

Th2A and Th17 cell frequencies and regulatory markers as follow-up biomarker candidates for successful multifoed oral immunotherapy

To the Editor,

In the context of food oral immunotherapy (OIT), the identification of biomarkers that can replace current standard burdensome and risky double-blind placebo-controlled food challenges (DBPCFC), which are used pre- and post-therapy,¹ would be of great value in clinical settings. With this goal in mind, we undertook a comprehensive immune response analysis on blood cells obtained from a sub-group of multi-allergic participants in a clinical multi-OIT study (ClinicalTrials.gov number, NCT02626611)² to evaluate potential biomarker candidates. In the open-label phase of the multi-OIT study ($n = 70$, age 5-22 years), participants received omalizumab (weeks 1-16) and multi-OIT (2-5 allergens; 1 g each; weeks 8-30), after which they were tested by food challenge (week 30). Subsequently, 60 eligible participants (excluding 10 drop-outs) were randomized 1:1:1 to receive in a blind manner either 0, 300 mg, or 1 g of food allergens (weeks 30-36). These participants were then tested again by food challenge at week 36.

We analyzed systemic changes in immune parameters, such as T helper (Th) cell and innate lymphoid cell (ILC) frequencies as well as dendritic cell (DC)-associated markers, occurring after 30-week multi-OIT, in a subset of 42 out of the 60 eligible patients. The demographics and baseline characteristics of patients are provided in Table S1. Peripheral blood mononuclear cells (PBMCs) were stained with fluorescently labeled antibodies as described in Supplementary Methods according to the gating strategy shown in Figure S1. We observed lower frequencies of both Th2A ($P < .05$) and Th17 ($P = .0504$) subsets in PBMCs at week 30 when compared to baseline (Figure 1A,B). For the sake of simplicity, we divided ages in two groups instead of three groups since only 3 participants were over 15-year-old. Noticeably, the decrease of Th2A cell frequencies was statistically significant ($P < .05$) only for patients aged 10 and over, although a positive trend was also detected in younger patients (Figure 1A, right panel). Additionally, multi-OIT markedly ($P < .05$) decreased Th17 cell frequencies in patient aged 10 and over, but no differences were seen for patients under 10-year-old. (Figure 1B, right panel). To further explore cellular immune responses, we performed a detailed analysis of changes in innate immune cells by flow cytometry. However, no alterations were observed between groups in terms of cell frequencies for both type 1, 2, and 3 innate lymphoid as well as for DC subsets (data not shown). In addition, we monitored PBMCs for expression of markers associated with type 1 (ie,

MX1), type 2 (ie, GATA3 and CD141), or regulatory (ie, C1q, STAB1 and *FcγRIIIa*) DCs previously identified to correlate with clinical efficacy of allergen immunotherapy (AIT)^{3,4} (See Supplementary Methods). We did not observe any changes in DC1- or DC2-associated markers. In contrast, 2 out of 3 DCre markers exhibited statistical differences before and after multi-OIT. The expression of STAB1 was significantly ($P < .05$) upregulated in PBMCs from multi-OIT patients at week 30 when compared to baseline (Figure 1C, left panel) particularly for patients aged 10 and over (Figure 1C, right panel). Multi-OIT did not alter the expression of *FcγRIIIa* in patients' blood when considering the whole subset (Figure 1D, left panel). However, *FcγRIIIa* expression was significantly ($P < .05$) increased in PBMCs from patients under 10 and correlated with a decrease of Th17 cell frequencies (Figure S2; center panel) but was unchanged in patients older than 10 years of age (Figure 1D, right panel).

At week 36, immune changes were also analyzed in sub-groups of patients receiving either 0 mg ($n = 12$), 300 mg ($n = 17$), or 1 g ($n = 13$) as aforementioned. No differences in terms of Th2A or Th17 cell frequencies (Figure S3) or STAB1 expression (Figure 2A, upper panel) were observed between baseline and week 36 within the 3 groups (0 mg, 300 mg, 1 g), likely due to a limited number of patients. It is thus difficult to conclude on the value of following such markers after cessation of OIT. Only on pooling data from the 300 mg and 1 g treated groups, STAB1 was observed to be significantly ($P < .05$) upregulated following a 36-week multi-OIT, particularly in PBMCs from patients aged 10 and over (Figure 2A, lower panel). Strikingly, *FcγRIIIa* expression was significantly ($P < .01$) increased in PBMCs from patients receiving 1 g of food allergens up to week 36 (Figure 2B, upper panel). On pooling data from the 300 mg and 1 g treated groups, we confirmed that this marker is significantly increased in PBMCs irrespective of age groups at W36 (but not at W30), suggesting that this DCre marker is regulated in a time- and age-dependent fashion (Figure 2B, lower panel).

These results establish for the first time that omalizumab-facilitated multi-OIT induces changes in immune polarization-based readouts. Multi-OIT promoted a decrease in Th2A and Th17 cell frequencies while increasing regulatory markers in blood, particularly evidenced for patients aged 10 and over for which desensitization has been successful. Such results will need to be confirmed with a larger cohort of patients in DBPC clinical trials since we acknowledge the limits of our study due to a low number of participants.

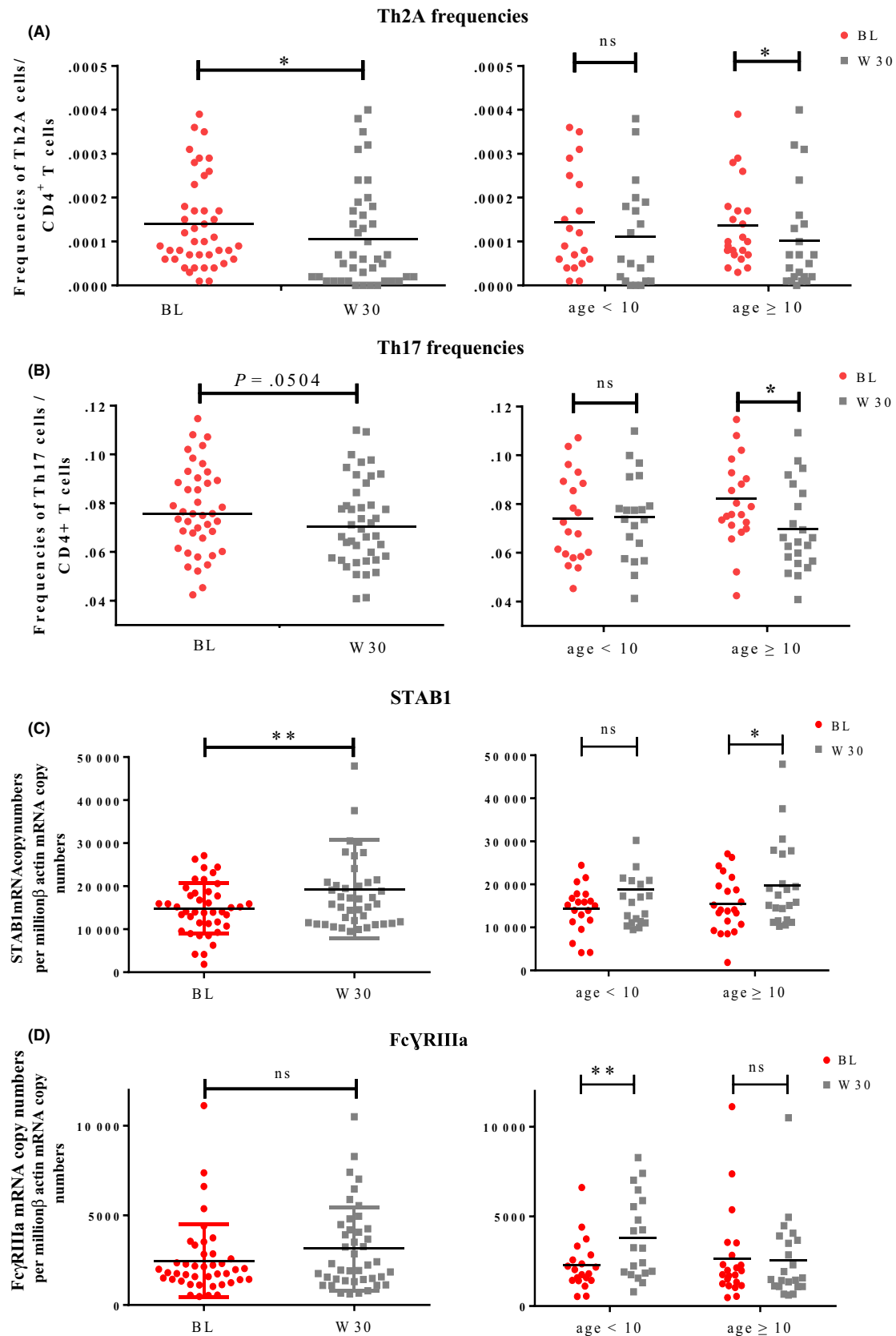


FIGURE 1 Th2A and Th17 cell frequencies decreased while the regulatory marker STAB1 and Fc γ RIIIa increased after multi-OIT. PBMCs from patients receiving multi-OIT ($n = 42$) were analyzed at baseline (BL) and week 30 (W30) for Th2A (A) and Th17 (B) cell frequencies by flow cytometry and for STAB1 (C) and Fc γ RIIIa (D) gene expression by quantitative PCR. Age-related factors were analyzed for patients under 10 and aged 10 and over. Wilcoxon tests were used for statistical analysis. * $P < .05$; ** $P < .01$; ns = nonsignificant

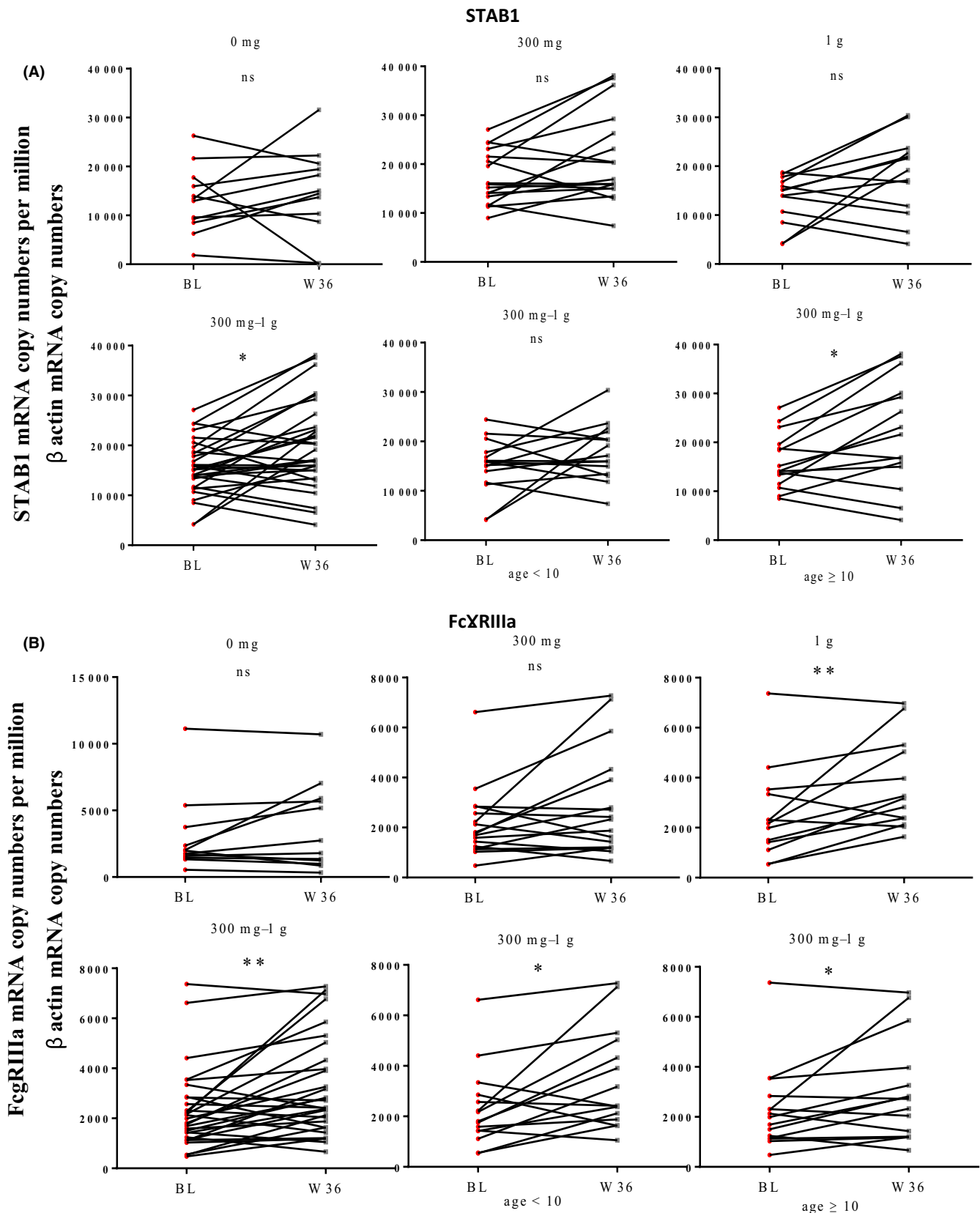


FIGURE 2 Regulatory markers STAB1 and FcγRIIIa are confirmed to be upregulated in PBMCs from patients after extension of multi-OIT. At week 30 (W30), patients receiving OIT were randomized in 3 groups: patients interrupting the treatment (0 g; n = 12), patients continuing OIT with 300 mg (n = 17) or 1 g (n = 13) until week 36 (W36). PBMCs from patients were analyzed at baseline (BL) and W36 for STAB1 (A) and FcγRIIIa (B) gene expression by quantitative PCR. Age-related factor was analyzed for patients under 10 and aged 10 and over. Wilcoxon tests were used for statistical analysis. * $P < .05$; ** $P < .01$; ns = nonsignificant

Additionally, long-term therapy should be considered as it may enable sustained effect and allow cessation of immunotherapy.² Our findings are fully aligned with previous reports monitoring follow-up candidate biomarkers in AIT studies for respiratory allergies.^{3,4} Although a surrogate biomarker of AIT efficacy has not been validated so far, immune changes documented in peripheral blood or mucosal tissues of patients include the downregulation of allergen-specific Th2 cells.^{5,6} On the other hand, a limited number of studies have analyzed a change in Th17 polarization with mixed conclusions.^{7,8} We also confirmed the interest of both STAB1 and FcγRIIIa as hallmark DCre markers in the mechanism of action of immunotherapy.^{3,4} This is consistent with observations that STAB1 is expressed by tolerogenic DCs/macrophages and that it contributes to fetal implantation in the human decidua.^{4,9} In contrast, the identification of FcγRIIIa as a DCre marker was less expected since this receptor is more involved in inflammatory mechanisms.⁴

Altogether, our findings pave the way for further understanding of the mechanisms involved in food immunotherapy. Positive outcomes on systemic immune parameters, as observed in this study, confirm the value of monitoring (particularly in PBMCs from pediatric patients), Th2/Th17 cell frequencies and DCre markers as potential replacements for oral food challenges. We also suggest that successful multi-OIT involve different immune pathways according to the maturation level of the immune systems during infancy.

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CONFLICT OF INTEREST

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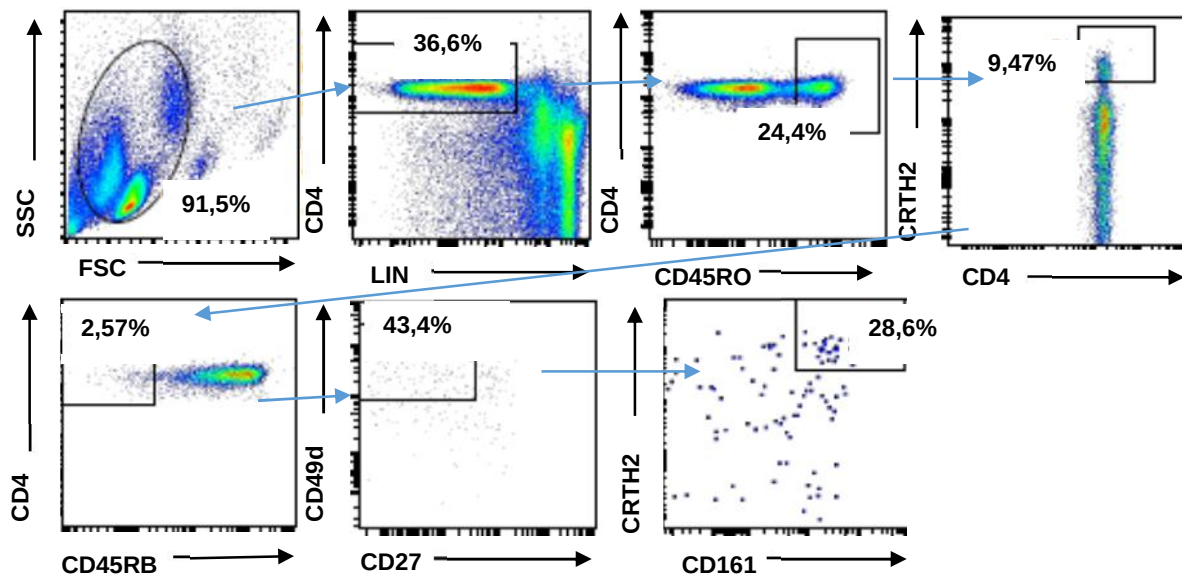
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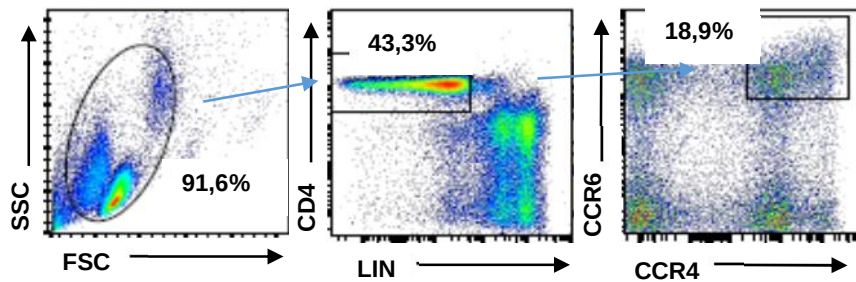
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

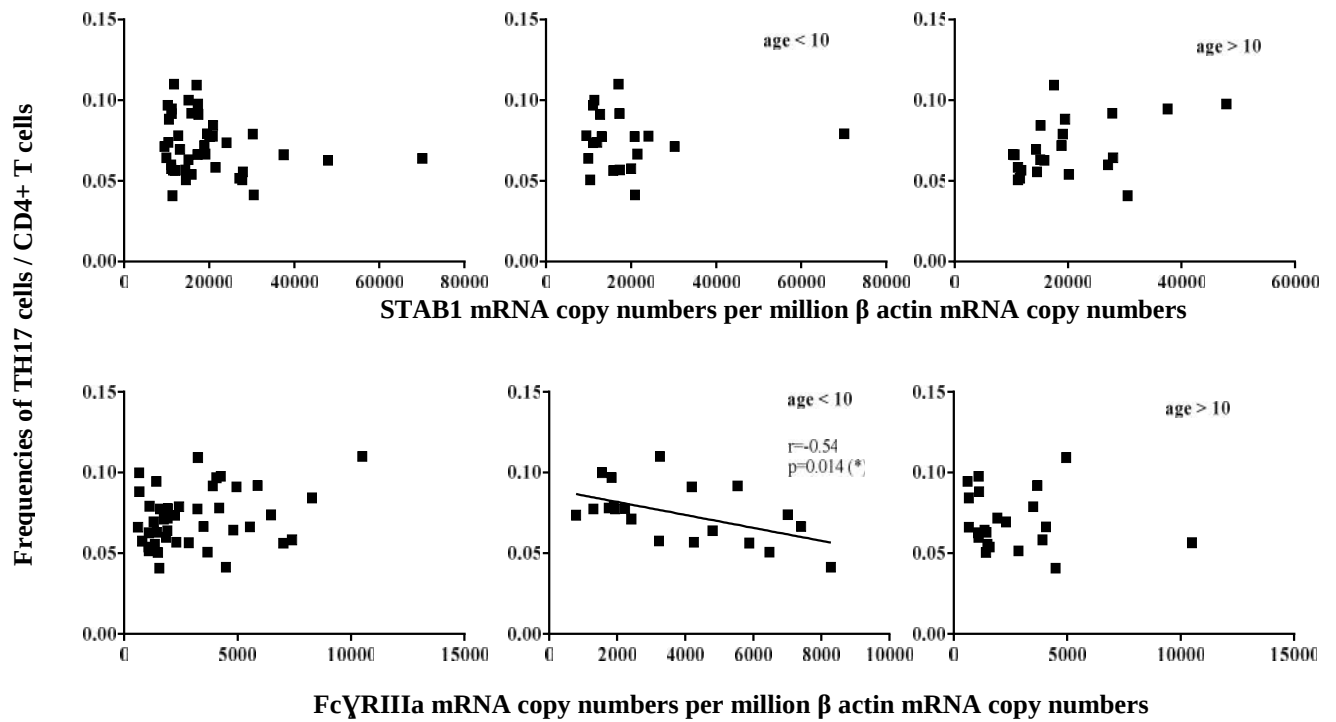
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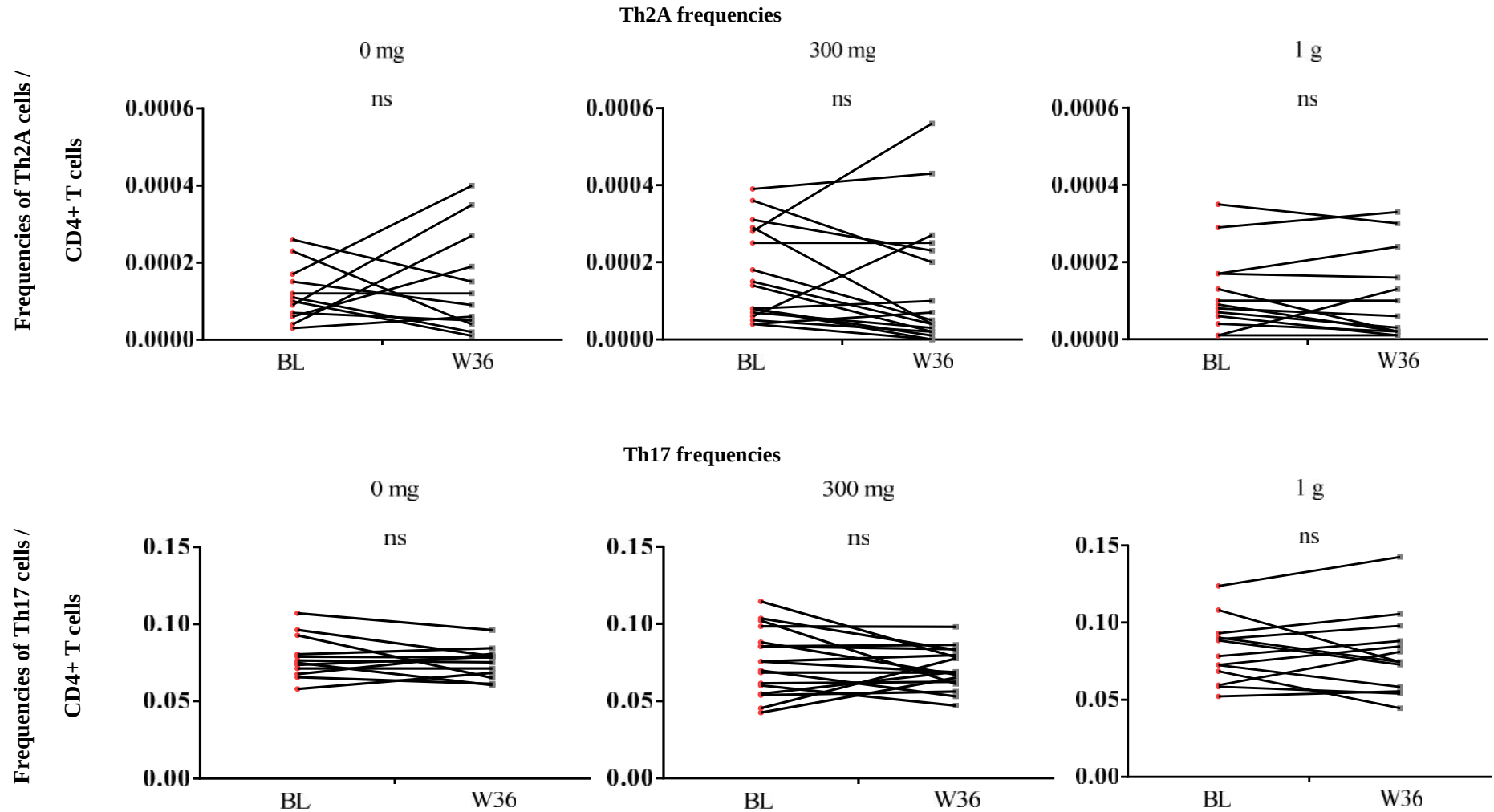
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Supplementary Figure S1: Gating strategy for flow cytometry analyses of Th2A and Th17 cell frequencies. Whole leukocytes population was identified within human PBMCs with the measurements of forward-scattered light (FSC) and side-scattered light (SSC). **(A)** Th2A cells were identified as CD4⁺, CD45RO⁺, cells negative for lineage-associated markers (Fixable Viability dye, CD8, CD11c, CD14, CD16, CD20, CD34, CD56, CD123 and TCR α 27j α 18), CD45RB⁻, CD27⁻, CD49d⁺ and CD161⁺. **(B)** Th17 cells were identified as CD4⁺, cells negative for lineage-associated markers (Fixable Viability dye, CD8, CD11c, CD14, CD16, CD20, CD34, CD56, CD123 and TCR α 27j α 18), CCR6⁺ and CCR4⁺. All gates were set up based on reactivity with relevant control isotype. Representative dot plots are shown.



Supplementary Figure S2: Correlation between frequencies of Th17 cells and FcγRIIIa mRNA copy numbers after W30 of multi OIT for patients under 10-year-old. Spearman correlations between Th17 cell frequencies and the regulatory marker STAB1 and FcγRIIIa in PBMCs from patients (n = 42) after W30 of multi OIT. Age-related factors were analysed for patients under 10 and aged 10 and over.



Supplementary Figure S3: Analyses of Th2A and Th17 cell frequencies by flow cytometry in PBMCs from patients at baseline (BL) and week 36 (W36). At week 30 (W30), patients receiving OIT were randomised in 3 groups: patients interrupting the treatment (0 g; n=12), patients continuing OIT (300 mg; n=17) and (1 g; n=13).

Supplementary Table 1: Demographics and clinical characteristics at baseline

	Total N = 42	0 mg N=12	300 mg N=17	1 g N=13	300-1000 mg N=30
Age in years, mean (SD)	9.9 (3.6)	10.3 (3.9)	10.5 (4.2)	8.5 (2.2)	9.7 (3.6)
Gender, n (%)					
male	25 (59.5%)	8 (66.6%)	7 (41.2%)	10 (76.9%)	17 (56.6%)
female	17 (40.5%)	4 (33.3%)	10 (58.8%)	3 (23.1%)	13 (43.3%)
Total IgE (KU/L), mean (SD)	890.9 (497.3)	908 (507.1)	836.2 (501.8)	946.1 (515.4)	883.8 (501.9)
History of comorbid conditions, n (%)					
Asthma	29 (69%)	7 (58.3%)	10 (58.8%)	12 (92.3%)	22 (73.3%)
Allergic rhinitis	37 (88%)	11 (91.6%)	14 (82.3%)	12 (92.3%)	26 (86.6%)
Atopic dermatitis	37 (88%)	11 (91.6%)	13 (76.5%)	13 (100%)	26 (86.6%)
Participants with..., n (%)					
2 allergens in OIT	12 (28.6%)	3 (25%)	7 (41.2%)	2 (15.4%)	8 (26.6%)
3 allergens in OIT	10 (23.8%)	3 (25%)	2 (11.8%)	5 (38.5%)	7 (23.3%)
4 allergens in OIT	10 (23.8%)	3 (25%)	4 (23.5%)	3 (23.1%)	7 (23.3%)
5 allergens in OIT	10 (23.8%)	3 (25%)	4 (23.5%)	3 (23.1%)	7 (23.3%)
Participants with..., n (%)					
Almond in OIT	4 (9.5%)	1 (8.3%)	1 (5.9%)	2 (15.4%)	3 (10%)
Cashew in OIT	26 (61.9%)	7 (58.3%)	11 (64.7%)	8 (61.5%)	19 (63.3%)
Egg in OIT	11 (26.2%)	5 (41.6%)	3 (17.6%)	3 (23.1%)	6 (20%)
Hazelnut in OIT	12 (28.6%)	3 (25%)	3 (17.6%)	6 (46.1%)	9 (30%)
Milk in OIT	13 (30.9%)	7 (58.3%)	3 (17.6%)	3 (23.1%)	6 (20%)
Peanut in OIT	36 (85.7%)	9 (75%)	16 (94.1%)	11 (84.6%)	27 (90%)
Pecan in OIT	9 (21.4%)	0 (0%)	5 (29.4%)	4 (30.8%)	9 (30%)
Sesame in OIT	6 (14.3%)	3 (25%)	2 (11.8%)	1 (7.7%)	3 (10%)
Shrimp in OIT	3 (7.1%)	1 (8.3%)	2 (11.8%)	0 (0%)	2 (6.6%)
Soy in OIT	1 (2.4%)	0 (0%)	1 (5.9%)	0 (0%)	1 (3.3%)
Walnut in OIT	20 (47.6%)	5 (41.6%)	7 (41.2%)	8 (61.5%)	15 (50%)
Wheat in OIT	3 (7.1%)	1 (8.3%)	2 (11.8%)	0 (0%)	2 (6.6%)

Results describing continuous variables are expressed as means (SDs). Results describing categorical variables are expressed as percentages relative to numbers of oral immunotherapy treated patients.

