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Letter to the Editor

SEMA4A promotes eosinophil survival and contributes to eosinophil-mediated allergic diseases

Dear Editor,

Semaphorins were originally identified as axon guidance factors involved in development of the neuronal system. Several semaphorins, so-called 'immune semaphorins', are crucially involved in various phases of both physiological and pathological immune responses. SEMA4A, a class IV semaphorin, is expressed in dendritic cells (DCs) and Th1-type T cells, and contributes to T cell priming and Th1/Th2 differentiation.^{1,2}

Eosinophils play a crucial role in pathogenesis in allergic diseases such as chronic rhinosinusitis (CRS) and asthma.³ CRS is clinically divided into two groups: CRS with nasal polyps (CRSwNPs) and CRS without nasal polyps (CRSsNPs).⁴ CRSwNPs exhibit substantial overlap with asthma in the pathogenesis of the Th2 inflammatory response.⁵

IL-5-mediated signal transduction is essential for eosinophil survival and effector function, and neutralizing IL-5 antibodies reduce severe exacerbations and improve lung function in poorly controlled steroid-resistant asthma patient by decreasing eosinophil abundance.^{3,6}

Despite the critical role of eosinophils in asthma, the functional role of SEMA4A in eosinophils remains to be elucidated. Previously, we reported that SEMA4A-deficient (KO) mice increase airway inflammation and hyper-reactivity in an OVA-induced asthma model, and that SEMA4A directly inhibits IL-4-producing OVA-specific CD4⁺ T cells.⁷ However, we did not address the role of SEMA4A in eosinophils in a clinical setting. Therefore, in this study, we investigated the function of SEMA4A in eosinophils and sought to determine how SEMA4A contributes to pathogenesis in allergic diseases.

Although SEMA4A is a transmembrane protein, it can be shed from several types of cells and detected in serum as the soluble form (sSEMA4A). To determine whether SEMA4A leads to pathogenesis of specific type of allergic disease, we examined serum sSEMA4A levels in patients with eosinophilic chronic rhinosinusitis (ECRS), non-eosinophilic chronic rhinosinusitis (NECRS), other sinus diseases (OSDs), and asthma. In Japanese criteria,⁸ CRS is further subclassified into ECRS and NECRS based on eosinophil counts, image evaluations, nasal polyposis, and laterality. ECRS is similar to CRSwNPs described in western countries, as both are characterized by Th2 inflammation. Although the backgrounds of all patient cohorts were similar (Supplementary Table 1), levels of serum SEMA4A were significantly higher in

patients with ECRS or asthma than in those with NECRS or OSDs (Fig. 1A).

According to receiver operating characteristic (ROC) curve analysis, a SEMA4A cutoff point for ECRS diagnosis of 307.5 yielded a sensitivity of 68% and a specificity of 100% (Supplementary Fig. 1A). In nasal polyps from ECRS patients, SEMA4A was strongly expressed in eosinophils, rather than T cells, but was not expressed in nasal polyps without eosinophils from NECRS patients (Fig. 1B, data not shown). Together, these observations suggest that eosinophils are a major source of sSEMA4A in ECRS patients. We then examined the mechanism of SEMA4A cleavage on eosinophils. To this end, we analyzed the level of SEMA4A on eosinophils by flow cytometry after treating with several sheddases, including a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4), thymic stromal lymphopoietin (TSLP), TNF α , IL-5, IL-33, and TNF- α converting enzyme (TACE). Although the level of SEMA4A was not changed upon IL-5 stimulation, it was decreased by ADAMTS4 (Fig. 1C). However, ADAMTS4 treatment did not change the mRNA level of SEMA4A in eosinophils (Supplementary Fig. 1B). Furthermore, to determine whether SEMA4A can support eosinophil survival, we stimulated healthy human peripheral blood eosinophils (PBEos) with recombinant SEMA4A. Of note, recombinant SEMA4A prolonged survival of PBEos, increasing their abundance relative to the control (Fig. 1D). These findings demonstrate that SEMA4A on eosinophils can be cleaved by ADAMTS4, and that the resultant increase in sSEMA4A may support eosinophil survival in ECRS and asthma patients.

To further investigate how SEMA4A functionally contributes to eosinophilic inflammation, we generated bone marrow-derived eosinophils (BMDEos) from wild-type (WT) and SEMA4AKO mice.⁹ Stimulation with recombinant IL-5 increased the expression of SEMA4A and Siglec-F in WT BMDEos (Fig. 2A). Consistent with the human result, BMDEos were less abundant in KO mice than in WT mice (Fig. 2B $p = 0.011$). In addition, the number of murine splenic eosinophils was lower in KO mice (Fig. 2C $p = 0.011$). Furthermore, BMDEos from KO mice exhibited higher rates of apoptosis (Fig. 2D, $p = 0.032$) and reduced proliferation (Fig. 2E, $p = 0.036$) relative to those from WT mice. By contrast, BMDEos of KO mice were morphologically similar to those of WT mice (data not shown).

Given that STAT5,⁶ a critical downstream signaling factor of the IL-5/IL-5R axis, provides the key signal for proliferation of

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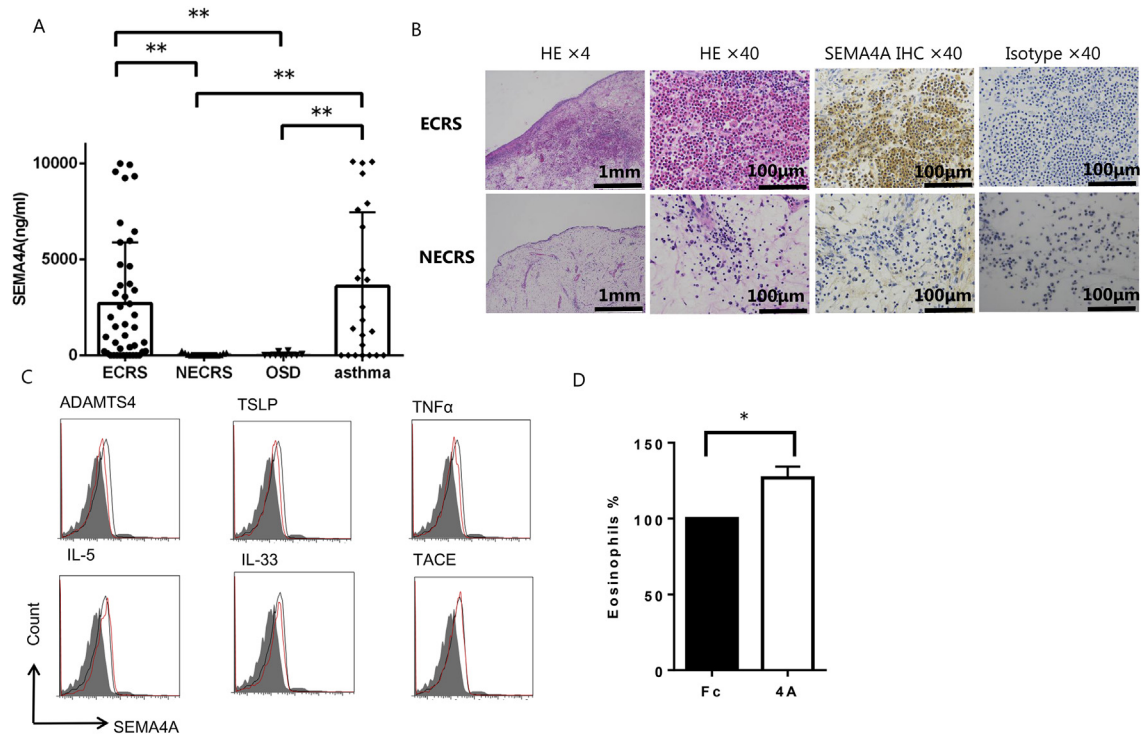


Fig. 1. Soluble SEMA4A promotes eosinophilic inflammation in patients with eosinophilic chronic rhinosinusitis or asthma. **(A)** Serum soluble SEMA4A (sSEMA4A) levels were measured in patients with eosinophilic chronic rhinosinusitis (ECRS, $n = 44$), non-eosinophilic chronic rhinosinusitis (NECRS, $n = 9$), other sinus disease (OSD, $n = 31$), or asthma ($n = 19$). * $p < 0.05$, ** $p < 0.01$, determined by one-way ANOVA. OSDs include paranasal fungal ball and post-operative paranasal sinus cyst. **(B)** Immunohistochemistry of SEMA4A in nasal polyps of ECRS and NECRS patients. **(C)** Histograms of cell surface expression of SEMA4A on eosinophils from healthy controls after the following treatments: TSLP 2 µg/ml, ADAMTS4 4.6 µg/ml, TNFα 10 µg/ml, IL-5 5 µg/ml, and TACE 10 µg/ml. Gray filled, isotype control; black, untreated; red, treated. Data are representative of three independent experiments. **(D)** PBEos from four independent healthy donors were co-cultured with Fc-control and recombinant sSEMA4A, and numbers of eosinophils were assessed by flow cytometry. Data are expressed as relative values, with Fc-control defined as 100%. Data are representative of three independent experiments.

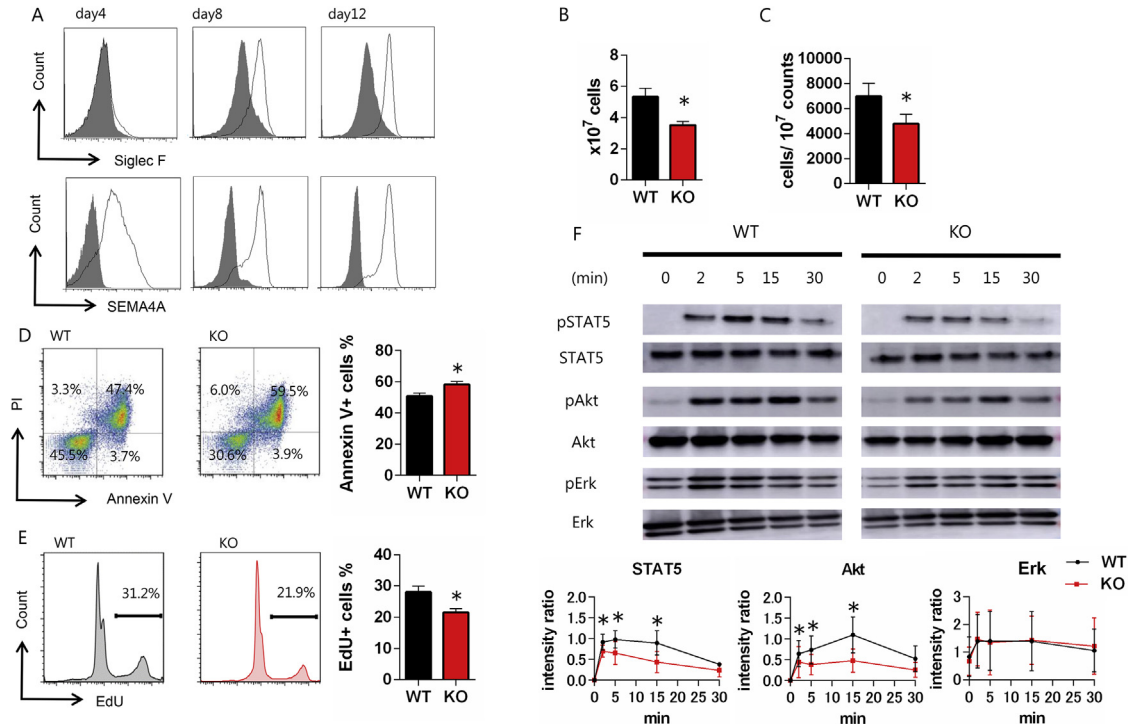


Fig. 2. SEMA4A supports survival of eosinophil in mice. **(A)** Representative flow cytometry data from wild-type bone marrow cells. Expression of SEMA4A and Siglec-F was followed over a time course of eosinophil induction with recombinant IL-5. Gray filled: isotype control. **(B, C)** Numbers of BMDEos (**B**: real number is multiplied by 10⁷) and splenic eosinophils (**C**: 10⁷ flow cytometry counts) were compared between wild-type mice (WT) and SEMA4A-deficient mice (KO). **(D)** BMDEos apoptosis was assessed by annexin V-propidium iodide assays on day 13. **(E)** BMDEos proliferation was assessed by EdU assay on day 9. **(F)** BMDEos prepared from WT or KO mice were cultured with 2 ng/ml recombinant IL-5 (2 ng/ml) for 0, 2, 5, 15, or 30 min. Phospho-STAT5, total STAT5, phospho-Akt, total Akt, phospho-Erk, and total Erk protein levels in BMDEos were evaluated by western blot (upper lane). Quantitative intensity ratio at each time point is shown below (lower lane). * $p < 0.05$, determined by Student's t-test. All data are representative of three independent experiments.

eosinophils, we evaluated IL-5-dependent signaling pathways. IL-5R expression levels were comparable between WT and KO mice (data not shown). However, upon stimulation with IL-5, up-regulation of phosphorylated STAT5 was moderately attenuated in SEMA4A-deficient BMDEos, and up-regulation of phosphorylated Akt was also reduced (Fig. 2F). We also calculated the intensity ratios; at 2, 5, and 15 min, the proportions of phosphorylated STAT5 ($p = 0.022, 0.016, 0.017$) and phosphorylated AKT ($p = 0.029, 0.021, 0.020$) were significantly reduced (Fig. 2F). By contrast, phosphorylation of Erk was not affected. Together, these data indicate that SEMA4A supports eosinophil survival and activation through the regulation of IL-5R/STAT5 pathway.

In this study, we found that the levels of sSEMA4A were significantly elevated in patients with CRSwNPs and asthma, and that SEMA4A was especially highly expressed in eosinophils in nasal polyps. We also demonstrated that SEMA4A in eosinophils can be cleaved by ADAMTS4, suggesting that eosinophils are a major source of SEMA4A in serum from these patients. However, a subset of patients with ECRS or asthma did not exhibit upregulation of sSEMA4A, suggesting that SEMA4A is not the sole factor responsible for regulation of eosinophilic inflammation, and that other factors such as periostin,¹⁰ which induces production of proinflammatory cytokines, could contribute to pathogenesis of these diseases.

In terms of the function of SEMA4A in allergic diseases, we found that SEMA4A positively regulates IL-5/STAT5 signaling pathway in eosinophils. Although IL-5R signaling has not been carefully studied in mouse eosinophils, it is known that human eosinophils stimulated with IL-5 up-regulate STAT5 phosphorylation.¹¹ Because specific types of semaphorins function as co-stimulatory molecules,¹ we speculate that SEMA4A serves as a co-stimulatory molecule on eosinophils.

Future studies will be needed to elucidate the crosstalk between SEMA4A and IL-5R/STAT5 signaling, as well as the detailed mechanisms underlying phosphorylation.

In conclusion, SEMA4A can promote eosinophil survival and may contribute to pathogenesis in both CRSwNPs and 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.2018.10.001>.

Conflict of interest

The authors have no conflict of interest to declare.

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Supplementary Methods

Mice

C57BL/6J mice were purchased from CLEA Japan. C57BL/6J SEMA4A-deficient (KO) mice were established as previously described (1) and bred at the Institute of Experimental Animal Sciences Faculty of Medicine. All animal procedures were approved by the Institutional Animal Care and Use Committee of Osaka University.

Human sample collection

Human samples were obtained from consenting subjects at Osaka University Hospital under institutional review board–approved protocol 14463. All cases were diagnosed as ECRS according to the JESREC study (2). Written informed consent was obtained from all subjects.

Bone marrow–derived eosinophils

Eosinophils were differentiated from BM of WT and KO mice according to a previously reported protocol (3). Briefly, BM cells were collected and cultured in the presence of stem cell factor (SCF) and Flt3L for 4 days. Thereafter, the medium was replaced with IL-5 for the rest of the culture (up to day 14). Recombinant mouse SCF and Flt3L were purchased from Peprotech, and recombinant mouse IL-5 was purchased from R&D Systems.

Flow cytometry analysis

Cells were prepared and stained for flow cytometry analysis of cell surface markers using the following fluorochrome-conjugated Abs diluted in PBS + 2% FBS: anti-human CCR3 (clone:5E8, Biolegend), anti-human Siglec8 (clone:7C9, Biolegend), anti-human and –mouse SEMA4A (clone:5E3, MBL), anti-mouse CD45 (clone:30-F11, Biolegend), anti-mouse Gr-1 (clone:RB6-8c5, Biolegend), anti-mouse B220 (clone:RA3-6B2, Biolegend), anti-mouse CD11b (clone:M1/70, Biolegend), and anti-mouse SiglecF (clone:HL3, Biolegend)

Cell proliferation analysis and ELISA

Cell proliferation was measured using the Click-iT EdU kit (Invitrogen). SEMA4A levels in serum were measured as previously described (4). Concentrations of human IL-5 in serum were determined by ELISA (R&D Systems). SEMA4A-Fc recombinant protein or control human IgG-Fc proteins were purchased from EMD Millipore.

Western blot

BMDEos were incubated with the indicated concentrations of IL-5 in RPMI 1640 medium

containing 20% FCS and antibiotics. Cells were collected and lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 250 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate, and 1 mM NaF). Lysates were subjected to immunoblot analyses, which were performed using standard protocols. For SDS-PAGE, samples were boiled for 5 min in SDS-PAGE sample buffer containing 0.125 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, and 10% 2-ME. Protein samples were fractionated on 4–15% Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories) and transferred to Trans-Blot Turbo Mini PVDF Transfer Packs (Bio-Rad Laboratories) using a Trans-Blot Turbo Transfer Starter System (Bio-Rad Laboratories). Membranes were incubated with specific primary Abs, and then with the appropriate secondary Abs. Antibodies for western blotting were all purchased from Cell Signaling Technology: anti-Phospho-STAT5 (Tyr694) (clone:D47E7), anti-STAT5 (CST#9363), and anti- β -actin (clone:13E5). Quantitation of intensity was done by ImageJ (ver.1.51u).

RT-PCR analysis

Total RNA was prepared using the RNeasy mini kit (Qiagen). cDNA synthesis was performed using Superscript III reverse transcriptase (Invitrogen). mRNA expression was quantified on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primer sets were purchased from Applied Biosystems.

Immunohistochemistry

Paraffin sections were stained by indirect immunohistochemistry. Briefly, deparaffinized and rehydrated tissue sections were blocked with 3% hydrogen peroxide. Sections were blocked with 10% normal serum, incubated with primary polyclonal rabbit anti-SEMA4A Ab (Abcam), overnight at 4°C, and then incubated with secondary antibody Simple Stain MAX-PO (Nichirei Bioscience) for 30 min. Signals were visualized using a 3,3'-diamino-benzidine kit (DAB Kit; Nichirei Bioscience) and counterstained with Mayer's hematoxylin.

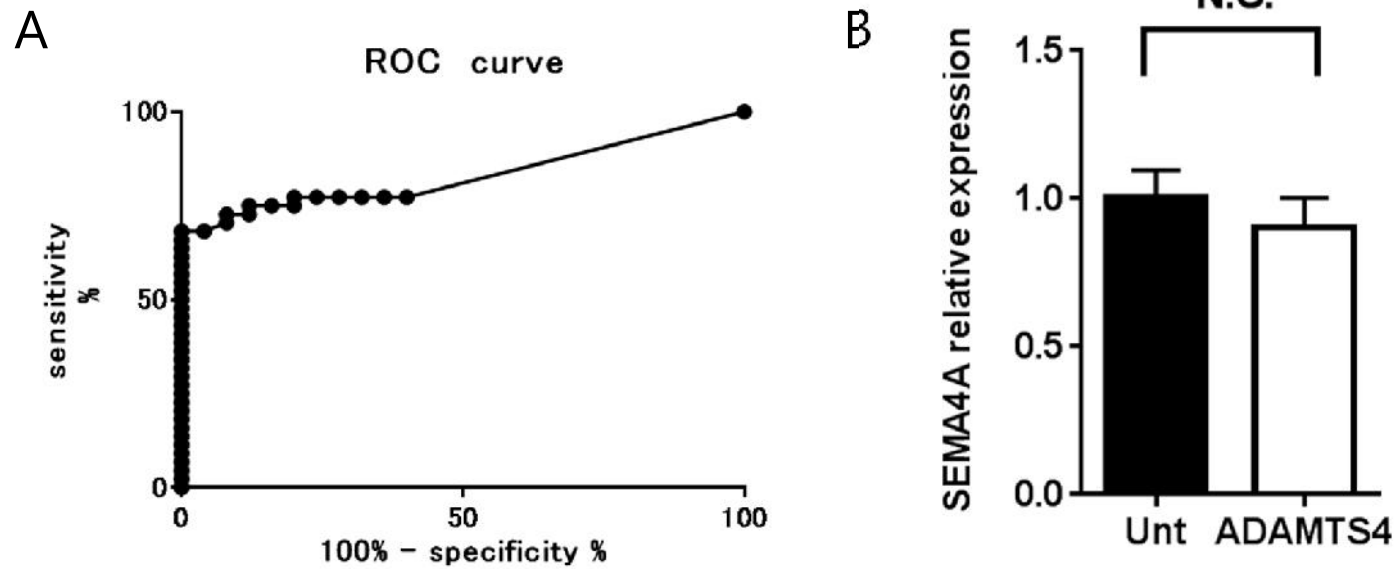
Statistical analysis

Data are expressed as means $\pm$ SD (Figure 1) or means $\pm$ SEM (Figure 2). Nonparametric Mann–Whitney U tests were used to compare two groups, and the Kruskal–Wallis test was used for comparisons among four groups. P values < 0.05 were considered significant.

Supplementary Table 1. Backgrounds of individual patients.

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Supplementary Fig. 1. Soluble SEMA4A is involved in promoting eosinophilic inflammation in patients with eosinophilic chronic rhinosinusitis or asthma.

(A) ROC curve of serum SEMA4A levels for predicting ECRS. AUC was calculated to be 0.831. **(B)** SEMA4A mRNA level in eosinophils untreated (Unt) or treated with ADAMTS4 (4.6 µg/ml).

Supplementary Table 1. Backgrounds of individual patients

	ECRS (n=44)	NECRS (n=15)	OSD (n=10)	asthma (n=23)
age	56.5±4.2	65±6.9	61±8.4	59±5.8
male(%)	32(72.7%)	11(73.3%)	6(60.0%)	7(30.4%)
WBC	7229±1779	6550±1537	6944±1697	6267±2146
Eos%	10.04±6.29	2.72±2.17	1.64±1.08	4.77±4.85
Eos	751±642	172±138	104±62	272±281
IgE	543.4±1176.7 (n=42)	220.7±330.6 (n=12)	18.4±15.6 (n=5)	795.2±1906.4 (n=23)
systemic steroid	3(6.8%)	none	none	8(18.1%)
local steroid	6(13.6%)	none	none	23(100%)