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Skin Surface Lipid-RNA Profile Obtained from Patients with Severe Asthma After Benralizumab Treatment

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Background: Examining human coding and non-coding RNAs present in skin surface lipids (SSL-RNAs) offers a promising approach to understanding the physiological state of the skin. Benralizumab treatment can reduce exacerbations and improve symptom control and quality of life in patients with severe eosinophilic asthma. Although this treatment effectively depletes peripheral blood eosinophils, the impact of benralizumab on SSL-RNA remains completely unknown.

Objective: To investigate the effects of benralizumab treatment on SSL-RNA profiles in patients with severe asthma.

Methods: Skin samples were non-invasively collected from patients before and after one year of benralizumab treatment. Sixteen patients were enrolled, but the SSL-RNA analysis was only feasible for five patients due to collection challenges, mainly in female participants.

Results: Following benralizumab treatment, asthma symptoms, exacerbation rates, and lung function parameters improved. Peripheral blood eosinophils were completely depleted and serum eotaxin-1 levels increased. SSL-RNA analysis revealed differential expression of 134 genes, with significant downregulation of immune-related pathways and genes associated with neutrophilic inflammation.

Conclusion: These findings suggest a suppression of both type 2 and non-type 2 inflammation in response to benralizumab treatment, with potential implications for asthma management. However, the limitations of the study include a small sample size and challenges in sebum collection, particularly among female participants. Although the noninvasive nature of this sampling method makes it attractive for both research and clinical applications, additional studies are needed to fully investigate the potential of SSL-RNA analysis as a noninvasive biomarker to assess treatment response in asthma.

Keywords: asthma, benralizumab, skin surface lipid, sebum, type 2 inflammation

Introduction

The delicate balance of various biomolecules maintains the molecular composition of healthy skin, and disease disrupts this balance. Skin diseases, such as cancer, psoriasis, and eczema, and diseases that affect internal organs, are responsible for altering the molecular composition of the skin.[1] Skin phenotypes, which include various components such as lipids, proteins, inflammatory mediators, and immune cells, provide information on skin conditions as well as conditions of the entire systemic body.[1,2] In addition, skin serves as the body's surface, enabling non-invasive collection of biomolecules from samples such as sweat, hair, and the stratum corneum. Consequently, the skin becomes a useful source of samples to monitor both skin and body conditions. Skin surface lipid-RNAs (SSL-RNAs) in skin surface lipids (SSLs), predominantly originate from the sebaceous glands, epidermis, and hair follicles. The surrounding lipids in SSLs play a role in

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inhibiting RNase, allowing the detection of substantial levels of human coding and non-coding SSL-RNAs.[3] Non-invasive collection of SSL-RNAs is possible using a single oil-blotting film. SSL-RNA detection can be achieved by applying AmpliSeq, a transcriptome method that specifically targets the sequencing of more than 20,000 human transcripts. Understanding the physiological state of the skin is achievable through the application of these techniques.[3] The analysis of SSL-RNAs in atopic dermatitis not only identified genes consistent with known pathophysiology, but also indicated mechanisms related to altered lipid metabolism.[3]

Variable airflow limitation and hyperresponsiveness to bronchial air are characteristic of asthma, which is one of the most common chronic diseases.[4,5] Asthma pathogenesis involves a complex interplay of immune responses, airway epithelium, genetics, and environmental factors. External agents such as environmental pollutants and allergens trigger both innate and adaptive immune responses, resulting in chronic inflammation, which contributes to goblet cell hyperplasia, disruption of tight junctions, tissue remodeling, variable airflow limitation, and hyperresponsiveness in the airways of patients with asthma.[4–9] More than 50% of patients with asthma are affected by eosinophilic asthma, among various asthma phenotypes/endotypes.[10,11] Inhaled corticosteroids (ICS) effectively treat eosinophilic asthma, as they induce eosinophil apoptosis.[12,13] However, the impact of ICS on asthma is limited due to steroid resistance in patients with the most severe clinical phenotype of eosinophilic asthma, which results in refractory asthma.[14] To address this, biologics with additional therapeutic benefits have been developed for intractable asthma, such as benralizumab, a humanized monoclonal IgG1 antibody against the anti-IL-5 receptor α subunit monoclonal IgG1 antibody. Through antibody-dependent cell cytotoxicity, benralizumab effectively reduces eosinophils in the peripheral blood and airway mucosa, with a notable impact on the depletion of eosinophils in the peripheral blood.[15–19] In this study, we used SSLs as samples to comprehensively and non-invasively analyze SSL-RNA in patients with severe asthma whose peripheral blood eosinophils were depleted to investigate the effects of benralizumab treatment on SSL-RNA in patients with severe asthma.

Methods

Study Participants

This study was a prospective observational study that enrolled patients with severe asthma with newly prescribed benralizumab from March 2018 to May 2019. Patients who had severe asthma and were 20 years or older, whose asthma symptoms and asthma exacerbations that required oral corticosteroid could not be controlled by existing treatment options despite high-dose ICS plus long-acting β2 agonists with another controller, and who required benralizumab treatment in insurance medical treatment were recruited from our outpatient clinic at Juntendo University Hospital (Tokyo, Japan). Asthma was diagnosed based on a clinical history of episodic symptoms with airflow limitation and variation in lung function monitored by forced expiratory volume in 1 second (FEV_1) or peak expiratory flow according to the Global Initiative for Asthma guidelines.[20] Patients with any of the following criteria were excluded: (1) a diagnosis of eosinophilic granulomatosis with polyangiitis, interstitial pneumonia, infectious disease, or cancer; (2) those administering other antibody preparations; (3) cases that were considered inappropriate by the study investigators; and (4) cases under treatment with omalizumab and mepolizumab with <1 month of the last dose and cases under treatment with other biologics. The present study, aligned with the Declaration of Helsinki, was reviewed and approved by the Juntendo University Research Ethics Committee (Tokyo, Japan). Written informed consent was obtained from each patient prior to participation in the study. The study was registered in the UMIN Clinical Trial Registry (UMIN000031905) on 23 March 2018 (http://www.umin.ac.jp/).

The asthma control test (ACT), lung function tests, oscillometry (also known as the forced oscillation technique), measurement of fractional exhaled nitric oxide (FeNO) levels, and blood sampling were performed on the date of initial administration of benralizumab, four months, eight months, and one year after administration. FeNO levels were measured according to the recommendations of the American Thoracic Society at a constant flow of 0.05 L/s against an expiratory resistance of 20 cm of water with an electrochemical handheld NO analyzer (NIOX VERO®; Aerocrine AB, Solna, Sweden).

Definition for Responders

The patients were classified as responders and non-responders according to changes in ACT score, lung function, and asthma exacerbations with reference to previous studies.[21–27] A responder to benralizumab treatment was defined as meeting two of the following three criteria after one year of benralizumab treatment without substantial deterioration in any other criterion: (1) improvement in the ACT score of at least three points (including patients who achieved an ACT score of 25 points). An increase in the ACT score of at least three points was previously suggested as the minimal clinically important difference;[28,29] (2) a reduction in the number of asthma exacerbations (including patients who had no exacerbations before and after treatment); and (3) improvement in FEV_1 of at least 100 mL.[27,30] The following criteria were considered to be associated with significant deterioration: (a) a decrease in the ACT score of at least three points; (b) an increase in the number of exacerbations; (c) a decrease in FEV_1 of at least 100 mL.

Quantification of Circulating Lymphocyte Frequency

Flow cytometry analysis was performed as previously described.[31,32] Briefly, peripheral venous blood samples were collected in tubes containing heparin and PBMCs (3×10^6/well) were purified by density gradient centrifugation using Ficoll–Paque Plus solution (Cytiva, Tokyo, Japan). Cells were stained with different combinations of the appropriate antibodies for 30 min at 4 °C. In this study, the following surface marker antibodies were used: anti-CD3-APC-H7, anti-CD4-FITC, anti-CD19-FITC, anti-CD56-PE-CF594, anti-CD117 (c-Kit)-PE-CF594 (BD Biosciences, San Jose, CA, USA), anti-BDCA2-FITC, anti-CD1a-FITC, anti-CD3-FITC, anti-CD11c-FITC, anti-CD14-FITC, anti-CD25-PE, anti-CD34-FITC, anti-CD123-FITC, anti-CD127 (IL-7Rα)-Brilliant Violet 421 and 605, anti-CD161-PerCPCy5.5, anti-CD183 (CXCR3)-APC, anti-CD194 (CCR4)-Brilliant Violet 510, anti-CD196 (CCR6)-PerCPCy5.5, anti-CD294 (CRTH2)-Brilliant Violet 421, and anti-FCεR1-FITC. Negative lineage markers (Lin^-) were defined as $CD1a^-$, $CD3^-$, $CD11c^-$, $CD14^-$, $CD19^-$, $CD34^-$, $TCR\gamma\delta^-$, $CD123^-$, $BDCA2^-$, and $FC\varepsilon R1^-$. Th1 cells were identified as $CD3^+$, $CD4^+$, $CCR4^-$, $CCR6^-$, and $CXCR3^+$ cells; Th2 cells as $CD3^+$, $CD4^+$, $CCR4^+$, $CCR6^-$, and $CXCR3^-$ cells; Th17 cells as $CD3^+$, $CD4^+$, $CCR4^+$, $CCR6^+$, and $CXCR3^-$ cells; Tregs as $CD3^+$, $CD4^+$, $CD25^+$, and $CD127^-$ cells; natural killer (NK) cells as $CD3^-$ and $CD56^+$ cells; innate lymphoid cell (ILC) 1 as Lin^-, $CD127^+$, $CD161^+$, $CD117^-$, and $CRTH2^-$ cells; ILC2 as Lin^-, $CD127^+$, $CD161^+$, and $CRTH2^+$ cells; ILC3 as Lin^-, $CD127^+$, $CD161^+$, $CD117^+$, and $CRTH2^-$ cells. Dead cells were identified using the Zombie Fixable Viability Kit (BioLegend), followed by doublet exclusion in forward scatter and side scatter. After overnight fixation, cells were analyzed using a fluorescence-activated cell sorting (FACS) LSRFortessa cell analyzer (BD Biosciences). The FACS data was evaluated using FlowJo software (version 9; BD Biosciences).

Quantification of Serum Eotaxin-1 Levels

The sera of the patients were collected after centrifugation of the blood samples and then frozen at −80°C. Subsequently, eotaxin-1 assays were performed using the MILLIPLEX multiplex assay, following the manufacturer's guidelines (Merck Millipore, Burlington, MA, USA). The working range of the eotaxin-1 assay ranged from 3.2 (lower limit of quantification) to 10,000.0 (upper limit of quantification).

Collection of SSLs, RNA Preparation, and AmpliSeq Transcriptomic Analysis

We collected SSLs from the face and purified SSL-RNAs according to previous reports.[3,33] In summary, sebum samples were collected from the entire faces of study participants by wiping the skin surface using a single sheet of oil-blotting film (5.0 × 8.0 cm; 3M Japan, Tokyo, Japan) per participant. RNA was extracted using QIAzol reagent (QIAGEN, Valencia, CA, USA) from the oil-blotting film, followed by purification using the RNeasy Mini Kit (QIAGEN). Sequence library preparation, template preparation, and sequencing were performed using the Ion AmpliSeq Transcriptome Human Gene Expression Kit, Ion Chef System, and Ion S5 XL System (Thermo Fisher Scientific, Waltham, MA, USA).

Sample Quality Control, Data Analysis, and Statistics

Data analyses were performed according to previous reports.[3,33] Normalization and statistical analysis of the AmpliSeq transcriptomic data were performed using the R language. Ion Torrent Suite software version 5.16 (Thermo Fisher

Scientific) was used to obtain read count data. The package R DESeq2 (Bioconductor) 25 was used for normalization and quality check of the read count data. We removed samples in which the proportion of genes detected among the 20,802 target genes analyzed in the Ion AmpliSeq Transcriptome Human Gene Expression Kit was less than 20% from the analysis. Only genes for which one or more reads were detected in ≥ at least 90% of the samples, were normalized by the size factor.

Differentially expressed genes (DEGs) between 0 month (pre-treatment) and 12 months (after 12 months of treatment) were analyzed using the likelihood ratio test, and those with a false discovery rate (FDR) were adjusted using the Benjamini–Hochberg method below the threshold value of 0.05. The gene ontology (GO) analysis of genes obtained by differential expression analysis was performed using Reactome (https://reactome.org/). To evaluate the eosinophil gene signature and the IL-13-related gene signature, gene set variation analysis (GSVA) was performed using various previously reported immune-related gene sets. Enrichment scores for each gene set were calculated at 0 months (before treatment) and 12 months (after 12 months of treatment). Comparisons of GSVA enrichment scores between 0 months (before treatment) and 12 months (after 12 months of treatment) were made using paired *t*-tests.

Results

Characteristics of Study Participants

Sixteen patients with severe asthma that was uncontrolled by existing treatment regimens were enrolled and treated with benralizumab. Although their sebum samples were collected, analysis of SSL-RNA before and after benralizumab administration was only feasible for five patients. Of these, four were male and one was female. In contrast, eight of the remaining 11 were females who, perhaps due to washing or makeup, were unable to collect enough sebum to analyze SSL-RNA. Furthermore, a case of switching from omalizumab treatment was excluded, and the remaining five patients had not received any biological treatment before. The clinical characteristics of the study participants are shown in Table 1. The median age (range) of the patients was 57 (41–71) years. Among them, three were ex-smokers, and two were nonsmokers. A patient was on a regular 10 mg/day prednisolone regimen. The median daily dose of ICS was 800 µg. A patient was unable to administer high-dose ICS due to side effects of hoarseness. The median duration of

Table 1 Baseline Characteristics

	Case 1	Case 2	Case 3	Case 4	Case 5	Median
Sex (M/F)	M	M	F	M	M	NA
Age (y)	48	57	41	61	71	57.0
Age at asthma onset (y)	29	50	37	29	70	37.0
Duration of asthma (y)	19	7	4	32	1	7.0
BMI (kg/m^2)	22.3	32.7	25.0	24.1	23.6	24.1
Smoking history (never/ex)	ex	never	never	ex	ex	NA
Atopic dermatitis	–	–	–	–	–	NA
Atopic rhinitis	+	+	+	+	–	NA
Atopic conjunctivitis	–	–	–	–	–	NA
Chronic sinusitis	+	+	+	–	–	NA
NERD	–	–	+	–	–	NA
Daily dose of ICS (FP equivalent dose, µg)	500	800	750	1000	1000	800.0
Daily dose of OCS (PSL equivalent dose, mg)	–	–	–	–	10	NA
Asthma exacerbations (/year)	24	5	0	3	4	4.0
Unscheduled visits (/year)	12	6	0	0	11	6.0
ACT score points	11	20	24	15	11	15.0
FeNO (ppb)	102	49	17	27	13	27.0
VC (L)	3.72	3.07	3.91	3.22	4.65	3.7
FVC (L)	3.22	2.79	3.7	3.25	4.33	3.3
%FVC (predicted, %)	70.4	73.9	118.6	95.7	115.7	95.7
FEV_1 (L)	1.79	1.54	2.96	2.14	2.92	2.1

(*Continued*)

Table 1 (Continued).

	Case 1	Case 2	Case 3	Case 4	Case 5	Median
%FEV_1 (predicted, %)	45.7	48.6	112	76.2	96.3	76.2
FEV_1% (%)	55.6	55.2	80	65.8	67.4	65.8
PEFR (L/s)	5.92	5.76	9.41	8.25	8.9	8.3
%PEFR (predicted, %)	57.7	64.7	136.4	99.5	106.1	99.5
MMF (L/s)	0.62	0.55	2.69	1	1.75	1.0
%MMF (predicted, %)	14.3	15	77.8	30.1	55.6	30.1
R5 (cmH20/L/S)	4.26	4.03	3.15	1.82	2.96	3.2
R20 (cmH20/L/S)	3.34	3.15	2.83	1.59	1.97	2.8
X5 (cmH20/L/S)	−1.34	−1.17	0.02	−0.16	−0.64	−0.6
Fres (Hz)	12.08	11.02	5.09	6.39	10.53	10.5
ALX (cmH20/L/S*Hz)	6.44	5.26	0.34	0.59	2.71	2.7
Peripheral neutrophils (%)	65.7	57.8	55.6	68.6	66.5	65.7
Peripheral neutrophils (cells/μL)	3022	4508	3781	5008	5985	4508.4
Peripheral eosinophils (%)	6.4	10.3	4.6	3	1.5	4.6
Peripheral eosinophils (cells/μL)	294	803	313	219	135	294.0
Peripheral basophils (%)	1.1	1.2	0.3	0.6	0	0.6
Peripheral basophils (cells/μL)	51	94	20	44	0	43.8
Peripheral lymphocytes (%)	19.8	22.6	34.7	18.6	27	22.6
Peripheral lymphocytes (cells/μL)	911	1763	2360	1358	2430	1762.8
Peripheral monocytes (%)	7	8.1	4.8	9.2	5	7.0
Peripheral monocytes (cells/μL)	497	575	341	653	355	497.0
Total IgE (IU/mL)	453	201	41	89	38	89.0
Eotaxin-1 (pg/mL)	37.3	71.6	28.7	65.1	137.8	65.1
Th1 cells (% of Th cells, %)	23.0	15.6	21.3	11.4	22.2	21.3
Th2 cells (% of Th cells, %)	9.0	6.6	3.8	6.2	4.7	6.2
Th17 cells (% of Th cells, %)	7.2	4.8	4.8	5.0	3.4	4.8
Treg cells (% of Th cells, %)	7.1	4.7	3.8	2.1	5.2	4.7
ILC1 (% of ILC cells, %)	85.9	84.7	79.8	57.9	78.8	79.8
ILC2 (% of ILC cells, %)	6.3	12.4	16.5	20.3	8.7	12.4
ILC3 (% of ILC cells, %)	7.9	2.9	3.6	21.9	12.5	7.9
NK cells (% of lymphoid cells, %)	9.3	24.9	7.8	12.8	9.0	9.3

Abbreviations: ACT, asthma control test; BMI, body mass index; FeNO, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 second; FEV_1%, FEV_1 second/forced vital capacity; FP, fluticasone propionate; FVC, forced vital capacity; ICS, inhaled corticosteroid; OCS, oral corticosteroid; IgE, immunoglobulin E; ILC, innate lymphoid cell; NA, not applicable, NERD, nonsteroidal anti-inflammatory drug-exacerbated respiratory disease; NK, natural killer; PSL, prednisolone; Th, helper T; Treg, regulatory T.

asthma was seven years, ranging from 1 to 32 years. The median FEV_1% was 65.8%, and the median peripheral eosinophil count was 294/μL. Four patients met the response criteria, defined by their ACT scores, exacerbation rates, and FEV_1 measurements.

Changes in Each Parameter One Year After Benralizumab Treatment

Following one year of benralizumab treatment, four of the five patients exhibited an improvement in ACT scores by a minimum of three points, which is recognized as the minimal clinically important difference, or achieved total control (Table 2).[28,29] The number of asthma exacerbations and unscheduled visits due to worsening asthma decreased (Table 2). Benralizumab treatment led to improvements in airflow limitation and reductions in peripheral blood neutrophil, eosinophil, and basophil counts. In all cases, peripheral blood eosinophils were completely absent, while basophils were nearly absent (Table 2). Furthermore, it resulted in increased serum levels of eotaxin-1 (Table 2). Next, we investigated the frequency of PBMCs in peripheral blood using flow cytometry. Supplementary Figure 1 shows the gating strategy for PBMCs. The frequencies of Th cells and ILCs were determined by their ratios to $CD3^+$ and $CD4^+$

Table 2 Change in Parameters Before and After 1 Year of Benralizumab Treatment

	Case 1	Case 2	Case 3	Case 4	Case 5	Median
Asthma exacerbations (/year)	−24	−5	0	−3	0	−3.0
Unscheduled visits (/year)	−12	−6	0	0	−7	−6.0
ACT score	13	5	1	10	−2	5.0
Responder/non-responder	Responder	Responder	Responder	Responder	Non-responder	NA
FeNO (ppb)	198	27	2	12	7	12.0
VC (L)	1.05	−0.05	−0.07	0.5	−0.24	0.0
FVC (L)	1.52	−0.09	−0.02	0.49	0.15	0.2
%FVC (predicted, %)	34.1	−2.5	0.5	14.9	4.3	4.3
FEV_1 (L)	1.44	0.22	0.03	0.01	−0.1	0.0
$\%FEV_1$ (predicted, %)	37.8	7	2.3	0.6	−2.8	2.3
$FEV_1\%$ (%)	12.5	10	1.3	−8.3	−4.5	1.3
PEFR (L/s)	4.23	0.6	−0.26	0.58	0.44	0.6
%PEFR (predicted, %)	42.5	7	−2.2	7.1	5.9	7.0
MMF (L/s)	1.22	0.36	0.31	−0.35	−0.42	0.3
%MMF (predicted, %)	29	10.1	9.9	−10.4	−12.6	9.9
R5 (cmH20/L/S), n = 5	−2.07	−0.84	−0.4	1.31	−0.27	−0.4
R20 (cmH20/L/S), n = 5	−1.61	−0.72	−0.28	1.04	−0.08	−0.3
X5 (cmH20/L/S), n = 5	1.28	0.47	−0.09	0.02	−0.02	0.0
Fres (Hz), n = 5	−6.76	−2.48	0.23	−0.34	0.45	−0.3
ALX (cmH20/L/S*Hz), n = 5	−6.14	−2.75	0.11	−0.12	0.24	−0.1
Peripheral neutrophils (cells/μL)	−187	−1340	884	977	−2795	−187.2
Peripheral eosinophils (cells/μL)	−294	−803	−313	−219	−135	−294.0
Peripheral basophils (cells/μL)	−51	−94	−13	−11	0	−13.1
Peripheral lymphocytes (cells/μL)	122	69	−213	311	−342	68.7
Total IgE (IU/mL)	−226	3	4	−6	126	3.0
Eotaxin-1 (pg/mL)	50.0	148.8	47.9	144.2	95.9	95.9
Th1 cells (% of Th cells, %)	−1.1	3.2	−3.4	−2.4	−2.4	−2.4
Th2 cells (% of Th cells, %)	−0.6	0.2	−0.4	1.6	0.5	0.2
Th17 cells (% of Th cells, %)	0.3	−0.2	−1.9	−0.2	1.5	−0.2
Treg cells (% of Th cells, %)	−1.6	1.2	−0.9	−0.2	−1.6	−0.9
ILC1 (% of ILC cells, %)	−7.2	5.7	−0.8	15.2	8.2	5.7
ILC2 (% of ILC cells, %)	−2.0	−5.7	−5.5	2.8	2.9	−2.0
ILC3 (% of ILC cells, %)	9.1	0.0	6.4	−18.1	−11.2	0.0
NK cells (% of lymphoid cells, %)	0.1	9.5	4.3	0.3	−0.1	0.3

Abbreviations: ACT, asthma control test; FeNO, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 second; $FEV_1\%$, FEV_1 second/forced vital capacity; FVC, forced vital capacity; IFN-γ, interferon-gamma; IgE, immunoglobulin E; IL, interleukin; ILC, innate lymphoid cell; IP, interferon-γ inducible protein; MAIT, mucosal associated invariant T; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; NA, not applicable, NK, natural killer; RANTES, regulated on activation normal T cell expressed and secreted; Th, helper T; Treg, regulatory T.

cells, and Lin^- $CD127^+$ and $CD161^+$ cells, respectively. Additionally, the frequencies of NK cells were represented by their ratios to lymphocytes. However, no significant changes were observed in the frequencies of Th cells and ILCs after one year, as indicated in Table 2.

Differential Expression Analysis Using SSL-RNAs in Patients with Severe Asthma Before and After One Year of Benralizumab Treatment

To gain insight into the biological characteristics of SSL-RNA profiles in patients with severe asthma after one year of benralizumab treatment, we performed a differential expression analysis. We compared patient profiles before and after treatment and established an FDR threshold of < 0.05, leading to the identification of 134 DEGs. These DEGs are detailed in Supplementary Table 1. Among these, seven genes were upregulated, and 127 genes were downregulated after one year of treatment (Figure 1). The GO analysis using Reactome revealed a significant decrease in the regulation of biological functions

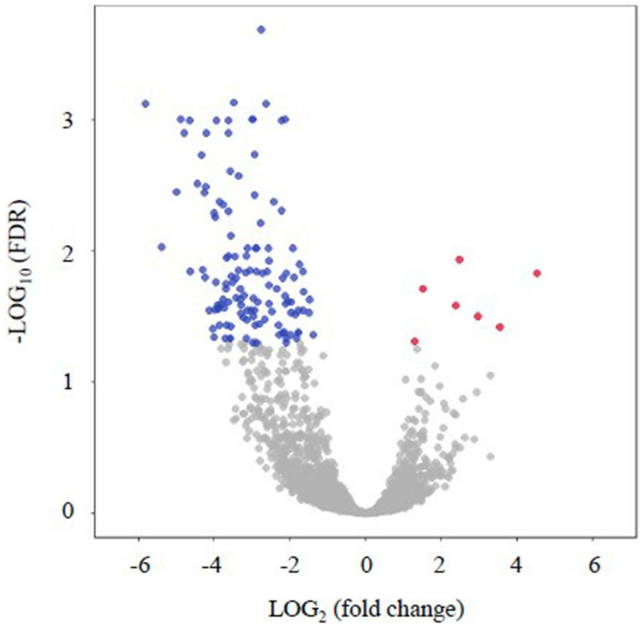

Figure 1 Volcano plot of differentially expressed genes between 12 months of benralizumab treatment and 0 months (pre-treatment). Upregulated genes in 12 months after benralizumab treatment show red spots and downregulated genes in 12 months show blue spots. (Benjamini–Hochberg's false discovery rate (FDR) < 0.05).

related to the immune system, neutrophil degranulation, interleukin (IL)-4 and IL-13 signaling, and interferon signaling in patients with severe asthma after one year of benralizumab treatment (Figure 2). Cytokines, IL-4 and IL-13, signaling and immune responses were suggested to be reduced, therefore, an enrichment score was calculated through GSVA using the eosinophil and IL-13-related gene sets used in previous reports to analyze immune-related changes with benralizumab treatment.[34,35] However, no changes were observed in the signature associated with eosinophils or IL-13, except for a trend toward a decrease in the IL-13-associated signature ($p = 0.059$) (Figure 3). In contrast, among DEGs, the expression of key genes involved in non-type 2 inflammation, including IL-8, IL-17RA, CXCR1, and CXCR2, was found to decrease in patients with severe asthma after one year of benralizumab treatment (Figure 4).

Discussion

In a very small number of patients with asthma in this study, benralizumab therapy improved airflow limitation while reducing the counts of peripheral blood neutrophils, eosinophils, and basophils. Furthermore, there was an increase in serum eotaxin-1 levels. To our knowledge, this study marks the first analysis of SSL-RNA before and after

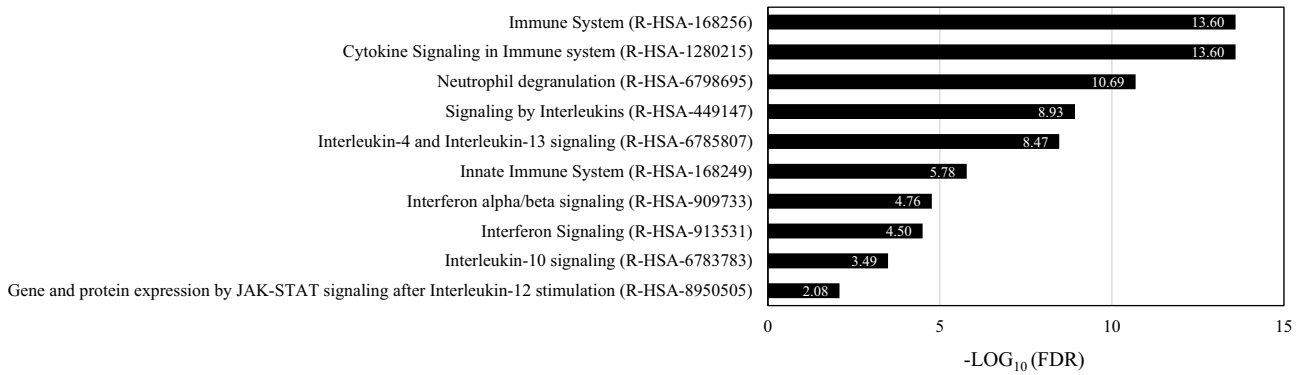

Figure 2 The result of enrichment analysis for REACTOME database in down-regulated genes 12 months after benralizumab treatment (Benjamini–Hochberg's false discovery rate (FDR) <0.05).

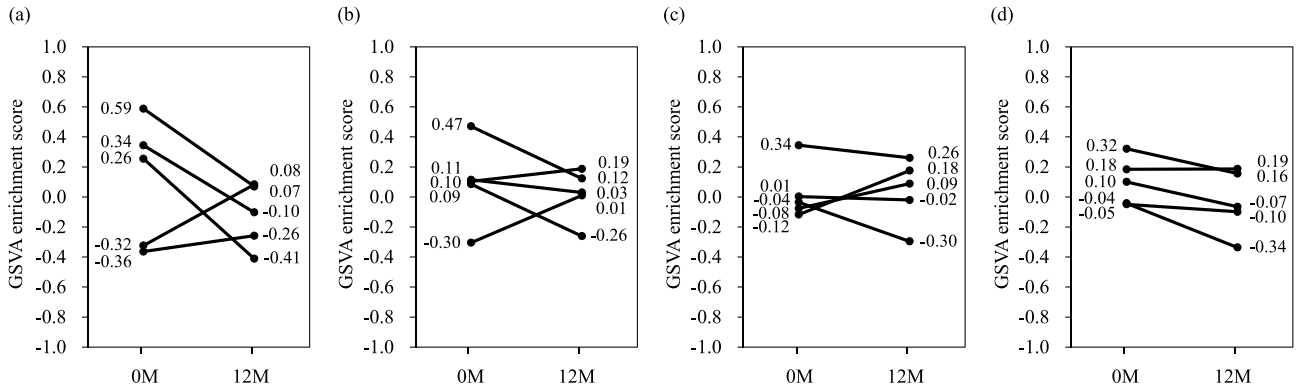

Figure 3 Changes in the signature of the genes related to eosinophils and IL-13 before and after 12 months of benralizumab treatment. GSVA signature scores for the three eosinophil-related genes (**a**–**c**) eosinophil signature #1, #2, #3, and (**d**) IL-13-related gene signature. GSVA: Gene Set Variation Analysis.

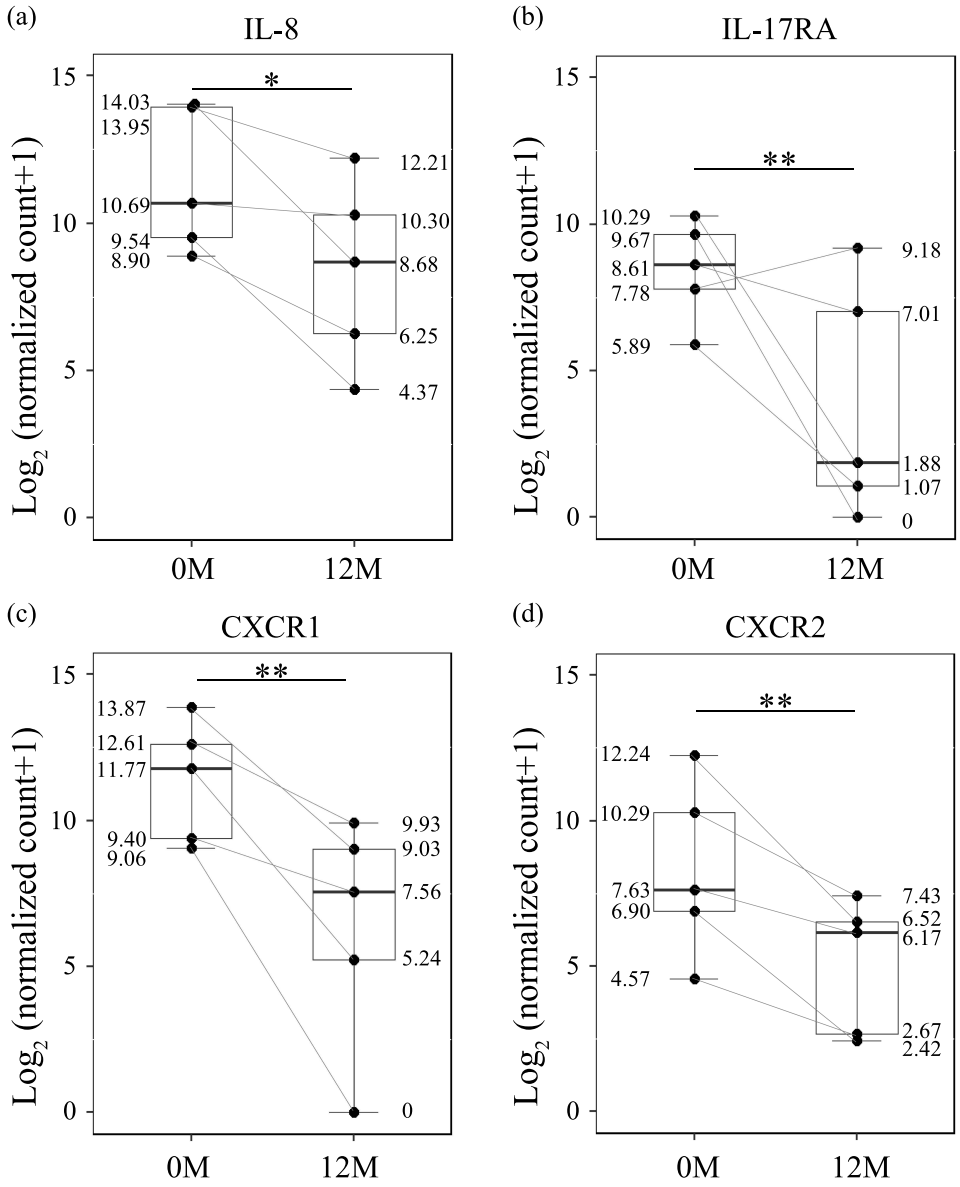

Figure 4 Alteration in non-type 2 inflammation-related gene expression before and after 12 months after benralizumab treatment. *: FDR <0.05, ** FDR <0.01, FDR: Benjamini–Hochberg's false discovery rate.

benralizumab treatment in patients with severe asthma. After one year of benralizumab treatment, 127 DEGs were downregulated, including crucial genes involved in non-type 2 inflammation, such as IL-8, IL-17RA, CXCR1, and CXCR2. GO analysis revealed a significant down-regulation of biological functions related to the immune system, neutrophil degranulation, IL-4 and IL-13 signaling, and interferon signaling, indicating a reduced immune response and possible suppression of inflammation due to treatment. These findings are consistent with decreased peripheral blood neutrophils, eosinophils, and basophils. Although our previous study that analyzed peripheral blood lymphocyte fractions showed increased Th2 cells and decreased Tregs after one year of benralizumab treatment, examination of local SSL-RNA profile suggests subsidence of both type 2 and non-type 2 inflammation.[36] These discrepancies highlight the differences between peripheral blood and local skin, implying that the observed changes in the circulating Th2 and Treg fractions may be the result of the subsiding of local inflammation. Notably, all validated patients in this study were responders to benralizumab treatment, affecting cutaneous RNA; however, airway and skin localization assessment is complex. Furthermore, the increase in serum eotaxin is in agreement with previous findings, indicating a feedback loop on peripheral blood eosinophil depletion.[36] However, despite achieving zero eosinophils in peripheral blood with benralizumab treatment, no eosinophil-related changes were observed in SSL-RNA. This could be due to the low proportion of eosinophils in the sebum, not captured by SSL-RNA or the absence of changes in eosinophils in the location of the skin. However, our findings in this study suggest a reduced immune response with benralizumab, likely reflecting the attenuation of inflammation in asthma. In particular, reduced expression of multiple genes linked to neutrophils indicates that benralizumab, designed to target eosinophils, could influence non-type 2 neutrophilic inflammation. The presence or absence of expression of the IL-5 receptor makes it unlikely that benralizumab directly suppresses neutrophilic inflammation, implying secondary effects or unidentified mechanisms related to the suppression of eosinophilic inflammation, which requires further investigation.

The primary limitation of this study is its exceptionally small sample size. Furthermore, the female participants visiting our hospital in central Tokyo faced challenges in refraining from face washing and makeup application before sebum collection. Despite our instructions to minimize these activities, the high rate of unsuccessful sample collection among female suggests the potential influence of face washing and makeup on the results. This result also corresponds to the limitation of this study, as only one of the five participants in this study was female, despite the fact that 70% of the participants in the previous study were female.[36] Sebum collection is non-invasive and an excellent candidate for biomarkers, but the collection method needs to be investigated. Furthermore, more studies are needed to determine whether local sebum information can reflect the entire body or local airways. Additionally, asthma is significantly influenced by external environmental factors such as pollutants and allergens, and the response to treatments with biologics can also be affected by geographic variation.[6–9] Although the participants in this study lived in Tokyo and its suburbs, minimizing geographic variation, the possibility remains that differences in indoor environments, air quality, surrounding vegetation, and proximity to major roads may have impacted asthma pathogenesis. This remains a common limitation in studies of this nature.

Conclusions

This study reports for the first time that SSL-RNA from patients with asthma who improve with benralizumab treatment exhibits type 2 and non-type 2 inflammation and immune responses, mainly in neutrophil-related genes. These findings imply that benralizumab treatment, designed to target eosinophils, could also influence non-type 2 inflammation associated with neutrophils. To our knowledge, this study is one of the few in this field, as the number of patients worldwide treated with benralizumab is limited, and the technology to measure SSL-RNA remains scarce. Further exploration is warranted to assess the potential utility of noninvasive SSL-RNA analysis as a biomarker.

Abbreviations

ACT, asthma control test; BMI, body mass index; FeNO, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 second; FEV_1%, FEV_1 second/forced vital capacity; FP, fluticasone propionate; FVC, forced vital capacity; ICS, inhaled corticosteroid; IgE, immunoglobulin E; ILC, innate lymphoid cell; NA, not applicable, NERD, nonsteroidal anti-inflammatory drug-exacerbated respiratory disease; NK, natural killer; PSL, prednisolone; Th, helper T; Treg, regulatory T.

Data Sharing Statement

The Juntendo University Research Ethics Committee does not permit the sharing of unprocessed raw data with researchers or companies outside the author's research group. The data presented in this study are available within the article, but the raw, non-statistically processed data are not publicly accessible due to privacy concerns.

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Disclosure

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