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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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IL-10 mRNA levels in whole blood cells correlate with house dust mite allergen immunotherapy efficacy

To the Editor,

Allergen immunotherapy (AIT) is recognized as a safe and efficacious treatment for IgE-mediated respiratory allergies to pollen and house dust mite (HDM) allergens.¹ Whereas patients' eligibility to AIT relies on symptoms severity, reactivity to skin prick tests, and/or high allergen-specific IgEs titers,¹ and patients would greatly benefit from the identification of biomarkers capable to predict the likelihood of clinical improvement during AIT. With this need in mind, we undertook a gene expression analysis on whole blood cells obtained from a sub-group of 152 Japanese patients included in a double-blind placebo-controlled clinical HDM AIT study (S-524101)² to search for predictive biomarker candidates.

Specifically, in this study, a total of 152 HDM allergic patients receiving daily either a 300 IR HDM ($n = 75$) or placebo ($n = 77$) tablet sublingually for 12 months were stratified into four subgroups based upon the median percentage (51.18%) of AASS improvement (cf. Methods S1), namely high active responders (ARs^{high} ; $n = 38$), low active responders (ARs^{low} ; $n = 37$), high placebo responders (PRs^{high} ; $n = 18$), and low placebo responders (PRs^{low} ; $n = 59$). The demographics and baseline characteristics of patients were similar in all groups (Tables S1 and S2). We evaluated polarization markers (ie, *Tbet*, *MX1*, *IFN- γ* , *GATA3*, *OX40L*, *RIPK4*, *IL-13*, *ROR γ -t*, *IL-23*, *IL-17*, *Foxp3*, *C1q*, *STAB1*, and *IL-10*) described to be involved in the allergic reaction or in its resolution³ as predictive markers of clinical responses to AIT. We thus monitored by qPCR the expression of the aforementioned markers and endogenous control β -actin gene in

whole blood cells collected before AIT, and we calculated the copy numbers of each gene per million copies of β -actin mRNA. Among these 14 gene expression analyses, we highlighted that *IL-10* mRNA levels are predictive of clinical responses to AIT.

As shown in Figure 1A, *IL-10* mRNA levels were significantly ($P < 0.01$) higher in whole blood cells from ARs^{high} than in ARs^{low} , whereas no such difference was observed in the placebo group between PRs^{high} and PRs^{low} . In addition, there was no potential bias due to differences in *IL-10* mRNA levels between the active and the placebo groups (Figure 1A). No statistical differences could be observed in the gene expression of other markers between ARs and ANRs (Figure S1). When plotted against percentages of AASS improvement (V17/V2), mRNA levels of *IL-10* were significantly correlated (with a Spearman correlation $R = 0.3509$, $P = 0.002$) with clinical benefit within patients from the active group (Figure 1B, upper panel), whereas no such a correlation was observed in the placebo group (Figure 1B, lower panel). We also measured *IL-10* expression by qPCR in whole blood collected after 2-month immunotherapy (V6) from 152 HDM allergic patients receiving either 300 IR HDM ($n = 75$) or placebo ($n = 77$) tablet sublingually, once a day, for 12 months. When plotted against percentages of AASS improvement (V17/V2) for each individual patient, levels of *IL-10* mRNA were significantly correlated (with a Spearman correlation $R = 0.3258$, $P = 0.0043$) with clinical benefit within patients from the active group (Figure S2, upper left panel), whereas no such a correlation was observed in placebo-treated patients (Figure S2, upper right panel). No such relationship could be

observed after 12-month immunotherapy (Figure S2, lower panels) suggesting an alteration of mRNA levels following HDM AIT.

To assess the advantage of evaluating *IL-10* mRNA levels in whole blood cells from HDM allergic patients undergoing AIT, we selected for further analyses 75% (114 out of 152) of HDM allergic patients exhibiting the highest levels of *IL-10* mRNA, defined as *IL-10*^{high} patients based on a copy number of *IL-10* mRNA per million copies of β -actin mRNA ≥ 94.455 . The 25% (38 out of 152) of remaining patients exhibiting the lowest levels of *IL-10* mRNA (*IL-10*^{low} patients) were excluded from subsequent analyses. As shown in Figure 2A,B, when compared to placebo-treated patients, improvement of AASS after 12-month AIT with 300IR HDM tablets in *IL-10*^{high} patients was much higher (−34.5%) than in the whole cohort a (−26%). If we set up the value to 50% of HDM allergic patients exhibiting the highest levels of *IL-10* mRNA, we now consider a target population of patients having a copy number of *IL-10* mRNA per million copies of β -actin mRNA ≥ 131.74 . When compared to placebo-treated patients, improvement of AASS after 12-month AIT with 300IR HDM tablets in these *IL-10*^{high} patients is still much higher (−41.3%; Figure 2C) than in the whole cohort (−26%; Figure 2A). As such, these data strengthen our previous results (value to 75%) that high *IL-10* mRNA levels in patient's blood correlate with clinical efficacy. In addition, as shown in Figure 2D, receiver operating characteristic curves of *IL-10* mRNA abundance confirmed that this marker has the highest predictive value (area under the curve = 0.7242; P -value = 0.0043) when the cutoff value of positive response to AIT is set at 25% of AASS improvement.

Noteworthy, among all the markers tested, qPCR analyses clearly established that *IL-10* mRNA levels are higher in whole blood cells from ARs^{high} relative to ARs^{low} and correlate significantly with clinical improvement following AIT. A limitation of our study is that the therapy lasted for 12 months and as such, this candidate biomarker may not be predictive of long-term efficacy.

IL-10 has been extensively analyzed as a follow-up rather than a predictive biomarker of AIT efficacy in several exploratory studies.^{4,5} Particularly, *IL-10* producing regulatory B- and T-cell specific for allergens were reported as being increased in the course of AIT or following natural allergen exposure,^{6–9} with the exception of only one study observing that *IL-10* secreting T cells were rather decreased after AIT.¹⁰ Recent data in experimental models also provided new insight on the critical role of *IL-10* directly regulating pathogenic Th2 cells

during HDM-induced airway inflammation,¹¹ and thus *IL-10* may act in a similar manner in HDM allergic patient receiving HDM AIT.

Since our analysis has been conducted in whole blood, the precise source of *IL-10* is still unclear. In our hands, however, we previously observed that myeloid and B cells are the main contributors of *IL-10* mRNAs expression in stimulated whole blood cells (Figure S3). As such, these observations are in agreement with previous studies

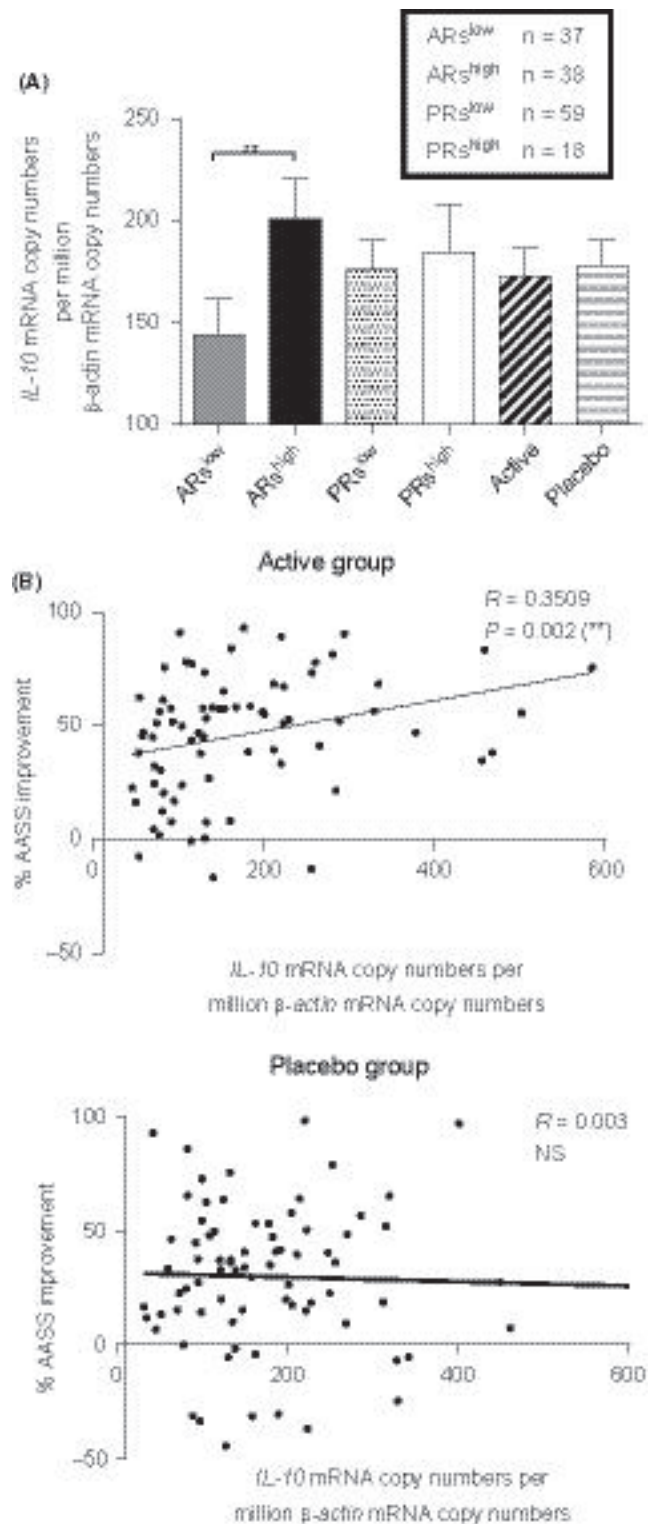


FIGURE 1 *IL-10* mRNA levels in whole blood cells from HDM allergic patients correlate with AIT clinical efficacy. A, Expression of the *IL-10* gene (mean $\pm$ SEM) was assessed at baseline by qPCR in whole blood cells from patients of the active and placebo groups (ARs^{high}, $n = 38$; ARs^{low}, $n = 37$; PRs^{high}, $n = 18$; and PRs^{low}, $n = 59$) (** $P < 0.01$, Mann-Whitney tests). B, Correlation of *IL-10* mRNA copy number per million copies of β -actin mRNA with percentage of improvement in AASS (V17/V2) in patients from the active (upper panel) and placebo (lower panel) groups (** $P < 0.01$, ns = nonsignificant; Spearman test)

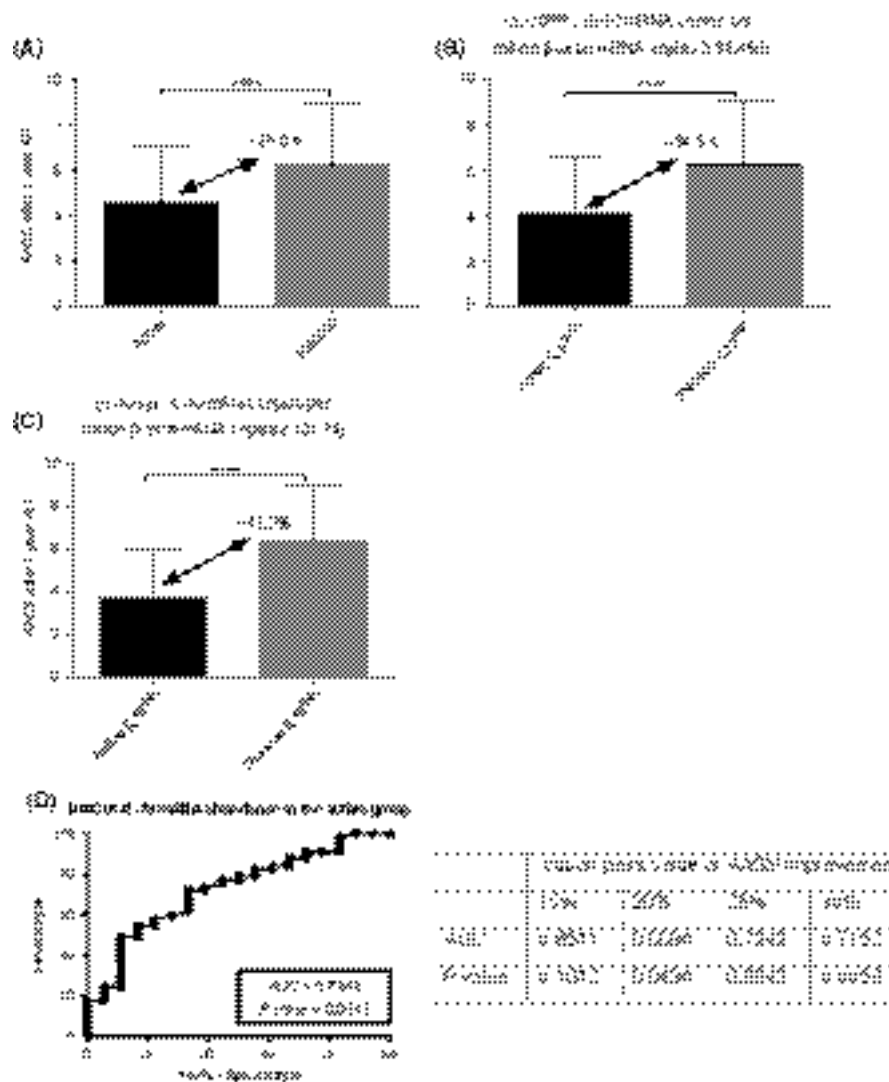


FIGURE 2 Patients with the highest levels of *IL-10* mRNA in whole blood cells exhibit higher improvement of clinical scores. A, Average Adjusted Symptom Scores (AASS; mean $\pm$ SD) are presented after 12 months of immunotherapy for 152 patients (75 receiving the active treatment and 77 receiving placebo) without using predictive biomarker criteria. B, AASS (mean $\pm$ SD) are presented after 12 months of immunotherapy for 114 patients exhibiting high levels of *IL-10* mRNA that is a copy number of *IL-10* mRNA per million copies of β -actin mRNA ≥ 94.455 (52 receiving the active treatment and 62 receiving placebo) ($***P < 0.0001$, Mann-Whitney tests). C, AASS (mean $\pm$ SD) are presented after 12 months of immunotherapy for 82 patients exhibiting high levels of *IL-10* mRNA that is a copy number of *IL-10* mRNA per million copies of β -actin mRNA ≥ 131.74 (38 receiving the active treatment and 44 receiving placebo) ($***P < 0.0001$, Mann-Whitney tests). D, Receiver operating characteristic (ROC) analysis of *IL-10* mRNA abundance in whole blood samples from patients of the active group with 25% AASS improvement as a cutoff to define responders versus non-responders (left panel). AUC and P-values with 15%, 20% 25%, and 30% of AASS improvement as a cutoff (right panel)

highlighting the tolerogenic role of monocytes, dendritic cells, and B cells in the course of AIT.^{3,8,9,12} Although we do not exclude T cells as a potential source of IL-10, since blood cells were not activated in our analyses, we postulate that the source of IL-10 rather relates to innate immune cells, that is likely monocytes and/or dendritic cells. A clinical implication of our finding would be to control *IL-10* mRNA levels before AIT in order to improve its efficacy.

Altogether, the present study, focused on the search of predictive biomarker of AIT efficacy, describes a new method to select patients eligible for AIT based on *IL-10* mRNA abundances. Confirmation that *IL-10* gene expression levels in whole blood represent a

candidate biomarker for predicting HDM AIT efficacy is currently ongoing in larger cohorts of patients receiving HDM AIT.

DISCLOSURE

Co-authors (CG, SL, VL, VBo, PM and LM) are or have been employees at Stallergenes Greer.

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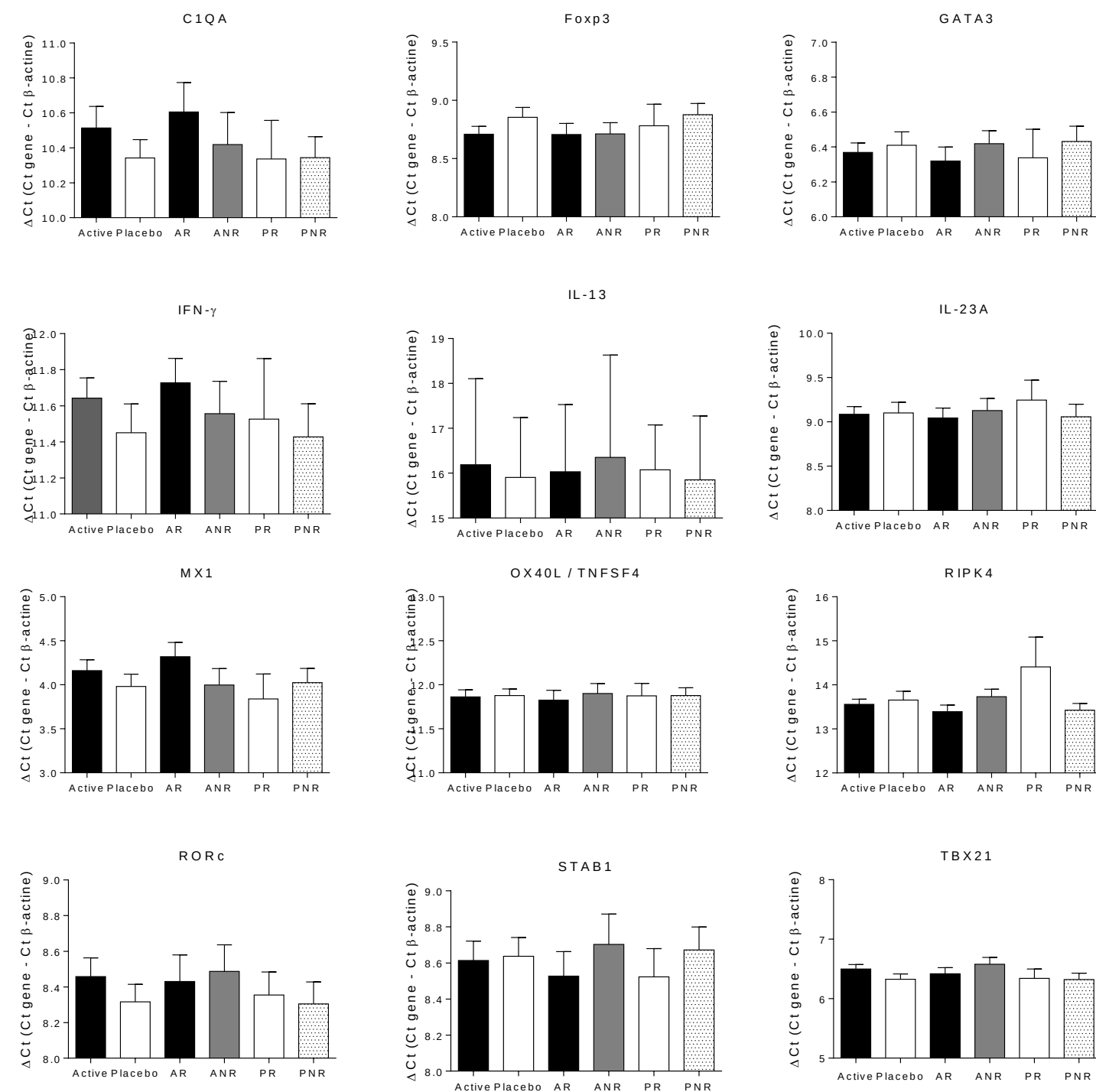
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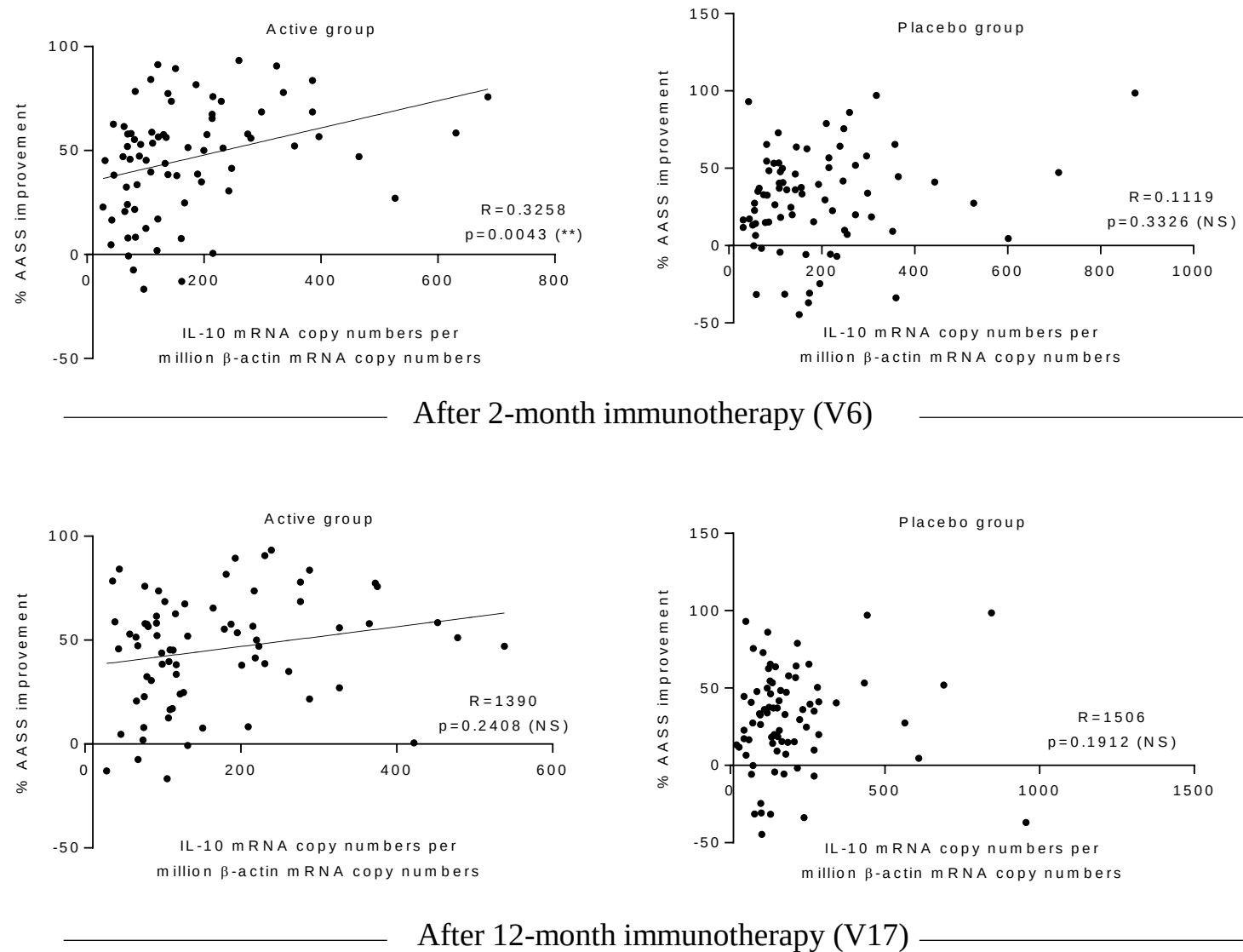
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SUPPORTING INFORMATION

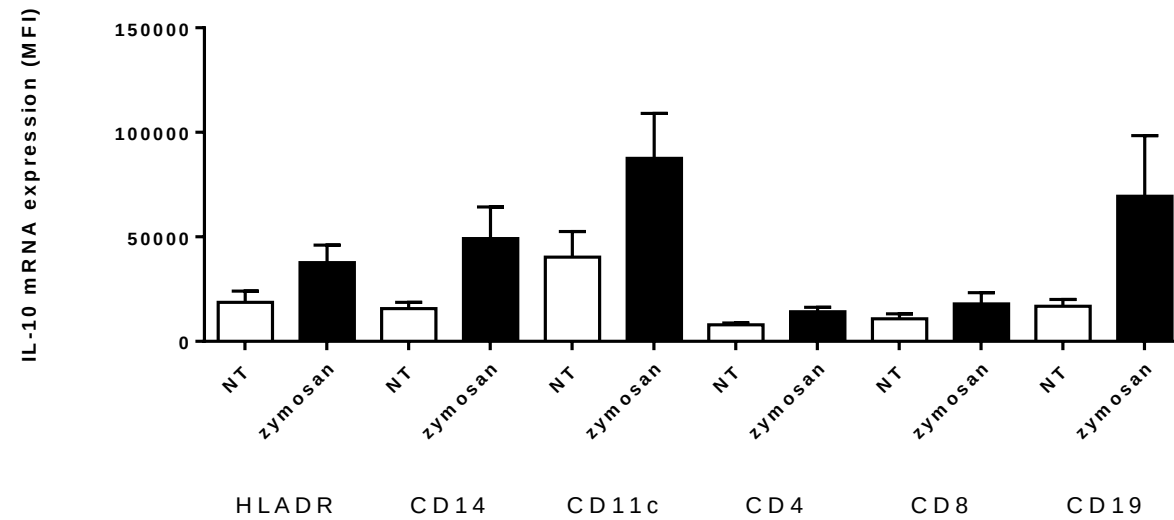
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Supplementary Figure 1: Gene expression (mean $\Delta\text{Ct} \pm \text{SEM}$) of C1QA, Foxp3, GATA3, IFN- γ , IL-13, IL23A, MX1, OX40L, RIPK4, RORc, STAB1 and TBX21 (T-bet) was assessed at baseline by qPCR in whole blood cells from patients of the active and placebo groups (AR, n = 38; AR, n = 37; PR, n = 18; and PNR, n = 59) (p = not significant, Mann-Whitney tests).



Supplementary Figure 2: IL-10 mRNA levels in whole blood after 2-month immunotherapy from HDM allergic patients correlate with AIT clinical efficacy. Spearman correlations at an individual patient level of IL-10 gene levels after 2-month (V6) and 12-month (V17) immunotherapy by plotting copy numbers of IL-10 mRNA per million copies of β -actin mRNA against percentage improvements in AASS (V17/V2) in patients from the active (*left panels*) and placebo (*right panels*) groups (** $p < 0.005$, ns = non-significant; Spearman test).



Supplementary Figure 3: Normalized MFI values for IL-10 expression following restimulation of whole blood cells by zymosan and evaluated in antigen presenting cells (HLA-DR⁺), monocytes (CD14⁺), dendritic cells (CD11c⁺), T (CD4⁺ and CD8⁺) and B (CD19⁺) lymphocytes.

Supplementary data Table SI:

Demographics and baseline characteristics of patient receiving immunotherapy or placebo

	Immunotherapy N = 75	Placebo N = 77
Age; mean years (SD)	33 (10.36)	33 (10.21)
Gender; male (%) : female (%)	38 (50.7%) : 37 (49.3%)	37 (48.1%) : 40 (51.9%)
AASS baseline; mean (SD)	8.6 (1.8)	9 (2)
D farinae IgE; mean Ua/mL (SD)	35.8 (68.4)	35.2 (59.2)
D pteronyssinus IgE; mean Ua/mL (SD)	35.1 (70.6)	33.3 (48)
D farinae IgG4; mean mgA/L (SD)	0.25 (0.21)	0.26 (0.21)
D pteronyssinus IgG4; mean mgA/L (SD)	0.31 (0.23)	0.33 (0.28)
Sensitization status with autumn allergies; n (%)	5 (6.7%)	9 (13%)

Results describing continuous variables are expressed as means with SDs. Results describing categorical variables are expressed as numbers of patients and percentages relative to numbers of immunotherapy or placebo-treated patients.

Demographics and baseline characteristics of patient receiving immunotherapy or placebo categorized into low and high responders

	Immunotherapy N = 75		Placebo N = 77	
	AR ^{high} N=37	AR ^{low} N=38	PR ^{high} N=19	PR ^{low} N=58
Age; mean years (SD)	32 (11)	34 (9.7)	40 (11.7)	31 (8.6)
Gender; male (%) : female (%)	20 (%) : 17 (%)	18 (%) : 20 (%)	5 (%) : 14 (%)	32 (%) : 26 (%)
AASS baseline; mean (SD)	8.6 (2)	8.6 (1.6)	9.3 (1.9)	8.9 (2)
D farinae IgE; mean Ua/mL (SD)	20.3 (21.1)	51.7 (93.1)	24.1 (21.3)	38.9 (67.3)
D pteronyssinus IgE; mean Ua/mL (SD)	21.8 (24.6)	48,8 (96.1)	24.6 (21.9)	36.3 (53.9)
D farinae IgG4; mean mgA/L (SD)	0.2 (0.13)	0.31 (0.26)	0.27 (0.19)	0.26 (0.22)
D pteronyssinus IgG4; mean mgA/L (SD)	0.26 (0.16)	0.37 (0.28)	0.32 (0.2)	0.33 (0.3)
Sensitization status with autumn allergies; n (%)	2 (5.4%)	3 (7.9%)	2 (10.5%)	7 (12.1%)

Results describing continuous variables are expressed as means with SDs. Results describing categorical variables are expressed as numbers of patients and percentages relative to numbers of immunotherapy or placebo-treated patients.

Supplementary Methods

Outcomes and clinical samples from the S-524101 house dust mite study

The randomized, double-blind, placebo-controlled, S-524101 study evaluated the safety and efficacy of a house dust mite tablet (300IR and 500IR, Stallergenes SA, Antony, France) over a 12-month course of sublingual allergen immunotherapy (AIT) {Okamoto, 2017 #1018}. Clinical responses were evaluated in each individual patient as percentages of improvement in Average Adjusted Symptom Score (AASS) which adjusts symptom scores for the use of rescue medication. In more detail, the Average Adjusted Symptom Score (AASS) was the average of the Adjusted Symptom Scores (ASSs) over the primary evaluation period. The ASS is based on ‘the highest observation carried forward basis’ approach and was calculated for each patient as follows. If a patient did not take rescue medication on the day of scoring or the day prior to that, the ASS was equal to the Rhinitis Total Symptom Score (RTSS). If a patient took rescue medication on the day of scoring, the ASS was equal to the day’s RTSS or the ASS of the previous day, whichever was higher. The ASS on the next day of rescue medication use was equal to the RTSS on the next day or the ASS from the prior day, whichever was higher. The RTSS (range: 0–15) was the sum of the four individual rhinitis symptom scores. The Total Rhinoconjunctivitis Symptom Score was the sum of the four nasal and two ocular individual symptom scores. A Combined Score (range: 0 to 2.875) was calculated as follows: $(RTSS/4 + \text{medication score})/2$.

For the ancillary study conducted in HDM allergic patients receiving daily either 300IR HDM (n = 75) or placebo (n = 77) tablets, the median percentage of AASS improvement in the active group after treatment was also used as a threshold to identify high clinical responders exhibiting a $\geq 51.18\%$ AASS improvement, from low-responders. Based on this threshold, 4 patient subgroups were defined, including high active responders (ARs^{high} , n = 38), low active responders (ARs^{low} , n = 37), high placebo responders (PRs^{high} , n = 18), and low placebo responders (PRs^{low} , n = 59). The expression of *IL-10* was assessed in whole blood cells from patients at baseline (Visit 2; V2) and after 2 (V6) and 12 months (V17) of AIT. Whole blood cells were collected by using PAXgene blood RNA tubes (Qiagen, Courtaboeuf, France), and kept frozen at -80°C until use. All biological analyses were performed on samples coded to the operators.

RNA isolation and quantitative real-time PCR analyses

Total RNAs were extracted with the nucleic acid purification kit ([PAXgene miRNA Kit, Qiagen](#)) by using a Qiacube robot (Qiagen) as per the manufacturer's instructions. cDNAs were synthesized using the SuperScript® VILO™ cDNA Synthesis kit (Applied Biosystems, Les Ulis, France) as per the manufacturer's instructions. mRNA expression was evaluated by quantitative PCR (qPCR) on a 7900HT Real-Time PCR system (Applied Biosystems) with predesigned TaqMan gene expression assays and reagents, according to the manufacturer's instructions. The expression of *IL-10* gene (*IL-10* – Hs00174066_m1) was assessed and compared with endogenous β -actin (Hs99999903_m1) as a control. The relative amounts of *IL-10* mRNAs corresponding to the target gene in each sample were calculated in comparison with the endogenous β -actin calibrator gene, using the following equation: $\Delta Ct = Ct_{IL-10} - Ct_{\beta\text{-actin}}$. The copy number of *IL-10* mRNA per million copies of β -actin mRNA was calculated using the formula $(2^{-\Delta Ct}) \times 10^6$.

PrimeFlow™ RNA Assay

Whole blood cells obtained from healthy donors at “Etablissement Français du Sang” (Rungis, France) were incubated in a 24-well plate in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 1% L-glutamine, 200 U/mL penicillin, and 200 μ g/mL streptomycin (all from Life Technology). Cells were stimulated or not by 100 μ g/mL Zymozan (InvivoGen, Toulouse, France) for 24 h at 37 °C and 5% CO₂. Thereafter, cells were harvested and labelled with antibodies : lin- (anti CD16, anti CD3, anti CD56-SB 702, anti CD20-BV 711), anti HLA DR-APC efluor 780, anti CD11c-PE, anti CD14-PE Cy7 for gating on antigen presenting cells; anti CD4-PE Cy7, anti CD8-APC Alexa Fluor 750 and anti CD19-PE for gating on B and T cells. Cells were washed in PBS and PrimeFlow™ assay was performed according to the manufacturer's instructions by using the human *IL-10* Type 1 probe (ref: VA1-10840, eBioscience, San Diego, CA) and RPL13A (ref : VA4-13187) as a control probe. Samples were analyzed with an Attune NxT (Thermo Fisher, Waltham, MA) apparatus, and data interpreted using the FlowJo software (TreesStar, Ashland, OR).

Receiver operating characteristic (ROC) analyses

Receiver operating characteristic (ROC) curves of *IL-10* mRNA abundance were performed with Prism6 software (GraphPad Software, Inc, La Jolla, CA) using 25% AASS improvement as an

60 optimal cut-off point. ROC curve represents a sensitivity/specificity pair by plotting the true
61 positive rate (Sensitivity) in function of the false positive rate (100-Specificity) for the cut-off
62 point. The area under the ROC curve (AUC) measures how well *IL-10* mRNA levels can
63 distinguish between AR^{low} and AR^{high}.