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

Claudia Petrarca[1], Paola Lanuti[1], Marianna Petrosino[2], Sabrina Di Pillo[2], Gianni Mistrello[3], Enrico Compalati[3], Takemi Otzuki[4], Marco Marchisio[1], Laura Pierdomenico[1], Roberto Paganelli[1], and Mario Di Gioacchino[1]

[1]University Gabriele d'Annunzio of Chieti and Pescara Department of Medicine and Aging Science
[2]University Hospital SS Annunziata
[3]Lofarma SpA
[4]Kawasaki Medical School

July 24, 2020

To the Editor.

The beneficial effects of Allergen Specific Immunotherapy (AIT) relies 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) (1). In its place, the surface antigen CD127, whose expression inversely correlates with FoxP3, conveniently identifies Tregs as $CD4^+CD25^+CD127^{neg}$cells (3) (2), 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) (3). Furthermore, HLA-DR expression is a monitor of Treg differentiation status and identifies a functionally and greatly suppressive population (1,5) (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) (3). $CD4^+CD25^{high}CD39^+CD127^{neg}$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) (6). This latter subtype includes terminally differentiated Tregs, the most highly suppressive (5) (7) (Supplementary Figure 1). They are different from secreting or type III Tregs expressing CD127 that represent a short-lived terminally differentiated population (5,6,8) . 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. All patients and parents signed a written informed consent after having been informed about the procedures of the study.

Twenty patients diagnosed with mite-allergic persistent rhinitis with or without asthma were enrolled. Allergic rhinitis (AR) was graded according to ARIA guidelines in 1) intermittent mild, 2) intermittent moderate/severe, 3) persistent mild and 4) 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 *online supplementary material* .

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. 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 3 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 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 ϱ=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 ϱ=0.54104, p= 0.01376) (Figure 1C). 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) (9). In AR children, there are evidences that Tregs have defect in suppressing IgE production and that they can be incremented by mite SLIT.

Next step of our study will be to evidence if such 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.

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:10.1186/s40413-015-0063-2.

2. Shevyrev D, Tereshchenko V. Treg Heterogeneity, Function, and Homeostasis. Front. Immunol. 2020;10:3100
3. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006;203:1701–1711
4. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood 2007;110:1225–1232
5. Cuadrado E, van den Biggelaar M, de Kivit S, et al. Proteomic Analyses of Human Regulatory T Cells Reveal Adaptations in Signaling Pathways that Protect Cellular Identity. Immunity. 2018;48:1046-59.e6. doi:10.1016/j.immuni.2018.04.008
6. Rosenblum MD, Way SS, Abbas AK. Regulatory T cell memory. *Nat Rev Immunol* 2016;**16** :90–101
7. Di Gioacchino M, Perrone A, Petrarca C, Di Claudio F, Mistrello G, Falagiani P et al. Early cytokine modulation after the rapid induction phase of sublingual immunotherapy with mite monomeric allergoids. Int J Immunopathol Pharmacol. 2008;21(4):969–76. doi:10.1177/039463200802100421.
8. Petrarca C, Lazzarin F, Pannellini T, Iezzi M, Braga M, Mistrello G et al. Monomeric allergoid intragastric administration induces local and systemic tolerogenic response involving IL-10-producing CD4(+)CD25(+) Regulatory T cells in mice. Int J Immunopathol Pharmacol. 2010;23(4):1021–31. doi:10.1177/039463201002300407.
9. Saad K, Zahran AM, Elsayh KI, Abdelmoghny A, Aboul-Khair MD. Variation of Regulatory T Lymphocytes in the Peripheral Blood of Children with Allergic Rhinitis. Arch Immunol Ther Exp. 2018;66(4):307–313. doi:10.1007/s00005-017-0498-y.

Authors

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

Affiliations

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

Conflict of interest:

Dr Gianni Mistrello and Enrico Compalati are employees of Lofarma.

Prof. Mario 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 SanofiGenzyme outside the submitted work.

Prof. Claudia Petrarca reports grants from Lofarma

The other authors have no conflict to declare

Corresponding Author

Claudia Petrarca

Associate Professor of Occupational Medicine

Unit of Nanoimmunotoxicology, Allergy and & Occupational Biorepository

DMSI-CAST

University "G. d'Annunzio" Chieti-Pescara

Via Luigi Polacchi, 11

66013 Chieti - Italy

phone +39.871.541.290/1

Mail:*claudia.petrarca@unich.it*

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

	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 (aTregs)	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

Figure legend

Figure

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.