DOI: 10.1111/all.14692

Peripheral effector memory regulatory T cells are incremented and functionally enhanced in successful mite monomeric allergoid sublingual immunotherapy

To the Editor,

The beneficial effects of Allergen-Specific Immunotherapy (AIT) rely on the induction of allergen-specific Regulatory T cells (Tregs).[1] Tregs, a subpopulation of $CD4^+CD25^+$ T cells expressing the specific transcription factor Foxp3, are not functionally homogeneous and their detection is complex and uncertain due to FoxP3 intracellular localization. Furthermore, $FoxP3^+$ Tregs might become unstable and halt the production of their functional suppressive cytokines in inflammatory conditions.[2] In its place, the surface antigen CD127, whose expression inversely correlates with FoxP3, conveniently identifies Tregs as $CD4^+CD25^+CD127^{neg}$ cells,[3] so surmounting the problems of FoxP3 stability and intracellular detection. Tregs also constitutively express the inhibitory antigen CD39, enhanced in highly suppressive memory Tregs.[4] Furthermore, HLA-DR expression is a monitor of Treg differentiation status and identifies a functionally and greatly suppressive population.[1,5] Lack of CD45RA characterizes memory T cells enabled to survive for long periods, even in absence of specific antigen, showing increased activity upon re-exposure and able to induce apoptosis in target cells.[6] $CD4^+CD25^{high}CD39^+CD127^{neg}$ cells are subtyped as Resting ($CD45RA^+$/HLA-DR^{neg}: rTreg), Activated ($CD45RA^{neg}$HLA-DR^{neg}: aTreg) and Effector ($CD45RA^{neg}$HLA-$DR^{low/high}$: eTreg) Tregs.[6] This latter subtype includes terminally differentiated Tregs, the most highly suppressive[5] (Supplementary Figure S1). They are different from secreting or type III Tregs expressing CD127 that represent a short-lived terminally differentiated population.[5] In order verify possible correlations between specific subsets of Treg and the effectiveness of AIT, we applied this analytical approach to study Treg profile in adolescents suffering from mite-allergic rhinitis, pre and 12 months post Sublingual Immunotherapy (SLIT) with mite monomeric allergoid, an acid-resistant allergen known to elicit early T reg-activation.[7,8] The study was approved by the Ethical Committee of University "G. d'Annunzio", Chieti-Pescara.

Twenty patients diagnosed with mite-allergic persistent rhinitis with or without asthma were enrolled. Allergic rhinitis (AR) was graded according to ARIA guidelines in a) intermittent mild, b) intermittent moderate/severe, c) persistent mild, and d) persistent moderate/severe. At the enrollment, each patient marked in a 100 mm visual analogic scale (VAS) the level of its health status related to allergy with 0 the best status and 100 the worst.

All patients were treated by SLIT with mite monomeric allergoid (LAIS - Lofarma, Milan, Italy) at 1000 UA four times/week every other day, for 12 months. No adverse local and systemic reactions were detected. The effectiveness of SLIT was established comparing VAS, ARIA grading, and ACT questionnaire performed after 12 months of treatment with their basal values. Two blood samples were drawn pre/post-SLIT to be analyzed for Regulatory T cells. Clinical and demographic details of the studied population, analytical methods, statistical approach, and the outline of the study are detailed in the supplementary Tables S1 and S4.

Rhinitis scores VAS and ARIA significantly decreased after SLIT (Table 1), with the same statistical significance (Wilcoxon z −3.7236; $P = 0.0002$). Improvement was evidenced also in the subgroup of asthmatic patients (n = 7) since ACT scores significantly increased from the baseline value of 18 (16–19) up to 24 (20–25) after 12 months of treatment (the low number of patients does not allow application of efficient statistics).

Tregs were analyzed as frequency of total Treg cells and their three subsets, namely Resting (rTregs), Activated (aTregs), and Effector (eTregs), within the parental population of $CD4^+$ cells (supplementary Figure S2a and b). Total Tregs did not change significantly; rTreg significantly decreased (Wilcoxon z-3.6214, $P < 0.0003$), while the abundance of aTregs and eTregs significantly incremented (Wilcoxon z-2.9011, $P < 0.05$ and z-3.077, $P = 0.002$, respectively) (Table 1). A significant negative correlation has been observed between the decrease in rTreg and the increase in aTreg (Spearman's ρ-0.69391, $P < 0.02$) and increase in eTreg cells (Spearman's ρ-0.56845, $P < 0.02$) (Figure S3 in supplementary material).

HLA-DR resulted significantly up-regulated in all Tregs from 4.93 ± 3.1 to 6.92 ± 5.1 MFI (Wilcoxon z-4.2026, $P < 0.00001$). HLA-DR increased on aTregs from 3.4 ± 3.03 to 4.91 ± 3.2 MFI (Wilcoxon z-3.2479, $P = 0.001$) and on eTregs from 1.54 ± 0.66 to 2.0 ± 1.45 MFI (Wilcoxon z-2.9664, $P = 0.005$). CD39 was found differently expressed in the three subsets of Tregs at baseline, with Resting < Activated < Effector. After 12 months of SLIT, CD39 surface expression was found significantly increased in all Tregs from

TABLE 1 Clinical parameters and Treg frequency of patients pre- and post-SLIT

	Pre-SLIT	Post-SLIT	Wilcoxon signed rank test
ARIA score	3.7 (2–4)	1.85 (0–3)	z–3.7236; $P = 0.0002$
ACT score in asthmatics (n7)	18.2 (16–19)	23.14 (20–25)	low n. of patients
VAS	7.8 (3–10)	4.3 (1–7)	z–3.7236; $P = 0.0002$
Resting Tregs	61.1 ± 9	59.1 ± 10	z–3.6214, $P < 0.003$
Activated Tregs	5.8% ±4.4	8.8% ± 4.7	z–2.9011, $P < 0.05$
Effector Tregs	31.4% ±10.3	37.6% ±8.9	z–3.077, $P = 0.002$
HLA-DR expression (aTregs)	4.93 ± 3.1	6.92 ± 5.1	z–4.2026, $P < 0.00001$
HLA-DR expression (eTregs)	1.54 ± 0.66	2.0 ± 1.45	z–2.9664, $P = 0.005$
CD39 expression (all Tregs)	6.9 ± 4	8.02 ± 5	z–3.1049, $P = 0.001$

Note: Tregs values represent the frequency of the parental $CD4^+$ population. The data are presented as median values together with their range (minimum-maximum) or as %±SD.

6.9 ± 4 to 8.02 ± 5 MFI (Wilcoxon z-3.1049, $P = 0.001$) (HLA-DR and CD39 changes are reported in Table 1). We found some interesting correlations between laboratory data and clinical parameters. Changes in eTregs significantly correlated with both ARIA (Spearman's ρ=0.58728, $P = 0.013$) (Figure 1A) and VAS (Spearman's $\rho = 0.49172$, $P = 0.044$) (Figure 1B) variations after SLIT. While a significant negative correlation was found between rTregs and clinical parameter changes after treatment (Spearman's ρ-0.48482, $P = 0.0491$). Changes in HLA-DR expression on all Treg cells significantly correlated with variation in VAS pre-/post-SLIT (Spearman's $\rho = 0.54104$, $P = 0.01376$) (Figure 1C). These Tregs, detected in mite allergoid-treated patients and associated with reduction of mite-related AR symptoms, are expected to be antigen-specific. A specific dedicated study will be performed to evidence the allergen specificity of such Treg cells. No other correlations were found except for the lowest increase (<8%) of memory Tregs ($CD45RA^{neg}$) detected in patients with the lowest levels of mite-specific serum IgE (not shown).

To our knowledge, this is the first report on successful SLIT being associated with re-patterning of the differentiation status of Tregs, with high rates of the most suppressive Treg subtypes: activated and effector, characterized by higher expression of HLA-DR and CD39 both playing inhibitory function in Tregs. Moreover, effective SLIT seems to be associated with the generation of cells lacking CD45RA that characterizes memory T cells with increased activity upon re-exposure to the antigen. Our results suggest that SLIT also induced empowerment of Treg inhibitory function, likely compensating the under-representation of Tregs observed in allergic patients.[9]

Results of our study seem to disagree with those obtained based on FoxP3 detection, showing an increase in total Tregs.[10] Actually, our Treg panel differs from those used by the other authors; therefore, it is not correct to directly compared them. In particular, $FoxP3^+$ Treg includes both the $CD127^{neg}$ subset (the focus of this study) and the $CD127^{pos}$ subset, short-lived cells not analyzed in our study. We demonstrated that focusing on $CD127^{neg}$ and memory ($CD45RA^{neg}$) cells, it is possible to show that the increase in Tregs is restricted to specific subpopulations. In particular, effector Tregs were incremented after 1-year SLIT suggesting that their regulatory activity (mostly exerted by cell-cell contact) persists and is maintained when the Th2 toward Th1 shift and the increase in IgG occur. On the other hand, our findings are in agreement with those described by Boonpiyathad[11] showing that increased number of Der p 1-specific $CD127^{neg}$ $FOXP3^+Helios^+$ T regulatory cell responses correlated with improved allergic symptoms in the long term; parallelly, the inactive Treg subsets (ie, dysfunctional/resting Treg) decreased in both studies. However, the two $CD127^{neg}$ populations are somehow functionally distinct: In fact, those Tregs are $IL\text{-}10^+$ while our Tregs presumptively perform mostly by cell-cell contact.

Next step of our study will be to evidence the allergen specificity of the AIT induced aTregs and eTregs and if the relationship between effective SLIT and Treg re-patterning is present in the first months of SLIT, with a view to profiling Tregs for the early identification of SLIT responders/non-responders by mean of a straightforward and non-invasive blood test.

CONFLICT OF INTEREST

Dr. Petrarca reports grants from Lofarma, outside the submitted work. Dr. Lanuti has nothing to disclose. Dr. Petrosino has nothing to disclose. Dr. Di Pillo has nothing to disclose. Dr Mistrello has nothing to discloseDr. Compalati reports other from Lofarma, outside the submitted work. Dr. Marchisio has nothing to disclose. Dr. Pierdomenico has nothing to disclose. Dr. Otsuki has nothing to disclose. Dr. Paganelli has nothing to disclose. Dr. Di Gioacchino reports grants and personal fees from GSK, grants from Novartis, grants, personal fees and non-financial support from Lofarma, personal fees from Menarini, grants from Sanofi-Genzyme outside the submitted work.

Claudia Petrarca[1,2]
Paola Lanuti[1,2]
Marianna Immacolata Petrosino[3]

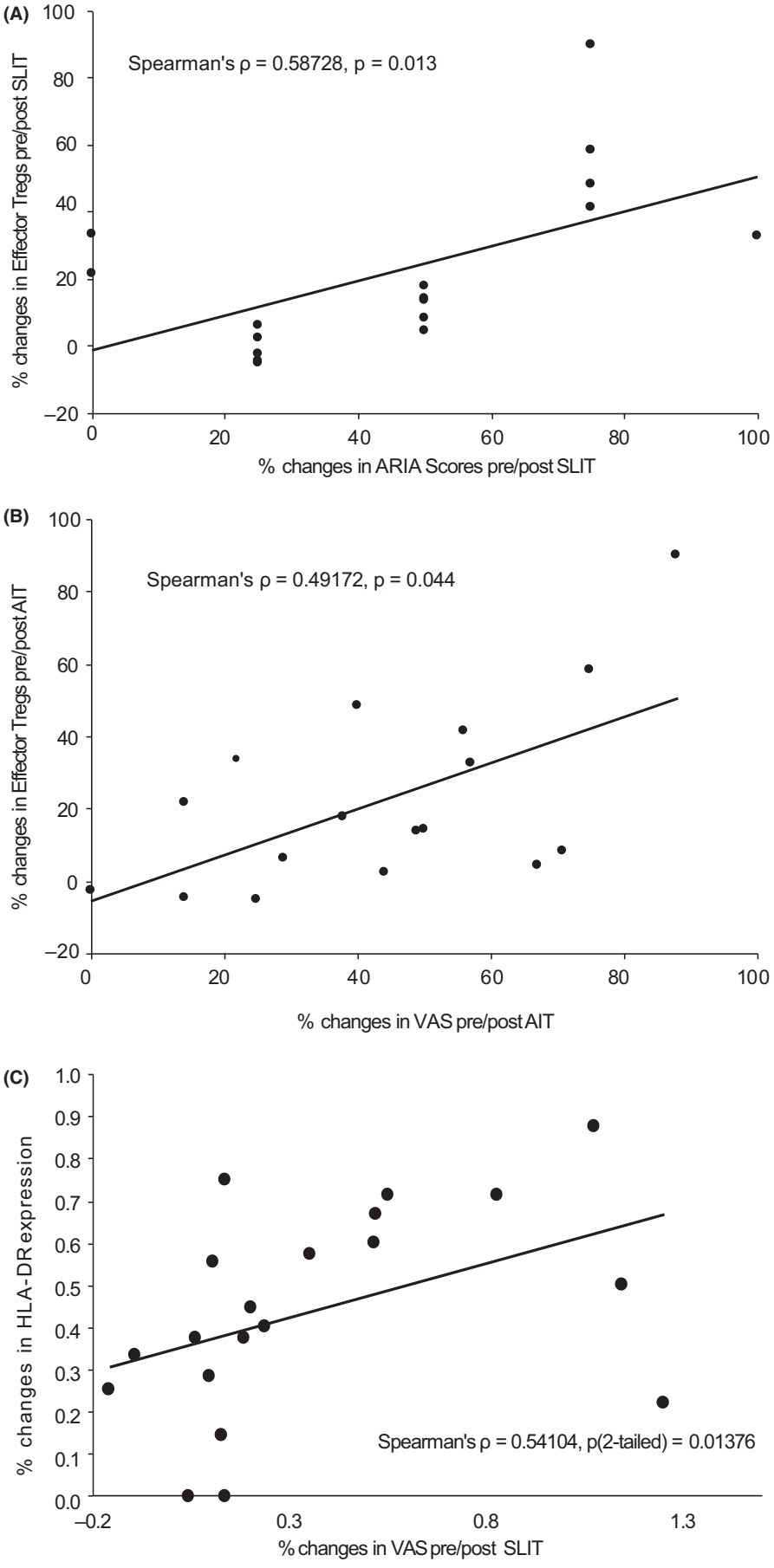

FIGURE 1 Correlation between clinical parameters and regulatory T cell changes pre/post-SLIT. Changes in Effector Regulatory T cells after mite allergoid SLIT significantly correlates with changes in Aria classification for Rhinitis (A) and VAS (B). Changes in HLA-DR expression on Regulatory T cells after mite allergoid SLIT significantly correlates with changes in VAS (C). Statistical significance: Spearman's Rank Correlation

Sabrina Di Pillo[3]
Gianni Mistrello[4]
Enrico Compalati[4]
Takemi Otzuki[5]
Marco Marchisio[1,2]
Laura Pierdomenico[1,2]
Roberto Paganelli[1,2]
Mario Di Gioacchino[1,2,6]

[1]*Department of Medicine and Science of Aging, G. d'Annunzio University, Chieti, Italy*
[2]*Center for Advanced Science and Technology (CAST), G. d'Annunzio University, Chieti, Italy*
[3]*Unit of Pediatrics, University Hospital, Chieti, Italy*
[4]*Lofarma Allergeni, SpA, Milan, Italy*
[5]*Department of Hygiene, Kawasaki Medical School, Okayama, Japan*
[6]*Foundation for Clinical Immunotherapy and Advanced Biological Treatments, Pescara, Italy*

Correspondence
Claudia Petrarca, Occupational Medicine, Unit of Nanoimmunotoxicology, Allergy and & Occupational Biorepository, DMSI-CAST, University "G. d'Annunzio" Chieti-Pescara, Via Luigi Polacchi, 11, 66013 Chieti - Italy
Email: claudia.petrarca@unich.it

ORCID
Claudia Petrarca https://orcid.org/0000-0002-6910-0160

REFERENCES

1. Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens. *World Allergy Organ J*. 2015;8(1):1-12.

DOI: 10.1111/all.14712

Tolerance of an immunotherapy switch between two aqueous hymenoptera venoms

To the Editor,

Immunotherapy with Hymenoptera venom is an effective treatment for patients with Hymenoptera venom allergy. At the end of 2019, the production of the aqueous, partly purified honey bee (HBV) and wasp venom (WV) Pharmalgen® (ALK-Abello, Horsholm, Denmark), was discontinued. The switch from the aqueous HBV and WV Venomil® (Bencard Allergie GmbH, Munich, Germany) to the depot preparation Alutard® (ALK-Abello Horsholm, Denmark) is mostly well tolerated.[1,2] However, a change to an aluminium-precipitated depot preparation is not suitable for all patients.[3] High-dose venom immunotherapy has not been evaluated specifically for aluminium toxicity.[4] The amount of aluminium is actually restricted to 1.25 mg per human dose in Europe.[5] Treatments with doses over 100 µg (e.g. in beekeepers or therapy-refractory cases) with Alutard® are off-label (this corresponds to a dose of more than 100,000 SQ-U Alutard® with 1.13 mg of the adjuvant aluminium according to