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Letter to the Editor

Expression of Siglec-8 is regulated by interleukin-5, and serum levels of soluble Siglec-8 may predict responsiveness of severe eosinophilic asthma to mepolizumab

Dear Editor,

Sialic acid-binding immunoglobulin-like lectin (Siglec)-8 is a transmembrane receptor that binds a sulfated glycan ligand.^{1,2} It is selectively expressed on human eosinophils as well as mast cells, and its engagement induces eosinophil/mast cell apoptosis.³ Siglec-8 also exists in a soluble form in human serum (sSiglec-8), and a recent report suggested that sSiglec-8 might be used as a biomarker for various eosinophil-associated diseases.⁴ However, the precise roles and mechanisms of cell surface-bound Siglec-8 and sSiglec-8 are poorly understood. Thus, we aimed to investigate the regulation of their expressions both *in vitro* and in patients undergoing asthma therapy using mepolizumab, a humanized monoclonal antibody that specifically targets an eosinophil-activating cytokine, interleukin-5 (IL-5).^{5,6} The protocol for this study was reviewed and approved by the Institutional Ethics Review Board of National Hospital Organization Tokyo National Hospital (Institutional approval number: No. 366). Informed consent was obtained from all blood donors. All statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA).

First, leukocytes from healthy subjects were collected from venous blood by dextran-sedimentation, stained with PE-conjugated anti-human Siglec-8 antibody (IgG1κ, clone 7C9) (BioLegend, San Diego, CA, USA) and analyzed for Siglec-8 expression by flowcytometry.

Leukocytes were stained with mouse anti-human antibodies against CD193 (CCR3) (clone 5E8-G9-B4), FcεRI (clone AER-37), CD14 (clone 61D3) and CD4 (clone RPA-T4) (all obtained from Thermo Fisher Scientific, Waltham, MA, USA), as well as CD8a (clone RPA-T8) and CD19 (clone H1B19) (both obtained from TONBO Biosciences, San Diego, CA, USA), for analysis by FACSVerse (BD Biosciences, Franklin Lakes, NJ, USA). Siglec-8 was strongly expressed by CD193-positive eosinophils, as previously reported³ (Fig. 1A), and weakly expressed by FcεRI-positive basophils and CD8-positive lymphocytes (data not shown).

Next, eosinophils were purified (>95% purity) by negative selection using anti-CD16 magnetic beads (Miltenyi BioTech, Bergisch-Gladbach, Germany) as previously described.⁷ Approximately $0.7\text{--}1 \times 10^5$ eosinophils were cultured in the presence and absence of recombinant human IL-5 (PeproTech, Rocky Hills, NJ, USA), with and without mepolizumab (GlaxoSmithKline, London, UK), for 24 h at 37 °C. IL-5 dose-dependently upregulated the expression of

Siglec-8 on the eosinophils (data not shown) and reached statistical significance at 1.0 ng/ml (Fig. 1B, C). However, that upregulation was significantly suppressed when 100 µg/ml mepolizumab was co-present with 0.1 ng/ml IL-5, and when 10 or 100 µg/ml mepolizumab was co-present with 1.0 ng/ml IL-5 (Fig. 1C). Interestingly, when the level of sSiglec-8 in the culture supernatants of eosinophils co-cultured with IL-5 and mepolizumab was measured using an sSiglec-8 ELISA kit (Elabscience, Houston, TX, USA; detection limit: 0.16 ng/ml), the results were completely the opposite from those for cell-surface Siglec-8 (Fig. 1D). That is, the level of sSiglec-8 was significantly suppressed by IL-5 at 1.0 ng/ml and tended to recover in the presence of mepolizumab, although without statistical significance (Fig. 1D). From these results, we conclude that the levels of Siglec-8 and sSiglec-8 were oppositely affected by IL-5, and mepolizumab counteracted the effects of IL-5.

Therefore, we next aimed to elucidate the potential of sSiglec-8 as a biomarker for asthma therapy with mepolizumab. Twelve severe eosinophilic asthma patients scheduled to receive mepolizumab were enrolled in this study. Clinical data as well as serum samples were collected at baseline and 1, 2 and 4 months after the first injection of mepolizumab. Interestingly, there were two distinct groups showing either low or high baseline serum levels of sSiglec-8: a low baseline sSiglec-8 group, i.e., below the detection limit of the sSiglec-8 ELISA kit, ≤ 0.16 ng/ml, and a high baseline sSiglec-8 group having a mean level of 1.79 ± 0.78 ng/ml. The difference between those values was statistically significant ($p = 0.031$) (Table 1). Table 1 shows the baseline characteristics of the 12 patients, stratified by the baseline serum sSiglec-8 level. Since Siglec-8 is produced mainly by eosinophils, we analyzed for a correlation between the peripheral blood eosinophil count and the sSiglec-8 level, but we found none (Spearman $R = 0.13$, $p = 0.68$). When %FEV1 (%) was compared, only the low baseline sSiglec-8 group showed significant elevation at 4 months compared to the baseline (Fig. 1E). FEV1 was also significantly improved at 4 months compared to the baseline only in the low baseline sSiglec-8 group (low baseline sSiglec-8 group: 1.60 ± 0.25 L at baseline vs 1.93 ± 0.24 L at 4 months, $p = 0.03$; high baseline sSiglec-8 group: 1.43 ± 0.21 L at baseline vs 1.52 ± 0.28 L at 4 months, $p = 0.47$). Similarly, the Asthma Quality of Life Questionnaire (AQLQ) overall score showed significant improvement at 4 months only in the low baseline sSiglec-8 group (Fig. 1F). When the baseline sSiglec-8 level was analyzed for correlations with changes in FEV1 and the AQLQ, the only statistically significant correlations found were with the change in the AQLQ at 2 (Spearman $R = -0.62$,

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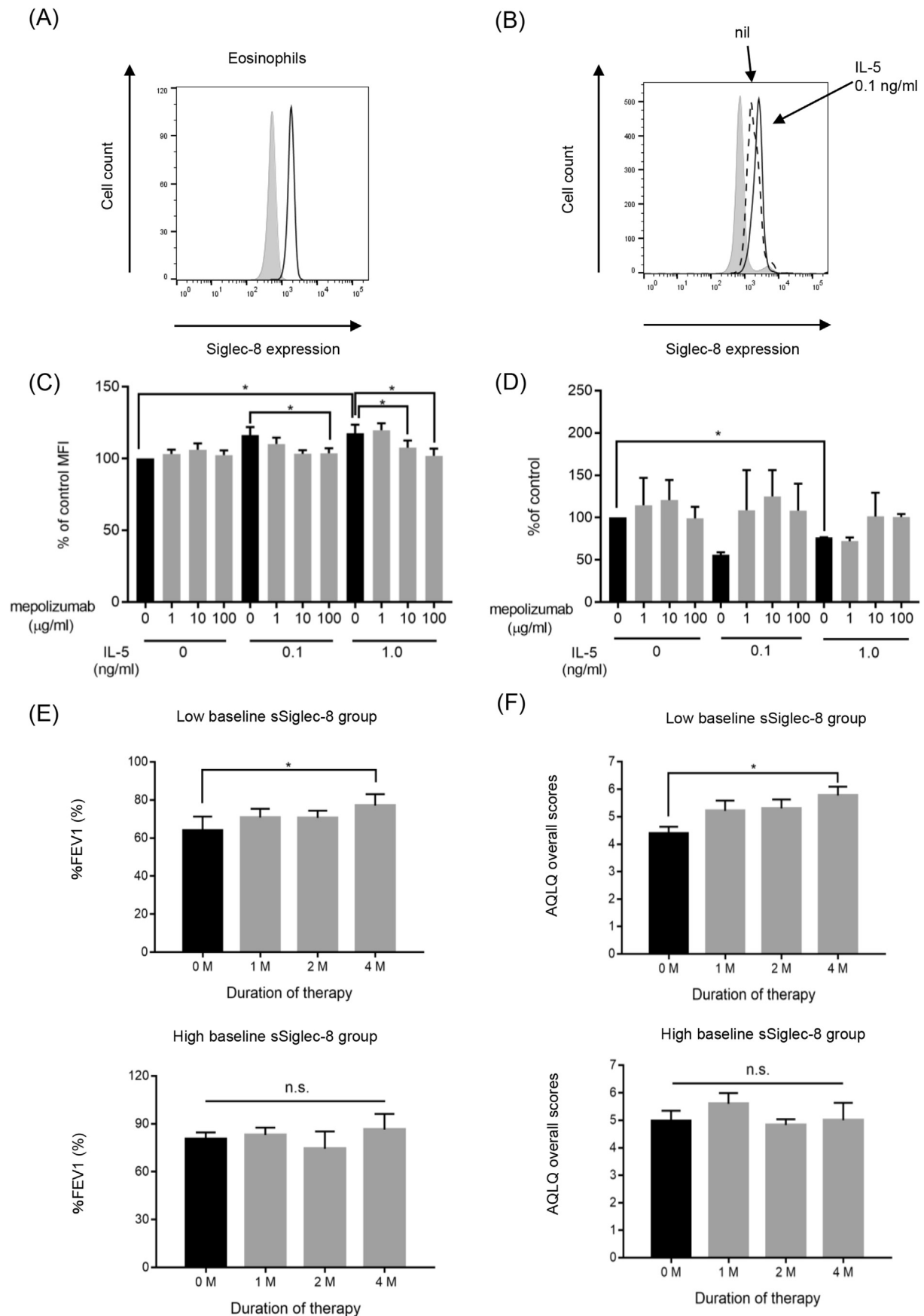


Table 1

Baseline characteristics of the patients, stratified by baseline serum sSiglec-8 level.

Characteristic	Low baseline sSiglec-8 group (n = 7)	High baseline sSiglec-8 group (n = 5)	Total (n = 12)	P-value
sSiglec-8 level (ng/mL)	≤0.16 ng/mL	1.79 ± 0.78	0.83 ± 0.39	0.031 [†]
Age (years)	50.1 ± 7.7	66.8 ± 4.89	57.1 ± 18.6	0.131 [†]
Gender (male/female)	3/4	1/4	4/8	0.407 [‡]
BMI (kg/m ²)	19.6 ± 0.82	26.3 ± 1.74	22.4 ± 4.48	0.003 [†]
Disease duration (years)	24.1 ± 6.75	26.4 ± 12.8	25.1 ± 21.7	0.869 [§]
Oral corticosteroid users (number, %)	3, 42.9	2, 40.0	5, 41.7	0.921 [‡]
Current or ex-smokers (number, %)	2, 28.6	1, 20.0	3, 25.0	0.735 [†]
FeNO level (ppb)	73.5 ± 27.1	121.5 ± 20.5	92.7 ± 60.2	0.237 [§]
Blood eosinophils (/μL)	481.9 ± 147.7	532.8 ± 103.7	503.1 ± 321.8	0.801 [§]
%FEV1 (%) (% predicted)	63.9 ± 7.37	82.8 ± 5.30	71.8 ± 18.8	0.084 [†]
FEV1 (L)	1.60 ± 0.25	1.43 ± 0.21	1.53 ± 0.56	0.636 [†]
AQLQ overall score at baseline	4.39 ± 0.25	4.98 ± 0.37	4.6 ± 0.72	0.206 [†]
Clinical outcome	Low baseline sSiglec-8 group (n = 7)	High baseline sSiglec-8 group (n = 5)	Total (n = 12)	P-value
Increase in FEV1 (L) at 4 months	0.33 ± 0.12	0.08 ± 0.11	0.23 ± 0.09	0.172 [†]
Increase in %FEV1 (%) at 4 months	13.11 ± 3.75	6.02 ± 5.88	10.16 ± 3.29	0.310 [†]
AQLQ improvement at 4 months	1.39 ± 0.40	0.08 ± 0.51	0.91 ± 0.36	0.076 [†]

Values are presented as mean ± SD.

FEV₁, forced expiratory volume in 1 s; AQLQ, Asthma Quality of Life Questionnaire.[†] Unpaired *t* test.[‡] χ^2 test.[§] Mann–Whitney *U* test.

$p < 0.05$) and 4 months (Spearman $R = -0.63$, $p = 0.04$). This showed that a low baseline value for sSiglec-8 in the serum was related to improvement in the AQLQ. In addition, although not statistically significant, only the Asthma Control Questionnaire (ACQ) score at 4 months in the low baseline sSiglec-8 group tended to show improvement (low baseline sSiglec-8 group: 2.04 ± 0.38 at baseline vs 0.85 ± 0.38 at 4 months, $p = 0.0507$; high baseline sSiglec-8 group: 1.45 ± 0.54 at baseline vs 1.26 ± 0.49 at 4 months, $p = 0.80$). On the other hand, the low and high baseline sSiglec-8 groups showed no significant differences in the clinical outcomes (Table 1).

In the present study, we found that IL-5 upregulated Siglec-8 surface expression by eosinophils, which was suppressed by mepolizumab (Fig. 1B, C). Conversely, IL-5 downregulated the level of sSiglec-8 in the eosinophil culture supernatant, which tended to be reinstated by mepolizumab (Fig. 1D). Although the precise role of sSiglec-8 has not yet been clarified, sSiglec-8 seemed to be controlled in reverse fashion to surface-bound Siglec-8. sSiglec-8 may serve as an inhibitory receptor to block Siglec-8 ligand and suppress the function of surface-bound Siglec-8. In addition, IL-5 and sSiglec-8 may have opposite roles. Indeed, our present data from the patients' samples showed good responses to mepolizumab in terms of pulmonary function as well as the QOL in the low baseline sSiglec-8 group, indicating that patients whose serum sSiglec-8 is suppressed by IL-5 might

respond well to anti-IL-5 therapy. However, we must note that whereas IL-5 significantly decreased the level of sSiglec-8 in the eosinophil culture supernatant (Fig. 1D), the baseline serum levels of IL-5 and sSiglec-8 in severe asthma patients did not show a significant negative correlation (Spearman $R = 0.21$, $P = 0.50$) (data not shown). It is also noteworthy that the low baseline sSiglec-8 group showed gradual elevation of serum sSiglec-8 during the therapy, although it was not statistically significant (≤ 0.16 ng/mL at baseline vs 0.71 ± 0.33 ng/mL at 4 months, $p = 0.14$). The precise mechanism(s) underlying the elevation of sSiglec-8 despite the disappearance of peripheral blood eosinophils due to mepolizumab therapy remains unclear. However, perhaps sSiglec-8 remains in the blood longer than eosinophils, or sSiglec-8 is produced by other cell types than eosinophils, such as basophils and mast cells.⁸ The change in the sSiglec-8 level may reflect the response to mepolizumab, or it may even be part of mepolizumab's mechanism of action. Further study with a larger number of patients is needed.

Siglec-8 has been suggested to be a good clinical target because it selectively induces eosinophil apoptosis.⁹ Although we found no correlation between the blood eosinophil count and the sSiglec-8 level, an earlier study indicated a high sSiglec-8 level in hypereosinophilic syndrome, suggesting a close relationship with the fate of eosinophils.⁴ In addition, in our present study the serum sSiglec-8 levels in the healthy control subjects ($n = 5$) were lower than the

Fig. 1. (A) Siglec-8 expression by peripheral blood eosinophils obtained from healthy subjects. Purified eosinophils (>95% purity) were stained with anti-Siglec-8 antibody (solid line) or isotype-matched control antibody (shaded area) and then analyzed by flowcytometry. Data are representative of three separate experiments showing similar results (B) Purified eosinophils (>95% purity) from healthy subjects were incubated with (solid line) and without (dotted line) IL-5 at 0.1 ng/mL for 24 h, and Siglec-8 expression was analyzed by flowcytometry. The shaded area indicates eosinophils stained with the control antibody. Data are representative of three separate experiments showing similar results (C) Purified eosinophils (>95% purity) from healthy subjects were incubated with IL-5 and mepolizumab at the indicated concentrations for 24 h at 37 °C, and Siglec-8 expression was analyzed by flowcytometry. Values for the % of control MFI without stimulus are shown. Bars represent the SEM ($n = 4$). * $p < 0.05$ by one-way ANOVA followed by Tukey–Kramer test (D) sSiglec-8 levels in the eosinophil culture supernatant. Purified eosinophils (>95% purity) from healthy subjects were incubated with IL-5 and mepolizumab at the indicated concentrations for 24 h at 37 °C, and the level of sSiglec-8 in each eosinophil culture supernatant was measured by ELISA. Values for the % of control without stimulus are shown. Bars represent the SEM ($n = 3$). * $p < 0.05$ by one-way ANOVA followed by Tukey–Kramer test (E, F) %FEV1 (%) (E) and AQLQ overall scores (F) at baseline and 1, 2 and 4 months after the first injection of mepolizumab in the low (upper panels) and high (lower panels) baseline sSiglec-8 groups are shown. Bars represent the SEM ($n = 5–7$). * $p < 0.05$ by one-way ANOVA followed by Tukey–Kramer test. n.s., not significant.

levels in the severe asthma patients ($n = 12$), although the difference did not reach statistical significance (0.51 ± 0.28 ng/ml in healthy controls vs 0.84 ± 0.39 ng/ml in severe asthma patients, $p = 0.62$). Since sSiglec-8 was found to be abundant in human serum, it may have great potential as a biomarker in diseases in which eosinophils play roles and in therapies targeting eosinophils. Further studies are needed to fully elucidate the precise roles of Siglec-8 and the mechanisms by which they are regulated.

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Conflict of interest

The authors have no conflict of interest to declare.

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