

The frequency of CD4 + CD25 + FoxP3 + CD127 – cells in Bet v 1 contiguous overlapping peptide immunotherapy as a putative marker of efficacy

To the Editor,

Allergen-specific immunotherapy (AIT) represents the only curative treatment for allergic diseases. Although recent research provides strong evidence on pivotal mechanisms of AIT,¹ the identification of robust biomarkers for the efficacy monitoring or stratification of potentially best clinical responders before the treatment still requires both validation and qualification. In this study, we aimed at investigation of the effect of AIT with *Bet v1*-derived contiguous overlapping peptides (COPs) on T-cell subsets frequencies and how this correlates with clinical efficacy in a double-blind, *placebo*-controlled phase IIb study in birch pollen-allergic patients (NCT02271009). Clinical efficacy of the product—*Bet v1*-derived COPs have been reported before.^{2,3}

Clinical efficacy of the treatment was evaluated by total nasal symptoms score (TNSS) measured at the 3rd hour of challenge in environmental exposure chamber (EEC-2 day regime, duration 6 hours, 12 time points) performed before and after AIT. Optimal time point for clinical efficacy evaluation in EEC was 3 hours, as assessed on the basis of TNSS saturation data for all patients. We observed a significant response to the EEC challenge in all patients, before and after AIT (Figure S1). We could not prove clinical efficacy as assessed by comparison of *verum* vs *placebo* groups. A nonsignificant ($P = .15$) trend in favor of *verum* group with reduction in TNSS score by 15.2% compared with *placebo* (Figure 1A) was shown. In addition, we observed significant reduction in TNSS in *verum* but not in *placebo* patients after AIT (Figure 1A).

This clear trend for reduction in TNSS in *verum* patients prompted the assessment of patients groups defined as responders (R—improved after AIT > 20% improvement in TNSS), nonresponders (NR—slight improvement 0%–20%, no response or worsening between 0% and 99% after AIT) and A—aggravators—significant worsening ≥100% in TNSS after AIT). The group of aggravators differed significantly from other populations investigated in this study and was characterized by a significantly lower TNSS in EEC response before AIT and a very strong increase in TNSS after AIT (Figure 1B). Interestingly R, NR and A populations were observed in both *verum* and *placebo* patients (Figure S2). To further characterize patient subpopulations and to find immune parameters which might dissect *verum* R patients from *placebo* R patients, the effect of AIT on frequencies of *Bet v1*-specific

enriched Th1, Th2, and CD4 + CD25 + FoxP3 + CD127 – cells subsets was analyzed. For this purpose, peripheral blood mononuclear cells (PBMCs) from birch pollen-allergic patients participating in the study (NCT02271009) were collected before, during and after AIT with *Bet v1* COPs. PBMCs were cryopreserved, and following

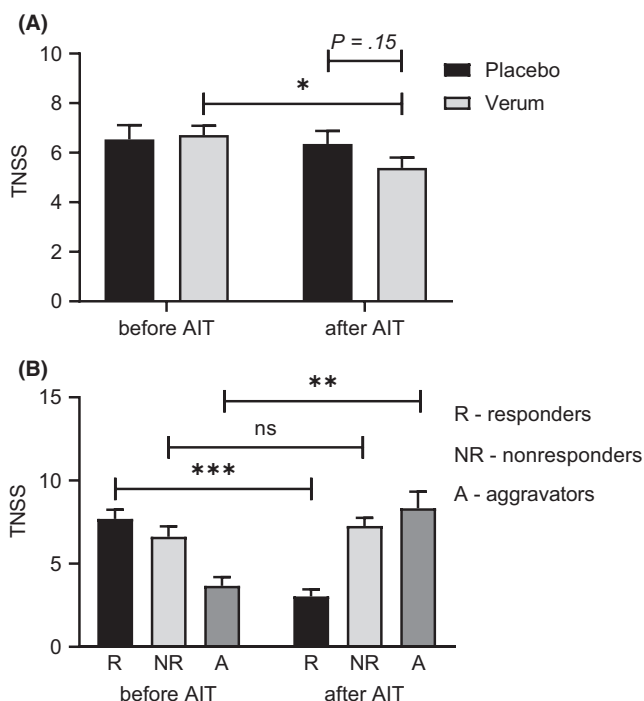


FIGURE 1 No statistically significant reduction ($P = .15$) in TNSS was shown for *placebo* vs *verum* patients with a trend towards clinical improvement. Significantly reduced TNSS after AIT for *verum* but not *placebo* patients was observed. A, A 15.2% nonsignificant difference in TNSS at optimal time point of efficacy assessment (3 h) after AIT was observed for *placebo* and *verum*. Comparisons were analyzed between visits and treatment types. R shows significant reduction in TNSS after AIT, while NR do not change in score and A shows significant increase in TNSS. B, Comparisons were analyzed between R/NR/A time points before and after AIT within each response group. Analyses were carried out via two-sample Student's *t* test or Mann-Whitney *U* test, depending on the normality of the samples. * P -value < 0.05, ** P -value < 0.01, *** P -value < 0.001

resuscitation was stimulated in batches with recombinant Bet v1 for 7 days. Procedure was performed within 4 weeks and used same reagents batches. Frequencies of Th1, Th2, and CD4 + CD25 +

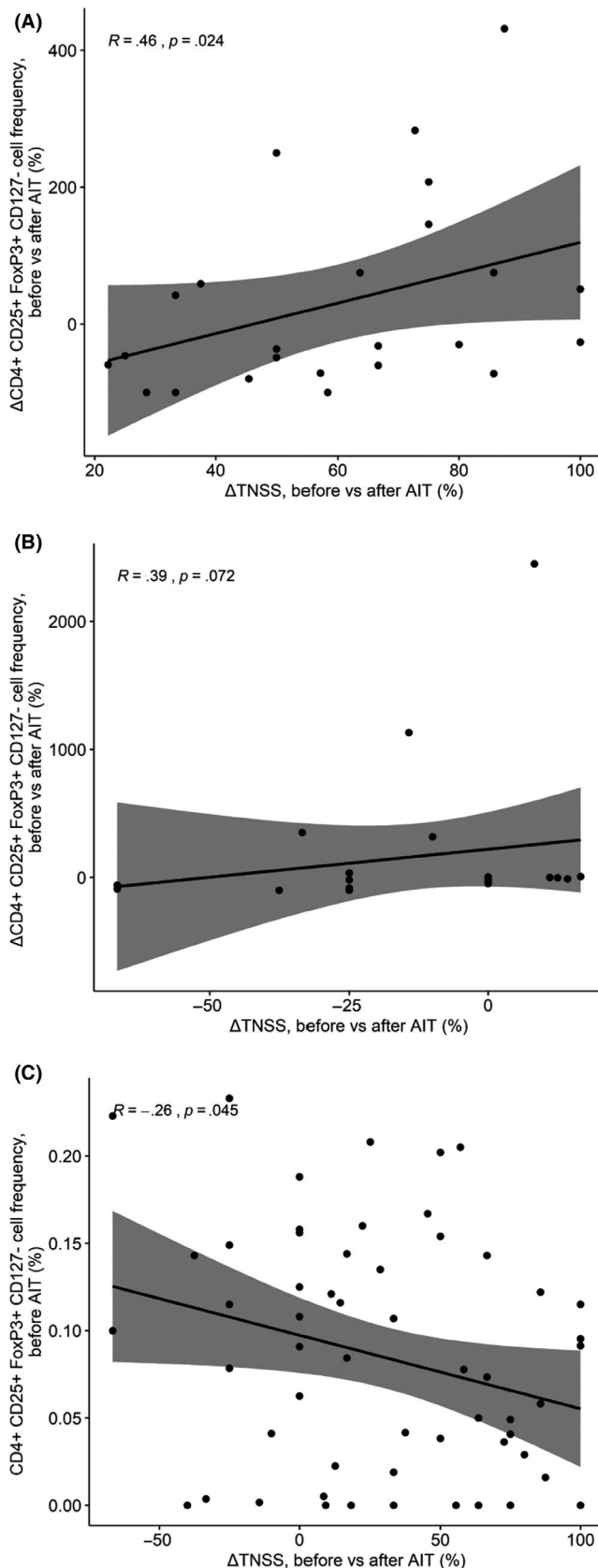


FIGURE 2 Correlation graphs showing interaction between Δ TNSS and Δ CD4 + CD25 + FoxP3 + CD127 - cell frequency before and after AIT for R. Improvement of TNSS is positively correlated with increase in CD4 + CD25 + FoxP3 + CD127 - cell frequency in R (A). No such correlation was observed for the NR group (B). The interaction between Δ TNSS and Δ CD4 + CD25 + FoxP3 + CD127 - cell frequency before AIT for combined R and NR. A positive correlation between CD4 + CD25 + FoxP3 + CD127 - cell levels before AIT and clinical efficacy of treatment was demonstrated (C). Δ TNSS compares improvement in TNSS before versus after AIT, at the 3 h time point. P-values shown in the graphs are based on the Pearson or Spearman correlation coefficient, dependent on normality of the samples

FoxP3 + CD127 - cells were measured using flow cytometry. In line with previous studies, well-known hallmarks of successful immunotherapy, namely decreased Th2 response (Th2 frequency reduction) and the increased CD4 + CD25 + FoxP3 + CD127 - cell frequency was shown (Figure S6). These findings were observed for vaccine doses of 10 and 25 μ g, but not for 50 μ g. In the *verum* group, we showed significant correlation between the increase in CD4 + CD25 + FoxP3 + CD127 - cell number and clinical efficacy assessed by TNSS decrease in the responders only (Figure 2 A,B). This trend was not observed in any other *verum* group, nor in *placebo* (Figure S4a-c). These results account for the relevance of changes in CD4 + CD25 + FoxP3 + CD127 - cell frequencies in the monitoring of AIT efficacy. Since this effect was not observed in the *placebo* responders, the additional advantage of CD4 + CD25 + FoxP3 + CD127 - cell measurements would be the possibility to dissect real response to AIT from the *placebo* effect. The group of aggravators is of major interest. These subjects showed significantly lower TNSS before AIT and significantly higher TNSS after AIT compared with responders and nonresponders (Figure 1B). This requires further research of the underlying immune mechanisms in these subjects. In addition, a low response to allergen challenge in EEC could be a contraindication for AIT.

Recent research also focused on finding of the predictive biomarkers for patient stratification into potential responders and nonresponders. Therefore, the level of CD4 + CD25 + FoxP3 + CD127 - cells at the onset of therapy was correlated with the clinical response to AIT. No significant differences in CD4 + CD25 + FoxP3 + CD127 - cell frequency at the onset of AIT were observed in *verum* vs *placebo* groups. A positive correlation between CD4 + CD25 + FoxP3 + CD127 - cell levels before AIT and clinical efficacy of treatment was demonstrated (Figure 2C). Patients are more likely to improve their TNSS score during AIT when their CD4 + CD25 + FoxP3 + CD127 - cell level at the onset of therapy is low. This effect was not observed in the *placebo* group (Figure S3).

The role of CD4 + CD25 + FoxP3 + CD127 - cells and the main immunosuppressive cytokines IL-10 and TGF β in successful AIT is well-defined.⁴ This report confirms previous findings that the increase in CD4 + CD25 + FoxP3 + CD127 - cell frequency is associated with successful AIT in a novel model of Bet v1 COPs vaccination. This report also shows that the assessment of allergen-specific

CD4 + CD25 + FoxP3 + CD127 – cell population might serve as a biomarker of treatment efficacy in AIT. In addition, this parameter can allow the clinically plausible dissection between real *verum* and *placebo* responders. Furthermore, this report indicates that low level of CD4 + CD25 + FoxP3 + CD127 – cells at the onset of AIT might correlate with a better efficacy of treatment. Biologically, this makes sense as subjects with the low levels of CD4 + CD25 + FoxP3 + CD127 – cells are more likely to benefit the most from an increase in circulating CD4 + CD25 + FoxP3 + CD127 – cells induced by AIT. This suggests that the subset of CD4 + CD25 + FoxP3 + CD127 –^{low} birch pollen-allergic patients should be targeted and encouraged to undergo AIT. The population of major interest is aggravators in whom mechanisms leading to more severe allergic response might be triggered by AIT. However, no correlation of clinical worsening with increased Th2 response (Figure S4) was found.

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CONFLICT OF INTEREST

Paweł Gajdanowicz, Dries Van Elst, Andrzej Eljaszewicz and Sylwia Smolińska have no conflict of interest to declare. Liam O'Mahony reports personal fees from Alimentary Health Ltd, grants from GSK, outside the submitted work. Alexander Kettner, reports personal fees from Anergis SA, during the conduct of the study. Marek Jutel, reports personal fees from ALK-Abello, personal fees from Allergopharma, personal fees from Stallergenes, personal fees from Anergis, personal fees from Allergy Therapeutics, personal fees from Circassia, personal fees from Leti, personal fees from Biomey, personal fees from HAL, during the conduct of the study; personal fees from Astra-Zeneca, personal fees from GSK, personal fees from Novartis, personal fees from Teva, personal fees from Vectura, personal fees from UCB, personal fees from Takeda, personal fees from Roche, personal fees from Janssen, personal fees from Medimmune, personal fees from Chiesi, outside the submitted work.

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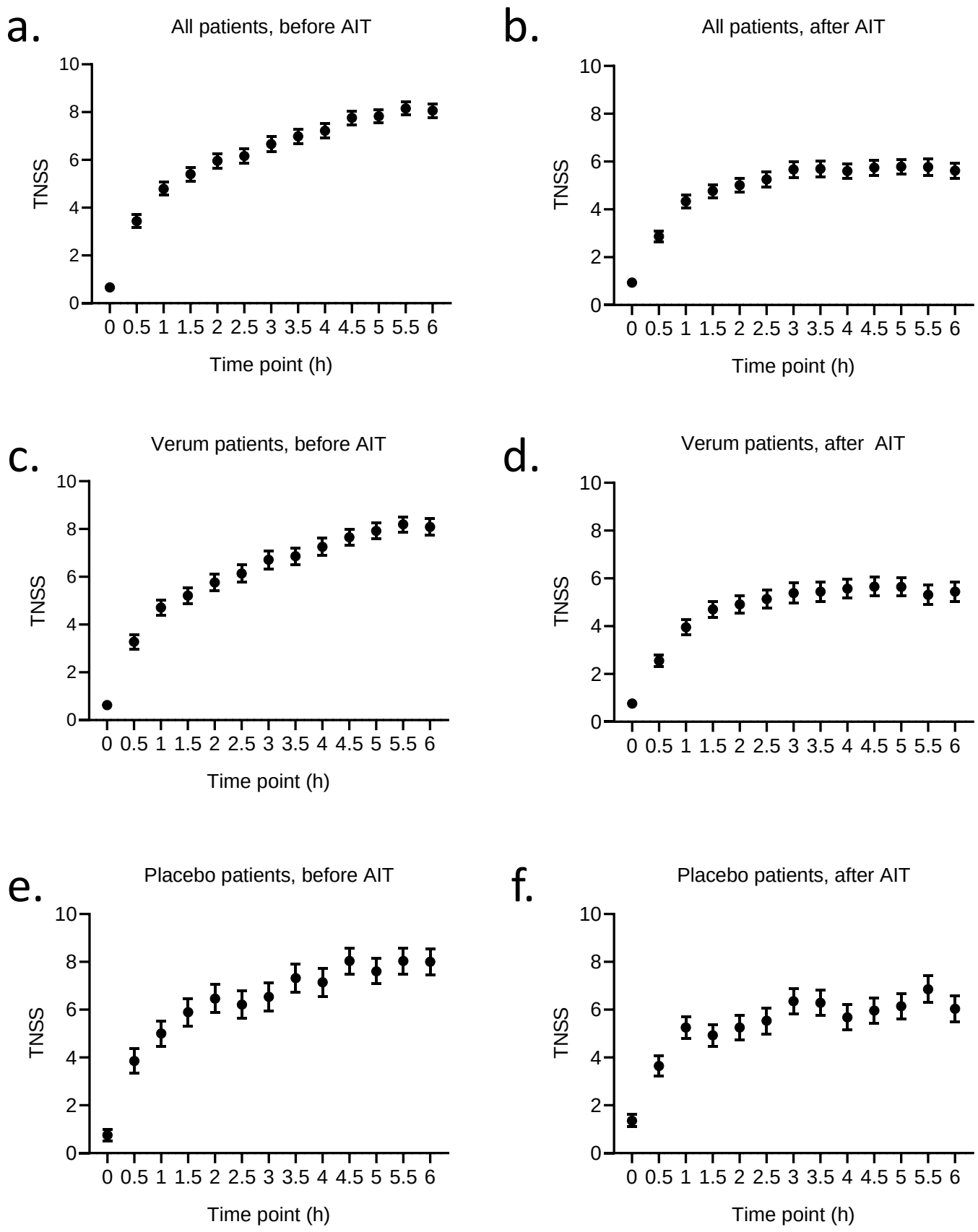
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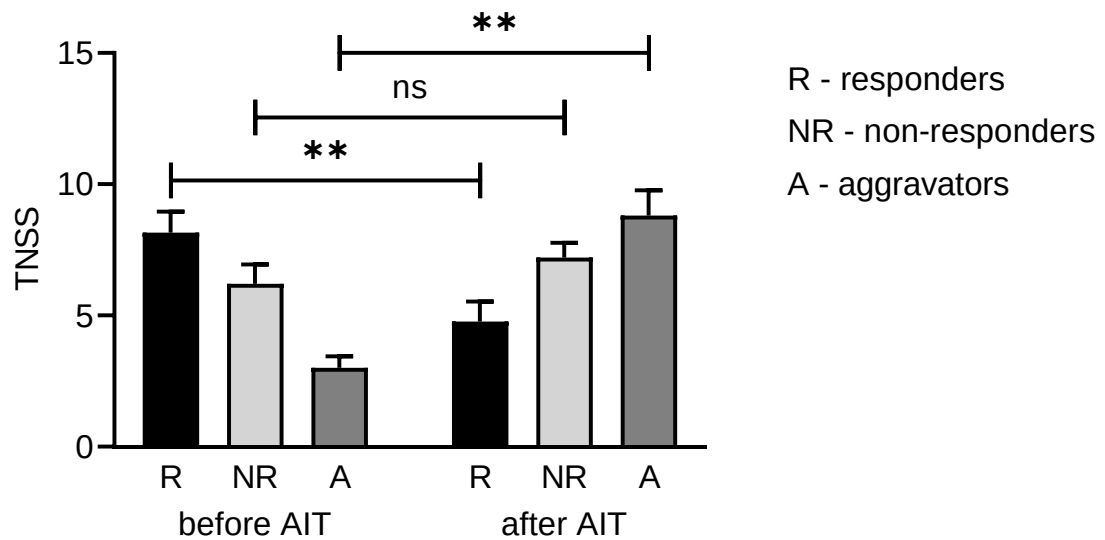
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SUPPORTING INFORMATION

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

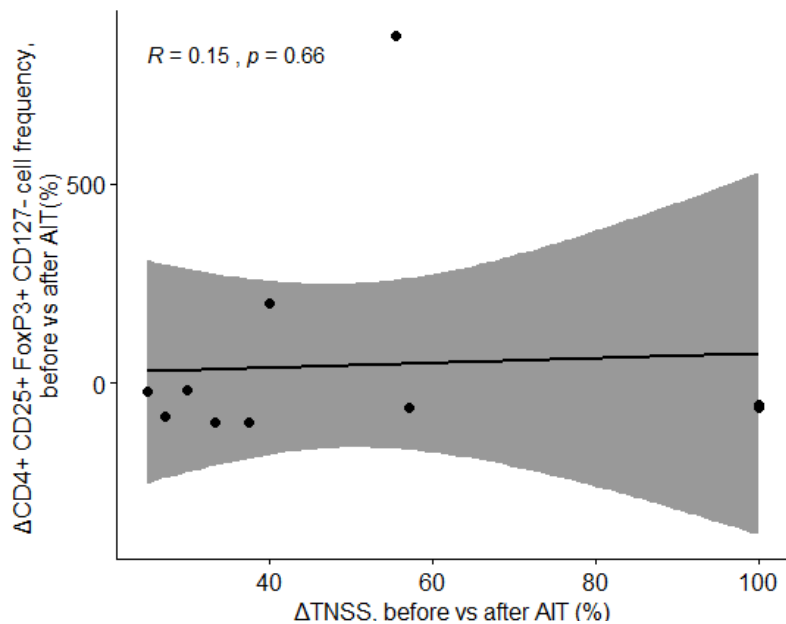


Supplemental figure S1. **Selection of the 3h timepoint as the optimal timepoint for clinical efficacy evaluation in the EEC.** TNSS kept rising during stay in EEC before AIT when checking all patients (a) and *verum* (c) patients whereas stagnation of TNSS occurs after 3 hours spent in the EEC for both all (b) and *verum* (d) patients. TNSS shows more fluctuation in *placebos* both before (e) and after (f) AIT.

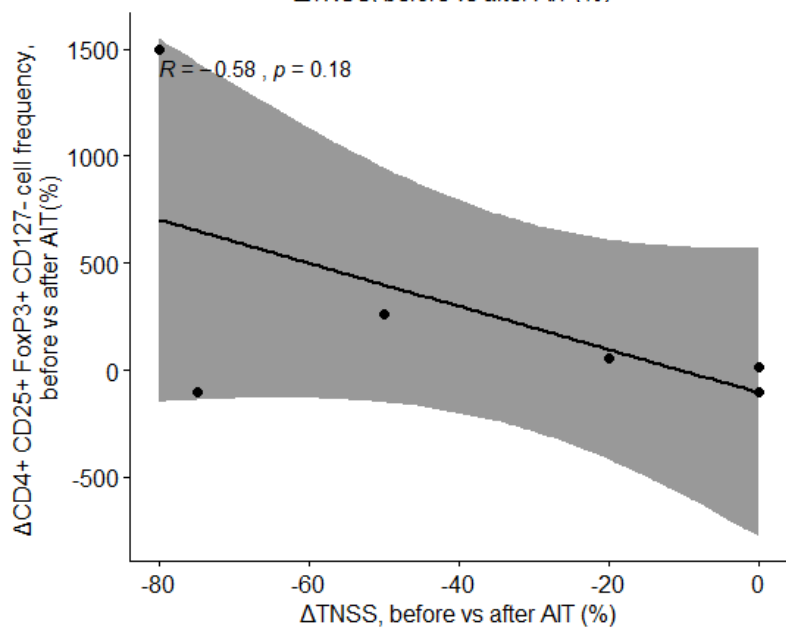


Supplemental figure S2. **TNSS shows significant reduction in R after AIT in *placebo* patients.** TNSS were compared at the 3h timepoint in the EEC. Comparisons were made between timepoints before and after AIT within each response group. Comparisons were carried out via two-sample student's t-test or Mann-Whitney U test, depending on the normality of the samples. * indicates p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

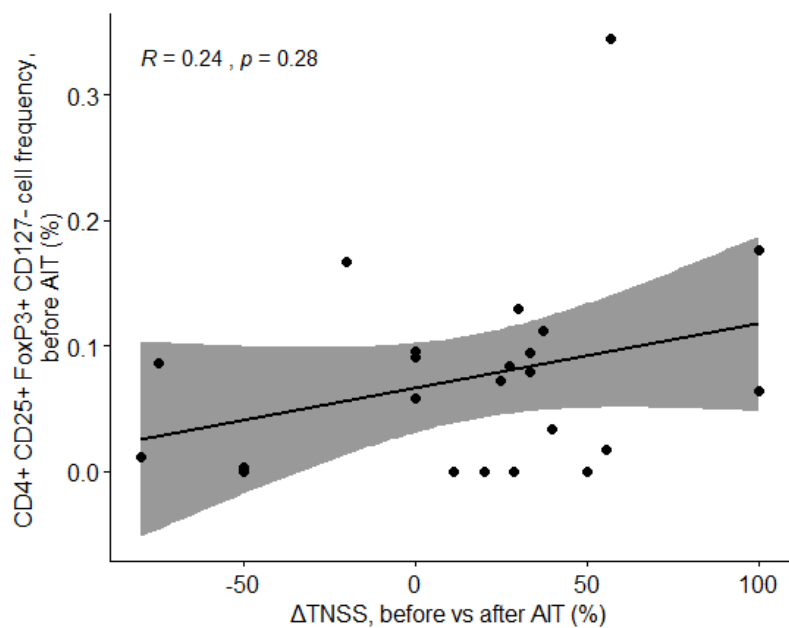
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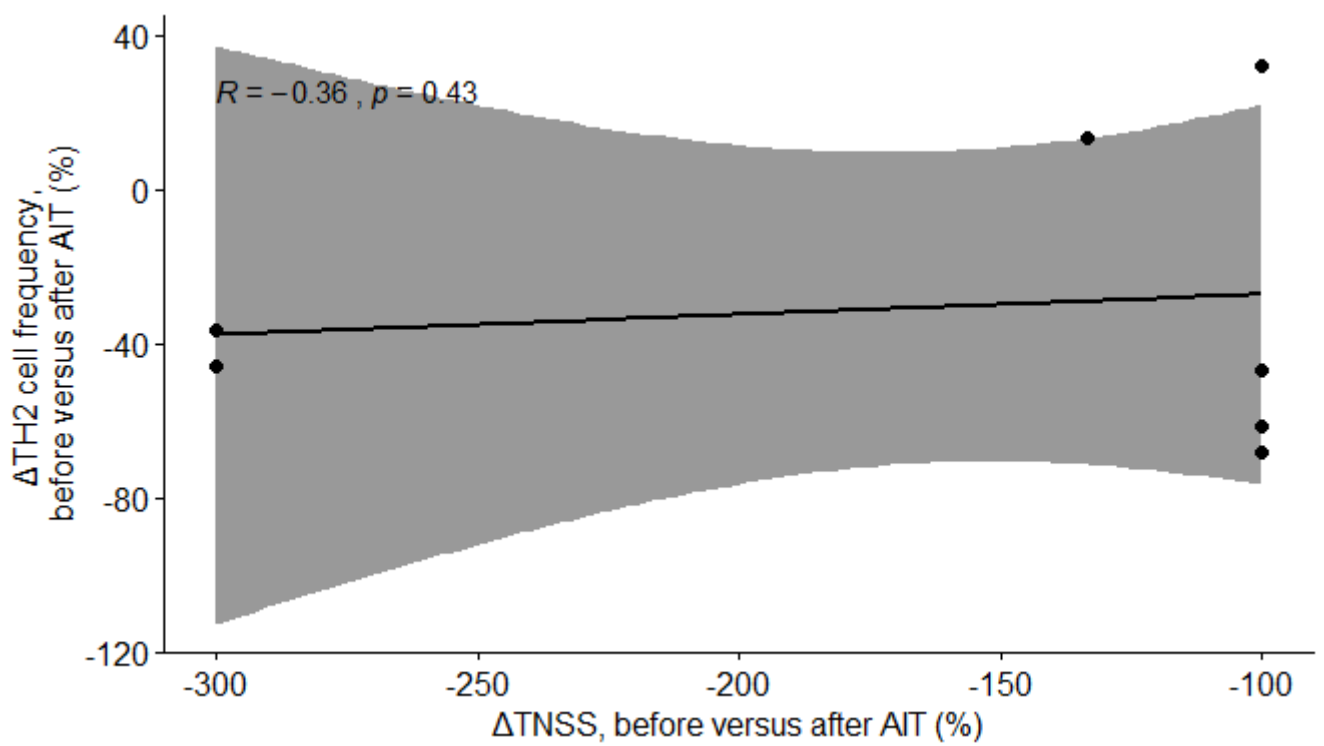
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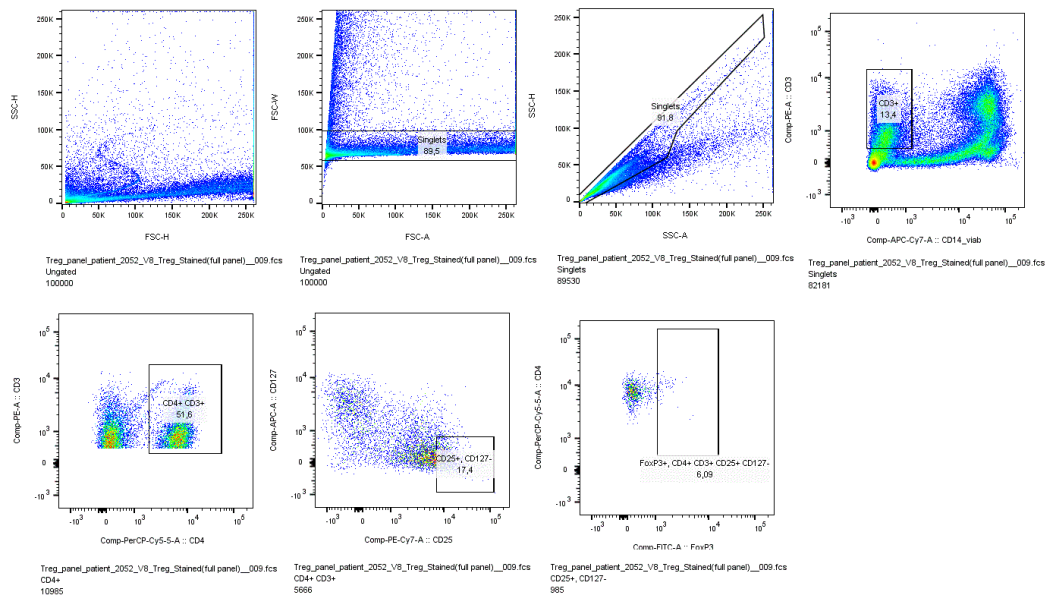
c.



Supplemental figure S3. **No correlations were detected between Δ TNSS and Δ CD4+ CD25+ FoxP3+ CD127- cell frequency before and after AIT for the *placebo* R (a) and NR (b) groups.** No correlation between CD4+ CD25+ FoxP3+ CD127- cell levels before the start of AIT and clinical efficacy of treatment (TNSS) was demonstrated in the *placebo* group combining R and NR (c). P-values shown in the graphs are based on the Pearson or Spearman correlation coefficient, dependent on normality of the samples.



Supplemental figure S4. **No correlation was detected between Δ TNSS and Δ Th2-cell frequency before and after AIT in *verum* aggravators.** P-value shown in the graph is based on the Spearman correlation coefficient.



Supplemental figure S5. Gating strategy for cytometric CD4+,CD25+, FoxP3+, CD127- cells.

