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ORIGINAL RESEARCH

The Effect of Mepolizumab on Blood Eosinophil Subtype Distribution and Granule Protein Gene Expression in Severe Eosinophilic Asthma

Airidas Rimkunas[1], Andrius Januskevicius[1], Egle Vasyle[1], Jolita Palacionyte[2], Virginija Kalinauskaite-Zukauske[2], Skaidrius Miliauskas[2], Kestutis Malakauskas[1,2]

[1]Laboratory of Pulmonology, Department of Pulmonology, Lithuanian University of Health Sciences, Kaunas, Lithuania; [2]Department of Pulmonology, Lithuanian University of Health Sciences, Kaunas, Lithuania

Correspondence: Airidas Rimkunas, Laboratory of Pulmonology, Department of Pulmonology, Lithuanian University of Health Sciences, A. Mickeviciaus st. 9, Kaunas, LT-44307, Lithuania, Email Airidas.rimkunas@lsmu.lt

Purpose: Mepolizumab, which causes a decrease in the number of blood eosinophils, is used to treat patients with severe eosinophilic asthma (SEA). However, there is a relative lack of data on dynamic changes in blood eosinophil subtype distribution and their granule protein expression following anti-interleukin (IL)-5 treatment. Our objective was to evaluate blood inflammatory-like (iEOS-like) and resident-like (rEOS-like) eosinophil subtype distribution and *CLC, EDN, EPX, ECP*, and *MBP* gene expression following up to 24 weeks of treatment with mepolizumab in SEA patients.

Patients and Methods: Ten free of oral steroids SEA patients and 9 healthy control subjects (HS) were included. Patients were treated with mepolizumab 100 mg subcutaneously/4 weeks, and investigation tests were performed at 0, 4, 12, and 24 weeks. Blood eosinophils were isolated by Ficoll centrifugation and magnetic separation, then subtyped using magnetic separation against CD62L. Gene expression investigation was done using quantitative real time-polymerase chain reaction analysis.

Results: Approximately three-quarters of isolated blood eosinophils were iEOS-like cells before mepolizumab treatment, $p<0.01$. Blood eosinophil granule protein gene expression was increased in SEA patients compared to the HS, $p<0.05$, and iEOS-like cells *EPX, MBP*, and *CLC* gene expressions were higher than rEOS-like cells, $p<0.05$. Following 4, 12, and 24 weeks of treatment with mepolizumab, residual blood eosinophils shifted towards rEOS-like cells, $p<0.05$, and *CLC, EPX, ECP*, and *MBP* gene expression of both eosinophil subtypes decreased to HS levels.

Conclusion: Treating SEA patients with mepolizumab shifts blood eosinophil subtype distribution towards rEOS-like cells and reduces granule protein gene expression levels to those of healthy individuals.

Keywords: anti-IL-5 treatment, severe asthma, eosinophil subtypes, gene expression

Introduction

Asthma is a heterogeneous respiratory disease, characterized by airway hyperreactivity and chronic airway inflammation. These features usually persist even without symptoms or normal lung function but can be normalized with treatment. In mild-to-moderate asthma, inhaled corticosteroids, bronchodilators, and self-management training are the mainstays of effective treatment.[1] However, approximately up to 10% of asthma patients present with a severe form that does not respond to standard high-intensity treatment.[2] Asthma is classified into endotypes and phenotypes to select individualized treatment and achieve effective responses properly. The most common endotype of asthma diagnosed in adults is T2 asthma, which is characterized by blood and/or airway eosinophilia and expression of the cytokine interleukin (IL)-4, IL-5, IL-13 and granulocyte macrophage-colony stimulating factor (GM-CSF) produced by type 2 helper lymphocytes (Th2) or type 2 innate lymphoid cells (ILC2).[3,4] Eosinophil differentiation, activation, and survival are primarily regulated by IL-5 and to a lesser extent by IL-3 and GM-CSF, whereas IL-4 and IL-13 stimulate B cells to produce immunoglobulin (Ig)E. Eosinophils are one of the main effector cells involved in the inflammatory response in the lungs and contribute to airway innervation[5] and structural changes,

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also known as remodeling.[6] Clinically, higher eosinophil levels often correlate with airway hyper-responsiveness, exacerbation number, airflow limitation, asthma control, and quality of life.[7,8]

The recent advances in our knowledge of the T2 inflammatory pathways have led to the development of biological therapies acting on the T2 cascade, introducing new treatment options to a subgroup of severe asthma patients with persistent airway eosinophilia despite using oral corticosteroids (OCS) or high doses of inhaled steroids.[9] Various studies have demonstrated the existence of distinct eosinophil subtypes - pro-inflammatory eosinophils (iEOS), largely involved in immune responses, and homeostatic or resident eosinophils (hEOS, rEOS), responsible for various organ homeostasis, metabolism and immunoregulation.[10,11] Similar receptor-expressing eosinophils have been identified in blood as iEOS-like and rEOS-like cells based on CD62L expression, and their distribution was revealed to be different between patients with asthma or chronic obstructive pulmonary disease.[12] Therefore, eosinophil subtype distribution has the potential to become a surrogate biomarker to characterize the predominant pathway in patients with eosinophilic inflammation and aid in selecting effective biological treatment.[13]

Eosinophils store and secrete various preformed cytokines, chemokines, enzymatic and nonenzymatic cationic proteins from their primary and secondary granules.[14] The main cationic proteins are eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), major basic protein (MBP), and eosinophil peroxidase (EPX).[15] In addition, eosinophil cytoplasm contains an autocrystallizing protein Galectin-10, which forms distinctive hexagonal bipyramidal Charcot-Leyden crystals (CLC) that are considered a hallmark of eosinophilic inflammation in body fluids and tissue.[16] Bronchial damage and structural changes caused by chronic eosinophilic inflammation are attributed to their degranulation when the previously mentioned granule proteins are released into the extracellular environment after stimulation by inflammatory mediators.[17] Higher mRNA levels of eosinophil granule proteins such as EPX and MBP have been attributed to airway hyperresponsiveness and airflow limitation and may have clinical potential to be used to assess asthmatic patient disease severity.[18] Thus, further eosinophil subtype and their granule protein research are required to unravel these cells' distinct roles in eosinophilic airway disease pathogenesis and clinical implications.

IL-5 is described as the crucial cytokine controlling eosinophil biological properties,[17] thus became one of the biological treatment targets of eosinophilic inflammation-driven diseases.[19,20] Mepolizumab is a monoclonal antibody against IL-5, which has been approved as a therapeutic agent for managing severe eosinophilic asthma. Mepolizumab prevents human IL-5 from binding to the α-chain of the IL-5 receptor complex on the cell surface, thus inhibiting the IL-5 signaling cascade and depleting eosinophils, but not basophils and T-cells.[21,22] This results in significant eosinophil count depletion, improved clinical symptoms, and reduced OCS usage.[19] In addition, treatment with mepolizumab has been shown to attenuate EDN and IL-3 receptor expression in eosinophils.[23] Nonetheless, more data should be available regarding residual eosinophil subtype distribution and biological activity following IL-5 pathway blockade. This understanding could provide information regarding disease monitoring, predicting treatment response to biological therapy, and potential broader health implications of eosinophil depletion.

This study investigated the blood eosinophil subtype distribution and granule protein *EPX, MBP/PRG2, EDN/RNASE2, ECP/RNASE3, CLC/GAL10 g*ene expression during 24 weeks of treatment with anti-IL-5 drug mepolizumab in patients with SEA.

Material and Methods

Ethics

The study complies with the Declaration of Helsinki. Each investigated subject was introduced with the research protocol confirmed by the Lithuanian University of Health Sciences (LUHS) Committee of Regional Biomedical Research Ethics (BE-2-58) and signed a written informed consent form. The study is registered in the U.S. National Institutes of Health ClinicalTrials.gov trial registry with the unique identifier NCT04542902.

Study Design and Experimental Plan

All study subjects were patients from the Department of Pulmonology, LUHS Hospital Kaunas Clinics. At the initial visit, each individual underwent a health history, physical examination, and spirometry to confirm the inclusion and exclusion criteria, presented in Figure 1. The inclusion criteria for SEA patients were based on biological anti-IL-5 drug

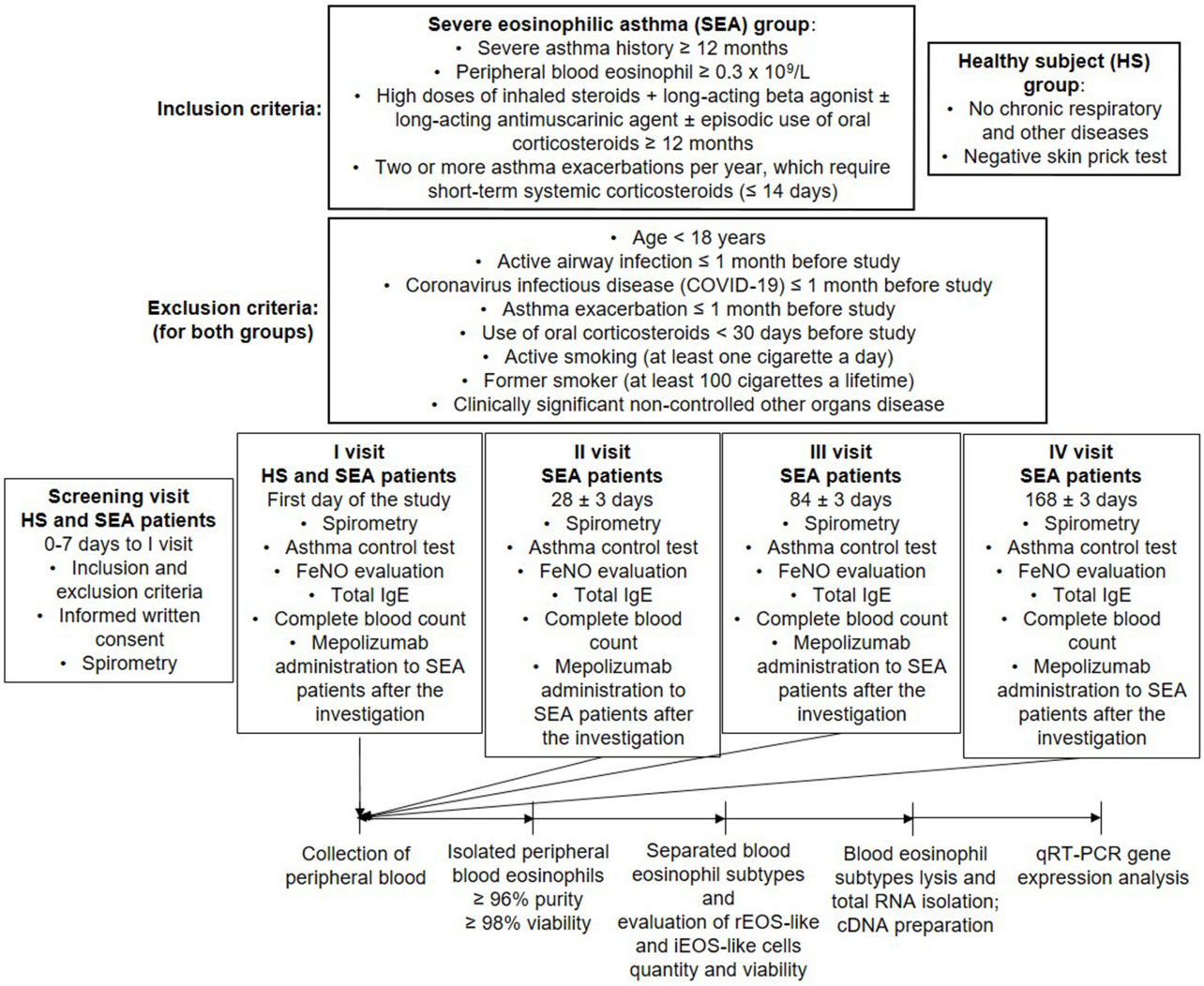

Figure 1 Inclusion, exclusion criteria, study design, and experimental plan.
Abbreviations: cDNA, complementary deoxyribonucleic acid; RNA, ribonucleic acid; qRT-PCR, quantitative real-time polymerase chain reaction.

mepolizumab prescription, administration and treatment guidelines. The healthy subject control group was comprised of non-atopic individuals with negative skin prick test. Afterward, the first experimental visit was arranged 0–7 days after inclusion into the study. During the experimental visit, blood samples were taken for complete blood count, total IgE, and eosinophil isolation. In addition, subjects underwent spirometry, fractional exhaled nitric oxide (FeNO) evaluation, and the biologic drug mepolizumab was administered (100 mg subcutaneously) to SEA patient group. Afterward, patients visited the hospital every 28 ± 3 days to continue the biological treatment, and the clinical and experimental examination was carried out as shown in Figure 1.

Complete Blood Count and Serum Total Immunoglobulin E

The investigated subjects' blood was collected in sterile vacutainers (BD Vacutainer Systems, Plymouth, UK) and transported directly to the LUHS Hospital Kaunas Clinics laboratory. The hematology analyzer UniCel® DxH 800 Coulter® Cellular Analysis System (Beckman Coulter, Miami, FL, USA) measured a complete blood count test, and the immunoassay analyzer AIA-2000 (Tosoh Bioscience, South San Francisco, CA, USA) measured serum total immunoglobulin E (IgE) concentration.

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Lung Function Testing

The investigated subjects underwent spirometry testing during each visit using an ultrasonic spirometer (Ganshorn Medizin Electronic, Niederlauer, Germany). Measured results of forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), and the FEV_1/FVC ratio were considered the largest of the three independent measurements, as previously described.[24]

Fractional Exhaled Nitric Oxide Measurement

During each experimental visit, FeNO was evaluated by a handheld Vivatmo-me device (Bosch Healthcare Solutions, Waiblingen, Germany) according to the manufacturer's instructions. Before each measurement, the device conducted a brief self-calibration. The individual was tasked to exhale through the mouthpiece in a steady flow for approximately 5 s, and the test was repeated twice. To avoid cross-contamination, the examining personnel was equipped with protective equipment, the Vivatmo-me device was sanitized between uses, and the mouthpiece was replaced after each use.

Peripheral Blood Eosinophil Isolation, Purification, and Subtyping

During the investigated individuals' experimental visit peripheral blood (approximately 30 mL) was drawn into sterile vacutainers with K_2EDTA (BD Vacutainers Systems). Red blood cell lysis, granulocyte isolation, eosinophil enrichment, purification, and subtyping were performed as previously described.[24] A flow cytometry dot plot of isolated blood eosinophils labeled with CD62L antibody is shown in Figure 2. After magnetic separation with CD62L magnetic beads, the total amount and viability of isolated eosinophils were assessed by ADAM (NanoEnTek Inc, Mountain View, CA, USA), while a flow cytometer FacsCalibur (BD Biosciences, San Jose, CA, USA) was used to confirm blood eosinophil subtypes. The isolated blood eosinophil sample was used after corresponding to viability (≥95%) and purity (>96%) criteria.

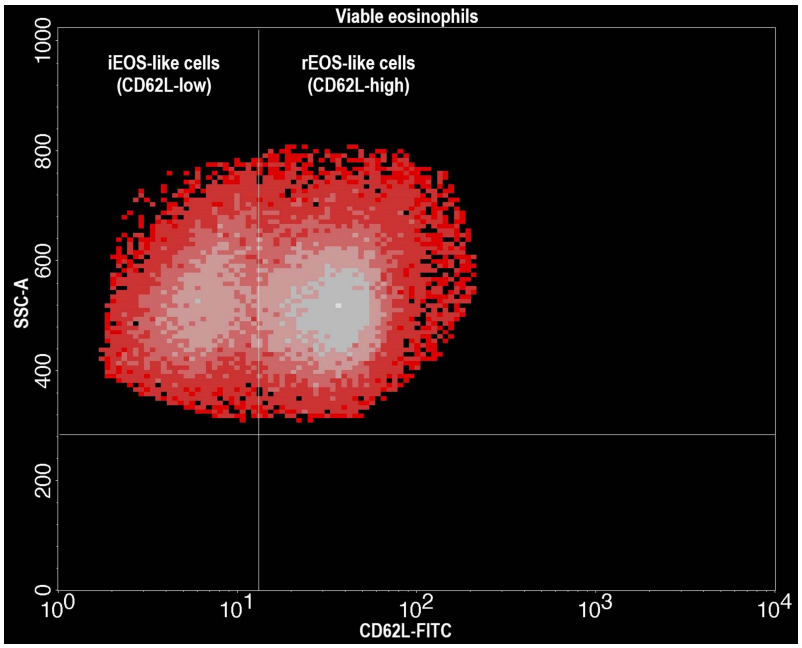

Figure 2 Isolated blood eosinophils. Eosinophils were labeled with CD62L-FITC antibody, excluding cell debris (with forward scatter/side scatter), and gated using a fixed threshold.

Abbreviations: SSC, side scatter; CD62L, L-selectin; FITC, Fluorescein isothiocyanate.

Eosinophil RNA Isolation and Gene Expression Analysis

Isolated blood eosinophil subtypes were lysed thoroughly by Trizol reagent (InvitrogenTM, UK), and RNA was purified with miRNeasy MiniElute kit (Qiagen, Maryland, USA) as stated by the manufacturer's protocol. During the isolation process, an additional DNA digestion step was included using an RNase-Free DNase Set (Qiagen). Isolated RNA purity and concentration were measured with a microvolume spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific, USA), and the sample RNA was converted to copy DNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems by Thermo Fisher Scientific).

Eosinophil subtype granule protein gene expression analysis was performed using quantitative real-time polymerase chain reaction (PCR) analysis with custom setup TaqMan® Array Fast Plates (Applied Biosystems by Thermo Fisher Scientific, Vilnius, Lithuania) in the Applied Biosystems 7500 Fast Real-Time PCR System according to the manufacturer's instructions. Actin beta (*ACTB*) and 18S ribosomal RNA (*18S*) were used as endogenous control genes for data normalization. The SEA patients' blood eosinophil subtype gene expression changes were evaluated based on a fold change compared to the HS group's respective eosinophil subtype gene expression. Normalized relative gene expression data are presented as relative fold changes, calculated from the $2^{-\Delta\Delta Ct}$ formula.

Statistical Analysis

Statistical data analysis and visualization used GraphPad Prism 8 for Windows (ver. 8.0.1, 2018; GraphPad Software Inc., San Diego, CA, USA). Results are presented as mean ± standard error of the mean. The Shapiro–Wilks normality test was implemented to examine the normality in data distribution. The data distribution did not pass the normality test; thus, non-parametric tests were used. The Mann–Whitney two-sided *U*-test evaluated distinctions between two independent groups. Meanwhile, the Wilcoxon matched-pairs signed-rank two-sided test was used for two dependent groups. A $p < 0.05$ value was considered statistically significant.

Results

Study Subject Characteristics

The study enrolled 10 SEA patients (1 male, 9 females) and 9 healthy control subjects (2 males, 7 females). SEA patients were older (58.3 ± 3.5 vs 29.3 ± 2.1 years), with a higher body mass index (BMI) (29.6 ± 2.5 vs 21.9 ± 0.9 kg/m2), and showed a worse lung function (1.51 ± 0.13 vs 3.96 ± 0.26 FEV_1/L; 58.7 ± 5.2 vs 103.2 ± 3.69 FEV_1, % of predicted) than the HS group, $p < 0.05$. In addition, blood eosinophil count (0.715 ± 0.129 vs 0.111 ± 0.011×109 cells/l), FeNO (56.4 ± 17.2 vs 10.0 ± 2.8, ppb), and total IgE (149.5 ± 48.8 vs 14.1 ± 3.9, IU/mL) in patients with SEA before treatment with mepolizumab were significantly elevated compared to the HS group, $p < 0.05$.

The clinical characteristics of the SEA group following treatment with mepolizumab are presented in Table 1. Following 4 weeks after the first dose of mepolizumab (V2), blood eosinophil count decreased by 85.6%. Similarly, after 12 and 24 weeks of treatment with mepolizumab, blood eosinophil count remained significantly reduced. Interestingly, after 12 weeks of treatment with mepolizumab (3 doses in total), blood eosinophil count further decreased, compared with 4 weeks of treatment (V2). In addition, four weeks after beginning treatment with mepolizumab, the asthma control test (ACT) scores and FEV_1 significantly increased, $p < 0.05$. These changes remained throughout the investigated treatment period, while FeNO and total IgE values stayed unaffected.

Blood Eosinophil Subtype Distribution Following Treatment with Mepolizumab

After isolating blood eosinophil subtypes from biologic-naive SEA patients, 72.9 ± 4.3% of total isolated eosinophils were iEOS-like cells compared to 27.1 ± 4.3% rEOS-like cells, $p < 0.0001$ (Figure 3a). Following treatment with mepolizumab, the residual blood eosinophils pool shifted towards rEOS-like cells - 62.1 ± 5.6% vs; 61.2 ± 4.4%; and 63.4 ± 4.3% of total isolated eosinophils after 4, 12, and 24 weeks of treatment, respectively, $p < 0.05$. In addition, individual SEA patients' blood eosinophil subtype distribution during each experimental visit is shown in Figure 3b and c. Comparing differences between investigated subject groups, we found that SEA patients' iEOS-like cell proportion was significantly higher at V1; however, after 12 and 24 weeks (V3-V4) of treatment with mepolizumab the remaining percentage of iEOS-like cells in SEA patients' was notably less than in healthy individuals, # $p < 0.05$. Similarly, SEA

Table 1 Clinical Characteristics of the SEA Group Following Treatment with Mepolizumab

Characteristic	Severe Eosinophilic Asthma Patients			
Number, n	10			
	V1 – baseline	V2 – 4 weeks	V3 – 12 weeks	V4 – 24 weeks
ACT, score	10.1 ± 0.7	16.5 ± 1.3 *	17.8 ± 1.3 **	17.6 ± 1.3 **
FEV_1, L	1.51 ± 0.13	1.95 ± 0.22 **	2.04 ± 0.18 **	2.00 ± 0.15 **
FEV_1, % of predicted	58.7 ± 5.2	76.2 ± 8.4 **	76.6 ± 6.6 **	78.3 ± 5.5 **
FeNO, ppb	56.4 ± 17.2	35.4 ± 7.8	49.8 ± 17.0	38.9 ± 10.6
Blood eosinophil count, $\times 10^9$ cells/l	0.715 ± 0.129	0.093 ± 0.022 **	0.054 ± 0.009 **	0.063 ± 0.017 **
Total IgE, IU/mL	149.5 ± 48.8	143.6 ± 47.5	129.2 ± 45.0	121.3 ± 43.7

Notes: * $p < 0.05$, ** $p < 0.01$, compared with V1 results. Data presented as mean ± standard error of the mean. Data presented as mean ± standard error of the mean.

Abbreviations: ACT, asthma control test; IgE, immunoglobulin E; FeNO, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 s; V1, first experimental visit before starting mepolizumab treatment; V2, visit 4 weeks after beginning mepolizumab treatment (after 1 mepolizumab dose); V3, visit 12 weeks after beginning mepolizumab treatment (after 3 mepolizumab doses); V4, visit 24 weeks after beginning mepolizumab treatment (after 6 mepolizumab doses).

rEOS-like cell proportion at V1 was noticeably lesser than the HS group, but significantly increased at V3 and V4, # $p < 0.05$. Lastly, SEA patients' blood eosinophil subtype proportions before treatment and 3, 12, and 24 weeks (V2-V4) after treatment changed considerably, * $p < 0.05$.

Blood Eosinophil Subtype Granule Protein Gene Expression Analysis

We investigated the gene expression of five eosinophil granule proteins - *EPX, ECP/RNASE3, EDN/RNASE2, MBP/PRG2*, and *CLC/GAL-10*, which are implicated in eosinophil contribution to airway inflammation, remodeling, and tissue damage.[14,15] At baseline, SEA patients' iEOS-like cells displayed enhanced granule protein gene expression compared to rEOS-like cells: iEOS-like cells *CLC* expression was 3.1 ± 0.5-fold vs 1.8 ± 0.2-fold, $p = 0.0411$ higher than rEOS-like cells; *EPX* (2.6 ± 0.3-fold vs 1.3 ± 0.2-fold, $p = 0.0411$), and *MBP* (2.5 ± 0.3-fold vs 1.7 ± 0.1-fold, $p = 0.0260$), with no significant differences in ECP (1.9 ± 0.3-fold vs 1.8 ± 0.2-fold, $p > 0.99$) and EDN (2.4 ± 0.3-fold vs 3.0 ± 0.4-fold, $p = 0.3$) expression. In addition, SEA patients' blood eosinophil subtypes investigated granule protein gene expression at V1 was significantly elevated compared with the HS group, # $p < 0.05$, as shown in Figure 4.

Following 4, 12, and 24-week treatment with mepolizumab (V2-V4), blood iEOS-like cells' *CLC, EPX, ECP, MBP*, and rEOS-like cells' *CLC, MBP*, and *EDN* gene expression decreased to HS group levels. Interestingly, iEOS-like cells' *EDN* (1.5 ± 0.1-fold, $p = 0.0313$) gene expression remained significantly higher than the HS group. In contrast, rEOS-like cells *EPX* (0.7 ± 0.1-fold, $p = 0.0313$) and *ECP* (0.4 ± 0.1-fold, $p = 0.0313$) expression diminished significantly below HS group levels.

Comparison between blood eosinophil subtypes throughout mepolizumab treatment revealed that iEOS-like cells' *EPX* and *ECP* expression were substantially higher than rEOS-like cells at V3, and iEOS-like cells' *ECP* expression remained higher at V4, $p = 0.0411$.

Discussion

This study found that the observed dominant blood eosinophil subtype in the biological-naive SEA patient group was iEOS-like cells, and their *EPX, MBP/PRG2*, and *CLC/GAL-10* gene expressions were higher than rEOS-like cells. In addition, SEA patients' blood eosinophil subtypes demonstrated significantly enhanced *EDN/RNASE2, EPX, MBP, ECP/RNASE3*, and *CLC* gene expression compared with the HS group. Treatment with mepolizumab resulted in significant blood eosinophil count depletion, improved FEV_1, and increased ACT scores, and this effect remained throughout all 24 weeks of treatment. Moreover, the distribution of remaining blood eosinophils shifted steadily towards rEOS-like

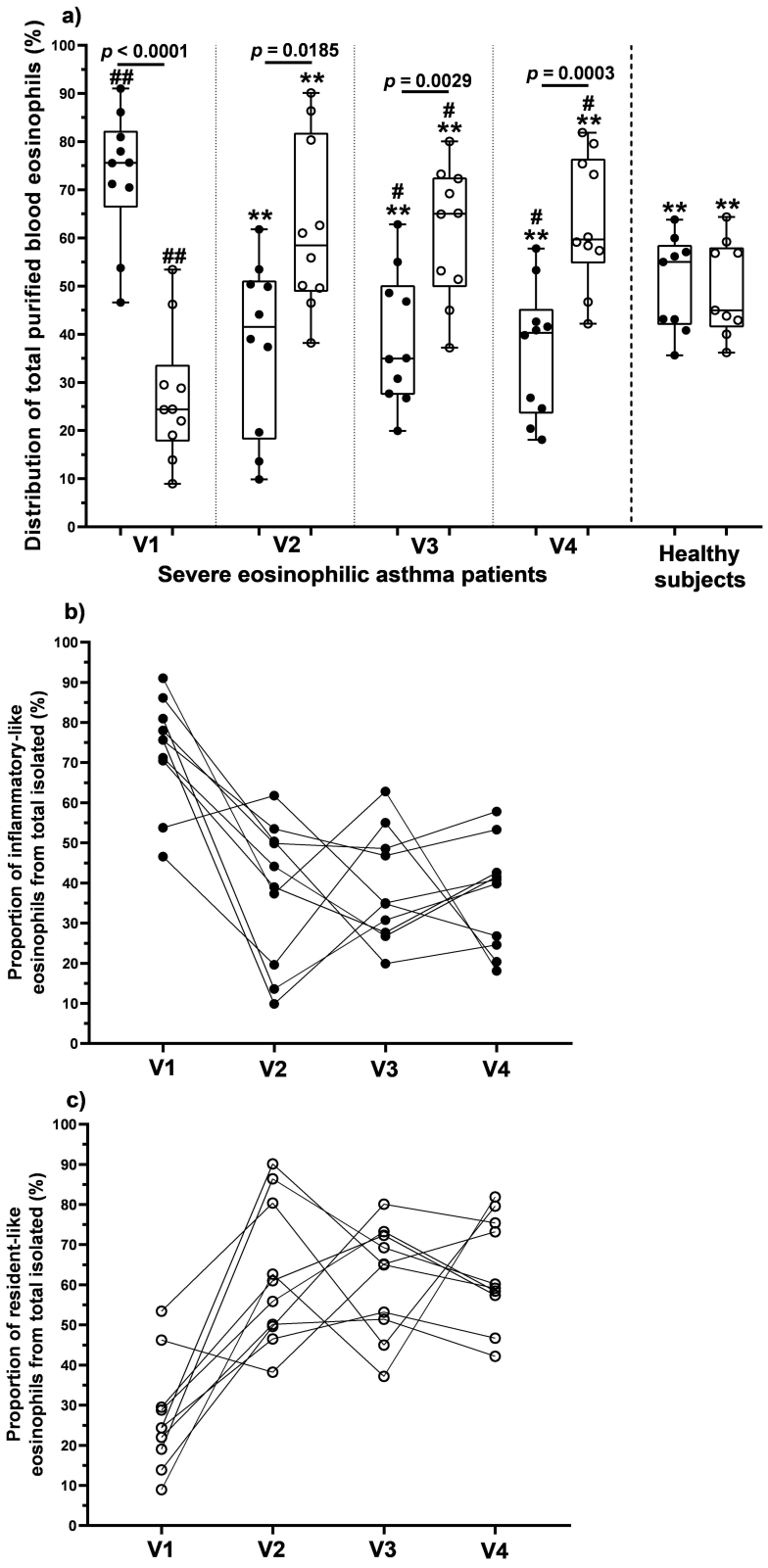

Figure 3 Isolated blood eosinophil subtypes. (**a**) Isolated blood eosinophil subtype proportions following mepolizumab-treated severe eosinophilic asthma patients and healthy control subjects. # $p < 0.05$; ## $p < 0.01$, compared with healthy subjects respective eosinophil subtype. ** $p < 0.01$, compared with SEA patients' V1 respective eosinophil subtype. (**b**) Individual SEA patients' blood inflammatory-like eosinophils distribution following treatment with mepolizumab. (**c**) Individual SEA patients' blood resident-like eosinophils distribution following treatment with mepolizumab.

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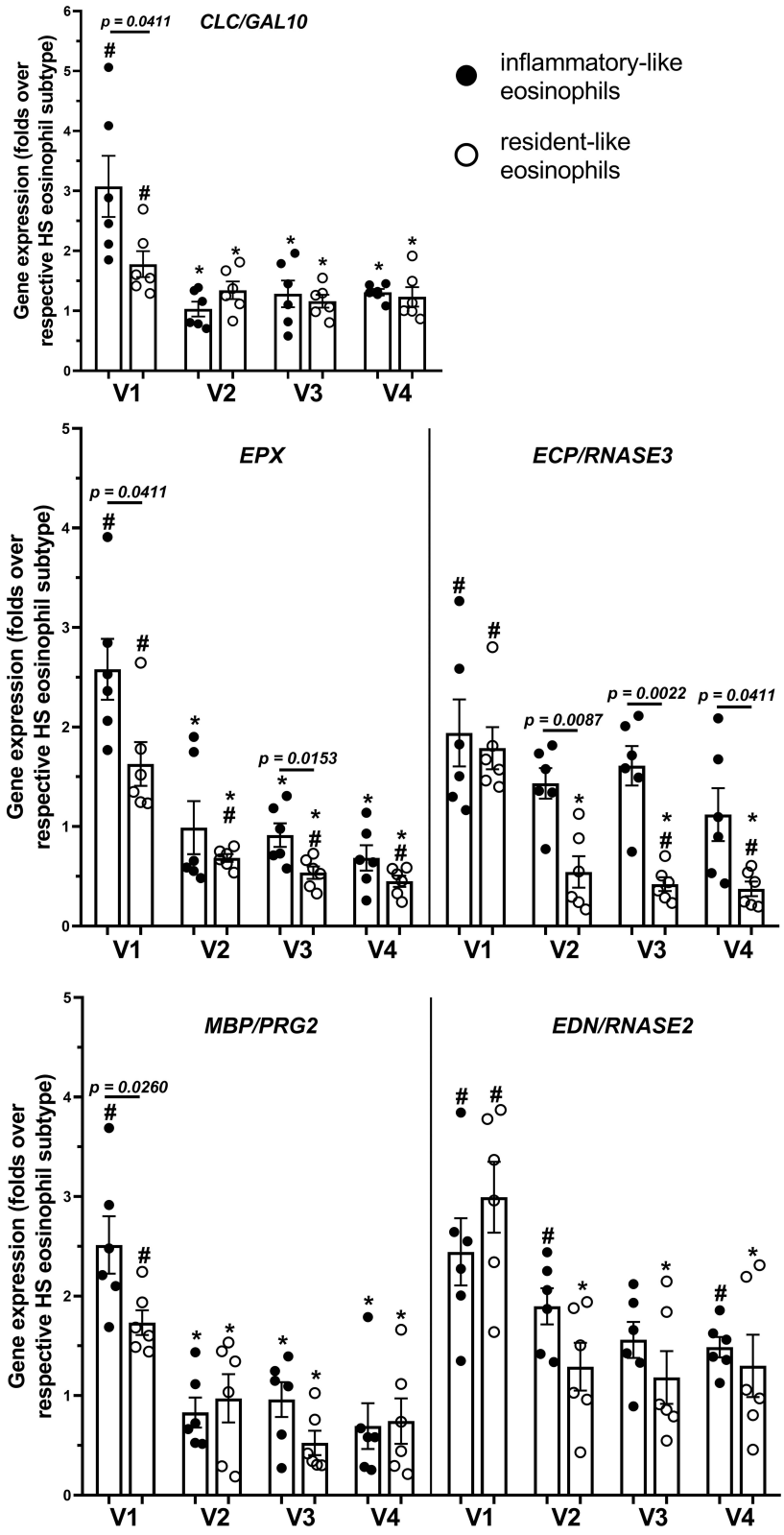

Figure 4 Blood eosinophil subtypes granule protein gene expression. Healthy subjects n = 6. * $p < 0.05$, compared with V1 respective eosinophil subtype; # $p < 0.05$, compared with healthy subjects' respective eosinophil subtype.

cells. The remaining SEA patients' iEOS-like cells *CLC, EPX, ECP, MBP*, and rEOS-like cells *CLC, MBP*, and *EDN* gene expression decreased to HS group levels. Lastly, iEOS-like cells' *EPX* and *ECP* expression remained significantly higher than rEOS-like cells.

Chronic airway inflammation is a hallmark feature of severe asthma, which results in elevated inflammatory cytokine, chemokine, and growth factor production by airway structural cells and immune cells,[25] subsequently stimulating eosinophil proliferation in the bone marrow and recruitment to sites of inflammation. Inflammatory-like eosinophils are the predominant, active eosinophil population primarily involved in allergic reactions, infections, and inflammation,[26] therefore are the main cells reactive to the inflammatory background in asthma. Recent studies have shown that higher proportions of circulating blood iEOS-like cells statistically significantly correlated with worse ACT scores, Asthma Control Questionnaire-5, and asthma exacerbations in biologic treatment-naive SEA patients.[27] A positive tendency between decreased iEOS distribution and increased spirometry values was noticed in SEA group following treatment with mepolizumab, although no statistically significant correlations between eosinophil subtype changes and asthma control test or FEV_1 values were obtained in this study. Nonetheless, we found that untreated SEA patients' blood eosinophils were primarily iEOS-like cells, and their *CLC, EPX*, and *MBP* gene expression were significantly higher than rEOS-like cells. Eosinophil-derived granule proteins, particularly ECP, MBP, and EPX, are toxic to helminths, cells, and tissues by disrupting the lipid bilayer membrane or altering enzyme activity.[15] The released mediators also prime migrated eosinophils to secrete and release their cytotoxic granule content locally, thus causing exacerbation of inflammation and tissue damage. Our findings suggest that patients with higher levels of circulating iEOS-like cells overexpressing cytotoxic granule proteins may be prone to worse clinical outcomes and increased risk of exacerbations.[27]

A subset of homeostatic or resident eosinophils (hEos, rEos), produced partially independently of IL-5, under baseline conditions, may exert crucial homeostatic processes in the gastrointestinal tract, lungs, adipose, thymus, and uterus.[10,11] In this study, biological-naive SEA patients' blood iEOS-like and rEOS-like cells *EDN, EPX, MBP, ECP*, and *CLC* gene expression was significantly higher than the HS group, signaling that both cell types may contribute to chronic airway inflammation via enhanced cytotoxic granule protein production. However, after treatment with mepolizumab, the investigated gene expression was significantly reduced, and the remaining blood eosinophil pool consisted mainly of rEOS-like cells. It has been previously reported that anti-IL-5 treatment may dampen blood eosinophils αMβ2 integrin and P-selectin glycoprotein ligand-1 expression, potentially lowering eosinophilia-related lung inflammation.[28] Moreover, E Kelly et al have shown that treatment with mepolizumab reduces the expression levels of IL-3Rα on circulating eosinophils but not airway eosinophils. In addition, eosinophils release of EPX and EPX-containing granules decreases in bronchial mucosa following 1 month after administrating mepolizumab.[23] Accordingly, this study observed a consistent decrease in eosinophil granule protein expression after 4 weeks of treatment with mepolizumab, and up to 32 weeks the effect remained similar. Our data are in agreement with recent studies which have demonstrated that anti-IL-5 mepolizumab treatment primarily depletes iEOS-like cells and reduces circulating eosinophil viability,[29] meanwhile, a steady pool of residual rEOS-like cells is spared. Transcriptomic analysis, published by V Hulst et al, suggests that remaining circulating eosinophils after mepolizumab treatment in severe asthmatics may resemble non-asthmatic control subjects.[30] In addition, serum analysis after biologic treatment revealed changes in circulating protein abundance similar to healthy controls involved in inflammatory pathways, extracellular matrix remodeling, lipid metabolism, and regulation of blood coagulation.[31] Moreover, MW Johansson et al demonstrated that patients with allergic asthma can have a strong non-T2-mediator airway response following a bronchoprovocation with allergen, proposing a reason for insufficient effect of biological T2 asthma treatment in clinical practice.[32] This strengthens our findings regarding decreasing SEA patients' eosinophil granule protein gene expression to healthy control subject levels throughout 24 weeks of mepolizumab treatment. Overall, it is evident that anti-IL-5 therapy provides a compound alleviation of chronic inflammation as shown by the reduction of circulating iEOS-like cells and their cytotoxic granule protein expression.

A potential limitation of our study is the small number of study participants. The primary focus of the research was to obtain initial insights into the research question regarding continuous anti-IL-5 treatment effect on blood eosinophil subtype distribution and biological properties and was reported as a pilot study. A larger study with more investigated individuals is needed to validate these results further. Nonetheless, following SEA patient treatment with mepolizumab, longitudinal changes were analyzed for each participant, thus minimizing the potential nuisance effect caused by heterogeneity among all participants. Another limitation is that after purifying blood eosinophils from granulocytes,

the rEOS-like ($CD62L^+$) cells were isolated using positive selection with magnetic beads, and unselected cells were deemed as iEOS-like ($CD62L^-$) cells. Recent blood eosinophil studies differentiate blood iEOS as $CD62L^{low}$, and rEOS as $CD62L^{bright}$ cells.[33] Meanwhile, for the magnetic separation method used in this study, the low amount of potentially expressed CD62L adhesive molecules, as shown in Figure 2, is insufficient for retention in the magnetic field, thus only a small fraction of iEOS-like cells could theoretically get caught in the magnetic column. Nevertheless, our reported results of iEOS-like cell distribution in SEA patients before and after anti-IL-5 treatment are in agreement with similar eosinophil subtype-based published studies.[29,33] In addition, we have shown that asthmatic patients' iEOS-like cells had elevated cytotoxic protein gene expression. Thus, we claim that these results are in line with the deemed "negative" $CD62L^{low}$ iEOS subtype, whose amount correlated with worse ACT scores and exacerbation history.[27] Another potential study limitation may be that patients in the SEA group were significantly older than the HS group. It has been shown that IL-5-stimulated eosinophil degranulation of EDN is decreased in older (55–80 years old) asthma patients; however, the total EDN in the eosinophils was found to be similar between the younger and older asthmatics.[34] Thus, we claim that the SEA patients' eosinophil granule protein gene expression is properly comparable with the HS group. Finally, a potential limitation of our eosinophil subtype granule protein gene expression analysis was that other methods did not further evaluate and confirm changes at the protein level. Larger study cohorts have reported a positive correlation between serum EDN, ECP and peripheral blood eosinophil count (PBEC) before biological treatment in SEA patients. Following 32-week treatment with mepolizumab, a decrease in serum EDN, ECP and PBEC was observed, attributing the changes in protein amount to blood eosinophils.[35] Our findings on diminished eosinophil granule protein gene expression are in line; however, the protein expression of selected analytes would give a more accurate result. In addition, it is presumed that the protein levels may not accurately correlate each time with the amount of transcripts. Nevertheless, previous studies have shown that differentially expressed messenger ribonucleic acids (mRNAs) correlate considerably more with the protein product compared to the non-differentially expressed mRNAs.[36]

Conclusion

Overall, the dominant circulating eosinophil subtype in the SEA patient group was iEOS-like cells, which displayed enhanced *EPX, MBP/PRG2*, and *CLC/GAL-10* gene expression over rEOS-like cells and healthy control subjects. The anti-IL-5 drug mepolizumab primarily depletes blood iEOS-like cells and also reduces remaining blood eosinophils' cytotoxic granule proteins gene expression to levels resembling those of healthy individuals. The changes in blood eosinophil subtype distribution and gene expression are noticeable after one course of biological treatment and remain stable over time following subsequent therapies. Current indications for administering biological treatment to SEA patients do not include eosinophil subset distribution as a treatment response predictor. Meanwhile, emerging evidence suggests that inflammatory eosinophils are a key subpopulation responsible for the pathogenesis of eosinophilia-driven diseases and are primarily abolished by anti-IL-5 treatment. Nonetheless, more extensive studies are required to investigate the biological features of blood eosinophil subtypes to understand their clinical implications better.

Abbreviations

ACT, asthma control test; BMI, body mass index; CLC, Charcot-Leyden crystal; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPX, eosinophil peroxidase; FeNO, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; GM-CSF, granulocyte macrophage-colony stimulating factor; hEOS, homeostatic eosinophils; HS, healthy subjects; iEOS-like, inflammatory-like eosinophils; IgE, immunoglobulin E; IL, interleukin; ILC2, innate type 2 lymphoid cell; LUHS, Lithuanian university of health sciences; MBP, major basic protein; mRNA, messenger ribonucleic acid; OCS, oral corticosteroids; PBEC, peripheral blood eosinophil count; PCR, polymerase chain reaction; rEOS-like, resident-like eosinophils; SEA, severe eosinophilic asthma; Th2, type 2 lymphocyte helper; V1, first experimental visit before starting mepolizumab treatment; V2, visit 4 weeks after beginning mepolizumab treatment (after 1 mepolizumab dose); V3, visit 12 weeks after beginning mepolizumab treatment (after 3 mepolizumab doses); V4, visit 24 weeks after beginning mepolizumab treatment (after 6 mepolizumab doses).

Data Sharing Statement

The authors of this manuscript declare no intention to share individual de-identified participant data. All relevant clinical data of the study participants are provided in the manuscript and no additional unpublished data from the study will be made available elsewhere.

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Disclosure

The authors report no conflicts of interest in this work.

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