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Original Article

Pretreatment circulating MAIT cells, neutrophils, and periostin predicted the real-world response after 1-year mepolizumab treatment in asthmatics

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ACT, asthma control test; AERD, aspirin-exacerbated respiratory disease; FP, fluticasone propionate; FVC, forced vital capacity; FEV1%, FEV1/forced vital capacity; ICS, inhaled corticosteroid; ICS, inhaled corticosteroids; ILC1, group 1 ILC; ILC3, group 3 ILC; IP, IFN-γ inducible protein; MAIT, mucosal associated invariant T; MCP, monocyte chemotactic protein; MHC, major histocompatibility complex; MIP, macrophage inflammatory protein; MR1, MHC class I-related protein 1; Mr1−/−, MR1-knockout mice; NA, not applicable; NK, natural killer; OCS, oral corticosteroids; RANTES, regulated on activation normal T cell expressed and secreted; TCR, T cell antigen receptor

ABSTRACT

Background: Mepolizumab treatment improves symptom control and quality of life and reduces exacerbations in patients with severe eosinophilic asthma. However, biomarkers that predict therapeutic effectiveness must be determined for use in precision medicine. Herein, we elucidated the dynamics of various parameters before and after treatment as well as patient characteristics predictive of clinical responsiveness to mepolizumab after 1-year treatment.

Methods: Twenty-seven patients with severe asthma were treated with mepolizumab for one year. Asthma control test scores, pulmonary function tests, fractional exhaled nitric oxide levels, and blood samples were evaluated. Additionally, we explored the role of CD69-positive mucosal-associated invariant T (MAIT) cells as a candidate biomarker for predicting treatment effectiveness by evaluating an OVA-induced asthma murine model using MR1 knockout mice, where MAIT cells were absent.

Results: The frequencies of CD69-positive group 1 innate lymphoid cells, group 3 innate lymphoid cells, natural killer cells, and MAIT cells decreased after mepolizumab treatment. The frequency of CD69-positive MAIT cells and neutrophils was lower and serum periostin levels were higher in responders than in non-responders. In the OVA-induced asthma murine model, CD69-positive MAIT cell count in the whole mouse lung was significantly higher than that in the control mice. Moreover, OVA-induced eosinophilic airway inflammation was exacerbated in the MAIT cell-deficient MR1 knockout mice.

Conclusions: This study shows that circulating CD69-positive MAIT cells, neutrophils, and serum periostin might predict the real-world response after 1-year mepolizumab treatment. Furthermore, MAIT cells potentially have a protective role against type 2 airway inflammation.

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Introduction

Asthma is one of the most common chronic diseases characterized by variable airflow limitation and bronchial hyperresponsiveness. It is deemed a condition in which heterogeneous chronic airway inflammation persists because of the complex involvement of host factors, including genetic and external factors.[1–3] Eosinophilic asthma is present in more than 50% of patients with asthma, and numerous eosinophils found in both the peripheral blood and the airways are associated with asthma exacerbations and airflow limitation.[4,5] Eosinophilic asthma is caused by a type 2 inflammatory mechanism, in which the activation of Th2 cells and ILC2 pathways play a central role.[6,7] These cells produce type 2 cytokines, including IL-4, IL-5, and IL-13. Among the cytokines and other inflammatory mediators produced by these cells, IL-5 is a pivotal cytokine involved in most eosinophil functions, including differentiation, survival, migration, activation, and effector function.[7]

As steroids induce apoptosis in eosinophils, eosinophilic asthma generally responds well to treatment with inhaled corticosteroids (ICS).[8,9] However, the effect of ICS can be limited because some patients who develop the most severe clinical phenotype of eosinophilic asthma also have steroid-resistant refractory asthma. Suppression of IL-5 can overcome steroid resistance in such severe cases with inadequate symptom management with existing therapies.[10] Available biologics targeting the IL-5 pathway include mepolizumab and reslizumab, which are anti-IL-5 neutralizing mAbs, and benralizumab, a monoclonal antibody that targets IL-5 receptor α. These biologics effectively reduce eosinophil count in the blood and airway tissue and inhibit eosinophilic airway inflammation.[11,12] Mepolizumab is the only humanized anti-IL-5 monoclonal IgG1 antibody available in Japan. Treatment of uncontrolled eosinophilic asthma with mepolizumab reduced exacerbations, emergency room visits, and hospitalizations; and improves asthma control, lung function, and oral corticosteroid (OCS)-sparing effects.[13–17]

Mepolizumab is used in clinical practice worldwide as an add-on biologic for patients with uncontrolled asthma who have peripheral blood eosinophil counts greater than 150 cells/μL at screening or greater than 300 cells/μL at some point in the previous year.[13–17] Although high peripheral blood eosinophil counts are known biomarkers for predicting the therapeutic effect of mepolizumab, certain patients with asthma do not respond to mepolizumab even though they have a high peripheral blood eosinophil count.[13,16] Therefore, there is a need for new biomarkers that are useful for predicting the effectiveness of treatment with mepolizumab. Since biologics are expensive, it is critical from the standpoint of the medical economy and the future of stratified medicine to identify responders before treatment administration. Additionally, the effect that suppressing IL-5 has on the immune system is unknown except for the decrease in eosinophil and basophil count in the peripheral blood. In the present study, we investigated whether mepolizumab therapy is associated with peripheral blood immunocompetent cells and searched for useful biomarkers to predict treatment effectiveness. The primary potential biomarkers we investigated were CD69-positive mucosal-associated invariant T (MAIT) cells, neutrophils, and serum periostin.

MAIT cells, a subset of innate-like T lymphocytes restricted by major histocompatibility complex-related molecule 1 (MR1), were named after their preferential location in mucosas.[18–20] MAIT cells are enriched in various tissues and in the peripheral blood of humans and recognize non-peptide antigens presented by the MR1 that include microbially-derived vitamin B2 (riboflavin) derivatives with MAIT cell-activating activity and vitamin B9 (folic acid) derivatives that are non-activating.[19–23] Activated MAIT cells produce cytokines, including IFN-γ, TNF-α, and IL-17.[24–27] It has been shown that patients with asthma have fewer peripheral blood MAIT cells than healthy individuals and is even lower in severe asthma and neutrophilic asthma.[28,29] In *Alternaria* and house dust mite-induced asthma model mice using the MAIT cell-deficient MR1 knockout mice, MAIT cells were reported to repress ILC2 responses and restrict allergic airway inflammation and AHR.[30] Because the role of $CD69^+$ MAIT cells in asthma, which we previously demonstrated lower in severe asthma and have listed as a candidate for biomarkers, is unknown, in this study, we investigated the role of MAIT cells in the OVA-induced asthma murine model which generates a robust adaptive type 2 immune response using mice deficient in MAIT cells.[31,32]

Methods

Patients

This study was a prospective, non-interventional, observational cohort study enrolling patients diagnosed with asthma and newly prescribed mepolizumab treatment. Patients who had severe asthma and were aged 20 years or older, whose asthma symptoms and asthma exacerbations requiring OCS could not be controlled by their existing treatment options despite treatment with high-dose ICS plus long-acting β2 agonists with another controller, and who required mepolizumab treatment in the insurance medical treatment were recruited from our outpatient clinic at Juntendo University Hospital (Tokyo, Japan). Asthma was diagnosed by a clinical history of episodic symptoms with airflow limitation and by either variation in pulmonary function monitored by FEV_1 or by peak expiratory flow per the Global Initiative for Asthma guidelines.[33] Patients having any of the following criteria were excluded: (1) cases with a diagnosis of eosinophilic granulomatosis with polyangiitis, interstitial pneumonia, infectious disease, or cancer, (2) cases under the administration of other antibody preparations, (3) cases which were judged as inappropriate by the investigators, and (4) cases under treatment with omalizumab with <1 month of the last dose and cases under treatment with other biologics. The present study was reviewed and approved by the Juntendo University Research Ethics Committee (Tokyo, Japan). Written informed consent was obtained from each patient before their participation in the study. This study was registered in the UMIN Clinical Trial Registry (UMIN000024886) from May 2016 through March 2019 (http://www.umin.ac.jp/).

The asthma control test (ACT), pulmonary function test, measurement of FeNO levels, and blood sampling were performed on the date of initial mepolizumab administration, and then at 3 months, 6 months, and 1 year after administration. FeNO levels were measured in accordance with the American Thoracic Society's recommendations at a constant flow of 0.05 L/s against an expiratory resistance of 20 cm water with an electrochemical hand-held NO analyzer (NIOX VERO®; Aerocrine AB, Solna, Sweden).

Criteria for responsiveness to mepolizumab

Patients were classified as responders according to changes in ACT score, lung function, and asthma exacerbations with reference to previous studies.[34–40] A responder was defined when 2 of

the following 3 criteria were met after one-year treatment with mepolizumab without significant deterioration in any other criterion: (1) ACT score improvement of at least 3 points (including patients who achieved an ACT score of 25 points). An increase in ACT score of at least 3 points, which was previously suggested as the minimal clinically important difference.[41,42] (2) reduction in the number of asthma exacerbations (inclusive of patients who had no exacerbations before and after treatment), and (3) improvement in the FEV_1 of at least 100 mL.[40,43] Significant deterioration of a criterion was determined as follows: (a) an ACT score decrease of at least 3 points, (b) number of exacerbations increasing, and (c) a decrease in the FEV_1 of at least 100 mL.

Quantification of the frequency of circulating lymphocytes in patients with asthma

Flow cytometry analysis was conducted as previously described.[32] In brief, peripheral venous blood samples were collected in heparin-containing tubes and peripheral blood mononuclear cells (PBMCs, at 3×10^6/well) were purified by density-gradient centrifugation using Ficoll–Paque Plus solution (Cytiva, Tokyo, Japan). The antibodies and reagents used for flow cytometry analysis are listed in Supplementary Table 1. The cells were stained with combinations of appropriate antibodies for 30 min at 4 °C. The specific markers for different immune subsets are shown in Supplementary Table 2. The following surface marker antibodies were used in this study: anti-CD3-APC-H7, anti-CD4-FITC, anti-CD19-FITC, anti-CD56-Alexa Fluor 700, anti-CD117 (c-Kit)-PE-CF594 (BD Biosciences, San Jose, CA, USA), anti-T cell antigen receptor (TCR)-Pan-γδ-FITC, anti-TCR-Pan-γδ-PE (Beckman Coulter, Miami, FL, USA), anti-BDCA2-FITC, anti-CD1a-FITC, anti-CD11c-FITC, anti-CD14-FITC, anti-CD25-PE, anti-CD34-FITC, anti-CD123-FITC, anti-CD127 (IL-7Rα)- Brilliant Violet (BV) 421, anti-CD127-BV605, anti-CD161-PerCPCy5.5, anti-CD183 (CXCR3)-APC, anti-CD194 (CCR4)-BV510, anti-CD196 (CCR6)-PerCPCy55, anti-CD294 (CRTH2)-BV421, anti-CD69-APC, anti-CD69-Alexa Flour 700, anti-FC-R1-FITC, anti-Vα7.2-PE (BioLegend, San Diego, CA, USA). The lineage negative (Lin^-) markers defined were $CD1a^-$, $CD3^-$, $CD11c^-$, $CD14^-$, $CD19^-$, $CD34^-$, $TCR\gamma\delta^-$, $CD123^-$, $BDCA2^-$, and $FC\text{-}R1^-$. The $CD4^+$ Th1 cells identified were $CD3^+$ $CD4^+$ $CCR4^-$ $CCR6^-$ $CXCR3^+$ cells; the Th2 cells were $CD3^+$ $CD4^+$ $CCR4^+$ $CCR6^-$ $CXCR3^-$ cells; the Th17 were $CD3^+$ $CD4^+$ $CCR4^+$ $CCR6^+$ $CXCR3^-$ cells; the Treg cells were $CD3^+$ $CD4^+$ $CD25^+$ $CD127^-$ cells; the γδT cells were $CD3^+$ $TCR\gamma\delta^+$ cells; the MAIT cells were $CD3^+$ Vα7.2 TCR^+ $CD161^{high}$ cells; the NK cells were $CD3^-$ $CD56^+$ cells; the ILC1s were Lin^- $CD127^+$ $CD161^+$ $CD117^-$ $CRTH2^-$ cells; the ILC2s were Lin^- $CD127^+$ $CD161^+$ $CRTH2^+$ cells; and the ILC3s were Lin^- $CD127^+$ $CD161^+$ $CD117^+$ $CRTH2^-$ cells. The activation marker CD69 was analyzed in total MAIT cells, NK cells, and ILCs. After overnight fixation, the cells were analyzed by fluorescence-activated cell sorting (FACS) (LSRFortessa cell analyzer, BD Biosciences). Flowcytometry gating strategies are shown in Supplementary Figure 1. The FACS data were analyzed with FlowJo software (Version 9, BD Biosciences). There were missing data in the Th cell fraction of one subject and the flow cytometric data one year later after collection, due to analyzer failure and human error.

Quantification of serum cytokines and chemokines levels

The sera of the patients were collected after density-gradient centrifugation of the blood samples and frozen at −80 °C. They were then assayed by multiplex bead array assay following the manufacturer's instructions (Bio-Plex, Bio-Rad Laboratories, Hercules, CA, USA). Serum periostin levels were measured using an ELISA (Shino test, Kanagawa, Japan), as described previously.[44] Serum tenascin-C was simultaneously quantified in thawed serum using the human tenascin-C ELISA kit (IBL, Gunma, Japan). The assay working range was determined between the lower and upper limits of quantification (LLOQ and ULOQ) (Supplementary Table 3). Because the serum IFN-γ, IL-5, IL-13, and IL-17 levels were below the detection limit, they were excluded from the analysis.

Mice

Female C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). $Mr1^{-/-}$ mice, with no MAIT cells, were provided by S. Gilfillan (Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA).[18] All mice were kept under specific pathogen-free conditions during the experiments. All animal experiments were approved by the Juntendo University Animal Experimental Ethics Committee and complied with the National Institutes of Health guidelines for animal care.

Induction of airway inflammation

Female $Mr1^{-/-}$ mice on the C57BL/6 background and wildtype littermate control $Mr1^{+/+}$ mice maintained in our facility and were used for in a murine asthma model. Mice were anesthetized by isoflurane and then immunized by intraperitoneal injection of 20 μg of OVA (Sigma-Aldrich) with 2 mg of adjuvant (Thermo Fisher Scientific, Yokohama, Japan) on day 0. On days 7, 8, 9, and 10, the mice were challenged intranasally with 10 μg of OVA alone. The negative control animals were i. p. injected with PBS/alum, and challenged with PBS, in a similar manner.

AHR measurements

Two days after the last challenge, mice were anesthetized with pentobarbital and xylazine, and then intubated with metal 18-gauge catheters via a tracheotomy. The needle was immediately connected to the flexiVent™ (SCIREQ, Montreal, QC, Canada). After measurement of baseline resistance (saline inhalation), the mice were challenged with increasing concentrations (0, 6, 12, 24, and 48 mg/mL) of methacholine aerosol generated with an in-line nebulizer and administered directly through the ventilator for 5 s.

Flow cytometric analysis of whole mouse lung cells

Mice lung tissue was removed and minced 2 days after the last injection. The samples were digested with collagenase and DNase I (Roche Biochemicals, Mannheim, Germany) in RPMI 1640 medium for 30 min at 37 °C, and then filtered to obtain single-cell suspensions. The antibodies and reagents used for flow cytometry analysis are listed in Supplementary Table 1. Cells were preincubated with unlabeled anti-CD16/32 mAb to avoid nonspecific binding of antibodies to the Fc receptor. Then, cells were stained with specific markers for different immune subsets which are shown in Supplementary Table 2. In brief, cells were stained with anti-CD11c-FITC, anti-Siglec F-PE (BD Biosciences), anti-CD45.2-BV510, anti-Ly-6G/Ly6C, Gr-1-BV605, anti-F4/80-

Table 1
Baseline characteristics of the study population.

	Number (%), mean ± SD or median (interquartile range), n = 27		Number (%), mean ± SD or median (interquartile range), n = 27
Sex (M/F)	6 (22.2%) /21 (77.8%)	Peripheral lymphocytes (%)	27.6 ± 6.1
Age (y)	55.6 ± 13.4	Peripheral lymphocytes ($\times 10^2$ cells/μL)	17.8 ± 4.9
Age at asthma onset (y)	38.4 ± 18.3	Total log IgE	2.4 ± 0.6
Duration of asthma (y)	17.2 ± 11.6	Periostin (ng/mL)	107.1 ± 46.2
BMI (kg/m^2)	24.2 ± 4.7	Tenascin-C (ng/mL)	37.0 ± 18.4
Smoking history (never/ex)	21 (77.8%)/6 (22.2%)	IL-4 (pg/mL), n = 26	1.1 (0.8–1.5)
AERD	3 (11.1%)	IL-8 (pg/mL), n = 22	7.4 (5.9–8.7)
Atopic dermatitis	10 (37.0%)	Eotaxin-1 (pg/mL)	73.1 (53.0–84.1)
Allergic rhinitis	20 (74.1%)	IP-10 (pg/mL)	600.7 ± 224.0
Chronic sinusitis	9 (33.3%)	MCP-1 (pg/mL), n = 25	20.5 (14.7–23.4)
Daily dose of ICS (FP equivalent dose, μg)	1000.0 (1000.0–1000.0)	MIP-1α (pg/mL)	1.4 (1.2–2.2)
Oral corticosteroid treatment	3 (11.1%)	MIP-1β (pg/mL)	74.5 (67.3–82.0)
Previous omalizumab treatment	16 (59.3%)	RANTES (ng/mL)	5.0 (4.6–5.8)
Asthma exacerbations (/year)	2.0 (0.0–3.0)	Th1 cells (% of Th cells, %), n = 26	18.2 (15.1–28.2)
Unscheduled visits (/year)	1.0 (0.0–2.0)	Th2 cells (% of Th cells, %), n = 26	5.7 ± 2.9
Hospitalizations (/year)	0.0 (0.0–0.0)	Th17 cells (% of Th cells, %), n = 26	5.4 ± 2.5
ACT score points	17.9 ± 5.3	Treg cells (% of Th cells, %), n = 26	5.4 (4.2–6.7)
FeNO (ppb)	25.0 (15.0–71.0)	ILC1 (% of ILC cells, %)	63.1 ± 11.8
FVC (L)	2.9 ± 0.7	ILC2 (% of ILC cells, %)	24.8 ± 11.9
%FVC (predicted, %)	98.4 (93.1–110.6)	ILC3 (% of ILC cells, %)	10.7 (6.6–13.4)
FEV_1 (L)	2.1 ± 0.7	$CD69^+$ ILC1 (% of ILC1, %)	10.5 ± 4.6
%FEV_1 (predicted, %)	89.1 ± 24.8	$CD69^+$ ILC2 (% of ILC2, %)	10.0 ± 3.2
FEV_1% (%)	71.7 ± 16.5	$CD69^+$ ILC3 (% of ILC3, %)	12.7 ± 5.1
Peripheral neutrophils (%)	61.5 (56.7–65.2)	NK cells (% of lymphoid cells, %)	14.1 ± 8.3
Peripheral neutrophils ($\times 10^2$ cells/μL)	40.0 ± 11.0	$CD69^+$ NK cells (% of NK, %)	9.5 (7.7–14.9)
Peripheral eosinophils (%)	4.1 (3.1–7.5)	γδT cells (% of $CD3^+$-cells, %)	2.5 (1.6–5.4)
Peripheral eosinophils (cells/μL)	269.0 (161.0–486.0)	MAIT cells (% of $CD3^+$-cells, %)	1.8 (0.9–2.6)
Peripheral basophils (%)	0.6 (0.4–0.8)	$CD69^+$-MAIT cells (% of MAIT cells, %)	32.5 ± 13.9
Peripheral basophils (cells/μL)	39.0 (21.6–50.4)		

Abbreviations for all tables: ACT, asthma control test; AERD, Aspirin-exacerbated respiratory disease; FEV1%, FEV1 second/forced vital capacity; FP, fluticasone propionate; FVC, forced vital capacity; ICS, inhaled corticosteroid; 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.

FITC, anti-CD3-BV605, anti-CD4-APC/Fire750, anti-CD69-PECy7 (BioLegend), and anti-mMR1 tetramers (5-OP-RU)-BV421 (NIH tetramer core facility at Emory University, Atlanta, GA, USA).[45] To detect intracellular cytokine production, these cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and ionomycin (1 μg/mL) for 4 h in the presence of a protein transport inhibitor containing brefeldin A (GolgiPlug) for the last 2 h. After stimulation and cell surface staining, the cells were fixed and permeabilized using the Cytofix/Cytoperm Kit, followed by intracellular staining with anti-IFN-γ, anti-IL-4, and anti-IL-17A-PE mAbs. Dead cells were identified using the Zombie Fixable Viability Kit, followed by doublet exclusion through both forward scatter and side scatter. Stained cells were analyzed by LSR Fortessa and data were processed by FlowJo software (Version 10, BD Biosciences). Flowcytometry gating strategies are shown in Supplementary Figure 2.

Statistical analysis

Sample normality was examined using the D'Agostino-Pearson test. Differences in parameters between populations were analyzed for significance using Welch's *t*-test, paired *t* test, Mann–Whitney *U* test, Wilcoxon signed-rank test, and Fisher's exact test as appropriate. Comparisons between multiple groups were made by one-way repeated ANOVA measurements with Tukey's multiple comparisons test and Friedman's test with Dunn's multiple comparisons test. A ROC curve analysis was performed to differentiate the responders and non-responders for mepolizumab. For correlation between variables, the Pearson's correlation coefficient and Spearman's rank correlation coefficient were used where appropriate. Differences were statistically significant when p values were 0.05 or less. Statistical analyses were performed using GraphPad Prism software, version 6 (GraphPad Software, San Diego, CA, USA).

Results

Baseline characteristics

We included 27 patients with severe asthma that was uncontrolled even by the existing treatment, who were then treated with mepolizumab. The baseline characteristics are shown in Tables 1 and 2. The mean (± standard deviation) age of the patients was 55.6 ± 13.4 years (Table 1). The median peripheral blood eosinophil count was 269/μL (Tables 1 and 2). Twenty-four patients (89%) had met a blood eosinophil count of 150/μL or higher at the start of the study or 300/μL or higher in the previous year, and the remaining three patients treated with omalizumab or OCS before mepolizumab treatment.

Changes in each parameter one year after mepolizumab treatment

After one year of mepolizumab treatment, ACT scores improved by at least 3 points (the minimal clinically important

Table 2
Baseline characteristics of the study population and kinetics of parameters in patients treated with mepolizumab.

n = 25	Baseline	1 year post treatment	p value
Asthma exacerbations (/year)	2.2 ± 2.2	1.0 (0.0–2.0)	0.04*
Unscheduled visits (/year)	1.0 (0.0–2.0)	0.0 (0.0–1.5)	0.04*
Hospitalizations (/year)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	1.00
ACT score points	18.4 ± 5.1	22.0 (20.0–25.0)	0.005*
FeNO (ppb)	25.0 (15.0–78.5)	25.0 (14.0–55.5)	0.23
FVC (L)	2.9 ± 0.7	3.0 ± 0.7	0.08
%FVC (predicted, %)	98.2 (92.8–110.7)	106.6 ± 16.7	0.02*
FEV_1 (L)	2.1 ± 0.7	2.2 ± 0.7	0.07
$\%FEV_1$ (predicted, %)	88.5 ± 25.7	93.3 ± 26.3	0.02*
$FEV_1\%$ (%)	71.1 ± 16.8	77.2 (61.9–84.7)	0.49
Peripheral neutrophils (%)	61.5 (56.4–65.3)	61.6 ± 10.2	0.70
Peripheral neutrophils ($\times 10^2$ cells/μL)	40.6 ± 11.3	39.1 ± 15.4	0.55
Peripheral eosinophils (%)	4.1 (3.1–7.4)	0.8 (0.4–1.3)	<0.001*
Peripheral eosinophils (cells/μL)	269.0 (161.0–528.5)	38.0 (28.0–72.0)	<0.001*
Peripheral basophils (%)	0.6 (0.4–0.8)	0.4 ± 0.2	<0.001*
Peripheral basophils (cells/μL)	39.0 (22.0–49.6)	23.5 ± 16.9	<0.001*
Peripheral lymphocytes (%)	27.8 ± 6.3	31.7 ± 9.7	0.02*
Peripheral lymphocytes ($\times 10^2$ cells/μL)	18.2 ± 4.9	18.8 ± 5.6	0.53
Total log IgE	2.4 ± 0.6	2.2 ± 0.7	0.04*
Periostin (ng/mL)	111.4 ± 45.3	86.0 (64.5–121.5)	0.03*
Tenascin-C (ng/mL)	37.6 ± 18.9	45.1 ± 25.2	0.09
IL-4 (pg/mL), n = 24	1.1 (0.8–1.5)	1.7 ± 1.0	0.049*
IL-8 (pg/mL), n = 19	7.4 (6.3–8.6)	9.8 ± 4.5	0.72
Eotaxin-1 (pg/mL)	73.1 (50.7–82.7)	94.0 ± 40.3	0.006*
IP-10 (pg/mL)	602.2 ± 231.5	572.1 ± 262.6	0.48
MCP-1 (pg/mL), n = 21	20.5 (13.8–23.3)	18.4 ± 6.7	0.16
MIP-1α (pg/mL), n = 24	1.4 (1.2–2.2)	1.7 (1.1–3.1)	0.85
MIP-1β (pg/mL)	74.5 (67.8–81.0)	79.0 ± 18.1	0.62
RANTES (ng/mL)	4.9 (4.6–5.6)	5.2 ± 1.2	0.92
Th1 cells (% of Th cells, %), n = 23	18.6 (15.9–27.8)	19.0 (16.2–28.2)	0.58
Th2 cells (% of Th cells, %), n = 23	4.9 (3.0–6.1)	5.0 (3.5–6.6)	0.15
Th17 cells (% of Th cells, %), n = 23	4.9 (3.1–6.0)	5.0 (4.2–6.3)	0.06
Treg cells (% of Th cells, %), n = 23	5.4 (4.2–6.6)	5.5 ± 1.9	0.38
ILC1 (% of ILC cells, %), n = 24	62.8 ± 12.0	66.3 ± 16.0	0.08
ILC2 (% of ILC cells, %), n = 24	25.0 ± 12.1	22.3 ± 14.8	0.08
ILC3 (% of ILC cells, %), n = 24	10.4 (6.8–13.4)	9.1 (5.3–13.3)	0.55
$CD69^+$ ILC1 (% of ILC1, %), n = 24	10.4 ± 4.0	6.9 ± 3.7	<0.001*
$CD69^+$ ILC2 (% of ILC2, %), n = 24	10.1 ± 3.3	7.2 (4.5–10.1)	0.056
$CD69^+$ ILC3 (% of ILC3, %), n = 24	13.0 ± 5.3	10.2 ± 6.4	0.047*
NK cells (% of lymphoid cells, %)	13.4 ± 8.5	12.4 ± 8.3	0.34
$CD69^+$ NK cells (% of NK, %), n = 24	9.9 (7.8–14.6)	7.8 (5.5–12.5)	0.001*
γδT cells (% of $CD3^+$ cells, %), n = 24	2.3 (1.5–5.2)	2.1 (1.3–4.3)	0.24
MAIT cells (% of $CD3^+$ cells, %), n = 24	1.9 (0.8–2.6)	1.9 ± 1.0	0.56
$CD69^+$ MAIT cells (% of MAIT cells, %), n = 24	30.3 ± 12.3	24.7 ± 12.1	0.01*

Data are presented as the mean ± standard deviation or the median (interquartile range) unless otherwise indicated.
*$p < 0.05$.

difference[42]) or (well-controlled' asthma was achieved in 18 (67%) of 27 patients. Owing to worsening asthma symptoms five months after the start of mepolizumab treatment, 2 of the 27 cases discontinued treatment and were excluded from the analysis. In the remaining 25 cases, ACT score, %FVC, and $\%FEV_1$ improved significantly, and the number of asthma exacerbations and unscheduled visits for worsening asthma decreased significantly, although the number of hospitalizations did not change (Table 2). Mepolizumab treatment significantly decreased peripheral blood eosinophil counts, basophil counts, serum periostin, and total IgE levels, and increased serum IL-4 and eotaxin-1 levels (Table 2). The frequencies of Th cells, ILCs, MAIT, and NK cells in peripheral blood did not significantly change after treatment (Table 2). However, the frequencies of $CD69^+$ group 1 innate lymphoid cell (ILC1), $CD69^+$ group 3 innate lymphoid cell (ILC3), $CD69^+$ NK cells, and $CD69^+$ MAIT cells was significantly decreased after one year of mepolizumab treatment (Table 2).

Parameters for predicting the effectiveness of mepolizumab treatment in patients with severe asthma

We divided the 27 patients into two subgroups according to the response for mepolizumab treatment (Table 3). The number of responders was 12 (44%) and responders had a significantly lower neutrophil and $CD69^+$ MAIT cell count in the peripheral blood and higher levels of serum periostin before mepolizumab treatment than non-responders (Table 3). There were more omalizumab users among non-responders than among responders and more non-responders received a daily dose of ICS before treatment (Table 3). The difference in daily ICS dose was due to the responders including two patients who were unable to use high-dose ICS due to their side effects. ROC curve analysis showed that the area under the curve of $CD69^+$ MAIT cells, neutrophil counts, and serum periostin levels were 0.81, 0.77, and 0.74, respectively (Table 4). The best cutoffs for predicting responders were peripheral neutrophil

Table 3
Baseline characteristics in responders or non-responders.

	Responder (n = 12)	Nonresponder (n = 15)	p value
Sex (M/F), n (%)	3 (25.0%)/9 (75.0%)	3 (20.0%)/12 (80.0%)	1.00
Age (y)	53.8 ± 13.6	56.9 ± 13.6	0.56
Age at asthma onset (y)	37.5 ± 17.7	39.2 ± 19.3	0.81
Duration of asthma (y)	16.3 ± 12.3	17.8 ± 11.5	0.75
BMI (kg/m^2)	25.1 ± 5.3	22.8 (21.1–25.1)	0.16
Smoking history (never/ex), n (%)	9 (75.0%)/3 (25.0%)	12 (80.0%)/3 (20.0%)	1.00
AERD, n (%)	0 (0.0%)	3 (20.0%)	0.23
Atopic dermatitis, n (%)	6 (50.0%)	4 (26.7%)	0.26
Allergic rhinitis, n (%)	9 (75.0%)	11 (73.3%)	1.00
Chronic sinusitis, n (%)	3 (25.0%)	6 (40.0%)	0.68
Daily dose of ICS (FP equivalent dose, μg)	875.0 ± 176.5	1000.0 (1000.0–1000.0)	0.03*
Oral corticosteroid treatment, n (%)	2 (16.7%)	1 (6.7%)	0.57
Previous omalizumab treatment, n (%)	4 (33.3%)	12 (80.0%)	0.02*
Asthma exacerbations (/year)	2.4 ± 2.5	1.8 ± 1.7	0.48
Unscheduled visits (/year)	1.5 ± 1.8	1.0 (0.0–3.0)	0.93
Hospitalizations (/year)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.23
ACT score points	15.8 ± 5.8	20.0 (17.0–23.0)	0.07
FeNO (ppb)	51.0 ± 45.7	23.0 (15.0–57.0)	0.59
FVC (L)	2.9 ± 0.5	3.0 ± 0.8	0.87
%FVC (predicted, %)	101.0 ± 13.0	100.4 (92.4–110.6)	0.57
FEV_1 (L)	2.1 ± 0.5	2.1 ± 0.9	0.99
%FEV_1 (predicted, %)	88.5 ± 17.6	89.6 ± 30.0	0.90
FEV_1% (%)	73.6 ± 14.8	70.2 ± 18.1	0.60
Peripheral neutrophils (%)	56.8 ± 8.3	64.0 (60.0–67.9)	0.02*
Peripheral neutrophils (× 10^2 cells/μL)	34.3 ± 10.0	44.6 ± 9.7	0.01*
Peripheral eosinophils (%)	7.3 ± 5.0	4.1 ± 1.9	0.055
Peripheral eosinophils (cells/μL)	472.5 ± 393.1	290.8 ± 157.2	0.15
Peripheral basophils (%)	0.6 (0.4–1.3)	0.5 (0.3–0.6)	0.28
Peripheral basophils (cells/μL)	52.5 ± 40.3	33.2 (22.4–50.4)	0.73
Peripheral lymphocytes (%)	29.7 ± 5.9	25.7 (22.1–30.4)	0.23
Peripheral lymphocytes (× 10^2 cells/μL)	17.4 ± 3.7	18.1 ± 5.8	0.74
Total log IgE	2.4 ± 0.8	2.4 ± 0.4	0.80
Periostin (ng/mL)	124.4 ± 44.8	86.0 (62.0–104.0)	0.03*
Tenascin-C (ng/mL)	40.2 ± 22.5	34.4 ± 14.7	0.45
IL-4 (pg/mL)	1.4 ± 0.9	1.0 (0.9–1.5), n = 14	0.97
IL-8 (pg/mL)	7.4 (6.4–12.6), n = 9	6.6 (5.2–8.4), n = 13	0.40
Eotaxin-1 (pg/mL)	71.9 (48.8–89.3)	70.8 ± 22.9	0.93
IP-10 (pg/mL)	583.0 ± 244.5	614.8 ± 213.8	0.73
MCP-1 (pg/mL)	21.8 (13.2–29.0)	20.2 ± 5.4, n = 13	0.84
MIP-1α (pg/mL)	1.6 (1.3–2.1)	1.8 ± 1.1	0.48
MIP-1β (pg/mL)	74.9 (66.8–93.1)	70.8 (67.3–80.1)	0.64
RANTES (ng/mL)	5.4 ± 1.3	5.1 ± 0.6	0.53
Th1 cells (% of Th cells, %)	21.5 ± 11.1	18.5 (15.7–26.2), n = 14	0.75
Th2 cells (% of Th cells, %)	4.4 (2.5–6.0)	6.1 ± 2.5, n = 14	0.23
Th17 cells (% of Th cells, %)	5.2 ± 3.0	5.0 (4.0–6.0), n = 14	0.50
Treg cells (% of Th cells, %)	5.7 (4.3–6.8)	5.6 ± 1.9, n = 14	0.89
ILC1 (% of ILC cells, %)	67.6 ± 10.4	59.4 ± 12.0	0.07
ILC2 (% of ILC cells, %)	22.7 ± 9.5	26.5 ± 13.6	0.41
ILC3 (% of ILC cells, %)	8.6 ± 4.6	11.9 (8.7–13.7)	0.08
CD69$^+$ ILC1 (% of ILC1, %)	9.8 ± 4.7	11.0 ± 4.6	0.50
CD69$^+$ ILC2 (% of ILC2, %)	10.2 ± 2.6	9.9 ± 3.7	0.83
CD69$^+$ ILC3 (% of ILC3, %)	14.0 ± 5.9	11.6 ± 4.2	0.25
NK cells (% of lymphoid cells, %)	12.9 ± 8.7	14.9 ± 8.2	0.55
CD69$^+$ NK cells (% of NK, %)	9.5 (6.6–15.1)	10.6 (8.2–14.9)	0.45
γδT cells (% of CD3$^+$ cells, %)	3.7 ± 2.9	2.1 (1.7–4.5)	0.82
MAIT cells (% of CD3$^+$ cells, %)	2.0 (1.3–2.6)	1.6 ± 0.9	0.28
CD69$^+$ MAIT cells (% of MAIT cells, %)	23.9 (17.8–30.1)	38.0 ± 12.3	0.005*

Data are presented as the mean ± standard deviation or the median (interquartile range) unless otherwise indicated.
*$p < 0.05$.
Responder was defined as at least 2 positive response criterion without significant deterioration in any other criterion.

counts of 4035 cells/μL (sensitivity, 75.0%; specificity, 73.3%), neutrophil frequencies of 61.6% of white blood cells (sensitivity, 75.0%; specificity, 66.7%), serum periostin levels of 92.5 ng/mL (sensitivity, 83.3%; specificity, 73.3%), and the number of CD69$^+$ MAIT cells being 29.0% of MAIT cells (sensitivity, 75.0%; specificity, 86.7%) (Fig. 1).

The findings in Table 2 that 1-year mepolizumab treatment decreased the frequencies of CD69$^+$ MAIT cells and the findings in Table 3 and Figure 1 that patients with low CD69$^+$ MAIT cells were responders of 1-year mepolizumab treatment seemed contradictory. Therefore, we compared changes in each parameter one year after mepolizumab treatment in responders and

Table 4
Receiver operating characteristic (ROC) curve.

	Area under ROC curve	*p* value
CD69$^+$ MAIT (% of MAIT cells, %)	0.81	0.006*
Peripheral neutrophils (cells/μL)	0.77	0.02*
Peripheral neutrophils (%)	0.76	0.02*
Serum periostin (ng/mL)	0.74	0.03*
Peripheral eosinophils (%)	0.66	0.15
Peripheral eosinophils (cells/μL)	0.60	0.39
Periostin/Neutrophils ratio	0.87	0.001*
Periostin/CD69$^+$ MAIT ratio	0.85	0.002*
Periostin/Neutrophils (%) ratio	0.79	0.01*
Eosinophils (%)/CD69$^+$ MAIT ratio	0.78	0.01*
Neutrophils (%)/CD69$^+$ MAIT ratio	0.74	0.03*
Eosinophils/CD69$^+$ MAIT ratio	0.72	0.06
Eosinophils (%)/Neutrophils (%) ratio	0.68	0.12
Eosinophils/Neutrophils ratio	0.67	0.14
Neutrophils/CD69$^+$ MAIT ratio	0.58	0.49

*$p < 0.05$.

non-responders and compared patients with more decreased CD69$^+$ MAIT cells with those without. In responders, the number of asthma exacerbations, ACT score, FVC, %FVC, FEV_1, and $\%FEV_1$ improved significantly, and the frequencies of peripheral blood lymphocytes and Th17 cells significantly increased and serum IP-10 levels and the frequencies of CD69$^+$ ILC1 significantly decreased after treatment (Supplementary Table 4). Mepolizumab treatment significantly decreased peripheral blood eosinophil and basophil counts, and the frequencies of eosinophils and CD69+ NK cells in both responders and non-responders. The frequencies of basophils and serum total IgE levels were significantly decreased, and serum tenascin-1, IL-4, and eotaxin-1 levels were significantly increased after treatment in non-responders, and similar trends were observed in responders, except IgE (Supplementary Table 4). Although the changes in the frequencies of CD69$^+$ MAIT cells tended to decrease in both responders and non-responders, there was no significant difference (Supplementary Table 4). Furthermore, we divided patients into two groups that decrease and did not decrease below the mean value of −5.6% in the CD69$^+$ MAIT cells difference between baseline and 1-year post-treatment. There were no significant differences in baseline characteristics, except that patients with below-mean decreasing CD69$^+$ MAIT cells had significantly more peripheral blood basophils (Supplementary Table 5). Moreover, one year of mepolizumab treatment significantly decreased eosinophil and basophil counts and the frequencies of eosinophils and CD69$^+$ ILC1 in patients with below and above mean decreasing CD69$^+$ MAIT cells (Supplementary Table 6). In patients with below-mean decreasing CD69$^+$ MAIT cells, the frequencies of basophils significantly decreased, and MAIT cells significantly increased after treatment (Supplementary Table 6). In patients with the above mean decreasing CD69$^+$ MAIT cells, ACT score, serum tenascin-C, IL-4, and eotaxin-1 levels significantly increased and serum total IgE, and periostin levels, and the frequencies of CD69$^+$ NK cells significantly decreased after treatment (Supplementary Table 6).

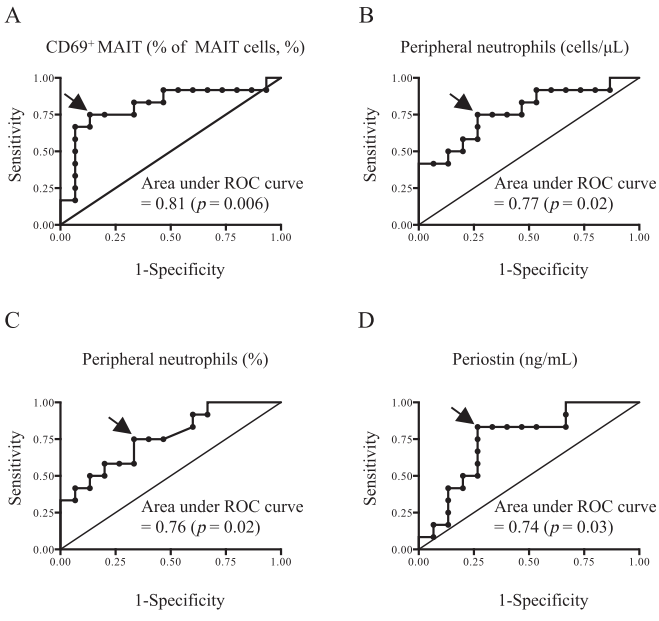

Fig. 1. ROC curve for predicting the effect of mepolizumab treatment in patients with severe asthma. Using Youden's index, the cut-off value for **(A)** frequency of CD69$^+$ MAIT cells of 29.0% of MAIT cells (sensitivity, 75.0%; specificity, 86.7%), **(B)** peripheral neutrophil counts of 4035 cells/μL (sensitivity, 75.0%; specificity, 73.3%), **(C)** neutrophil frequencies of 61.6% of white blood cells (sensitivity, 75.0%; specificity, 66.7%), and **(D)** serum periostin levels of 92.5 ng/mL (sensitivity, 83.3%; specificity, 73.3%) are indicated with arrows.

Association of type 2 and non-type 2 biomarker levels with the characteristics of patients with severe asthma

Activated MAIT cells were reported to be two types into cells that primarily produce Th1 cytokines including IFN-γ and cells that predominantly produce Th17 cytokines including IL-17A.[24,46–49] Neutrophils and activated MAIT cells expressing membrane protein CD69, were considered non-type 2 biomarkers. We investigated whether type 2 biomarker and non-type 2 biomarker levels, including the frequency of CD69$^+$ MAIT cells and neutrophils in peripheral blood, were associated with clinical asthma parameters.

The frequency of CD69$^+$ MAIT cells was positively correlated with the frequency of CD69$^+$ ILC1 ($r = 0.45$, $p = 0.02$) and the frequency of CD69$^+$ NK cells ($r = 0.39$, $p = 0.048$) (Table 5). The peripheral neutrophil counts were negatively correlated with the frequency of peripheral lymphocytes ($r = -0.63$, $p < 0.001$) (Table 5). Serum periostin levels were positively correlated with FeNO levels ($r = 0.52$, $p = 0.005$), peripheral blood eosinophil counts ($r = 0.51$, $p = 0.006$), tenascin-C ($r = 0.42$, $p = 0.03$), serum IFN-γ-inducible protein 10 (IP-10) levels ($r = 0.45$, $p = 0.014$), and the frequency of CD69$^+$ ILC3 ($r = 0.42$, $p = 0.03$); but were negatively correlated with %FVC ($r = -0.41$, $p = 0.03$), $\%FEV_1$ ($r = -0.53$, $p = 0.004$), and $FEV_1\%$ ($r = -0.49$, $p = 0.01$) (Table 5).

Additionally, we investigated whether the ratio of non-type 2 biomarkers (circulating CD69$^+$ MAIT cells and neutrophils) to the type 2 biomarker periostin was useful as a biomarker to predict the effectiveness of mepolizumab treatment. ROC curve analysis revealed the area under the curve to be 0.85 for the periostin/CD69$^+$ MAIT ratio and 0.87 for the periostin/neutrophils ratio (Table 4).

MAIT cell-deficiency in mice exacerbated eosinophilic airway inflammation in the murine asthma model

Because our findings suggested that CD69$^+$ MAIT cells could be biomarkers for predicting the effect of mepolizumab treatment in patients with severe asthma, we investigated the role of CD69$^+$ MAIT cells in the OVA-induced asthma murine model which

Table 5
Correlation of type 2 biomarker and non-type 2 biomarker levels with baseline characteristics.

	CD69+MAIT cells (n = 27)		Peripheral neutrophils (n = 27)		Periostin (n = 27)		Peripheral eosinophils (n = 27)		FeNO (n = 27)		Total log IgE (n = 27)	
	r	*p* value	r	*p* value	r	*p* value	r	*p* value	r	*p* value	r	*p* value
Age (y)	−0.017	0.93	0.018	0.93	0.297	0.13	0.232	0.24	0.261	0.19	−0.110	0.58
Duration of asthma (y)	0.261	0.19	0.258	0.19	0.142	0.48	0.084	0.68	0.276	0.16	0.145	0.47
BMI (kg/m^2)	0.172	0.39	−0.054	0.79	0.123	0.54	0.185	0.36	0.097	0.63	0.203	0.31
Daily dose of ICS (FP equivalent dose, μg)	0.360	0.07	0.338	0.08	−0.178	0.37	−0.181	0.36	−0.368	0.06	−0.118	0.56
Unscheduled visits (/year)	−0.023	0.91	−0.111	0.58	−0.147	0.46	−0.077	0.70	−0.272	0.17	−0.157	0.43
Asthma exacerbations (/year)	−0.194	0.33	−0.038	0.85	0.030	0.88	−0.078	0.70	−0.311	0.11	−0.519	0.006*
Hospitalizations (/year)	0.303	0.12	0.091	0.65	−0.227	0.25	−0.015	0.94	−0.432	0.02*	−0.091	0.65
ACT score points	−0.043	0.83	0.318	0.11	0.270	0.17	0.203	0.31	0.405	0.04*	0.207	0.30
FeNO (ppb)	−0.128	0.53	0.049	0.81	0.520	0.005*	0.529	0.005*	NA	NA	0.325	0.10
FVC (L)	0.261	0.19	−0.065	0.75	−0.062	0.76	0.087	0.67	0.093	0.65	0.066	0.74
%FVC (predicted, %)	−0.004	0.98	−0.136	0.50	−0.411	0.03*	0.049	0.81	0.086	0.67	−0.183	0.36
FEV_1 (L)	0.138	0.49	−0.233	0.24	−0.367	0.06	−0.260	0.19	−0.237	0.23	−0.030	0.88
%FEV_1 (predicted, %)	−0.087	0.67	−0.381	0.050	−0.530	0.004*	−0.387	0.046*	−0.363	0.06	−0.185	0.35
FEV_1% (%)	−0.071	0.73	−0.367	0.06	−0.490	0.01*	−0.404	0.04*	−0.447	0.02*	−0.071	0.72
Peripheral neutrophils (%)	0.066	0.74	0.364	0.06	−0.365	0.06	−0.294	0.14	−0.171	0.39	0.049	0.81
Peripheral neutrophils (cells/μL)	0.349	0.07	NA	NA	0.096	0.63	0.310	0.12	0.049	0.81	−0.045	0.83
Peripheral eosinophils (%)	−0.125	0.53	0.058	0.77	0.473	0.01*	0.941	<0.001*	0.603	<0.001*	0.064	0.75
Peripheral eosinophils (cells/μL)	0.052	0.80	0.310	0.12	0.513	0.006*	NA	NA	0.529	0.005*	0.027	0.89
Peripheral basophils (%)	0.026	0.90	−0.047	0.82	0.243	0.22	0.380	0.050	0.005	0.98	0.235	0.24
Peripheral basophils (cells/μL)	0.133	0.51	0.231	0.25	0.302	0.13	0.488	0.01*	0.073	0.72	0.149	0.46
Peripheral lymphocytes (%)	−0.234	0.24	−0.630	<0.001*	0.128	0.53	−0.164	0.41	−0.007	0.97	−0.204	0.31
Peripheral lymphocytes (cells/μL)	0.182	0.36	0.222	0.27	0.277	0.16	0.209	0.30	0.048	0.81	−0.235	0.24
Total log IgE	0.302	0.13	−0.045	0.83	0.083	0.68	0.027	0.89	0.325	0.10	NA	NA
Periostin (ng/mL)	−0.052	0.80	0.096	0.63	NA	NA	0.513	0.006*	0.520	0.005*	0.083	0.68
Tenascin-C (ng/mL)	0.184	0.36	−0.012	0.95	0.415	0.03*	0.056	0.78	0.209	0.30	0.205	0.31
IL-4 (pg/mL), n = 26	0.105	0.61	−0.120	0.56	0.050	0.81	0.135	0.51	0.294	0.14	0.093	0.65
IL-8 (pg/mL), n = 22	−0.104	0.65	−0.012	0.96	−0.183	0.41	−0.039	0.86	0.261	0.24	0.143	0.53
Eotaxin-1 (pg/mL)	0.198	0.32	−0.163	0.42	0.254	0.20	−0.030	0.88	0.214	0.28	0.245	0.22
IP-10 (pg/mL)	0.169	0.40	0.266	0.18	0.466	0.01*	0.279	0.16	−0.057	0.78	−0.234	0.24
MCP-1 (pg/mL), n = 25	−0.073	0.73	−0.137	0.51	0.271	0.19	0.224	0.28	0.516	0.008*	0.127	0.55
MIP-1α (pg/mL)	−0.002	0.99	−0.093	0.64	0.218	0.28	0.110	0.58	0.281	0.16	−0.019	0.93
MIP-1β (pg/mL)	0.220	0.27	0.158	0.43	0.349	0.07	0.412	0.03*	0.229	0.25	−0.085	0.67
RANTES (pg/mL)	0.085	0.67	0.014	0.94	−0.006	0.98	0.367	0.06	0.519	0.006*	0.155	0.44
Th1 cells (% of Th cells, %), n = 26	0.040	0.85	−0.044	0.83	0.087	0.67	0.066	0.75	0.032	0.88	−0.268	0.19
Th2 cells (% of Th cells, %), n = 26	0.067	0.74	0.138	0.50	−0.250	0.22	0.032	0.88	0.009	0.97	0.083	0.69
Th17 cells (% of Th cells, %), n = 26	−0.106	0.61	0.090	0.66	−0.016	0.94	0.036	0.86	0.256	0.21	0.215	0.29
Treg cells (% of Th cells, %), n = 26	−0.283	0.16	−0.283	0.16	0.069	0.74	−0.252	0.21	0.219	0.28	0.201	0.33
ILC1 (% of ILC cells, %)	−0.048	0.81	−0.128	0.52	−0.209	0.30	−0.240	0.23	−0.093	0.64	0.207	0.30
ILC2 (% of ILC cells, %)	−0.059	0.77	0.084	0.68	0.075	0.71	0.188	0.35	0.013	0.95	−0.180	0.37
ILC3 (% of ILC cells, %)	0.194	0.33	0.092	0.65	−0.238	0.23	0.039	0.85	0.055	0.79	−0.118	0.56
CD69$^+$ ILC1 (% of ILC1, %)	0.448	0.02*	−0.030	0.88	−0.062	0.76	−0.356	0.07	−0.275	0.16	0.319	0.11
CD69$^+$ ILC2 (% of ILC2, %)	0.181	0.37	0.105	0.60	0.241	0.23	0.097	0.63	0.083	0.68	0.427	0.03*
CD69$^+$ ILC3 (% of ILC3, %)	0.143	0.48	0.233	0.24	0.422	0.03*	0.069	0.73	0.192	0.34	0.451	0.02*
NK cells (% of lymphoid cells, %)	−0.161	0.42	0.041	0.84	0.143	0.48	0.185	0.35	0.094	0.64	0.023	0.91
CD69$^+$ NK cells (% of NK, %)	0.385	0.048*	0.035	0.86	−0.139	0.49	−0.225	0.26	−0.190	0.34	0.317	0.11
γδT cells (% of CD3$^+$ cells, %)	−0.019	0.93	−0.197	0.32	−0.193	0.33	−0.312	0.11	−0.076	0.70	−0.013	0.95
MAIT cells (% of CD3$^+$ cells, %)	−0.265	0.18	−0.156	0.44	−0.060	0.77	−0.161	0.42	−0.117	0.56	0.077	0.70
CD69$^+$ MAIT cells (% of MAIT cells, %)	NA	NA	0.349	0.07	−0.052	0.80	0.052	0.80	−0.128	0.53	0.302	0.13

*$p < 0.05$.

generates a robust adaptive immune response. CD69 has been known as an early activation marker of lymphocytes and plays crucial roles in the infiltration and/or maintenance of inflammatory cells in inflamed tissues.[50] We found that the MAIT cell counts and $CD69^+$ MAIT cell frequencies and counts increased in the lungs of OVA-sensitized/challenged female C57BL/6 mice, which were used as the wild-type control mice (Fig. 2). MAIT cell-deficient $Mr1^{-/-}$ mice showed exacerbated eosinophilic airway inflammation when compared with control mice in the murine asthma model (Fig. 2). However, there were no differences in AHR (Fig. 2). Furthermore, intracellular cytokine staining assay showed that intracellular IFN-γ, IL-4, and IL-17 expressing Th cells increased in lung tissue from OVA-sensitized/challenged $Mr1^{-/-}$ mice compared with $Mr1^{+/+}$ mice (Fig. 3).

Discussion

After one year of treatment with mepolizumab, the number of asthma exacerbations, unscheduled visits for worsening asthma, ACT score, and airflow limitation improved for the majority of patients. In general, peripheral blood eosinophil and basophil counts, serum periostin and total IgE levels, and frequencies of $CD69^+$ ILC1, ILC3, NK, and MAIT cells all decreased significantly while serum IL-4 and eotaxin-1 levels increased. This suggests that one-year treatment with mepolizumab for patients with severe asthma reduces the number of $CD69^+$ innate immune cells in the peripheral blood. Patients categorized as responders to mepolizumab treatment had significantly lower neutrophil and $CD69^+$ MAIT cell count in the peripheral blood and higher levels of serum periostin before mepolizumab treatment and fewer of them were omalizumab users before treatment than the non-responders. A low frequency of peripheral $CD69^+$ MAIT cells and neutrophils, low neutrophil counts, and high serum periostin levels before treatment could be biomarkers for predicting the effect of mepolizumab treatment in patients with severe asthma. Additionally, ROC curve analysis revealed that circulating $CD69^+$ MAIT cells, neutrophils, serum periostin levels, periostin/$CD69^+$ MAIT cell ratio, and periostin/neutrophils ratio might also be predictive biomarkers for mepolizumab treatment in patients with severe asthma. The recent study has reported that baseline characteristics in responders to mepolizumab treatment, who were defined as having $\geq$50% reduction in exacerbations, included the presence of nasal polyposis, lower baseline Asthma Control Questionnaire 6, a lower BMI, and a lower dose OCS.[51] Because patients in our study were not evaluated for their nasal polyps and there were few OCS users, and it is reported that the Japanese patients with severe asthma had a low BMI, our study differs from this recent study.[51,52] Regarding ACT scores as an asthma control questionnaire in our study, although there was no significant difference in baseline ACT scores between responders and non-responders, baseline ACT scores were lower in responders, as in this recent study.

In previous studies, blood eosinophils were a useful biomarker for predicting the effects of mepolizumab[53]; however, it is difficult to predict the effectiveness of mepolizumab treatment from peripheral blood eosinophil counts alone in the real-world setting.[54] In the present study, although more than half of the patients who treated mepolizumab treated with omalizumab or OCS before mepolizumab therapy, these patients (except for three patients pre-treated with omalizumab or OCS) had met eosinophil counts with a minimum threshold of 150 eosinophils/μL before prescription or 300/μL in the previous year. However, these previous treatments may have contributed that peripheral blood eosinophil counts did not function as a biomarker for predicting the effectiveness of mepolizumab treatment. Alternatively, unlike peripheral blood eosinophil counts, serum periostin levels are stable and less susceptible to treatment, which is required for a reliable biomarker.[55] In a phase IIb study of lebrikizumab, the mean coefficient of variation for peripheral blood eosinophil counts and serum periostin levels during the run-in period was 21.3% and 5.0%, respectively.[56] Therefore, although it is the same type 2 marker, it is possible that serum periostin was more useful as a type 2 marker than eosinophil counts were. In our study, it is deemed a useful finding that clarified the differences in the characteristics of each type 2 biomarker from the difference in the effect of treatment and showed the importance of using it.

The significant increase in eotaxin-1 level in the present study might be attributable to low eosinophil counts induced by biologics targeting the IL-5 pathway, which also upregulates IL-4 and eotaxin-1 for the recovery of eosinophils. Previous phase II studies in patients with eosinophilic asthma demonstrated that benralizumab treatment increased the serum eotaxin-1 and eotaxin-2 levels.[57–59] Furthermore, the expression of CD69, a member of the C-type lectin super-family, suggests the activation of each innate subset of lymphoid cells.[60–62] Since our prior study showed that $CD69^+$ ILC1, ILC2, ILC3, NK, and MAIT cells were positively correlated with each other and associated with airflow limitation in patients with asthma,[32] the reduction in the number of these circulating cells may reflect an improvement in asthma pathology following mepolizumab treatment. Although this decrease in the frequencies of $CD69^+$ MAIT cells seemed contradictory with the findings that patients with low $CD69^+$ MAIT cells were responders of 1-year mepolizumab treatment and that $CD69^+$ MAIT cells might have a suppressive role in a murine asthma model, decreases in the frequency of $CD69^+$ MAIT cells were no significant difference in both responders and non-responders and changes in each parameter in each patient with the below-mean or above-mean decrease in the frequency of $CD69^+$ MAIT cells were independent of the intensity of $CD69^+$ MAIT cell decreasing. The findings in this study suggested that there might be no association between the reduction in $CD69^+$ MAIT cells one year after mepolizumab treatment and the effectiveness of mepolizumab for asthma, and at least suggesting that the reducing $CD69^+$ MAIT cells may not be involved in the effectiveness of mepolizumab treatment. Moreover, although as a result of mepolizumab treatment for more than 1-year, peripheral blood $CD69^+$ MAIT cells may decrease further and there may be some patients for whom mepolizumab is effective even among non-responders, it is ethically difficult to conduct studies to determine whether there are effective cases among long-term treatment with mepolizumab for more than 1 year.

Although the main cause of inflammation in asthma was thought to be the type 2 immune response due to the acquired immune response, innate immunity is attracting attention as a mechanism by which non-antigen-specific stimuli (e.g. viral infection, tobacco smoke, and climate change) exacerbate asthma. The lymphocytes involved in innate immunity are broadly divided into a group without a TCR and a group with an invariant TCR. The former group is ILCs, and the latter consists of innate-like lymphocytes, including γδ T cells and MAIT cells. Our previous study suggested that circulating activated MAIT cells were low in number in severe asthma,[32] and the present study suggests that low circulating activated MAIT cells might predict

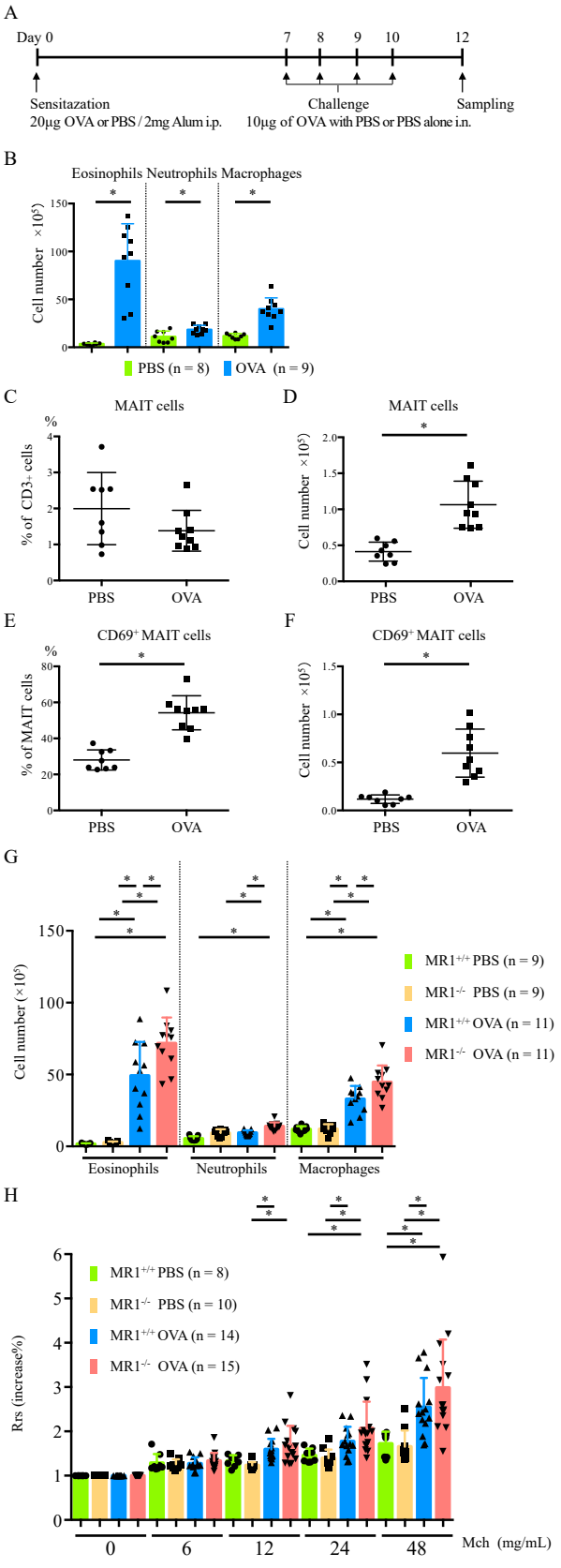

Fig. 2. MAIT cells in the whole lung in the OVA-induced asthma murine model. **(A)** For sensitization, Female C57BL/6 mice, Mr1−/− mice, and Mr1+/+ mice received an i. p injection of OVA or PBS with alum adjuvant on days 0. From days 7–10, the mice were challenged intranasally with 10 μg of OVA or PBS per day. Samples were collected on day 12. **(B)** The number of macrophages, eosinophils, and neutrophils in the whole lung from the OVA-induced asthma murine model (OVA) and control mice (PBS) are shown. **(C–F)** The number and the frequency of MAIT cells and CD69$^+$ MAIT cells after intranasal challenge with OVA are shown. The data represent the means ± SD. n = 8 to 9 mice per group. *$p < 0.05$. *Mr1*$^{-/-}$ mice lacked MAIT cells and *Mr1*$^{+/+}$ mice were the wildtype littermate controls. **(G)** The number of macrophages, eosinophils, and neutrophils in the whole lung from the OVA-induced asthma murine model (OVA) and control mice (PBS) in *Mr1*$^{-/-}$ mice and *Mr1*$^{+/+}$ mice. The data represent the means ± SD. n = 9 to 11 mice per group. **(H)** AHR results from 2 days after the last challenge. Results are presented as the mean respiratory system resistance (Rrs) ± SD in each group after exposure to increasing concentrations of inhaled methacholine (Mch). n = 8 to 15 mice per group. *$p < 0.05$ compared with control mice.

the real-world response of mepolizumab treatment. Since activated MAIT cells are known to mainly secrete Th1/Th17 cytokines,[24,46–49] it is assumed that MAIT cells antagonize or suppress type 2 inflammation. Previously, our group demonstrated that MR1 deficiency reduced the disease severity of oxazolone colitis and spontaneous lupus mouse model, and reduced T cell and innate T cell responses in the spontaneous lupus mouse model.[63,64]. However, in experimental autoimmune encephalomyelitis as a mouse model for multiple sclerosis, MR1 deficiency exacerbated the severity, and T cells proliferated more and produced more Th1 cytokines.[65] In the ILC2-mediated type 2 response asthma murine model by Alternaria or house dust mite, MR1 deficiency promoted ILC2 response and exacerbated allergic airway inflammation and AHR.[30] This report showed that repeated intranasal administration of allergens, including house dust mites, cockroaches, Alternaria, and Aspergillus resulted in a significant decrease in lung MAIT cells.[30] The role of MAIT cells in each inflammation may be different, and the precise role of MAIT cells in adaptive type 2 immune response is unknown.

Unlike the above antigen-induced and challenged asthma murine model, in which ILC2 is activated, reported by Longyun Ye *et al.*, our adaptive type 2 immune response asthma murine model by OVA stimulation with low ILC involvement revealed that lung MAIT cell and CD69$^+$ MAIT cell number increased. Although AHR tended to be suppressed in our adaptive type 2 immune response model, no significant difference was observed. Differences between models with high and low ILC2 involvement were the decrease and increase of lung MAIT cells, respectively, and the inhibitory effect on AHR. The cause of these discrepancies is not clear but may involve a balance between the inhibitory and stimulatory effects of MAIT cells on inflammation. However, mice deficient in MAIT cells showed exacerbated eosinophilic airway inflammation in both models with high and low ILC2 involvement, and lung Th cells produced more IFN-γ, IL-4, and IL-17 in the adaptive model. Altogether the results of this study and the report by Longyun Ye *et al.* suggested that mouse MAIT cells suppress both acquired and innate immunity involved in eosinophilic airway inflammation and asthma.[30] These findings suggested that decreased MAIT cells, which can suppress asthma, may exacerbate asthma. Therefore, the results of mepolizumab reducing suppressive CD69$^+$ MAIT cells in peripheral blood may suggest that CD69$^+$ MAIT cells may have migrated at the local site of inflammation or that the need for suppressive immune cells may reduce due to the stabilization of airway inflammation during mepolizumab treatment. However, it has been reported that both peripheral blood and local bronchial MAIT cells in patients with severe asthma were lower than in healthy individuals, as well as the results from the ILC2-mediated type 2 response asthma murine model.[29,30] In addition, this study did not show a relationship between the CD69$^+$ MAIT cell-reducing effect of mepolizumab and its effectiveness against asthma and it is still unclear whether human MAIT cells can

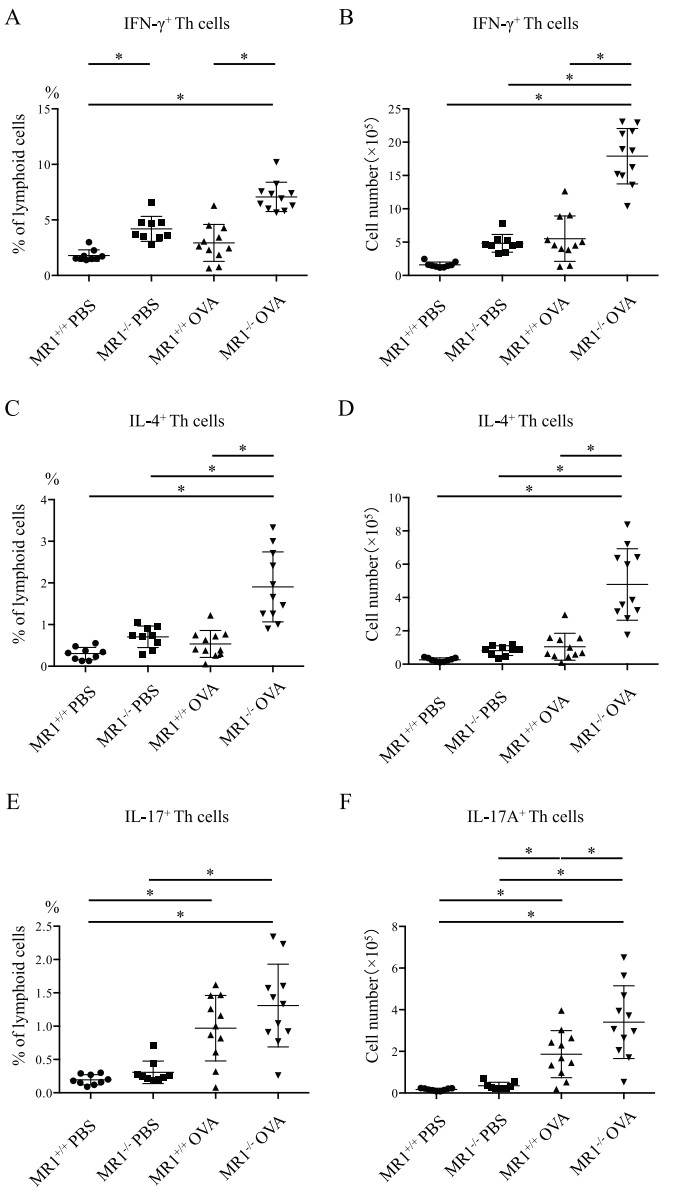

Fig. 3. Intracellular cytokine production in lung CD4$^+$ helper T cells of *Mr1*$^{-/-}$ mice. Single-cell suspensions were stained intracellular with anti-IFN-γ **(A, B)**, anti-IL-4 **(C, D)**, anti-IL-17A-PE mAbs **(E, F)** to detect intracellular cytokine production after intranasal OVA challenge. The data represent the means ± SD. n = 9 to 11 mice per group. *$p < 0.05$ compared with control mice.

migrate into the inflammation site of human lung tissue and have an inhibitory effect on asthma.

A notable limitation of this study is that it was a single center, single-arm, open-label, observational study, and the sample size was small. Many of the changes over the one-year period are uncertain and phenomenological, therefore further research is needed to confirm these findings. Moreover, the components in the expert consensus-based criteria of the clinical responders in this study have not yet had a consensus and will likely evolve and change over time. The major limitation of this study is that the frequency of circulating CD69$^+$ MAIT cells and serum periostin levels are unavailable as clinical biomarkers in practice. Additionally, the findings from the murine asthma models do not necessarily apply to human MAIT cells and asthma; further exploration of this subject is required.

In conclusion, to our knowledge, this is the first study to show that mepolizumab treatment decreases circulating CD69$^+$ ILC1, ILC3, NK cell, and MAIT cell counts. Furthermore, this study showed that CD69$^+$ MAIT cells, neutrophils, serum periostin, periostin/neutrophils ratio, and periostin/CD69$^+$ MAIT cell ratio might predict the effectiveness of mepolizumab treatment in patients with severe asthma. The study also showed that MAIT cells may play a protective role against type 2 airway inflammation in an OVA-induced asthma murine model. Additionally, although mouse MAIT cells inhibit asthma in murine models, it is unknown whether human MAIT cells have any effect on asthma. Although mepolizumab treatment was effective in patients with poorly controlled severe asthma with low counts of CD69$^+$ MAIT cells, additional studies are needed to investigate whether CD69$^+$ MAIT cells and/or a combination with other markers make more useful biomarkers in patients with asthma.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2023.06.001.

Conflict of interest

KI reports grants from eduring the conduct of the study and has patents licensed in Japan and United States managed by Shino-Test, Saga University, and Kurume University. The rest of the authors have no conflict of interest.

Authors' contributions

HS, NH, AC, HA, RA, KI, SM, and KT participated in the design of the study and drafted the manuscript. HS, NH, SH, TT, YS, YT, AI, KM, TN, and JI contributed to data collection. HS, NH, SH, TT, and YS carried out the flow cytometric analysis, molecular studies and mouse studies. HS, NH, SH, KI, SM, and KT performed the statistical analysis and interpretation of the results. All authors have read and approved the final manuscript.

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