneal and oral sensitization. (Fig. 1) [26, 27]. The same response outcome could not be achieved by an MHC-I binding OVA peptide (OT-I), confirming the importance of MHC-II interactions in selecting T-cell epitopes to treat food allergy.[27]

In the current study, we asked whether our tolerogenic nanoparticle platform could be used to intervene in the generation of anaphylaxis, following oral sensitization by a crude peanut protein extract (CPPE). We hypothesized that the selection of dominant T-cell epitopes from the major peanut allergen, Ara h 2, could provide tolerogenic cargo to accomplish durable suppression of allergic inflammation, mast cell release, and anaphylaxis in response to CPPE [20]. Use of the Immune Epitope Database (IEDB) and NetMHCII-2.3 to provisionally select MHC-II binding Ara h 2 epitopes, which were further refined by peptide selection for optimal particle loading and screening for the ability to generate CD4$^+$/Foxp3$^+$ Tregs *in vivo*. The dominant epitope (amino acids 59–73) was subsequently used to study the impact on the generation of anaphylaxis by CPPE in C3H/HeJ mice. Prophylactic administration was shown to be effective for at least two months. Controls included purified Ara h 2 protein, which was moderately effective, and a control sequence from an IgE binding sequence, which had no effect. We further demonstrated that the response to the purified Ara h 2 protein and the dominant epitope was successful in generating TGF-β producing CD4$^+$CD25$^+$FOXP3$^+$ Tregs. All considered, these results demonstrate the possibility of using a dominant Ara h2 T-cell epitope to obtain durable control of anaphylaxis in response to a crude peanut extract.

METHODS

Antigen cargo selection and synthesis of liver-targeting PLGA nanoparticles

The NIAID Immune Epitope Database (IEDB) and Analysis Resource (http://tools.iedb.org/mhcii/) was used to find possible T-cell epitopes for a dominant peanut allergen based on the ranking score of peptide interactions with murine MHC-II alleles. The IEDB recommends making selections based on consensus percentile ranking of the top 10% of predictions, performed by the “IEDB Recommended” approach that is premised on tools such as NetMHCII 1.1, NetMHCII 2.3, CombLib and Sturniolo as well as NetMHCIIpan. By entering the complete amino acid (aa) sequence, for the *Arachis hypogaea* protein, Ara h2, into the database, a consensus percentile ranking score for a series of 15-mer peptides were obtained for initial prediction making. The focus on Ara h 2 is based on its identification as the dominant peanut allergen that is recognized by IgE antibodies in the majority of peanut allergy patients [23]. Four top-ranking 15-mer peptide candidates with the highest binding affinity for murine MHC-II alleles (H2-IAb, H2-IEd and H2-IAd) were selected. The IEDB selections were also compared to the predicted interactions of these peptide sequences with the H2-IAk and H2-IEk alleles in the C3H/He mouse strain (peanut anaphylaxis model), making use of the online tool, NetMHCII-2.3 (https://services.healthtech.dtu.dk/service.php?NetMHCII-2.3). For comparative analysis, we also selected a control peptide that is frequently characterized as an IgE binding domain but cannot be categorized as a T-cell epitope [37]. Additional peptide analysis was undertaken to assess encapsulation

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efficacy by poly (lactide co-glycolic acid) (PLGA) based on peptide solubility and charge criteria. These physicochemical properties were assessed by using an online tool (https://web.expasy.org/protparam/) to calculate the isoelectric point (pI) at neutral particle pH, as well as peptide "grand average hydropathicity values" (GRAVY). Positive GRAVY values are indicative of more hydrophobic sequences, while negative values indicate hydrophilicity. Generally speaking, hydrophilic sequences with an isoelectric point (pI) that deviates from 7.4 are predictive of peptides that exhibit higher loading capacity.

Nanoparticles were synthesized using the double-emulsion (w/o/w) method combined with solvent evaporation, as previously described and explained in the Supplementary material (Fig. S1C) [26, 27]. Particles were separated and washed by centrifugation (15,000g, 10min). Hydrodynamic size and surface potential were measured using dynamic light scattering (DLS), with visualization of the particle size and morphology by scanning electron microscopy (SEM). Covalent surface conjugation of the stabilin-1 binding ApoB peptide sequence (RLYRKRGLK, containing a GGC tag) to allow LSEC targeting was performed as previously described [26, 27]. ApoB conjugation was verified by performing 1H NMR, aimed at detecting the presence of tyrosine residue in the attached ApoB peptide. In addition to the T-cell epitope loading, we also synthesized particles encapsulating the purified Ara h2 protein (Indoor Biotechnologies), the control peptide (CP) and a crude peanut protein extract (CPPE). The CPPE was prepared as described previously [38]. The allergen loading capacity of the particles (i.e., T-cell epitopes, CPPE, and purified Ara h2) as well as the conjugation efficacy of the ApoB ligand was determined by a bicinchoninic acid assay (BCA assay) (Fig. 2). The particle allergen loading capacity was determined by incubating 2 mg lyophilized particles in 1 mL of 0.1M NaOH solution under gentle shaking overnight. Allergen concentration in the solution was determined by BCA assay according to the manufacturer's instructions. Allergen dissolved in 0.1M NaOH solution was used to establish a standard curve, and blank PLGA nanoparticles were used as the control. Particle stability was assessed by incubating the particles in PBS at 4 °C for up to five weeks, during which aliquots were removed periodically to assess particle size by dynamic light scattering.

Assessment of the tolerogenic nanoparticles in an oral sensitization and anaphylaxis model

Six-week-old female C3H/HeJ mice, purchased from Jackson Laboratory (Bar Harbor, ME) were maintained in pathogen-free facilities at the Division of Laboratory Animal Medicine at UCLA. Animal experimentation followed the guidelines established by the National Society for Medical Research, as in "Principles of Laboratory Animal Care".

In the prophylactic approach, animals were divided into 5 groups (n=6). Three of these groups represent sensitized animals, prior treated with particles encapsulating CPPE, Ara h2, and Arah2 epitope (#4). The other two groups were comprised of sensitized animals not receiving pretreatment, as well as a control group that was unsensitized (Fig. 4A). Oral sensitization to CPPE was performed by oral gavage, administering 200 μL of PBS containing 2 mg CPPE and 10 μg cholera toxin, once a week for 3 weeks. This was followed by 300 μL of the suspension containing 5 mg CPPE and 10 μg cholera toxin in week 4 before animals were challenged intraperitoneally (IP) with 200 μg CPPE (in PBS) on week

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6. IP instead of oral challenge was used to obtain a more predictable early-onset response [38, 39]. Prophylactic treatment was administered intravenously (IV) by injecting 500 μg of a particle suspension containing 25 μg, 4 μg and 4 μg of CPPE, Ara h2, and Ara h2 epitope (#4), respectively, on two occasions, seven days apart (Fig. 4A). Anaphylaxis was assessed by recording core temperatures with a rectal probe (Kent Scientific) as well as performing visual scoring (by three independent observers) of physical manifestations of anaphylaxis, using a 0–5 point grading scale (shown in Fig. S2A) [40, 41]. Blood was collected for measurement of peanut-specific IgE, IgG1, IgG, and IgG2b serum antibody titers, using an ELISA procedure, previously reported [27, 41]. Peritoneal lavage fluid was collected after 48 hours to assess the late-phase pro-inflammatory response by measuring IL-4, IL-5, and TGF-β levels by an ELISA (R&D) procedure [42, 43]. We also assessed mouse Mast Cell Protease-1 (mMCPT-1) levels by an ELISA procedure, using the manufacturer's instructions (Thermo Fisher). It has previously been demonstrated that mMCPT-1 levels can be measured for a sustained period after the acute phase anaphylactic response due to plasma protein binding [27, 44–46].

Therapeutic intervention in already-sensitized animals (n=6) followed the same outline, except that tail vein injection of 500 μg nanoparticles, containing 25 μg CPPE or 4 μg each of Ara h2, Arah2 epitope, and the control peptide (CP), were administered during weeks 4 and 5, prior to peritoneal challenge (Fig. 5A). Anaphylaxis monitoring and endpoint analysis proceeded as above.

To assess the durability of the tolerogenic response, mice (n=6) were prophylactically injected with 500 μg PLGA particles containing 4 μg of Ara h2 epitope ($NP^{Ara\ h2\ Epi(\#4)}$/ApoBP) on two occasions during weeks 1, 2, and 4, in advance of sensitization. The mice were subsequently challenged and assessed as described above.

Assessment of the generation $CD4^+CD25^+$ $FOXP3^+$ Regulatory T-cells

Mice (n=4) received one-time IV administration of 500 μg of $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP, $NP^{Ara\ h2\ Epi(\#2)}$/ApoBP and NP^{Arah2}/ApoBP and were sacrificed for collection of splenocytes after 7 days. Splenocytes were magnetic bead sorted using the $CD4^+CD25^+$ Regulatory T-Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). For the performance of flow cytometry, the sorted cells were used to assess the percentage of $CD4^+CD25^+$ T-cells with $FoxP3^+$ expression, using a cellular staining procedure with anti-FoxP3-PE, anti-CD25-APC and anti-CD4-AlexaFluor488 antibodies and flow cytometry. For the performance of ELISPOT assays, sorted cells were used for seating onto tissue culture plates to quantify the frequency of TGF-β producing cell clusters according to the manufacturer's instructions (R&D).

Statistical analysis

Statistical analysis was performed by one-way ANOVA or the Student t test on GraphPad Prism 7 software (GraphPad Software, La Jolla, CA) to determine the statistical differences between different groups. The results were expressed as mean ± SEM and statistical significance thresholds were set at *$p<0.05$; **$p<0.01$; ***$p<0.001$.

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RESULTS

Synthesis and characterization of tolerogenic PLGA nanoparticles

We used the IEDB resource to identify 15-mer peptides with binding affinity for the murine MHC-II alleles. The IEDB currently recommends making selections based on a consensus percentile rank of the top 10%. When using the IEDB recommended method for the murine alleles, H2-IAb, H2-IEd and H2-IAd, including the use of NetMHCII 1.1 and NetMHCII 2 analyses for obtaining 50% inhibition of binding to a peptide probe, we obtained 475 peptide sequences, from which four top-ranking, non-overlapping Ara h2 sequences were selected based on IC50 values for scoring interference in the binding of a fluorescent probe (NetMHCII 1.1 and NetMHCII 2 analyses) (Fig. 2A). These peptides were regarded as prospective T-cell epitopes, which were synthesized and also used to confirm binding affinity to H2-IAk and H2-IEk alleles expressed in C3H/He mouse, using the online tool, NetMHCII-2.3 (https://services.healthtech.dtu.dk/service.php?NetMHCII-2.3). We also selected a 10-mer peptide (HASARQQWEL) for synthesis and inclusion as a control peptide (CP) sequence, which also appears in a previously characterized IgE binding domain (Fig. 2A) [37]. Additional GRAVY analysis and assessment of peptide isoelectric point (pI) were used to evaluate PLGA encapsulation efficacy, which is premised on peptide solubility and charge. These analyses demonstrated that the Ara h2 epitopes #2 (LRNLPQQCGLRAPQR) and #4 (SYGRDPYSPSQDPYS) as well as CP exhibit favorable loading characteristics, which was confirmed by particle BCA analysis (see inserted table, Fig. 2A). Noteworthy, epitope (#4) overlaps with a human peptide sequence DSYERDPYSPSQDPYSPSPY, delineated by Prickett *et al.* as exhibiting high binding affinity for the HLA-DRB3*01:01 allele [23]. Epitopes #2 and #4, as well as the CP were then used for particle encapsulation, using the double emulsion synthesis method (Fig. S1C). We also synthesized nanoparticles encapsulating CPPE as well as purified Ara h2. All the particles were subsequently used for covalent attachment of the LSEC-targeting ApoB-100 peptide sequences, as previously described by us (Fig. S1C) [26]. This yielded nanoparticle batches that were designated: NP^{CPPE}/ApoBP, $NP^{Ara\ h2}$/ApoBP, $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP, $NP^{Ara\ h2\ Epi(\#2)}$/ApoBP and $NP^{Ara\ h2\ CP}$/ApoBP (Fig. 2B). Fig. 2B displays the physicochemical characteristics of the particles, including size, size distribution, zeta potential, ligand, and cargo peptide contents (BCA assay). Fig. 2C demonstrates the uniformity of particle morphology and size by SEM characterization. The 200 nm particle size and negative charge are important for LSEC targeting and uptake by receptor-mediated uptake in clathrin-coated vesicles, as previously described by us [26]. The stability of synthesized tolerogenic NPs remained high for up to 35 days, without showing any changes in hydrodynamic sizes (Fig. S1D). Particle stability was also maintained during lyophilized storage for several months. Fig. 2D shows the assessment of the proton NMR spectra of the particles, including the presence of the lactide (−CH and $-CH_3$) and glycolide ($-CH_2$) peaks in the PLGA backbone, along with the presence of an ApoB tyrosine peak (~7 ppm) to confirm successful peptide conjugation. The ligand density in these particles has been chosen based on previous discoveries to achieve optimal LSEC targeting *in vivo* [26]. This is clarified by new IVIS imaging and confocal data shown in Figure S1B, which depicts data obtained in 2 animals, 24 h after IV injection of 500 μg ApoB-decorated PLGA nanoparticles containing 25 μg Dylight680-labeled antigen. Animals were sacrificed before explanting hearts, livers, spleens, lungs,

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and kidneys for *ex vivo* imaging, as previously described [26, 27]. The panel at the top of Figure S1B depicts the *in vivo* and *ex vivo* IVIS imaging data, while the confocal images at the bottom depict particle localization in the LSECs. In this particular experiment, we obtained a co-localization index of 60%, which is considerably higher than our historical co-localization index of 21% for non-ApoBP decorated particles [26, 27].

Flow cytometry and ELISPOT assays demonstrate $CD4^+CD25^+$ $FOXP3^+$ Treg generation

Previous studies looking at OVA-induced allergic inflammation and anaphylaxis demonstrated that T-cell epitope delivery by tolerogenic nanoparticles was equally or more effective than the intact egg white allergen in promoting Treg recruitment and TGF-β release in the lung and peritoneal lavage fluid [26, 27]. To determine whether targeted nanoparticles delivering purified Ara h2 or Ara h2 epitopes (#2 and #4) can generate Tregs in C3H/HeJ mice, $CD4^+CD25^+$ T-cells were isolated from the spleen, using magnetic bead separation (Fig. 3A). To assess the percent enrichment of $FoxP3^+$ cells in the positively selected $CD4^+CD25^+$ cells, flow cytometry was used to assess the percent enrichment of Tregs in the spleen. The results demonstrated significant increases in the % $FoxP3^+$ cells in animals treated with $NP^{Ara\ h2\ Epi(\#2)}$/ApoBP ($p<0.001$), $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP ($p<0.001$), and NP^{Arah2}/ApoBP ($p<0.01$), respectively (Fig. 3B). We also used sorted cells to assess the number of TGF-β spot-forming colonies in Experiment #1 on an ELISPOT plate (Fig. 3B). This demonstrated a significant increase in the number of TGF-β spot-forming colonies in response to the Ara h2 T-cell epitope ($p<0.001$) as well as purified Ara h2 ($p<0.01$) (Fig. 3B). Due to the discontinuation of the ELISPOT reagent during the Covid-19 pandemic, it was not possible to assess TGF-β production for epitope #2

Prophylactic effect of tolerogenic nanoparticles on peanut-induced anaphylaxis

An oral anaphylaxis model was established, using CPPE sensitization and boosting, as shown in Fig. 4A [38]. Animals receiving IV administration of NPs incorporating CPPE, purified Ara h2 and Ara h2 epitope (#4), on 2 occasions 7 days apart, were subsequently sensitized by CPPE gavage. Following the intraperitoneal challenge with CPPE on week 6, anaphylaxis was monitored by recording rectal temperatures every 10~20 min, in addition to employing three independent observers to record anaphylaxis scores. The scoring criteria were as follows: 0 = no symptoms; 1 = scratching and rubbing of the nose and head; 2 = puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with an increased rate of breathing; 3 = wheezing, labored respiration, and cyanosis around the mouth and the tail; 4 = no activity after prodding or tremor and convulsion; 5 = mortality. The sensitized animals receiving no pre-treatment experienced a 6°C core body temperature drop after 60 minutes (Fig. 4B). In contrast, no hypothermia was seen in the non-sensitized (control) group or animals prior treated with the Ara h2 epitope nanoparticles. While there was a lesser ($p<0.01$) hypothermic response to purified Ara h2, encapsulated CPPE had no protective effect. Monitoring of the physical manifestations of anaphylaxis further demonstrated significant improvement ($p<0.001$) of the anaphylaxis score during prophylactic treatment with the Ara h2 epitope (#4) compared to sensitized animals receiving no pretreatment (Fig. 4C). Only one animal in the prophylactic group demonstrated nose and head rubbing during maximal hypothermia (60 min), compared to untreated animals, exhibiting lack of activity after prodding, wheezing and labored

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respiration (median score of 3.5). In contrast, animals prior treated with CPPE and Ara h2 NPs showed lesser but significant ($p<0.05$) improvement in anaphylaxis scores (medium scores of 2–3) (Fig. 4C). These treatment responses also agreed with the anaphylaxis scores at 30 and 100 min (Fig. S2A).

In addition to impacting physical disease manifestations, pretreatment with $NP^{Ara\ h2}$/ApoBP and $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP significantly reduced the respective peanut-specific IgE levels ($p<0.01$ and $p<0.05$, Fig. 4D). In addition, $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP administration also decreased peanut-specific IgG1, IgG, and IgG2b, antibody titers (Fig. S2B–C). Assessment of mast cell protease (mMCPT-1) release to the serum also showed that $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP pretreatment could prevent mast cell degranulation in mice experiencing an anaphylactic response ($p<0.001$), with lesser effectiveness of encapsulated Ara h2 and CPPE (Fig. 4E). Please note that mMCPT-1 levels remain elevated in the bloodstream compared to other mast cell release products (e.g., tryptase) as a result of mMCPT-1 binding to plasma serpins [44–46]. Two days after the anaphylactic episode, the animals were sacrificed and peritoneal lavage was performed to assess the delayed phase TH2 cytokine and TGF-β levels. Compared to the high IL-4 values (Fig. 4F) in sensitized non-TNP treated mice, pretreatment with $NP^{Ara\ h2}$/ApoBP and $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP showed significant reductions ($p<0.001$) in cytokine levels (Fig. 4F). The same was true for IL-5, except that the response was not as dramatic for Ara h2 (Fig. S2D). Pertaining to TGF-β, pretreatment with $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP had a highly significant effect ($p<0.001$) on increasing peritoneal fluid levels, which was slightly less impactful ($p<0.01$) than during $NP^{Ara\ h2}$/ApoBP (Fig. 4G). These results are in agreement with the impact of the same nanoparticles on Treg generation in the spleen, using ELISPOT assays (Fig. 3 B–C). We suggest that TGF-β release to the peritoneal fluid reflects Treg activity in the interest ions and abdominal cavity.

Post-sensitization impact of the tolerogenic nanoparticles on peanut-induced anaphylaxis

In this experiment, particle treatment commenced after sensitization to CPPE (Fig. 5A). The study included the addition of a new group, namely the administration of nanoparticles encapsulating a peptide that overlaps with an IgE epitope, thereby acting as a control for the T-cell epitope. Following CPPE challenge, the same anaphylactic and biomarker manifestations were assessed as described above. This demonstrated that post-sensitization treatment with $NP^{Ara\ h2\ Epi\ (\#4)}$/ApoBP had comparable but lesser efficacy than prophylactic administration, while other treatments were much less effective. Noteworthy, there was no adverse reaction in response to the IgE binding peptide, which lacks a tolerogenic effect. Core temperature recording demonstrated that while $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP treatment effectively prevented hypothermia, while other treatments had no preventative effect (Fig. 5B). This was also reflected in anaphylaxis scores at the time of maximum hypothermia (60 min), with 3 $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP treated animals exhibiting facial puffiness and reduced activity (level 2), while the rest had no anaphylactic manifestations (level 0) (Fig. 5C). Other treatments solicited level 3 anaphylactic responses, compared to level 4 in non-treated animals (Fig. 5C). Comparable anaphylaxis scores were recorded at 30 and 100 mins (Fig. S3A).

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In agreement with the anaphylactic manifestations, mice treated with $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP showed a significant reduction in mMCPT-1 release ($p<0.001$), while NP^{CPPE}/ApoBP ($p<0.05$) and $NP^{Ara\ h2}$/ApoBP, ($p<0.01$) also exerted impactful effects (Fig. 5D). In addition, TGF-β levels were significantly ($p<0.01$) increased by $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP compared to other treatment groups (Fig. 5E). A less pronounced (compared to prophylactic treatment) but still a significant decline in peanut-specific IgE, IgG, and IgG1 antibody titers and TH2 cytokine (IL-4, IL-5) levels were observed in response to post-sensitization $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP administration (Fig. S3B–D).

Durable tolerogenic effect of prophylactically administered nanoparticles

Durable immune tolerance is the ultimate goal for food allergy immunotherapy. In order to explore the duration of the prophylactic response to $NP^{Ara\ h2\ Epi(\#4)}$/ApoBP, the nanocarrier was injected twice during weeks 1, 2 and 4 in advance of commencing CPPE sensitization and anaphylaxis challenge (Fig. 6A). The results demonstrated protection against the development of hypothermia and anaphylactic manifestations in all the pretreated groups, with marginally higher anaphylactic scores in animals receiving pre-treatment 4 weeks prior to the commencement of sensitization (Fig. 6B and C). Two out of six mice in the latter treatment group showed a mild reduction in activity, with wheezing and labored respiration, compared to zero or minor manifestations in other pre-treatment groups. This was comparable to anaphylactic scores at 30 and 100 mins (Fig. S4A). All treatment groups showed reduced mMCPT-1 release to the serum, with animals receiving particle administration 1 week prior to commencement of sensitization, exhibiting the most prominent decline (Fig. 6D). Similar results were also obtained in the assessment of anti-peanut antibody titers, TH2 cytokine, and TGF-β production (Fig. S4B–E). Thus, although there is a tendency for response decline with lengthening of the pre-treatment interval, there is a statistically significant suppression of anaphylaxis for at least two months between the time of initial nanoparticle administration and allergen challenge.

DISCUSSION

In this communication we demonstrate that encapsulated delivery of an Ara h2 T-cell epitope to the liver is capable of triggering a linked tolerogenic response, leading to successful suppression of the anaphylactic response to CPPE in an oral challenge model. Moreover, we demonstrate that the tolerogenic nanoparticle platform is capable of generating Tregs and TGF-β production, with a significant impact on allergic inflammation and mast cell release. The epitope-induced response was more robust than encapsulated delivery of purified Ara h2, a control peptide and CPPE. Prophylactic administration protected against anaphylaxis for at least two months.

While a number of NP platforms, including PLGA nanoparticles, have emerged for therapeutic intervention in autoimmune and allergic disorders [47], our approach of targeted antigenic cargo delivery to LSECs is unique [26, 27]. Major LSEC functions include the removal of macromolecules and particulate antigens from the blood, in addition to the ability to act as tolerogenic APC, capable of generating $CD25^{+}$ $FoxP3^{+}$ Tregs [32, 35]. While clearance of waste products from the blood has traditionally been attributed to the

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phagocytic capabilities of Kupffer cells[48], we now understand that LSECs also exhibit particle uptake through clathrin-mediated endocytosis that involves stabilin, mannose and scavenger receptors [49, 50]. Our nanocarrier targets the stabilin-1 receptor via the attached ApoB peptide ligand, as demonstrated by previous IVIS imaging and confocal studies [26, 27] as well as the new, previously unpublished data conveyed in Fig. S1B. Although it was shown that non-targeted PLGA nanoparticles in the size range 200–300 nm can be taken up by both Kupffer and LSECs [50, 51], we confirmed that covalent attachment of ApoBP (Fig. 2D) improves the co-localization index with stabilin-1 expressing LSECs *in vivo* (Fig. S1B) [26], The natural tolerogenic effect of these cells, including their ability to bind exogenous TGF-β, allows more robust $FoxP3^+$ Treg generation in response to antigenic cargo [35]. This agrees with the data demonstrating the generation of Ara h2-specific Tregs and TGF-β production (Fig. 3). While also effective in generating tolerogenic effects, the full-length Ara h 2 protein is not as effective as the epitope, which may be a reflection of more effective antigen presentation by MHC-II complex, in addition to the fact that the relative abundance of particular peptide sequence has a 10-fold molar excess in the epitope nanoparticles compared to the content in the intact protein.

IEDB-based predictions to identify potentially useful peptide sequences for therapeutic intervention in peanut allergy in humans have been published for dominant peanut allergens, including Ara h1 and h2 [21–25, 52]. This includes prediction making of lead peptide sequences with high binding affinity for HLA-DR, -DP and -DQ to achieve widespread population coverage, as well as avoiding IgE binding sequences that could adversely impact therapeutic safety [21, 23, 24, 37]. The initial proof-of-principle for the translational use of allergen-specific T-cell epitopes was originally provided by the performance of immunotherapy experiments in animals sensitized to house dust mite and cat allergen proteins [53, 54]. Noteworthy, these studies demonstrated that administration of a single dominant T-cell epitope can introduce specific non-responsiveness to repetitive intradermal injections of the same peptide, as well as the complete allergen extract [22, 53]. The robustness of the T-cell epitope approach has also been validated in other allergen models, evolving to the use of short peptide mixtures for intradermal administration [53, 55]. Currently, a phase 1 clinical trial is ongoing in Australia to assess the safety and tolerability of PVX108 (Aravax), an intradermal vaccine delivering a mixture of Ara h 1 and h2 epitope sequences (Australian New Zealand Clinical Trials Registry ACTRN 12617000692336) [56]. While the efficacy and durability of this vaccine still await the outcome of the clinical trial, there has been a tendency in previous clinical trials for the allergen immunotherapy response to decline after treatment discontinuation, in addition to the tendency to become re-sensitized to the same allergen [57]. Similarly, it is necessary during peanut oral immunotherapy (e.g., Palorzia) to provide daily peanut allergen ingestion to maintain the desensitized state [13, 15].

We propose that the use of a liver-targeting platform could provide a more durable and robust state of non-responsiveness to food allergens by engaging a natural tolerogenic environment that facilitates Treg regeneration. The unique properties of LSECs, which occupy a large surface area in contact with blood coming from the portal and systemic circulations, as well as acting as natural tolerogenic APC, hold great promise for a sustained immune suppressive effect. This is supported by the disappearance of oral-induced

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tolerance to food allergens after portocaval shunting [34]. While our results show that it is possible to prevent anaphylaxis for up to two months, additional research is required to address the duration of post-sensitization tolerance, the number of doses that need to be administered and the dosing frequency to maintain a tolerogenic effect. Our platform is quite adaptable, with the possibility to improve response duration, where required, by co-delivery of pharmaceutical agents that augment Treg stability/potency, as well as changing the polymer composition for slowing cargo release for weeks to months [58, 59]. We have also documented that the tolerogenic platform is very safe, with no evidence of cytotoxic or systemic effects by any of the particle components, active pharmaceutical ingredients or surface composition. In consideration of the clinical translation of the platform, it will be necessary to consider the differences between the murine and human immune system, in particular, the impact of the three highly polymorphic HLA loci (HLA-DR, HLA-DP, HLA-DQ), by which MHC II-mediated T-Tcell epitope presentation in humans [1, 2]. Thus, to identify lead peptides to include as potential tolerogenic cargo, we will consider the extensive epitope mapping and peripheral blood T-cell studies undertaken in humans to make the best choices. We have already referred to the choices made by Aravax in selecting a mixture of Ara h 1 and h2 epitope for subcutaneous vaccination [56]. While the exact number of peptides to include in the tolerogenic nanoparticles vaccine remains to be determined, our technology allows peptide combinations, which will also be investigated in animal studies. It is also possible to use multiple epitopes spliced together with appropriate linkers and splicing elements.

A shortcoming of our study is the lack of assessing other major peanut allergens and their corresponding epitopes, e.g., Ara h 1 and Ara h 6, due to the logistical constraint of the number of animals that can be investigated in the labor-intensive oral anaphylaxis model. However, we have identified additional T-cell epitopes in these allergens and will perform further comparative studies, including the encapsulation of epitope combinations. This may require an adaptation of the techniques to assess the generation of multiple epitope-specific Tregs. These studies can also be supplemented by the exploration of additional antigen-specific immune suppressive cells that play a role in the suppression of allergic effects, other than Tregs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Ara h 2	*Arachis hypogaea* 2
CPPE	Crude peanut protein extract
PLGA	Poly (lactide-co-glycolide acid)
Tregs	Regulatory T-cells
LSECs	Liver sinusoidal endothelial cells
APC	Antigen-presenting cell

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OVA	Ovalbumin
MHC II	Major histocompatibility type II
ApoBP	Apo B100 peptide
IEDB	Immune Epitope Database
GRAVY	Grand average hydropathicity values
pI	Isoelectric point
DLS	Dynamic light scatterin
SEM	Scanning electron microscopy
BCA	Bicinchoninic acid
mMCPT-1	Mouse Mast Cell Protease-1

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Highlights:

- Epitope mapping was used to identify T-cell epitopes in the dominant peanut allergen, Ara h2, for poly(lactide-co-glycolide acid) (PLGA) nanoparticle encapsulation and subsequent delivery to liver sinusoidal endothelial cells through a ligand that targets stabilin receptors.
- Targeted delivery of the dominant Ara h2 T-cell epitope to liver sinusoidal endothelial cells LSEC) was capable of inducing antigen-specific $FoxP3^{+}$ regulatory T cells.
- LSEC-targeting nanoparticles could intervene in the generation of anaphylaxis in a peanut oral anaphylaxis animal model, prophylactically as well as therapeutically.
- The tolerogenic effect lasts at least two months, offering a therapeutic intervention approach to alleviate peanut anaphylaxis in the clinic.

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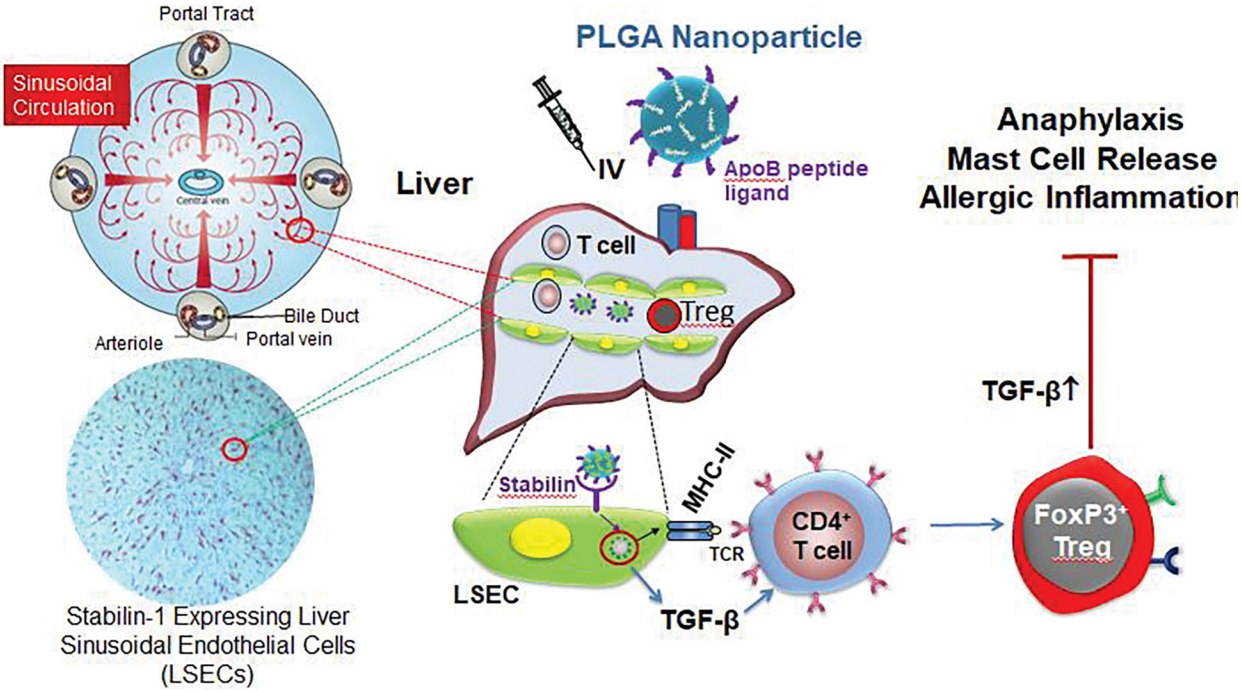

Figure 1. Scheme to explain the features of the tolerogenic nanoparticle platform in terms of its intended use to treat peanut anaphylaxis.

Previous studies have demonstrated that following the encapsulation of whole OVA or a MHC-II bound OVA peptide in PLGA nanoparticles, the carriers can be targeted to LSECs by surface attachment of an ApoB peptide sequence.[26, 27] These results also confirmed by the data presented in Fig. S1C and Fig. 2D. The ApoB ligand allows particle uptake by stabilin-1 receptors, which are expressed on LSECs. The degradation in the endocytic compartment allows the release of hydrolyzed peptides and epitopes to reticuloendothelial vesicles, carrying MHC-II molecules to the cell surface. T-cell epitope presentation to the TCR of naïve $CD4^{+}CD25^{-}$ T-cells, is capable of inducing their differentiation to antigen-specific $CD4^{+}/CD25^{+}/FoxP3^{+}$ regulatory T-cells (Tregs) (Fig. 3A). The APC function of the LSECs is assisted by tolerogenic cytokines (IL-10, TGF-β) produced or recruited to the cell surface. Subsequent Treg release and recruitment to the lung or gut-associated lymphoid tissue has proven quite effective for suppressing allergic lung inflammation and anaphylaxis in response to OVA or OVA epitopes, both prophylactically or post-sensitization [26, 27]. Our hypothesis is that the same outcome could be achieved through the encapsulation of peanut allergen epitopes. Permissions were granted to publish the sinusoidal circulation and stabilin-1 expressing LSEC images from Nature Reviews.

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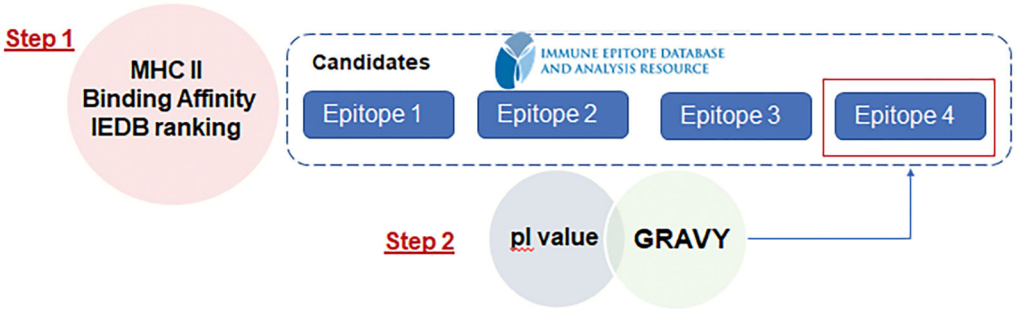
A

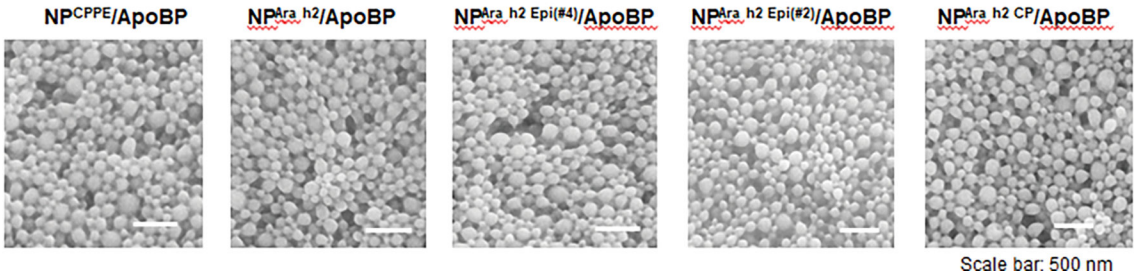


T-cell Epitope	Ara h2 Region	Sequence	pI	GRAVY	Loading capacity
#1	10-24	LALFLLAAHASARQQ	11.1	0.767	1.62 ± 2.11
#2	145-159	LRNLPQQCGLRAPQR	12.2	-1.027	3.22 ± 1.96
#3	1-15	MAKLTILVALALFLL	10.1	2.467	1.31 ± 1.72
#4	59-73	SYGRDPYSPSQDPYS	3.9	-1.820	8.68 ± 2.04
Control	18-27	HASARQQWEL	7.8	-1.250	4.75 ± 0.98

B

	Group	Nanoparticle Size (nm)	PDI	Zeta potential (mV)	Allergen content (μg/mg NP)	Ligand content (μg/mg NP)	mol% PLGA
1	NP^{CPPE}/ApoBP	210.5 ± 3.12	0.063	-38.30 ± 0.11	52.48 ± 1.39	11.45 ± 0.32	5.1
2	$NP^{Ara\ h2}$/ApoBP	207.3 ± 1.81	0.053	-38.16 ± 0.23	9.31 ± 2.66	11.82 ± 0.53	5.1
3	$NP^{Ara\ h2\ Epi(\#4)}$/ApoBP	205.9 ± 2.67	0.081	-33.28 ± 0.49	8.68 ± 2.04	11.55 ± 0.46	5.1
4	$NP^{Ara\ h2\ Epi(\#2)}$/ApoBP	203.3 ± 1.90	0.109	-36.22 ± 0.82	3.22 ± 1.96	11.47± 0.57	5.1
5	$NP^{Ara\ h2\ CP}$/ApoBP	202.8 ± 5.06	0.098	-30.39 ± 0.52	4.75 ± 0.98	11.32 ± 0.79	5.1

C



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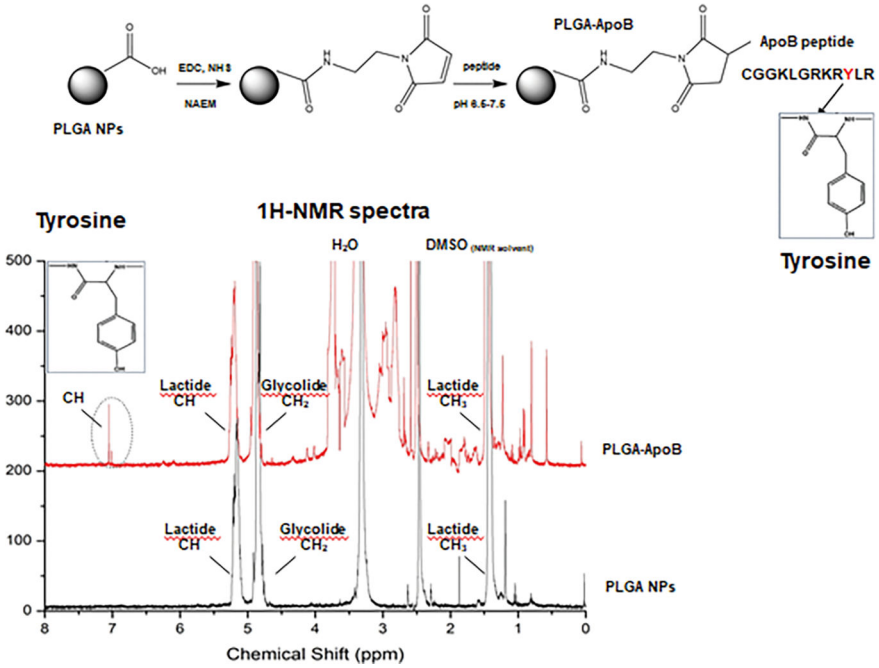

Figure 2. Epitope selection and encapsulation in liver-targeting PLGA nanoparticles.

Ara h2 epitope selection was initially carried out using the IEDB resource to make predictions of epitopes likely to have high affinity binding less < 50 nM) for murine H2-IAb, H2-IEd and H2-IAd alleles (Step 1). Four distinct 15-mer epitopes were identified, as shown in the table. Results were confirmed in the NetMHCII 2.3 database, which can be interrogated for epitope binding to the H2-IAk and H2-IEk alleles of C3H/HeJ mice. We also selected a peptide sequence serving as a comparative control for the T-cell epitopes, which overlaps with an IgE binding site. Step 2 was to determine the encapsulation efficiency of epitopes in PLGA nanoparticles, using GRAVY and assessment of peptide pI. Peptides with negative GRAVY values (indicative of high peptide solubility) facilitate peptide encapsulation. Based on these parameters, we selected Ara h2 epitopes #2 and #4, as well as a control peptide for nanoparticles encapsulation. Two additional particle batches were synthesized to encapsulate purified Ara h2 or CPPE. **B**. Characterization of the PLGA nanoparticles for hydrodynamic size, polydispersity index (PDI), zeta potential, allergen content, ligand content, and the coupling density of the ligand compared to the PLGA weight (mol/%). **C**. SEM showing the morphology and size distribution of the synthesized PLGA nanoparticles. **D**. The upper panel show the covalent attachment strategy of the ApoB peptide to the particle surface, as described previously (adapted from Ref. 26). The lower panel demonstrates the ^{1}H NMR spectra of the synthesized PLGA particles with and without ApoBP conjugation, to confirm successful conjugation of the peptide at 7 ppm.

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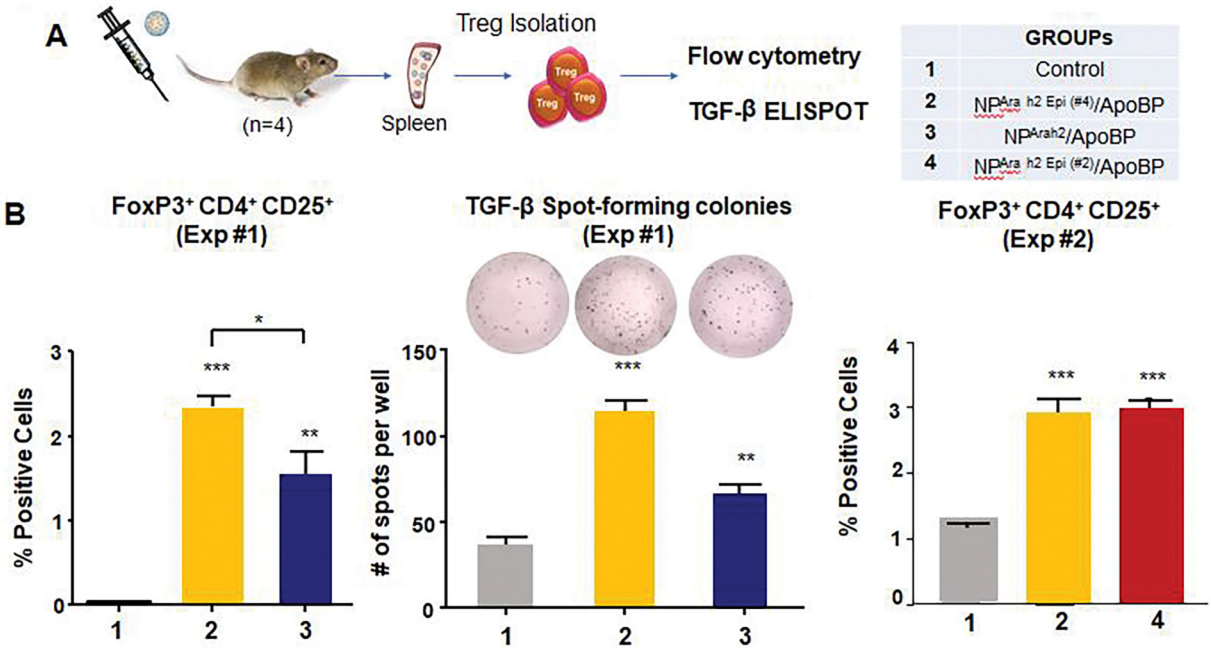

Figure 3. Nanoparticle-induced Treg generation.

A. The experimental protocol was to use a one-time IV injection of a particle dose of 500 μg, which delivers 4 μg purified Ara h2 or its respective epitopes (#2 and #4) to 6–8 week old C3H/HeJ mice (n=4). The animals were sacrificed 7 days after the injections. Spleens were collected and single-cell suspensions prepared for Treg isolation, as described in methods. Two experiments were performed to allow comparison of all the treatment modalities, in which we could only examine three conditions in the same experiment due to the limited number of magnets available for $CD4^+/CD25^+$ T-cell sorting. **B.** Flow cytometry analysis to identify $CD4^+CD25^+FoxP3^+$ Tregs was performed by intracellular FoxP3 staining and the Flowjo software (Ashland, OR) was used to calculate both the % positive cells as well as mean fluorescence intensity. ELISPOT colony counts (number of spots per 3×10^5 $CD4^+/CD25^+$ T-cells per well) were used to quantify the frequency of TGF-β producing cell clusters in the first experiment. The number of spots was quantified under a dissecting microscope, in addition to the capture of visual images with an ImmunoSpot Analyzer (Cleveland, OH). **C.** Flow cytometry analysis to identify $CD4^+CD25^+FoxP3^+$ Tregs was performed as described in B.

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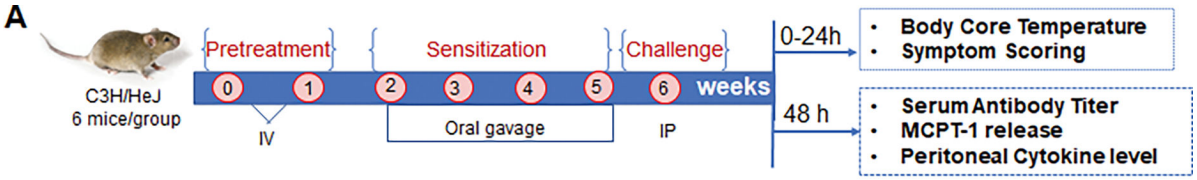
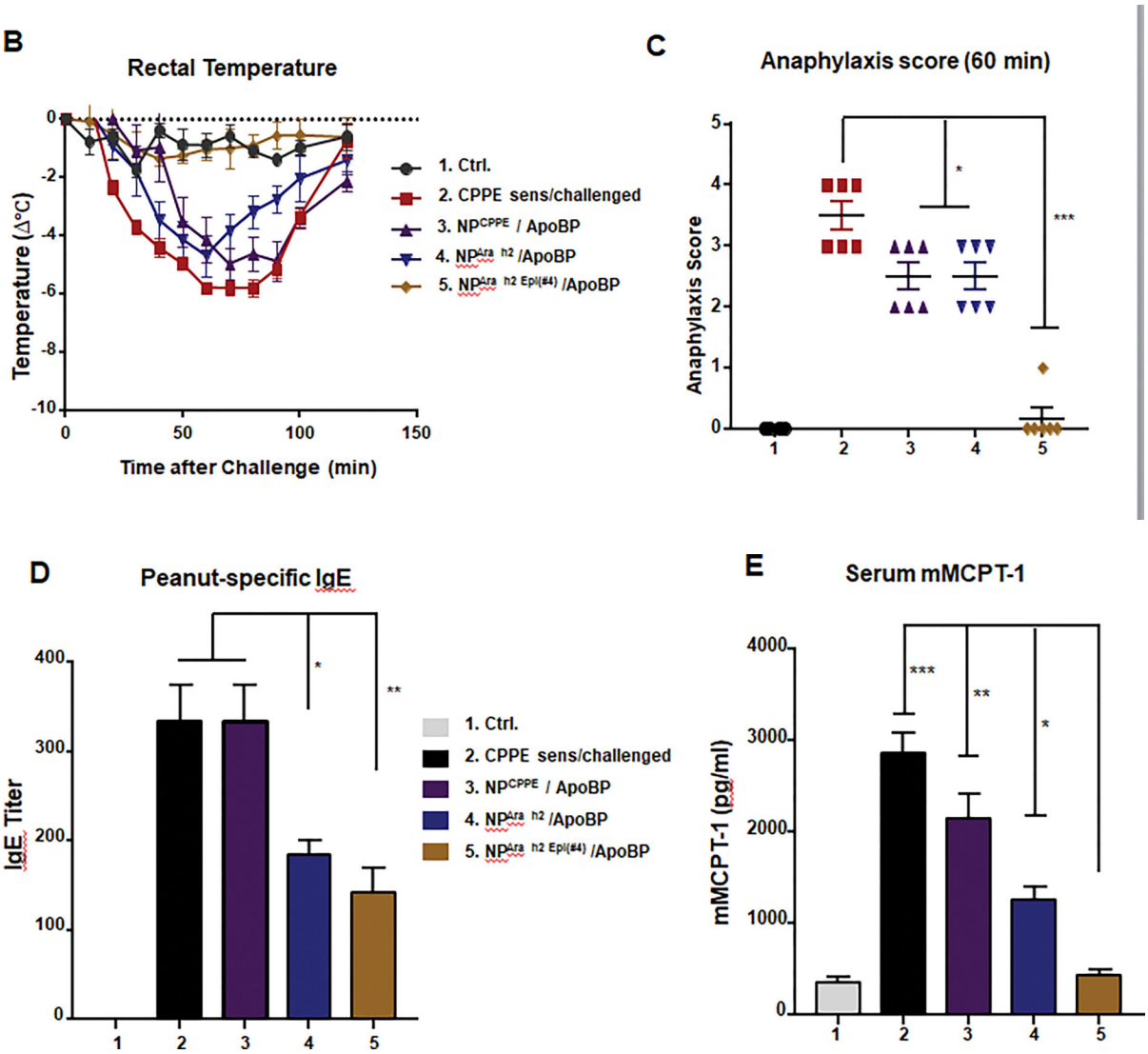


	GROUP	CPP/ Arah2	NPs	PBS	
1	Non-sensitized control	0	0	0	Control
2	CPPE sens/challenged	0	0	0	No NPs
3	NP^{CPPE}/ApoBP	25 µg	500 µg	100 µL	Pre-treatment
4	$NP^{Ara\ h2}$/ApoBP	4 µg			
5	$NP^{Ara\ h2\ Epi(\#4)}$/ApoBP	4 µg			



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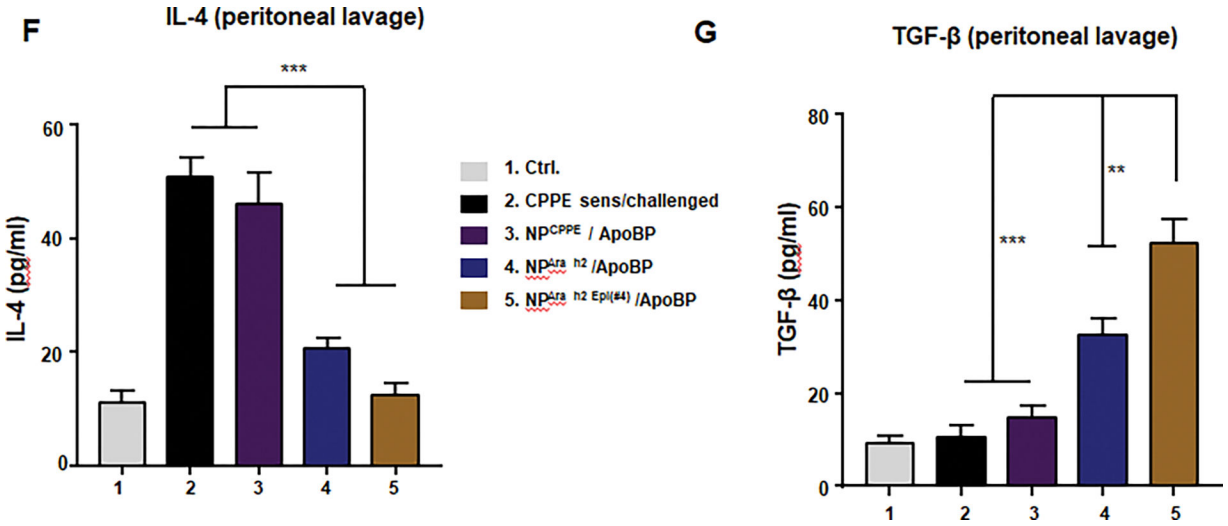

Figure 4. The effect of prophylactic administration of tolerogenic PLGA nanoparticles on anaphylaxis induction by a crude peanut protein extract (CPPE).

A. Experimental protocol outline showed that C3H/HeJ mice (n = 6) were used for IV NP injection on two occasions, 7 days apart. The particle dosimetry is discussed in Methods. Animal sensitization commenced a week later and was carried out at weekly intervals for four weeks, using CPPE and cholera toxin for oral gavage, at doses described in Methods. On week 6, mice were injected with 200 μg CPP into the peritoneal cavity to trigger an immediate anaphylactic response that is monitored. (B) Core body temperature assessment for 120 min. (C) Anaphylaxis scoring at 60 min post-challenge (maximum temperature drop). **D-G**. Assessment of antigen-specific IgE and mast cell protease levels in the serum, as well as cytokine release in the peritoneal fluid, using ELISA.

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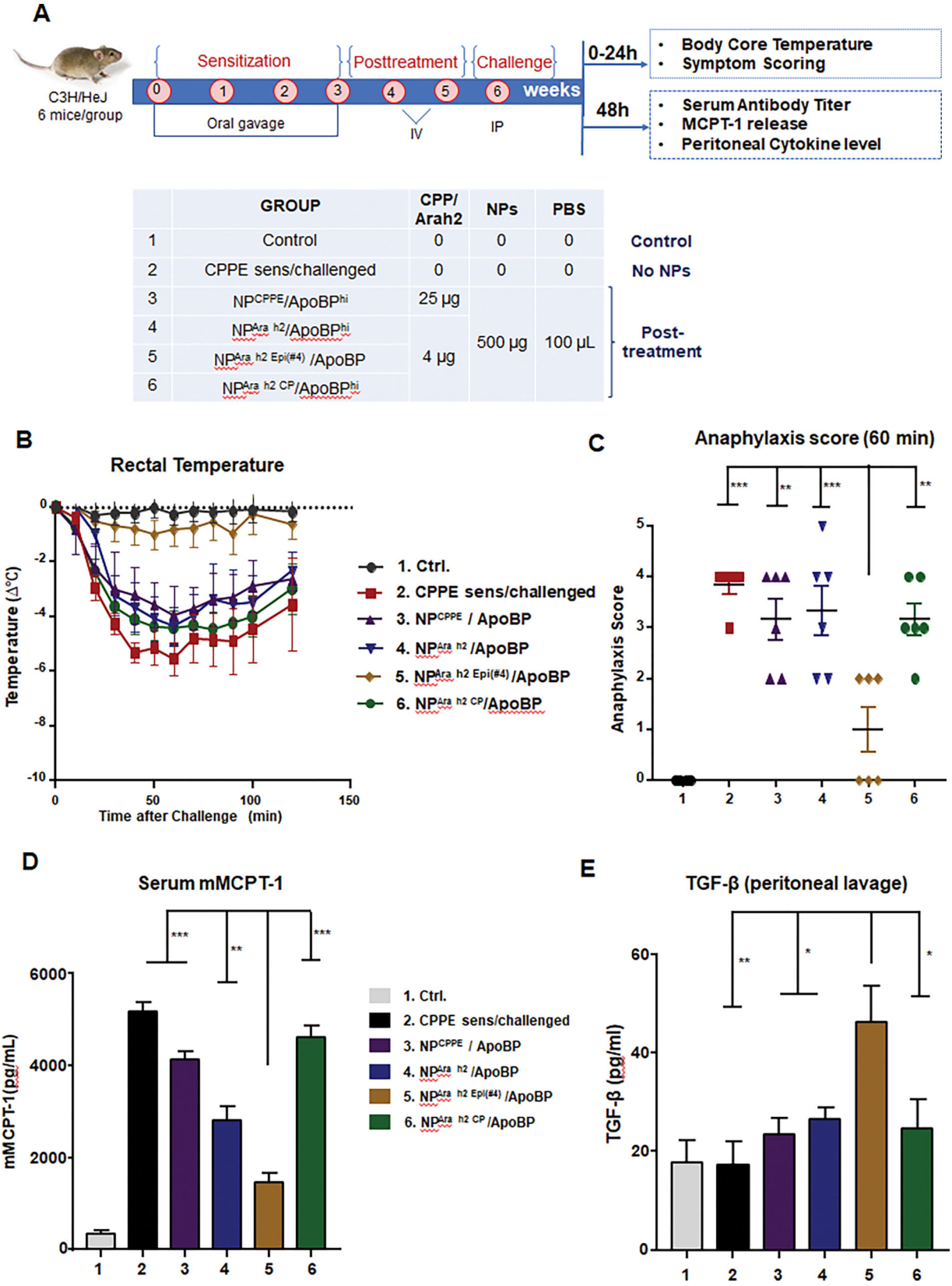

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Figure 5. Post-treatment efficacy of the tolerogenic PLGA nanoparticles in suppressing the anaphylactic response.

A. Outline of the experimental animal protocol. The animals were sensitized by oral gavage as described in Figure 4. On weeks 4 and 5, C3H/HeJ mice received IV injection of the tolerogenic nanoparticles. Mice received peritoneal injection with 200 μg CPP on week 6. Body temperatures and anaphylaxis scores were performed as described in Figure 4. **B**. Body core temperature, as determined by a rectal probe. **C.** Anaphylaxis scores at 60 min. **D.** Serum mMCPT-1 level determined by ELISA. **E**. TGF-β levels in the peritoneal lavage fluid.

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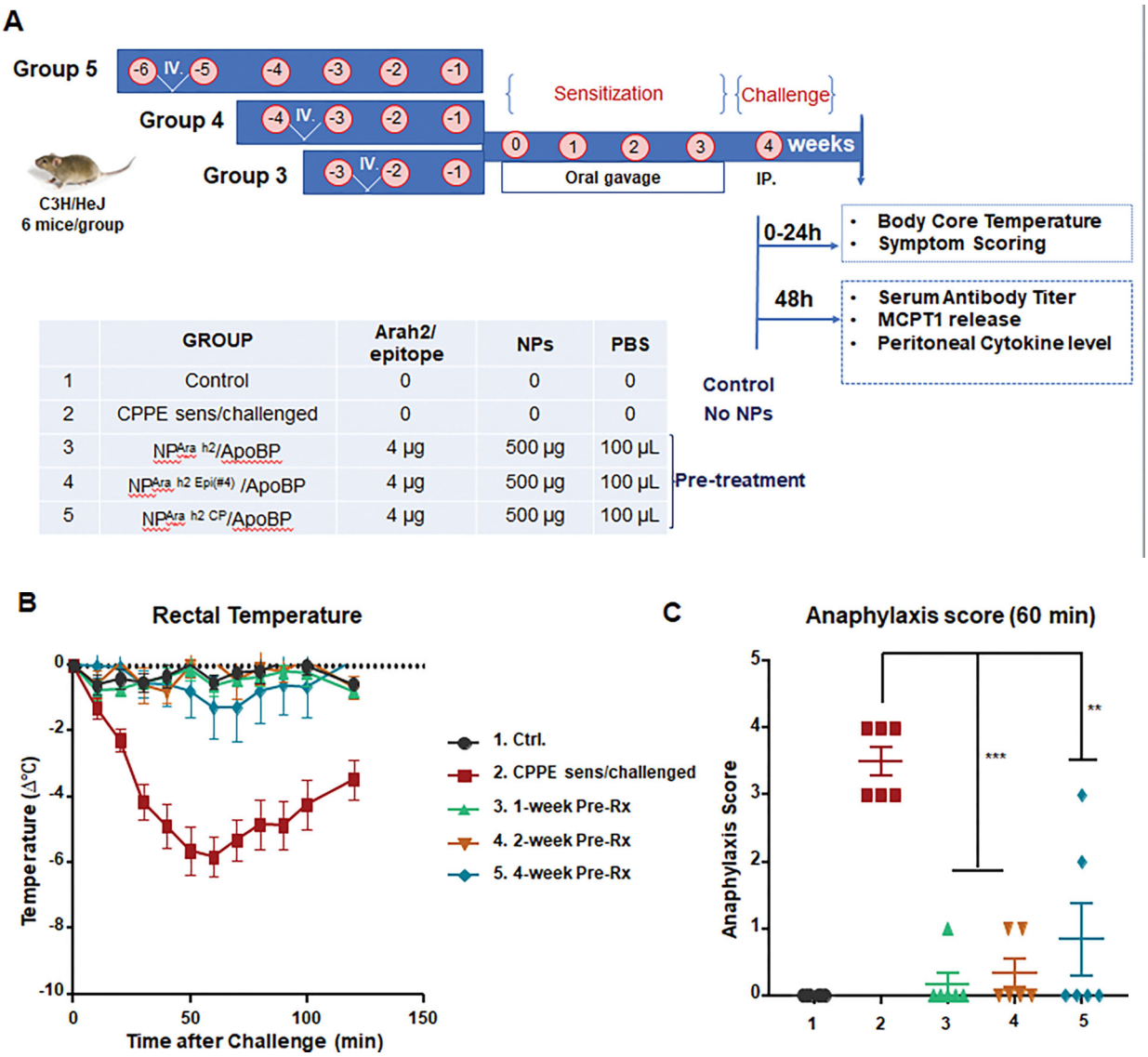

	GROUP	Arah2/ epitope	NPs	PBS
1	Control	0	0	0
2	CPPE sens/challenged	0	0	0
3	$NP^{Ara\ h2}$/ApoBP	4 μg	500 μg	100 μL
4	$NP^{Ara\ h2\ Epi(\#4)}$ /ApoBP	4 μg	500 μg	100 μL
5	$NP^{Ara\ h2\ CP}$/ApoBP	4 μg	500 μg	100 μL

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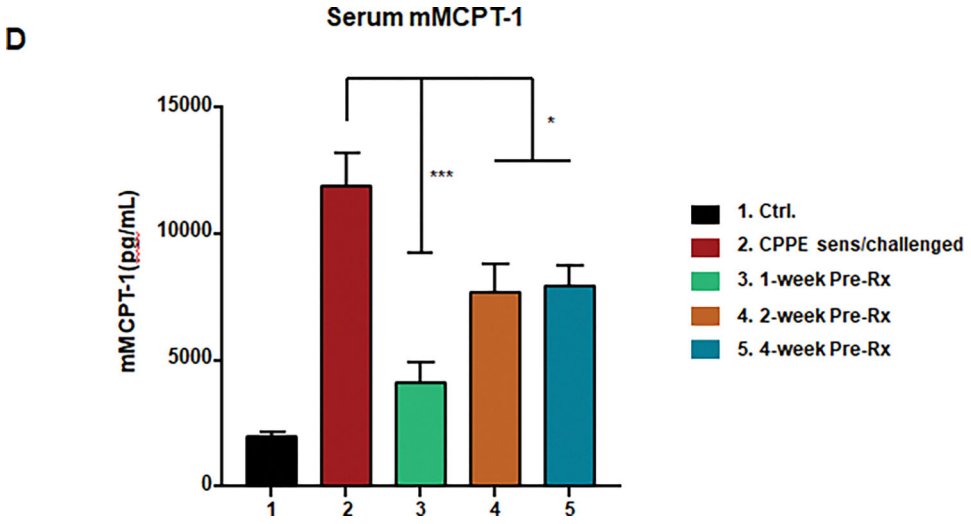

Figure 6. Duration of the tolerogenic effect, as determined by prophylactic particle administration.

A. Experimental design to show that PLGA nanoparticles were injected on three different occasions prior to the onset of sensitization, as described above. After the challenge, the animal response parameters including core body temperature, anaphylactic scores, as well as the assessment of antibody titers, MCPT-1 release and cytokine levels were performed as described in Figure 4.