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Decrease in CD38⁺ TH2A cell frequencies following immunotherapy with house-dust mite tablet correlates with humoral responses

Short title: CD38⁺ TH2A cells decrease after allergen immunotherapy

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Disclosure

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Author contribution

LM and SL designed the research. LM, VB and TB supervised the research. SL performed the experiments. SL, LM, AP, VB and ML analysed the data. LM wrote the paper.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Key words: CD38⁺ TH2A, house dust mite, sublingual immunotherapy

Key messages:

- The use of CD38 activation marker in TH2A cell allows to better identify and follow pro-allergenic cell changes in allergic individuals.
- Specifically, individuals suffering from house dust mite allergy exhibited a reduction in circulating CD38⁺ TH2A cells following 1-year HDM tablet immunotherapy.
- Correlated cellular (*ie.* decreased frequencies in CD38⁺ TH2A) and humoral (*i.e.* increased IgG4 or IgE antibody levels) responses emphasize that coordinated pathways occur in adaptive responses during AIT.

ABSTRACT

Background:

In line with evidence for a role of pathogenic TH2A in seasonal allergies, we previously showed that individuals suffering from food allergy exhibited a decrease in circulating TH2A cells following multi-food immunotherapy. Herein, we aim to confirm the decline of TH2A cells in individuals undergoing house dust mite immunotherapy (HDM-AIT) and extend our observation to a new subset of CD38 expressing activated TH2A cells.

Methods:

The frequencies of TH2A and CD38⁺ TH2A cells were analysed by flow cytometry in blood cells from 182 Japanese HDM-allergic individuals included in a 1-year clinical trial assessing the efficacy of HDM tablets. Interrelationship between these cellular responses and humoral mite-specific IgE and IgG4 levels was further explored.

Results:

A decrease in TH2A cells was observed in both active and placebo groups. Interestingly, CD38⁺ TH2A cell frequencies significantly decreased only in active groups. In younger individuals (16 to 30 years), both TH2A and CD38⁺ TH2A cells were significantly reduced in active groups but not in the placebo group. Significant inverse correlations were observed in the course of HDM-AIT between changes in TH2A or CD38⁺ TH2A frequencies and IgG4 antibody levels.

Conclusions

We confirm the value of monitoring TH2A cell frequencies in allergic individuals and extend this observation to perennial allergy to HDM. We highlight the interest of CD38 to better identify the subset of TH2A cell downregulated by AIT. Finally, correlated cellular and humoral responses observed in immunoreactive individuals stress that coordinated pathways occur in the adaptive responses during AIT.

INTRODUCTION

Allergen immunotherapy (AIT) performed *via* the subcutaneous or sublingual route is a well-recognized treatment of IgE-mediated respiratory allergies to pollen and house dust mite (HDM) allergens^{1, 2}. AIT is considered the only causative treatment as opposed to symptomatic drugs, with the promise of promoting long-lasting effects, persisting even after discontinuation of the therapy³⁻⁶.

Although no surrogate biomarker of AIT clinical efficacy has been validated so far⁷, immunological changes occur in peripheral blood or mucosal tissues of treated patients and include the induction of allergen-specific regulatory T cells (Tregs) along with an increase of allergen-specific blocking IgG4 antibodies competing with IgE for allergen binding⁸⁻¹¹. The induction of innate immune regulatory responses (*e.g.* induction of C1q and Stabilin-1) detected in peripheral blood mononuclear cells (PBMCs) also correlates with early onset of AIT clinical efficacy, thus emphasizing a critical role for Tregs in AIT¹²⁻¹⁴.

Less attention has been paid off to T helper (TH) 2 cell subsets until recently, when TH2 or the so-called TH2A cells or pathogenic TH2 cells (*i.e.* producing high levels of IL-4, IL-5 and IL-13)¹⁵ have been shown to be down-regulated in the course of AIT^{14, 16, 17}. CD38, a cell-surface activation marker¹⁸ has been observed to be up-regulated in allergen-specific TH2 / TH2A cells from allergic individuals upon alder or grass pollen exposure^{15, 19}. Starting out from this observation, we made the hypothesis that CD38⁺ TH2A cells would better define pathogenic TH2 cells, particularly for individuals allergic to perennial allergens such as house dust mite.

Herein, we assess the frequencies of both TH2A and CD38⁺ TH2A cell subsets in PBMCs obtained from a sub-group of 182 Japanese patients included in a double-blind placebo-controlled clinical HDM AIT study (1207D1731; JapicCTI-121917)²⁰. Using flow cytometry analyses, we provide evidence for a decrease in CD38⁺ TH2A cell frequencies in PBMCs after treatment, particularly in younger participants aged 16 to 30 years. We also observe an interrelationship between humoral and cellular responses at an individual participant level, with significant inverse correlation between CD38⁺ TH2A frequencies and mite-specific IgG4 antibodies titers.

METHODS

Study treatment and samples

The randomized double-blind placebo-controlled 1207D1731 study was conducted to assess the safety and efficacy of 300 IR and 500 IR house dust mite tablet (Stallergenes SAS, Antony, France/Shionogi & Co. Ltd., Osaka, Japan) over a 12-month course of sublingual allergen immunotherapy²⁰. All participants 16 years and older gave their written consent (or assent for subjects 15 years or younger) to participation in the study, as did the legal representatives for underage subjects, after having been informed of the study's objectives and procedures. The study complied with the International Conference on Harmonization Good Clinical Practice (GCP) guidelines and was approved by the Institutional Review Board of each study site. Herein the clinical response is defined for each individual patient as the percentage of improvement in Average Adjusted Symptom Score (AASS) which adjusts symptom scores for the use of rescue medication. In the context of this clinical trial, ancillary biomarker analyses were conducted in a subset of 182 HDM allergic patients out of the 968 patients enrolled in the 1207D1731 clinical trial and having received daily either 300 IR (n = 58), 500 IR (n = 63) HDM or placebo (n = 61) tablets. Sera samples and PBMCs were collected at baseline (Visit 2; V2) and after 12 months (V17) and kept frozen at -80°C until use. All biological analyses were performed on samples coded to the operators.

Analysis of cell-surface markers

For immunofluorescence staining of TH2A or CD38⁺ TH2A cells, PBMCs were thawed, then washed in PBS and incubated for 20 min at 4°C with a lineage conjugated cocktail encompassing Fixable Viability dye, anti-CD8, anti-CD11c, anti-CD14, anti-CD16, anti-CD20, anti-CD34, anti-CD56, anti-CD123 and anti-TCRva24ja18 monoclonal antibodies as well as anti-CD4, anti-CD45RO, anti-CD45RB, anti-CD27, anti-CD49d, anti-CD161, anti-CD294, anti-CD38, anti-CD69 antibodies.

Antibodies used for flow cytometry analysis of human TH2A or CD38⁺ TH2A cells

Specificity	Fluorochrome	Clone	Supplier
CD4	AF700	RPA-T4	Life Technologies
CD8	BV510	RPA-T8	BD horizon

CD11c	BV510	S-HCL-3	Ozyme
CD14	Viogreen	TÜK4	Miltenyi
CD16	BV510	3G8	BD horizon
CD20	Viogreen	LT20	Miltenyi
CD27	BV605	M-T271	BD horizon
CD34	BV510	581	Biolegend
CD38	APC	HIT2	BD horizon
CD45RB	APC-Vio770	REA119	Miltenyi
CD45RO	FITC	REA611	Miltenyi
CD49d	Vioblue	MZ18-24A9	Miltenyi
CD56	BV510	NCAM HCD56	Ozyme
CD69	BV711	FN50	Biolegend
CD123	BV510	6H6	Ozyme
CD161	PE	DX12	BD horizon
CRTH2	PerCP-Cy5.5	BM16	BD horizon
Fixable Viability dye	eFluor 506	N/A	Life Technologies
TCRab	BV510	T10B9.1A-31	BD horizon
TCRva24ja18	BV510	6B11	Ozyme

Cells were stained with corresponding control isotype antibodies and analysed with an Attune NxT flow cytometer (Life Technologies). Data were analysed with the FlowJo software (TreeStar, Ashland, OR).

Humoral responses

Levels of serum IgE and IgG4 specific to *D. farinae* and *D. pteronyssinus* were determined at baseline (V2) and week 52 (V17) (BML Inc., Kawagoe, Japan) as previously described ²⁰.

Statistical analyses

Statistical and graphical analyses were performed by using Prism 6 software (GraphPad, La Jolla, CA). Statistical differences between groups were assessed by using 2-tailed Wilcoxon or Mann-Whitney nonparametric tests to compare paired or independent data, respectively, with p -values below 0.05 considered significant. Correlation analyses were performed by using the nonparametric Spearman test. Analyses with mite-specific IgE and IgG4 levels were performed on log-transformed values.

RESULTS

A decrease in CD38⁺ TH2A cell frequencies better reflects the immunological effect of AIT than TH2A cell frequencies

In this study, we undertook a comprehensive TH2 immune response analysis on blood cells obtained from 182 HDM allergic participants enrolled in the 1207D1731 clinical trial and having received daily either HDM (300 IR (n = 58) or 500 IR (n = 63)) or placebo (n = 61) tablets sublingually for 12 months²⁰. Herein, visit 2 (V2) and visit 17 (V17) refer to baseline and 1 year of AIT, respectively. The demographics and baseline characteristics of participants of the study population are provided in **Table 1A**.

We first analysed systemic modifications in adaptive immune parameters, particularly frequencies of pro-allergenic T helper (TH) type 2A cell, observed after 1-year AIT in the whole or age-stratified study population. Peripheral blood mononuclear cells (PBMCs) were stained for TH2A with fluorescently labeled antibodies as described in **Methods** and analysed by flow cytometry according to the gating strategy shown in **Supplementary Figure S1**. We observed statistically significant lower frequencies of TH2A cells in PBMCs in the total active (300 IR and 500 IR) as well as 300 IR and 500 IR groups at V17 when compared to V2 ($p < 0.0001$) or to placebo group at V17 ($p < 0.01$, $p < 0.05$ and $p < 0.01$, respectively) (**Figures 1A and B**). However, TH2A cell frequencies were significantly ($p < 0.001$) decreased between V2 and V17 in the placebo group thus impeding the relevance of the preceding observation.

For age-stratified analysis, patients were categorized into three groups *i.e.* participants aged 16 to 30, 31 to 40 and 41 to 61. The demographics and baseline characteristics of participants of the study population stratified by age are provided in **Table 1A**. As shown in **Figures 1C and D** and **Table 1B**, we observed a statistically significant decrease in TH2A cell frequencies in PBMCs in the total active (300 IR and 500 IR), 300 IR and 500 IR groups at V17 when compared to V2 ($p < 0.0001$, $p < 0.0001$ and $p < 0.01$, respectively) and to placebo at V17 ($p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively) only for participants aged between 16 and 30. Interestingly, for this age group, difference in TH2A cell frequencies between V2 and V17 in the placebo-receiving patients was not significant.

CD38, defined as an activation marker¹⁸ with long-term persistence in chronic diseases (*e.g.* HIV or *P. falciparum* infection) was observed to be up-regulated in allergen-specific TH2 / TH2A cells

in individuals upon alder or grass pollen exposure^{15, 19} and as such may represent a marker to be added when monitoring TH2A cells in the blood of HDM allergic individuals for whom allergens airborne exposure is sustained. Therefore, we performed the same analysis with CD38 included in the cytometry panel as described in **Methods** and **Supplementary Figure S1**. We observed significantly lower frequencies of CD38⁺ TH2A cells in PBMCs from participants either in the total active (300 IR and 500 IR), 300 IR or 500 IR groups at V17 when compared to V2 ($p < 0.0001$, $p < 0.0001$ and $p < 0.001$, respectively) or to the placebo group at V17 ($p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively) while frequencies of CD38⁺ TH2A cells in the placebo group at V17 remained similar to V2 (**Figures 2A and B**). These statistically significant decreases were mostly seen in younger participants aged 16 to 30, for whom lower CD38⁺ TH2A cell frequencies were observed in all the active groups studied (*i.e.* total active (300 IR and 500 IR), 300 IR or 500 IR groups) at V17 when compared to V2 ($p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively) or to placebo group at V17 ($p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively) (**Figures 2C and D**). Importantly, change in CD38⁺ TH2A cell frequencies in the placebo group for participants aged 16 to 30 between V2 and V17 was not significant. In our stratified-age analysis, we also observed that CD38⁺ TH2A cell frequencies were decreased for participants aged from 31 to 40 in all the active groups studied (*i.e.* total active (300 IR and 500 IR), 300 IR and 500 IR groups) at V17 when compared to the same groups at V2 ($p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively) (**Figures 2C and D**).

A decrease in TH2A or CD38⁺ TH2A cell frequencies does not correlate with a clinical benefit

As we observed statistically significant lower frequencies of TH2A or CD38⁺ TH2A cells in PBMCs from participants in the total active group after 1-year of AIT, we performed a correlation analysis between differences in TH2A or CD38⁺ TH2A cell frequencies and clinical responses expressed as percentages of improvement $((V2 - V17)/V2)$ in Average Adjusted Symptom Score (AASS) which adjusts symptom scores for the use of rescue medication^{20, 21}. Unfortunately, when plotted against percentages of AASS improvement, changes (V17 - V2) in TH2A (**Figure 3A**) and CD38⁺ TH2A (**Figure 3B**) cell frequencies were not correlated with clinical benefit for patients on active treatment. As a control, no correlation was observed in placebo-recipients (**Figures 3C and D**).

Changes in TH2A and CD38⁺ TH2A cell frequencies correlate with humoral, mostly IgG4, responses

The levels of immunoglobulin E (IgE) and G4 (IgG4) specific to HDM increased in the active groups (*i.e.* total active (300 IR and 500 IR), 300 IR or 500 IR groups) between V2 and V17, while there were no marked changes in the placebo group (²⁰, data not shown). We confirm that changes in levels of *Dermatophagoides farinae* (Dfar) and *D. pteronyssinus* (Dpt) specific serum IgE or IgG4 antibodies did not correlate with clinical benefit within patients from the active groups (**Supplementary Figure 2, left and right panels, respectively**). However, we observed a correlation between mite allergen-specific IgE and IgG4 antibody levels at baseline (V2) (**Supplementary Figure 3**). In addition, in all groups a correlation between changes in levels of mite allergen-specific IgE and IgG4 antibodies was observed, thus showing an interrelationship between mite allergen-specific IgE and IgG4 responses at an individual participant level (**Supplementary Figure 4**).

We therefore analysed whether such an interrelationship could be observed between humoral and cellular responses. Changes (V17 - V2) in TH2A cell frequencies much better inversely correlate with changes in mite allergen-specific IgG4 than IgE levels in the active groups (**Supplementary Figures 5 and 6, respectively**). For the CD38⁺ TH2A cell subset, changes (V17 - V2) in cell frequencies not only correlate with changes in levels of mite allergen-specific IgG4 (Spearman correlation $r = 0.4106$ and $r = 0.4293$, $p < 0.001$, for Dfar and Dpt, respectively) (**Figure 4**) but also with those of specific IgE (Spearman correlation $r = 0.3570$, $p < 0.01$ and $r = 0.2725$, $p < 0.05$ for Dfar and Dpt, respectively) in the 500 IR group, for which higher increases of specific IgE and IgG4 antibody levels have been observed (**Figure 5**).

DISCUSSION

The search for surrogate biomarker candidates of AIT efficacy has raised a lot of interest but none has been validated yet ⁷. Nonetheless, immunological changes in peripheral blood or mucosal tissues of patients have been well-documented in various AIT studies and include an increase of regulatory responses (*e.g.* innate markers, Treg cell, IgG4, blocking factors) and a concomitant decrease of pro-allergenic immune responses (*e.g.* basophils, mastocytes, innate markers, TH2, IgE) ⁸⁻¹¹.

Recently, the key pathogenic role of TH2A cells was recently highlighted in peanut and grass allergic individuals ^{15, 22}. The study by Wambre *et al.* also further documented that CD38 activation marker was up-regulated on this cell subset in grass pollen-allergic individuals during the pollen season ¹⁵. In the study, monitoring TH2A cell frequencies without any additional marker was not sufficient to assess the effect of AIT since both the active and placebo groups exhibit a decrease of this cell subset when considering the whole cohort population aged from 16 to 61 years old. Adding the CD38 activation marker to the flow cytometry panel enabled to significantly discriminate between the active and the placebo groups not only for the total active group but also for each of the 300 IR and 500 IR groups. Our results establish for the first time that CD38⁺ TH2A cell frequencies decrease in HDM-allergic individuals upon immunotherapy with approved house dust mite (HDM) tablets, while this cell subset remains stable in the placebo group. We therefore confirm the interest to follow activation markers such as CD38 and extend previous observations by Wambre *et al.* to perennial allergy ^{15, 19}.

The age-stratified analysis also confirmed the value of monitoring CD38⁺ TH2A cell frequencies in youngest population. The inclusion of CD38 to the flow cytometry panel improved statistical significance between the 300 IR and placebo groups for participants aged 16 to 30 when compared to the analysis of TH2A cell frequencies in the same subgroup population. The fact that CD38⁺ TH2A frequency is significantly decreased in the active groups versus placebo most particularly in participants aged from 16 to 30-year-old suggests that this cell subset is regulated in an age-dependent manner. In the oldest age groups, the absence of a significant decrease of this TH2 cell subset after HDM-AIT may be due to an increase in the cellular TH2 bias observed with ageing ²³. An alternate hypothesis is that continuous exposure to HDM promoting IgE polysensitization to multiple HDM allergens according to age ²⁴, could dampen the decrease of TH2 immune responses.

Apart from the study by Wambre *et al.* ^{15, 19}, our findings are fully aligned with other previous reports monitoring follow-up type 2 candidate biomarkers in AIT studies for both respiratory and food allergies ^{13, 14, 17}. For example, CD27⁺CD161⁺ and ST2⁺CD45RO⁺ cells producing TH2 cytokines were significantly decreased in PBMCs sample from patients undergoing a 1-year HDM AIT ¹⁷. Particularly, in this study, our research group previously reported that both CD27⁺CD161⁺ and ST2⁺CD45RO⁺ cells were detected among proliferating T cells from PBMCs after an *in vitro* stimulation with natural HDM extract. As such these *in vitro* assays are likely difficult to standardize. Our present data are also in agreement with our recent report in which individuals aged between 10 and 22 years old exhibited a statistically significant decrease of TH2A cell frequencies following 30 weeks of multi-food oral immunotherapy ¹⁴.

In this HDM-AIT study, clinical responses were defined *post hoc* as percentages of improvement in Average Adjusted Symptom Score (AASS) ^{20, 21}. Changes in CD38⁺ TH2A (or TH2A) cell frequencies assessed before and after 1-year HDM AIT (V17 - V2) did not correlate with clinical effect evaluated as percentages of AASS improvement ((V17 - V2)/V2). However, changes (V17 - V2) in CD38⁺ TH2A cell frequencies did correlate with changes (V17 - V2) in levels of both mite allergen-specific IgG4 and IgE antibodies in the active 500 IR group exhibiting highest levels of mite specific IgE and IgG4 antibodies. These results confirm the difficulty to identify marker easily detectable in patients' blood that correlate with clinical efficacy. Given the capacity of CD4⁺ T cells to extravasate at sites of inflammation, changes in TH2 cell frequencies after AIT may appear rather within target organs, as opposed to peripheral blood where only in-transit T cells are present ⁸⁻¹¹. Due to these dynamic features of the immune system's response, previous studies already suggested that clinical responders included both immunoreactive patients exhibiting strong increases of allergen specific IgG and patients with no measurable antibody responses ²⁵. Interestingly, we clearly observed a statistically significant inverse correlation in immunoreactive patients between cellular and humoral responses. We acknowledge however that r correlation values are rather moderate, ranging from -0.2725 to -0.4296 (Figures 4 and 5). Another consideration for identifying candidate biomarkers is that their expression may be time dependent. We already established that innate immune markers (*e.g.* C1q, Stabilin-1, FcγRIIIa, GATA3 and CD141) associated with dendritic cell responses occur within months after the beginning of immunotherapy either with respiratory (*i.e.* grass pollen) ^{12, 13} or multifoood ¹⁴ allergen

immunotherapy, whereas adaptive (B and T cell) responses may take longer to be observed in AIT-treated patients and that more than two years is mandatory to see any clear correlation with clinical.

Altogether, our findings pave the ground for further understanding of the mechanisms involved in allergen immunotherapy. Positive outcomes on systemic immune parameters, as observed in this study, confirm the value of monitoring in the course of immunotherapy TH2A and, to a greater extend, CD38⁺ TH2A cell frequencies, particularly in the blood from young individuals as mechanistic biomarkers. The inverse correlation between cellular and humoral responses observed in immunoreactive patients highlights the consistency of adaptive immune responses during AIT. Our study also stresses that TH2 subpopulations such as TH2A and CD38⁺ TH2A subsets represent potential targets for immune intervention to further enhance tolerance induction in individuals suffering from respiratory and/or food allergies.

Figure legends

Figure 1: A decrease of TH2A cell frequencies after HDM-AIT is mostly detected in youngest participants.

PBMCs from participants receiving a placebo tablet ($n = 61$) and those receiving AIT ($n = 121$), including 300 IR ($n = 58$) and 500 IR ($n = 63$) tablets, were analysed at baseline (V2 – red dot) and after 12 months (V17 – grey square) by flow cytometry for TH2A cell frequencies expressed in number of cells per million CD4⁺ T cells **(A)** and **(B)**. 3 age groups were analysed: $16 \leq \text{age} \leq 30$ ($n = 45$, $n = 23$, $n = 22$ and $n = 28$ for total active, 300 IR, 500 IR and placebo, respectively); $31 \leq \text{age} \leq 40$ ($n = 39$, $n = 21$, $n = 18$ and $n = 21$ for total active, 300 IR, 500 IR and placebo, respectively) and $41 \leq \text{age} \leq 61$ ($n = 37$, $n = 14$, $n = 23$ and $n = 12$ for total active, 300 IR, 500 IR and placebo, respectively) **(C)** and **(D)**. The bar represents the median for each group. Wilcoxon tests for paired data and Mann-Whitney tests for unpaired data were used for statistical analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns: non-significant.

Figure 2: A decrease of CD38⁺ TH2A cell frequencies after HDM-AIT is observed at a whole population level although more pronounced in youngest participants.

PBMCs from participants receiving a placebo tablet ($n = 61$) and those receiving AIT ($n = 121$) including 300 IR ($n = 58$) and 500 IR ($n = 63$) tablets, were analysed at baseline (V2 – red dot) and after 12 month (V17 – grey square) by flow cytometry for CD38⁺ TH2A cell frequencies expressed in number of cells per million CD4⁺ T cells **(A)** and **(B)**. 3 age groups were analysed: $16 \leq \text{age} \leq 30$ ($n = 45$, $n = 23$, $n = 22$ and $n = 28$ for total active, 300 IR, 500 IR and placebo, respectively); $31 \leq \text{age} \leq 40$ ($n = 39$, $n = 21$, $n = 18$ and $n = 21$ for total active, 300 IR, 500 IR and placebo, respectively) and $41 \leq \text{age} \leq 61$ ($n = 37$, $n = 14$, $n = 23$ and $n = 12$ for total active, 300 IR, 500 IR and placebo, respectively) **(C)** and **(D)**. The bar represents the median for each group. Wilcoxon tests for paired data and Mann-Whitney tests for unpaired data were used for statistical analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns: non-significant.

Figure 3: Absence of correlation between changes in TH2A and CD38⁺ TH2A frequencies and clinical effect of HDM-AIT.

Spearman correlation at an individual participant level between changes in TH2A **(A)** or CD38⁺ TH2A **(B)** frequencies (expressed in number of cells per million CD4⁺ T cells) and percentage of

improvement in AASS in participants from the total active (n = 121) and placebo (n = 61) groups.
ns: non-significant.

Figure 4: Correlation between changes in CD38⁺ TH2A frequencies and changes in mite-specific IgG4 antibody levels in the 500 IR group after HDM-AIT.

Spearman correlation at an individual participant level of changes in CD38⁺ TH2A frequencies (expressed in number of cells per million CD4⁺ T cells) with changes in *D. farinae* (Dfar) (A) and *D. pteronyssinus* (Dpt) (B) log-transformed (log) IgG4 levels upon HDM-AIT in the total active (n = 121), 300 IR (n = 58), 500 IR (n = 63) and placebo (n = 61) groups. ** $p < 0.01$; *** $p < 0.001$; ns: non-significant.

Figure 5: Correlation between changes in CD38⁺ TH2A frequencies and changes in mite-specific IgE antibody levels in the 500 IR group upon HDM-AIT.

Spearman correlation at an individual participant level of changes in CD38⁺ TH2A frequencies (expressed in number of cells per million CD4⁺ T cells) with changes in *D. farinae* (Dfar) (A) and *D. pteronyssinus* (Dpt) (B) log-transformed (log) IgE levels upon HDM-AIT in the total active (n = 121), 300 IR (n = 58), 500 IR (n = 63) and placebo (n = 61) groups. * $p < 0.05$; ** $p < 0.01$; ns: non-significant.

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Table 1A: Demographics and characteristics of patients receiving immunotherapy or placebo

	Total active				300 IR				500 IR				Placebo			
	all		age		all		age		all		age		all		age	
	patients		16 to 30	31 to 40	41 to 61	patients		16 to 30	31 to 40	41 to 61	patients		16 to 30	31 to 40	41 to 61	
	n = 121	n = 45	n = 39	n = 37	n = 58	n = 23	n = 21	n = 14	n = 63	n = 22	n = 18	n = 23	n = 61	n = 28	n = 21	n = 12
Gender;																
male	53	25	17	11	31	15	9	7	22	10	8	4	31	20	9	2
(%)	(43.8%)	(55.5%)	(43.6%)	(29.7%)	(53.5%)	(65.2%)	(42.9%)	(50.0%)	(34.9%)	(45.4%)	(44.4%)	(17.4%)	(50.8%)	(71.4%)	(42.9%)	(16.6%)
Female	68	20	22	26	27	8	12	7	41	12	10	19	30	8	12	10
(%)	(56.2%)	(44.5%)	(56.4%)	(70.3%)	(46.5%)	(34.8%)	(57.1%)	(50.0%)	(65.1%)	(54.6%)	(55.6%)	(82.6%)	(49.2%)	(28.6%)	(57.1%)	(83.4%)
Age; mean years	34	23	35	46	33	22	35	47	35	23	36	45	32	24	35	47
(SD)	(10.38)	(4.32)	(2.50)	(4.47)	(10.73)	(4.60)	(2.41)	(5.34)	(9.99)	(3.90)	(2.63)	(3.89)	(9.80)	(4.09)	(2.84)	(4.34)
AASS baseline; mean	8.50	8.73	8.50	8.27	8.55	8.36	8.65	8.70	8.46	9.03	8.34	8.02	9.03	9.03	9.33	8.63
(SD)	(1.8)	(2.0)	(1.6)	(1.6)	(1.8)	(2.0)	(1.6)	(1.9)	(1.8)	(2.0)	(1.7)	(1.4)	(1.8)	(1.8)	(1.9)	(1.6)
Total IgE baseline	139.78	182.25	104.83	136.13	162.27	222.30	133.93	129.02	121.84	148.07	77.454	140.45	197.69	265.04	196.95	100.41
IU/mL																
<i>D. farinae</i> IgE baseline;	12.22	21.84	10.18	7.43	12.94	20.60	12.02	6.73	11.59	23.22	8.16	7.86	22.72*	36.04	18.05	11.58
geometric mean Ua/mL																
<i>D. pteronyssinus</i> IgE	11.76	22.38	10.07	6.60	12.33	21.07	12.04	5.28	11.26	23.85	7.56	7.51	21.28*	36.07	16.56	9.64
baseline; geometric																
mean Ua/mL																
<i>D. farinae</i> IgG4	0.21	0.22	0.16	0.23	0.19	0.23	0.18	0.16	0.22	0.22	0.16	0.28	0.20	0.20	0.19	0.22
baseline; geometric																

mean mgA/L

D. pteronyssinus IgG4

baseline; geometric mean mgA/L	0.25	0.27	0.21	0.25	0.24	0.29	0.23	0.18	0.25	0.25	0.20	0.31	0.25	0.26	0.23	0.26
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Results describing continuous variables are expressed as means (SDs). Results describing categorical variables are expressed as numbers of patients and percentages relative to numbers of immunotherapy or placebo-receiving patients

(*) Note: some placebo patients have very high values.

Table 1B: Frequencies of TH2A and CD38+ TH2A cells in patients receiving immunotherapy or placebo

		Total active				300 IR			500 IR			Placebo					
		all		age		all		age		all		age		all		age	
		patients				patients				patients				patients			
		16 to 30	31 to 40	41 to 61		16 to 30	31 to 40	41 to 61		16 to 30	31 to 40	41 to 61		16 to 30	31 to 40	41 to 61	
		n = 121	n = 45	n = 39	n = 37	n = 58	n =23	n = 21	n = 14	n = 63	n =22	n = 18	n =23	n = 61	n =28	n = 21	n = 12
Frequencies of	Baseline	6.74	7.87	5.3	5.62	6.69	8.43	6.26	4.48	6.79	7.28	5.35	6.29	8.23	9.26	6.46	8.90
TH2A cells /		(6.0)	(6.9)	(5.8)	(5.0)	(6.7)	(8.5)	(5.7)	(3.5)	(5.3)	(4.9)	(5.0)	(5.7)	(6.9)	(6.3)	(5.1)	(10.2)
CD4 ⁺ T cells;	After	4.61	4.67	5.12	4.01	4.72	4.62	5.10	4.31	4.52	4.72	5.15	3.84	6.99	8.52	5.35	6.27

mean $\times 10^{-4}$ (SD)	AIT	(4.1)	(4.0)	(4.3)	(4.2)	(4.5)	(4.8)	(3.7)	(5.2)	(3.8)	(3.0)	(4.9)	(3.5)	(6.4)	(7.7)	(4.2)	(6.2)
Frequencies of CD38 ⁺ TH2A	Baseline	1.39 (1.6)	1.77 (1.8)	1.55 (1.8)	0.97 (1.0)	1.31 (1.5)	1.67 (2.0)	1.37 (1.3)	0.63 (0.6)	1.47 (1.6)	1.65 (1.6)	1.67 (2.1)	1.17 (1.2)	1.74 (1.9)	1.77 (1.4)	1.50 (1.7)	2.06 (3.0)
cells / CD4 ⁺ T	After	0.84	0.87	0.87	0.79	0.83	0.87	0.74	0.89	0.86	0.86	1.02	0.73	1.43	1.74	1.12	1.23
cells; mean $\times 10^{-4}$ (SD)	AIT	(1.0)	(0.9)	(0.9)	(1.2)	(1.2)	(1.1)	(0.6)	(1.9)	(0.9)	(0.8)	(1.2)	(0.7)	(1.3)	(1.4)	(1.2)	(1.4)

Results are expressed as means (SDs).

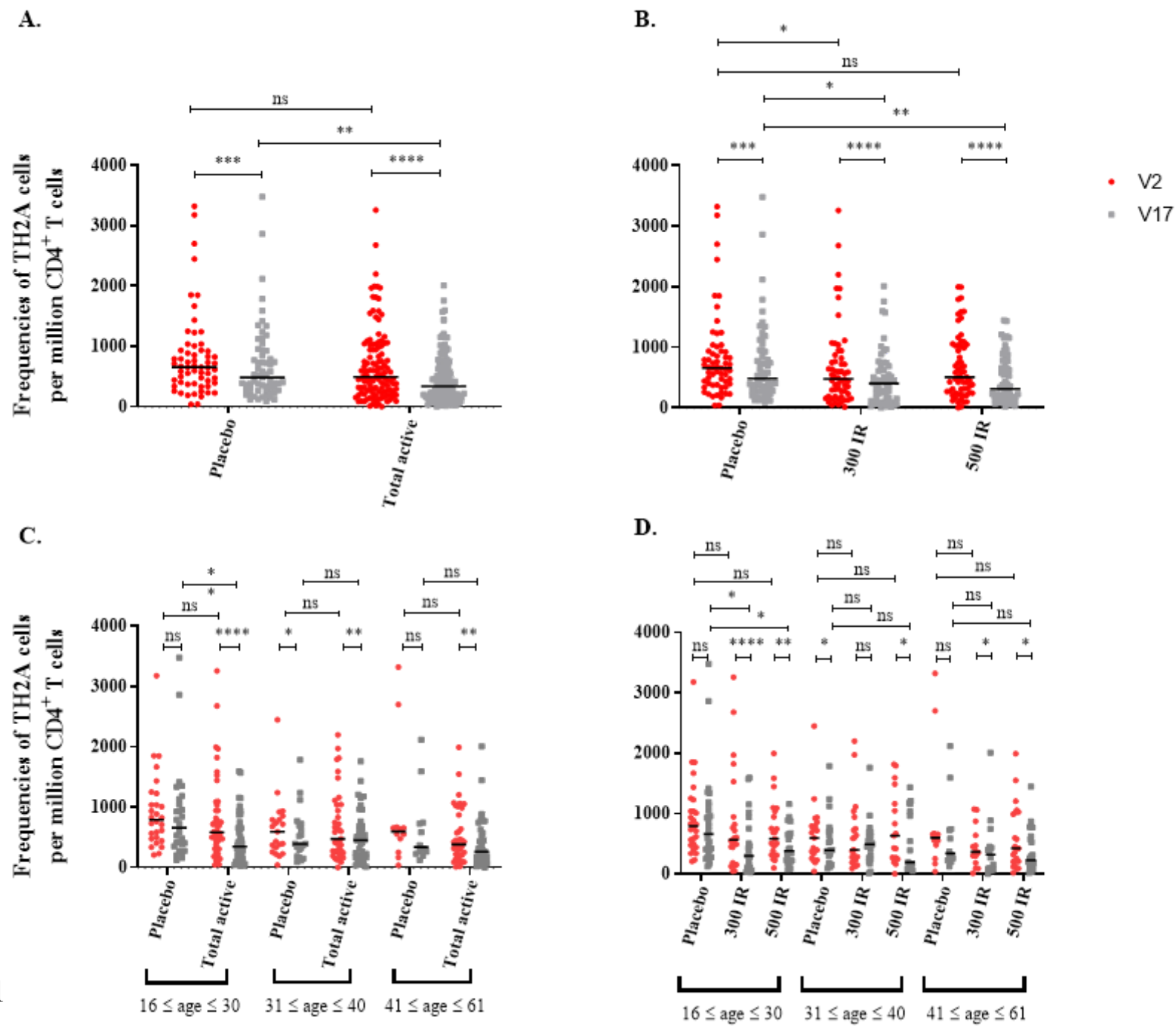


Figure 1

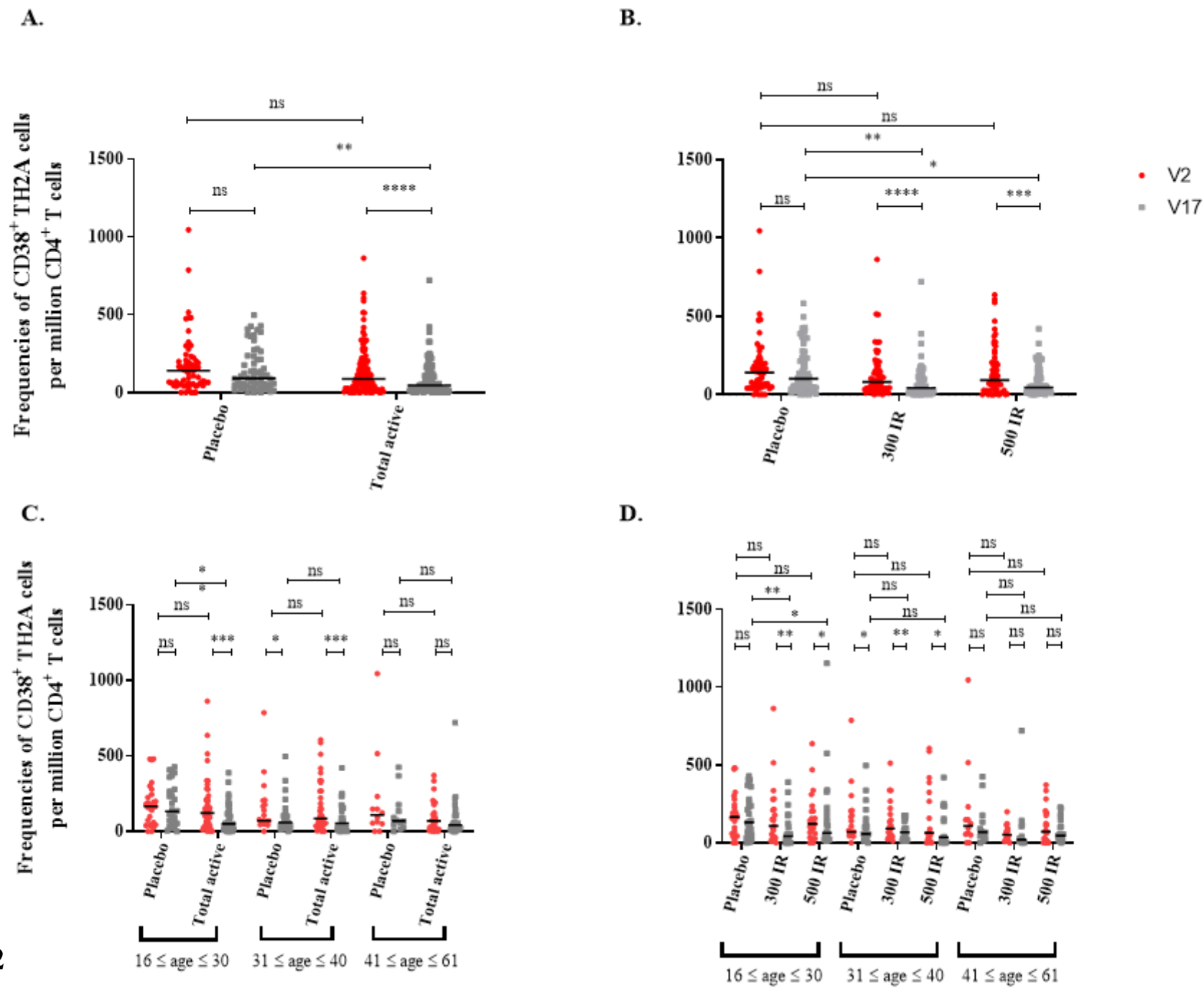


Figure 2

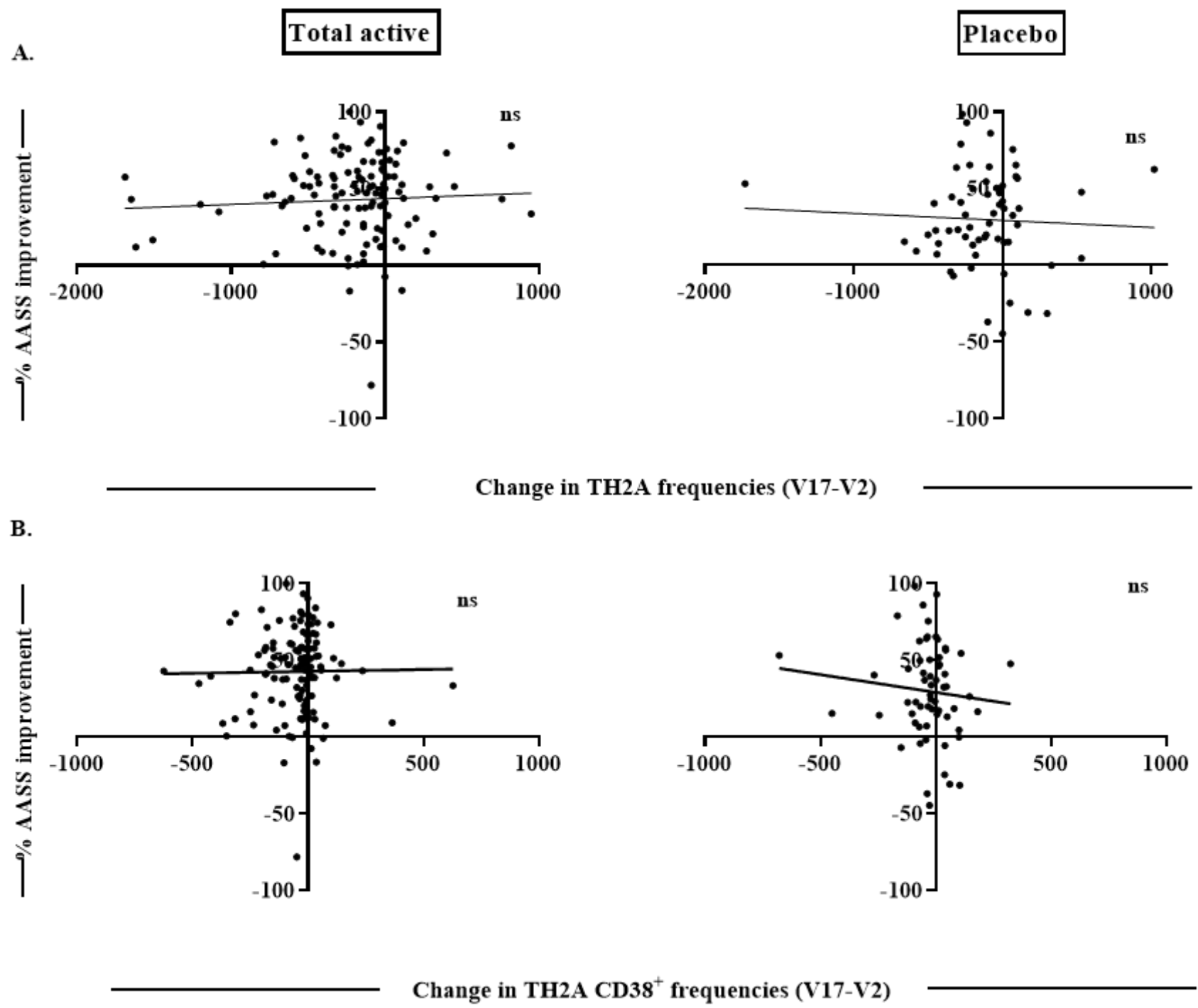


Figure 3

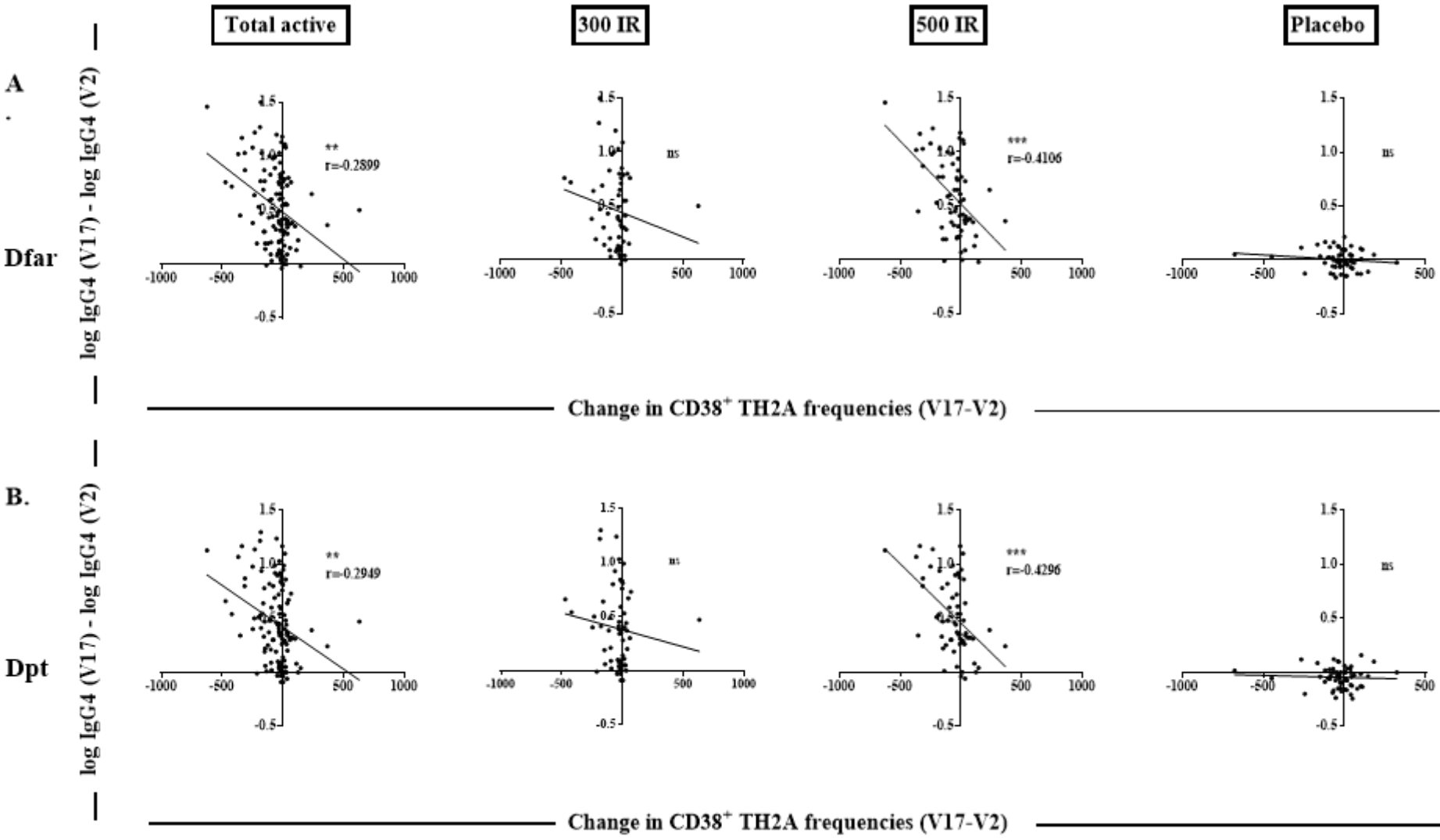


Figure 4

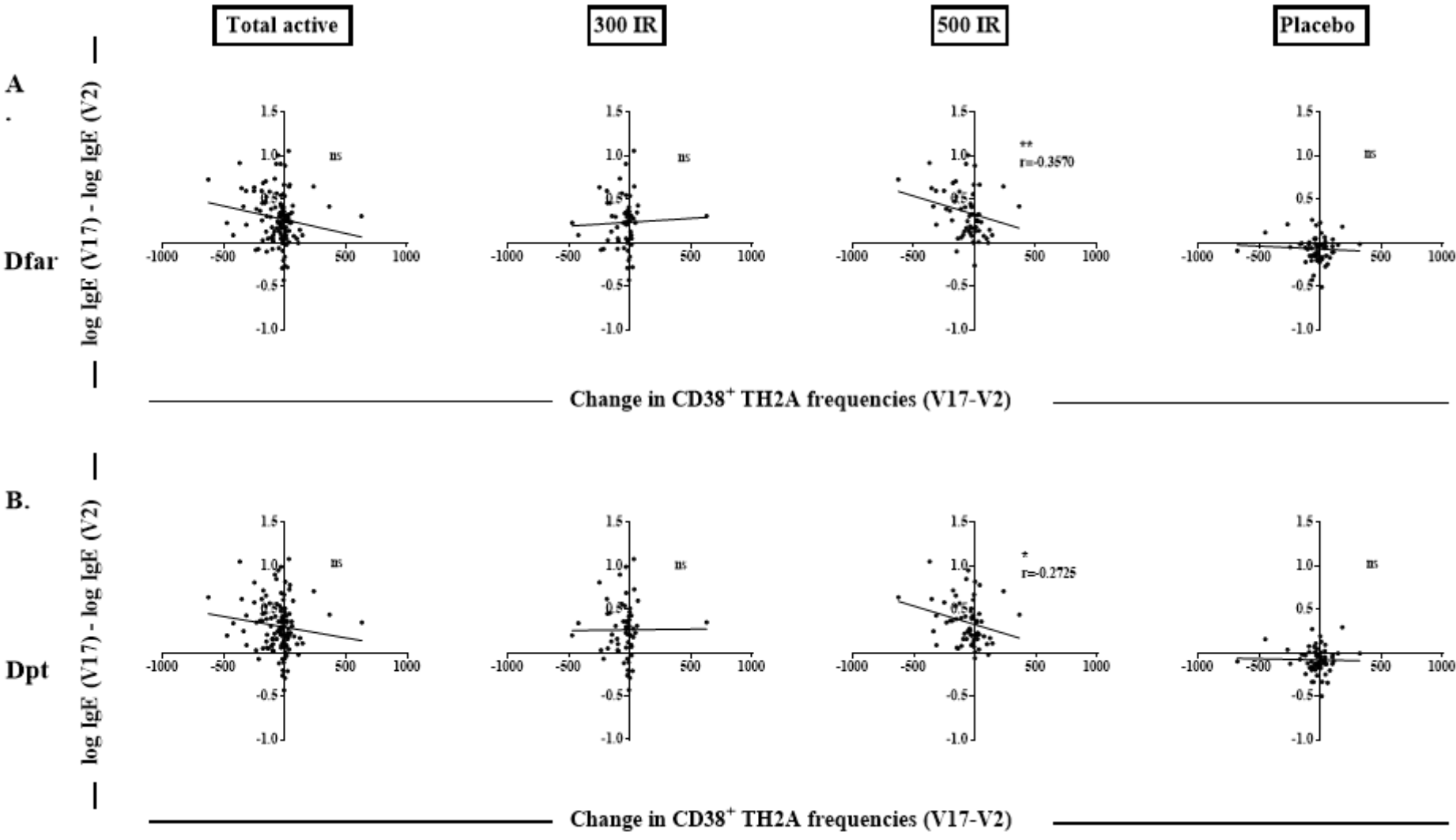
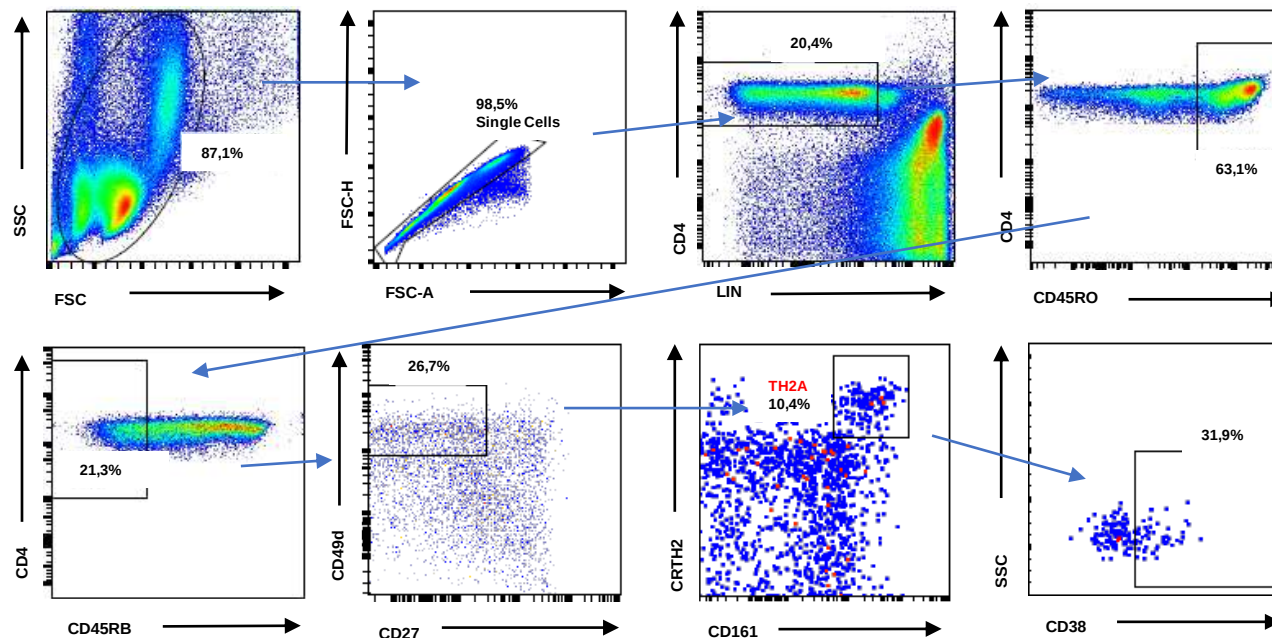
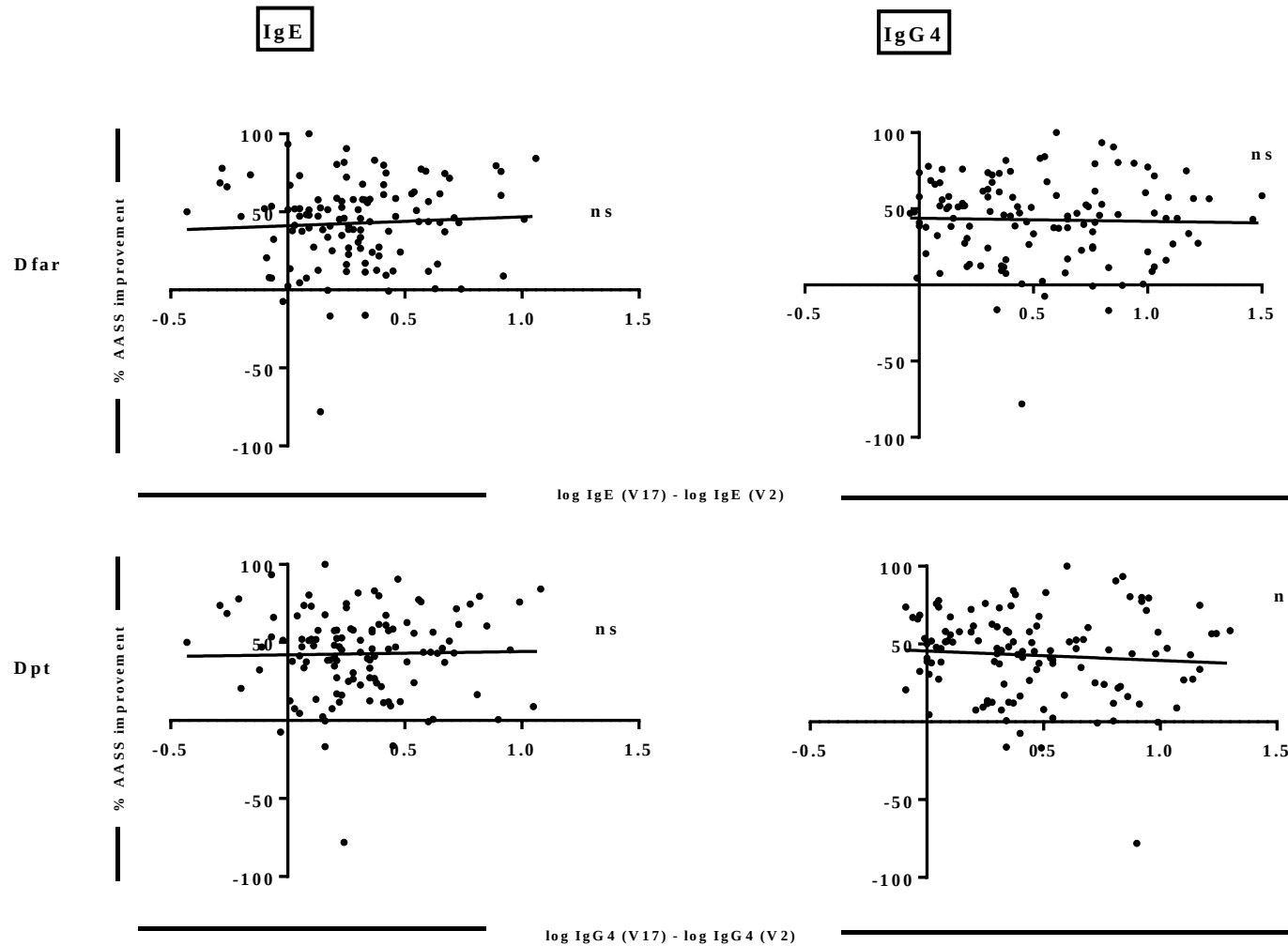


Figure 5

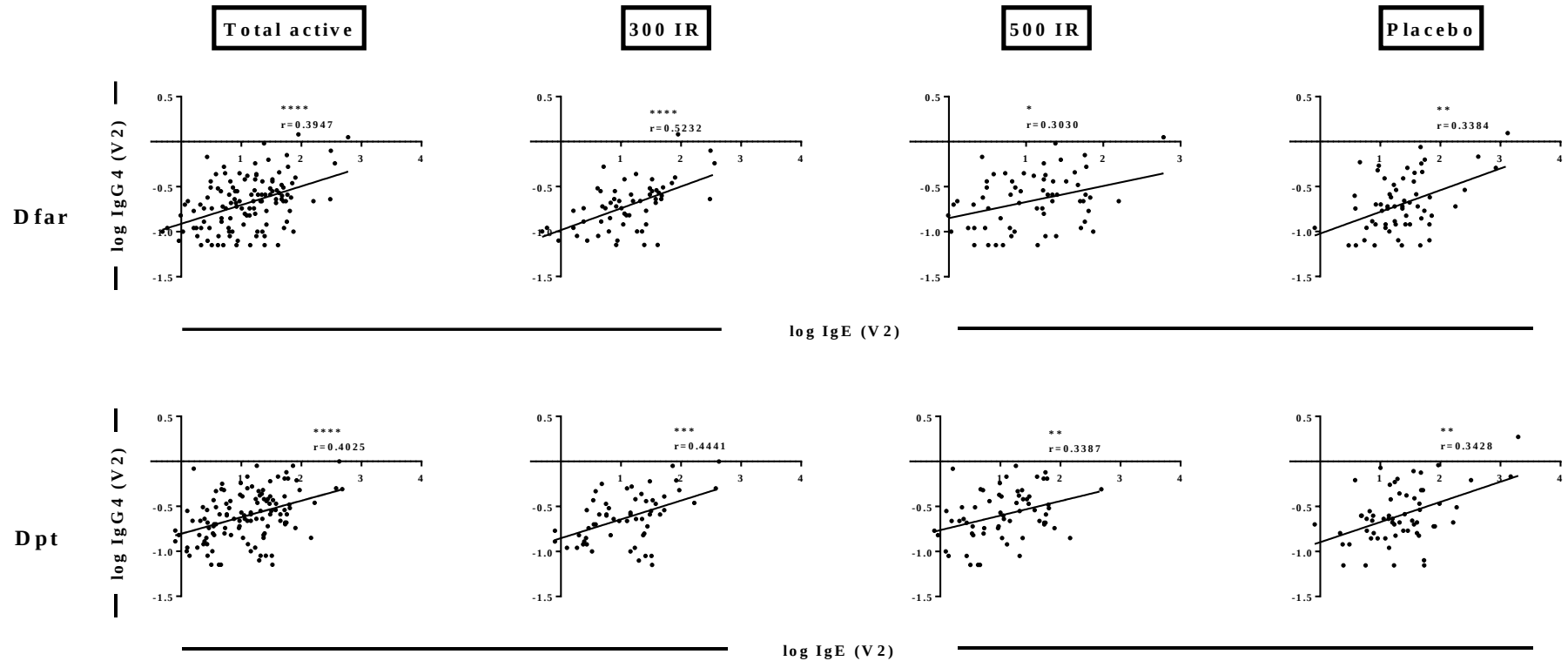


Supplementary Figure S1: Gating strategy for flow cytometry analyses of TH2A and CD38⁺ TH2A cells frequencies. Whole leukocytes population was identified within PBMCs with the measurements of forward-scattered light (FSC) and side-scattered light (SSC). TH2A cells were identified as CD4⁺, CD45RO⁺, cells negative for lineage-associated markers (Fixable Viability dye, CD8, CD11c, CD14, CD16, CD20, CD34, CD56, CD123 and TCRva24ja18), CD45RB⁻, CD27⁻, CD49d⁺, CRTH2⁺ and CD161⁺. CD38⁺TH2A cells are identified by adding CD38 in the gating strategy. All gates were set up based on reactivity with relevant control isotype.



Supplementary Figure S2: Absence of correlation between changes in IgE or IgG4 antibody levels and clinical effect.

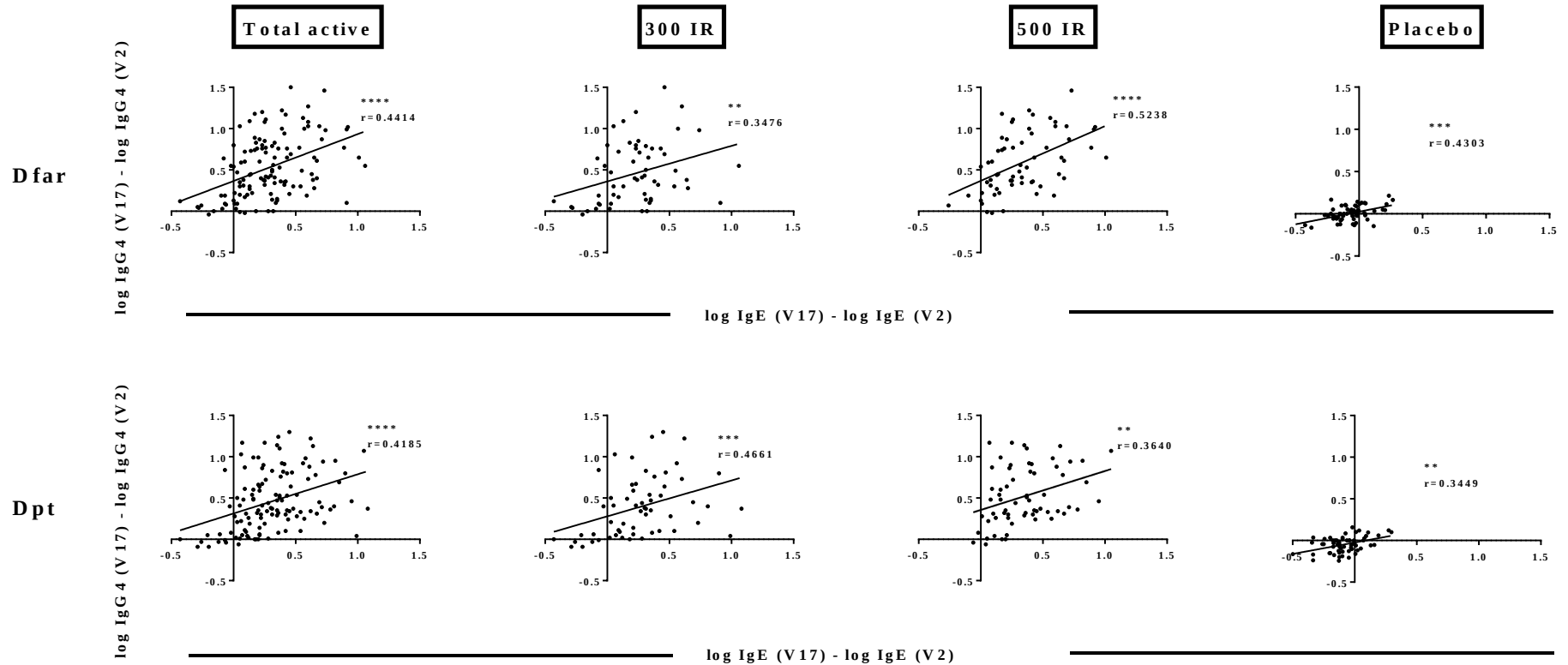
Spearman correlation of changes in log-transformed (log) *D. farinae* (Dfar) and *D. pteronyssinus* (Dpt) IgE levels with percentage improvements in AASS upon HDM-AIT in the total active ($n = 121$) group including 300 IR ($n = 58$), and 500 IR ($n = 63$) groups. ns: non-significant.



Supplementary Figure S3: Correlation between IgE and IgG4 antibody levels at baseline (V2).

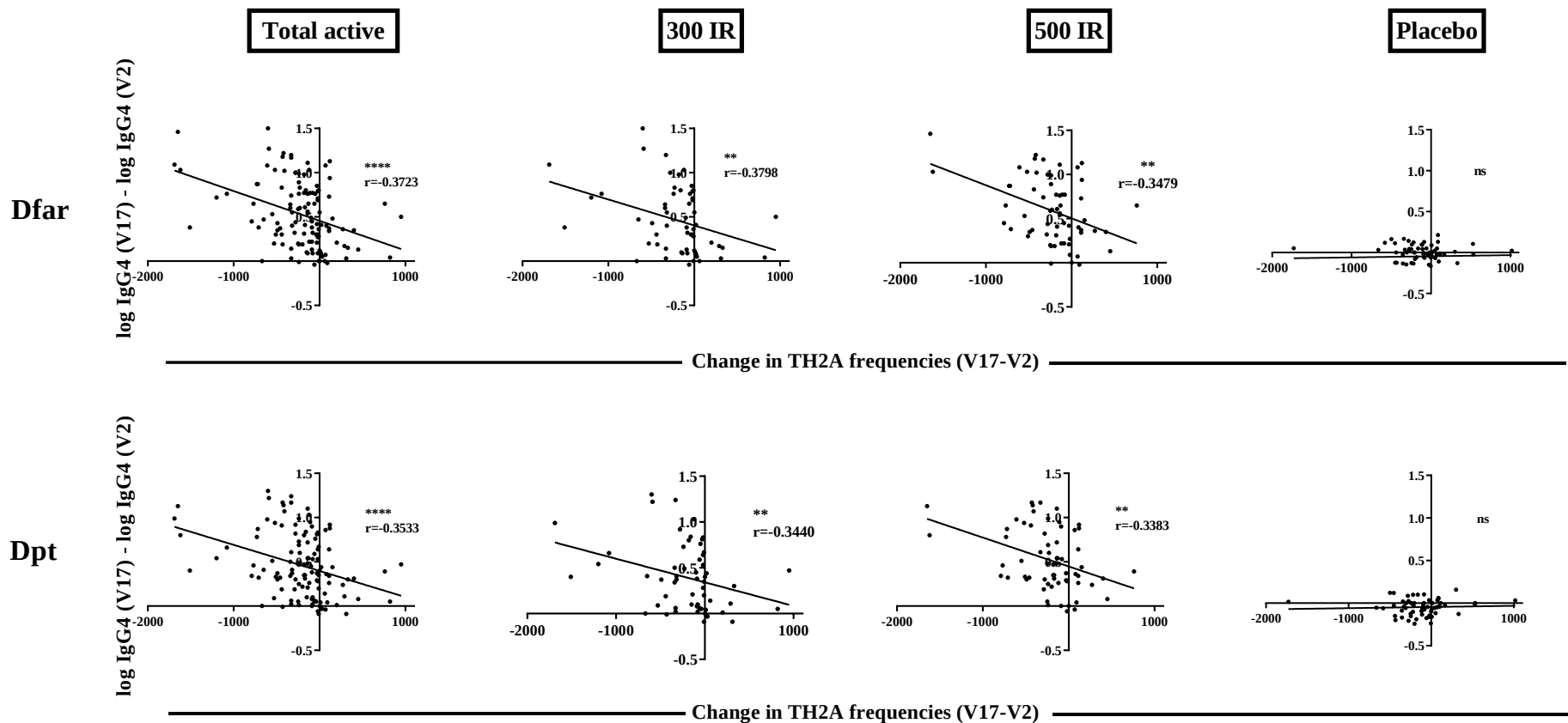
Spearman correlation of log-transformed (log) *D. farinosa* (Dfar) and *D. pteronyssinus* (Dpt) IgE levels with, respectively, log Dfar and log Dpt IgG4 levels at baseline (V2) in the total active (n = 121), 300 IR (n = 58), 500 IR (n = 63) and placebo (n = 61) groups.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.



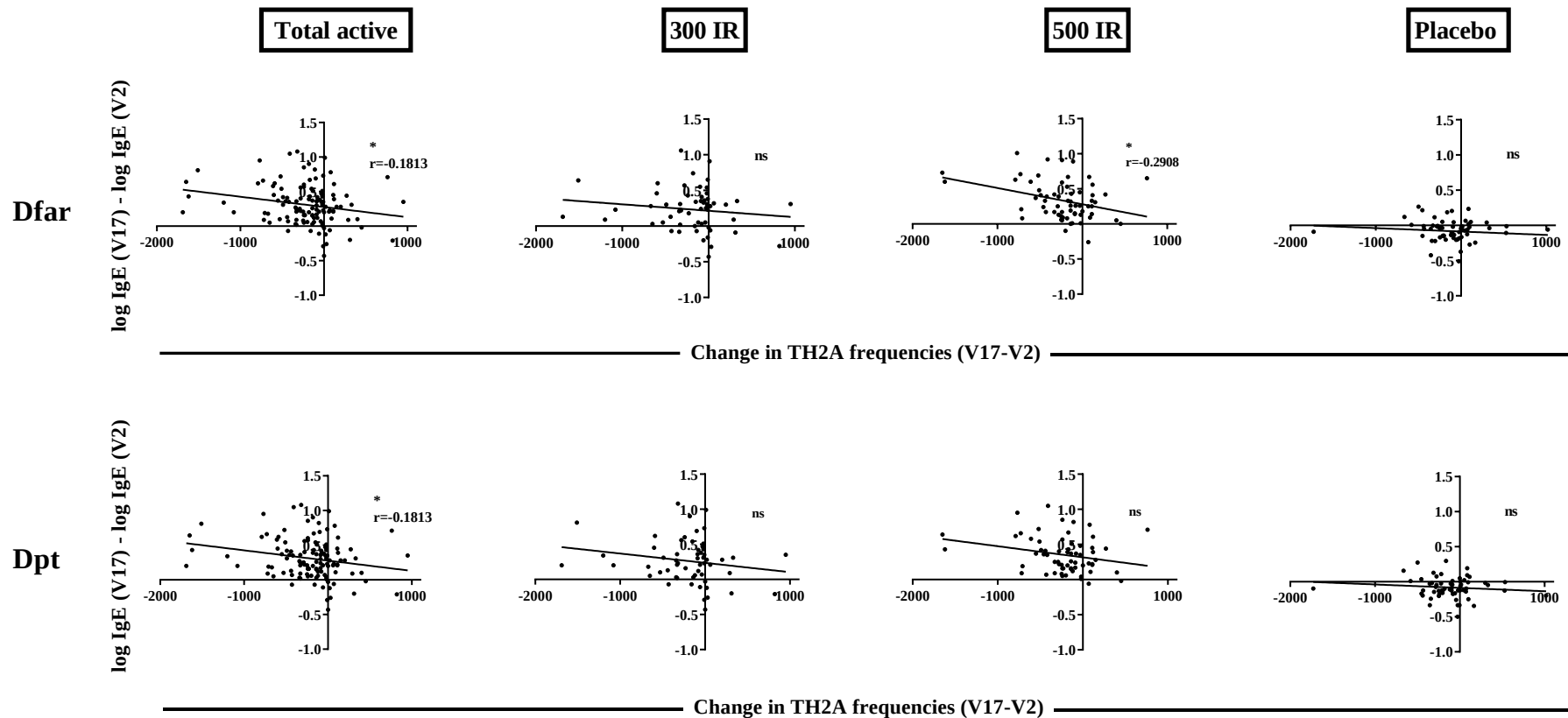
Supplementary Figure S4: Correlation between changes in IgE and IgG4 antibody levels after HDM-AIT.

Spearman correlation of changes in log-transformed (log) *D. farinae* (Dfar) and *D. pteronyssinus* (Dpt) IgE levels with, respectively, changes in log Dfar and Dpt IgG4 levels upon HDM-AIT in the total active (n = 121), 300 IR (n = 58), 500 IR (n = 63) and placebo (n = 61) groups. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.



Supplementary Figure S5: Correlation between changes in TH2A frequencies and IgG4 antibody levels after HDM-AIT.

Spearman correlation of changes in TH2A frequencies (expressed in number of cells per million CD4⁺ T cells) with changes in log-transformed (log) *D. farinae* (Dfar) and *D. pteronyssinus* (Dpt) IgG4 levels upon AIT in the total active (n = 121), 300 IR (n = 58), 500 IR (n = 63) and placebo (n = 61) groups. ** $p < 0.01$; **** $p < 0.0001$; ns: non-significant.



Supplementary Figure S6: Weak correlation between changes in TH2A frequencies and IgE antibody levels after HDM-AIT.

Spearman correlation of changes in TH2A frequencies (expressed in number of cells per million CD4⁺ T cells) with changes in log-transformed (log) *D. farinae* (Dfar) and *D. pteronyssinus* (Dpt) IgE levels upon HDM-AIT in the total active (n = 121), 300 IR (n = 58), 500 IR (n = 63) and placebo (n = 61) groups. * $p \leq 0.05$; ns: non-significant.