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SUPPORTING INFORMATION

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Innate lymphoid cells type 2 in LTP-allergic patients and their modulation during sublingual immunotherapy

To the Editor,

Lipid transfer protein allergy induces complex clinical manifestations, often severe or life-threatening in food allergy (FA).[1] It is characterised by an immunologic tolerance breakdown to ingested food associated with the immunological response type 2. The innate lymphoid cell type 2 (ILC2) have emerged as key in FA, mainly in animal models.[2] ILC2 share morphological characteristics with lymphocytes, with no expression of allergen recognition receptors, with expression of transcription factor GATA-3, and receptors for IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). ILC2 are activated by these cytokines, originating their expansion and production of Th2-cytokines.[3] Furthermore, a distinct subset of ILC2 with regulatory function with IL-10 production, which can be activated by different immunological pathways and by retinoic acid, has been characterised.[4] However, the innate immune cell involvement in the allergic disease remains to be elucidated, specifically ILC2 implication in loss of tolerance to food allergens in humans.

There is a great interest in exploring how ILC2 can be affected by allergen-specific immunotherapy (AIT), postulating them as a biomarker predictor of responses.[5] We demonstrated the safety and effectiveness of sublingual immunotherapy (SLIT) using Pru p 3-enriched extracts (SLIT-Prup3),[6] showing allergen-specific Th2-cell modulation, involving dendritic cell (DC) activity and inducing a regulatory pattern.[7] However, the effect of SLIT-Prup3 on ILC2 is yet to be determined.

We report for the first time ILC2 implication in LTP allergy and SLIT-Prup3 ability to modulate effector Th2 cells and ILC2 in LTP-allergic patients (LTP-AP). This study included LTP-AP and tolerant controls without FA (TC) (Table 1). From these, seven LTP-AP were chosen for presenting more allergic episodes after peach intake and systemic symptoms (Table 1), treated with (SLIT-Prup3-LTP-AP) for 1 year and evaluated at different time points (T0, T1, T6, T12 months). From all subjects, peripheral blood mononuclear cells (PBMCs) were obtained, stimulated and phenotypically characterised by flow cytometry as ILC2 and T cells (Figures S1-S2) and the possible relationship of ILC2 with T cells and clinical parameters analysed. Further methods details are included in the OS.

Our results suggest that ILC2, IL-4$^+$ and IL-13$^+$ILC2 (Figure 1A) as well as IL-4$^+$ and IL-13$^+$ Th2 cell percentages (Figure S3A) were significantly higher in LTP-AP than in TC.[8] This could indicate that the type 2 immune response in FA is mediated by an apparent action of Th2 cells and ILC2. Moreover, there was a positive correlation between both cell types, significant for IL-13$^+$ILC2 *vs* IL-13$^+$Th2 cells (Figure S3B).

Furthermore, IL-4$^+$ and IL-13$^+$ILC2 frequencies in LTP-AP had a strong positive correlation with skin prick test and Pru p 3-sIgE levels (Figure 1B). Although limited by the sample size, LTP-AP with anaphylaxis symptoms had higher IL-4$^+$ and IL-13$^+$ILC2 levels compared with LTP-AP with mild symptoms (urticaria and angioedema) (Figure 1C). These observations, together with the link between ILC2 and the IgE-mediated adaptive response, represent an important advance in the ILC2 role in FA, suggesting them as a potential therapeutic target

TABLE 1 Clinical characteristics and allergological work-up results

N. subjects	Age	Sex	Pollen/mite sensitisation	Rhinitis	Rhinitis severity (Aria)	Asthma/atopic dermatitis	Reaction type	OPS	N. episodes	SPT (mm^2)	sIgE (kU/L)
LTP-AP1	**34**	**F**	**Grass/olive**	**Yes**	**Persistent-mild**	**No**	**Anaph**	**5**	**>3**	**8**	**6.96**
LTP-AP2	**44**	**M**	**Grass**	**Yes**	**Persistent-moderate**	**No**	**U**	**17**	**>3**	**8**	**20.6**
LTP-AP3	**39**	**F**	**–**	**No**	**–**	**No**	**U/Ang**	**15**	**>3**	**9**	**10.9**
LTP-AP4	**32**	**F**	**Grass/olive**	**Yes**	**Intermittent-mild**	**No**	**U**	**12**	**>3**	**7**	**34.4**
LTP-AP5	**48**	**M**	**Olive**	**Yes**	**Persistent-moderate**	**No**	**Anaph**	**15**	**>3**	**8**	**3.58**
LTP-AP6	**27**	**F**	**Grass/olive**	**Yes**	**Persistent-mild**	**No**	**Anaph**	**2**	**>3**	**10**	**5.26**
LTP-AP7	**31**	**F**	**–**	**No**	**–**	**No**	**U**	**8**	**>3**	**10**	**7.85**
LTP-AP8	28	F	–	No	–	No	U/Ang	5	1	8	1.05
LTP-AP9	37	M	–	No	–	No	Ang	30	1	7	8.16
LTP-AP10	49	F	Grass	Yes	Persistent-mild	No	Anaph	10	1	13	13.2
TC1	35	F	–	No	–	No	T	–	–	–	<0.35
TC2	48	M	–	No	–	No	T	–	–	–	<0.35
TC3	46	M	HDM	Yes	Persistent-moderate	No	T	–	–	–	<0.35
TC4	48	M	Olive	Yes	Persistent-mild	No	T	–	–	–	<0.35
TC5	22	F	–	No	–	No	T	–	–	–	<0.35
TC6	39	F	HDM	Yes	Persistent-moderate	No	T	–	–	–	<0.35
TC7	26	F	Olive	Yes	Persistent-mild	No	T	–	–	–	<0.35
TC8	46	M	Olive	Yes	Persistent-moderate	No	T	–	–	–	<0.35
TC9	48	M	HDM	Yes	Persistent-mild	No	T	–	–	–	<0.35
TC10	39	M	HDM	Yes	Persistent-moderate	No	T	–	–	–	<0.35

Note: OPS, Onset of Peach Symptoms is the time (min) that has passed from the first reaction with peach to the patient evaluation in the consultation. Marked in bold letters, patients who were treated with SLIT-Prup3.

Abbreviations: Anaph, anaphylaxis; HDM, House Dust Mite; LTP-AP, Lipid transfer protein-allergic patients; N, number; SPT, Skin prick test and sIgE to Pru p 3; T, tolerant; TC, tolerant control; U/Ang, urticaria and/or angioedema.

for FA in clinical practise. Moreover, the different ILC2 phenotype frequencies and their correlations with aeroallergen-sIgE in LTP-AP and TC with or without rhinitis demonstrated that the differences found between LTP-AP and TC are mediated by Pru p 3 and not by the respiratory symptoms (data not shown).

We characterised the ILC2 profile during SLIT-Prup3 in PBMCs from LTP-AP treated for 1 year (Table S2 and Figure S4A). Results showed a significant decrease in total ILC2 during treatment and a reduction in IL-4^+ and IL-13^+ILC2 frequency at T12; being significantly lower in SLIT-Prup3-treated LTP-AP compared with non-treated LTP-AP, and tending to TC levels (Figure 1D). Moreover, T cell modulation by SLIT-Prup3 showed: i) a significant increase in IFNγ^+Th1 and Treg cells and a significant decrease in IL-4^+Th2 cells at T12 (Figure S3C)[7]; and ii) a positive correlation after 1 year of treatment between the Th2 cells and ILC2, being significant for IL-13^+ILC2 *vs* IL-13^+Th2 cells (Figure S3D). Furthermore, ILC2 were not capable of producing IFNγ, but they did produce IL-10 only in treated LTP-AP (Figure 1D). Indeed, we considered that IL-10^+ILC2 was an independent phenotype without IL-4 production by ILC2 (Figure S1); and after 1 year of treatment the IL-10 frequency was more elevated than IL-4^+ and IL-13^+ frequencies in the ILC2 (Figure 1E). These results suggested that SLIT-Prup3 could induce a new immunomodulatory function of activated IL-10^+ILC2. Although not directly studied in this work, immunological pathways, implicating the retinoic acid or IFN_γ production, could be associated with downregulated pro-inflammatory genes distinct from those that induce IL-4^+ or IL-13^+ILC2.[4,9] Moreover, we observed a significant negative correlation between IL-13^+ILC2; and IL-4^+ILC2 with the regulatory pattern of ILC2 in LTP-AP treated for T12 (Figure S5A). These results suggest that SLIT could modulate the regulatory ILC2 phenotype. Furthermore, our results showed the significant positive correlation of Treg cells *vs* IL-10^+ILC2 stimulated by SLIT-Prup3 in LTP-AP at T12 (Figure S5B). The correlation analysis between the regulatory pattern (Treg

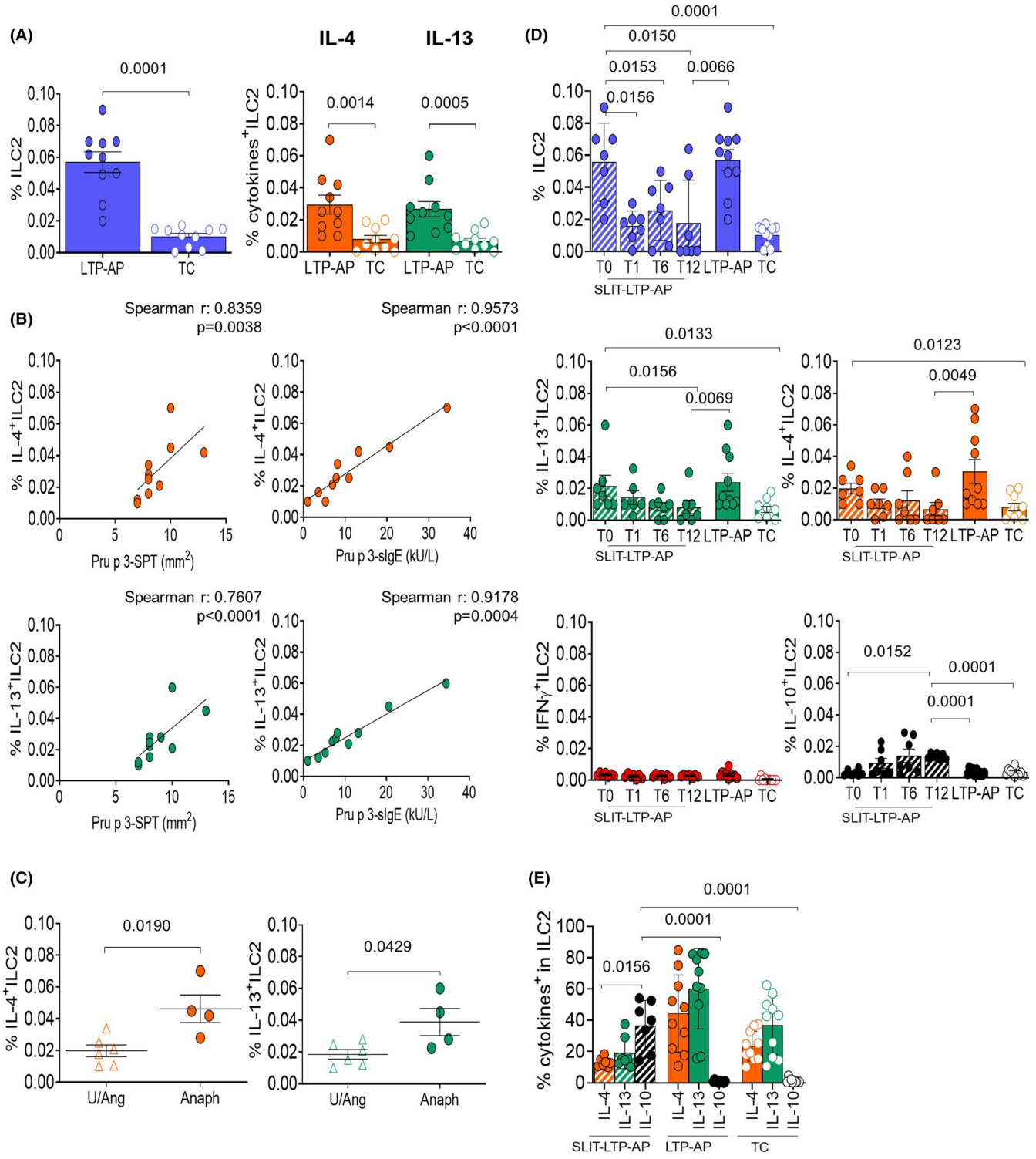

FIGURE 1 ILC2 analysis. (A) ILC2 percentages in LTP-allergic patients (LTP-AP, *N* = 10) and tolerant controls (TC, *N* = 10); (B) Correlation of the IL-4$^+$ and IL-13$^+$ILC2 percentages *vs* clinical parameters. (C) ILC2 percentages in LTP-AP with Urticaria and/or Angioedema (without colour, U/Ang, *N* = 6) and Anaphylaxis (with colour, Anaph, *N* = 4); (D) Changes in the total ILC2 and their phenotypes during SLIT-Prup3 in treated LTP-AP (*N* = 7) (T0, T1, T6 and T12), in non-treated LTP-AP, and TC at only one point. (E) IL-4, IL-13 and IL-10 percentages in ILC2 from treated LTP-AP (at T12), from non-treated LTP-AP, and TC at only one point. Bars plus symbols represent the mean and SEM. The symbols indicate individual data points for the different groups of study. The significant differences are represented as *p*-values <0.05

cells and IL-10$^+$ILC2) and clinical characteristics after T12 showed positive correlations (Figures S4B-S5C), significant for Treg cells *vs* Pru p 3-sIgG4. Although most of these correlations were not significant, probably because of the sample size, suggested the possible implication of regulatory cells in the tolerance response induced by AIT.

Our results report the first evidence of the presence of ILC2 in FA patients and their modulation by specific immunotherapy. Overall, we have identified and characterised peripheral ILC2 in LTP-AP, with frequencies closely related to clinical parameters (in vivo) together with Pru p 3-sIgE levels and Th2 cells. Furthermore, SLIT-Prup3 could change their type 2 response phenotype frequencies in peripheral blood towards a regulatory phenotype, associated with regulatory pattern of the adaptive T cells, suggesting their contribution to clinical and immunological tolerance. We are aware of the need of more studies, if the changes in the ILC2 frequency are maintained in the time; and on a larger population for AIT with Pru p 3 and specific cytokines to stimulate the ILC2 and analysing the $IL10^{+}$ILC2 functionality in the immunomodulation. Even so, these findings offer new information on ILC2 participation in loss of tolerance in FA, postulating them as therapeutic targets and biomarkers to predict food-immunotherapy responses.

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CONFLICT OF INTEREST

The authors declare that they have no relevant conflicts of interest.

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