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Benralizumab affects NK cell maturation and proliferation in severe asthmatic patients

Laura Bergantini [1,*], Miriana d'Alessandro [1], Tommaso Pianigiani, Behar Cekorja, Elena Bargagli, Paolo Cameli

Respiratory Disease Unit, Department of Medical Sciences, University Hospital of Siena (Azienda Ospedaliera Universitaria Senese, AOUS), Viale Bracci, 53100 Siena, Italy

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ABSTRACT

Introduction: The mechanism of action of benralizumab is determined by its afucosylated constant fragment that binds CD16a receptors on the membrane of natural killer cells. Here we analysed changes in Natural Killer and T-cells in Severe asthmatic patients, before and after benralizumab.

Methods: Natural Killer and T-cell subsets were detected through multiparametric flow cytometry. The concentrations of serum cytokines levels were detected through multiplex assay. Functional proliferation assay was performed in follow-up samples in severe asthmatic patients.

Results: At baseline, severe asthmatic patients showed higher percentages of immature Natural Killer cells when compared with healthy controls. We demonstrate the proliferative capacity of these cells and their activation after benralizumab administration. Benralizumab shifted Natural Killer cell phenotypes towards maturity. Correlation between the Natural Killer cells and functional parameters and with steroid-sparing was observed.

Conclusion: Together this data contributes to our understanding of the mechanisms of action of benralizumab in the resolution of inflammation in severe asthma patients.

1. Introduction

Severe asthma is defined as asthma that remains uncontrolled despite maximal optimized high-dose inhaled corticosteroids and long-acting beta agonists and treatment of comorbidities, or asthma that worsens when high-dose treatment is decreased [1,2]. Asthma is classically thought to be a T helper 2 (TH2)-cell driven inflammatory disease, characterized by eosinophilic inflammation, TH2-cell associated cytokine production and airway hyperresponsiveness [3].

In the last decade, new biologics have been developed to treat patients suffering from severe asthma, especially those with evidence of type-2 dominant inflammatory endotype [4]. Among these, two monoclonal antibodies targeting interleukin (IL)-5 mepolizumab (Nucala) and reslizumab (Cinqair), and one targeting IL-5Rα, benralizumab (Fasenra), is currently approved for the treatment of severe eosinophilic asthma [5,6]. IL-5 signaling is intimately involved in eosinophil biology, including the differentiation, survival and proliferation of these cells [7,8].

Benralizumab is a humanized IL-5Rα-specific monoclonal antibody that effectively improves severe eosinophilic asthma by inducing rapid and nearly complete depletion of eosinophils [7,9]. A particular mechanism of action of this drug is determined by its afucosylated constant fragment (Fc) that binds FcγRIIIa receptors on the membrane of natural killer (NK) cells. After this interaction, NK cells activate antibody-

Abbreviations: NK, natural Killer; HC, healthy controls; SA, severe asthmatic patients; M/M, mild to moderate patients; NK, natural killer cells; IL, interleukin; ADCC, antibody-dependent cell-mediated cytotoxicity; Fc, afucosylated constant fragment; CD, cluster of differentiation; Th, T helper; TCM, T central memory; TEM, T effector memory; TEMRA, T effector memory expressing CD45RA; DP, double positive NK cells; mem.like, memory Like NK cells; term.diff, terminally differentiated NK cells; PCA, principal component analysis; IFN, Interferon; OCS, oral corticosteroid; ICS, inhaled corticosteroid; EOS, eosinophils in peripheral blood; PFTs, pulmonary function tests; FEV1, Forced Expiratory Volume in the 1st second; FVC, forced vital capacity; ACT, asthma control test; ACQ, asthma control questionnaire.

* Corresponding author at: Department of Medical Sciences, Surgery and Neurosciences, Respiratory Disease and Lung Transplant Unit, Siena University, 53100 Siena, Italy.

E-mail address: laura.bergantini@unisi.it (L. Bergantini).

[1] These authors equally contributed to the study.

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dependent cell-mediated cytotoxicity (ADCC) through release of pro-apoptotic proteins, such as perforin and granzyme B, leading to apoptosis of eosinophils [10,11]. Although the role of NK cells in asthmatics is still debated, recent data sustains the hypothesis that severe asthmatic airways are characterized by depleted NK cell cytotoxicity, characterized by the release of IFN-γ [12]. Moreover, corticosteroids, which are commonly used as anti-inflammatory therapy in asthma, further disable NK cell functions, paradoxically contributing to persistent airway inflammation [12]. Dysregulated NK cell activity may also interfere with inflamed lung clearance, contributing to chronic airway inflammation and inducing remodelling processes [13].

In vitro studies have shown the strength and rapidity of antibody-dependent cell-mediated cytotoxicity (ADCC) induced by benralizumab treatment and its association with eosinophil depletion of patients in vivo[14]. However, no studies have investigated the change in NK and T cell markers during follow-up of drug administration and no data is available regarding NK cell subsets and function in treated patients. Considering the crucial role of T cell subtypes in the pathogenesis and severity of asthma, here we also evaluated the cross-talk between NK and T cell subsets. Regarding T cell compartment, functionally, TCM and TEM cells both produce effector cytokines (especially IL-5, IL-4 and IL-13) when stimulated [15]. TCM cells show lymphoid homing profiles and high proliferative capacity, while TEM cells produce more effector cytokines [16]. A dysregulated response of immune system in severe asthmatic patients was also described. In particular an inefficient production of anti-inflammatory cytokines, first of all IL-10 and TGF-β, mainly produced by T regulatory cells, was reported [17,18]. Furthermore, Fas-Fas ligand interaction is strictly correlated with the development of T2-mediated chronic airway inflammation [19]. The functional soluble form of Fas (sFas), and FasL (sFasL), was also identified in asthma, showing a dysregulation in their levels [20].

Here we analysed specific changes in different markers expressed by NK and T cells, before and after treatment of a population of severe asthmatics with benralizumab. We considered markers that modulate NK cell maturation and activation.

Moreover we also evaluated the concentration of pro and anti-inflammatory cytokines release after benralizumab administration.

We confirmed previous data regarding the imbalance in NK cell subsets in asthma patients [12]. We demonstrate for the first time the in vivo proliferative capacity of these cells and their activation after benralizumab administration. Benralizumab shifted NK cell phenotypes towards maturity and towards release of cytotoxic mediators responsible for apoptosis. Although the imbalance of T cell subsets was already presents after 6 months of treatments. We also evaluated correlations of NK cells subsets and clinical improvement.

2. Methods

2.1. Study population

Nineteen patients diagnosed with asthma and monitored at the Respiratory Diseases Unit of Siena University Hospital, were prospectively enrolled in the study from January 2019 to January 2020. Nine had mild-to-moderate asthma (M/M) (mean age 46.2 ± 18.6 years, 33% males, 66% never smokers) and 10 had severe asthma (SA) (mean age 52.4 ± 18.8, 60% males, 60% never smokers) according to Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) guidelines [2]. Fifteen healthy subjects (HC) (mean age 41.4 ± 16.2, 33% males, 73% of never smokers) were also enrolled in the study. To be included in the study, patients had to have predominantly eosinophilic inflammation, namely peripheral eosinophil count >300 cell/mm^3 not >6 months prior to enrolment. All SA patients were treated with benralizumab at the approved dose for eosinophilic asthma (i.e. 30 mg every 4 weeks for the first three administrations, then every 8 weeks) according to local clinical practice.

Clinical control of SA patients was assessed by the 6-item asthma control questionnaire (ACQ-6) and the asthma control test (ACT), at baseline (T0) and after 1 and 6 months of treatment (T1 and T6, respectively), together with pulmonary function tests and immunological analysis of serum and peripheral blood samples. Exacerbation rate, severity and changes in therapy, including oral steroid dose, were recorded throughout the observation period.

The control group had no history of asthma or allergy and was not on any type of medication. They were monitored for at least 12 months after inclusion in the study and did not develop any disease. They did not show changes in blood count and had no current or recent 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. All were expressed as percentages of predicted values and ml.

2.3. PBMC collection and handling of cells

Peripheral blood mononuclear cells and serum samples were separated in the laboratory of the Respiratory Diseases 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. Serum samples were also collected for all 34 subjects (HC, MM and SA) and during follow-up of benralizumab for SA group. Serum samples were frozen at −80 °C until analysis.

2.4. NK and T cells cytometric analysis

Briefly, cells were washed with wash buffer (HBSS−/− with 2% FBS) and incubated with the mAb mix for 30 min in the dark at RT. 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 [21]. Forward versus side scatter gating was used to identify lymphocytes, and the entire NK cell population was found negative for CD3$^-$CD14$^-$CD19$^-$ CD56 was plotted against CD16 in order to obtain immature (CD56^{br}CD16^{neg}) and mature (CD56$^{dim/neg}$CD16$^+$) phenotypes of NK cells. A series receptor, including NKG2A, NKG2C, CD57 and KIR, was evaluated on the CD56$^{dim/neg}$CD16$^+$ population. For T cells, the subsets were detected on the basis of CD62L and CD45RA expression: T central memory (TCM) (CD62L$^+$CD45RA$^-$), effector memory T cells (TEM) (CD62L$^-$CD45RA$^-$), naive T cells (T naive) (CD62L$^+$CD45RA$^+$) and memory T effector memory RA (TEMRA) (CD62L$^-$CD45RA$^+$). To observe the activity of T and NK cells, CD137-APC was added to the tubes. The first gate was set to discriminate singlets (FSC-H vs FSC-A), followed by lymphocytes based on FSC-A vs SSC-A. A third gate discriminated viable cells using 7AAD as viability dye. All monoclonal antibodies used in flow cytometry to detect surface markers are described in suppl. Table 1.

2.5. CellTrace™ Violet proliferation assay

Peripheral blood mononuclear cells were isolated from fresh whole blood using a density gradient centrifuge (Ficoll-Paque® Plus, GE Healthcare). Cells were thawed and resuspended in Dulbecco's phosphate-buffered saline (DPBS) (GIBCO), stained for 20 min with 1 μM CellTrace™ Violet (Invitrogen), quenched for 5 min with one

volume of Fetal Bovine Serum, and resuspended in Cell wash 1× (BD Biosciences). The specific subpopulations induced to proliferate by benralizumab treatment were characterized by multicolour flow cytometry using monoclonal antibody conjugates against human CD3 APC-Cy7, CD4 FITC, CD8 PE-Cy7 and CD56 PerCP-Cy5.5.

2.6. Immunoassays

Serum concentrations of biomarkers including TGF-β, IL-5, IL-10, sFAS, sFASL and IL-33 were quantified by bead-based multiplex LEGENDplex™ analysis (LEGENDplex™ Custom Human Assay, Biolegend) according to the manufacturer's instructions. Serum samples from stored aliquots were used. Reactions were run in duplicate with a BD FACSLyrics flow cytometer (BD-Biosciences, San Jose, CA, USA). The data was processed with Legendplex V8.0 software (Biolegend), and concentrations were expressed in pg/mL.

2.7. IFN-γ detection

The detection of interferon-gamma (IFN-γ) was performed using a commercial enzyme-linked immunosorbent assay (ELISA) (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The limit of detection of IFN-γ is 0.065 IU/ml.

2.8. 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 normal distribution of the data. The Chi-squared test was used for categorical variables. A one-way ANOVA non-parametric test (Kruskal–Wallis test) and Dunn test or Friedman test were performed for multiple comparisons. A p value <0.05 was considered statistically significant. Statistical analysis and graphic representation of the data were performed by GraphPad Prism 9.0 software (Graphpad Holdings, LLC, San Diego, CA, USA). For correlation analysis, the Spearman test was applied. Jamovi Free software (Jamovi 1.6.23) was used to run the test. Supervised principal component analysis (PCA) was employed to reduce the dimensions of the data hyperspace and to cluster samples on the basis of cell subsets. For the multivariate analysis, the percentage of differential surface markers of NK and T cell subsets, together with the serum concentrations of proteins detected in the overall cohort, were used to perform supervised heatmap analysis; this analysis visualizes the percentages of differential cell markers in each patient. Clustering was performed on the basis of Spearman rank correlation and K-means. The above analyses and corresponding figures were obtained using free software: MORPHEUS (https://software.broadinstitute.org/morpheus/) and ClustVis (http://biit.cs.ut.ee/clustvis).

3. Results

3.1. Clinical and functional characteristics of HC, M/M and SA

No differences in the distribution of sex, mean age and smoking habits emerged between the control, M/M and SA groups. The main characteristics of the SA subgroup at T0, T1 and T6 are reported in Table.1.

After the beginning of benralizumab treatment, we observed a significant improvement in the clinical control of asthma and PFTs in SA patients. In particular, FEV1, FVC and FEV1/FVC ratio were significantly higher after 6 months of therapy as were ACT and ACQ-6 scores. In line with the literature, eosinophil depletion emerged already after one month of therapy. The daily dose of oral and inhaled corticosteroids was significantly lower after 6 months of therapy.

Table 1
Clinical and functional characteristic of severe asthmatic patients before treatment (T0), at one (T1) and six months (T6) after benralizumab administration.

	T0	T1	T6	p
%FEV1	87,84 ± 14.55	90,90 ± 16,38	95,85 ± 20,78	0,03
% FVC	106,53 ± 15.50	111,07 ± 15,64	113,67 ± 13,1	0,02
Daily oral glucocorticoid dose (mg/day)	2,91 ± 4,50	1,44 ± 3,35	0,5 ± 1,58	<0,0001
Inhaled glucocorticoid dose (mcg/day)	611,6 ± 281,14	541,6 ± 314,2	410,8 ± 263,06	<0,0001
ACQ-5 score	11 ± 7,90	7,14 ± 3,71	2,77 ± 2,27	<0,0001
ACT score	15,66 ± 6,16	21 ± 2,59	24,44 ± 1,01	<0,0001
Blood eosinophil count (cells/MM^3)	864,44 ± 476,34	0	15,71 ± 41,57	<0,0001

3.2. Mild/moderate and severe asthma patients showed differential cell subsets and cytokine profiles compared to HC

Based on the flow cytometry data of NK and T cell subsets and serum concentrations of cytokines, we performed supervised principal component analysis (PCA) analysis of control, M/M and SA parameters. The PCA analysis was used in order to observe if the cellular subsets analysed, well clustered our 3 group of subjects, HC, MM and SA respectively.

The PCA plot showed that samples from different groups clustered well, corroborating the evidence that differential cell and cytokine profiles were characteristic of the three groups (Fig. 1a). Furthermore, M/M asthmatics clustered close to controls, whereas SA patients were located on the opposite side of the graph, distant from the control and M/M groups (Fig. 1a). For PCA analysis, Unit Variance Scaling was applied to rows to calculate the principal components. The X and Y axes show principal components 1 (PC1) and 2 (PC2) that explained 24,5% and 16,9% of the total variance, respectively. Fig. 1b shows the heatmap on cell surface markers of NK and T cells detected in the three groups. It is based on hierarchical clustering and the Spearman rank correlation. Like for PCA analysis, the general trend separated samples into two main groups, A and B, as indicated by the dendrogram at the top of the matrix. Interestingly, group A was mainly composed of mature phenotypes of NK cells (including CD56^{dim} and CD56^{dim}KIR$^+$) and TEMRA subsets. Group B included mainly naïve T and immature NK cells. K-mean clustering was also applied to partition observations according to expression of cell surface markers (Fig. 1b).

3.3. NK cells subsets of severe asthma patients proved to have limited cytotoxic activity, whereas T cells contributed to lung inflammation

Gating strategy of NK cells was reported in Fig. 2a. Total NK cell percentages obtained by excluding CD3, CD19 and CD14 from PBMCs the same in the three groups.

Among CD56 cell subsets, CD56$^{dim/neg}$CD16^{br}, the subset responsible for cytotoxic functions of NK cells, was lower in the SA than in the control and M/M groups. The same pattern was found for the more mature subset memory-like NK cells (which express NKG2C) and the maturing phenotypes defined as NKG2A$^+$ and KIR$^-$. On the contrary, double positive (NKG2A$^+$KIR$^+$) NK cells, which express NKG2A and KIR, were more abundant in SA patients (Fig. 2b).

Gating strategy of T cells was reported in Fig. 3a. Dysregulation of the T cell compartment also emerged from our data. Different T-cell maturation phases were detected by flow cytometry, based on expression of CD62L and CD45RA T cells. Significantly lower CD45$^+$CD3$^+$, T naïve and TEMRA percentages were found for CD4 and CD8 in the SA group than in the other groups. On the contrary, higher TCM and TEM percentages for CD4$^+$ and CD8$^+$ cells, which contribute to the

Fig. 1. a) PCA was applied to percentages of NK and T cell subsets in the three groups. For PCA, Unit Variance Scaling was applied to 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) Heatmap of cell surface markers of NK and T cells in our cohort (patients with mild to moderate and severe asthma, and healthy controls (HC)). Hierarchical clustering is based on the Spearman rank correlation. K-means were used to detect clusters on the basis of expression of surface markers. Abbreviations: PC: principal component, HC: healthy controls, M/M: mild to moderate, Severe Baseline: Severe asthmatic patients before benralizumab administration.

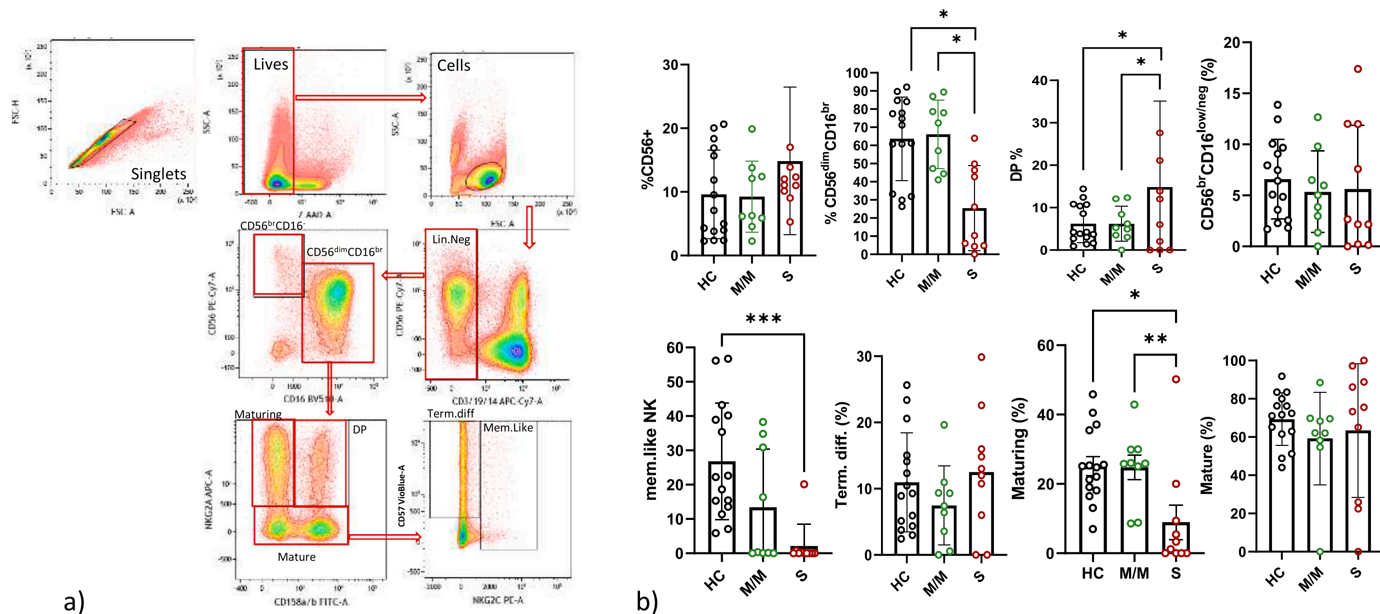

Fig. 2. a) Gating strategy for NK cells applied in flow cytometry to detect different NK subsets. b) Histograms indicate the distribution of NK subsets among the three groups: healthy controls (HC; $n = 15$), mild-to-moderate patients (M/M $n = 9$) and severe asthma patients (SA; $n = 10$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: CD: cluster of differentiation, HC: healthy controls, M/M: mild to moderate, SA: severe asthmatic patients, DP: double positive Nk cells, mem.like: memory Like NK cells, term.diff: terminally differentiated NK cells, lin.neg: lineage neg.

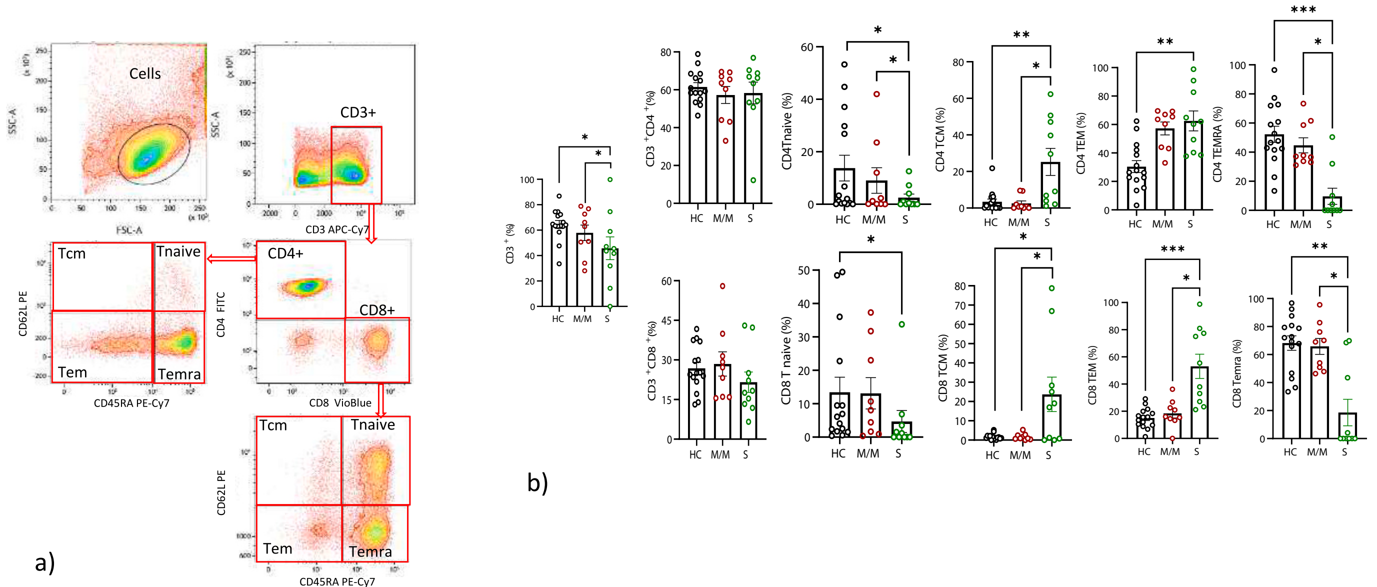

Fig. 3. a) Gating strategy for T cells in flow cytometry to detect different T cell subsets. b) Histograms indicate the distribution of T cell subsets among the three groups: healthy controls (HC; n = 15), mild-to-moderate patients (M/M n = 9) and severe asthma patients (SA; n = 10). *p < 0.05, **p < 0.01, ***p < 0.001. Abbreviations: CD: cluster of differentiation, HC: healthy controls, M/M: mild to moderate, SA: severe asthmatic patients, TCM: T central memory, TEM: T effector memory, TEMRA: T effector memory expressing CD45RA.

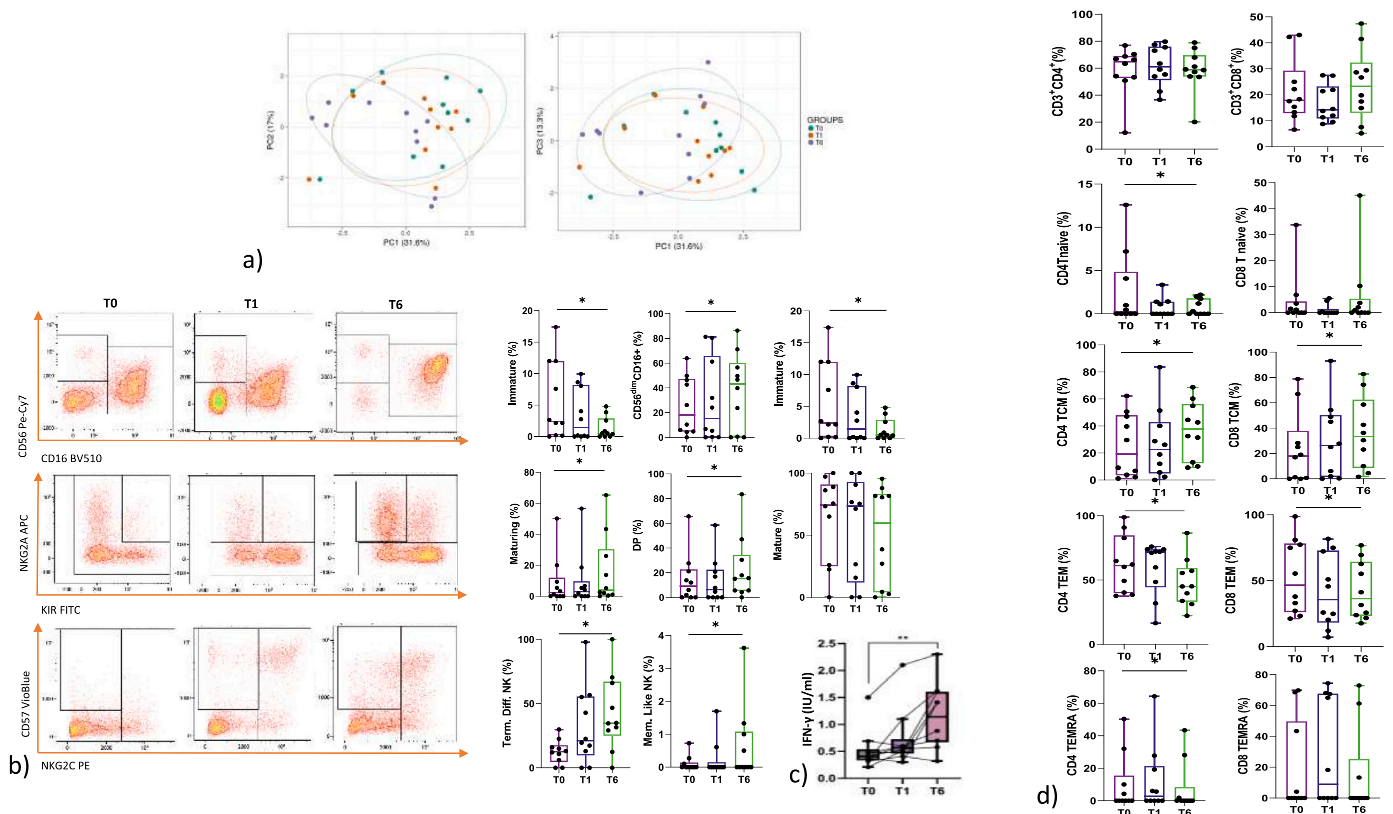

Fig. 4. a) PCA was applied to percentages of NK and T cell subsets in the severe asthma group, before (T0), after 1 month (T1) and after 6 months (T6) of treatment with benralizumab. For PCA analysis, Unit Variance Scaling was applied to rows to calculate the principal components. The X and Y axes show principal components 1 (PC1) and 2 (PC2). Prediction ellipses include 0.95% of the data points. b) Dot plots and histograms of different NK subsets of patients with severe asthma at T0, T1 and T6. c) IFN-γ detected in serum of severe asthma patients at T0, T1 and T6. d) Histograms of T cell maturation in severe asthma patients at T0, T1 and T6. *p < 0.05 Abbreviations: CD: cluster of differentiation, HC: healthy controls, M/M: mild to moderate, SA: severe asthmatic patients, TCM: T central memory, TEM: T effector memory, TEMRA: T effector memory expressing CD45RA, DP: double positive Nk cells, mem.like: memory Like NK cells, term.diff: terminally differentiated NK cells, PC: principal component, IFN: Interferon.

persistence of inflammation in the lung, were recorded in SA patients (Fig. 3b).

3.4. PCA revealed an immune rebalancing trend after benralizumab administration

Based on the flow cytometry data of the NK and T cell subsets, we performed supervised PCA analysis of SA patients at T0, T1 and T6. The PCA plot showed that samples from different groups clustered together at T0 and T6 (Fig. 4a). For PCA analysis, Unit Variance Scaling was applied to the rows to calculate principal components. The X and Y axes show principal components 1 (PC1), 2 (PC2) and 3 (PC3) which explain 31,6%, 17% and 13,3% of the total variance, respectively. The prediction ellipses enclosed 0.75% of the points (Fig. 4a).

3.5. NK cells acquired cytotoxic potential and were activated after benralizumab administration

In order to establish whether immunological pathways tended to rebalance after benralizumab treatment in SA patients, we analysed the cell subsets at T1 and T6. Interestingly, total NK cell count was enhanced after drug administration. Immature $CD56^{br}CD16^{neg}$ phenotypes decreased in favour of the cytotoxic phenotype $CD56^{dim}CD16^{br}$, which increased in SA patients, together with the maturest memory-like and terminally differentiated NK phenotypes, characterized by expression of NKG2C and CD57 (Fig. 4b). These results were also supported by the observation of higher serum concentrations of IFN-γ at T6 (Fig. 4c) (suppl.table 2).

Regarding T cell subsets, a rebalancing of T naive and TEM subsets of CD4 and CD8 was recorded at T6, while TCM continued to increase after administration of drug. No changes were observed in CD4 and CD8 TEMRA subsets (Fig. 4d). To investigate activation of CD4, CD8 and CD56, expression of CD137 on the cell surface of these subsets was evaluated. Interestingly, CD4 and CD8 T cells showed significantly enhanced expression of CD137 at T6, whereas for CD56, expression of this marker was already significantly enhanced at T1 and further increased at T6 (Fig. 5a) (suppl.table 3).

3.6. NK- and T-cell proliferative capacity increased in SA patients after administration of benralizumab

Since expression of CD137 on cell surfaces is known to induce cell proliferation, we also evaluated the proliferation rate of T and NK cell subsets. The probe CellTrace™ Violet was used to assess the SA cohort at T0, T1 and T6 for $CD3^{+}CD4^{+}$, $CD3^{+}CD8^{+}$ T, NK and $CD56^{dim}$ NK cells. As reported in Fig. 5b, increased proliferative capacities were found at T6. $CD56^{dim}$ NK cells were the phenotype undergoing the greatest proliferation.

3.7. Cytokine dysregulation in asthma patients

Multiplex analysis revealed that cytotoxic protein TGF-beta and death proteins sFas and sFasL, part of the fundamental axis inducing apoptosis in target cells, were significantly depleted in both groups of asthma patients, as was IL-10, a typical regulatory protein of inflammation. On the other hand, IL-33 was greatly enhanced only in the M/M group (Fig. 6a). After benralizumab therapy, no clear pattern emerged regarding serum concentrations of these proteins. Those of TGF-beta and sFas decreased between T0 and T6, while IL-5 and sFas dropped between T1 and T6, demonstrating a reduced pro-inflammatory cytokines after benralizumab treatments. Interestingly, a regulatory protein IL-10, mainly produced by TREG cells, decreased at T1, however it resulted to increase at T6. IL-33 was the only protein that increased significantly at T6 (Fig. 6b).

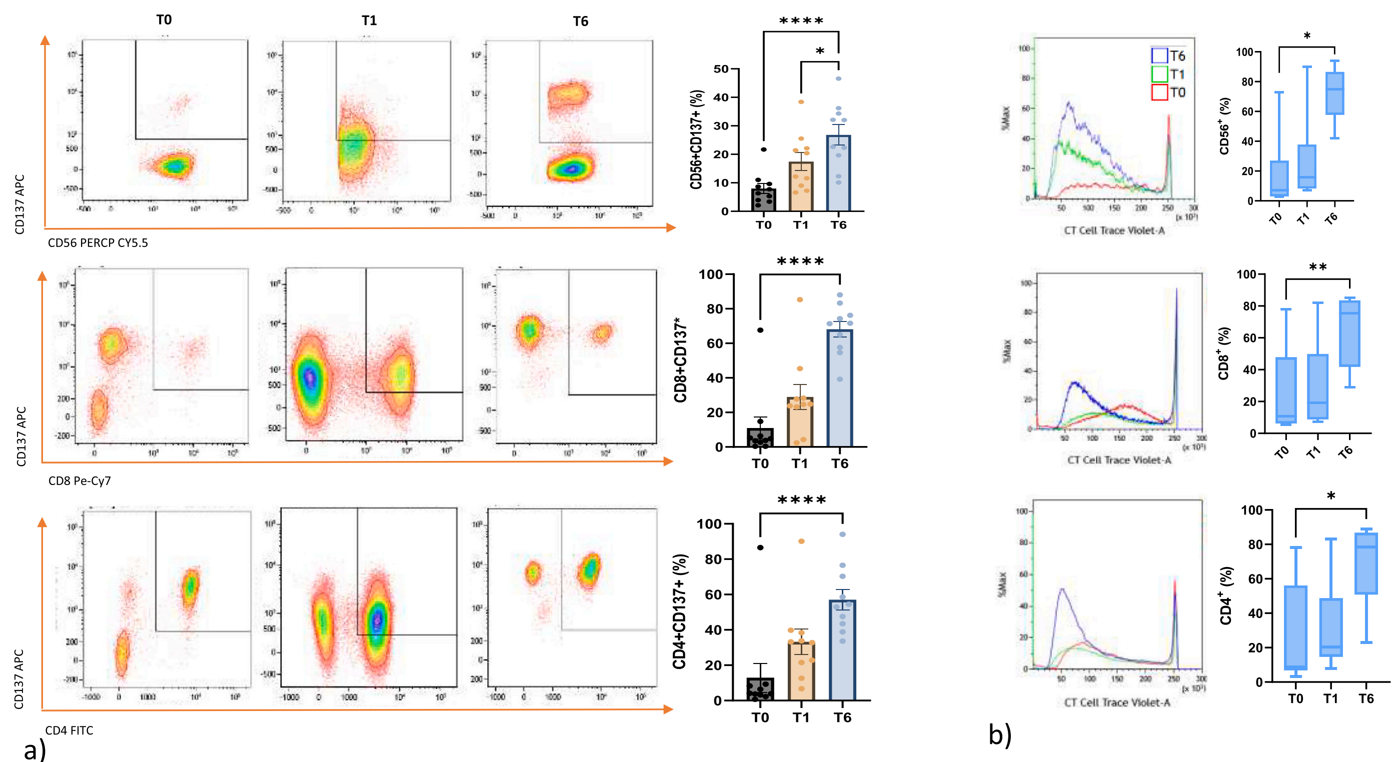

Fig. 5. a) Flow cytometric analysis of CD137 expressed on the surface of NK, CD4 and CD8 T cells. b) Proliferation assay with the probe Cell Trace Violet combined with other surface markers (CD4, CD8 and CD56) to determine the proliferation rate of these cell phenotypes in severe asthma patients before (T0) and after (T1 and T6) administration of benralizumab. Abbreviations: CD: cluster of differentiation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

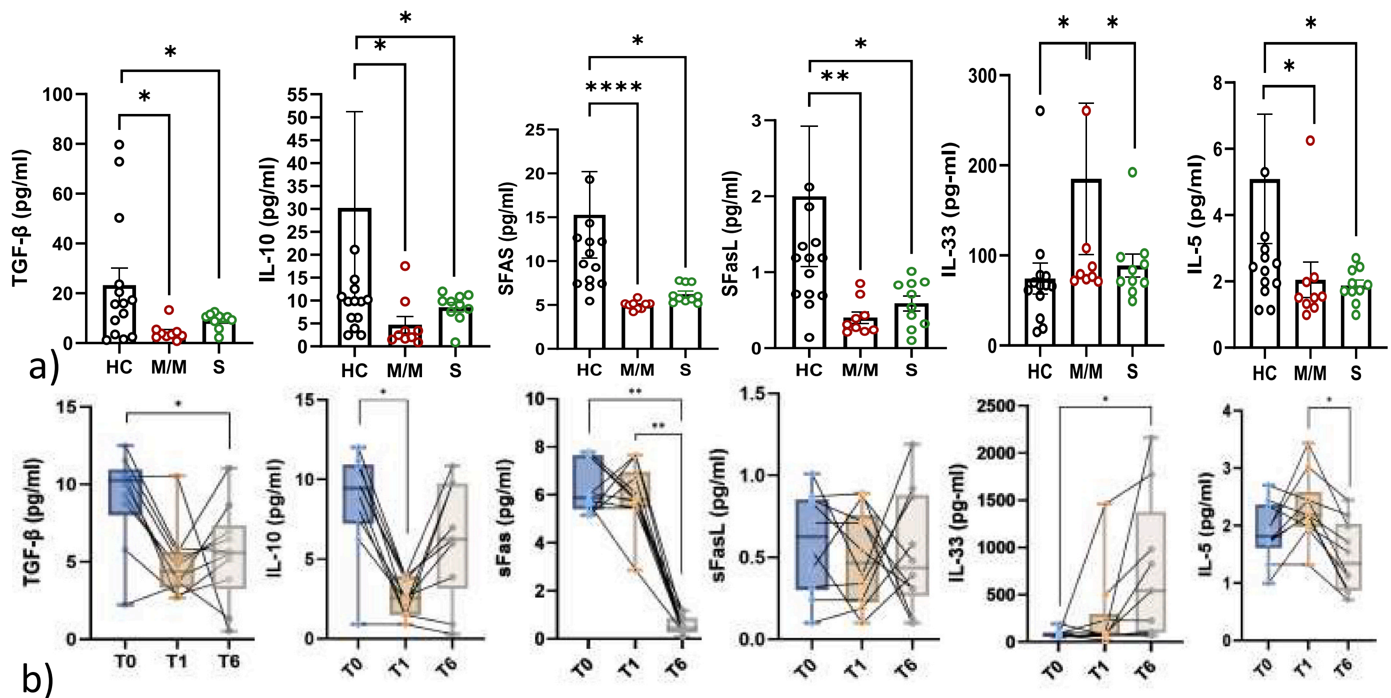

Fig. 6. a) Histograms of different cytokines detected by multiplex assay in the serum of our cohort. b) The same cytokines were also detected in severe asthma patients at T0, T1 and T6. The results are expressed in pg/ml. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$. Abbreviations: Sfas: soluble FAS, Sfasl: soluble FAS ligand, TGF: tumor growth factor, IL: interleukin.

3.8. Correlation analysis of clinical and lung function parameters with immunological aspects

In order to evaluate the clinical utility of changes in cell subsets during benralizumab treatment, we looked for correlations of PFTs and the classical biomarkers used to monitor response to treatment (such as eosinophil count and percentages) with the cell subsets. It emerged that the change in $CD3^+$ cells was directly correlated with changes in FEV1 (% and ml), FVC (%), and FEV1/FVC ratio. Activation of NK cells expressed as $CD56^+CD137^+$ NK cell percentage was directly correlated with changes in FEV1%, FEV1/FVC ratio and CD3 percentages. The change in CD56 proliferation was indirectly correlated with oral corticosteroid dose. Finally, changes in IL-33 were indirectly correlated with changes in peripheral eosinophil percentages (Fig. 7).

4. Discussion

A common phenotype among SA patients is high levels of eosinophils in serum and sputum, associated with frequent moderate-to-severe exacerbations and a good response to oral corticosteroid treatment. Considering the high health risks and social costs associated with severe eosinophilic asthma, the three currently approved drugs which target the IL-5 pathway (mepolizumab, benralizumab and reslizumab) have demonstrated reliable cost-effectiveness, significantly improving clinical control of the disease, risk of exacerbation/hospitalization and reducing the use of oral corticosteroids [22]. Benralizumab is the only biologic that acts as a receptor antagonist, binding IL-5Rα which is expressed on the surface of eosinophils and basophils. However, the immunological effects of blocking the IL-5 pathway in SA patients remain largely unknown.

Our study provides a new perspective on benralizumab's anti-eosinophilic activity, since it focused on immunological modifications of NK and T cells and their correlations with clinical outcomes in peripheral blood of severe asthma patients. Fig. 8 reported the mechanisms of action evidenced in the present study.

It is intuitive that benralizumab may influence the cytotoxicity of NK cells: its mechanism of action is closely linked to the afucosylated Fc domain in the RIIIa region of the Fcy receptor on NK cells, macrophages and neutrophils, thus strongly inducing ADCC in circulating and tissue-resident eosinophils [7,10]. Most recent evidence describes complex cross-talk between eosinophils and NK cells: in healthy subjects, NK cells exposed to innate cytokines (also produced by eosinophils) may amplify their effector functions and release IFNγ, auto-promoting their maturation [11]. Formation of an eosinophil/NK cell synapse enhances NK cytotoxicity, allowing stimulator signals to bypass inhibitor receptors, such as KIR [23,24]. On this topic, our study showed that NK cell activity is significantly impaired in SA patients, characterized as these patients are by less cytotoxic activity with reduced $CD56^{dim}CD16^{br}$ subsets. The pathogenic significance of this alteration is also confirmed by the inverted trend of these cell subsets after administration of benralizumab, a trend accompanied by a decrease in the immature subset $CD56^{br}CD16^{neg}$ which has immunoregulatory functions. The proliferative potential and activation status of NK cells, evaluated via expression of CD137 on the membrane surface after benralizumab administration, provided further insights. CD137 is a validated marker of NK cell activation, known to be upregulated after binding of the FcRγIII receptor (CD16) with the Fc portion of monoclonal antibodies. In cancer, CD137 is demonstrated to enhance the function of mAb-mediated antibody-dependent cell-mediated cytotoxicity [25]. CD137 activation may also limit IL-5–mediated antiapoptosis of eosinophils [26]. The absence of the CD137 anti-inflammatory mechanism may further increase eosinophil numbers at inflammatory sites in asthma patients [27]. Our data also sustains the crucial role of CD137 expression for activation of antibody-dependent cell-mediated cytotoxicity in SA patients treated with benralizumab, suggesting that the drug may directly influence NK activation status. Our findings suggested that benralizumab induces expression of CD137 on activated NK cells and this may favour apoptosis of eosinophils [26]. The results regarding apoptosis was also confirmed through the detection of sFas and sFasL in the sera of our group of patients. Apoptosis was triggered upon Fas/FasL binding. Fas-mediated signaling is defective in asthma, leading to delayed resolution of inflammation [28]. The decrease level of sFas and sFasL support the

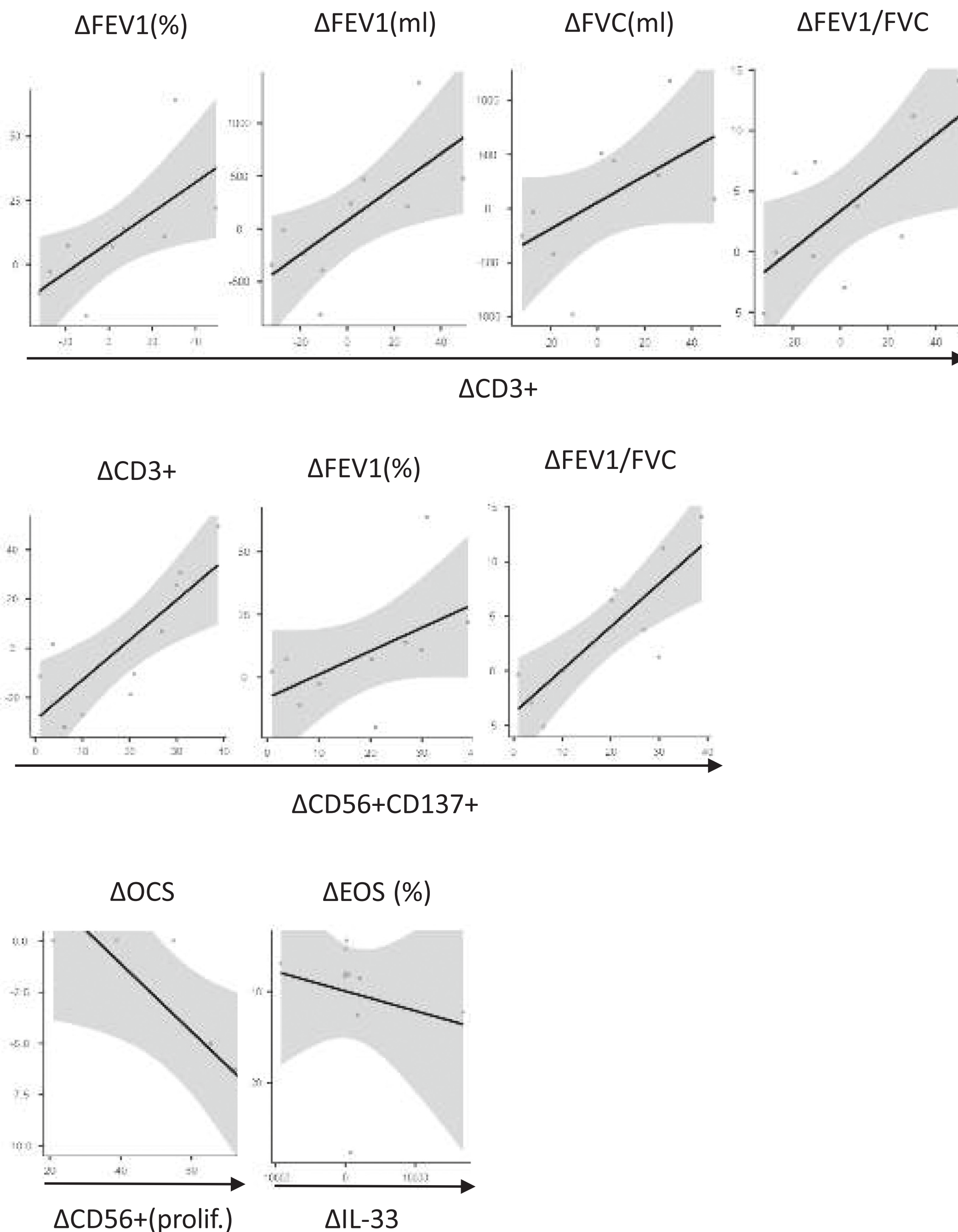

Fig. 7. Spearman correlation analysis with rho coefficients of clinical changes and changes in cell subsets or proliferation, all expressed as Δ (T6-T0). Abbreviations: OCS: oral corticosteroid, EOS: eosinophils in peripheral blood, FEV1: Forced Expiratory Volume in the 1st second, FVC: forced vital capacity.

inflammatory status through T cell activation and inhibition of cell death.

CD137 is also considered a critical receptor for stimulation of T cells that mediate cross-talk between innate and adaptive immunity by promoting T cell proliferation and cytokine production [29]. Considering the crucial role of T cell subtypes in the pathogenesis and severity of asthma, here we also evaluated the cross-talk between NK and T cell subsets. NK cells interact with T cell activation while NK cells can kill activated $CD4^+$ T cells by virtue of their cytotoxic properties [30]. When primed by various soluble factors, NK cells boost the maturation and activation of T cells through a combination of cell surface receptors and cytokines, first and foremost IFN-γ [31,32]. These assumptions/hypotheses are in line with our findings, since a relevant increase in IFN-γ levels was detected after 6 months of benralizumab therapy, associated with a substantial, albeit partial, restoration of the balance of T cell subtypes.

In our SA patients, an unbalanced distribution of T cell subtypes emerged, particularly concerning the percentages of TEM and TCM subsets. After administration of monoclonal antibody, TEM cells decreased and release of effector cytokines (such as IL-5 and TGF-β) attenuated, while TCM showed an increasing trend. Functionally, TCM and TEM cells both produce effector cytokines when stimulated [15]. TCM cells show lymphoid homing profiles and high proliferative capacity, while TEMcells produce more effector cytokines [16]. Our findings therefore suggest that the effectiveness of benralizumab in improving clinical control of asthma and lung function goes beyond "simple" depletion of eosinophils in blood and tissues, since there is evidence that it modified the immune profile of SA patients. Moreover, a

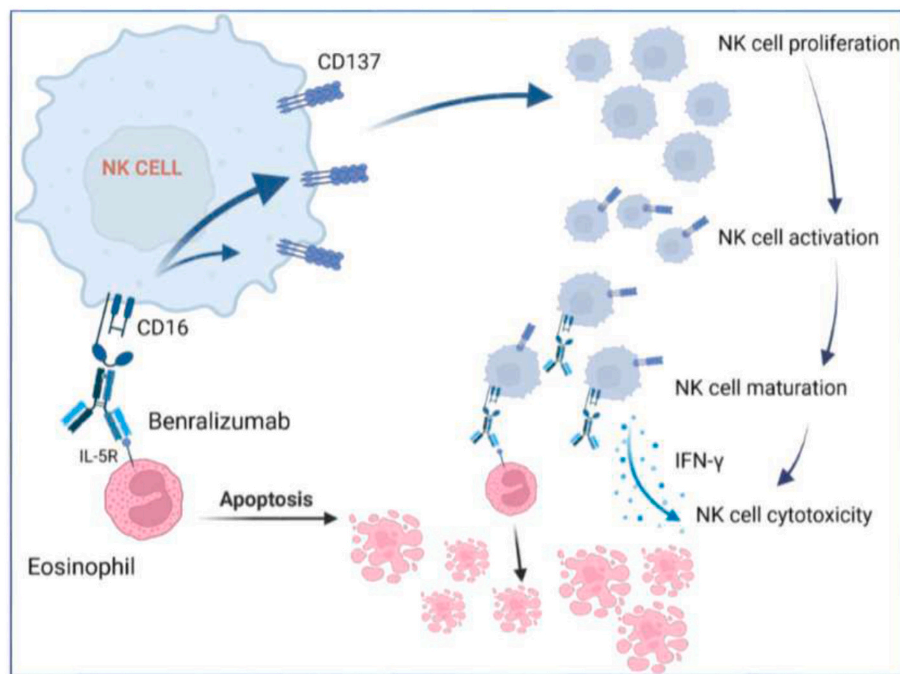

Fig. 8. Graphic representation of the main mechanisms evaluated by analysis of blood from severe asthma patients treated with benralizumab Abbreviations: CD: cluster of differentiation.

regulatory protein IL-10, mainly produced by TREG cells, resulted to increase after 6 months of bernalizumab treatment, suggesting a possible rebalancing of regulatory T cells functions, merits of further experiments in a larger cohort.

Interestingly, some of these immunological modifications during benralizumab treatment appeared to be significantly correlated with the change in clinical and lung function parameters observed in our patients, suggesting that these "background" effects may be useful in the clinical management of SA. In particular, the change in CD3 percentages was directly correlated with improvement in FEV1, FVC and the FEV1/FVC ratio, and with modifications in activated NK cells (i.e. $CD56^{+}CD137^{+}$). Furthermore, the change in CD3 was also correlated with expression of this NK cell phenotype, highlighting the role of both cell lines in the response to treatment. Intriguingly, we observed an inverse association between the daily dose of oral corticosteroids and the proliferative capacity of NK. Corticosteroids, commonly used as anti-inflammatory agents in SA, also appeared to further disable NK cell functions, paradoxically contributing to persistent airway inflammation [12,33]. Conversely, when oral corticosteroids were decreased or suspended on the basis of clinical judgment, NK cells tended to proliferate. Although investigation of the effects of oral corticosteroids on NK cells in SA is beyond the scope of this study, our findings are interesting since they suggest that oral corticosteroids not only counteract immune imbalance in eosinophilic SA, but may also cause detrimental effects in this setting, beyond the well-known adverse effects and safety issues related to this drug regimen. In our opinion that change in NK cells is due to an interplay of OCS dose and benralizumab administration.

Together this data contributes to our understanding of the mechanisms of action of benralizumab in combination with decrease use of OCS in severe asthmatic patients. It was observed a shifting of NK cells towards the most mature phenotypes, similar to those reported in HC. The activation and proliferation of NK cells allow depletion of eosinophils.

4.1. Study limitations

In recent times, the number of monoclonal antibodies for the treatments of SA was increased. However, no clear strategy regarding the choose of these drugs were done. This is also the result of of relative novel availability of these moAbs. This is the first study that consider cohort of SA patients treated with benralizumab where several aspects of immune system were analysed. Although, the limit of our study comprises the monocentric nature of the research, small sample size, as well as the missing of unresponsive patients to benralizumab treatments that can further explain the cell subsets that may contribute to the mechanisms of action of benralizumab, allowing the clinician to make a better management of these complex kind of patients.

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None.

Informed consent and local ethics committee

All subjects gave their written informed consent to participation in the study, which was approved by our Local Ethics Committee CEAVSE (code number 180712 and Markerlung 17431). This study was performed in accordance with the Declaration of Helsinki.

Author contribution statement

LB, MdA and PC designed the study, PC and TP wrote the study protocol, and gained ethical approval for the study. PC, TP, EB and BC recruited all the patients and carried out the study. LB, MdA performed the experiments. TP performed the analysis. LB and MdA wrote the manuscript. All authors critically revised the manuscript together.

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 a 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2023.109680.

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