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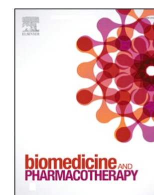

The effect of anti-IL5 monoclonal antibodies on regulatory and effector T cells in severe eosinophilic asthma

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ABSTRACT

Introduction: Biological treatments have redesigned the clinical management of severe eosinophilic asthmatic (SA) patients. Despite emerging evidence supporting the role of natural Killer (NK), and T regulatory cells (Treg) in the pathogenesis of asthma, no data is available on the effects of anti-IL5/IL5R therapies on these cell subsets.
Methods: We prospectively enrolled fourteen SA patients treated with benralizumab ($n = 7$) or mepolizumab ($n = 7$) and compared them with healthy controls (HC) ($n = 11$) and mild to moderate asthmatic (MM) patients ($n = 9$). Clinical parameters were collected at baseline (T0) and during follow-up. Cellular analysis, including the analysis of T/NK cell subsets, was determined through multicolor flow cytometry.
Results: At T0, SA patients showed higher percentages of CD4 TEM (33.3 ± 17.9 HC, 42.6 ± 16.6 MM and 66.1 ± 19.7 in SA; $p < 0.0001$) than HC and MM patients. With different timing, the two drugs induce a reduction of CD4 TEM (76 ± 19 T0; 43 ± 14 T1; 45 ± 23 T6; 62 ± 18 at T24; $p < 0.0001$ for mepolizumab and 55 ± 21 T0; 55 ± 22 T1; 43 ± 14 T6; 27 ± 12 at T24; $p < 0.0001$ for benralizumab) and an increase of Treg cells (1.2 ± 1.3 T0; 5.1 ± 2.5 T1; 6.3 ± 3.4 T6; 8.4 ± 4.6 at T24; $p < 0.0001$ for mepolizumab and 3.4 ± 1.7 T0; 1.9 ± 0.8 T1; 1.9 ± 1 T6; 5.1 ± 2.4 at T24; $p < 0.0001$ for benralizumab). The change of $CD56^{dim}$ $PD\text{-}1^{+}$ significantly correlated with FEV1% ($r = -0.32$; $p < 0.01$), while Treg expressing PD-1 correlates with the use of oral steroids ($r = 0.36$ $p = 0.0008$) and ACT score ($r = 0.36$ $p = 0.0008$) $p < 0.001$)
Conclusions: Beyond the clinical improvement, anti-IL-5 treatment induces a rebalancing of Treg and T effector cells in patients with SA

1. Introduction

Severe asthma (SA) is defined as asthma that is only controlled by inhaled maximally optimized high-dose corticosteroids (ICS) plus a second controller, or which is not 'controlled' even by this therapy [1]. A better understanding of the immunological processes behind airway inflammation in SA has led to the development of biological treatments, mainly for the type 2 inflammatory endotype. Two drugs target the IL-5 axis: mepolizumab, a humanized monoclonal antibody that binds circulating interleukin-5 (IL-5), and benralizumab, a humanized monoclonal antibody against IL-5 receptor α. Both drugs effectively improve the control of severe asthma by inducing fast, nearly complete depletion of eosinophils [2-4].

Airway inflammation is mediated by T/B cells, eosinophils, and activated complement fragments, which play a critical role in tissue injury and remodeling [5]. Emerging evidence shows the complexity of asthma development, especially the underlying interactions between innate and adaptive immune mechanisms [6]. Regarding T cells, T central memory (TCM) and T effector memory (TEM) cells are key players in severe asthma inflammation. Stimulated TCM and TEM

Abbreviations: SA, severe eosinophilic asthma; IL, interleukin; NK, Natural Killer; Treg, regulatory T cell; HC, healthy controls; MM, mild to moderate asthmatic; ICs, immune checkpoints; PD-1, programmed cell death; TIGIT, T cell immunoreceptor with Ig and ITIM domains; LAG-3, Lymphocyte-activation gene 3; CTLA4, Cytotoxic T-Lymphocyte Antigen 4; TCM, T central memory; TEM, T effector memory; TEMRA, T effector memory expressing CD45RA.

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produce effector inflammatory cytokines such as IL-13, IL-4, and IL-5. While TEM has rapid effector function, "terminally differentiated" effector memory (TEMRA) cells and immature T elements, such as T naïve, do not play a significant role in the cytokine release that contributes to airway inflammation. These reactions are normally managed by regulatory T cells (Treg), which maintain airway tolerance [7]. Tregs are the main negative regulatory cells of the immune response [8]. They are characterized by the secretion of regulatory cytokines, IL-10 and TGF-β, to suppress immune responses and trigger inducible Treg expansion [9]. In severe asthma patients, decreased levels of Treg cells sustain the concept of immune effector and regulatory imbalance [10]. The exhausted phenotype of Treg cells has also been observed in severe asthma patients by analysis of immune checkpoints (ICs) on the surface of Treg cells, first of all, PD-1 [11,12]. In T-cell biology, 'exhaustion' is recognized as a dysfunctional T-cell state. The term 'exhaustion' is mainly used for effector T cells have reduced capacity to secrete cytokines and increased expression of inhibitory receptors but was recently also described for Treg cells [13].

Although some of these immunological aspects have been analyzed in relation to SA, no data is yet available on their behavior after anti-IL-5 target therapies. The aim of our study was to analyze T cell maturation, evaluating ICs expression and regulatory T cell properties in these patients before and after 24 months of biological therapy with benralizumab and mepolizumab. We then correlated immunological data with clinical improvement induced by the drugs in order to link immunological changes with the treatments. Our results offer insights into immunological changes induced by these new monoclonal antibody target therapies.

2. Materials and methods

2.1. Study design and cohort

We enrolled 34 subjects in our study: 23 had asthma and 11 were healthy controls (HC). Nine patients had mild-to moderate (MM) and 14 had severe eosinophilic asthma (SA). The latter were treated with biologic therapies: seven with mepolizumab (median age 60 ± 10.13; 85 % males) and seven with benralizumab (median age 50.57 ± 17.42; 71 % males). The SA patients, recruited by prospective enrollment from January 2019 to August 2022, were monitored at the Respiratory Disease Unit, Siena University Hospital. Diagnosis of SA was based on Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) guidelines and all the patients included had peripheral eosinophilia (eosinophil count > 300 cell/mm^3), detected no more than 6 months prior to the start of treatment. Clinical control was poor in all cases as shown by at least two moderate exacerbations in the year preceding the beginning of biological therapy.

The benralizumab-treated group was given 30 mg every 4 weeks for the first three injections and every 8 weeks for subsequent injections, the approved dose for eosinophilic asthma, while the mepolizumab group was given 100 mg subcutaneously every 4 weeks, the approved dose for eosinophilic asthma.

We used the Asthma Control Test (ACT) and Asthma Control Questionnaire (ACQ-6) to evaluate the degree of control at baseline (T0) and after 1 month and 6 and 24 months of treatment (T1, T6, T24). The scores were entered in a database with exacerbation rate, use of ICS or oral corticosteroids (OCS) and lung function tests. Immunological analysis of serum was also recorded throughout the observation period.

The healthy subjects enrolled as control group were normal to a physical examination; they had no history of asthma or allergy and were not on any type of medication. They were monitored for at least 24 months after inclusion in the study and did not develop any disease, nor did they show any changes in blood count or develop infections.

2.2. Lung function tests

The following lung function parameters were recorded according to ATS/ERS standards using a Jaeger body plethysmograph with corrections for temperature and barometric pressure: forced vital capacity (FVC), forced expiratory volume in the first second (FEV1) and FEV1/FVC ratio.

2.3. PBMC collection and handling of cells

Peripheral blood mononuclear cells were separated in the laboratory of the Respiratory Disease Unit, Siena University Hospital (Italy) in the period January 2019 to December 2022. Peripheral blood samples were drawn into a tube containing EDTA anticoagulant (BD Vacutainer® EDTA tubes, BD Biosciences, CA, USA) and processed within 8 h. PBMCs were then separated by gradient centrifuging (Ficoll Histopaque®-1077, Sigma-Aldrich), washed twice, resuspended in 80 % RPMI 1640, 10 % FBS and 10 % dimethyl sulfoxide (DMSO) at 2×10^6 cells per vial, and stored in liquid nitrogen until analysis.

2.4. Flow cytometric analysis

For multiparametric flow cytometric analysis, a standard staining protocol for extracellular markers was used. Briefly, cells were washed with wash buffer ($HBSS^{-/-}$ with 2 % FBS) and incubated with the mAb mix for 30 min in the dark at room temperature. Samples were acquired using a BD FACS Lyrics (BD Biosciences) flow cytometry system. The optimal concentration of all mAbs used in the study was defined by titration experiments. According to the guidelines for accurate multicolor flow cytometry, fluorescence minus one (FMO) controls were used.

T cell subsets were detected on the basis of CD62L and CD45RA expression: T central memory cells (TCM) (CD62L+CD45RA-), effector memory T cells (T_{EM}) (CD62L-CD45RA-), naive T cells (Tn) (CD62L+CD45RA+) and T effector memory RA cells (T_{EMRA}) (CD62L-CD45RA+). The first gate was set to discriminate singlets (FSC-H vs FSC-A), followed by lymphocytes based on FSC-A vs SSC-A.

For exhausted T cells, LAG-3, TIGIT, CTLA4 and PD-1 were detected on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells and NK cells.

For detecting T regulatory cells, a cocktail containing anti-human CD4 FITC (clone SK3), anti-human CD25 PE-Cy™7 (clone 2A3) and anti-human CD127Alexa Fluor® 647 (clone hIL-7R-M21) was used and the cells were defined as $CD4^+CD25^+CD127^{low}$. PD-1PE moAb was added to the Treg cocktail to detect exhausted T regulatory cells.

2.5. Statistical analysis

The results were expressed as means and standard deviations (M ± SD) or medians and quartiles (25th and 75th percentiles) for continuous variables, as appropriate. The Shapiro-Wilk test was used to test for the normal distribution of the data. The Chi-squared test was used for categorical variables analysis.One-way ANOVA non-parametric test (Kruskal–Wallis test) and the Dunn test or Friedman test were used for multiple comparisons. For paired variables, the Wilcoxon test was performed during the follow-up of therapy (T0, T1, T6, and T24). For correlation analysis, the Spearman test was applied. A p-value < 0.05 was considered statistically significant.

Unsupervised hierarchical clustering analysis with the K means algorithm was applied to the control group and severe asthmatic patients in order to distinguish and cluster the behavior of different cell markers. The distance measure was Euclidean and the clustering method was ward.D2. The plots were dendrograms, heatmaps and cluster plots.

Principal component analysis (PCA) was also employed to reduce the dimensions of the data hyperspace and to cluster samples on the basis of cell subsets. Two different PCA multivariate analysis models were also built among controls, MM and SA patients: the first comprised T cell maturation phenotypes including TEM, TEMRA, TCM and T naïve

(related to CD4 and CD8), and the second included expression of immune checkpoints in CD4, CD8 and NK cell subsets.

In order to evaluate rebalancing of T cells, PCA analysis was used during the follow-up of patients, comparing the results obtained with the two therapies (mepolizumab and benralizumab) with the control group. In particular, for PCA we used the percentages of T cell subsets (CD4 and CD8), including TEM, TEMRA, TCM and T naïve. PCA was performed according to Kassambara and Mundt (2020) (Factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R Package Version 1.0.7. https://CRAN.R-project.org/package=factoextra) with the unsupervised K means clustering approach. The analysis was performed using all cell subsets revealed during follow-up, including T-cell maturation and exhaustion markers. Statistical analysis and graphic representation of the data were performed by Jamovi free software (Jamovi 1.6.23).

3. Results

3.1. Baseline clinical characteristics of SA cohort showed no differences between benralizumab- and mepolizumab-treated groups

No differences in the distribution of sex, mean age or smoking habits emerged between the control, MM and SA groups (suppl. tab.1). At baseline, the mepolizumab and benralizumab-treated groups did not show any significant differences in clinical scores, respiratory functional data, eosinophil count, ICS and OCS dosage, or co-existence of comorbidities, such as allergic rhinitis and nasal polyposis (suppl.tab.2).

3.2. T effector cells were elevated in the peripheral blood of SA patients before treatment

In order to evaluate the immunological state of SA before treatment with anti-IL-5 target therapies, we analyzed the function, maturation, and exhaustion state of T and NK cell compartments and compared them with HC and MM patients' samplings. As expected, the first results showed a misbalance of T cells between HC and MM and the group of SA patients. SA patients showed higher percentages of effector T cells including TEM and TCM derived from CD4- and CD8-positive T cells (suppl. Tab.3). An opposite behavior was reported for terminally differentiated T cells (TEMRA) of CD4- and CD8-positive T cells, which were significantly depleted in the SA group with respect to controls and MM patients (Fig. 1a) (suppl. tab. 3). Naive T cells of both CD4- and CD8-positive T cells showed a decreased percentage in SA patients compared with the other two groups (HC and MM), although without reaching statistical significance (suppl. Tab. 3). When PCA cluster analysis was applied, SA patients were clearly separated from the other two groups (yellow ring). Although MM (blue ring) had asthma, they clustered close to HC (grey ring) and were located on the opposite side of the graph in respect to SA (Fig. 1b).

3.3. LAG-3 and TIGIT overexpression on CD4, CD8 and NK cells was a feature of SA

Markers of cell exhaustion, including PD-1, CTLA4, LAG-3 and TIGIT, were detected in CD4 and CD8 T cells and in the NK cell compartment. The latter was stratified on the basis of CD56 expression by CD56^{dim} mature NK phenotype or CD56^{br}immature NK phenotype. T cell and CD56^{dim} NK from SA patients showed overexpression of LAG-3 and TIGIT, while no differences were reported for PD-1. CTLA-4 showed increased expression on CD4, CD8, and NK cell subsets of the MM group. Unsupervised hierarchical clustering for heatmaps showed clear clustering of SA which proved to be characterized by high expression of TCM, TEM, LAG-3 and TIGIT (Fig. 1c). When the unsupervised machine learning algorithm, K-means clustering, was applied to our cell subsets, the best number of clusters was two: the first cluster composed of 80 % MM and HC and the second preferentially of 76 % SA group (Fig. 1d). This demonstrated that cell exhaustion and maturation were radically different in SA patients with respect to HC and MM.

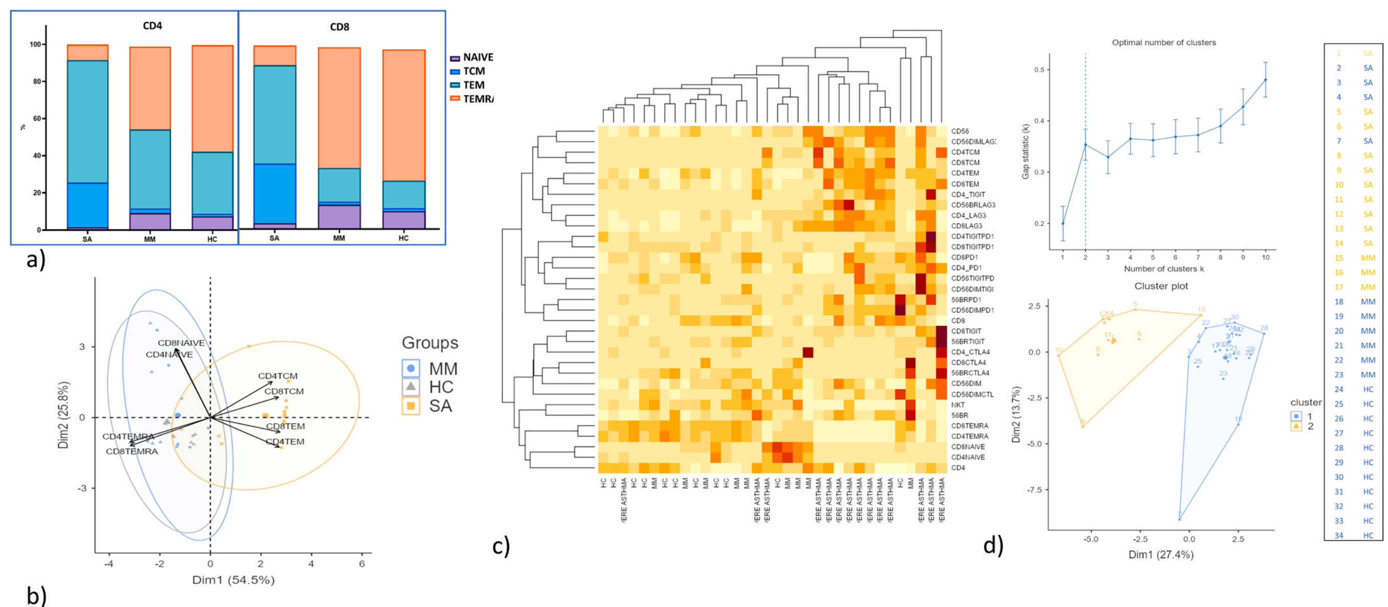

Fig. 1. a) Histograms showing percentages of T cell subsets (TCM, TEM, TEMRA and T naïve) of CD4- and CD8-positive cells in the three groups: healthy controls (controls; n = 11), mild-to-moderate patients (MM n = 9) and severe asthma patients (SA; n = 14). b) Supervised PCA analysis of controls, MM and SA on the subset variables TCM, TEM, TEMRA and T naïve of CD4- and CD8-positive cells. Unit Variance Scaling was applied to the rows to calculate the principal components. The X and Y axes show principal components 1 (PC1) and 2 (PC2). Prediction ellipses include 0.95 % of the data points. c) Heatmap depicting unsupervised hierarchical clustering in rows and columns. Hierarchical clustering is based on the Spearman rank correlation. d) The unsupervised machine learning algorithm, K-means clustering, was also applied to our cell subsets, including T cell maturation subsets, and expression of LAG-3. PD-1, TIGIT and CTLA-4 in CD4, CD8, CD56^{dim} and CD56^{br}.

3.4. Patients treated with mepolizumab and benralizumab showed clinical and functional improvement

Clinical and functional data collected during therapy at pre-established times (1 month and 6, and 24 months from the beginning of therapy) showed that mepolizumab- and benralizumab-treated SA patients improved in functional data and clinical control of asthma, namely fewer exacerbations, improved clinical scores and reduced use of oral corticosteroids (Table 1).

Mepolizumab and benralizumab treatment significantly improved the clinical control of asthma as assessed by ACT, ACQ and reduction of OCS dosage (Table 1).

Although from clinical point of view these patients resulted very similar, many differences emerged from immunological data at baseline. First of all, in respect with benralizumab, mepolizumab-treated patients showed decreased percentages of Treg cells, together with an increase expression of PD-1 on Treg cells (suppl. tab. 4).

Moreover, in mepolizumab subgroup, we also observed higher percentages of LAG-3 and TIGIT on CD4 cells, as well as on NK cells and a higher expression of PD-1 on CD4+ cells and CD56^{dim}TIGIT+ (suppl. tab. 4). No differences were reported for T cell subsets, including TEM, TCM Naive and TEMRA between mepolizumab and benralizumab-treated patients (suppl.tab.4).

3.5. Re-balancing of effector T cell subsets after benralizumab and mepolizumab treatment

We clustered SA T0 and during benralizumab and mepolizumab therapy on the basis of T cell subsets. Based on the flow cytometry data of T cell subsets (TEM, TEMRA, TCM and T naïve for CD4 and CD8), we performed PCA of controls and the SA group, stratified by mepolizumab and benralizumab treatment. The SA group results were considered at T1, T6, and T24. For benralizumab, a rebalancing emerged at T24; indeed, the PCA plot shows that samples clustered at T0, T1 and T6 (Fig. 2a) on the basis of expression of memory T cell subsets (TCM and TEM), while samples at T24 clustered close to HC on the opposite side of the graph, on the basis of expression of T naïve and TEMRA. The X and Y axes show that principal components 1 (PC1) and 2 (PC2) explained 49.9 % and 25.9 % of the total variance, respectively.

For mepolizumab-treated patients, the clustering was less clear, with controls partially overlapping at T24. The X and Y axes show that principal components 1 (PC1) and 2 (PC2) explained 54.4 % and 27.6 % of the total variance, respectively. These results were further confirmed by comparison (Fig. 2a). Interestingly, mepolizumab-treated patients already showed an increase in TEMRA percentages of CD4 and CD8 at T1, while with benralizumab this increase appeared at T24. A similar trend was evident for TCM, which already showed a decline at T1, whereas with benralizumab a decreasing trend was evident at T24 (Fig. 2b–c).

3.6. A rebalancing of Treg cells emerged after 24 months of anti-IL-5 therapy

From a regulatory point of view, Treg cell percentages increased at T24 for both treatments, however with a different timing: in the case of mepolizumab, a significant increase is already reached starting from T6 and it is maintained at T24. While for benralizumab, the statistical significance is reached at T24 (Fig. 3a–b). Concerning the degree of exhaustion of these cells, assessed through the expression of PD-1 on Treg surface, it was clear that these cells tend to be activated during the administration of drugs: in fact the expression of PD-1 progressively decreases during the follow-up of these patients, reaching the statistical significance at T24 (Fig. 3a–b). Interestingly, applying unsupervised statistical test of K means clustering method and using as a variables subsets of T cells, the expression of ICs (including TIGIT, LAG-3, CTLA-4 and PD-1) and Treg cell percentages, including Treg expressing PD-1, the better number of cluster correspond to four: as reported in Fig. 3c mepolizumab and benralizumab subgroups at T24 were in different clusters (blue and purple cluster, respectively) The other two clusters include the majority of T0M and some T0B, T1B and T6B (green cluster), and benralizumab treated patients at T0, T1 and T6 (red cluster). Overall, although it was applied an unsupervised approach, the four timepoints analyzed for cellular subsets can be considered representative of the different behavior of these cell subsets in mepolizumab and benralizumab-treated patients.

Table 1

Clinical and functional characteristic of severe asthmatic patients before treatment (T0) and after one month (T1), six months (T6) and 24 months (T24) of mepolizumab and benralizumab treatment. Abbreviations: T0M: baseline values for mepolizumab treated patients, T1M: values after 1 month of mepolizumab treatment, T6M: values after 6 months of mepolizumab treatment, T24M: values after 24 months of mepolizumab treatment, T0B: baseline values for benralizumab treated patients, T1B: values after 1 month of benralizumab treatment, T6B: values after 6 months of benralizumab treatment, T24B: values after 24 months of benralizumab treatment, OCS: oral corticosteroid, ICS: inhaled corticosteroids, FEV1: Forced expiratory volume in the first second FVC: forced vital capacity, ACT: asthma control test, ACQ: Asthma control questionnaire. P values reported in the table referred to one-way ANOVA (Kruskal-Wallis) test.

	T0B	T1B	T6B	T24B	P values	T0M	T1M	T6M	T24M	P values
%FEV1	92.5 ± 37.6	87.43 ± 15.45	89.78 ± 17.97	95.14 ± 25.02	**0.051**	83.83 ± 29.2	84.95 ± 30.78	90.8 ± 26.76	97.57 ± 23.3	**0.048**
% FVC	108.4 ± 18.3	107.24 ± 16.91	108.67 ± 10.60	116.17 ± 36.56	0.587	97.33 ± 13.6	99.64 ± 21.33	101.42 ± 1.71	104.57 ± 14.77	0.576
FEV1/FVC	67.7 ± 7	68.93 ± 10.03	70.73 ± 10.2	67.93 ± 10.9	0.876	57.93 ± 8.4	64.55 ± 11.23	62.99 ± 11.58	70.61 ± 12.8	0.928
OCS dose(mg/day)	6 ± 10.8	2.17 ± 4.02	0.714 ± 1.89	0	**0.03**	3.75 ± 4.84	6.43 ± 9.34	0	0	**0.028**
ICS dose(µg/day)	475.1 ± 317.4	453.71 ± 359.44	368 ± 225.22	383.33 ± 222.86	0.098	533.71 ± 20.45	475.14 ± 317.4	446.57 ± 291.2	418 ± 283.36	0.989
ACQ-6 SCORE	2.02 ± 1.26	1.3 ± 0.9	0.5 ± 0.4	0.6 ± 0.4	**0.087**	3.2 ± 1.7	1.3 ± 0.9	1.1 ± 1.07	0.71 ± 0.9	**0.022**
ACT SCORE	16.6 ± 6.3	21.83 ± 2.48	24.5 ± 1.22	24.6 ± 0.54	**0.010**	14.28 ± 5.3	20.14 ± 3.3	21.86 ± 3.53	23.43 ± 2.15	**0.006**
Eosinophils (cells/mm$^{3)}$	756.43.44 ± 245.95	12.4 ± 19.54	0	3.8 ± 3.2	< 0.0001	894.44 ± 414.34	0	0	6.71 ± 30.57	< 0.0001
Treg Mean (SD)	1.2 (1.3)	5.1 (2.5)	6.3 (3.4)	8.4 (4.6)	< 0.001	3.4 (1.7)	1.9 (0.8)	1.9 (1.0)	5.1 (2.4)	< 0.001
TregPD1 (Mean (SD)	68.3 (29.6)	42.0 (22.0)	29.3 (24.9)	18.9 (15.7)	< 0.001	20.0 (7.9)	9.7 (8.1)	6.1 (5.8)	2.1 (2.6)	< 0.001

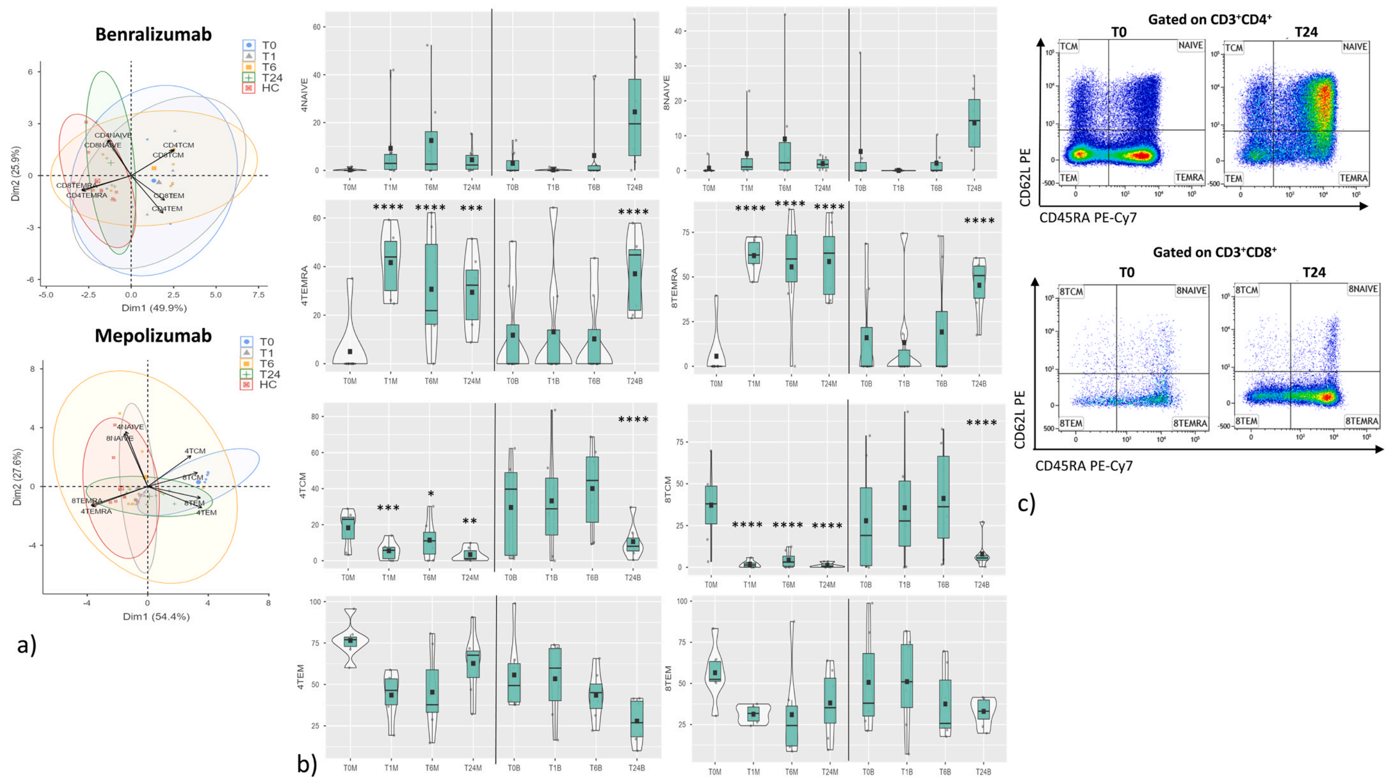

Fig. 2. a) Supervised PCA analysis of SA patients treated with mepolizumab and benralizumab stratified at times T0 (before starting treatment), T1 (after one month of treatment), T6 (after 6 months after of treatment) and T24 (after 24 months of treatment). The variables used for analysis were TCM, TEM, TEMRA and T naïve of CD4- and CD8-positive cells. For PCA, Unit Variance Scaling was applied to the rows to calculate the principal components. The X and Y axes show principal components 1 (PC1) and 2 (PC2). Prediction ellipses include 0.95 % of the data points. b) Histograms showing the percentages of T cell subsets (TCM, TEM, TEMRA, and T naïve) of CD4- and CD8-positive cells in severe asthma patients before starting therapy (T0) and after 1 month (T1), 6 months (T6) and 24 months of therapy (T24). c) Representative flow cytometric plots at T0 and T24. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and **** $p < 0.0001$ with respect to T0. Unless otherwise indicated, p values are not significant. Individual values, mean (center bar) ± SEM (upper and lower bars).

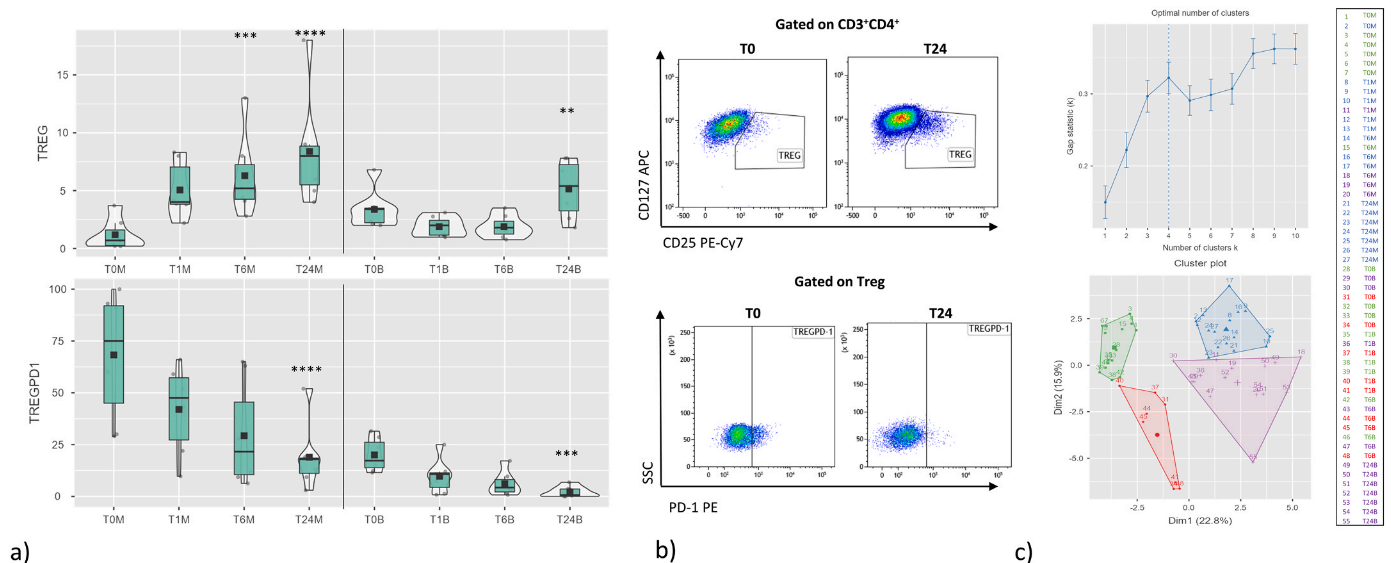

Fig. 3. a) Histograms showing regulatory T cells and Treg cells expressing PD-1, stratified by treatment (mepolizumab and benralizumab) at baseline (T0) and during follow-up (T1, T6 and T24). b) Representative flow cytometric plots at T0 and T24. c) The unsupervised machine learning algorithm, K-means clustering, was applied to our cell subsets, including T cell maturation subsets, and expression of LAG-3, PD-1, TIGIT and CTLA-4 in CD4-, CD8-, $CD56^{dim}$- and $CD56^{br}$-positive cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and **** $p < 0.0001$ with respect to T0. Unless otherwise indicated, p values are not significant. Individual values, mean (center bar) ± SEM (upper and lower bars).

3.7. Differences in T cell exhaustion in SA, before and during treatment with benralizumab and mepolizumab

Regarding ICs expression, a similar trend emerges between CD4 and CD8. No differences were reported for CTLA4 expression on CD4 and CD8 positive T cells for both treatments. Regarding TIGIT expression, an increased level emerged for benralizumab treated patients at T24, while, in case of mepolizumab, the significance was evident already at T6, and it was maintained at T24. For LAG-3 ICs, an increased expression was observed in mepolizumab-treated patients while, on the contrary, a total depletion was reported in benralizumab group. The benralizumab-treated group also recorded an increase in PD-1 expression at T24, which was not detected in mepolizumab patients (Fig. 4a–b). From these data, an exhausted phenotype of CD4 and CD8 cells were reported after the administration of both treatments, although with some differences regarding the expression of LAG-3. For innate NK immune cells, we found a change in CTLA4 expression with higher concentrations in CD56^{br} and CD56^{dim} NK cells after both treatments (Fig. 5a–b). In this case, LAG3 expression was totally depleted at T24 of both treatments. Regarding expression of PD-1, a decreased percentage of CD56^{dim}PD-1^{+} emerged during follow-up, with both treatments. An increase in TIGIT expression on CD56^{dim} emerged in benralizumab and mepolizumab-treated patients, while for CD56^{br}PD1^{+}, a decrease appeared only in case of mepolizumab administration at T24. TIGIT and PD-1 were the only ICs to co-express at T24, with increased percentages for CD56, CD4 and CD8 (Fig. 6a–b) (suppl. Tab.5).

3.8. CD56^{dim}PD-1^{+} and TregPD-1^{+} showed correlations with clinical parameters

When we looked for correlations between clinical and functional parameters we observed correlations between OCS, ACT, ACQ and Treg expressing PD-1. TregPD-1^{+} in particular showed a direct correlation with use of OCS and with ACQ score, and an inverse correlation with ACT score (Fig. 7a). Other cell subsets that correlated with clinical parameters resulted CD56^{dim}PD-1^{+}. CD56^{dim}PD-1^{+}showed an indirect correlation with functional parameters including FEV1 and FVC, and with ACT score when all populations were analyzed and also in the case of therapy stratification and at different time points (Fig. 7b).

4. Discussion

T effector cells and Treg cells play an important role in driving and damping airway inflammation in patients with asthma. Although new monoclonal antibodies have demonstrated unprecedented clinical efficacy for the treatment of SA [14,15], very little is known about the potential effects of these drugs in adaptive immunity.

In the present study, we investigated this specific issue enrolling a cohort of SA patients treated for 24 months with anti-IL-5/IL-5R therapies. To our knowledge, this is the first study to evaluate changes in T cell maturation subsets, including effector and regulatory T cells, and their state of exhaustion, evaluated in terms of elevated expression of ICs proteins, and to compare the potential effects between mepolizumab and benralizumab The latter point was made possible by the observation of a good response in all our patients in terms of clinical and functional improvement, associated with a significant depletion of eosinophils. Therefore, this substantial homogeneity of clinical outcome allowed us to suppose that the immunological modifications could be mainly related to the specific drug and not to a different response to the treatment.

Firstly, it was observed a misbalance of T effector cells in SA patients when compared with a cohort of HC. This was expected due to the well-known described mechanisms of T2-high diseases including SA. In fact, this disease is characterized by an hyperactivation of effector cells,

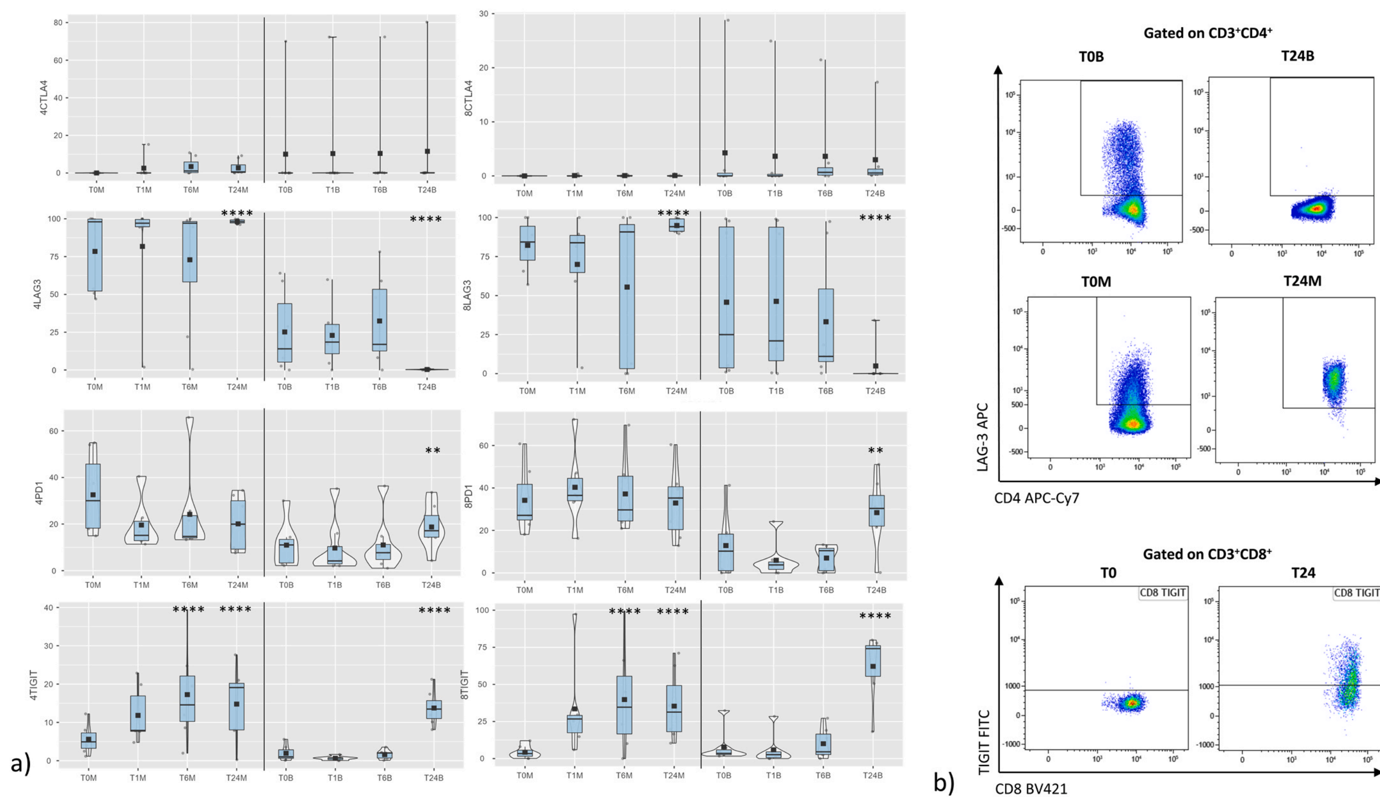

Fig. 4. a) Histograms showing expression of PD-1, LAG-3, CTLA-4 and TIGIT on CD4- and CD8-positive T cells stratified by treatment (mepolizumab and benralizumab) at baseline (T0) and during follow-up (T1, T6 and T24). B) Representative flow cytometric plots at T0 and T24. **p < 0.01 and **** p < 0.0001 with respect to T0. Unless otherwise indicated, *p* values are not significant. Individual values, mean (center bar) ± SEM (upper and lower bars).

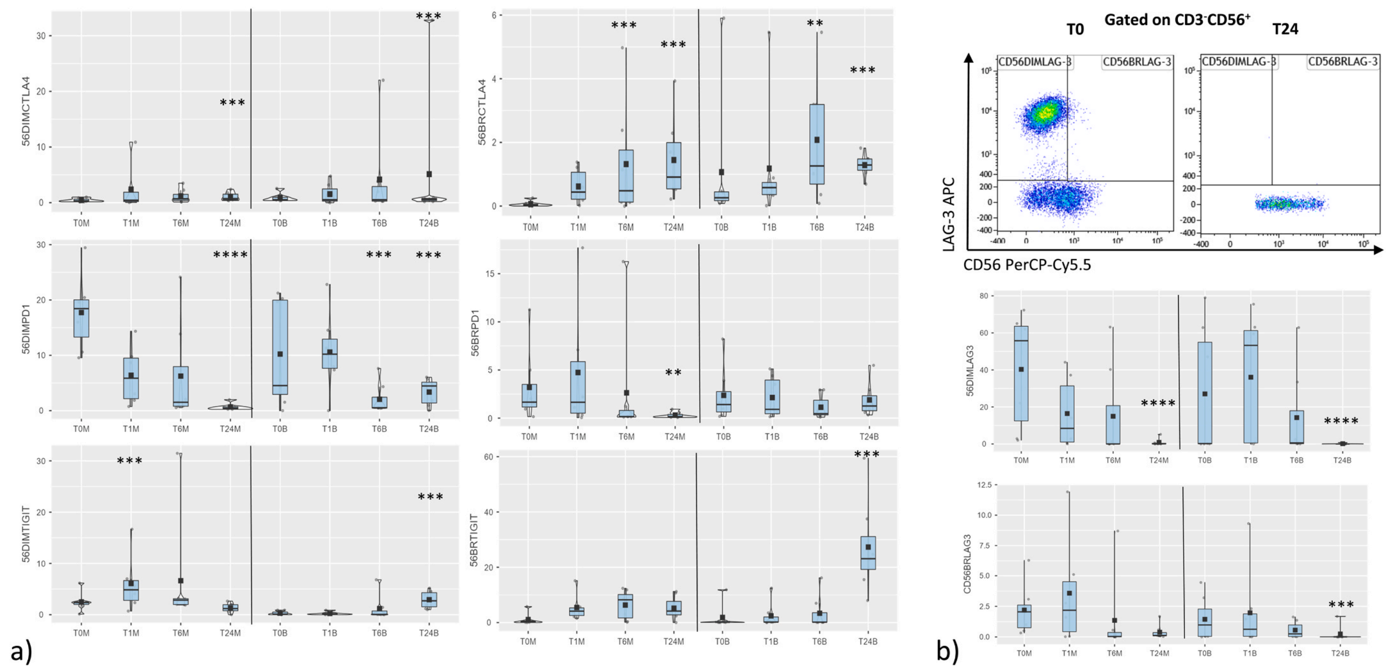

Fig. 5. a) Histograms showing expression of PD-1, LAG-3, CTLA-4 and TIGIT on $CD56^{dim}$ and $CD56^{br}$ cells stratified by treatment (mepolizumab and benralizumab) at baseline (T0) and during follow-up (T1, T6 and T24). B) representative flow cytometric plots at T0 and T24. **p < 0.01 ***p < 0.001 and **** p < 0.0001 with respect to T0. Unless otherwise indicated, *p* values are not significant. Individual values, mean (center bar) ± SEM (upper and lower bars).

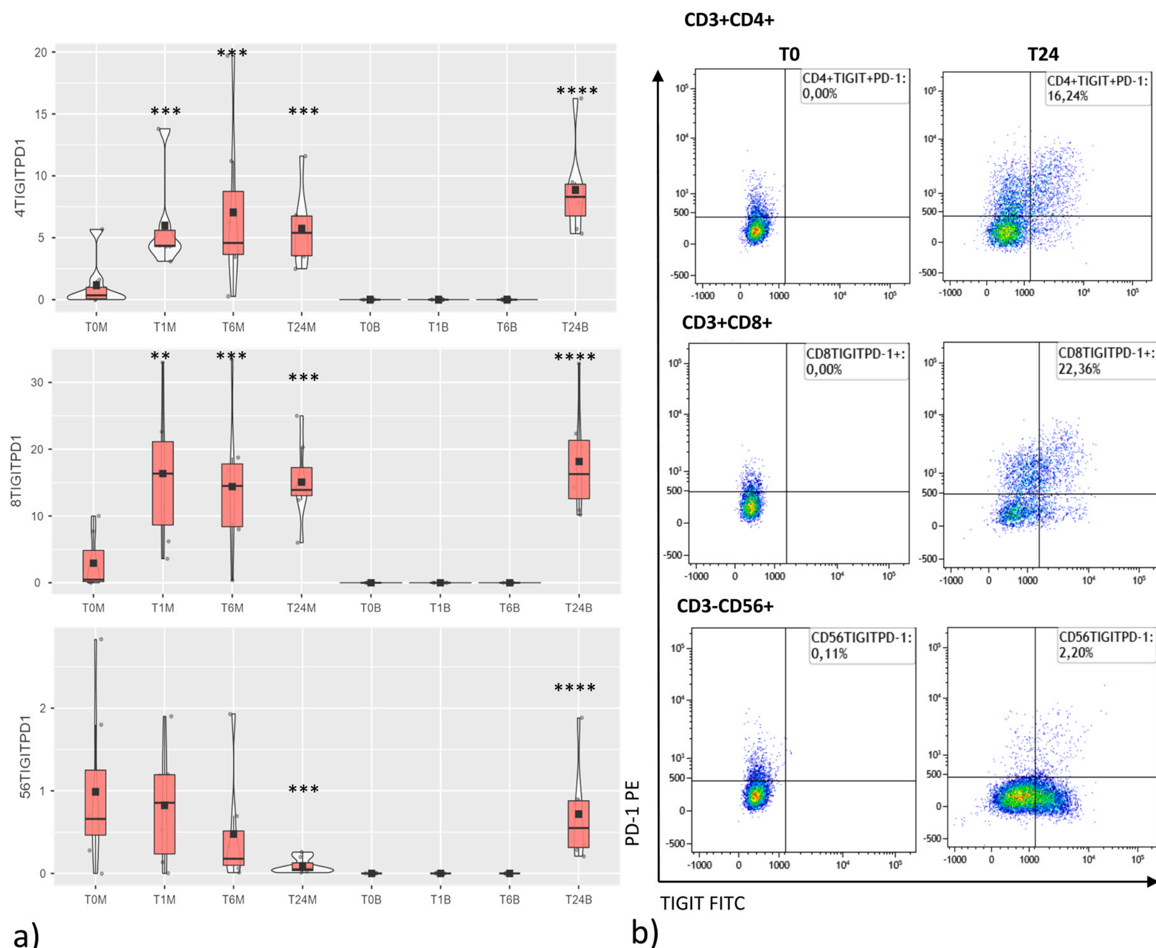

Fig. 6. a) Histograms showing double expression of $PD\text{-}1^{+}TIGIT^{+}$ on CD4, CD8 and CD56 cells. b) Representative flow cytometric plots at T0 and T24. ***p < 0.001 and **** p < 0.0001 with respect to T0. Unless otherwise indicated, *p* values are not significant. Individual values, mean (center bar) ± SEM (upper and lower bars).

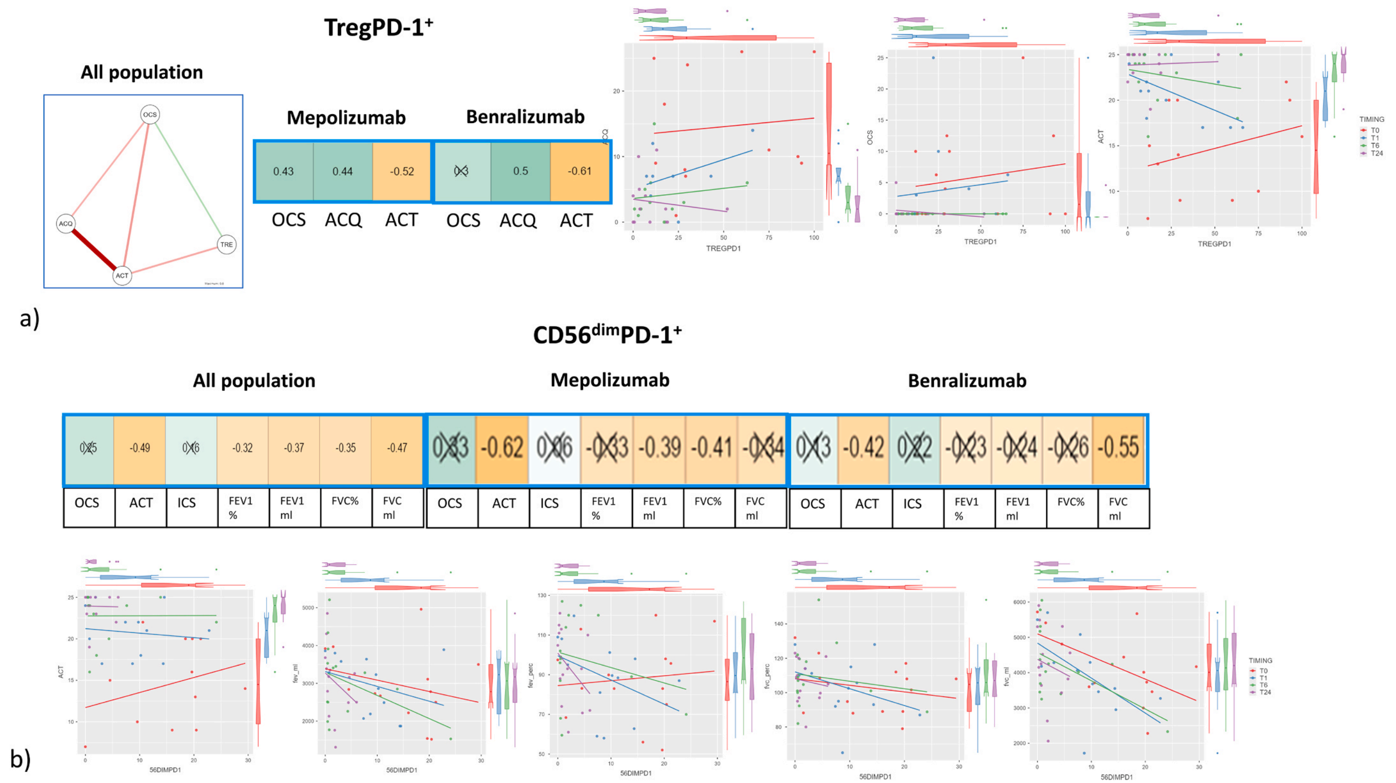

Fig. 7. a) Spearman correlation analysis of TregPD-1 cells with clinical parameters OCS, ACT and ACQ. b) Spearman correlations stratified by time points (T0, T1, T6 and T24). c) Spearman correlation analysis of CD56^{dim}PD-1^{+} cells with clinical parameters OCS, ICS, ACT, FEV1 (% and ml) and FVC (% and ml). d) Spearman correlations stratified by time points (T0, T1, T6 and T24). ***$p < 0.001$ and **** $p < 0.0001$ with respect to T0. Unless otherwise indicated, p values are not significant. Individual values, mean (center bar) ± SEM (upper and lower bars).

characterized by an abundant release of cytokines, such as IL-5, IL-4 or IL-13, not by chance representing the target of biologic therapies such as mepolizumab and benralizumab [16,17].

Moreover, from the analysis of baseline values of mepolizumab and benralizumab treated groups, several differences emerged regarding immunologic aspects, even though these two groups were very similar from clinical point of view. In particular, mepolizumab treated patients showed an higher degree of impairment of Treg compartments in terms of percentage and exhaustion state, associated to a more pronounced exhaustion of CD4, CD8 and NK cells, as well.

Although it surely impairs the homogeneity of study population and limits our findings, this observation is also meaningful: it suggests that the actually recommended clinical and biomarkers-driven categorization of SA patients may not be so accurate in detecting also differences of adaptive immunological behavior. The clinical implications are currently unknown and go beyond the scope of this paper: however, these findings are surely interesting worthy to be further investigated in a larger cohorts of patients with SA.

We also observed a complete rebalancing of T cell subsets after 24 months of treatment with both mepolizumab and benralizumab: on one hand, we reported a decrease in TEM and TCM, which represents the T cells phenotypes responsible for the release of IL-5 and other pro-inflammatory cytokines. On the other hand, T naïve and TEMRA, T cell subsets that do not mediate effector immune responses, together with Treg cells, proved to be greatly increased. The rebalancing of T cells subsets induced by the IL-5 blockade was evident not only numerically but also in qualitative terms: throughout the follow-up, Treg cells showed a progressively decreased expression of T exhaustion marker PD-1, allowing the regulatory compartment to explicate more efficiently its immunomodulatory functions. In fact, the primary role of PD-1 is regulation of T cell responses via promotion of apoptosis in effector T cells and inhibition of apoptosis in Treg cells after antigen clearance [18,19]; overexpression of PD-1 leads to anergy, exhaustion and tolerance of CD4^{+} and CD8^{+} T cells [20]. These observations are intriguing since an impairment of Treg cells percentage and activity in SA patients has been described and associated with severity of disease [21,22]. Overall, our findings suggest that mepolizumab and benralizumab induce a substantial restoration of T cell balance in patients with SA: interestingly, these effects appear to be time-dependent, since both percentage and PD-1 expression of T-reg showed a homogeneous trend during the time of observation. Considering that recent evidences have demonstrated that a prolonged exposure to anti-IL-5 treatment led to an ever greater clinical benefit [23,24], our data are intriguing as they suggest a potentially crucial role of adaptive immunity determining the long-term effectiveness of these drugs in SA.

On this regard, we also observed some significant differences between mepolizumab and benralizumab: at T24, PD1 showed an increase in percentages in CD4^{+} and CD8^{+} T effector cells only in benralizumab-treated patients, suggesting that the inhibition of IL-5 receptor may lead to a deeper exhaustion of the capacity of these cells to release effector cytokines (such as IL-5) in respect with the inhibition of circulating IL-5. Standing that baseline PD-1 expression on CD4^{+} and CD8^{+} T effector cells was different between the two subgroups, these findings might be questionable. However, it is meaningful that a similar opposite behavior was observed also for other ICs included in the immunological analysis. In fact, LAG-3 expression showed opposite patterns in SA patients treated with benralizumab and mepolizumab as we observed a total depletion with the former and an increase with the latter. The mechanisms and signaling mediated by LAG-3 are still unclear [25] and, also in other diseases it has been reported that LAG-3 behaves in the opposite manner to other ICs, as we observed in our cohort [26]. LAG-3 is considered a negative regulator of T cell expansion: its blockade result in persistent expansion of CD8^{+} and CD4^{+} T cells and, for this reason, it is considered a promising target for cancer immunotherapy [27–29].

Moreover, LAG-3 is required for Treg- mediated control of T-cell homeostasis [29]. As for PD-1, the clinical relevance of this observation is uncertain, as both mepolizumab and benralizumab-treated patients shows a similar numerical decrease of $CD4^+$ and $CD8^+$ T cell subsets: however, it could be worthy to further investigate in larger cohorts of SA patients, both for evaluating the potential correlations with clinical outcomes and for detecting drug-specific immunological alterations.

On the other hand, other ICs on $CD4^+$ and $CD8^+$ T effector cells shows a similar response to mepolizumab and benralizumab, even though with a different timing. Indeed, an increase in percentages of NK and $CD4^+$T cells expressing TIGIT was recorded in benralizumab subgroup at T24. This enhancement was also found in mepolizumab-treated patients already at T1. TIGIT is a co-inhibitory receptor specifically expressed in immune cells such as activated T cells and NK cells [30–32]. In addition to its direct inhibitory role in NK and effector T cells, TIGIT also inhibits immune responses by promoting Treg cell function [33]. Importantly, we learn from the literature that TIGIT-dependent induction leads to selective sparing of Th2 cell responses and strongly suppresses pro-inflammatory Th1 and Th17 cell responses [34]. Simultaneous expression of PD-1 and TIGIT also emerged after 24 months of therapy. Dual expression of PD-1 and TIGIT has already been described in the literature [35]. These results suggest a more immunosuppressive microenvironment in SA patients after therapy through the promotion of anti-inflammatory pathways, although a deeper analysis of T2 cells expression and activity need to be performed in order to improve the knowledge of these receptors in SA.

Focusing on the innate aspects of this response, also the $CD56^{dim}$ subset showed features of exhaustion with upregulation of PD-1. These cell subsets were strongly correlated with functional parameters in our SA cohort.

NK cell exhaustion is associated with poor long-term survival in other diseases [23]. NK cell immunology in the airways of SA also features lower NK cell cytotoxicity, which is exacerbated by corticosteroids that further disable NK cell function [36]. These failed resolution mechanisms may contribute to persistent airway inflammation [30]. In line with this evidence, our findings showed an exhausted CD56 phenotype before therapy. The activation of NK cells in patients with SA may be insufficient during some respiratory infections: the exhausted phenotype may participate in NK cell impairment and aggravation of virus-induced asthma exacerbation in these patients [31].

The clinical implications of these differential effects between mepolizumab and benralizumab are unknown and our study is not powered for this purpose: however, even though they required to be confirmed on larger cohorts, our findings are surely intriguing in an optic of personalized medicine.

Obviously, it is limited by some issues: first of all, the small sample size, therefore, the potential impact of some clinical on our findings is not to be underestimated. Second, our study is designed as monocentric: even though this aspect guarantees the standardization of patients' enrollment flow cytometry procedures, in a high-experienced lab. Moreover, further data can improve our understanding regarding the role of ICs (and particularly PD-1, TIGIT and LAG-3) in response of these therapies, due to its opposite behavior after treatments.

It could be useful understand what kind of CD4 T cells subsets are mainly involved in these mechanisms by deeply analyzing Th2 and regulatory T cell subsets. In fact, we did not evaluate T helper cell subsets and relative expression of ICs. Several novel cell surface markers can also help to define the role of regulatory T cells functions in response to anti IL-5 treatments.

In conclusion, the present study provides a thorough overview of the effect of anti-IL5 treatment both on innate and adaptive immunological compartments. Both mepolizumab and benralizumab appeared to induce an overall rebalancing of T cells and NK cells activity and maturation, similar to non-asthmatic subjects, suggesting that the clinical efficacy of these drugs is not simply a consequence of eosinophil depletion. Standing the substantial homogeneity of the two subgroups in terms of conventional T2 biomarkers and severity assessment, the differences observed between mepolizumab and benralizumab patients are surely intriguing as they suggest the existence of different drug-specific immunological effects, especially concerning NK cells and ICs expression. These results, if confirmed in larger cohorts, may pave the way to the identification of novel and more specific markers for the prediction and evaluation of anti-IL5 treatment response in patients with SA.

Informed Consent and Local Ethics Committee

All subjects gave their written informed consent to participate in the study, which was approved by our Local Ethics Committee CEAVSE (Code no. 180712 and Markerlung 17431). This study was performed in accordance with the Declaration of Helsinki.

Disclosure of Potential Conflicts of Interest

PC served as a speaker and consultant and advisory board member for Astra Zeneca, Sanofi, Novartis, and GSK. PC, EB, MdA, and LB are investigators for current research financed by AstraZeneca (grants paid to his institution). All other authors declare no conflict of interest.

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None.

CRediT authorship contribution statement

LB, TP, PC: Conceptualization, Data curation, Formal analysis, Writing – original draft. **MD, BC:** Investigation. **MD, SG:** Methodology. **EB:** Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

PC served as a speaker and consultant and advisory board member for Astra Zeneca, Sanofi, Novartis, and GSK. PC, EB, MdA, and LB are investigators for current research financed by AstraZeneca (grants paid to his institution). All other authors declare no conflict of interest.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115385.

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