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RESEARCH LETTER

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Plant-based enveloped Ara h 2 bioparticles display exceptional hypo-allergenicity

To the Editor:

Since the early nineties several approaches have been tested to develop safe and effective allergen immunotherapy (AIT) for the treatment of peanut allergy, starting with subcutaneous AIT with aqueous extract, followed by hypo-allergenic major allergens produced in bacteria that were administered rectally. These approaches were abandoned, mainly because of too many severe side-effects. Other administration routes that are considered to be safer, like sublingual and epicutaneous, have not yet reached the market. The only treatment that did receive market authorization is oral immunotherapy (OIT). It is effective for desensitization, disappointing for sustained efficacy, and side-effects occur frequently.[1] Furthermore, the most sensitive patients being at risk of severe life-threatening allergic reactions are unlikely to be helped with this approach, as side-effects may include severe anaphylaxis.[2] Therefore, there is an urgent need to provide all patients, including the severest ones, with a safe and effective treatment. Recently, nano- and micro-particulate strategies, such as virus-like particles, have been proposed as potentially safe and effective approaches. For example, a virus-based particle expressing the peanut allergens Ara h 1 or 2, based on the Cucumber Mosaic Virus, was shown to be hypo-allergenic in a mouse model.[3] Here we report the ground-breaking hypo-allergenicity of a novel non-virus-based microparticle produced in plants, that is, enveloped bioparticles (*e*BPs) expressing ~3000 copies of Ara h 2 on the surface of each particle. Ara h 2 *e*BPs were generated by transfection of *Nicotiana benthamiana* with *Agrobacterium tumefaciens* carrying Ara h 2 cDNA constructs followed by oligomerization and membrane sequences, as described before for Der p 2 *e*BPs.[4] Quantification of Ara h 2 was performed by immuno-slot blot using an in-house polyclonal rabbit anti-nAra h 2 serum and by dot-blot using serum from an Ara h 2-sensitized patient (Figure 1A, B). Dilutions of Ara h 2-BPs were compared by densitometric scanning to a standard curve of titrated natural purified Ara h 2 (nAra h 2) that had been quantified by a protein assay (BCA). The number of Ara h 2 molecules per bioparticle was calculated based on the number of particles, determined by tunable resistive pulse sensing (TRSP), and the concentration of Ara h 2, determined by SLOT-BLOT.[4]

The *e*BP platform, first tested with house dust mite (Der p 2) and cat dander (Fel d 1) allergens, demonstrated significantly stronger immunogenicity than alum-adsorbed allergen, and close to a 1000-fold reduction in allergenicity shown by the basophil activation test (BAT) and the rat basophilic leukaemia (RBL) cell test.[4,5] In the present study, hypo-allergenicity of Ara h 2 *e*BPs was first evaluated by reduction of IgE binding using ImmunoCAP inhibition. A pool of sera from eight Ara h 2-sensitized patients was incubated with dilution series of either nAra h 2 or the Ara h 2 *e*BP, followed by quantification of IgE binding to the recombinant Ara h 2 ImmunoCAP (f423; Thermo Fisher Scientific, Uppsala, Sweden). The Ara h 2 concentration of the bioparticles on the X-axis in Figure 1C was directly based on the quantitative SLOT-BLOT (Figure 1A). Collection of sera was approved by the Institutional Review Board of the University of Colorado, Denver, all subjects or their guardians signed informed consent and, for minors, assent. The Ara h 2 *e*BP showed a 10,000-fold reduction in IgE binding potency compared to nAra h 2 (Figure 1C). Additionally, the degree of functional hypo-allergenicity was assessed using either $CD34^+$ stem-cell-derived human mast cells or RBL cells which were loaded with human IgE from nine Ara h 2-sensitized patients, and subsequently incubated with soluble nAra h 2 or with Ara h 2 *e*BPs. Both assays demonstrated around a 10,000-fold reduction of β-hexosaminidase release, a measure for degranulation, for Ara h 2 *e*BPs compared to nAra h 2 (Figure 2A, B). In the case of the RBL assay, concentrations of Ara h 2 required to induce half-maximal release could be calculated, showing that the *e*BPs are >14,000-fold less allergenic than nAra h 2 (Figure 2C). To take patient-associated effector-cell properties into account, a BAT with basophils from five peanut allergic patients was performed, demonstrating a similar degree of hypo-allergenicity, using the established activation markers CD203c and CD63 (Figure 2D). Altogether, these data demonstrate a very substantial reduction in the capacity of Ara h 2 *e*BPs to induce effector cell driven allergic responses.

Another aspect of hypo-allergenicity, rarely taken into account, is IgE-facilitated allergen presentation by B cells to allergen-specific Th2 cells, contributing to sustained allergen-specific Th2 responses.[6] Here, we evaluated the capacity of Ara h 2 *e*BPs to form immune complexes with Ara h 2-specific IgE that can bind to the low-affinity IgE receptor (CD23) on CD23+ EBV-transformed B cells, serving as a surrogate read-out for the capacity of B cells to present allergens to allergen-specific T cells.[7] Either nAra h 2 or Ara h 2 *e*BPs were pre-incubated with patient serum at 37°C, followed by addition of the EBV-transformed B cells at 4°C (to avoid internalization). When IgE forms a complex with Ara h 2, accessible IgE Fc regions present in the complex can be detected by flow cytometry on the B cells ($CD23^+IgE^+$ B cells). Soluble nAra h 2 pre-incubated with

patient sera resulted in the presence of $CD23^{+}IgE^{+}$ B cells already at much lower concentrations of allergen than observed with the Ara h 2 *e*BPs, either after incubation with a patient serum pool of nine Ara h 2-sensitized patients or with individual sera from two different patients (Figure 2E). These results indicate that the *e*BPs have a > 10,000-fold reduced capacity to form IgE-Ara h 2 complexes and subsequently activate Th2 cell responses via IgE-facilitated allergen presentation by B cells.

Together, these data demonstrate an impressive degree of hypo-allergenicity of Ara h 2 *e*BPs, at the level of IgE binding, allergic effector cell activation, and IgE-facilitated allergen-presentation. Interestingly, the IgE-binding capacity during ImmunoCAP inhibition assays was enhanced for Der p 2 *e*BPs and unaffected for Fel d 1 *e*BPs (unpublished data). These differences compared to Ara h 2 *e*BPs may be explained by the localization of dominant IgE epitopes, which either remain available or become shielded off in the one-directional positioning of the allergens on the surface of the particles. Nevertheless, all three allergen *e*BPs are exceptionally hypo-allergenic in functional assays with allergenic effector cells (mast cells, RBL, BAT).[4,5] This may be caused by insufficient FcεR cross-linking due to the restricted freedom of movement of the allergens on the *e*BPs, while soluble allergen can freely move around. Moreover, there is limited space for the 150–200 nm *e*BPs to bind to the surface of a ~ 1.5 μm large cell, decreasing effective FcεR cross-linking by steric hindrance.[8]

In conclusion, plant-produced Ara h 2 *e*BPs have a very promising safety profile that has great potential to be used as a safe subcutaneous treatment, possibly even in patients with the highest sensitivity. As a next step, a skin prick test study will have to establish whether the same degree of hypo-allergenicity is indeed confirmed in vivo.

Key messages

- Ara h 2 *e*BPs show reduced IgE binding, effector cell activation, and IgE-facilitated antigen presentation compared to nAra h 2
- Ara h 2 *e*BPs are >10,000-fold hypoallergenic compared to nAra h 2
- Ara h 2 *e*BPs are a promising candidate for peanut allergen immunotherapy

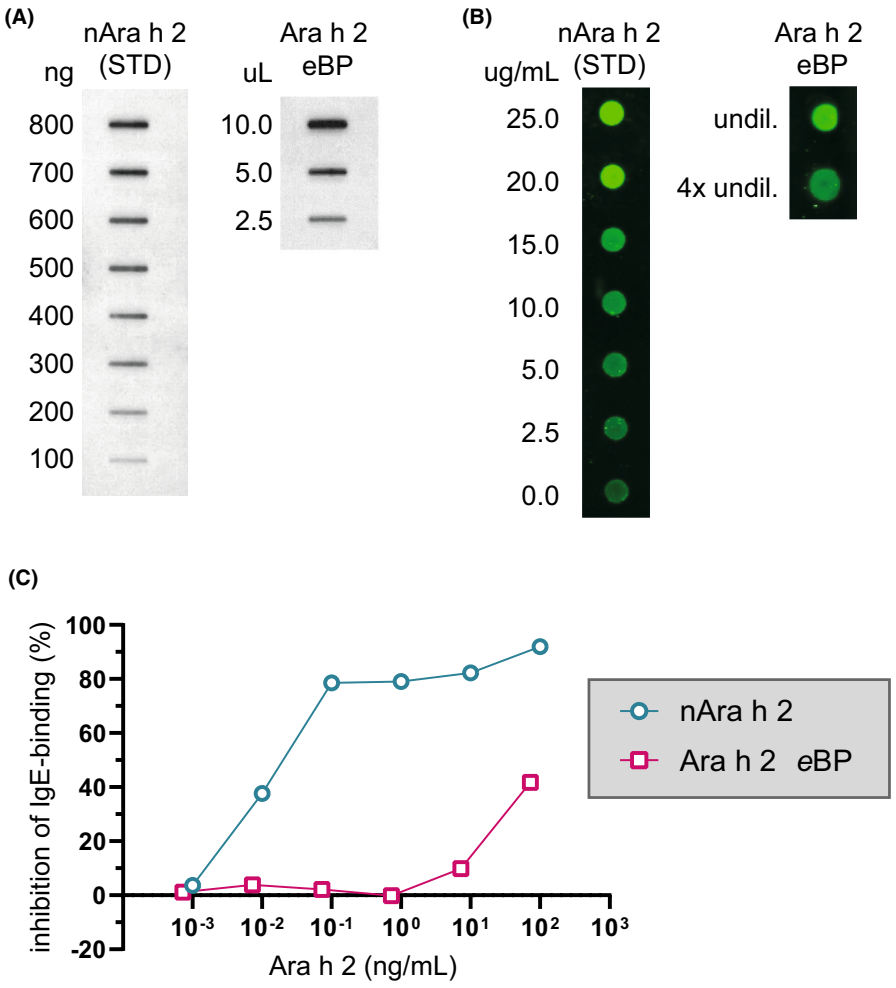

FIGURE 1 *e*Bioparticle quantification of Ara h 2 and IgE binding capacity. Quantification of Ara h 2 on the *e*Bioparticle was performed using an immuno-slot blot (A) or a dot-blot (B). A titration of nAra h 2 (calibration line) or Ara h 2 *e*BP (sample) was spotted, followed by detection using an in-house polyclonal rabbit anti-nAra h 2 serum for the immuno-slot blot or serum IgE from an Ara h 2-sensitized patient for the dot-blot. (C) ImmunoCAP inhibition with either nAra h 2 or the Ara h 2 *e*BPs. A pool of sera from eight Ara h 2-sensitized peanut allergic donors was incubated with a titration of nAra h 2 or the Ara h 2 *e*BP for 1 h. Subsequently, IgE binding was measured using ImmunoCAP (Thermo Fisher Scientific).

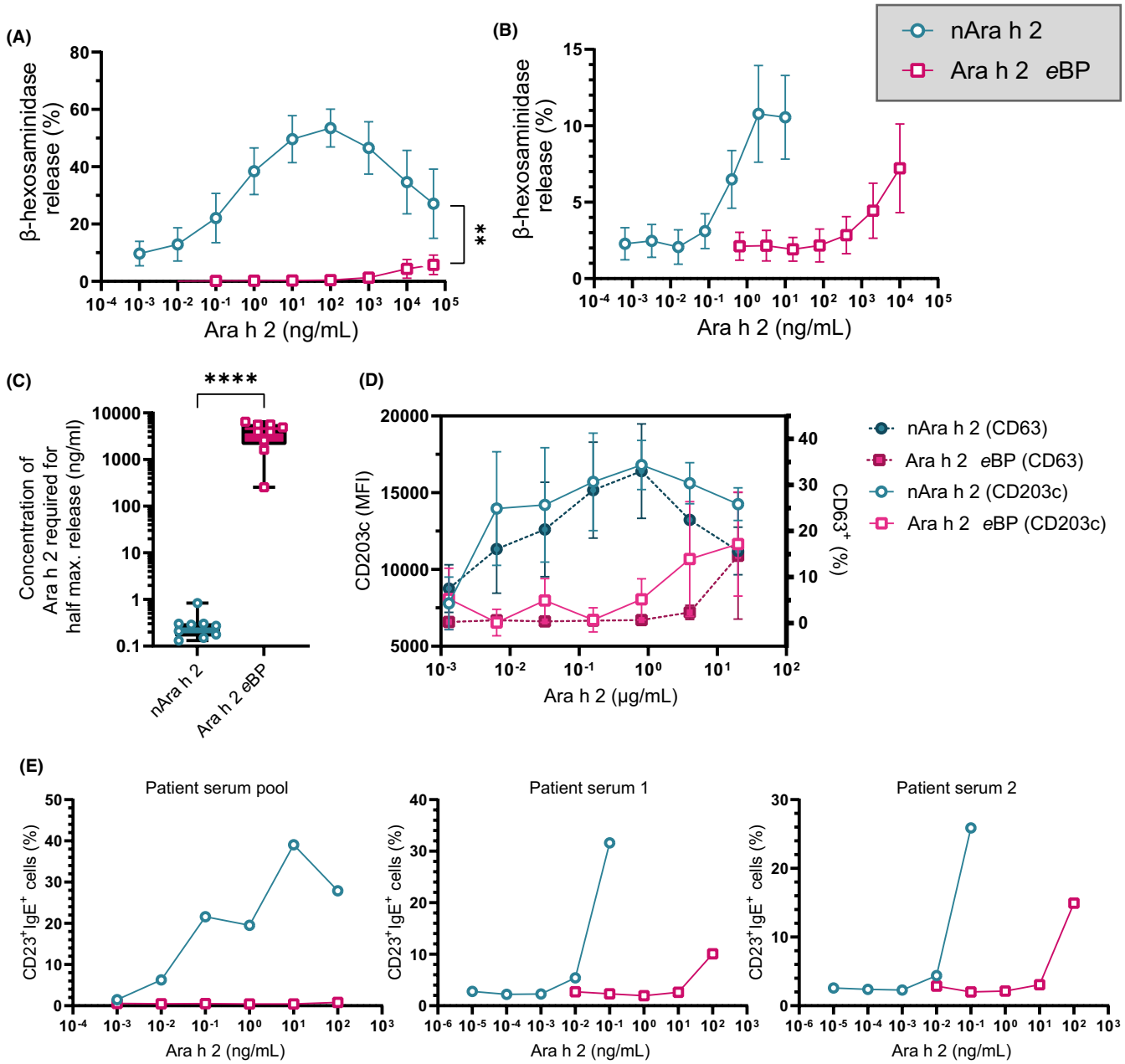

FIGURE 2 Modulation of allergen effector cell activation by the Ara h 2 *e*Bioparticle. (A) Human CD34$^+$ stem cell derived mast cells ($n = 8$) or (B) rat basophil leukaemia cells expressing the human FcεRI ($n = 9$) were sensitized with serum from Ara h 2-sensitized peanut allergic patients. The cells were stimulated with a titration of either nAra h 2 or Ara h 2 *e*BPs and β-hexosaminidase release was measured, representing the level of degranulation. (two-way ANOVA, ** $p < .005$, all concentrations tested had a $p < .005$ or less) (C) The concentration of Ara h 2 necessary to obtain half maximal mediator release in the rat basophil leukaemia cells calculated for each response curve per patient, showing a > 14,000-fold decrease in concentration. ($n = 9$, two-tailed paired t-test with transformed data, **** $p < .00005$) (D) Basophil activation test. Whole blood from five Ara h 2-sensitized patients was stimulated with nAra h 2 or the Ara h 2 *e*BP, after which CD203c and CD63 expression was measured using flow cytometry. (E) Facilitated antigen binding assay (FAB). nAra h 2 or the Ara h 2 eBP were incubated with serum of Ara h 2-sensitized peanut allergic patients and CD23$^+$ EBV-transformed B cells were added, after which the CD23$^+$IgE$^+$ B cell population was measured using flow cytometry. Left: FAB performed with patient serum pool of nine Ara h 2-sensitized patients, middle and right: FAB performed with patient serum from two individual Ara h 2-sensitized patients.

AUTHOR CONTRIBUTIONS

C.Castenmiller and R. van Ree conceptualized the study and wrote, revised, and edited the manuscript. C.Castenmiller, J.H. Akkerdaas, S. Versteeg, B.R. Blokhuis, M.E. Kirpas and M. Stigler designed experiments and/or acquired, interpreted, and analysed the data. L. Auger, R. Desgagnés, C. Martel, L. Mirande, B. Morel, J. Roberge, V. Stordeur, G. Tropper, L.P. Vézina, and V. Gomord contributed to the concept design, development, or manufacturing of the plant-derived bioparticle platform. S.C. Dreskin provided peanut allergic patient serum. E.C. de Jong, W.G. Shreffler, F. Redegeld, L. Aglas and R. van Ree supervised experiments. R. van Ree supervised the study. All authors critically reviewed the manuscript.

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KEYWORDS

allergen immunotherapy, bioparticle, food allergy, hypoallergenicity, peanut allergy, plant-based particle

CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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