

ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

Siglec-7 on peripheral blood eosinophils: Surface expression and function

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Abstract

Background: Siglec-7 is an inhibitory receptor (IR) expressed on human blood eosinophils. Whereas activation of other IRs, including Siglec-8 and CD300a, has been shown to downregulate eosinophil function, little is known about the role of Siglec-7 on human eosinophils.

Objective: To examine Siglec-7 expression and function in eosinophils from normal (ND) and eosinophilic (EO) donors.

Methods: Eosinophil expression of Siglec-7 was quantified by flow cytometry and quantitative PCR. Soluble Siglec-7 (sSiglec-7) levels were measured by ELISA in serum. The effect of Siglec-7 on eosinophil viability and degranulation was assessed in vitro by AnnexinV-FITC/7-AAD staining and by measuring GM-CSF-induced mediator release in culture supernatants. Signal transduction was studied by Western blot.

Results: Siglec-7 was expressed ex vivo on blood eosinophils from all eosinophilic and normal individuals studied. Siglec-7 surface, but not *SIGLEC-7* mRNA expression, was correlated with absolute eosinophil count (AEC). Siglec-7 was upregulated on purified eosinophils after in vitro stimulation with GM-CSF or IL-5. Serum sSiglec-7 was detectable in 133/144 subjects tested and correlated with AEC. Siglec-7 cross-linking inhibited GM-CSF-induced release of eosinophil peroxidase, TNF- α , and IL-8 ($n = 7-8$) but did not promote eosinophil apoptosis ($n = 5$). Finally, Siglec-7 cross-linking on GM-CSF-activated eosinophils induced phosphorylation of SHP-1 and de-phosphorylation of ERK1/2 and p38.

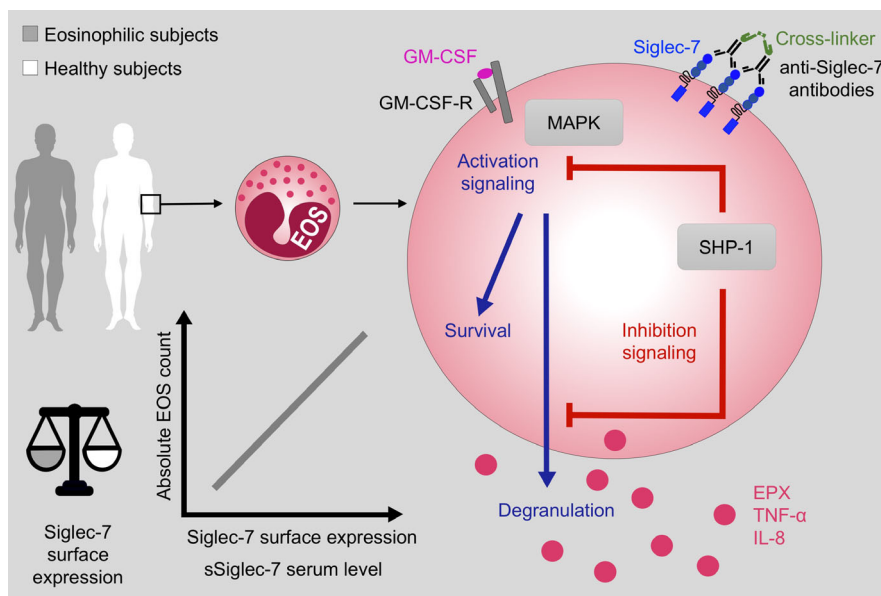
Conclusions: Siglec-7 is constitutively expressed on human eosinophils and downmodulates eosinophil activation. Targeting of Siglec-7 on eosinophils might enhance treatment efficacy in eosinophil-driven disorders. Conversely, therapeutic interventions that inhibit Siglec-7 could have unanticipated consequences and promote eosinophilic inflammation.

KEYWORDS

apoptosis, eosinophilia, eosinophils, hypereosinophilic syndrome, ITIM, Siglec-7, Siglec-8, signaling

Abbreviations: AEC, absolute eosinophil count; CL, cross-linker; *E. Coli*, *Escherichia coli*; EO, eosinophilic donors; Eos, eosinophils; EPX, eosinophil peroxidase; GM-CSF, granulocyte-macrophage colony-stimulating factor; GM-CSF-R, GM-CSF receptor; GM, geometric mean; HES, hypereosinophilic syndromes; IL, interleukin; IR, inhibitory receptor; ITAM, immunoreceptor tyrosine-based activating motifs; ITIM, immunoreceptor tyrosine-based inhibitory motif; mAbs, monoclonal antibodies; MAPK, mitogen-activated protein kinase; ND, normal donors; NK, natural killer cells; Rh, recombinant human; RT, room temperature; SEB, *Staphylococcus aureus* enterotoxin B; SHP-1, Src homology-2 (SH2) domain-containing protein-tyrosine phosphatase-1; Siglec, sialic acid binding Ig-like lectin; TNF- α , tumor necrosis factor alpha.

Legrand and Landolina contributed equally.
Klioni and Levi-Schaffer jointly supervised this work.



GRAPHICAL ABSTRACT

Siglec-7 is expressed at comparable levels on blood eosinophils from normal and eosinophilic subjects. Absolute eosinophil counts correlate with surface expression of Siglec-7 on blood eosinophils and soluble Siglec-7 levels in serum. Siglec-7 inhibits eosinophil activation but does not affect survival. EOS: eosinophil, EPX: eosinophil peroxidase, GM-CSF: granulocyte-macrophage colony-stimulating factor, GM-CSF-R: GM-CSF receptor, IL-8: interleukin-8, MAPK: mitogen-activated protein kinase, Siglec-7: sialic acid binding Ig-like lectin-7, SHP-1: Src homology-2 (SH2) domain-containing protein-tyrosine phosphatase-1, TNF- α : tumor necrosis factor alpha.

1 | INTRODUCTION

Eosinophils are main effector cells in allergic inflammation and have been implicated in the late and chronic phases of the allergic response. Their recruitment and activation are regulated by a complex network of activating and inhibitory signals that represent key therapeutic targets.^{1,2} Initial attention focused primarily on the IL-5 axis in eosinophil-associated disorders, leading to the recent approval of mepolizumab, reslizumab, and benralizumab for the treatment of eosinophilic asthma^{3,4} and mepolizumab for the treatment of eosinophilic granulomatosis with polyangiitis.⁵ The role of inhibitory receptors (IRs), many of which are shared by eosinophils, basophils, and mast cells, has been less well-explored.

Inhibitory receptors, including members of the Sialic acid binding Ig-like lectin (Siglec) family⁶ and the Ig-like superfamily member CD300a,⁷ are characterized by immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail, which, when phosphorylated, decrease cellular activation and/or induce apoptosis. The precise effects of an individual IR may vary depending on the cell type. For example, cross-linking of Siglec-8, an IR expressed on eosinophils,⁸ mast cells,⁹ and basophils,¹⁰ leads to apoptosis of eosinophils,^{11,12} but inhibits degranulation of mast cells without promoting cell death.⁹ In contrast, CD300a activation causes decreased function and apoptosis of both mast cells and eosinophils in the presence of their respective growth factors.^{13,14}

Originally detected on Natural Killer cells (NK cells) and monocytes,^{15,16} Siglec-7 (p75/AIRM) is an additional inhibitory member of the Siglec family that is expressed on a wide variety of cells, including human eosinophils,¹³ mast cells, and basophils.¹⁷ Siglec-7 displays two signaling motifs: a membrane-proximal ITIM and a membrane-distal motif (ITIM-like).¹⁵ On NK cells, the Siglec-7 cytoplasmic domain associates with the protein-tyrosine phosphatases Src homology-2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1), which mediates the Siglec-dependent inhibitory signal.¹⁸

In contrast to Siglec-8 which binds sialylated ligands on human airways cells,¹⁹ Siglec-7 binds preferentially to α 2,8-disialyl and branched α 2,6-sialyl carbohydrate structures,²⁰ which are expressed on various tumor cells and normal colonic epithelial cells.²¹ The function of Siglec-7 has been best studied in the context of tumor biology, where expression of Siglec-7 ligands on tumor cells suppresses NK cell function leading to increased metastatic potential.²² These findings have stimulated the development of novel strategies to inhibit Siglec-7 function, including the development of low molecular weight, high-affinity Siglec-7 ligands that block the inhibitory effects of tumor cells on NK cells in vitro.²³ Such strategies may have unanticipated consequences on cells other than NK cells that express Siglec-7, including the cells involved in allergic inflammation. Although activation of Siglec-7 on mast cells can inhibit IgE-mediated mast cell and basophil activation,¹⁷ the function of Siglec-7 on eosinophils is unknown. The aim of the present study was to characterize the expression of Siglec-7 on eosinophils from normal

donors (ND) and subjects with eosinophilia (EO) and to explore the functional consequences of Siglec-7 engagement on eosinophils.

2 | MATERIALS AND METHODS

2.1 | Study subjects

Eosinophilic subjects (EOS) underwent detailed clinical evaluation at the NIH Clinical Center as part of an NIAID Institutional Review Board (IRB)-approved protocol to study eosinophilic disorders (NCT00001406). For the purposes of this study, hypereosinophilic syndrome (HES) is defined as hypereosinophilia (AEC $\geq 1500/\mu\text{L}$) with evidence of eosinophil-associated clinical manifestations. Eosinophilic subjects included subjects with idiopathic HES ($n = 32$), myeloid HES ($n = 19$), lymphocytic HES ($n = 15$), overlap HES ($n = 30$), hypereosinophilia of unknown significance ($n = 8$), familial eosinophilia ($n = 3$), episodic angioedema and eosinophilia ($n = 3$), and hypereosinophilia associated with neoplasia ($n = 5$), immunodeficiency ($n = 3$), and atopy ($n = 1$). Forty-seven subjects were untreated at the time of analysis (geometric mean (GM) AEC: $2505/\mu\text{L}$, range 470–68 $220/\mu\text{L}$) and 72 were receiving treatment at the time of the study (GM AEC: $871/\mu\text{L}$, range 30–26 $420/\mu\text{L}$). See Table S1 for details. Normal donors (ND) were recruited under NIAID IRB (NCT00090662) and Hadassah-Hebrew University Human Experimentation Helsinki Committee approved protocols for in vitro research. All participants gave written informed consent.

2.2 | Eosinophil purification

Eosinophils were purified by sedimentation on Ficoll-Hypaque and magnetic bead purification, as previously described.^{13,24} Eosinophil purity was $>98\%$ in all experiments with a viability of $>98\%$, as assessed by trypan blue staining.

2.3 | Real-time quantitative PCR

Total RNA was extracted from 10^7 purified eosinophils using TriZol (Invitrogen, Carlsbad, CA) prior to cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA), according to the manufacturer's protocol. *SIGLEC-7* and *SIGLEC-8* mRNA, and 18S rRNA were amplified in a 96-well plate using the following commercially available TaqMan primers (Applied Biosystems) in a final volume of $10 \mu\text{L}$: Hs01100854_m1, Hs00274289_m1, and X03205.1, respectively. Each sample was run in triplicate, and cycle threshold levels were normalized using the 18S cycle threshold values from corresponding samples. mRNA levels are expressed in arbitrary units ($\times 10^{-5}$) related to 18S rRNA.

2.4 | Assessment of surface receptor expression by flow cytometry on whole blood

Surface expression of Siglec-7 was quantified by multiparameter flow cytometry ex vivo on eosinophils ($\text{CD}45^+\text{CD}16^-$ granulocytes) in

whole blood as previously described²⁴ and in vitro using purified blood eosinophils ($2 \times 10^6/\text{mL}$) incubated with or without 10 ng/mL of rhIL-5 (R&D, Minneapolis, MN) or rhGM-CSF (PeproTech, Rocky Hill, NJ). The antibodies used are provided in Data S1.

2.5 | Modulation of Siglec-7 expression on purified eosinophils in vitro

Purified eosinophils ($2 \times 10^6/\text{mL}$ /wells) were incubated overnight with or without 10 ng/mL of recombinant human cytokines (rhGM-CSF, rhIL-5, rhIL-33, rhIFN- α , rhIFN- γ), SEB ($5 \mu\text{g/mL}$), or *E coli* extracts ($4 \mu\text{g/mL}$). Surface expression of Siglec-7 was determined by flow cytometry on viable eosinophils (7-AAD^{neg}), and RNA was extracted from TriZol and mRNA levels assessed after overnight incubation with and without GM-CSF (10 ng/mL).

2.6 | Measurement of soluble Siglec-7 receptor levels in serum

Serum levels of soluble Siglec-7 (sSiglec-7) were quantified using a commercially available sandwich ELISA (Human Siglec-7/CD328 Duo-Set ELISA, R&D) according to the manufacturer's instructions. All assays were performed in singlicate using undiluted serum, and values were calculated on the basis of a recombinant standard curve. The minimal level of detection of sSiglec-7 in serum was 125 pg/mL .

2.7 | In vitro eosinophil stimulation and inhibition assays

Freshly isolated eosinophils ($1.5 \times 10^5/150 \mu\text{L}$ in culture medium) were blocked in 5% goat serum in PBS prior to incubation for 30 minutes at 4°C in 96-well U shape plates (Nunc, Roskilde, Denmark) in the presence of anti-Siglec-7 (QA79, eBiosciences, ThermoFisher, Waltham, MA), anti-Siglec-8 (7C9, Biolegend, San Diego, CA), or matched control antibodies (mouse IgG1k isotype control, eBiosciences) ($0.62\text{--}5 \mu\text{g/mL}$). After washing, cross-linker (F(ab')₂ fragment goat anti-mouse IgG (H+L) ($10 \mu\text{g/mL}$), Jackson Laboratories, West Grove, PA) and rhGM-CSF (50 ng/mL) were added simultaneously and the cells were incubated for 40 minutes (eosinophil peroxidase, EPX release) or overnight (CD69 expression and cytokine release) at 37°C , 5% CO_2 in phenol-free RPMI 5% FCS. Supernatants were collected and stored at -20°C . Anti-CD300a ($1 \mu\text{g/mL}$, from hybridoma #12, produced at the Hebrew University of Jerusalem, Francesca Levi-Schaffer's lab) was used as a positive control for inhibition in all experiments.

2.8 | Measurement of EPX and cytokines in supernatants

Eosinophil peroxidase (EPX) release was measured by colorimetric assay using freshly prepared peroxidase substrate solution containing o-phenylenediamine (OPD) as described previously.²⁵ A standard curve was constructed using purified human EPX (provided by Dr.

Gerald J. Gleich, University of Utah Health Sciences Center, Salt Lake City, UT). The reaction was stopped by the addition of 100 μ L of 4 mmol/L sulfuric acid (BDH), and absorbance was determined at 492 nm in a spectrophotometer (PowerWave XS; Bio-Tek Instruments, Winooski, VT). IL-8 and TNF- α levels were detected in supernatants by ELISA (Peprotech, minimum detectable level 16 pg/mL). Data are expressed as % of control, calculated by dividing the concentration in the antibody-treated well by that in the corresponding uninhibited control well (GM-CSF only) and multiplying by 100.

2.9 | Assessment of eosinophil apoptosis

Eosinophil cell death was quantified by flow cytometry using AnnexinV-FITC/7-AAD staining (BD). Briefly, after overnight incubation with the cytokines (GM-CSF or IL-5 at 10 ng/mL Peprotech), anti-Siglec-7, anti-Siglec-8, or their respective isotype controls were added for another overnight incubation at 37°C, 5% CO₂. The cells were then washed in PBS 1X, stained with AnnexinV-FITC and 7-AAD probes (5 μ L/tube) in a binding buffer (BD) during 15 minutes room temperature. The samples were acquired on a LSRII flow cytometer (BD).

2.10 | Western blot

Cross-linking with anti-Siglec-7, anti-CD300a, or matched control antibody was performed on freshly isolated peripheral blood eosinophils ($2 \times 10^6/200 \mu$ L) in culture medium for 30 minutes at 4°C as described above. Cells were activated for the indicated times in Eppendorf tubes in a 37°C water bath in the presence of rhGM-CSF (50 ng/mL) and sheep anti-mouse F(ab')₂ cross-linker antibodies (CL) (10 μ g/mL). Activation was stopped by adding ice-cold PBS to the pellet followed by centrifugation. Cell pellets were lysed using a cell lysis buffer (#7018 Cell Signaling Technology, Danvers, MA) supplemented with protease inhibitor cocktail (1:100, Sigma-Aldrich, Rehovot, Israel), and cell debris was removed from the lysates by centrifugation (14 000 g, 1 minute 4°C). Western blot analysis was performed using standard techniques (sData S1).

2.11 | Statistical analysis

Group means were compared using the Mann-Whitney U test, ANOVA (for multiple group comparisons) and Wilcoxon signed rank test (for paired analyses). Spearman rank was used for determination of correlation and a custom rank trend test for Western blot analysis (Data S1). *P* values less than 0.05 were considered significant for all analyses.

3 | RESULTS

3.1 | Eosinophil expression of Siglec-7

Siglec-7 expression was detected on eosinophils from all 23 ND and 31 EO subjects tested. Geometric mean (GM) Siglec-7 expression

showed a tendency toward an increase in EO subjects compared to ND with GM Δ MFI of 183 vs 105, respectively, (*P* = 0.065; Figure 1A) and was significantly correlated with absolute eosinophil count (AEC) (*n* = 52, *P* < 0.05, Figure 1B). Siglec-7 was expressed on blood eosinophils at lower levels than the inhibitory receptors, CD300a and Siglec-8 (GM Δ MFI 271 vs 667 and 980, respectively, *n* = 20 EO; GM Δ MFI 307 vs 852 vs 837, *n* = 11 ND; Wilcoxon test, *P* < 0.05 to *P* < 0.0001; Figure 1C) and was positively correlated with Siglec-8 expression (*r* = +0.52, *P* < 0.0001, *n* = 47; Figure 1D) and CD300a (*r* = +0.7, *P* < 0.0001, *n* = 27, Figure S1).

SIGLEC-7 mRNA was quantified in purified peripheral blood eosinophils from 45 subjects (13 ND and 32 EO) and levels were detectable in all subjects tested (data not shown). *SIGLEC-7* mRNA levels were not correlated with the surface Siglec-7 levels measured on blood eosinophils by flow cytometry (Figure 1E). Moreover, contrary to what was seen at the cell surface levels by flow cytometry (Figure 1D), levels of mRNA for *SIGLEC-7* and *SIGLEC-8* were not correlated (Figure 1F).

3.2 | Measurement of soluble Siglec-7 receptor levels in serum

sSiglec-7 was measured by ELISA in the serum of normal and eosinophilic donors (*n* = 16 and 126) and was detectable in 133 of 142 subjects tested. Compared to ND (GM 270 pg/mL; *n* = 16) and treated EOS patients (GM 366 pg/mL; *n* = 81), serum sSiglec-7 levels were increased in untreated EOS subjects (GM 501 pg/mL; *n* = 45) (Figure 2A). Untreated EO subjects also had higher GM AEC than ND or treated patients (GM 2281 vs 250 vs 907 Eos/ μ L, Figure 2B). Serum levels of sSiglec-7 were weakly but significantly correlated with AEC (*n* = 135, *r* = +0.23, *P* < 0.001, Figure 2C). However, there was no significant correlation between sSiglec-7 levels in serum and surface expression of Siglec-7 on blood eosinophils for the 32 subjects tested including 11ND and 21EOS (*r* = -0.14, *P* = 0.42; Figure 2D). Levels of serum sSiglec-7 were comparable in untreated subjects of different HES subtypes, with the exception of MHES subjects who had significantly higher sSiglec-7 levels than ND (942 vs 270 pg/mL, respectively, Figure 2E). Of note, GM AEC was the highest in this HES subgroup (Figure 2F), and sSiglec-7 levels and AEC were correlated (*r* = +0.6, *P* < 0.05, *n* = 15, data not shown).

3.3 | Modulation of Siglec-7 expression in vitro

Geometric mean surface expression of Siglec-7 on purified blood eosinophils increased from an Δ MFI of 67.3-101.1 and 100.8 after an overnight incubation with GM-CSF or IL-5 (10 ng/mL), respectively (*P* < 0.05, *n* = 7; Figure 3A). Other stimuli, including IL-33, SEB (Figure 3A), *E. Coli* extracts, rhIFN- α , and rhIFN- γ , had no detectable effect on Siglec-7 surface expression (*n* = 6, Figure S2). Similarly, *SIGLEC-7* mRNA levels significantly increased from 7.0 to 14.5 A.U. $\times 10^{-5}$ in purified eosinophils incubated overnight with GM-CSF (*P* < 0.05; *n* = 7; Figure 3B).

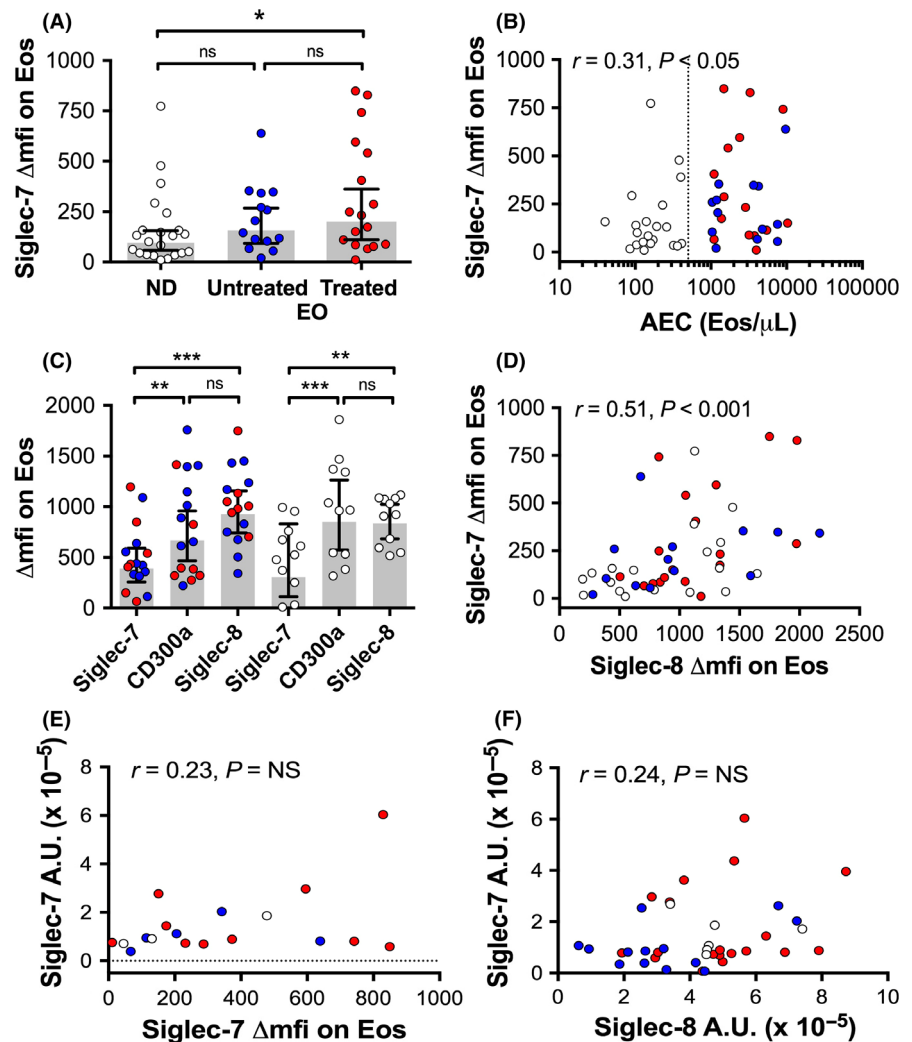


FIGURE 1 Siglec-7 expression on peripheral blood eosinophils. A, Siglec-7 expression (expressed as Δ mfi) on eosinophils in normal donors (ND) and eosinophilic subjects (EO), untreated and treated. B, Correlation between Siglec-7 expression and AEC. C, Comparison with eosinophil expression of Siglec-8 and CD300a. (D-F), Correlation between Siglec-7 and Siglec-8 surface expression (D), *SIGLEC-7* mRNA levels and cell surface expression (E), and *SIGLEC-7* and *SIGLEC-8* mRNA levels (F). Symbols represent individual subjects (white = ND, blue = untreated EOS, red = EOS on treatment). EOS subjects were comprised of subjects with MHES ($n = 6$), LHES ($n = 6$), overlap HES ($n = 5$), IHES ($n = 9$), and other eosinophilic disorders ($n = 3$). The gray bars indicate geometric mean. Error bars represent 95% CI. The vertical dotted line in panel B is drawn at the laboratory cutoff for eosinophilia (500/ μ L). * $P < 0.05$; ** $P < 0.001$, *** $P < 0.0001$ [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | Effect of Siglec-7 engagement on eosinophil viability and function

In contrast to anti-Siglec-8 antibodies, which induced eosinophil apoptosis, as assessed by total AnnexinV staining, after overnight priming with GM-CSF or IL-5 (10 ng/mL), anti-Siglec-7 antibodies had no effect on eosinophil apoptosis under the same conditions ($n = 5$; Figure 4). The percentage of 7-AAD⁺ eosinophils was also unchanged in response to anti-Siglec-7 (data not shown).

Cross-linking of Siglec-7 in the presence of GM-CSF reduced eosinophil activation in vitro, as assessed by surface expression of CD69 (Figure 5A, $n = 6$, $P < 0.05$). Cross-linking of Siglec-7, but not Siglec-8, on eosinophils also significantly inhibited the release of EPX, TNF- α , and IL-8 induced by GM-CSF (50 ng/mL) compared to the isotype control (mIgG1) ($n = 7-8$; Wilcoxon test, Figure 5B,C,D).

3.5 | Activation of Siglec-7 on eosinophils induces SHP-1 phosphorylation and decreases MAPK phosphorylation in a time-dependent fashion

Siglec-7 cross-linking of GM-CSF-activated eosinophils showed a trend toward induction of a time-dependent phosphorylation

of SHP-1 (Figure 6A,B; $P = 0.054$, rank trend test). Conversely, GM-CSF induction of phosphorylation of ERK1/2 and p38 (Figure 6C,D,E) was reduced by Siglec-7 cross-linking in a time-dependent manner, although this was not statistically significant. Notably, eosinophils incubated with anti-Siglec-7 alone displayed a basal phosphorylated state. Activation of CD300a (positive control) produced a pattern similar to that seen with Siglec-7.

4 | DISCUSSION

Inhibitory receptors (IRs) play important roles in modulating the function of innate immune cells, including eosinophils and mast cells, and have been proposed as therapeutic targets in allergic inflammation.²⁶ Siglec-7 is an IR expressed on a wide variety of human hematopoietic cells including NK cells, monocytes,²⁷ mast cells, basophils, and eosinophils.^{13,17} Decreased expression of Siglec-7 on selected cell populations has been reported in obesity²⁸ and diabetes²⁹ and has been associated with impaired NK cell function during chronic viral infections.^{30,31} Although Siglec-7 expression was

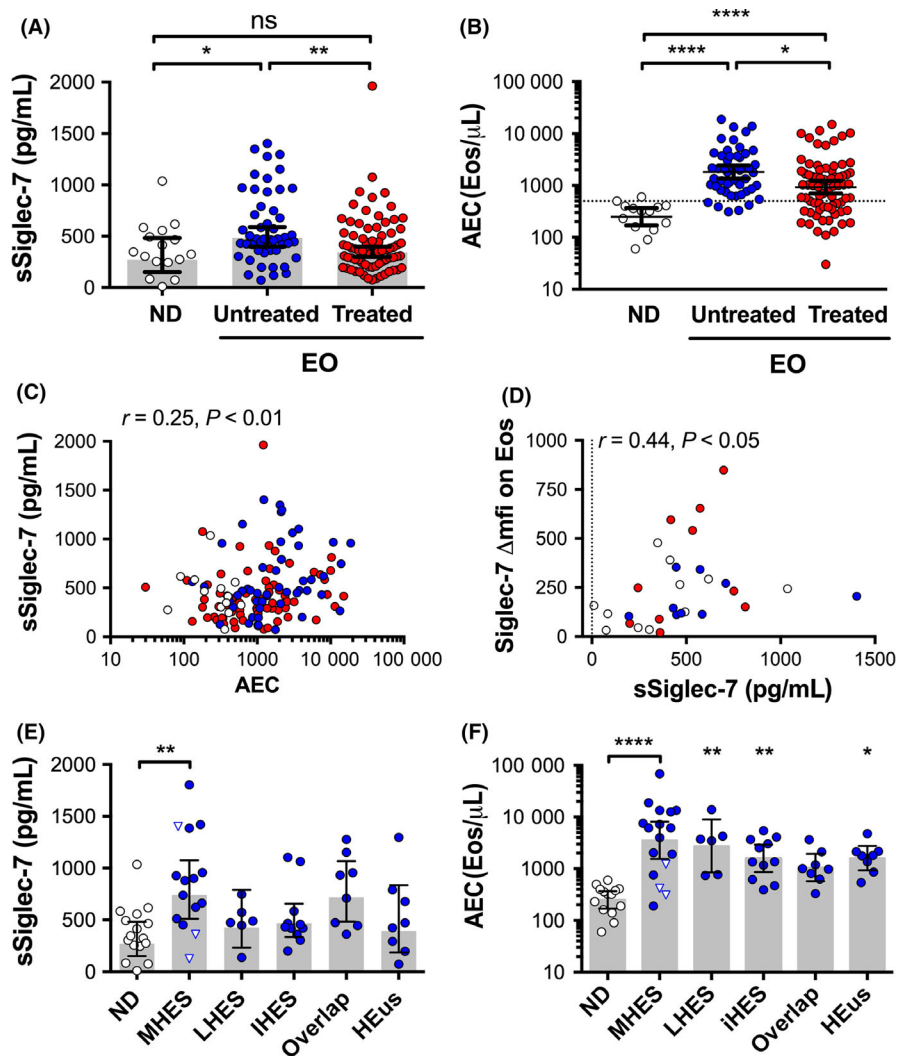


FIGURE 2 Serum levels of soluble Siglec-7. (A and B), sSiglec-7 levels in serum (left) and AEC (right) from ND and EO subjects. (C and D), Comparison of sSiglec-7 levels (left) and absolute eosinophil count (AEC) (right) between eosinophilic donors (EO) clinical subgroups in untreated patients (blue), inverted triangles indicate *FIP1L1-PDGFR α* ^{neg} MHES. Normal donors (ND) is shown as a control. E, Correlation of sSiglec-7 levels with AEC. F, Correlation with surface expression of Siglec-7 quantified on blood eosinophils by flow cytometry. Symbols represent individual subjects (white = ND, blue = untreated EO, red = EO on treatment). The gray shading indicates GM. Error bars represent 95% CI. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$, **** $P < 0.00001$ [Colour figure can be viewed at wileyonlinelibrary.com]

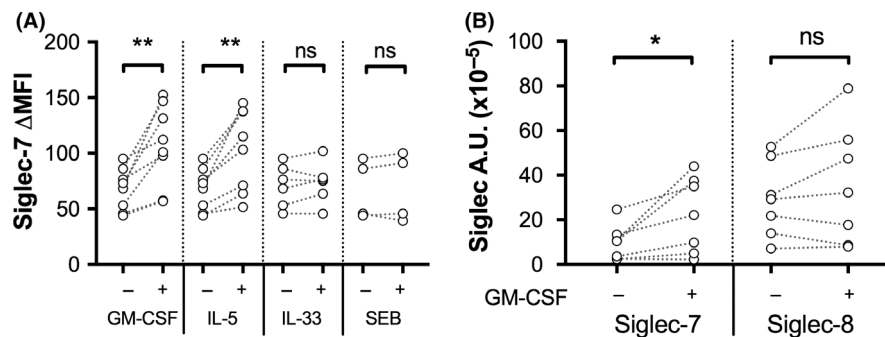


FIGURE 3 Siglec-7 is upregulated by stimulation with GM-CSF or IL-5. A, Surface expression of Siglec-7 after overnight incubation in media (–) or media supplemented with GM-CSF, IL-5, IL-33, or *Staphylococcus aureus* enterotoxin B (SEB) at 10 ng/mL (+). B, *SIGLEC-7* and *SIGLEC-8* mRNA levels in eosinophils after overnight incubation in media (–) or media supplemented with GM-CSF at 10 ng/mL (+). * $P < 0.05$; ** $P < 0.01$ ($n = 7$). Symbols represent individual subjects [white = normal donors (ND)]

previously demonstrated on eosinophils,¹³ the regulation of eosinophil expression of Siglec-7 in eosinophilic disorders and the functional consequences of Siglec-7 engagement on eosinophils has been largely unexplored.

In the present study, Siglec-7 expression was examined in normal donors and a large, heterogeneous cohort of eosinophilic subjects.

Unlike Siglec-8, surface expression of Siglec-7 on eosinophils was positively correlated with absolute eosinophil count and was upregulated in vitro by IL-5 and GM-CSF, cytokines involved in eosinophilopoiesis and survival, but not by any of the other stimuli examined. These data, together with the finding that serum levels of sSiglec-7 are increased in eosinophilic donors, suggest that expression of

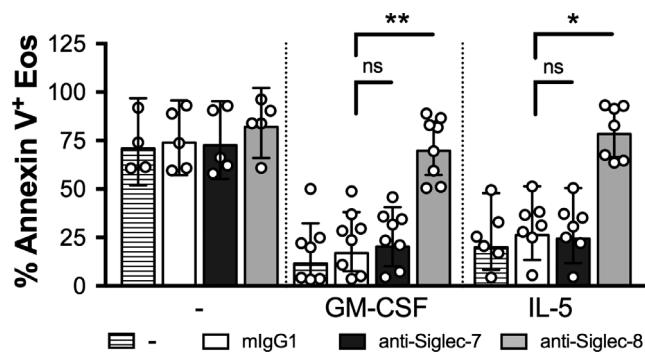


FIGURE 4 Siglec-7 does not induce eosinophil cell death. After overnight incubation with GM-CSF or IL-5, eosinophils were incubated with anti-Siglec antibodies (10 μ g/mL) for 18 h. Cell death was evaluated by AnnexinV/7-AAD staining and FACS analysis. Data are expressed as % geomean with 95% CI ($n = 8$) $**P < 0.01$. Symbols represent individual subjects (white = ND) $*P < 0.05$; $**P < 0.01$.

Siglec-7 is actively regulated on the surface of eosinophils and may play a role in down-modulating eosinophil activation in the setting of allergic inflammation.

In support of this hypothesis, antibody-induced Siglec-7 cross-linking inhibited GM-CSF-mediated release of EPX, IL-8, and TNF- α . This suppression of mediator release is similar to what has been described in response to cross-linking of Siglec-7 on anti-IgE-activated human cord blood-derived mast cells¹⁷ and may be important in the

context of the "allergic effector unit" where both mast cells and eosinophils interact via physical and soluble mechanisms.³² A similar inhibition of mediator release has been reported with activation of the inhibitory receptor, CD300a, on eosinophils¹³ and may be explained by the ability of these two IRs to phosphorylate common pathways with a critical role in signal transduction, such as SHP-1. In this context, the failure of Siglec-7 to induce eosinophil apoptosis (unlike CD300a) may be due to relatively weaker SHP-1 recruitment by Siglec-7.¹⁸ In contrast to Siglec-7 and CD300a, Siglec-8, which signals through a pathway involving Akt, p38, and c-Jun N-terminal kinase 1, appears to function predominantly as an activating receptor on IL-5 primed eosinophils leading to increased release of ROS and eosinophil apoptosis.³³

The major limitation of the present study was the technical challenges of working with human eosinophils, which are present in relatively low numbers in peripheral blood of normal individuals and do not survive in culture without the addition of exogenous cytokines. Moreover, eosinophils purified from different individuals exhibit differences in the basal activation state. Unfortunately, Siglec-E, the murine ortholog of Siglec-7 and Siglec-9, is not expressed on murine eosinophils³⁴ and humanized mouse models expressing Siglec-7 on eosinophils are unavailable at the present time.

In summary, Siglec-7 was constitutively expressed on eosinophils from all normal and eosinophilic donors tested, and expression was

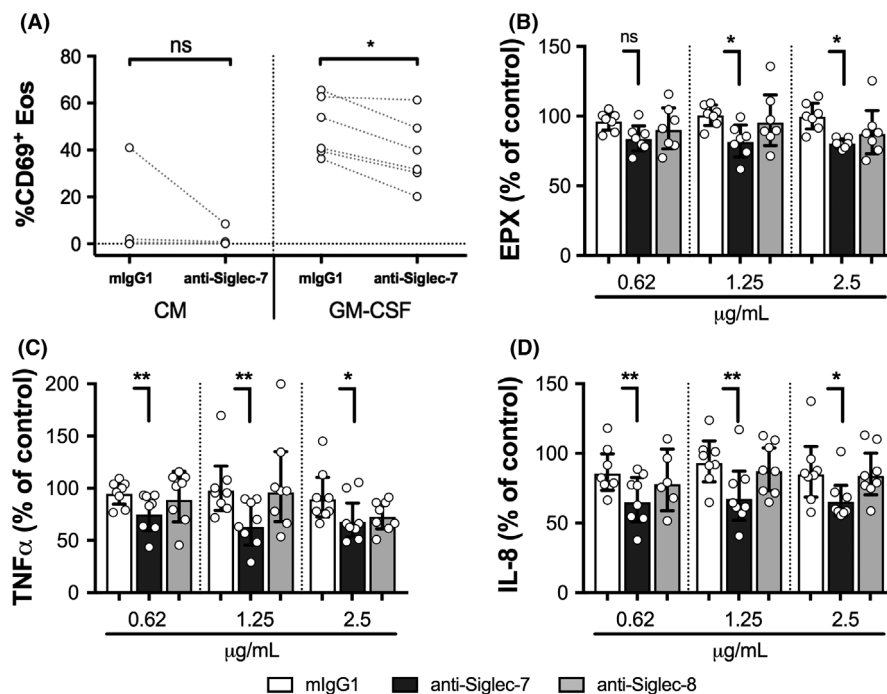


FIGURE 5 Cross-linking of Siglec-7 inhibits GM-CSF-induced eosinophil activation. A, Eosinophil surface expression of CD69 after overnight incubation in culture medium (CM) $\pm$ GM-CSF (50 ng/mL). The antibodies were cross-linked with F(ab')₂ goat anti-mouse (10 μ g/mL). Measurement of EPX (B), TNF- α (C), and IL-8 (D) in culture supernatants following cross-linking of isotype control (white bars), anti-Siglec-7 (dark gray bars), or anti-Siglec-8 (light gray bars) at antibody concentrations ranging from 0.62 to 2.5 μ g/mL on eosinophils for 40 min (EPX), or overnight (TNF- α and IL-8) in the presence of GM-CSF ($n = 7-8$). Mediator release is expressed as the % of the level measured in the control well containing 50 ng/mL GM-CSF alone. Data are expressed as % GM with 95% CI. $*P < 0.05$; $**P < 0.001$; Wilcoxon matched pairs signed rank test. Symbols represent individual subjects (white = ND)

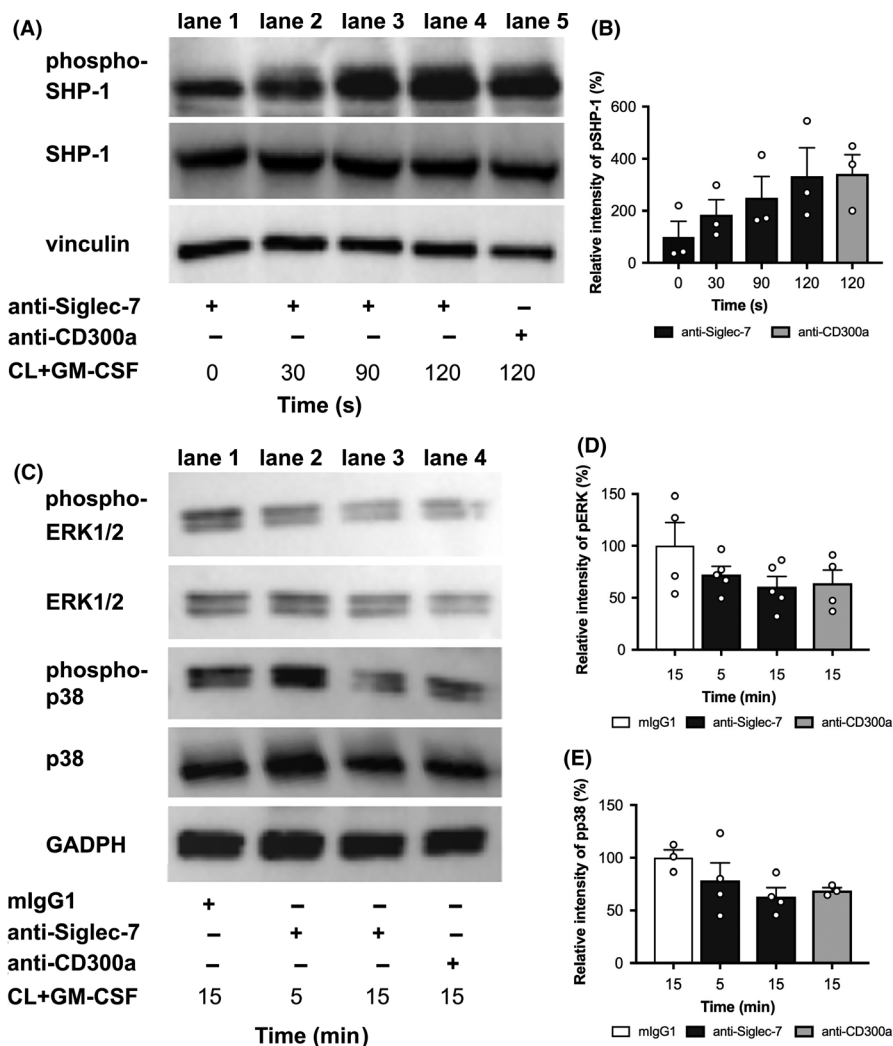


FIGURE 6 Activation of Siglec-7 on eosinophils induces SHP-1 phosphorylation and reduces mitogen-activated protein kinase (MAPK) phosphorylation in a time-dependent fashion. Western blot detecting phosphorylation of SHP-1 (A and B), ERK1/2 and p38 (C, D, and E). Eosinophils were incubated with IgG1, anti-Siglec-7, or CD300a antibodies (10 μ g/mL) followed by cross-linking (CL) in the presence of GM-CSF ($n = 4$). Phosphorylation of SHP-1 and MAPK (ERK1/2 and p38) was determined in the time interval of 0–120 s and 5–15 min, respectively. Vinculin and GAPDH were used as loading controls. Western blots shown are representative of four independent experiments performed on normal donors; pooled data are shown in the densitometry graphs. Relative intensity of phospho-SHP-1 (B), phospho-ERK1/2 (D), and phospho-p38 (E) is shown. Symbols represent individual subjects (white = normal donors [ND])

increased in the context of eosinophilia and by stimulation with cytokines that lead to eosinophilia and eosinophil activation.

Although the effect of Siglec-7 on eosinophil function was relatively modest, it appeared to be increased by eosinophil activation and was similar to the effect previously described on mast cell activation.¹⁷ Whether targeting Siglec-7 (alone or in combination with other IRs) would be useful as an additional means of reducing allergic inflammation remains unknown. Conversely, the effects of agents currently in development designed to block Siglec-7 function in the setting of tumors may have unexpected effects on eosinophil and mast cell activation.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

F.L., N.L., I.Z., R.O.E., and E.C. performed all the laboratory experiments, and F.L. and N.L. analyzed the data and drafted the manuscript. A.K. and F.L.S. designed the study revised and corrected the manuscript, obtained funding for the study. All authors reviewed and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary Materials and Methods

Assessment of surface receptor expression by flow cytometry

Antibodies used for eosinophil gating and surface staining in whole blood were the following. CD45-PerCP (clone 2D1), CD16 APC-H7 (clone 3G8), CD9 FITC (clone M-L13), mIgG1-FITC and mIgG1-APC (clone MOPC-21) are from BD. Anti-Siglec-7 PE (clone eBioQA79) and mIgG1 PE (clone P3.6.2.8.1) are from eBiosciences. Anti-Siglec-8 PE (clone 7C9) is from BioLegend and anti-CD300a PE (clone E59.126) is from Beckman Coulter. Anti-CD69-FITC (clone FN50, BioLegend) was used on purified eosinophils to assess activation.

Western Blot

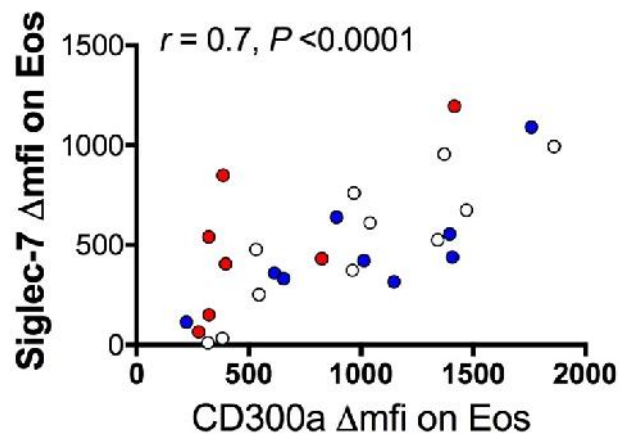
Protein content of each sample was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Bonn, Germany). The supernatants (containing 20 µg of protein) were boiled for 10 minutes in loading buffer 1x and run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were electrotransferred to Protran BA83 Nitrocellulose Blotting Membrane, 0.2 µm (GE Healthcare Life Sciences). Thereafter, membranes were blocked for one hour in 5% 1X TBST with 5% w/v nonfat dry milk (RT) and incubated overnight (4°C) with the following primary Abs: anti-SHPTP1 (1:1000, 14Tyr 536), anti-SH-PTP1 (1:1000, C-19), anti-GAPDH (1:1000, 6C5) (all from SantaCruz Biotechnology, Santa Cruz, CA, USA); anti-phospho- p44/42 MAPK (1:1000, T202/Y204), anti-p44/42 MAPK(1:1000, 137F5), anti-phospho-p38 and anti-p38 (1:2000, 1:1000, 28B10), (all from Cell Signaling Technology) and anti-vinculin (1:1000, EPR8185, Abcam Cambridge, UK). After this step, membranes were washed and incubated with anti-mouse-HRP or anti-rabbit-HRP, as

appropriate (Jackson Laboratories) before development using enhanced chemiluminescence (ECL) substrate (Luminata Crescendo Western HRP Substrate, Millipore). Image J Software was used for quantification of band intensity in western blot analysis. Data plotted in B, D, and E show relative intensity of phosphor-protein/total protein. Then, the data were normalized by the intensity of the bands obtained for the loading control (anti-vinculin (for SHP-1) or anti-GAPDH (for ERK1/2 and p38) at each time point.

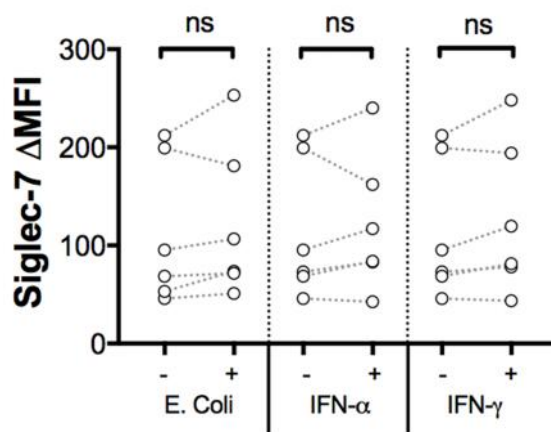
The test for a time trend for the Western blot analysis was done using the Monte Carlo permutation test with 1000,000 replications. For each replication, the values within each Western blot were permuted, and for each of those permuted data sets, trend was measured by first ranking the 4 values within each Western blot (to be robust outliers), then calculating the slope of the average ranks over time. Calculations were done in R (Version 3.5.1).

Supplementary Figures

Supplementary Figure 1. Siglec-7 is correlated with CD300a. Surface expression of Siglec-7 and CD300a were measured on blood eosinophils from 11 normal donors (white dots) and 16 eosinophilic subjects (untreated=blue; on treatment=red). Spearman correlation test.



Supplementary Figure 2. Siglec-7 is not upregulated by stimulation with E.coli extracts, IFN- α or IFN- γ . Surface expression of Siglec-7 after overnight incubation in media (-) or media supplemented with E.coli extracts (4 μ g/mL), IFN- α or IFN- γ at 10ng/ml (+). (n=6) Wilcoxon matched-pairs signed rank test.



Supplementary Tables.

Table S1. Subject characteristics by experiment.

	Surface Fig1 WB Flow	mRNA Fig1E PCR-WB Flow	mRNA Fig1F PCR	sSiglec-7 Fig2 ELISA	sSiglec-7 Fig 2D ELISA-WB Flow	Total Fig1-3 WB Flow-PCR- ELISA
<i>n</i>	53	18	38	139	32	144
ND	22	3	6	16	11	25
EO	31	15	32	123	21	119
<i>HES subtypes</i>						
MHES	6	4	7	15	5	19
LHES	5	3	5	18	5	15
Overlap HES	6	3	5	31	4	30
IHES	9	5	12	35	4	32
Other	5	0	3	25	3	23
<i>Treatment</i>						
YES	17	10	19	74	12	72
Glucocorticoid	10	4	8	54	6	52
Hydroxyurea	2	1	2	5	2	5
Interferon-alpha	3	3	5	4	1	4
Imatinib	1	0	0	6	0	5
Other	4	4	6	18	2	21
NO	14	5	13	49	9	47

WB: whole blood; Fig: Figure; ND: normal donor; EO: eosinophilic donor; HES: Hypereosinophilic syndrome; MHES: myeloid HES; LHES: Lymphoid HES; IHES: idiopathic HES.