

The study was supported by grants from the Swedish Research Council (grant no. K2011-56X-21854-01-06), the Ekhaga Foundation, the Olle Engkvist Foundation, the Cancer and Allergy Association, and the University Hospital of Linköping, Sweden.

Disclosure of potential conflict of interest: J. Mjösberg has received research support from the Swedish Research Council and the Swedish Cancer Society. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online March 14, 2014.
<http://dx.doi.org/10.1016/j.jaci.2014.01.027>

Siglec-7 is an inhibitory receptor on human mast cells and basophils

To the Editor:

Allergic effector cells, such as mast cells (MCs), eosinophils, and basophils, are key cellular targets whose activation must be inhibited to resolve or prevent allergic reactions. One approach to inhibiting these cells is to activate inhibitory receptors (IRs) expressed on their surface.¹ Sialic acid-binding Ig-like lectin 7 (Siglec-7, also called p75/AIRM1 and CD328) was first described in human natural killer (NK) cells² and later in granulocytes, monocytes, a minor subset of CD8⁺ T cells,³ and most recently in eosinophils.⁴ Siglec-7 preferentially binds to α 2,8-disialyl and branched α 2,6-sialyl carbohydrate structures,⁵ and association of its immunoreceptor tyrosine-based inhibition motif with the inhibitory phosphatase Src homology region 2 domain-containing phosphatase-1 (SHP-1) transduces an inhibitory signal.² Here, we show for the first time that Siglec-7 is also expressed on human MCs and basophils; functionally, our data suggest that Siglec-7 plays a significant role in inhibiting IgE-mediated MC activation, but only moderately affects IgE-mediated activation in primary human basophils.

Flow cytometric (FC) analysis showed that Siglec-7 was expressed on fully differentiated human cord blood-derived MCs (CBMCs) from all 10 donors (Fig 1, A) as well as on isolated human foreskin MCs and isolated human peripheral blood basophils (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Interestingly, Siglec-7 appeared on immature CBMCs as early as week 4 of culture

(data not shown), and this level of expression was maintained thereafter throughout the 8-week maturation period. Siglec-7 expression was also detected to varying degrees on the leukemic MC cell lines HMC-1 and LAD-2 (Fig 1, A). Immunofluorescent microscopy for Siglec-7 expression confirmed the FC data (Fig 1, B).

Siglec-7, originally identified on NK cells as a 75-kDa protein,² consists of a 46-kDa peptide backbone modified by extensive glycosylation and therefore may range in size from 46 to at least 75 kDa. To verify the form(s) of Siglec-7 present on MCs and basophils, Siglec-7 was immunoprecipitated (IPed) and resolved by a reducing SDS-PAGE, followed by Western blot analysis. CBMC-associated Siglec-7 migrated as a 75-kDa protein (Fig 1, C), with basophil-associated Siglec-7 migrating within the same size range (see Fig E1, B).

We next tested the ability of Siglec-7 to inhibit MC degranulation induced by Fc ϵ RI-dependent stimulation using an activating anti-Siglec-7 mAb. Because IRs were shown to optimally inhibit Fc ϵ RI activation-mediated tyrosine kinase signaling cascades on co-cross-linking both the IR and Fc ϵ RI,¹ we incubated IgE-sensitized CBMCs with mouse anti-human IgE mAb with or without mouse anti-human Siglec-7 mAb or its isotype-matched negative control antibody (mouse IgG₁) at various concentrations, and then added the goat F(ab')₂ antimouse IgG antibody to co-cross-link and activate Siglec-7 and Fc ϵ RI (see Methods in this article's Online Repository at www.jacionline.org). Co-cross-linking Siglec-7 with Fc ϵ RI significantly inhibited Fc ϵ RI-dependent CBMC activation and the release of the degranulation-associated mediators tryptase (Fig 2, A), β -hexosaminidase (Fig 2, B), and the *de novo*-synthesized lipid mediator PGD₂ (Fig 2, C) in a manner dependent on the dose of anti-Siglec-7 mAb. Next, to evaluate whether co-cross-linking Siglec-7 with Fc ϵ RI could also affect cytokine production/release in CBMCs, we activated CBMCs as above for 24 hours and found that anti-Siglec-7 specifically inhibited GM-CSF release (Fig 2, D).

Overall, our data showing an effect of Siglec-7 activation on MCs are similar to those observed when activating Siglec-8,⁶ although Siglec-7 has a slightly more potent inhibitory effect on cytokine release. To determine the signaling molecules mediating the inhibitory effect of Siglec-7 in CBMCs, we IPed Siglec-7 and checked for co-IP of SHP-1, which was previously found to participate in Siglec-7 function in NK cells.² Indeed, we observed that tyrosine-phosphorylated SHP-1 co-IPed with Siglec-7 in CBMCs (Fig 2, E). However, although treatment with orthovanadate—a tyrosine phosphatase inhibitor—significantly increased Siglec-7 tyrosine phosphorylation (Fig 2, E, p-Tyr 75-kDa band), the amount of phosphorylated SHP-1 co-IPed with Siglec-7 increased only slightly in CBMCs (Fig 2, E), indicating that SHP-1 might not be the main signal transduction molecule responsible for mediating the signals downstream of activated Siglec-7 in CBMCs.

Co-cross-linking Siglec-8 with Fc ϵ RI is not required for its inhibitory function in MCs.⁶ To test whether this was also the case for Siglec-7, IgE-sensitized CBMCs were preincubated with mouse anti-Siglec-7 mAb or its isotype control, followed by simultaneously cross-linking anti-Siglec-7 mAb (using a goat F(ab')₂ anti-mouse IgG antibody) and activating Fc ϵ RI using a polyclonal rabbit anti-human IgE, which could not be cross-linked by the F(ab')₂ anti-mouse IgG. Cross-linking Siglec-7 alone did not inhibit Fc ϵ RI-induced degranulation

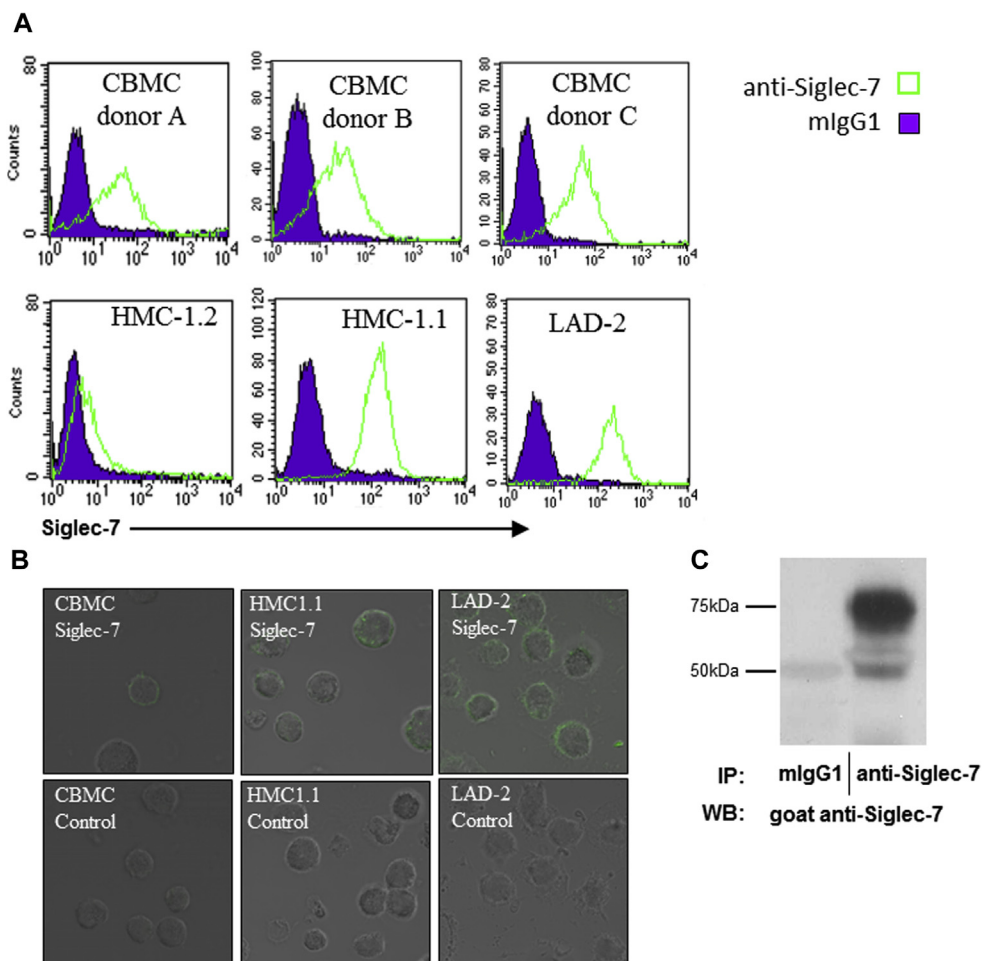


FIG 1. **A**, FC analysis of Siglec-7 expression in CBMCs and MC cell lines. **B**, IF of Siglec-7 expression (green fluorescence on cell surface) on slide-fixed CBMCs and MC cell lines. **C**, CBMC proteins were IPed with an anti-Siglec-7 or isotype-matched antibody (mouse IgG₁ [mIgG₁]), resolved by SDS-PAGE, and analyzed by WB using a polyclonal goat anti-Siglec-7 antibody. Protein marker sizes are indicated. Representative results are shown for n = 10 (Fig 1, A), 2 (Fig 1, B), and 3 (Fig 1, C) separate experiments performed with different donors/cell batches. *IF*, Immunofluorescence; *WB*, Western blot.

(Fig 2, F), demonstrating that Siglec-7 must be coupled to FcεRI by co-cross-linking to effectively inhibit mediator release from CBMCs. We speculate that this observed distinction between Siglec-7 and Siglec-8 in terms of their requirement for co-cross-linking with FcεRI is based on their use of different intracellular signal transduction pathways.

It is possible that the requirement of Siglec-7 to co-cross-link with FcεRI to inhibit CBMC activation *in vitro* is also true *in vivo*. On tissue-resident MCs *in vivo*, ligated FcεRI (and potentially other activating receptors) and Siglec-7 (and other IRs) likely comigrate to specific membrane sublocations where mutual activation and inhibition can occur. In addition, it is possible that intercellular interactions that occur during allergic inflammation can induce robust Siglec-7 clustering strong enough to induce self-activation without the requirement for co-cross-linking.

We next tested whether Siglec-7 inhibition of FcεRI-induced degranulation was a mechanism unique to MCs or whether it could also occur in basophils. In contrast to MCs, engagement of Siglec-7 co-cross-linked with FcεRI activation in basophils only

weakly inhibited IgE-mediated activation (see Fig E1, C). This moderate inhibitory activity was similar to the inhibition triggered by the CD300a IR but contrasted with the strong inhibition observed after CD200R engagement in basophils, as we recently reported.⁷ This difference in the degree of Siglec-7-mediated inhibition between MC and basophils could be due to the relatively low Siglec-7 expression in basophils. Indeed, IP analysis of Siglec-7 showed that basophils have 33% lower total Siglec-7 protein content than do CBMCs (assessed by quantifying band intensities in Fig E1, B; data not shown). Nevertheless, FC analysis showed that surface Siglec-7 expression was similar among fresh primary basophils, CBMCs, and fresh monocytes (see Fig E1, A). Therefore, the difference in Siglec-7 potency between MCs and basophils is likely due to their different intracellular signal transduction pathways.

We show here for the first time that surface Siglec-7 is expressed at high levels and functions as an IR in MCs. Using gene arrays, Yoki et al⁸ previously demonstrated very low Siglec-7 mRNA levels in peripheral blood-derived MCs (matured for 14-15 weeks in culture). The apparent difference between

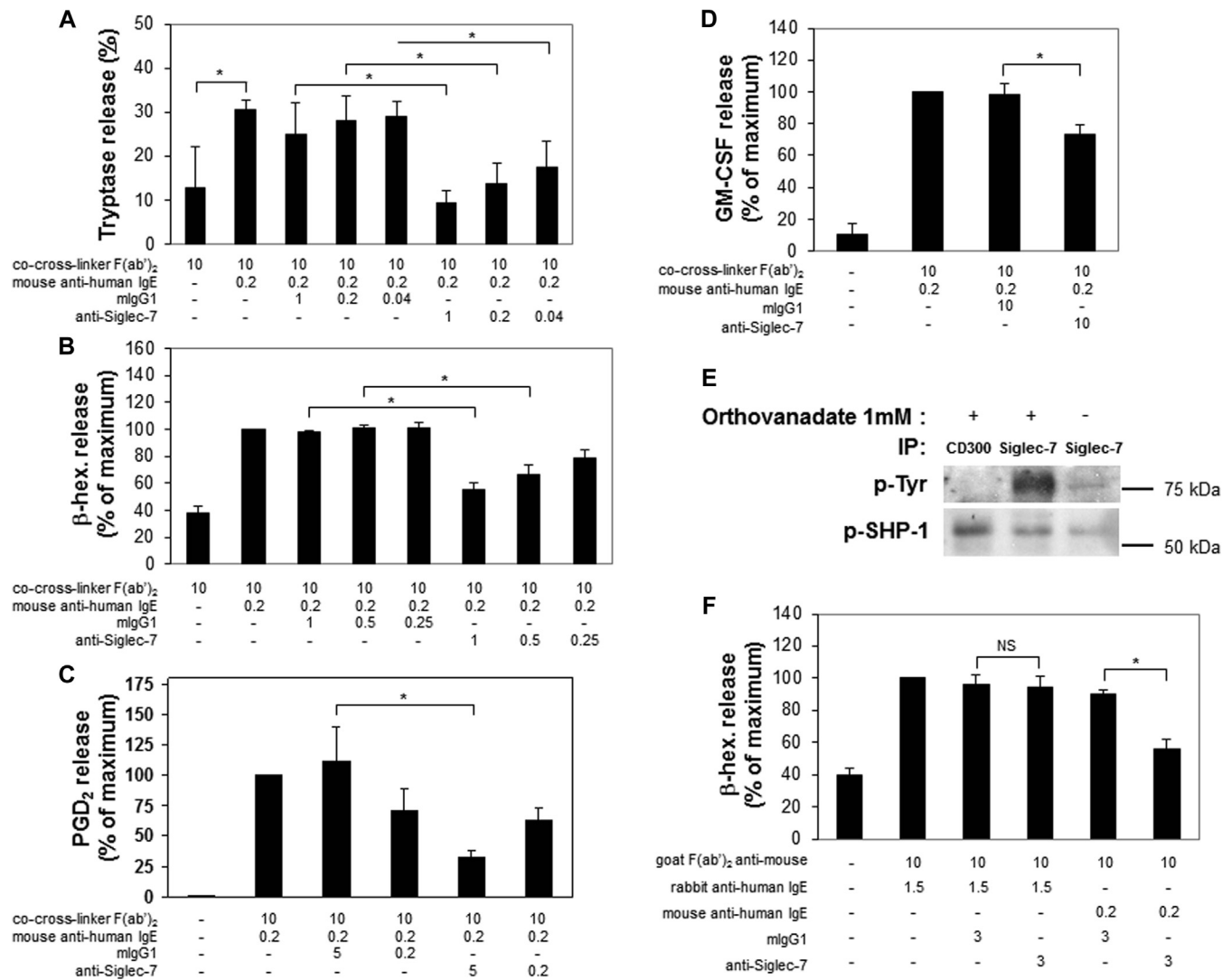


FIG 2. Effects of Siglec-7 stimulation on IgE-dependent mediator release from CBMCs. Data are shown as the mean \pm SEM from $n = 3$ separate experiments. Data shown are tryptase release (%) (A), β -hex (B), PGD₂ (C), and GM-CSF (D) (see Mediator assays and calculations in the Methods section in this article's Online Repository). E, IP-WB analysis showing Siglec-7, CD300a, and co-IP of phosphorylated SHP-1 expressed in CBMCs incubated with or without orthovanadate (representative of $n = 3$ experiments). F, Effect of Siglec-7 stimulation on IgE-dependent β -hex release from CBMCs without co-cross-linking. Percentage of maximum release is shown, and data are expressed as the mean \pm SEM from $n = 2$ separate experiments (see the Methods section in this article's Online Repository). Antibody concentrations are expressed as μ g/mL. β -hex, β -Hexosaminidase; mIgG₁, mouse IgG₁; WB, Western blot. * $P < .03$.

their data and ours may be explained by the different methods used for detecting Siglec-7 expression, the source of MCs, and/or the culture time and conditions used for cell maturation.

Together with the known expression of Siglec-7 on eosinophils,⁴ our results revealing a strong inhibitory effect of Siglec-7 on MCs and a moderate inhibitory effect on basophils suggest that Siglec-7 is involved in the inhibition and/or resolution of T_H2 responses. This finding is strengthened by the observation that suppressor of cytokine signaling 3—a pro-T_H2 regulator⁹—is directed toward proteasomal degradation after Siglec-7 activation, which may shift cell responses toward T_H1.¹⁰ Collectively, these lines of evidence suggest that targeting Siglec-7 in allergic diseases may be a powerful tool to limit allergic reactions by directly inhibiting allergic effector cell responses.

We thank Dr David Mankuta (Department of Gynecology, Hadassah Hospital, Jerusalem, Israel) and Mrs Sheli Friedman from our laboratory for collecting cord blood. Also, special thanks to Mrs Madelyn Segev for secretarial help.

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The authors acknowledge financial support for this study from a "Kamin" grant of the Israel Ministry of Industry and Trade (Grant no. 46223) and The Aimwell Charitable Trust Foundation (UK) to F.L.-S.; European Cooperation in Science and Technology COST Action BM1007 "Mast Cells and Basophils - Targets for Innovative Therapies" facilitated the collaboration between F.L.-S. and B.F.G. in support of this study. F.L.-S. is a member of the Dr Adolph and Klara Brettler Center for Research in Molecular Pharmacology and Therapeutics and the David R. Bloom Center for Pharmacy at The Hebrew University of Jerusalem's School of Pharmacy.

Disclosure of potential conflict of interest: S. Mizrahi has received travel support from the European Cooperation in Science and Technology COST Action BM1007. B. F. Gibbs has received start-up funds from and is employed by the University of Kent, has received payment for lectures from the University of Virginia and ALK Abello, and has received travel support from EU/ESF COST Action BM1007. F. Levi-Schaffer has received research support from the Israeli Ministry of Industry and Trade (Grant No. 46223); is a member of the Committee for Human Experimentation of New Drugs for the Israeli Ministry of Health; and has received travel support from EU/ESF COST Action BM1007. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online May 5, 2014.
<http://dx.doi.org/10.1016/j.jaci.2014.03.031>

Occurrence of B-cell lymphomas in patients with activated phosphoinositide 3-kinase δ syndrome

To the Editor:

Activated phosphoinositide 3-kinase δ syndrome (APDS) is a novel autosomal-dominant primary immunodeficiency (PID) caused by a heterozygous gain-of-function mutation in the *PIK3CD* gene encoding the p110δ protein, the catalytic subunit of phosphoinositide 3-kinase δ (PI3Kδ).¹ The c.3061G>A mutation results in a substitution of a glutamic acid by a lysine at position 1021 (E1021K). This new PID is characterized by recurrent respiratory infections, leading to bronchiectasis, progressive lymphopenia, and defective antibody production. Both T- and B-cell compartments are affected as shown by the

propensity of CD4⁺ and CD8⁺ T cells to die after *in vitro* stimulation and their poor capacity for cytokine production, as well as an immunoglobulin class switch recombination defect (CSR-D). Most of the cases have an increase in serum IgM levels and a decrease in IgG₂ isotype, while total IgG and IgA levels can be either normal or strongly decreased. The clinical presentation is variable, ranging from combined immunodeficiency requiring hematopoietic stem cell transplantation to an isolated primary antibody deficiency that can be well controlled by IgG substitution. To identify new patients with APDS, we genotyped the *PIK3CD* gene at position c.3061G as described previously¹ in a cohort of 139 patients with immunologic phenotype of immunoglobulin CSR-D. We found 8 new patients with APDS with the E1021K heterozygous mutation in the *PIK3CD* gene (see Tables E1 and E2 in this article's Online Repository at www.jacionline.org) in addition to the 17 described previously,¹ bringing the total number of known patients carrying this *PIK3CD* mutation to 25. We noticed that among these 8 new patients with APDS, 2 developed B-cell lymphomas, suggesting that a constitutively active PI3Kδ predisposes to malignancies. These 2 cases are herein reported (Tables I and II).

Patient 1 has no familial history of PID, but his mother died at age 35 years of subarachnoid hemorrhage. He was referred to our hospital at the age of 2 years with recurrent bronchopulmonary infections, lymphadenopathy, hepatosplenomegaly, and liver disease (elevated transaminases and portal septal fibrosis at liver biopsy). He had increased serum IgM levels (4.25 g/L), normal IgG levels (5.7 g/L), and decreased IgA levels (0.65 g/L), compatible with the diagnosis of CSR-D. The CD40L and CD40 defects were excluded and intravenous IgG substitution was initiated. At age 8 years, he developed a high-grade diffuse large B-cell lymphoma (DLBCL, World Health Organization classification) of the biliary tract (Fig 1, A-C). *In situ* hybridization for EBV was negative, and Bcl-6 was expressed as shown by immunohistochemistry. The patient recovered after 9 courses of chemotherapy (UKCCSG 9002 protocol^{E1}). At age 19 years, under IgG substitution, he again developed a high-grade EBV(-) DLBCL of the colon, which was found to be Bcl-6 negative (Fig 1, D-F). He received cyclophosphamide, vincristine, steroids plus rituximab. He died from large-bowel perforation and bleeding 12 days after the third course of chemotherapy.

Patient 2 belongs to a family in which 2 siblings were reported as suffering from a CSR-D (data from the affected sister P7; see Tables E1 and E2). From the age of 5 months, he suffered recurrent upper (recurrent acute otitis media) and lower respiratory tract infections complicated by bronchiectasis, chronic noninfectious diarrhea with malabsorption syndrome, and failure to thrive. Other infections were also noticed, including pericarditis caused by Echo virus infection and recurrent synovitis. The diagnosis of CSR-D was made according to his familial history and IgG substitution was started. At age 6 and 8 years, he displayed episodes of massive enlargement of lymph nodes (cervical and mesenteric) with no malignant feature at biopsy. Serum immunoglobulin levels revealed an increase in IgM (4.5 g/L at 5 years and 13 g/L at 11 years) levels and a decrease in IgG (<1.9 g/L) and IgA (0.41 g/L) levels. At age 11 years, he had a new episode of cervical lymph nodes enlargement that led to the diagnosis of Hodgkin disease, histologic type nodular sclerosis, stage III, with localization to cervical, mediastinum, retroperitoneum, and spleen (the EBV status was unknown and could not be studied retrospectively) (Fig 1, G-I). The patient

METHODS

Cells

After approval by the ethical committee (written informed consent was obtained according to the guidelines of the Hadassah-Hebrew University Human Experimentation Helsinki Committee [7-14.01.05]), CBMCs were obtained as previously reported.^{E1} Briefly, human umbilical cord blood was obtained from normal births after informed consent. Mononuclear cells were enriched by Ficoll-density centrifugation and grown in MEM- α medium supplemented with stem cell factor (a kind gift of Sobi, Stockholm, Sweden), IL-6 (PeproTech, Rocky Hill, NJ), and PGE₂ (Sigma, St Louis, Mo). CBMCs cultured for 8 weeks with more than 90% mature MCs were used (mature MCs were positive for acidic toluidine blue staining). Tissue-resident mature MCs were obtained from infant foreskin as previously reported^{E2} with the addition of an MC isolation step using the CD117 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) after tissue digestion into a single-cell suspension.

After approval by the ethical committee (National Health Service-National Research Ethics Service project reference 12/WM/0319), human primary basophils were purified from buffy coats purchased from National Health Service Blood and Transfusion Service consisting of leukocyte concentrates from healthy donors undergoing routine blood donation. Basophils were enriched by Ficoll-density centrifugation followed by negative selection by magnetic cell sorting using a commercial Basophil Isolation Kit (StemCell Technologies, Vancouver, British Columbia, Canada) as previously described.^{E3}

Cell treatments

CBMCs were sensitized for 4 days with 300 ng/mL human IgE (Calbiochem, EMD Millipore, Billerica, Mass) in the presence of 10 ng/mL human IL-4 (PeproTech). On day 4, cells were blocked with 5% goat serum in PBS for 10 minutes on ice. Thereafter, cells were incubated in Tyrode's buffer^{E4} containing the indicated concentrations of mouse anti-human IgE (clone 4C3, Serotec, Oxford, United Kingdom) together with mouse anti-human Siglec-7 (clone eBioQA79, eBioscience, San Diego, Calif) or its isotype-matched negative control (mouse IgG₁ [mIgG₁], eBioscience) for 30 minutes on ice. Afterwards, cells were centrifuged and activated for 30 minutes at 37°C by resuspension in Tyrode's buffer containing 0.9 mM MgCl₂, 1.8 mM CaCl₂, and 10 mg/mL of the co-cross-linker F(ab')₂ goat anti-mouse IgG antibody (Jackson Immuno-Research Laboratories, West Grove, Pa). For the non-co-cross-linked assay (Fig 2, F), CBMCs were sensitized and blocked under the same conditions as above, but preincubated only with mouse anti-Siglec-7 or mIgG₁ antibody. Afterwards, a goat anti-mouse cross-linker was added to cross-link anti-Siglec-7 or mIgG₁ antibody, and cells were activated with 1.5 μ g/mL rabbit anti-human IgE polyclonal antibody (Dako, Glostrup, Denmark). For GM-CSF release, CBMCs were treated and activated as above for 24 hours in CBMC medium. For detecting phosphorylated SHP-1 and general tyrosine phosphorylation, 6 \times 10⁶ CBMCs/sample were incubated with 1 mM sodium orthovanadate for 10 minutes at 37°C. Purified basophils were activated similar to CBMCs except for IgE sensitization (because these primary cells are naturally sensitized, unlike CBMCs). The supernatants and the cell pellets were collected and kept frozen for subsequent analysis of mediator release.

Mediator assays and calculations

For the release of tryptase, β -hexosaminidase, and histamine, both supernatants and cell pellets were used in colorimetric enzymatic assays^{E4} (for measuring tryptase and β -hex) or a fluorometric assay (for measuring histamine).^{E3} For these mediators, the percentage of mediator release was calculated by dividing the amount of mediator detected in the supernatant by the total amount present in the cell pellet and supernatant, followed by multiplying by 100. For analyzing PGD₂ and GM-CSF release, their content in the supernatant (pg/mL) was evaluated using commercial ELISA kits (Cayman Chemical Co, Ann Arbor, Mich, and Peprotech, respectively).

FC analysis

For detecting Siglec-7 expression in different MC types, cells were blocked with 5% goat serum and resuspended in FC buffer (0.5% BSA, 0.05% sodium azide in PBS) containing an anti-Siglec-7 mAb (5 μ g/mL) or its isotype

control. Thereafter, cells were centrifuged, washed, and incubated with FC buffer containing fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ goat anti-mouse IgG (1:200, Jackson ImmunoResearch Laboratories).

For detecting Siglec-7 expression levels on basophils, NK cells, or monocytes, freshly isolated PBMCs or IL-2-supplemented NK cells (NK cell line) were blocked and stained for Siglec-7 as described above using an Alexa Fluor 647-conjugated goat anti-mouse antibody as the secondary antibody. Thereafter, cells were centrifuged and incubated with FITC-conjugated mouse anti-human CD3 and phycoerythrin-conjugated mouse anti-human CD56 for detecting Siglec-7 on CD56⁺CD3⁻ NK cells; FITC-conjugated mouse anti-human CD14 for monocytes; and phycoerythrin-conjugated mouse anti-human CD203C for basophils. The relevant isotype-matched controls were used (all from BioLegend, San Diego, Calif).

In all FC experiments, cells were washed once with FC buffer and analyzed by a FACSCalibur flow cytometer equipped with CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, Calif).

Immunoprecipitation and Western blot

For detecting Siglec-7, phosphorylated SHP-1 (p-SHP-1), or phosphorylated tyrosines in general in MCs, 6 \times 10⁶ CBMCs/sample were lysed using 0.5% NP-40 lysis buffer,^{E5} precleared, and washed extensively with 0.1% TBST buffer (0.1% v/v Tween 20 in TBS). Afterwards, proteins were co-IPed from the lysates using protein A/G beads (Santa Cruz Biotechnology, Dallas, Tex) conjugated to Siglec-7 or mIgG₁ (eBioscience). IPed proteins were resolved by a reducing SDS-PAGE, transferred to a nitrocellulose membrane, and blocked with BSA. For detecting Siglec-7, p-SHP-1, or phosphorylated tyrosines in general, membranes were incubated with a goat anti-human Siglec-7 polyclonal antibody (R&D Systems, Minneapolis, Minn), mouse anti-phosphotyrosine (Santa Cruz), or a rabbit anti-p-SHP-1 polyclonal antibody (ECM Biosciences, Versailles, KY), respectively, followed by the relevant horseradish peroxidase-conjugated secondary antibody.

For comparing Siglec-7 content in CBMCs and basophils, 100 ng/mL of either anti-Siglec-7 or isotype control antibody in PBS was added to a 96-well ELISA plate and incubated overnight at 4°C. After removing unbound antibody, the plate was blocked with 2% BSA in PBS for 2 hours before washing twice in 0.1% TRIS-buffered saline [solution] with Tween (TBST). Then, 1 \times 10⁶ CBMCs/sample were lysed with 0.5% NP-40 lysis buffer,^{E5} and lysates were heated at 95°C for 4 minutes and added into the appropriate wells. The plate was then incubated for 3 hours at room temperature with rocking and subsequently washed 3 times with TBST, followed by adding 20 μ L of glycine buffer (pH 2.0) into each well. After 5 minutes, proteins were transferred to vials containing 20 μ L of Laemmli reagent and then analyzed by SDS-PAGE and Western blot using an anti-Siglec-7 antibody (eBioscience). Secondary horseradish peroxidase-conjugated antibodies were then used according to the origin of the primary antibody, and the presence of the desired protein was detected by chemiluminescence.

Immunofluorescent staining

CBMCs, LAD-2, and HMC-1.1 cells were adhered onto poly-lysine-coated slides and fixed using 4% paraformaldehyde. Thereafter, the cells were washed, incubated with mouse anti-human Siglec-7 (eBioscience), washed again, and incubated with Alexa Fluor 647-conjugated goat anti-mouse Ab (1:50, Life Technologies, Carlsbad, Calif). Immunostained cells were mounted with aqueous mounting medium (Southern Biotech, Birmingham, Ala) and analyzed using a Zeiss LSM 710 Axio Observer Z1 confocal laser-scanning microscope equipped with LSM ZEN software (Zeiss, DE, Göttingen, Germany).

Statistical analysis

Statistical significance was evaluated by 1-tailed and equal-variance Student *t* test. *P* values are indicated in figure legends.

For tryptase (Fig 2, A) or histamine release (see Fig E1, C), the mean \pm SEM was calculated from 3 or 5 separate experiments, respectively. The mean percentage maximum release \pm SEM values were obtained from 3 separate experiments for Fig 2, B-D, and from 2 separate experiments for

Fig 2, *F*. These values were calculated by normalizing the values obtained in each experiment to the respective release induced by anti-IgE alone (positive control: taken as maximal release, 100%).

In all experiments, “n” represents the number of separate experiments performed with different CBMCs/basophils/MC cell lines/donors/cell batches. All CBMC functional assays were performed in triplicate. In all basophil experiments, each treatment group was tested in a single sample because of the extremely low number of cells that could be obtained from each donor.

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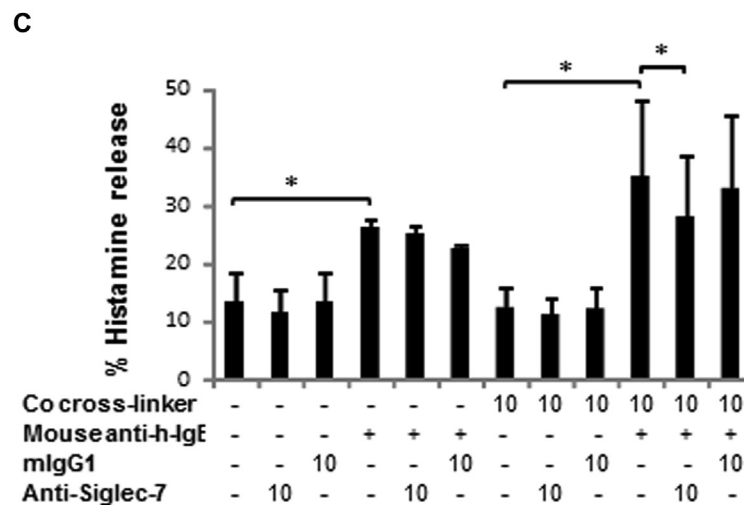
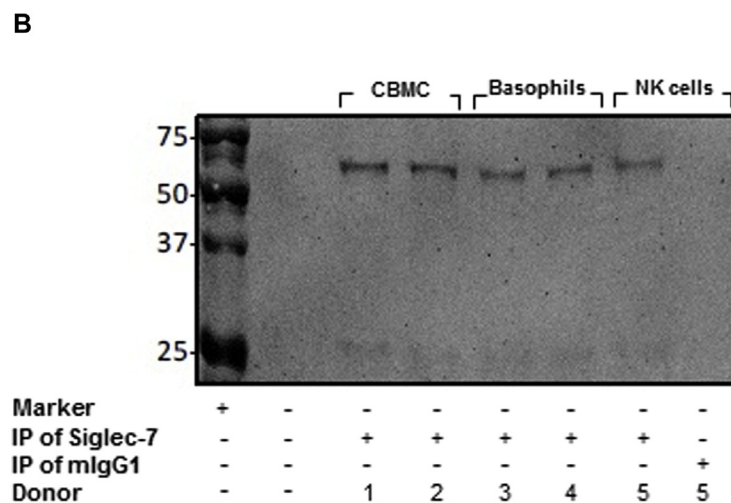
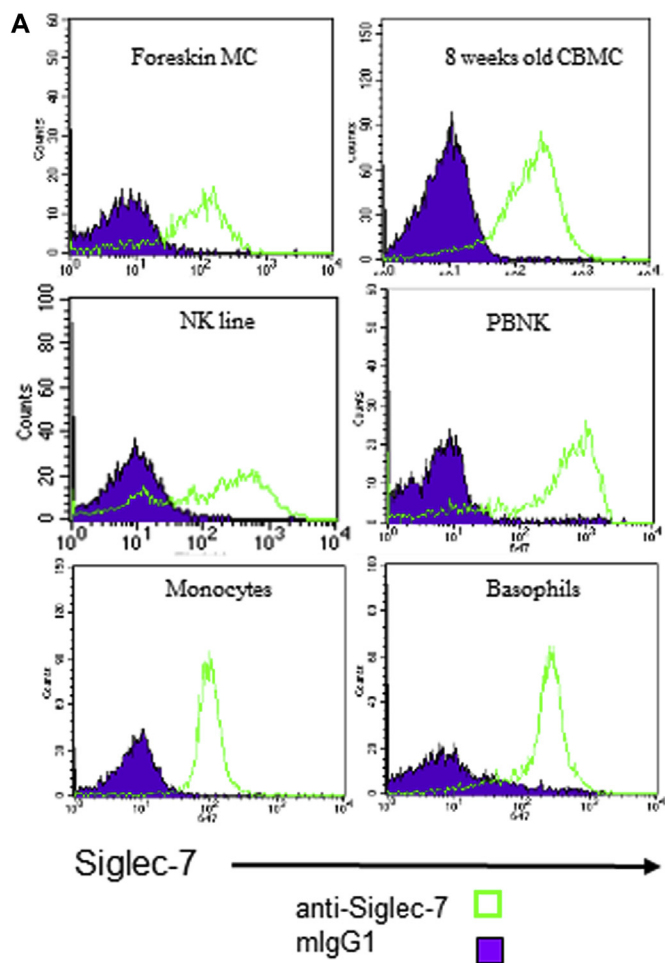


FIG E1. A, FC analysis of Siglec-7 expression on human foreskin MC, CBMCs, NK cell line, peripheral blood NK cells, monocytes, and basophils (representative data of $n = 3$ experiments performed with different donors). **B,** WB analysis of Siglec-7 IPed from 2 different donors for CBMCs and basophils, and 1 NK cell line. **C,** Effects of Siglec-7 stimulation on IgE-dependent histamine secretion from primary human basophils. Data are shown as the mean \pm SEM for $n = 5$ separate experiments (see “Cell treatments” section above). WB, Western blot; * $P \leq .04$.