

LETTER

***Lactococcus lactis*-derived microparticles *ad mixed* with allergen shift immune responses towards a regulatory profile in tree-nut allergic patients**

To the Editor

Sublingual immunotherapy (SLIT) is a disease-modifying intervention that offers a potential long-lasting therapeutic effect in food allergy (FA). However, there are unsolved issues regarding the long treatment period of minimum 3 years required to achieve greater efficacy and long-lasting immune tolerance, highlighting an unmet need for a more successful therapy with faster onset.¹ A possible explanation for the slow onset of SLIT could be the lack of proper signals to optimize regulatory immune reactions in the early phases of immunotherapy.² In this study, we analyse the application of a next-generation approach based on non-living microparticles derived from the probiotic *Lactococcus lactis*, termed tolerogenic bacterial particles (TBP).³

We studied immunomodulatory responses induced by TBP in peripheral blood mononuclear cells (PBMC) from tree-nut allergic patients (tree-nut-AP) and healthy controls (HC) (Table 1). PBMC were stimulated with walnut extract (WE), TBP and both (TBP + WE). Cell proliferation was determined by 5,6-carboxyfluorescein diacetate N-succinimidyl ester dilution assay (Figures S1 and S2), and the immunological pattern of response was assessed determining intracellular cytokine by flow cytometry (Figure S3) and cytokine secretion using a human multiplex immunoassay kit. Further details are included in the Appendix S1.

This study included ten tree-nut-AP, 70% of them presented symptoms of anaphylactic reactions to one or more tree-nuts, 30% referred oral allergy syndrome symptoms and 10% urticaria and angioedema induced by either tree and/or peanut. All tree-nut-AP presented positive skin tests and sIgE for at least one tree-and/or peanut tested, and/or for LTPs (Table 1 and results from Appendix S1).

Results show that, compared to unstimulated cells, WE induced a type 2 (T2) immune response confirmed by the proliferative response of IL-4⁺Th2 and IgE⁺PC and no proliferation of IFN γ ⁺Th1 and IL-10⁺Treg-cells in tree-nut-AP (Figure 1). Moreover, data of cytokine production in supernatants from the proliferation cultures indicated that the stimulation with WE induced a significant increase in IL-4 levels and a decrease in IFN γ and IL-10 in tree-nut-AP confirming the T2 immune response (Figures 2A). The results in the percentages of IFN γ , IL-4 and IL-10 production in proliferating CD4⁺T- and of

IgE production in proliferating CD19⁺B-cells confirmed the above-described data (Figure 1B).

Stimulation with TBP and WE *ad mixed* reduced the IL-4⁺Th2 and IgE⁺PC proliferation in tree-nut-AP compared to WE alone, being significant for IgE⁺PC. Furthermore, TBP + WE significantly increased IL-10⁺Treg-cell proliferation (Figure 1A). We observed significantly increased IFN γ and IL-10 levels, and decreased IL-4 levels when stimulating with TBP + WE compared to WE alone in tree-nut-AP (Figure 2A). Interestingly, the IFN γ /IL-4 and IL-10/IL-4 ratios were also significantly higher in TBP + WE-stimulated-PBMC than WE alone (Figure 2B). These results show good correlation with the percentages of IFN γ , IL-4 and IL-10 production in proliferating CD4⁺T- and IgE production in proliferating CD19⁺B-cells (Figure 1B).

No significant changes in the proliferation of cell subpopulation and cytokine production (from cell supernatants and proliferating cells) were observed in HC after stimulation with WE or TBP + WE (Figure S5A–C), except for IFN γ , which increased after TBP + WE stimulation compared to unstimulated condition (Figure S5B). Proliferation and cytokine production from supernatant or proliferating cells data after stimulation with TBP alone did not show any significant changes in tree-nut-AP and HC (Figures S4A–C and S6A–C).

Our results report evidence that TBP, in combination with the specific stimulation of tree-nut extract, triggers the inhibition of T2 immune responses, determined in both T- and B-cells, and increases regulatory response. These features resemble the property of several compounds known to affect dendritic cell function such as dexamethasone plus 1,25-dihydroxy vitamin D3, live bacterial probiotic strains as well as toll-like receptor 2 and 4 ligands.^{4,5} These molecules and bacteria share a property to act as Treg or Th1/Treg stimulators, suggesting that TBP act via similar pathways in combination with allergen. In support of this hypothesis, we recently demonstrated in a FA model in pigs an enhanced and prolonged allergen presentation using a combination of a mucoadhesive patch and proper stimulation of a privileged tolerogenic immune compartment by TBP, which translated into an efficient short-course SLIT approach.³ In spite of further studies with larger sample size and more detailed characterization of TBP mode of action are needed,

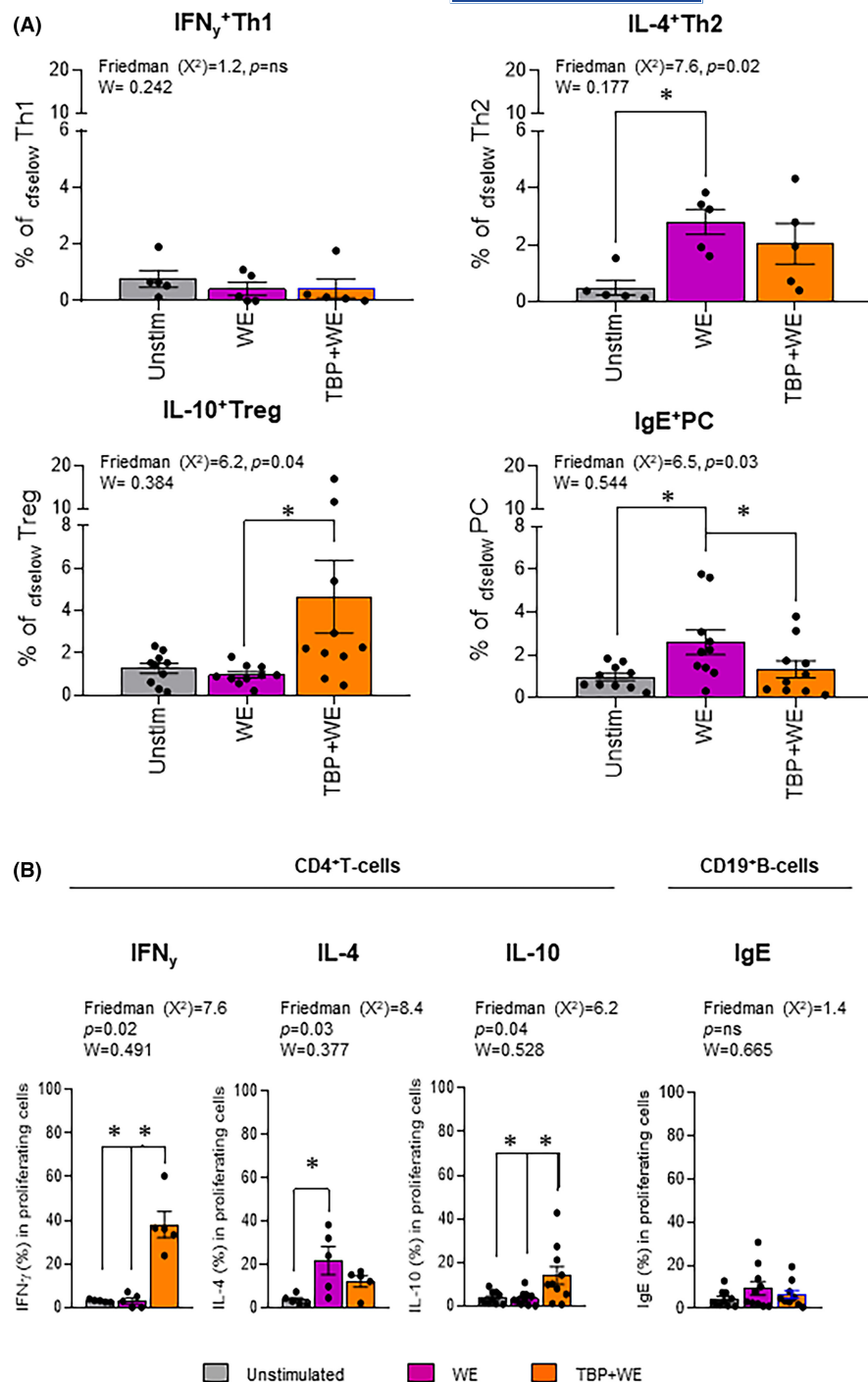
TABLE 1 Clinical features of food allergic patients and tolerant controls

AP	Sex	Age	Clinical symptoms	slgE to tree-and/or peanut purified allergen or extract	SPT positive
AP1	F	43	Contact urticaria: peach peel. Anaphylaxis: Tree-nuts.	Pru p 3: 7.58 Peanut: 1.19 Ara h 9: 6.18 Ara h 2: <0.35 Walnut: 1.41 Almond: 0.73	Peach-Peel Peanut
AP2	M	26	Anaphylaxis: Tree-nuts	Peach: 6.90 Peanut: 3.60 Walnut: 1.75 Almond: 1.32 HazelNut: <0.35	Peach-Peel
AP3	M	42	Anaphylaxis: Walnut. OAS: Peanut, almond and hazelnut.	Peach: 11.00 Peanut: 7.94 Walnut: 14.80 Almond: 6.11	Peach-Peel Peanut Walnut HazelNut
AP4	F	54	OAS: Peanut, almond, hazelnut, walnut, peach and melon.	Peach: 14.20 Peanut: 4.78 Walnut: 6.87 Almond: 0.68	Peach-Peel Peanut Walnut HazelNut Almond Melon
AP5	F	46	Anaphylaxis: Peach-peel. Urticaria and angioedema: Peanut, hazelnut and walnut.	Pru p 3: 23.70 Peanut: 6.80 Walnut: 14.80 HazelNut: 2.92 Almond: 3.47	Peach-Peel Peanut Walnut HazelNut
AP6	F	23	OAS: Unpeel peach and Walnut.	Pru p 3: 48.90 Peanut: 20.50 Walnut: 41.50	Peach-Peel Walnut
AP7	F	31	Anaphylaxis: Walnut. Contact urticaria: Peach-peel	Pru p 3: 12.70 Ara h 9: 9.56 Ara h 2: <0.35	Peach-Peel Walnut
AP8	F	43	Anaphylaxis: Almond. Avoid other nuts and peach	Almond: 9.60 Peanut: 16.60 HazelNut: 8.52	Peach-Peel Peanut Walnut HazelNut Almond
AP9	F	42	Anaphylaxis: Almond, walnut, hazelnut. Contact urticaria: Peach-peel	Pru p 3: 23.70 Peanut: 13.90 Ara h 9: 23.20 Ara h 2: <0.35 Almond: 2.18 Cor a 8: 3.21	Peach-Peel Peanut Walnut HazelNut Almond
AP10	M	21	Anaphylaxis: Almond walnut. Avoid other nuts and peach	Peanut: 5.69 Almond: 1.98 Peach: 20.70 Cor a 8: 4.45	Peanut Walnut HazelNut
HC1	F	37	-	<0.35	-
HC2	M	29	-	<0.35	-
HC3	F	25	-	<0.35	-
HC4	F	25	-	<0.35	-
HC5	M	46	-	<0.35	-

Note: Ara h 9: peanut allergen; Pru p 3: peach allergen; Cor a 8: hazelnut allergen.

Abbreviations: AP: Allergic patients; HC: Healthy control; OAS: Oral allergy syndrome; SPT: Skin prick test.

FIGURE 1 (A) Percentages (%) of different CFSE low cell subpopulations, and (B) % of cytokines (IFN γ , IL-4 and IL-10) production in proliferating CD4 $^{+}$ T-cells and % of IgE production in proliferating CD19 $^{+}$ B-cells after stimulation with WE (25 μ g/ml) and combined with TBP (TBP+WE, at 7.5×10^6 particles + 25 μ g/ml) in tree-nut-AP Unstim: Unstimulated cells. WE: Walnut extract. TBP: Tolerogenic bacterial particles. The bars with symbols represent mean and SEM of CFSE low % and the percentages of cytokines and IgE production in proliferating cells. Five tree-nut-AP were used to determine the IFN γ Th1 and IL-4 $^{+}$ Th2 proliferation, and an $N = 10$ for the rest of the cell populations. CFSE: 5,6-carboxyfluorescein diacetate N-succinimidyl ester. Friedman test was used to detect differences in the different condition experimental across multiple comparisons in related sample and Kendall's coefficient of concordance (W) to calculate the effect size, showing the Friedman χ^2 , p - and W-values, respectively. Wilcoxon test was used for pairwise comparisons in related samples, representing significant p -values as * ($p < .05$)



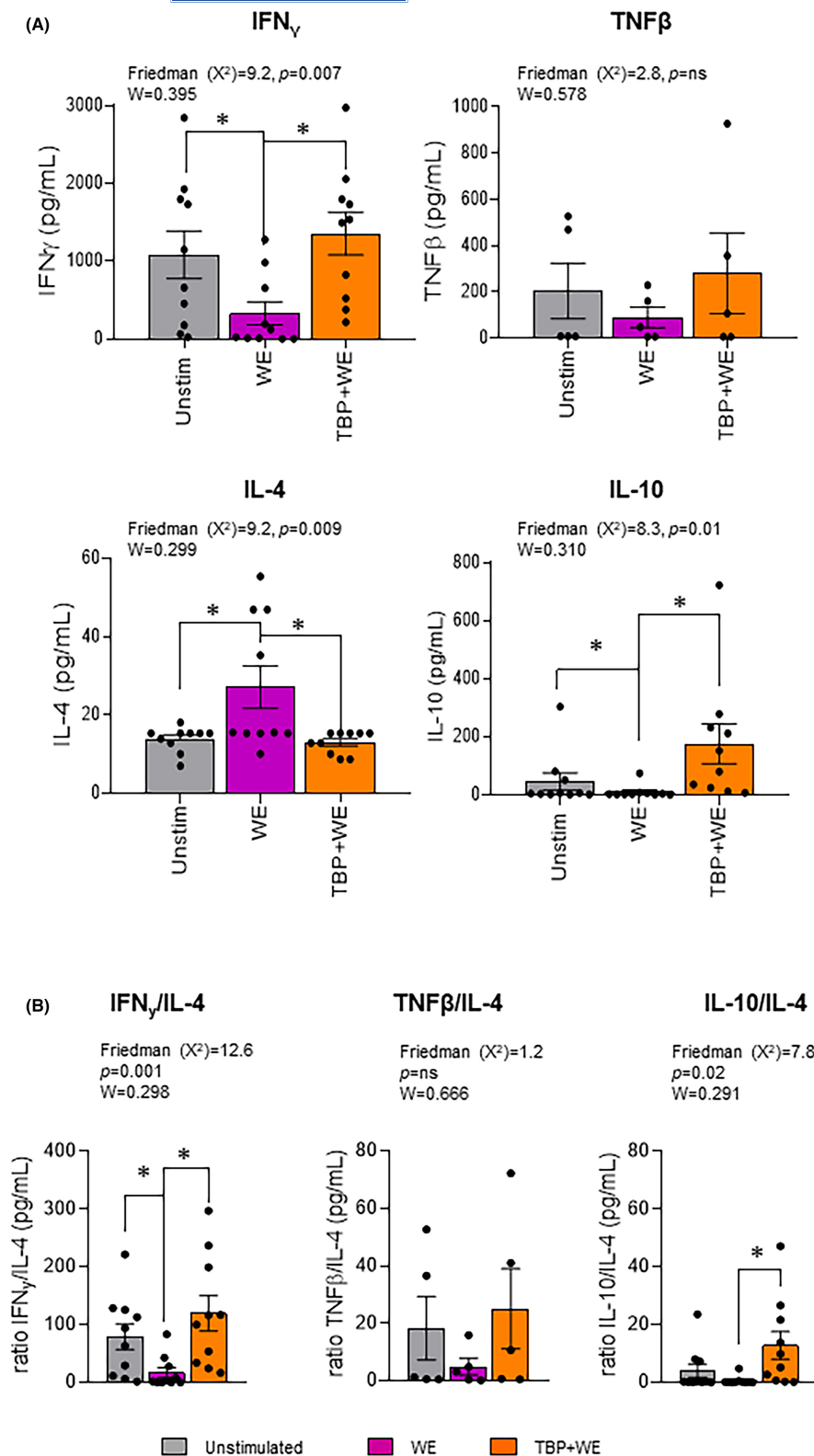


FIGURE 2 (A) Cytokine levels, and (B) ratio of them produced by PBMC after stimulation with WE (25 µg/ml) and TBP+WE (at 7.5×10^6 particles + 25 µg/ml). Unstim: Unstimulated cells. WE: Walnut extract. TBP: Tolerogenic bacterial particles. The bars with symbols represent mean and SEM of concentration of each cytokine. Five tree-nut-AP were used to determine the TNF β levels, and an $N = 10$ for the rest of cytokine levels. Friedman test was used to detect differences in the different condition experimental across multiple comparisons in related sample and Kendall's coefficient of concordance (W) to calculate the effect size, showing the Friedman X^2 , p - and W-values, respectively. Wilcoxon signed-rank test was used for pairwise comparisons in related samples, representing significant p -values as * ($p < .05$).

we suggest TBPs as new candidate tolerogenic immunomodulators for a more effective next-generation AIT approach, avoiding the use of live recombinant bacteria.

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CONFLICT OF INTEREST

PS and KL are shareholders in Allero Therapeutics. FP, CM, NPS, FG and MJT declare they have no relevant conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.