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Article type : Letter to the Editor

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

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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 cells 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

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acid, has been characterised (4). However, the innate immune cells 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-cells 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 one 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 online supplementary 1 (OS1)-OS2) 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-cells percentages (Figure OS3A) 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 OS3B).

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 for FA in clinical practice. 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 one year (Table OS2 and Figure OS4A). 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-cells modulation by SLIT-Prup3 showed: i) a significant increase in IFN γ ⁺Th1- and Treg-cells and a significant decrease in IL-4⁺Th2-cells at T12 (Figure OS3C)(7); and ii) a positive correlation after one year of treatment between the Th2-cells and ILC2, being significant for IL-13⁺ILC2 vs IL-13⁺Th2-cells (Figure OS3D). Furthermore, ILC2 were not capable of producing IFN γ , 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 OS1); and after one-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 OS5A). 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 OS5B). The correlation analysis between the regulatory pattern (Treg-cells and IL-10⁺ILC2) and clinical characteristics after T12, showed positive correlations (Figure OS4B-OS5C), 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 STATEMENT.

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

Table 1. Clinical characteristics and allergological work-up results

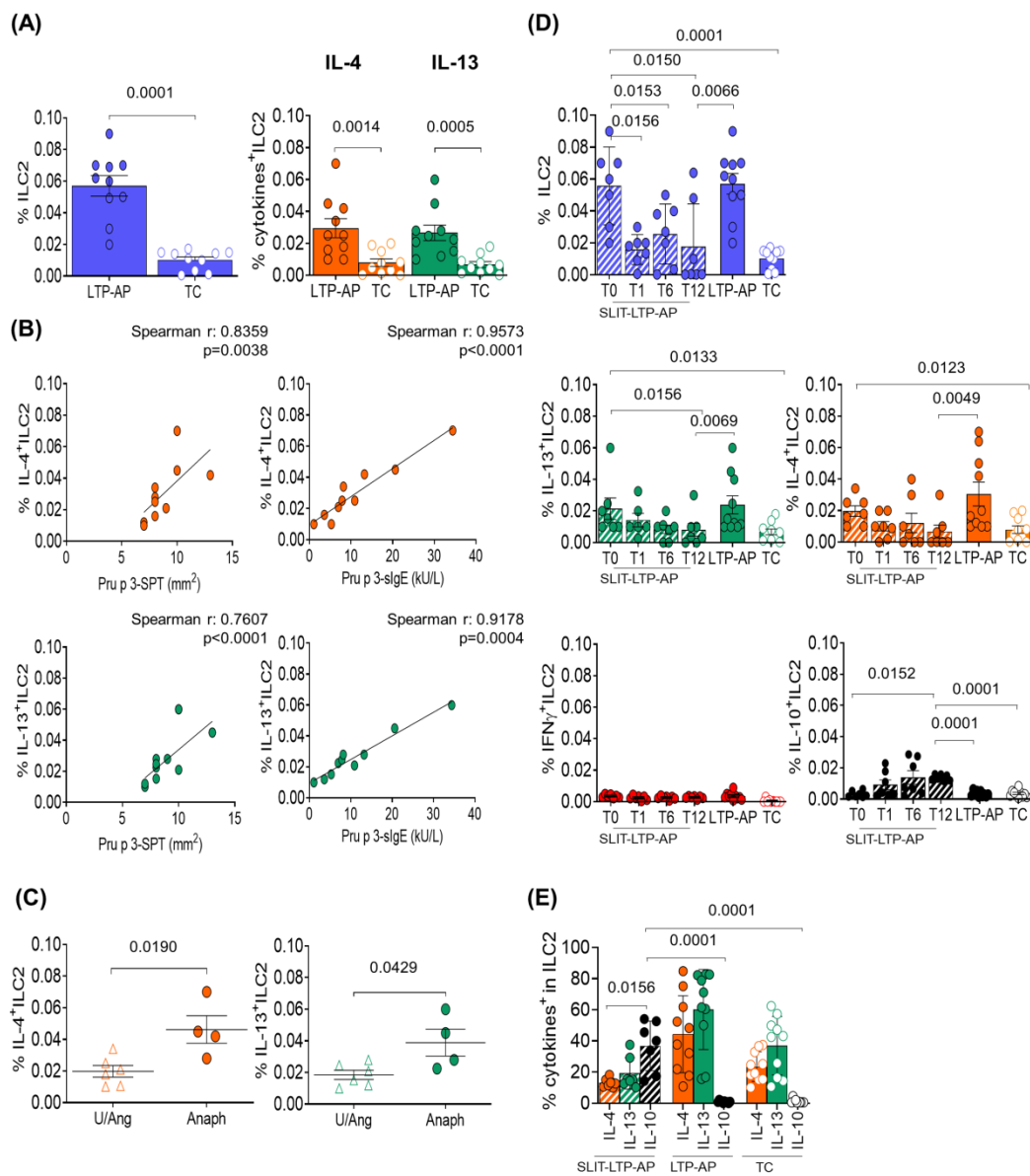
N. Subjects	Age	Sex	Pollen/Mite sensitisation	Rhinitis	Rhinitis severity (Aria)	Asthma/atopic dermatitis	Reaction type	OPS	N. episodes	SPT (mm ²)	sIgE (kU/L)
LTP-AP1	34	F	Grass/olive	yes	Persistent-Mild	no	Anaph	5	>3	8	6.96
LTP-AP2	44	M	Grass	yes	Persistent-Moderate	no	U	17	>3	8	20.6
LTP-AP3	39	F	-	no	-	no	U/Ang	15	>3	9	10.9
LTP-AP4	32	F	Grass/Olive	yes	Intermitent-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

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

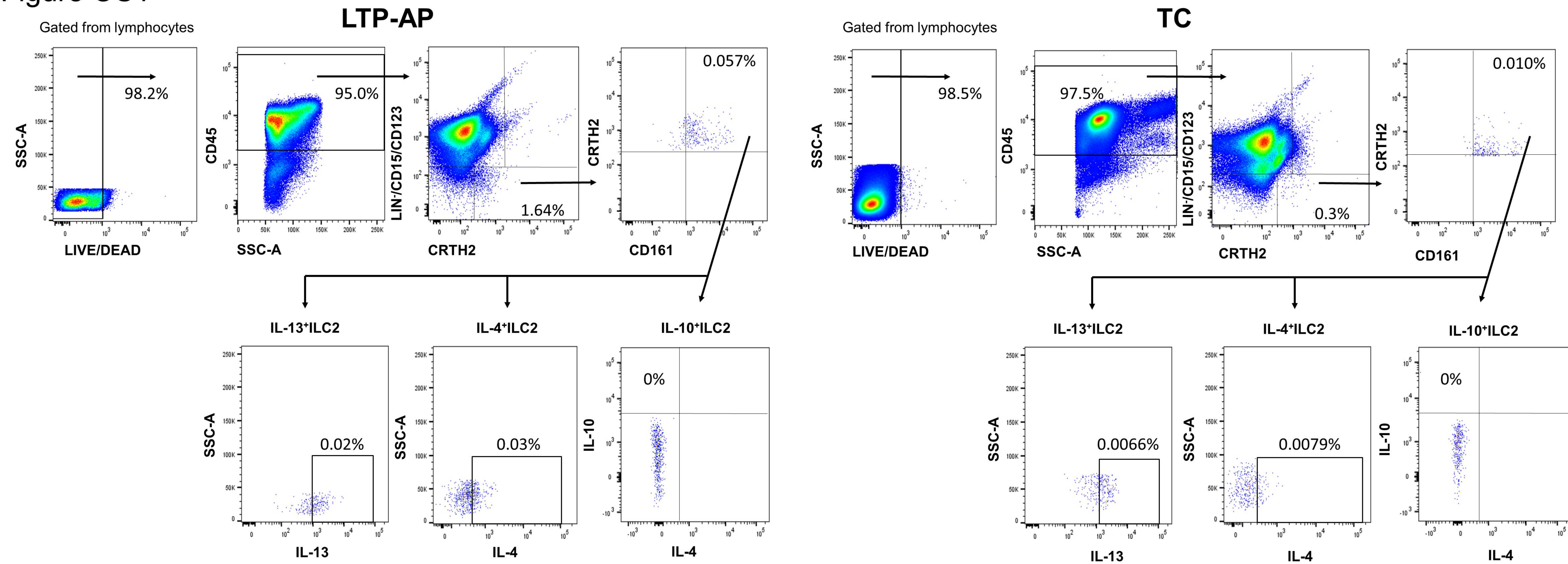
FIGURE LEGEND

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.



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Figure OS1



SLIT-LTP-AP at T12

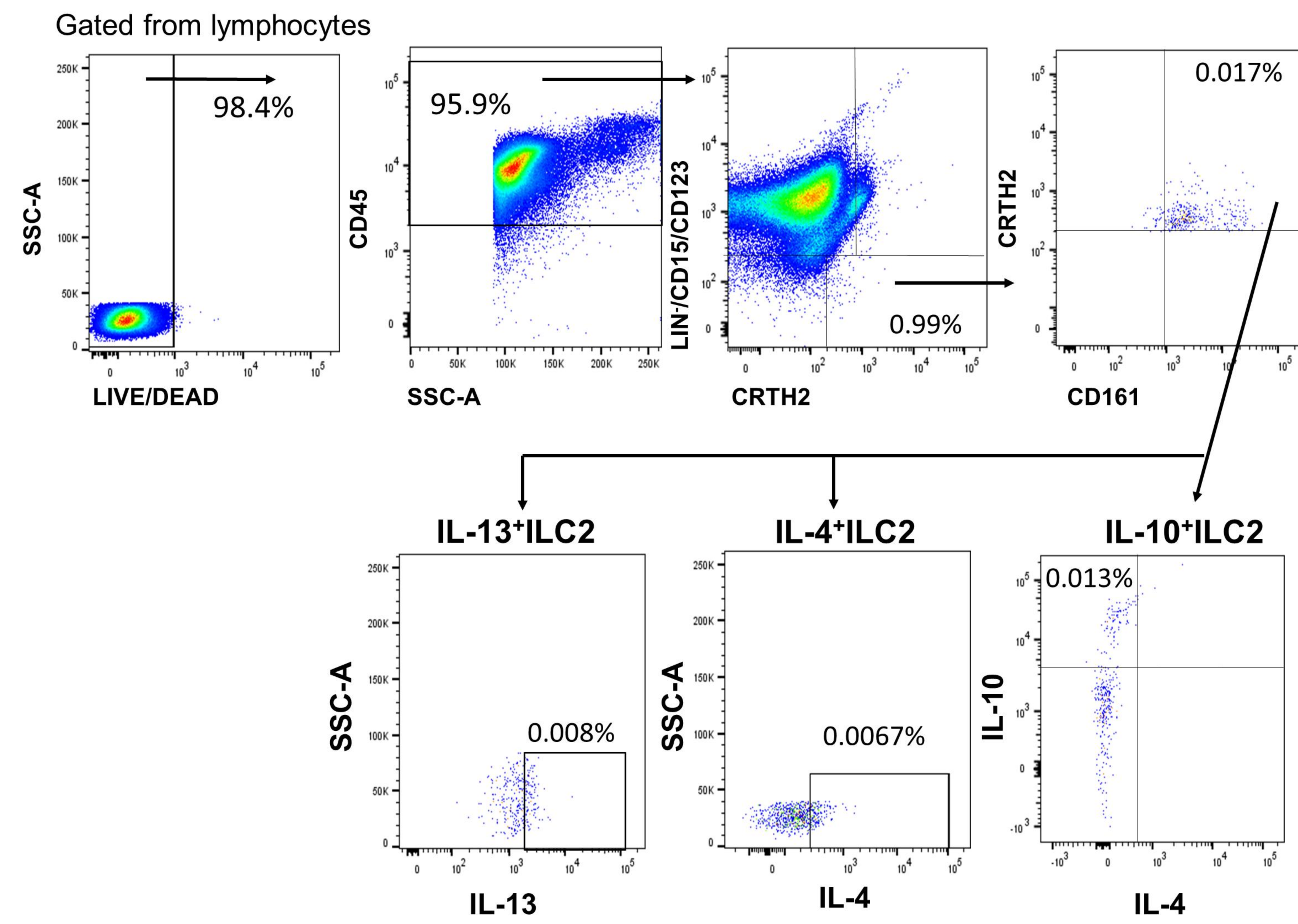


Figure OS2

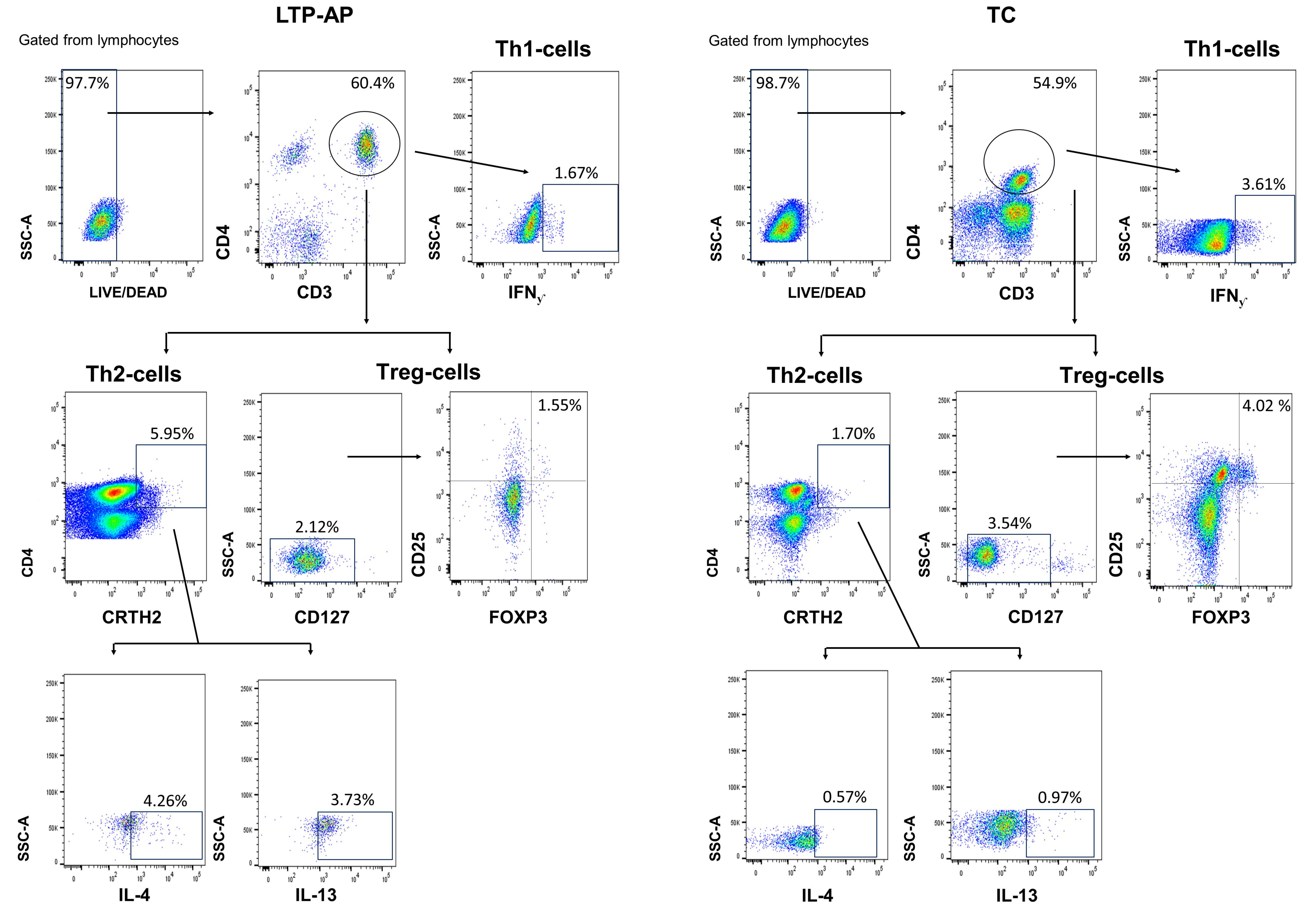
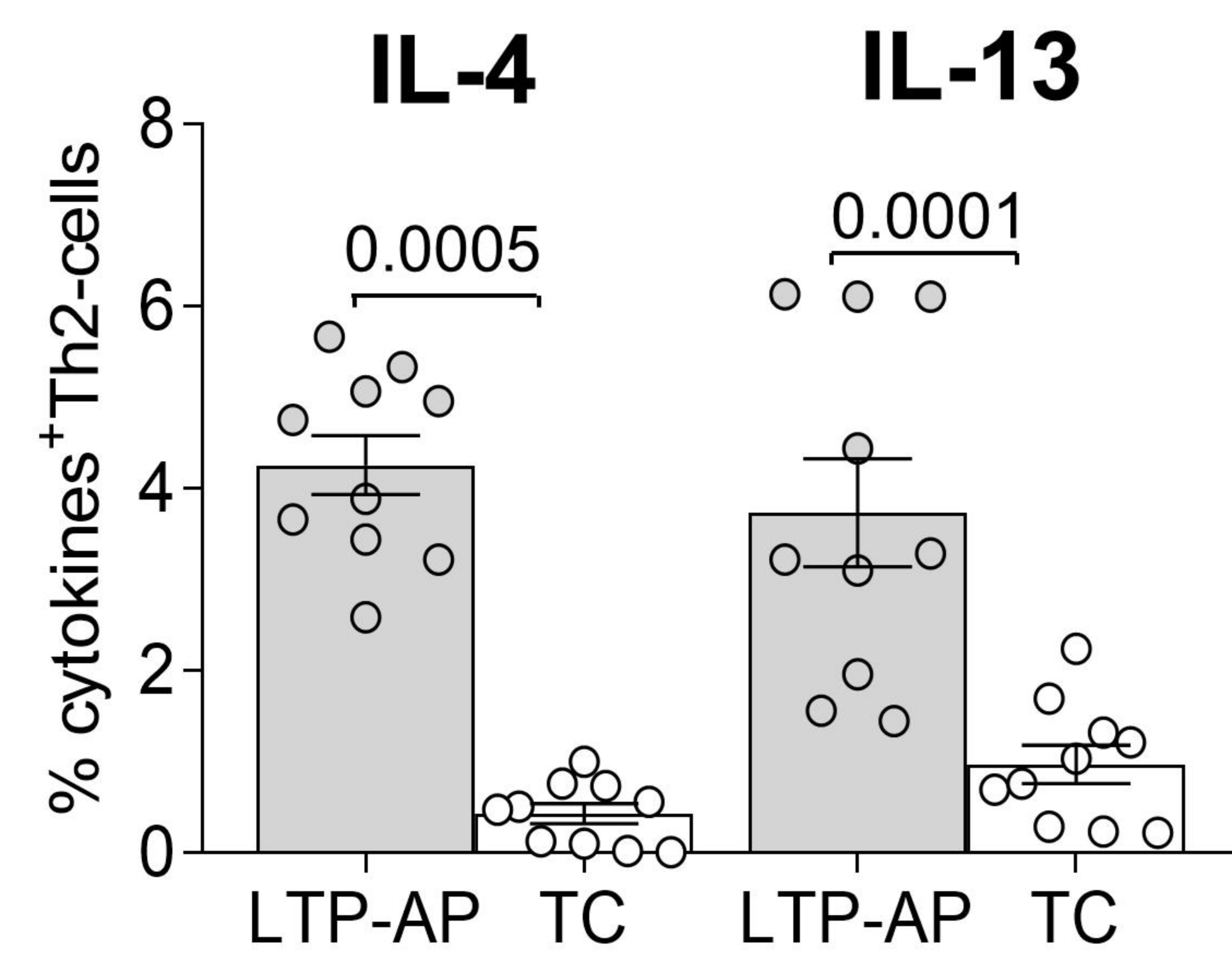
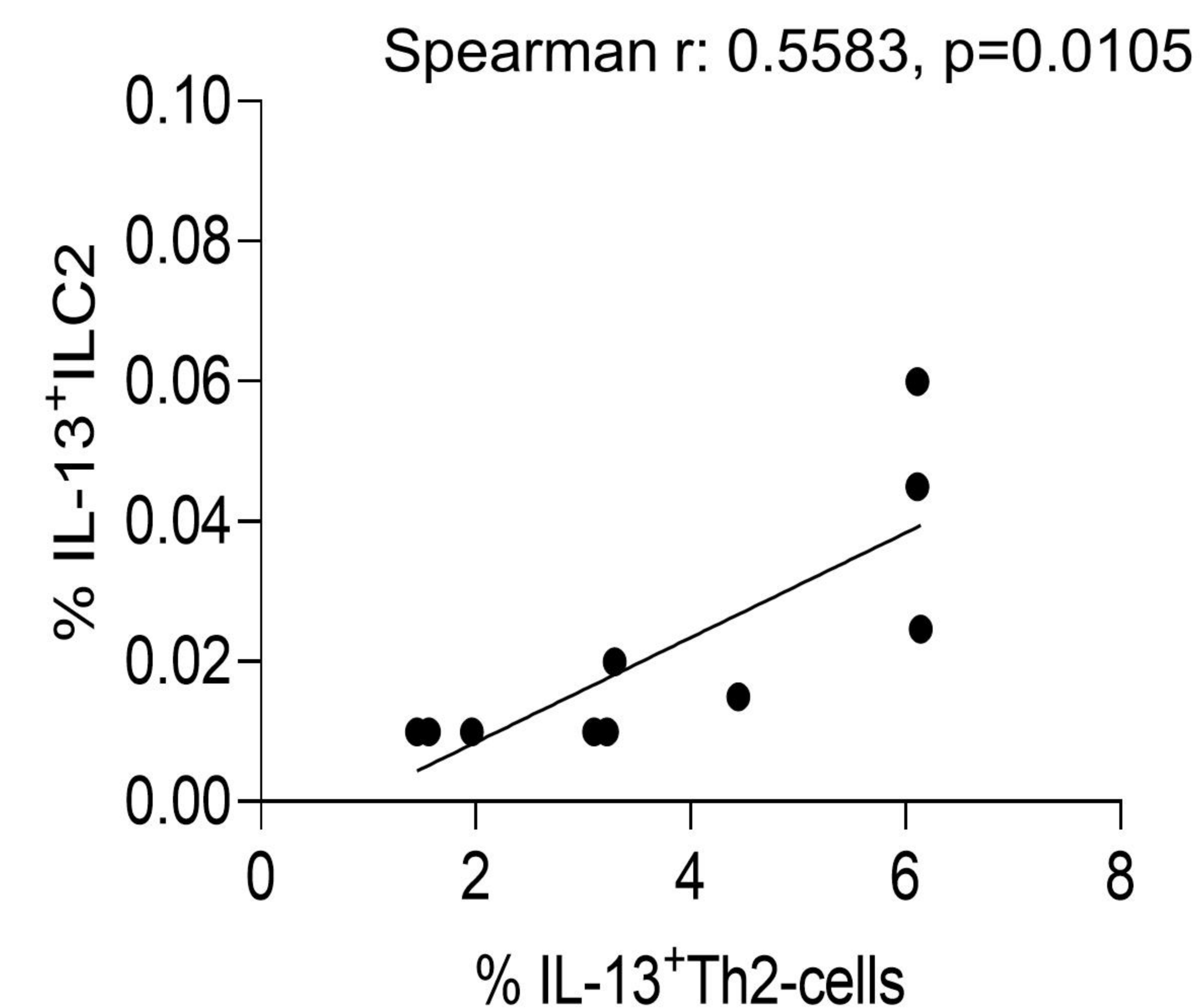
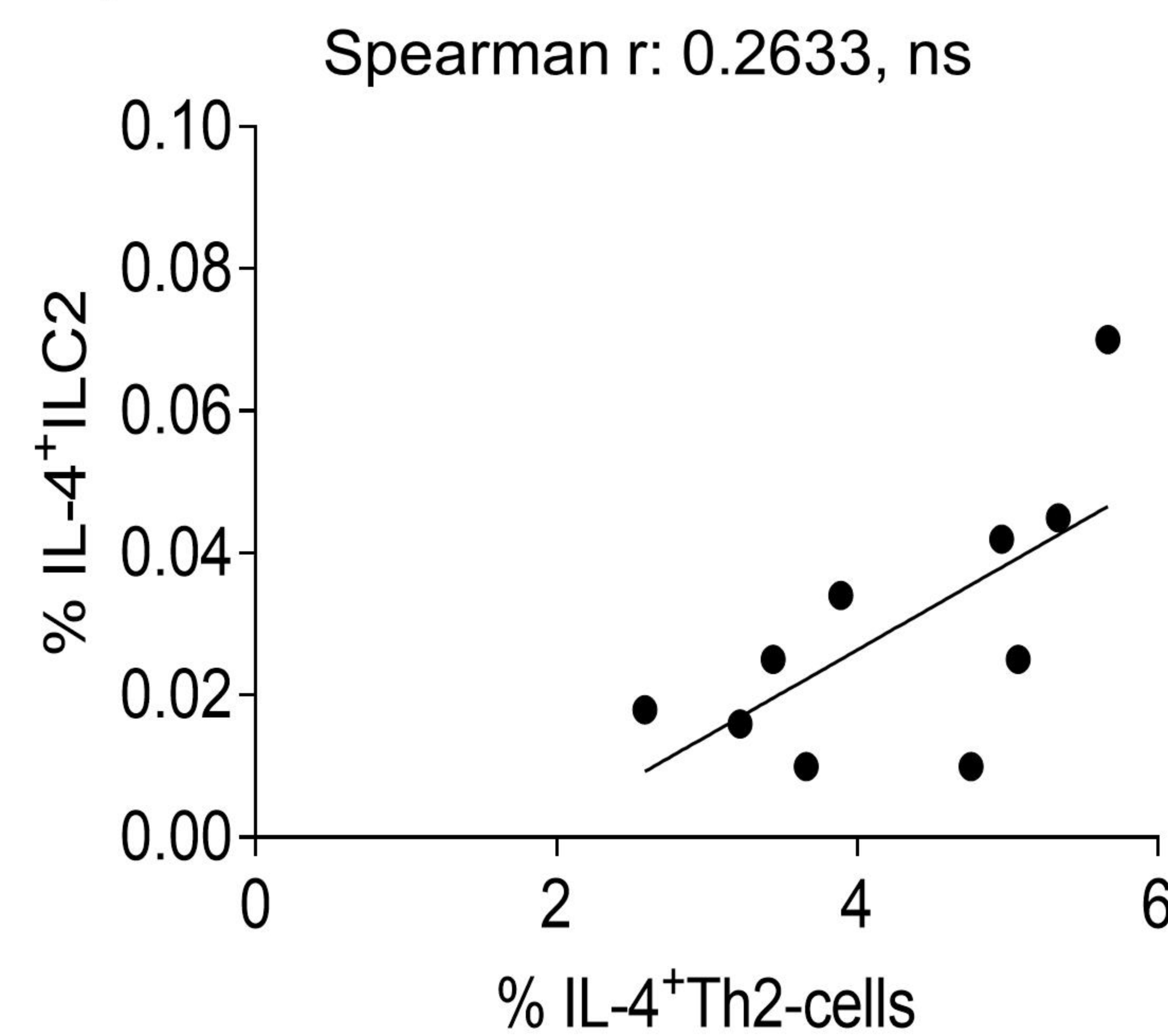


Figure OS3

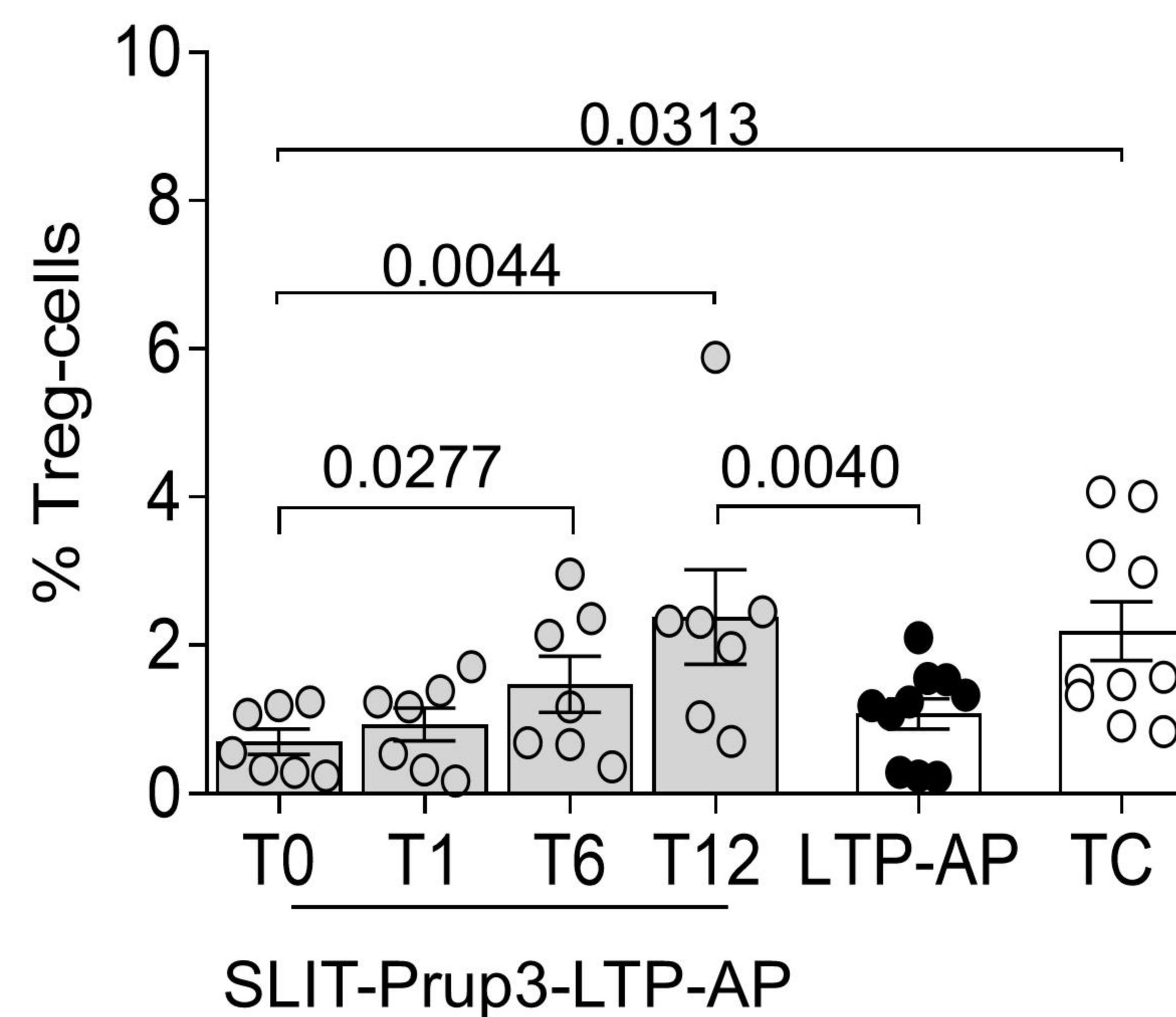
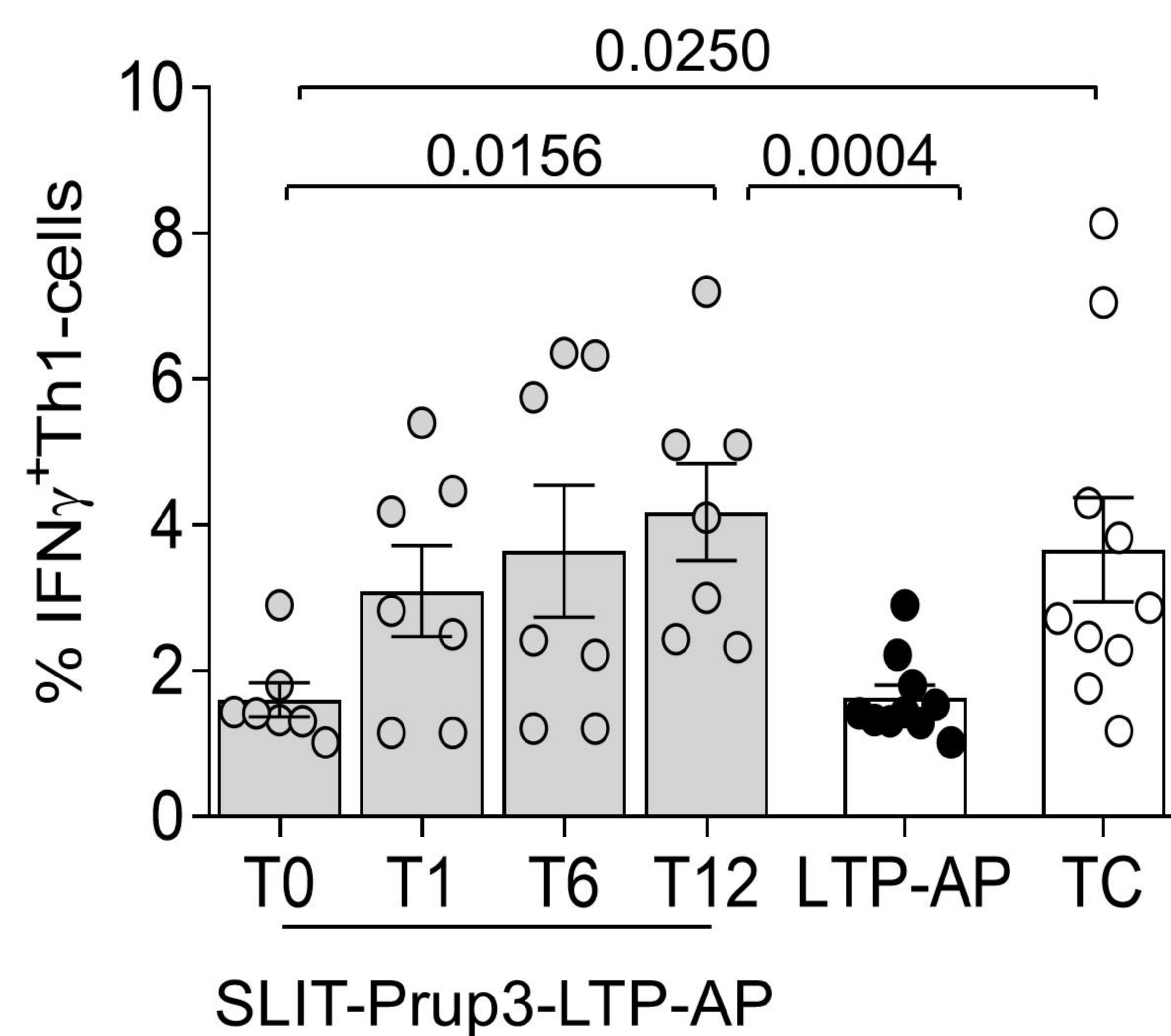
A)



B)



C)



D)

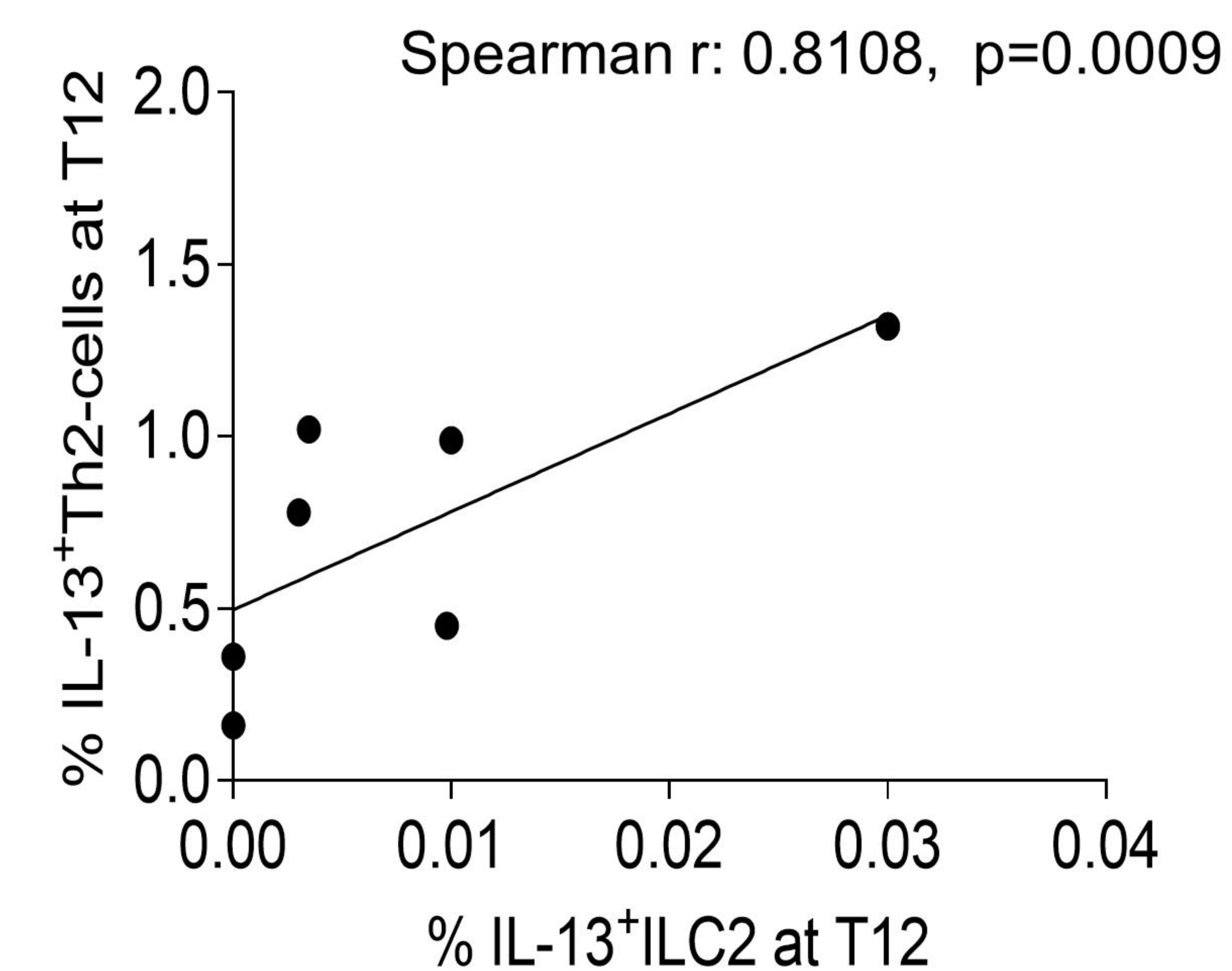
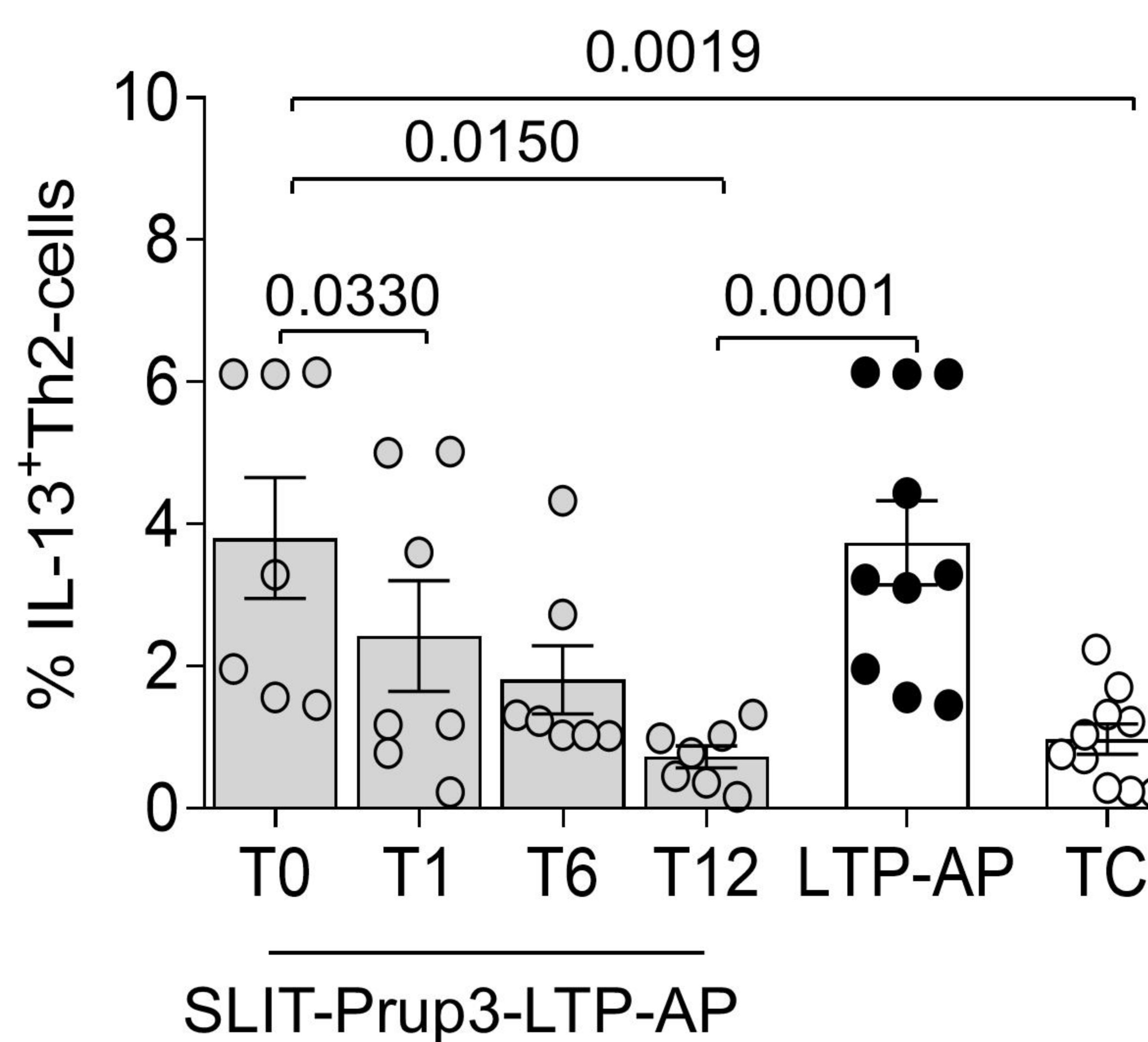
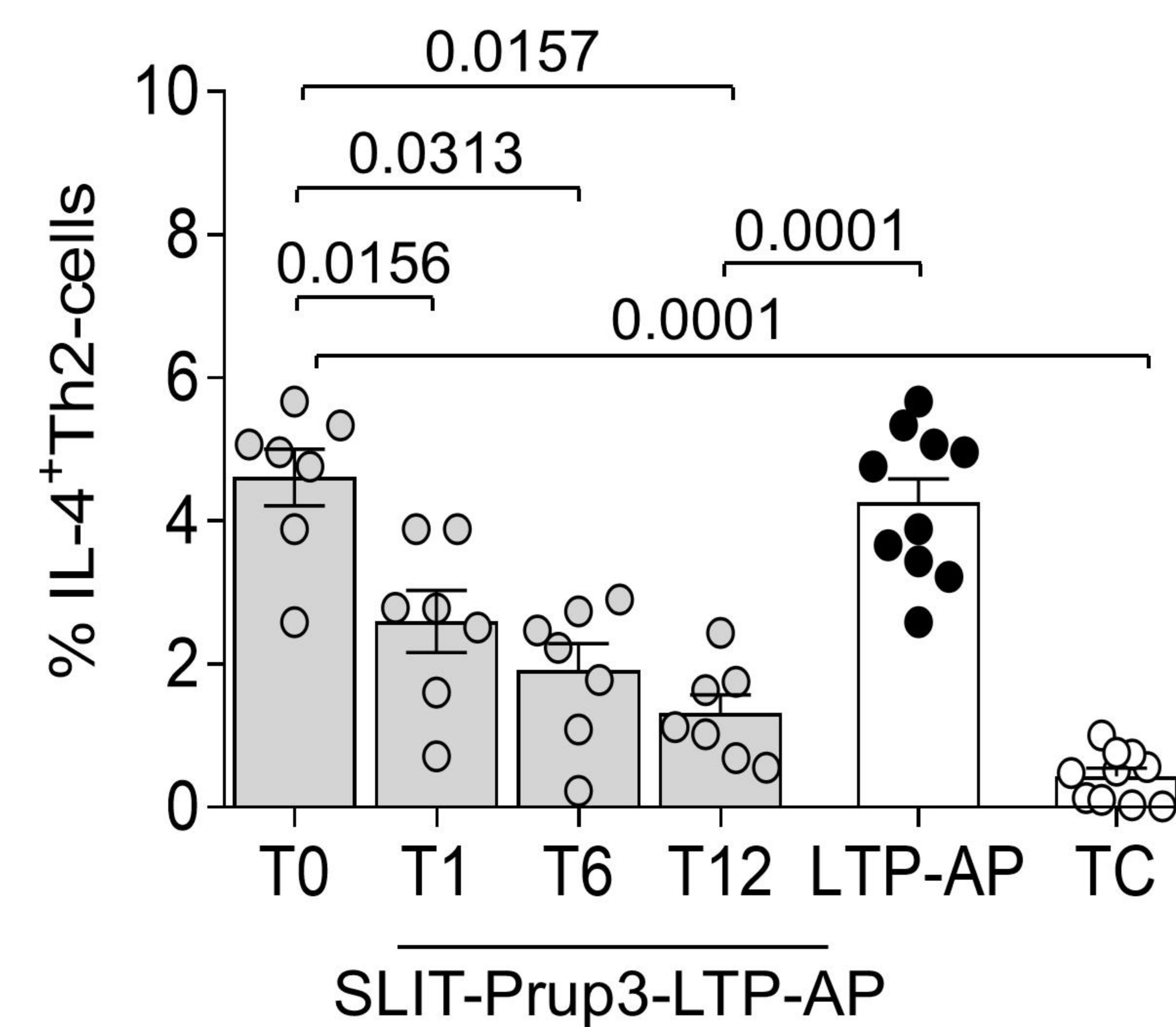
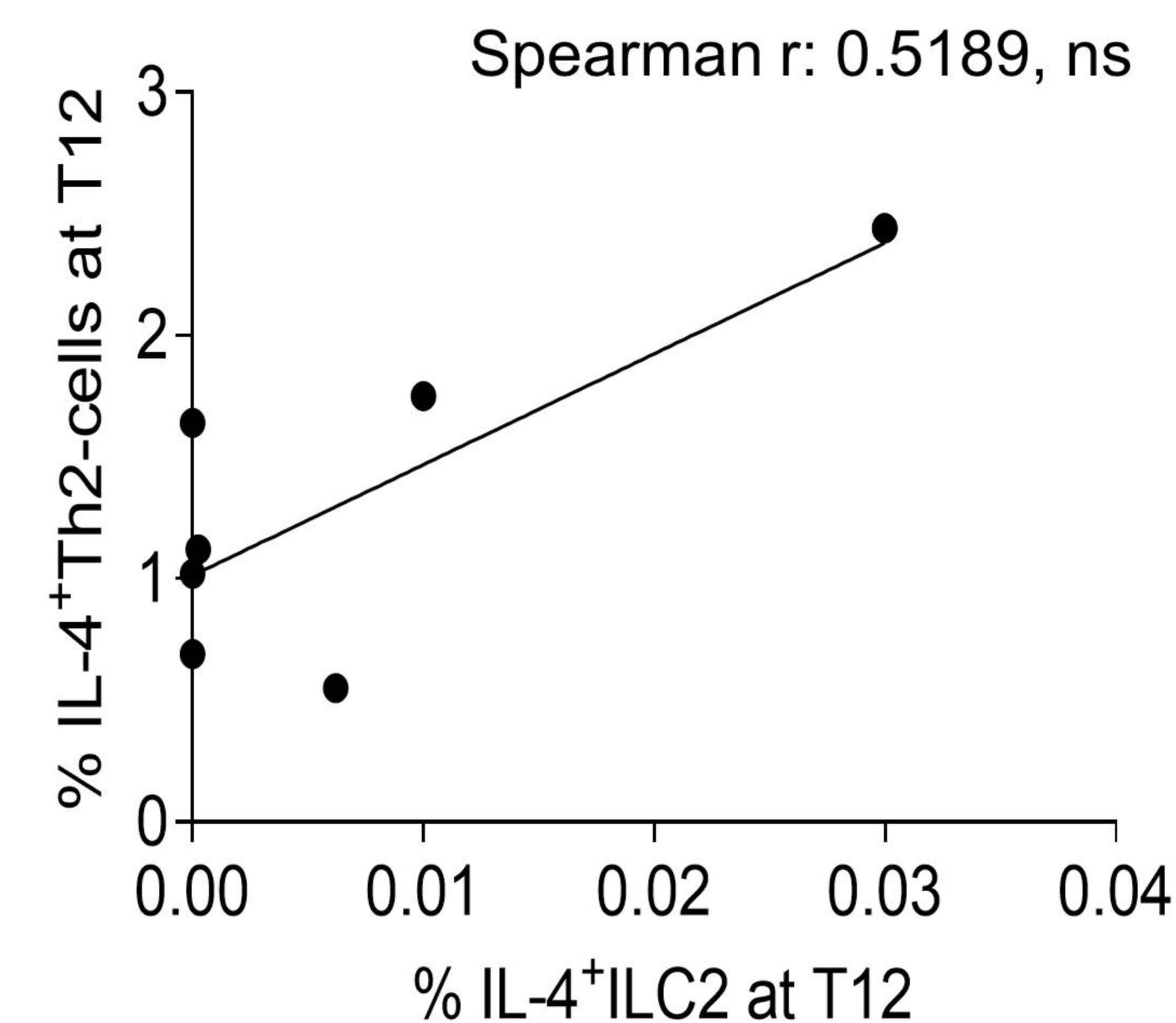
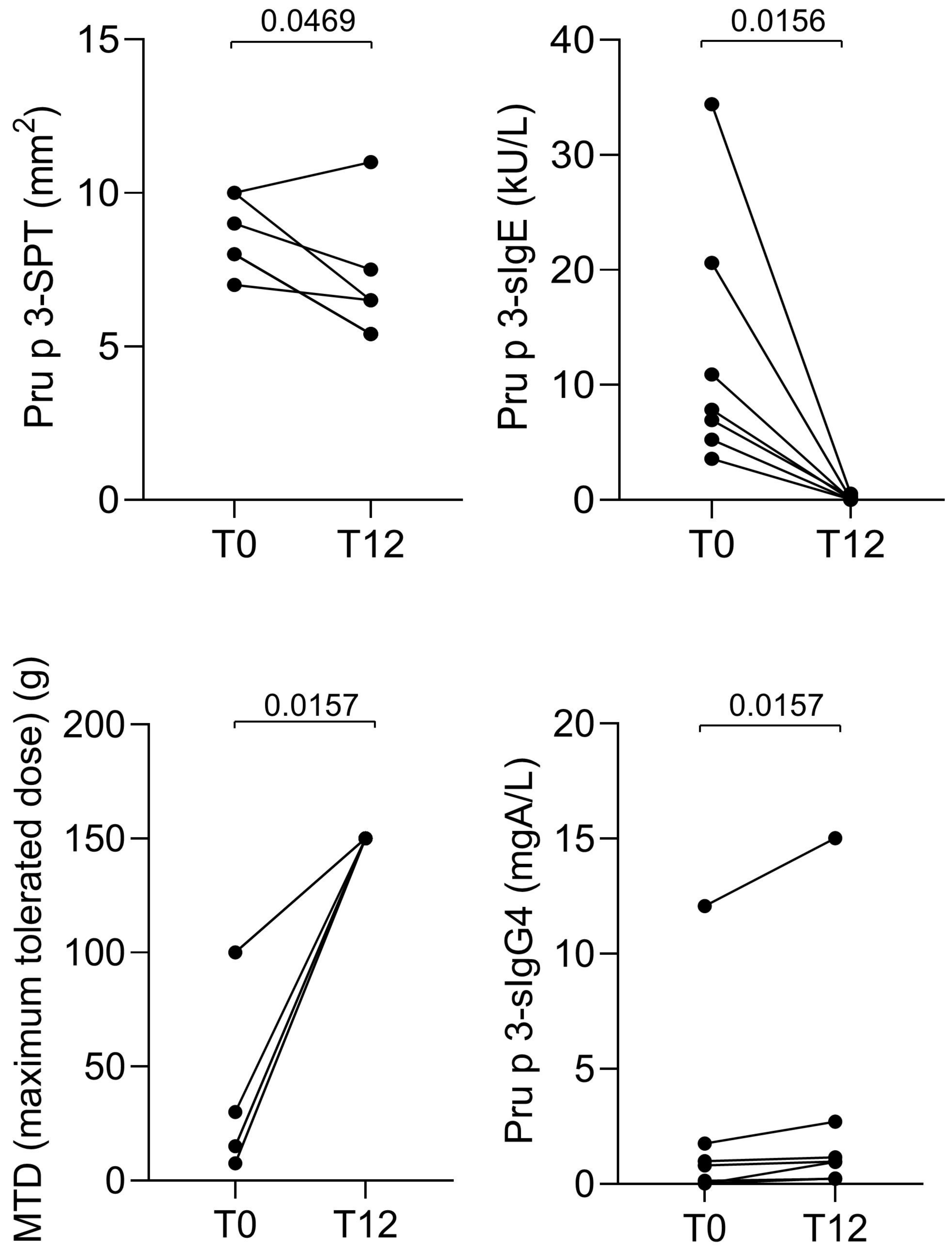


Figure OS4

A)



B)

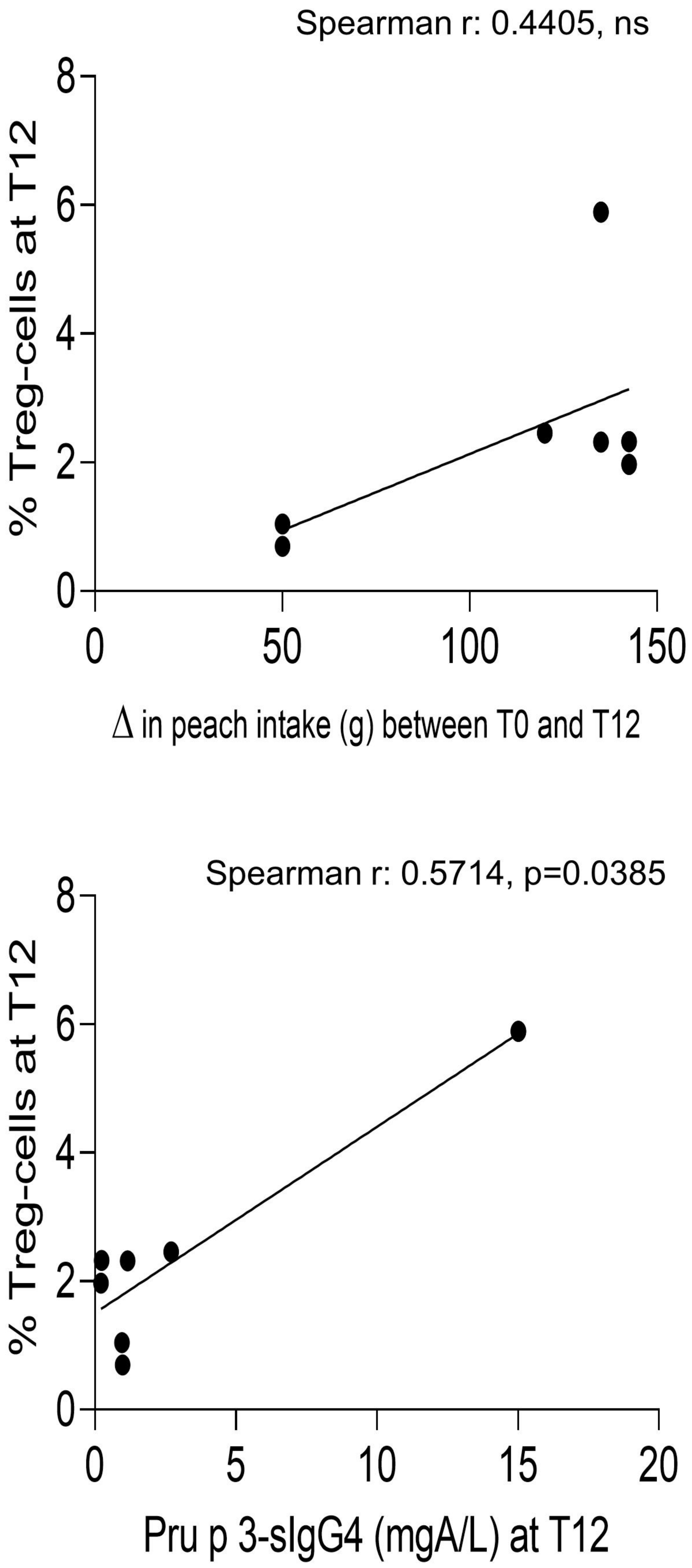
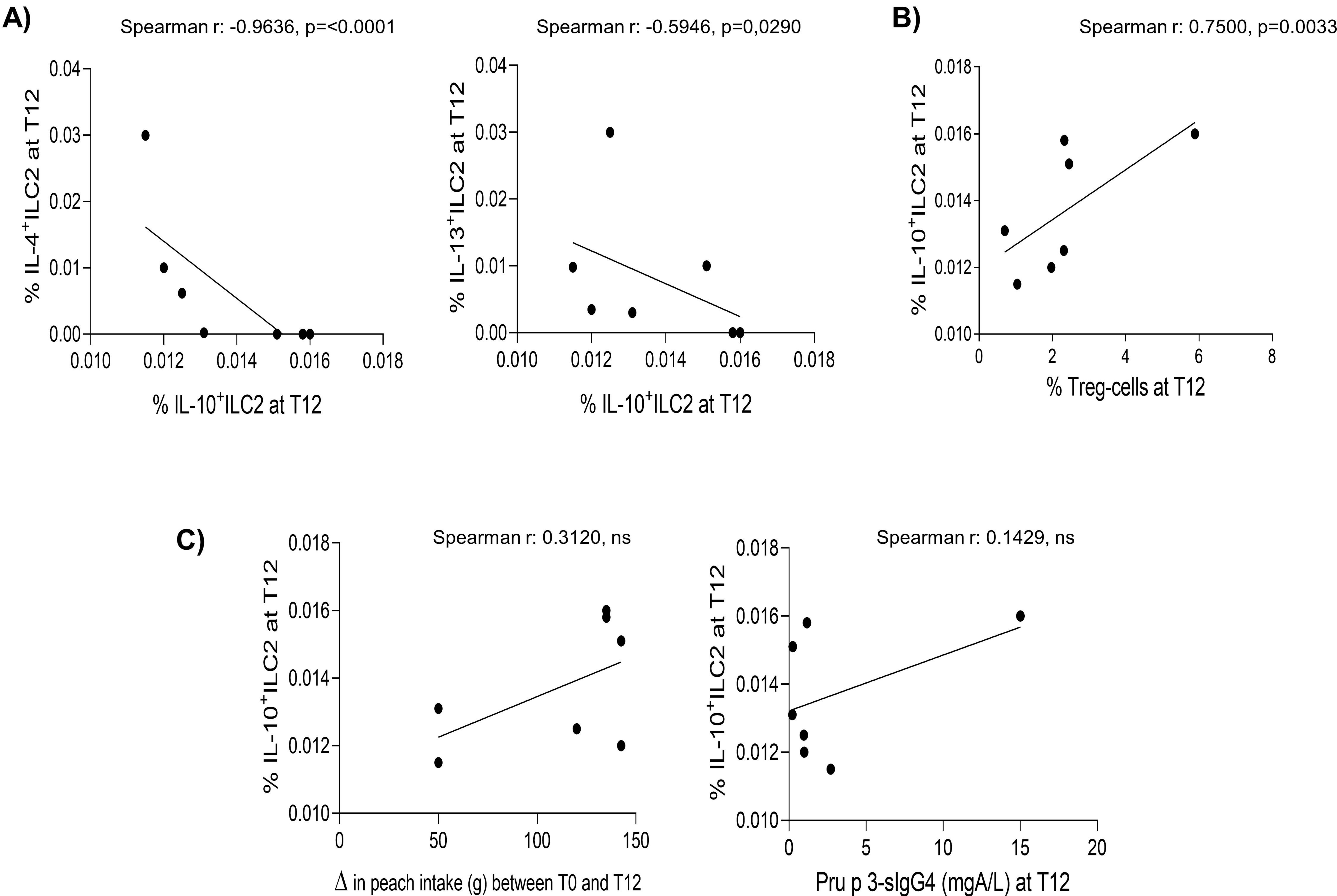


Figure OS5



ONLINE SUPPLEMENTARY

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

DETAILED EXPLANATIONS OF METHODS

Allergic Patients and Tolerant Controls.

This study included LTP-allergic patients (LTP-AP) with convinced clinical history of peach allergy, a positive skin prick test (SPT), specific IgE (sIgE) to peach LTP (Pru p 3), and/or a positive double-blind placebo-controlled food challenge (DBPCFC) to unpeeled-peach. DBPCFC was not performed in patients having suffered more than two episodes of anaphylaxis after peach ingestion in the two years preceding the study. Peach allergy severity was classified according to the criteria of Brown et al (1). LTP-AP with urticaria and/or angioedema and anaphylaxis were included. No patients with OAS exclusively were included in this study

A tolerant control (TC) group of subjects without food-allergy, negative SPT and sIgE to Pru p 3, and confirmed tolerance to unpeeled-peach was also included.

In addition, most LTP-allergic patients and tolerant controls presented respiratory allergy diseases, according to ARIA classification as described in Table 1. Sample obtainment was performed out of seasonal period for both groups.

The study was conducted at the Allergy Service and Research Laboratory of the Hospital Regional Universitario de Málaga-Instituto de Biomedicina de Málaga (HRUM-IBIMA), Spain, in accordance with the Declaration of Helsinki. The study was approved by the local ethics committee (MV18/00014). All participants signed informed consent forms before the study.

Allergic patients receiving sublingual immunotherapy.

Seven LTP-AP (diagnosed as described) with symptoms of anaphylaxis and urticaria, including angioedema according to Brown et al (1) were treated with a Pru p 3-enriched-SLIT (SLIT-Prup3) (50 µg/mL) (ALK-Abelló, Madrid, Spain) using a conventional schedule (2). All subjects were prospectively evaluated for one year at several time points: before starting treatment (T0), and 1 (T1), 6 (T6), and 12 months (T12) after starting SLIT-Prup3 treatment. These patients were chosen because they presented a greater number of allergic episodes after peach intake and developed systemic symptoms (Table 1).

Skin prick tests.

SPT were performed according to European guidelines (3) using commercialised extracts of nsLTP (Pru p 3-enriched) (from ALK-Abelló). The response was considered positive if the wheal size > 3 mm² than the one induced by a negative saline control.

Double-blind placebo-controlled food challenge.

DBPCFC was performed at T0 and T12, not during pollen season, as described (2) and following European guidelines (4).

Pru p 3-specific immunoglobulin (Ig) determination.

Serum sIgE and sIgG4 antibodies levels to Pru p 3 were determined by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) following the manufacturer's recommendations. Results were expressed as kU/L for IgE and as mgA/L for IgG4.

Sample Collection and Storing.

Peripheral blood was obtained by venopunction. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient and stored in liquid nitrogen. Biological samples, PBMCs and serum, were coded and biobanked according to HRUM-IBIMA Biobank, Andalusian Public Health System Biobank standard procedures.

Stimulation of immune cells.

PBMCs (average of 15×10^6 cells) from LTP-AP and TC were incubated in RPMI-1640 (Lonza, Basel, Switzerland) plus 10% foetal bovine serum (Gibco; Thermo Fisher Scientific), and treated for 2 h with 1/1000 of phorbol myristate acetate (100 µg/mL) plus a dilution 1/1000 of ionomycin (100 µg/mL) (Sigma-Aldrich, St. Louis, MO) in tubes (Falcon Round-Bottom Polypropylene (Francisco Soria Melguizo S.A, Madrid, Spain)). Following that, 1/1000 of Brefeldin A (5 mg/mL) (Sigma-Aldrich) was added for 3 h. Afterwards and without any cell pre-selection, the PMBCs were separated into different fractions (average of 3×10^6 PBMCs/tube to determinate the ILC2 and their phenotypes and an average of 1×10^6 PBMCs/tube to phenotype the T-cells). The cells were fixed with BD Cytofix/CytoPerm Fixation/Permeabilization solution kit (BD Bioscience, Franklin Lakes, NJ). For Treg-cells staining, human FOXP3 buffer was used (BD Bioscience).

Analysis of the frequency of different cell subpopulations.

ILC2 frequency, different lymphocyte subpopulations from LTP-AP and TC, and different time points during SLIT-Prup3 were analysed by flow cytometry using a FacsCanto II (BD Bioscience). The cell frequency analysis was performed using the FlowJo software (BD Bioscience-FlowJo LLC, Ashland, OR) as described (5). Both, isotype controls (data not shown) and un-stimulated cells, were considered controls for flow cytometric analyses. All monoclonal antibodies (MoAbs) used for ILC2 and T-cells phenotyping were from BD Bioscience, except for CD161 and IL-4, which were from BioLegend (BioLegend, San Diego, CA), and CRTH2, IL-10 and IFN γ , from Miltenyi Biotec (MACS-Miltenyi Biotec, Bergisch Gladbach, Germany). The Lineage cocktail 1 (Lin $^{-}$) included CD3, CD14, CD16, CD19, CD20, and CD56. The fluorescent dye-antibodies were: anti-Lin-FITC; anti-CD15-FITC; anti-CD123-FITC; anti-CRTH2-PE; anti-CD161-PE-Cy5; anti-CD45-Per-CP or -APC; anti-IL-4-APC-Cy7; anti-IL-10-APC; anti-IL-13-APC; anti-CD3- Per-CP; anti-CD4-FITC; anti-CD25-Pe-Cy7; anti-

CD127-PE; anti-IFN γ -Pe-Cy7 and anti-FOXP3-Alexa Fluor 647. The LIVE/DEAD fixable green and fixable near-IR dead cell stain kits were used to determine the viability of cells (Thermo Fisher Scientific). Cell subpopulation phenotypes were defined as: CD45⁺Lin⁻CD15⁻CD123⁻CD161⁺CRTH2⁺ as **ILC2**; IL-4⁺ILC2 as **IL-4-producing ILC2**; IL-13⁺ILC2 as **IL-13-producing ILC2**; IFN γ ⁺ILC2 as **IFN γ -producing ILC2**; CD3⁺CD4⁺IFN γ ⁺ as **Th1-cells**; CD3⁺CD4⁺CRTH2⁺IL-4⁺ as **IL4-producing Th2-cells**; CD3⁺CD4⁺CRTH2⁺IL-13⁺ as **IL13-producing Th2-cells**; CD3⁺CD4⁺CD127^{-/low}CD25⁺FOXP3⁺ as **Treg-cells**; IL-10⁺ILC2 as **IL-10-producing ILC2**. In addition, the determination of the phenotypes of ILC2, IL-4⁺ILC2 and IL-13⁺ILC2 were performed as described (6-8). The gate strategies followed to assess ILC2 and T-cells using flow cytometry are shown in Figure OS1 and Figure OS2. Results were expressed as percentage of ILC2 (IL-4⁺ILC2, IL-13⁺ILC2, and IL-10⁺ILC2) respect to CD45⁺cells, and of T-cells (Th1-, Th2, Treg-cells) respect to lymphocytes.

Statistical analysis.

All variables were analysed using Mann-Whitney U test between non-related samples (LTP-AP vs TC), being differences significant when a p-value lower than 0.05 was obtained. Moreover, for the analysis of LTP-AP treated with SLIT-Prup3, all variables between non-related groups (SLIT-Prup3-LTP-AP vs LTP-AP non-treated; and vs TC) were analysed using Kruskal-Wallis test, considering differences significant when a p-value lower than 0.05 was obtained. Additionally, in cases with significant differences, Mann-Whitney U test between pairs for non-related samples was also performed. For longitudinal analysis, comparisons of the different times points (related samples) were performed using Friedman test, defining as significant differences a p-value threshold of 0.05. In the case of significant differences, this was followed by the Wilcoxon signed-rank test between the different pairs of time points (T0 vs T1, T0 vs T6 and T0 vs T12). Moreover, the data were analysed using the Shapiro-Wilk test to determine the normal distribution but the most variables were fitted to non-parametric distribution. The correlation analyses were analysed using the Spearman test showing the lineal regression. All statistical analyses were performed using GraphPad Prism 7.04 version for windows. All reported p-values represent 2-tailed tests.

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Table OS2. Clinical characteristics of treated LTP-AP with SLIT-Prup3 at T0 and T12

N. Subjects	SPT (mm ²)		sIgE (kU/L)		sIgG4 (mgA/L)		MTD of peach in DBPCFC (g)	
	T0	T12	T0	T12	T0	T12	T0	T12
LTP-AP1	8	5.4	6.96	0.27	1.76	2.7	15	150
LTP-AP2	8	5.4	20.6	0.10	12.08	15.02	100	150
LTP-AP3	9	7.5	10.9	0.05	0.02	0.24	7.5	150
LTP-AP4	7	6.5	34.4	0.56	0.99	1.16	15	150
LTP-AP5	8	5.4	3.58	0.05	0.14	0.22	100	150
LTP-AP6	10	6.5	5.26	0.01	0.01	0.96	30	150
LTP-AP7	10	11	7.85	0.05	0.81	0.98	7.5	150

SPT: Skin prick test, sIgE and sIgG4 to Pru p 3 MTD: Maximum tolerated dose of peach in DBPCFC (Double blind placebo-controlled food challenge).

FIGURES AND FIGURE LEGENDS

Figure OS1. Gating strategy using flow cytometry pseudo-colour (with outliers) to determine the phenotype of ILC2 in LTP-AP, TC and LTP-AP treated with SLIT-Prup3 at T12. Gate strategy showed the different ILC2 phenotypes on stimulated PBMCs. The percentages showed have been obtained with respect to CD45⁺ cells.

Figure OS2. Gating strategy using flow cytometry pseudo-colour (with outliers) to determine the phenotype of T-cells in LTP-AP (left) and TC (right) using flow cytometry. Gate strategy showed the different T-cells phenotypes on stimulated PBMCs. The percentages showed have been obtained with respect to lymphocytes.

Figure OS3. T-cells analysis. A) IL-4⁺ and IL-13⁺Th2-cells percentages in LTP-allergic patients (LTP-AP, N=10) and tolerant controls (TC, N=10). **B)** Correlation analysis between IL-

4⁺ILC2 vs IL-4⁺Th2-cells and IL-13⁺ILC2 vs IL-13⁺Th2-cells levels in LTP-AP. **C)** Changes in the percentages of different T-cells phenotypes during SLIT-Prup3 (four time points) in LTP-AP (N=7), in non-treated LTP-AP (LTP-AP, N=10), and in TC (N=10) at one time point. **D)** Correlation analysis of the percentages of (IL-4⁺Th2-cells vs IL-4⁺ILC2) and (IL-13⁺Th2-cells vs IL-13⁺ILC2) in SLIT-Prup3 treated LTP-AP at T12 (N=7). 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.

Figure OS4. A) Clinical changes due to SLIT-Prup3 at T0 and T12 for treated patients (N=7). Changes in wheal diameter after skin prick test (SPT) with peach, changes in serum Pru p 3-sIgE and Pru p 3-sIgG4 and in the peach threshold after DBPCFC. **B)** Correlation analysis between the percentages of Treg-cells at T12 vs increment in the peach intake between T0 and T12 and vs Pru p 3-sIgG4 in serum in treated LTP-AP (N=7). The significant differences are represented as p-values<0.05.

Figure OS5. Correlation analysis of the percentages at T12 of **A)** IL-13⁺ILC2 and IL-4⁺ILC2 vs IL-10⁺ILC2; of **B)** IL-10⁺ILC2 vs Treg-cells; and **C)** IL-10⁺ILC2 vs clinical parameters in treated LTP-AP. The significant differences are represented as p-values<0.05.