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Siglec-9 is an inhibitory receptor on human mast cells *in vitro*

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Abstract

Background: Mast cell activation is critical for the development of allergic diseases. Ligation of Sialic acid-binding immunoglobin-like lectins (Siglecs) such as CD33, Siglec-6, -7 and -8 have been shown to inhibit mast cell activation. Recent studies showed that human mast cells express Siglec-9, an inhibitory receptor also expressed by neutrophils, monocytes, macrophages, and dendritic cells.

Objective: We aimed to characterize Siglec-9 expression and function in human mast cells *in vitro*.

Methods: We assessed the expression of Siglec-9 and Siglec-9 ligands on human mast cell lines and human primary mast cells by qPCR, flow cytometry and confocal microscopy. We used a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein

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Author contributions:

S.D.M. provided skin tissues. C.A.O. and Y.H advised and provided guidance with the generation of human skin-derived cultured mast cells. I.M., N.B.S., and A.M.P designed the experiments. I.M., N.B.S., A.J.S., J.F. and J.S. performed the experiments. I.M., N.B.S., and A.M.P. analyzed the results, and wrote the manuscript. All authors reviewed the final version of the manuscript.

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9 (Cas9) gene editing approach to disrupt the *SIGLEC9* gene. We evaluated Siglec-9 inhibitory activity on mast cell function by using native Siglec-9 ligands, glycophorin A (GlycA) and high molecular weight hyaluronic acid (HMW-HA), a monoclonal antibody against Siglec-9, and co-engagement of Siglec-9 with the high affinity receptor for IgE (FcεRI).

Results: Human mast cells express Siglec-9 and Siglec-9 ligands. *SIGLEC9* gene disruption resulted in increased expression of activation markers at baseline and increased responsiveness to IgE-dependent and IgE-independent stimulation. Pre-treatment with GlycA or HMW-HA followed by IgE-dependent or independent stimulation had an inhibitory effect on mast cell degranulation. Co-engagement of Siglec-9 with FcεRI in human mast cells resulted in reduced degranulation, arachidonic acid production, and chemokine release.

Conclusions: Our study indicates that Siglec-9 and its ligands play an important role in limiting human mast cell activation *in vitro*.

Clinical implications—Targeting Siglec-9 may limit mast cell contribution to allergic disease by inhibiting mast cell ability to release pro-inflammatory mediators.

Capsule summary

Human mast cell expression of functional Siglec-9 limits mast cell activation and mediator release *in vitro*.

Keywords

Mast cells; Siglec-9; sialic acids; CRISPR/Cas9; FcεRI

INTRODUCTION

Mast cells are hematopoietic progenitor-derived, granule-containing immune cells that are widely distributed in tissues that interact with the external environment, such as the skin and mucosal tissues[1]. Mast cells have been proposed to contribute to defense against pathogens, wound healing, and tumor surveillance by responding to a broad array of activating signals, secreting a wide range of inflammatory mediators, and recruiting and activating immune cells[2–5]. Despite these beneficial roles, numerous preclinical and clinical studies recognize mast cells as key effector cells in urticaria, mastocytosis and allergic disease[6–10]. These studies have shown that the dysregulated expansion and/or activation of mast cells have detrimental consequences in these disorders. Therefore, there is a need for effective new strategies that inhibit mast cell function and/or deplete active mast cells for the treatment of mast cell driven disorders.

Siglecs are a family of single-pass cell surface receptors characterized by a N-terminal domain that binds sialylated glycans[11]. Most Siglecs have one or multiple immunoreceptor tyrosine-based inhibitory motifs (ITIM) on the C-terminus that trigger inhibitory signals through the recruitment of tyrosine and inositol phosphatases[12]. Siglecs are predominantly found on immune cells, with each cell expressing a unique combination of Siglecs that allows them to respond to distinct sialylation patterns[13, 14]. Prior studies have shown that human mast cells express CD22/Siglec-2, CD33/Siglec-3, Siglec-5, Siglec-6, Siglec-7,

Siglec-8, and Siglec-10[15]. Importantly, it has been shown that Siglecs can reduce the release of mast cell mediators[16–19], attenuate mast cell-dependent anaphylaxis[18], limit growth of human mast cell lines in a mouse model of mastocytosis[20], ameliorate mast cell activation and inflammation in mouse models of non-allergic airway inflammation[21], and reduce mast cell numbers in a mouse model of eosinophilic gastrointestinal disease[22]. Thus, the capability to limit mast cell activation and expansion has made Siglecs an attractive therapeutic target to downregulate mast cell function in allergic and non-allergic diseases.

Siglec-9 is an inhibitory receptor broadly expressed by neutrophils, monocytes, macrophages, dendritic cells, and subsets of B cells, T cells, and natural killer (NK) cells[23–26]. Studies of Siglec-9 have been mainly focused on its detrimental effects including dampening the innate immune response to certain pathogens[27–30] and impairing immune surveillance in certain cancers[31–34]. However, recent studies support the use of Siglec-9 engagement to inhibit excessive immune cell activation and limit the magnitude of the inflammatory response during arthritis[35], colitis[36], and severe COVID-19[37].

Siglec-9 expression and function in mast cells has been largely unexplored. Publicly available published datasets and online databases indicate that *SIGLEC9* mRNA expression levels are low[38] or not detected in peripheral blood mononuclear cell derived-cultured mast cells (PBCMCs)[39]. Similarly, *SIGLEC9* mRNA was undetected (Lungmap database) or very low (CZ CellxGene data portal) in human lung mast cells. Based on the CZ CellxGene data portal, human skin mast cells expressed *SIGLEC9* mRNA at low levels. However, the FANTOM Consortium database reports that human skin mast cells express higher mRNA levels of *SIGLEC9* than *SIGLEC7* or *SIGLEC8*[40]. Recent reports support Siglec-9 protein expression in human skin[18] and lung[41] mast cells. Here, we confirmed that human mast cell lines and human primary mast cells express Siglec-9. Kinetic assessment of surface expression showed that Siglec-9 expression paralleled the expression of FcεRI during mast cell differentiation. Based on this evidence, we decided to investigate the functional relevance of Siglec-9 expression on human mast cells. Siglec-9 deletion by a CRISPR-Cas9 approach significantly increased the expression of activation markers on mast cells at baseline and mast cell ability to undergo a more robust activation when compared to unedited cells. Importantly, mast cells exhibited a marked reduction in mast cell degranulation when Siglec-9 was engaged with native ligands prior to IgE-dependent and IgE-independent activation. Furthermore, co-aggregating Siglec-9 with FcεRI resulted in decreased degranulation and reduced production of arachidonic acid metabolites and chemokines.

METHODS

Ethics statement

All animal experiments were approved by the Seattle Children's Research Institutional Animal Care and Use Committee (Protocol #IACUC00020) and performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (8th Edition).

Mice

C57BL/6J mice were purchased from Jackson Laboratories and bred and maintained at the Seattle Children's Research Institute Vivarium. Mice with Siglec-E-deficiency[42] on the C57BL/6 background were kindly provided by Dr. Paul R. Crocker (University of Dundee, Scotland, UK).

Generation of bone marrow derived-cultured mast cells (BMCMCs)

Femoral bone marrow cells from C57BL/6J mice were maintained *in vitro* for ~6 weeks in Dulbecco's Modified Eagle Medium (DMEM) containing 10 ng/ml recombinant mouse (rm) interleukin (IL)-3 (Peprotech, Rocky Hill, NJ) until the mast cells represented >95% of the total cells defined as c-Kit and FcεRIα positive cells when analyzed by flow cytometry.

Generation of fetal skin derived-cultured mast cells (FSCMCs)

Fetal skin cells from C57BL/6J mice were obtained as described[43] and maintained *in vitro* for 4–6 weeks in DMEM containing 10 ng/ml rmIL-3 and 10 ng/ml rm stem cell factor (SCF) (Peprotech) until the mast cells represented >95% of the total cells defined as c-Kit and FcεRIα positive cells when assessed by flow cytometry.

Human mast cell lines

The human MCL-derived cell line HMC-1, subclone HMC-1.2 harboring *KIT V560G* and *KIT D816V* was kindly provided by Dr. J.H. Butterfield (Mayo Clinic, Rochester, MN)[44] and was grown in Iscove's Modified Dulbecco Media (IMDM) supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin. LUVA cells were generously provided by Dr. John Steinke (University of Virginia, Charlottesville, VA)[45] and maintained in StemPro-34 serum-free medium supplemented with StemPro-34 nutrient supplement (catalog number 10639011, Thermo Fisher Scientific, Waltham, MA), 2 mM L-glutamine, and 1% penicillin/streptomycin. LAD2 cells were kindly provided by Dr. Arnold Kirshenbaum (Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD)[46] and maintained in the same media as LUVA cells with the addition of 100 ng/ml recombinant human (rh) SCF.

Human primary mast cells

Human peripheral blood was obtained from anonymous donors to Bloodworks Northwest (Seattle, WA) in accordance with established institutional guidelines, and mast cells were generated as described[47]. Briefly, $CD34^+$ hematopoietic progenitors were isolated from the peripheral blood mononuclear fraction by immunomagnetic positive selection (catalog number 17856, StemCell Technologies, Vancouver, BC, Canada). $CD34^+$ cells were cultured in StemSpan SFEM II media (StemCell Technologies) supplemented with 10 μg/ml ciprofloxacin (Sigma-Aldrich, St. Louis, MO), 50 ng/ml rhIL-6 (Peprotech), and 3% vol/vol of conditioned medium from Chinese hamster ovary (CHO) cell transfectants secreting murine SCF kindly donated by Dr. Patrice Dubreuil (INSERM, CNRS, Aix-Marseille Universite, Marseille, France) for at least 8 weeks. During the first week of culture, 50 ng/ml rhIL-3 (Peprotech) was added to the culture media. A mature mast

cell population containing at least 90% c-Kit and FcεRIα positive cells as assessed by flow cytometry, was observed after 5 weeks of culture. Human skin derived-cultured mast cells (HSCMCs) were generated from mononuclear cells isolated from discarded tissues that were collected at the time of mastectomies or purchased from the National Cancer Institute-supported Cooperative Human Tissue Network kindly provided by Dr. Oskeritzian (University of South Carolina). Mast cell isolation procedure was approved by the Seattle Children's Research Institute Human Subjects Review Committee and by the University of South Carolina Institutional Review Board. Skin mast cells were harvested and cultured as previously described[48]. Briefly, the subcutaneous fat was removed from skin specimens and the resultant skin was cut into small pieces and enzymatically digested in Hanks balanced salt solution (HBSS) containing 10% FBS, 10 mM HEPES, 0.035% sodium bicarbonate, 0.5% amphotericin B and 1% penicillin/streptomycin (Thermo Fisher Scientific) and supplemented with 0.7 mg/ml hyaluronidase (Sigma-Aldrich), 592.5 U/ml collagenase type II (Thermo Fisher Scientific), 0.15 mg/ml DNase type I (Sigma-Aldrich), and 1 mM $CaCl_2$ (Sigma-Aldrich). The specimens were digested on a shaker at 37°C for 1 h. The digested mixture was filtered through a 70-μm cell strainer, and the remaining tissue was collected for two additional digestions. Collected cells were washed, spun down, filtered through a 40-μm filter, and washed again. The collected pellets from the three digestions were combined and layered over 75% Percoll in HBSS cushion and centrifuged at 800x g at room temperature for 20 min. Nucleated cells were collected from the buffer/Percoll interface, washed, and plated at a concentration of 5×10^5 cells/ml in serum-free X-Vivo 15 media (catalog number 04–418Q iLonza, Basel, Switzerland) supplemented with 100 ng/ml rhSCF (Peprotech). A mature mast cell population containing at least 90% c-Kit and FcεRIα positive cells as assessed by flow cytometry, was observed after 8 weeks of culture.

Lung mast cells were enriched from healthy tissues provided by the National Disease Research Interchange (NDRI) program as previously described[49, 50]. Briefly, lung fragments of 0.5–2.0 mm^3 were washed twice with DMEM containing 2% FCS (DMEM/FCS) before incubation in the same buffer (1 g tissue/ 4 ml buffer) containing 1.5 mg/ml collagenase type IA, 0.75 mg/ml hyaluronidase, and 0.02 mg/ml DNAse. The specimens were digested on a shaker at 37°C for 1h. The digested mixture was filtered through a 70-μm cell strainer and washed twice with DMEM/FCS. The filtered cell suspension was resuspended in 30% Percoll (one phase) and centrifuged at 780x g for 12 min. Excess Percoll was removed by washing three times in HBSS containing 2% FCS. Siglec-9 expression was assessed by flow cytometry in the enriched lung mast cell population identified as c-Kit and FcεRIα positive cells.

Human neutrophils

Nucleated cells were isolated from the blood of healthy anonymous donors (Bloodworks Northwest) using HetaSep™ (catalog number 07906, StemCell Technologies) following manufacturer instructions. Neutrophils were identified as CD45, CD11b and CD66b positive cells as assessed by flow cytometry.

Gene Editing

To delete Siglec-9 expression, LAD2 cells were transfected with Siglec-9-targeting ribonucleoproteins using Lipofectamine CRISPRMAX reagent (catalog number CMAX00001, Thermo Fisher Scientific). Transfection was performed based on the manufacturer recommendations as follows. Cells were plated at 3×10^5 cells/well of a 24-well dish and transfected with equimolar (12 pmol) Cas9 (catalog number 1081058, Integrated DNA Technologies, Coralville, IA) and sgRNA (5'-GACGAUGCAGAGUUCCGUGA-3') (catalog number A35533, Thermo Fisher Scientific) complexed with 4 μl Cas9Plus reagent and 1.5 μl CRISPRMAX reagent according to the manufacturer's mixing instructions. Editing efficiency was evaluated by protein expression measured by flow cytometry.

qPCR

RNA was isolated from primary human mast cells using the RNeasy Plus Micro kit (catalog number 74034, Qiagen, Hilden, Germany). RNA was isolated from mast cell lines using the RNeasy Mini kit (catalog number 74104, Qiagen), converted to first-strand cDNA (SuperScript™ VILO™ cDNA Synthesis Kit, Invitrogen), and cDNA was analyzed for quantitative expression levels with the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) on a Step One Plus Real-Time PCR System Thermal Cycling Block (Applied Biosystems, Waltham, MA). Results were analyzed using the dCt method normalized to *GAPDH* Ct. The primers used were as follows: *SIGLEC7* forward, 5'-GCCATAAGTTTGCAGCATCTC-3'; *SIGLEC7* reverse, 5'- GCCATTGGAAGCTCTATCTGC-3'; *SIGLEC8* forward, 5'-GTTGGGGGTGAAGTCAGAAAAG-3'; *SIGLEC8* reverse, 5'- GGGTGGGAATCTGGATGAGTT-3'; *SIGLEC9* forward, 5'- AATCTGACCTGCTCTGTGCC-3'; *SIGLEC9* reverse, 5'- AAGTTCTGAGGCGGGTAGGA-3'; *ST3GAL1* forward, 5'- CCGCTGTGGTCATTTAGGAA-3'; *ST3GAL1* reverse, 5'- TCCATCTCTGGTCCCCAAAT-3'; *ST3GAL3* forward, 5'- CCGCTGTGGTCATTTAGGAA-3'; *ST3GAL3* reverse, 5'- GGGGTGAGCTAGAGTGACTA-3'; *ST3GAL4* forward, 5'- CTCCCGGGAAGACAGTTTTT-3'; *ST3GAL4* reverse, 5'- GTAAGCAGATGGCGTCTTGA-3'; *ST3GAL5* forward, 5'- CTCTGTGGCTGCTCTTGTCA-3'; *ST3GAL5* reverse TGGTGAGGAGGAGGGAGATG-3'; *ST3GAL6* forward, 5'- TTGCGAACAGAGGGTCTTTAG-3'; *ST3GAL6* reverse, 5'- AAGACAGCACTCAGGAATATGG-3'; *GNE* forward, 5'- TGACGGCGTCTGGAACTCTA-3'; *GNE* reverse, 5'-GTAGCAACACAAACCCGCAG-3'; *GAPDH* forward, 5'-GAGTCAACGGATTTGGTCGT-3'; *GAPDH* reverse, 5'-TTGATTTTGGAGGGATCTCG-3'.

Flow cytometry

Single human cell suspensions were stained with a combination of the following antibodies: Alexa Fluor-700-conjugated anti-human Siglec-6 (clone 767329, catalog number FAB2859N-025, R&D Systems, Minneapolis, MN); PE-conjugated anti-human Siglec-7 (clone 194211, catalog number FAB11381P, R&D Systems); BV421-conjugated anti-human Siglec-8 (clone 837535, catalog number 747875, BD Biosciences, Franklin Lakes, NJ); BV421-conjugated anti-human Siglec-9 (clone E10–286, catalog number 743363, BD Biosciences); APC-conjugated anti-human Siglec-9 (clone 191240, catalog number FAB1139A, R&D Systems); BV605-conjugated anti-human c-Kit (clone 104D2, catalog number 562687, BD Biosciences); FITC-conjugated anti-human FcεRIα (clone CRA-1, catalog number 11-5899-42, Invitrogen, Waltham, MA); APC/Cy7-conjugated anti-human lysosomal-associated membrane protein 1 (LAMP-1) (clone H4A3; catalog number 328629, Biolegend); FITC-conjugated anti-human CD45 (clone 2D1, catalog number 11-9459-41, Invitrogen); APC/Cy7-conjugated anti-human CD11b (clone ICRF44, catalog number 301341, Biolegend); BV421-conjugated anti-human CD66b (clone 6/40c, catalog number 392915, Biolegend).

Single mouse cell suspensions were stained with a combination of the following antibodies: anti-APC/Cy7-conjugated anti-mouse c-Kit (clone 2B8, catalog number 105825, Biolegend); PE-conjugated anti-mouse FcεRIα (clone MAR-1, catalog number 134307, Biolegend); BV421-conjugated anti-mouse Siglec-E (clone 750620, catalog number 748154, BD Biosciences). 4,6-Diamidino-2-phenylindole (DAPI) (catalog number D9542, Sigma Aldrich) was used to exclude dead cells.

To determine whether anti-Siglec-9 antibodies can block binding of native Siglec-9 ligands to Siglec-9, LAD2 cells were incubated with 5 μg/ml mouse anti-human Siglec-9 (clone 191240, catalog number MAB1139100, R&D Systems) or mouse IgG2a isotype control antibody (catalog number MAB003, R&D Systems) for 10 min on ice followed by two washes to remove excess antibody. Then, cells were incubated with 50 μg/ml of GlycA (catalog number G5017, Sigma Aldrich) for 30 min on ice. Unbound GlycA was washed off and cells were fixed with 4% paraformaldehyde for 10 min on ice. GlycA bound to mast cells was stained with APC-conjugated anti-human GlycA (clone HIR2, catalog number 306607, Biolegend) and analyzed by flow cytometry.

For Siglec-9 and LAMP-1 staining in PBCMCs and HSCMCs, cells were untreated or stimulated with 100 ng/ml anti-human FcεRIα (clone CRA-1, catalog number 14-5899-82, Invitrogen) for 30 min at 37°C.

In a separate group of experiments, LAD2 cells and HSCMCs were incubated with 10 mU/ml sialidases (catalog number 11080725001, Roche, Indianapolis, IN) for 1 h at 37°C as previously described[51] followed by LAMP-1 staining.

Receptor endocytosis was determined by delayed secondary staining as previously described[52]. Briefly, Siglec-9 was bound with 5 μg/ml unconjugated mouse anti-human Siglec-9 (clone 191240, R&D Systems) or mouse IgG2a isotype control antibody (catalog number MAB003, R&D Systems) for 20 min on ice, two washes to remove unbound

antibody, followed by incubation at 37°C for 1, 24, or 48 h. At indicated time points, 1:1000 dilution of an Alexa Fluor-594-conjugated donkey anti-mouse IgG antibody (catalog number A21203, Invitrogen) was added to assess the remaining Siglec-9 on the cell surface by flow cytometry. To assess total Siglec-9 levels, cells were stained with either APC-conjugated anti-human Siglec-9 (clone 191240, catalog number FAB1139A, R&D Systems) or APC-conjugated mouse IgG2a antibody (catalog number IC003A, R&D Systems) for 20 min on ice, unbound antibody was washed off, followed by incubation at 37°C for 1, 24, or 48 h.

The percentage of signal lost was calculated as follows: 100 × (1 – mean intensity fluorescence (MFI) at a specific time/MFI at time 0)

To determine whether native Siglec-9 ligands can cause Siglec-9 to internalize, LAD2 cells were incubated with either 50 μg/mL of GlycA (catalog number G5017, Sigma Aldrich) or 50 μg/ml of HMW-HA (catalog number 53747, Sigma Aldrich) for 30 min on ice. Unbound ligands were washed off and cells were incubated at 37°C for 1 h or 24 h. Cells were fixed with 4% paraformaldehyde for 10 min on ice, stained for Siglec-9 expression using PE-conjugated anti-human Siglec-9 antibody (clone E10–286, catalog number 564251, BD Biosciences), and analyzed by flow cytometry. Cells fixed before the addition of ligands were used as control cells (T= 0).

To determine expression of Siglec-9 ligands, untreated or sialidase-treated mast cells were incubated with 10 μg/ml rhSiglec-9 Fc chimera protein (catalog number 1139-SL-050, R&D Systems) for 30 min on ice followed by incubation with a 1:200 dilution of an Alexa Fluor 488-conjugated anti-human IgG secondary antibody (catalog number 709-546-149, Jackson ImmunoResearch, West Grove, PA) for 30 min on ice. Baseline staining was obtained using PBS followed by the fluorescently labeled secondary antibody.

Mast cell apoptosis was determined in cells cultured with 5 μg/ml mouse anti-human Siglec-9 (clone 191240) or mouse IgG2a isotype control for 20 min on ice followed by washing and incubation at 37°C in warmed media for 1, 24, or 48 h. At indicated time points, cells were collected, stained with propidium iodide (catalog number 421301, Biolegend) and FITC-conjugated Annexin V (catalog number 640905, Biolegend) in Annexin V binding buffer (catalog number 422201, Biolegend), and analyzed by flow cytometry.

Data were acquired on a BD LSR II with the FACSDIVA software and analyzed using FlowJo software (for Windows, version 10, Becton, Dickinson, and Company, Ashland, OR).

Microscopy

For Siglec-9 imaging, cells were plated on chamber slides (catalog number 154526, Thermo Fisher Scientific) coated with 40 μg/ml human plasma fibronectin (catalog number F2006, Sigma Aldrich) for 1 h at 37°C. Cells were fixed with 3% paraformaldehyde for 15 min at room temperature, washed 3 times with PBS, permeabilized with 0.5% Triton X-100 for 10 min, and blocked with 5% normal goat serum for 1 h at room temperature. Cells were then incubated overnight with either mouse anti-human Siglec-9 (clone 191240) or mouse

IgG2a isotype control antibody (catalog number MAB003, R&D Systems) at 4°C, washed 3 times with PBS, incubated with 1:200 Alexa Fluor 594-conjugated donkey anti-mouse IgG (catalog number A21203, Invitrogen) for 30 min at room temperature, and washed 3 times with PBS. Samples were mounted using Prolong Diamond DAPI containing mounting media (catalog number P36966, Invitrogen).

For Siglec-9 imaging upon mast cell activation, LAD2 cells were sensitized with 2 μg/ml of human IgE (catalog number ab65866, Abcam) overnight at 37°C. Cells were then washed with PBS and plated on chamber slides (catalog number 154526, Thermo Fisher Scientific) coated with 40 μg/ml human plasma fibronectin (catalog number F2006, Sigma Aldrich) with or without 500 ng/ml anti-human IgE (clone B3102E8, catalog number ab99804, Abcam) at 37°C for 1 h before proceeding with staining as above.

For analysis of endocytosis upon Siglec-9/FcεRI co-crosslinking, cells were fixed, permeabilized, and stained as above, but in suspension rather than adhered to slides and finally resuspended in SlowFade DAPI-containing mounting media (catalog number S36968, Invitrogen) before imaging. Endocytosis analysis was performed with the following additional antibodies: rabbit anti-Rab5a (catalog number 2143S, Cell Signaling Technology, Danvers, MA), rabbit anti-Rab7 (clone D95F2, catalog number 9367S, Cell Signaling Technology), Alexa Fluor 594-conjugated donkey anti-rabbit IgG (catalog number ab150076, Abcam, Cambridge, UK), and Alexa Fluor 488-conjugated donkey anti-mouse IgG (A21202, Invitrogen).

Siglec-9 expression images were acquired on a Leica TCS SP5 confocal inverted-base microscope (Leica Microsystems, IL) with a 63x oil objective and analyzed using ImageJ (NIH).

For Siglec-9 ligand expression images, cells tested for Siglec-9 ligand expression by flow cytometry were attached to a slide by cytospin (500x g, 5 min), fixed using 4% paraformaldehyde for 10 min, washed with PBS, and imaged under oil immersion at 63x using a Leica DM6000 (Leica, Wetzlar, Germany) microscope.

Siglec-9 engagement with native ligands

Mock- and *SIGLEC9*-edited LAD2 cells sensitized overnight with 2 μg/ml of human IgE (catalog number ab65866, Abcam) and HSCMCs were cultured with either 20–100 μg/ml GlycA (catalog number G5017, Sigma Aldrich) or 20–100 μg/mL HMW-HA (catalog number 53747, Sigma Aldrich) for 20 min. The cells were then activated with the indicated concentrations of stimulants for 30 min at 37°C. LAD2 cell and HSCMC activation was assessed by β-hexosaminidase release and increase in LAMP-1 expression, respectively. Percentage of inhibition of mast cell degranulation was calculated as follows: (% of β-hexosaminidase release or LAMP-1 expression in stimulated cells- β-hexosaminidase release or LAMP-1 expression in untreated cells) - (% of β-hexosaminidase release or LAMP-1 expression in stimulated cells treated with Siglec-9 ligand - β-hexosaminidase release or LAMP-1 expression in untreated cells) / (% of β-hexosaminidase release or LAMP-1 expression in stimulated cells - β-hexosaminidase release or LAMP-1 expression in untreated cells).

Siglec-9 engagement with anti-Siglec-9 antibody

Mast cells were pre-incubated with 2.5–5 μg/ml mouse anti-Siglec-9 (clone 191240) or mouse IgG2a isotype control antibody for 30 min at room temperature prior to activation with the indicated concentrations of stimulants for 30 min at 37°C.

In a separate set of experiments, PBCMCs incubated with anti-Siglec-9 or isotype control were exposed to 5 μg/ml goat anti-mouse IgG (Fc specific) F(ab')2 fragment antibody (catalog number M0284, Sigma-Aldrich) for 2 min to cross-link Siglec-9 prior to activation.

LAD2 cell activation was assessed by β-hexosaminidase release, and PBCMC and HSCMC activation was assessed by LAMP-1 staining.

Co-crosslinking of FcεRI and Siglec-9

PBCMCs and HSCMCs were incubated with 100 ng/ml anti-human FcεRIα plus 5 μg/ml mouse isotype control antibody (catalog number MAB003, R&D Systems) or anti-Siglec-9 mAb (clone 191240) for 2 min at 4°C. Cells were washed and then incubated with 5 μg/ml goat anti-mouse IgG (Fc specific) F(ab')2 fragment antibody for 2 min. After an additional PBS wash, cells were resuspended in warm complete medium and incubated for 30 min at 37°C to assess mast cell activation by LAMP-1 staining. Cell supernatants were collected at 20 min and after overnight stimulation to measure the levels of arachidonic acid metabolites and chemokine/cytokines, respectively.

β-hexosaminidase release assay

LAD2 cells were sensitized with 2 μg/ml of human IgE by overnight incubation at 37°C. The cells were then washed with Tyrode's buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% glucose) and 1×10^5 cells/well were added to a 96-well V-bottom plate. Next, equal volume of a 2x concentration of stimulus (500 ng/ml anti-human IgE (clone B3102E8, catalog number ab99804, Abcam), 10 μM compound 48/80 (c48/80) (catalog number C2313, Sigma Aldrich) or 1 μM A23187 (catalog number C7522, Sigma Aldrich) was added to the appropriate wells, and incubated at 37°C for 1 h. After centrifugation, supernatants were collected, and pellets were lysed with 0.5% Triton X 100. β-hexosaminidase release was quantified by enzyme immunoassay with *p*-nitrophenyl-*N*-acetyl-β-d-glucosamine (catalog number N9376, Sigma-Aldrich) substrate, as follows: 10 μl of culture supernatant or lysate was added to the wells of a 96-well flat-bottom plate; 50 μl of 1.3 mg/ml *p*-nitrophenyl-*N*-acetyl-β-d-glucosamine solution in 100 mM sodium citrate, pH 4.5, was added, and the plate was incubated at 37°C for 1 h. Next, 150 μl of 200 mM glycine, pH 10.7, was added to stop the reaction, and the optical density (OD_{405}) was determined.

Prostaglandin (PG)D_2 and cysteinyl leukotriene (cys-LT) release assays

PGD_2 and cys-LT levels were measured in supernatants by ELISA per manufacturer's instructions (catalog numbers 512031 and 500390 for PGD_2 and cys-LT, respectively, Cayman Chemical, Ann Arbor, MI). The assay detection limits were 19.5 pg/ml and 8.6 pg/ml for PGD_2 and cys-LT, respectively.

Chemokine release assay

IL-8 and monocyte chemoattractant protein (MCP)-1 levels were measured in cell supernatants using the human ProcartaPlex Multiplex ImmunoAssay (catalog number PPX-07-MXH6CA4, Thermo Fisher Scientific). The assay detection limits were 2.49 pg/ml for IL-8 and 4.16 pg/ml for MCP-1.

Statistical Analyses

Data are presented as means ± SEMs unless otherwise indicated. Statistical significance was determined by Mann Whitney *U* test, Wilcoxon matched pair signed rank test, or ANOVA and Tukey multiple comparisons test as appropriate, using GraphPad Prism version 9 (San Diego, CA, USA). When appropriate, single outliers were identified and removed from the data using the ROUT outlier test. Statistical differences were considered significant at $P < 0.05$.

RESULTS

Human mast cells express Siglec-9

First, we compared the surface level expression of Siglec-9 across a variety of human mast cell types. We found that human mast cell lines (Fig. E1A), PBCMCs, HSCMCs, and lung mast cells express Siglec-9 (Fig. 1A). Human neutrophils isolated from peripheral blood were used as a positive control for Siglec-9 protein expression (Fig. 1A). When compared to other Siglecs, human primary mast cells expressed Siglec-9 levels comparable to Siglec-7 and Siglec-8 (Fig. 1B). Moreover, Siglec-9 was the only Siglec expressed by all the human mast cell lines tested (Fig. E1B–C). At the mRNA level, human mast cell lines, PBCMCs, and HSCMCs expressed *SIGLEC9* mRNA levels comparable to Siglec-7 and Siglec-8 (Fig. E2A–C).

As previously reported for Siglec-6 and Siglec-8[15], our kinetics studies showed that Siglec-9 appeared on the cell surface in parallel with FcεRIα expression (Fig. 1C), indicating that Siglec-9 expression increases with mast cell maturation.

Confocal microscopy for Siglec-9 localization in HSCMCs (Fig. 1D) and LAD2 cells (Fig. E3) showed a punctate distribution of Siglec-9, which has previously been described in other cell types[25, 33, 53]. Given the punctate intracellular staining of Siglec-9 in HSCMCs, we hypothesized that there is a potential intracellular pool of Siglec-9. To test this, we stimulated HSCMCs in an IgE-dependent and IgE-independent manner and assessed surface expression of Siglec-9. HSCMCs (Fig. 1E) showed a significant increase in Siglec-9 expression after undergoing degranulation. Interestingly, confocal microscopy showed that there was not a significant reduction in punctuate staining for Siglec-9 in LAD2 cells upon IgE-dependent activation (Fig. E3B). These data suggest that a possible translocation of Siglec-9 to the cell surface may not be the only mechanism by which Siglec-9 surface expression increases upon mast cell activation. Together, these data demonstrate that human mast cells express Siglec-9 on their cell surface and intracellularly.

Siglec-9 is an endocytic receptor

Siglec-6 and Siglec-8 are internalized after antibody engagement of the receptor on eosinophils and mast cells[14, 16]. The endocytic capacity of Siglec-9 has only been described in cancer cells[54]; where up to 90% of Siglec-9 was internalized after treatment with anti-Siglec-9 antibodies. Therefore, we first tested whether Siglec-9 ligation causes a decrease in Siglec-9 surface expression using the previously described delayed secondary staining method[14]. After ligation of Siglec-9 with an anti-Siglec-9 monoclonal antibody (clone 191240), there was a gradual decrease in surface Siglec-9 staining on HSCMCs (Figs. 2A and 2B). As shown in Fig. 2C, approximately 30% of the Siglec-9 signal was lost at 24 h after antibody engagement when compared with Siglec-9 signal detected at time 0. Siglec-9 surface expression decreased more rapidly in LAD2 cells (Fig. E4A–B), which exhibited approximately a 30% loss in Siglec-9 surface expression at 1 h after Siglec-9 ligation (Fig. E4C). The MFI slightly increased and the percentage in surface Siglec-9 loss decreased at 24 h after Siglec-9 ligation, suggesting that a small portion of the original Siglec-9 started returning to the LAD2 cell surface (Figs. E4B and E4C). Together, this evidence shows that Siglec-9 surface expression decreases after receptor engagement.

To further characterize whether the Siglec-9 loss was due to endocytosis, we assessed the expression of surface and internalized Siglec-9 (Total Siglec-9) by using a fluorophore-conjugated anti-Siglec-9 antibody. HSCMCs exhibited a significant loss in total Siglec-9 signal at 1 h after antibody engagement (Fig. 2D). Loss in total Siglec-9 signal was more pronounced in LAD2 cells which exhibited an almost complete loss in total Siglec-9 signal at 24 h after Siglec-9 ligation (Figs. E4D–F). In contrast, LAD2 cells incubated with isotype control did not exhibit any loss in total Siglec-9 expression during the times tested (Fig. E4G). Based on these data, we hypothesized that Siglec-9 was degraded after endocytosis. To address this hypothesis, we examined whether Siglec-9 co-localizes with markers of the endosomal pathway in LAD2 cells after Siglec-9 engagement. Confocal images showed that Siglec-9 co-localized with Rab5, a marker of early endosomes, and with Rab7, a marker of late endosomes, at 1 h and 24 h after Siglec-9 engagement, respectively (Fig. E5). In contrast, Siglec-9 did not co-localize with Rab5 or Rab7 in LAD2 cells treated with isotype control at any timepoint tested (Fig. E5).

Together, these data suggest that Siglec-9 is internalized and degraded upon engagement with anti-Siglec-9 antibodies.

Siglec-9 interactions with sialic acids in *cis* dampen mast cell susceptibility to activation

Next, we decided to generate a human mast cell line deficient in Siglec-9 to assess the role of Siglec-9 in mast cell function. For this purpose, we deleted Siglec-9 in LAD2 cells using a CRISPR/Cas9 approach. By using a CRISPR/Cas9 approach we were able to generate a large population of LAD2 cells that does not express Siglec-9 (Fig. 3A). In contrast, Siglec-6, Siglec-7, and Siglec-8 expression levels were unaffected by Siglec-9 deletion in LAD2 cells (Fig. 3B). First, we observed that naïve Siglec-9-deleted LAD2 cells exhibited increased cell surface expression levels of LAMP-1 which indicates granule mobilization towards the plasma membrane (Fig. 3C). Moreover, Siglec-9-deleted LAD2 cells were more susceptible to degranulation induced by IgE-dependent (Fig. 3D) and IgE-independent

stimuli (Figs. 3E and 3F). These observations support the presence of sialic acid ligands with the ability to trigger an inhibitory signal in mast cells via Siglec-9 engagement. To address this hypothesis, we directly probed mast cells for the presence of Siglec-9 ligands using a recombinant chimera of Siglec-9 (Fc-Siglec-9) as previously described[55]. HSCMCs showed expression of Siglec-9 ligands by flow cytometry (Figs. 3G and 3H). Imaging of the Siglec-9 ligand expression in HSCMCs showed a punctate pattern of expression, suggesting there is an enrichment of these ligands in certain areas of the cell membrane (Fig. 3I). As shown in Figs. E6A and E6B, LAD2 cells also express significant levels of Siglec-9 ligands when compared with cells treated with secondary antibody alone. The staining for Siglec-9 ligands in LAD2 cells was significantly reduced when cells were treated with sialidases to remove potential Siglec ligands, demonstrating that Fc-Siglec-9 chimeras specifically bind to sialic acid residues on the cell membrane (Figs. E6A and E6B). Notably, Siglec-9-deleted LAD2 cells showed significantly higher staining for Siglec-9 ligands (Figs. E6A and E6B) suggesting that binding of Siglec-9 to sialic acids may interfere with Siglec-9 ligand detection by Fc-Siglec-9 chimeras in unedited LAD2 cells.

These findings prompted us to explore whether mast cells express enzymes involved in sialic acid biosynthesis. Sialic acid biosynthesis starts with the formation of cytidine monophosphate N-acetylneuraminic acid (CMP-Neu5Ac)[56] as an activated sugar donor for the transfer of sialic acids by sialyltransferases (SiaT) to the terminal glycosyl group of glycoproteins and glycolipids as acceptor molecules. As shown in Fig. E7A, we found that human mast cells express mRNA for UDP-GlcNAc 2-epimerase/ManNAc-6 (GNE), the first enzyme in the pathway to generating CMP-Neu5Ac[57]. Moreover, human mast cells express mRNA for SiaTs that can catalyze the formation of glycosidic linkages found in Siglec-9 ligands including ST3GAL1, ST3GAL3, ST3GAL4, ST3GAL5 and ST3GAL6 (Figs. E7B–F). Siglec-9-deleted LAD2 cells did not exhibit alterations in the expression of mRNA for GNE and SiaTs indicating that Siglec-9 expression does not regulate the expression of enzymes involved in sialic acid biosynthesis (Fig. E7G). Overall, this evidence supports the expression and biosynthesis of Siglec-9 ligands in human mast cells.

To address whether the presence of these sialic acid ligands can trigger an inhibitory signal in mast cells via Siglec-9 engagement, we examined LAMP-1 expression in mast cells devoid of potential Siglec ligands. As shown in Fig. 3J, unedited but not Siglec-9-deleted LAD2 cells exhibited increased LAMP-1 expression after sialidase treatment. Increased LAMP-1 expression was also observed when HSCMCs were treated with sialidases, indicating that sialic acid ligands also limit the extent of human primary mast cell activation (Fig. 3K). Together, these data show that mast cells express Siglec-9 ligands *in vitro* that can engage Siglec-9 to promote mast cell quiescence.

Siglec-9 ligands reduce mast cell activation

GlycA and HMW-HA have been recognized as ligands for Siglec-9 that can downregulate the activation of immune cells[27, 58]. Therefore, we assessed the effects of GlycA and HMW-HA on mast cell activation. As shown in Fig. 4A and Figs. E8A–D, optimal concentrations of both ligands inhibited β-hexosaminidase release from LAD2 cells stimulated by IgE-dependent and IgE-independent stimuli. Both GlycA and HMW-HA were also able to

inhibit PBCMC degranulation as assessed by a decrease LAMP-1 expression levels upon stimulation by IgE-dependent (Fig. 4B and Figs. E8E and E8F). In contrast, Siglec-9 ligands did not significantly inhibit PBCMC degranulation induced by IgE-independent stimuli (Fig. 4B and Figs. E8G–H).

It has been shown that anti- human Siglec-9 antibodies can block the interactions between HMW-HA and Siglec-9[27]. Similarly, Fig. E9A shows a significant reduction in GlycA binding to LAD2 cells pre-treated with anti-human Siglec-9 antibodies. Importantly, Siglec-9 deleted LAD2 cells did not exhibit a reduction in their ability to release β-hexosaminidase when incubated with Siglec-9 ligands (Fig. E9B). This evidence suggests that GlycA and HMW-HA inhibit mast cell activation by specifically engaging Siglec-9.

To assess whether Glyc A and HMW-HA cause Siglec-9 internalization, we assessed Siglec-9 expression in LAD2 cells at 1 h and 24 h after treatment with native ligands. As shown in Fig. E10, LAD2 cells treated with either GlycA or HMW-HA exhibited similar Siglec-9 expression to cells cultured in medium alone, suggesting that GlycA and HMW-HA were unable to induce Siglec-9 internalization.

These results point to naturally occurring Siglec-9 ligands like GlycA and HMW-HA being able to further dampen mast cell activation through their engagement of Siglec-9.

Antibody engagement of Siglec-9 inhibits mast cell activation

Antibodies against Siglecs have been used successfully to engage these inhibitory receptors and modulate immune and inflammatory responses[20–22, 59, 60]. To determine whether antibody engagement of Siglec-9 reduces mast cell activation, we incubated LAD2 cells with an anti-Siglec-9 monoclonal antibody (clone 191240) for 30 min prior to mast cell stimulation. Siglec-9 antibody engagement slightly inhibited mast cell degranulation induced by IgE-dependent (Fig. E11A) and IgE-independent stimuli (Fig. E11B) in LAD2 cells. In contrast, PBCMCs and HSCMC did not exhibit a reduction in their ability to degranulate when treated with anti-Siglec-9 antibodies (Figs. E11C and E11D). PBCMC degranulation was also not inhibited when anti-Siglec-9 antibodies were crosslinked with a secondary antibody prior to activation to enhance the inhibitory signal (Fig. E11E). As Siglec-9 engagement can induce apoptosis in neutrophils[24], we also examined PBCMC survival after Siglec-9 engagement with anti-Siglec-9 antibodies. As shown in Fig. E12, there was not significant decrease in PBCMC viability at any timepoint tested in cells treated with anti-Siglec-9 antibodies or isotype control conditions.

Due to the lack of anti-Siglec-9 inhibitory effect on human primary mast cell activation, we assessed whether co-engaging Siglec-9 with FcεRI could enhance Siglec-9 inhibitory function as shown for Siglec -3, -6, -7 and -8 on mast cells[16–18, 61]. For this purpose, we co-aggregated Siglec-9 and FcεRI on PBCMCs and HSCMC using a secondary cross-linking antibody that recognizes both anti-Siglec-9 and anti-FcεRIα antibodies. We observed a significant reduction in PBCMC (Fig. 5A) and HSCMC (Fig. 5B) activation after Siglec-9 co-engagement that was even more pronounced in HSCMCs than in PBCMCs (Fig. 5C). Cross-linking of Siglec-9 with FcεRIα also resulted in reduced production of the arachidonic acid metabolites cys-leukotrienes (cys-LT) (Fig. 5D and Figs. E13A and

E13D) and prostaglandin (PG)D_2 (Fig. 5E and Figs. E13B and E13E) in both PBCMC and HSCMC. Finally, we tested the effect of Siglec-9-FcεRI co-engagement on mast cells' ability to generate chemokines upon IgE-dependent activation. As shown in Fig. 5F and Figs. E13C and E13F, both PBCMC and HSCMC released significant reduced amounts of IL-8 when Siglec-9 was cross-engaged with FcεRIα. Moreover, HSCMCs but not PBCMCs were inhibited in their ability to release MCP-1 upon Siglec-9 and FcεRI co-engagement (46.5% ± 12.5% inhibition, $P < 0.005$ vs. cells treated with isotype control, $n = 6$ experiments with cells obtained from individual donors). Overall, these data suggest that Siglec-9 proximity to FcεRI increases the effectiveness of the Siglec-9 mediated inhibitory signal.

DISCUSSION

In this study, we report for the first time that human mast cells express functional Siglec-9 *in vitro*. Specifically, we show that Siglec-9 can inhibit human mast cell degranulation upon engagement with Siglec-9 ligands, GlycA and HA-HMW. Moreover, we observed that co-engagement of Siglec-9 with FcεRI can reduce human mast cell degranulation and limit their ability to produce arachidonic acid metabolites, MCP-1, and IL-8.

Prior studies reported that freshly isolated human skin[18] and lung[41] mast cells express surface Siglec-9. In the current study, we confirmed that Siglec-9 is expressed in freshly isolated human lung mast cells, HSCMCs, and PBCMCs (Fig. 1A). The levels of Siglec-9 mRNA and protein expression were comparable to those found for Siglec-7 and Siglec-8 in the human mast cells tested (Fig. E2). Previous studies showed that cord blood cultured mast cells and PBCMC do not express mRNA for Siglec-9[15]. We cannot fully explain the discrepancy between these data and the findings from our groups and others. However, we observed that the kinetics of expression of Siglec-9 parallels the expression of FcεRI in $CD34^+$ cells as they differentiate into mast cells *in vitro* (Fig. 1C). This observation suggests that variations in the culture conditions for $CD34^+$-derived cultured mast cells may affect the levels of Siglec-9 expression levels detected by different labs. The correlation between Siglec-9 expression and cell maturation is not unique to mast cells as it has been noted previously in other immune cells like NK cells[31]. Importantly, Siglec-6 and Siglec-8 expression also matches an increase in CD51 and FcεRIα expression and histamine content in PBCMCs[15] suggesting that mast cells upregulate the expression of a set of inhibitory receptors capable to engage an array of potential Siglec ligands during maturation to maintain mast cell quiescence. We addressed this hypothesis in part by showing that Siglec-9 deletion by a CRISPR-editing approach led to an increase in LAMP-1 expression in LAD2 cells at steady state (Fig. 3C). Moreover, Siglec-9-deleted LAD2 cells were more susceptible to IgE-dependent and IgE-independent stimuli (Figs. 3D–F). To our knowledge, there are no reports on how Siglec-9 deficiency can alter the function of human immune cells. However, studies using mice deficient in Siglec-E, the murine homologue of Siglec-9, suggest that Siglec-E-deficiency can impact the activation status of immune cells as observed for Siglec-9-deficient mast cells. For example, it has been shown that microglia from Siglec-E-deficient mice displays aggravated pro-inflammatory characteristics when exposed to neural debris damage-associated molecular patterns (DAMPs)[62] or lipopolysaccharide (LPS)[63]. Similarly, Siglec-E-deficient neutrophils exhibited increased

ability to migrate to the lung in an acute lung airway inflammation model induced by aerosolized LPS[42]. We could not examine whether Siglec-E-deficiency can impact mast cell function as we found that bone marrow-derived cultured mast cells (BMCMCs), peritoneal mast cells, and fetal skin-derived cultured mast cells showed very low surface expression of Siglec-E (Fig. E14A). These observations are in agreement with the RNAseq database from The Immunological Genome Project (ImmGen, http://rstats.immgen.org/Skyline/skyline.html) showing that mast cells from the peritoneum, esophagus, trachea, tongue, or skin express very low gene expression levels of Siglec-E (Fig. E14B) when compared with blood neutrophils.

Siglec ligands can act in *trans*, on adjacent cells, and in *cis*, where they cluster Siglecs on the same cell's membrane and maintain a basal level of inhibitory signaling that increases the threshold for immune cell activation. Depletion of Siglec ligands in *cis* by sialidases or oxidative cleavage has been linked to increased activity of B cells[64], macrophages[65], microglia[66], and monocyte-derived dendritic cells[67], and prolonged inhibition of sialic acid biosynthesis renders phagocytes more prone to activation[68]. The increased expression of activation markers in Siglec-9-deleted LAD2 cells points to a possible role for Siglec-9 interactions with sialic acid in *cis* in mast cell homeostasis. Previously, the ligands for Siglec-9 had only been found on endothelial cells[69], red blood cells[58], the upper airway[70], and the human aorta[71]. In this report, we demonstrate for the first time that human mast cells express ligands for Siglec-9 (Figs. 3G & E6). We also report that human mast cells highly express mRNA for enzymes involved in sialic acid biosynthesis and sialylation (Fig. E7). Importantly, sialic acid removal in LAD2 cells, and to a lesser degree in HSCMCs, led to an increase in the expression of mast cell activation markers as we observed in Siglec-9-deleted mast cells (Fig. 3J–K) supporting our hypothesis that Siglec-9 interactions with sialic acids in *cis* contribute to mast cell quiescence.

Despite the progress made to identify Siglec ligands, their carrier proteins and their tissue expression patterns, the identities and physiological roles of endogenous Siglec ligands relevant to mast cell biology are unknown. Using synthetic glycan arrays, it has been shown that Siglec-9 bind preferentially to sulfated sialylated trisaccharide structure (NeuAc α2,3 Gal β1,4 6-SO_3-GlcNAc). The search for endogenous ligands with the sulfated sialylated trisaccharide structures required for binding to Siglec-9 has been most successful for this receptor. In human upper airway tissues, Siglec-9 can bind to the glycans of the mucin MUC5B[70]. Siglec-9 can also bind the erythrocyte sialoglycoprotein, GlycA in a sialic acid dependent manner[58]. Importantly, Siglec-9-GlycA interactions have been shown to have immunosuppressive effects on neutrophils including decreased degranulation, reactive oxygen species (ROS) and neutrophil extracellular trap (NET) production, chemotaxis, and bacterial killing[58]. Siglec-9 mediated inhibition of neutrophils was also observed after treatment with the glycosaminoglycan, HMW-HA[27]. In contrast to GlycA, HMW-HA binding to Siglec-9 has been shown to be sialic acid-independent[27]. Based on this evidence, we investigated GlycA and HMW-HA effects on mast cell function. We observed that mast cell treatment with either GlycA or HMW-HA, was able to decrease mast cell activation by IgE-dependent or IgE-independent stimuli (Fig. 4A). Although mast cells express CD44, the primary receptor of HMW-HA[72], the inhibitory effect of HMW-HA was specific to Siglec-9 in our system since LAD2 cells deficient in Siglec-9 showed no decrease in their ability

to degranulate when activated in the presence of HMW-HA. Overall, these *in vitro* data demonstrate that Siglec-9 and its ligands, whether acting in *trans* or *cis*, play an important role in maintaining mast cell quiescence and limiting their activation.

Siglecs are endocytic receptors that either constitutively cycle between the cell surface and intracellular endosomes, or can undergo endocytosis upon ligation by antibody or multivalent ligands[11]. Compared to the endocytic capacity of Siglec-6 and Siglec-8 on human skin mast cells[16], the internalization kinetics of Siglec-9 closely resembled Siglec-6, with most of the receptor remaining on the cell surface at 24 h after Siglec-9 ligation. Contrastingly, 50% of Siglec-8 is endocytosed at 2 h post engagement and almost none of the receptor could be detected on mast cell surface at 24 h post engagement[16]. In contrast, there was no detectable Siglec-9 internalization after engagement of Siglec-9 with GlycA or HMW-HA (Fig. E10). Additional studies are required to understand the mechanisms involved in the regulation of endocytosis and expression of different Siglecs on mast cell surface upon engagement. These studies have the potential to further advance the use of Siglecs as antigen delivery therapeutics to target mast cells based on the internalization kinetics of different Siglecs[73].

The use of antibodies against Siglecs[20–22, 59, 60] and glycomimetic Siglec ligands on multivalent scaffolds, like nanoparticles, liposomes, or polymers that aggregate Siglec receptors[16, 37, 73] has demonstrated that Siglec engagement can be leveraged to modulate immune and inflammatory responses. Here, we report that Siglec-9 co-engagement with FcεRI can reduce human mast cell degranulation, arachidonic acid production, and chemokine release (Fig. 5). Similarly, several studies have demonstrated that clustering of CD33[18], Siglec-6[16], Siglec-7[17], or Siglec-8[19] with FcεRI is necessary for optimal inhibition of mast cells responses *in vitro*. Prior studies have shown that several CD33-related Siglecs, namely CD33, Siglec-6, Siglec-7, and Siglec-8, can prevent the release of mast cell mediators, mast cell-dependent anaphylaxis or inflammation in mouse models of disease[74]. Based on these findings, studies are on their way in our lab to address the impact of Siglec-9 inhibitory effects on mast cell function *in vivo*.

The possible addition of Siglec-9 to the repertoire of inhibitory receptors that can modulate mast cell function is significant for two reasons. First, mast cells may exhibit differential responsiveness to Siglec modulation depending on the tissue where they reside or the inflammatory microenvironment that may change over time. Accordingly, a broad repertoire of functional Siglecs may be needed for mast cell targeting in different conditions. The rationale for this is that inflammatory environmental changes are known to affect the expression of Siglecs and Siglec ligands. For example, it has been shown that patients with COPD[75], chronic rhinosinusitis[70], and rheumatoid arthritis[76] exhibited an up-regulation in the expression of Siglec-9 and Siglec-9 ligands. In relation to mast cells, our study shows that Siglec-9 inhibitory effects were more pronounced in HSCMC than in PBCMCs which may correlate to the expression levels of Siglec-9 and its ligands in these cells (Fig. 5). Second, certain Siglecs may play an important role in cell homeostasis that may preclude the use of a Siglec-targeted therapy in certain conditions. For example, Siglec-6 is specifically expressed on mast cells when compared with other immune cells[16], but it is also expressed on trophoblast cells of the placenta[77]. Importantly, placental Siglec-6 expression correlates

with preterm preeclampsia[78] suggesting that Siglec-6 may contribute or represent a response to preeclampsia pathogenesis. This study highlights the need for additional studies to better understand the role of Siglecs in non-immune cells whose function may be negatively impacted by anti-Siglec therapies potentially causing undesired effects.

It is important to point out that downmodulation of mast cell function via Siglec-9 targeting has the potential to negate the beneficial effects of these cells. Strong evidence of immune subversion by bacterial exploitation of Siglec-9 function has been reported[27–29]. With relevance to mast cells, it has been shown that the sialylated capsule of Group B *Streptococcus* is recognized by Siglec-9 resulting in dampening of the innate immune response and increased GBS colonization[28, 79]. We have shown that mast cells protect against GBS in models of systemic infection and pre-term birth[80–82]. Accordingly, studies are underway to determine how interactions between GBS and Siglec-9 may affect mast cell ability to protect against this bacterium. In the context of cancer, the expression levels of Siglec-7 and Siglec-9 ligands are greatly increased on a variety of cancer cells, which decreases their susceptibility to killing by NK cells[31, 83]. However, the impact of a Siglec-9 targeted therapy on mast cell contribution to cancer is uncertain as it may largely depend on unknown conditions that lead to a pro-tumoral or anti-tumoral mast cell phenotype[84].

In summary, our study provides evidence that human mast cells express Siglec-9 with inhibitory capabilities that may impact mast cell function in normal conditions and disease. Overall, we think that further understanding of Siglec-9 and other Siglecs' contribution to mast function in different compartments, in normal conditions and disease contexts may be needed to tailor potential Siglec targeted therapies for treatment of mast cell associated disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BMCMCs	bone marrow-derived cultured mast cells
Cas9	CRISPR associated protein 9
CD	cluster of differentiation
CHO	Chinese hamster ovary

CMP-Neu5Ac	cytidine monophosphate N-acetylneuraminic acid
CRISPR	clustered regularly interspaced short palindromic repeats
Cys-LT	cysteinyl leukotriene
DAMPS	damage-associated molecular patterns
DAPI	diamidino-2-phenylindole (DAPI)
DMEM	Dulbecco's Modified Eagle Medium
FBS	fetal bovine serum
FcεRIα	alpha subunit of the high affinity receptor for IgE
FcεRI	high affinity receptor for IgE
GlycA	glycophorin A
GNE	UDP-GlcNAc 2-epimerase/ManNAc-6
HBSS	Hanks balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMW-HA	high molecular weight hyaluronic acid
HSCMCs	human skin derived-cultured mast cells
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's Modified Dulbecco Media
ITIM	immunoreceptor tyrosine-based inhibitory motifs
LAMP-1	lysosomal-associated membrane protein -1
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
MFI	mean intensity fluorescence
NETs	neutrophil extracellular traps
NK	natural killer cells
PBCMCs	human peripheral blood mononuclear cell-derived cultured mast cells
PG	prostaglandin
Rh	recombinant human

Rm	recombinant mouse
ROS	reactive oxygen species
SiaT	sialyltransferases
Siglec	sialic acid-binding immunoglobin-like lectins
SCF	stem cell factor

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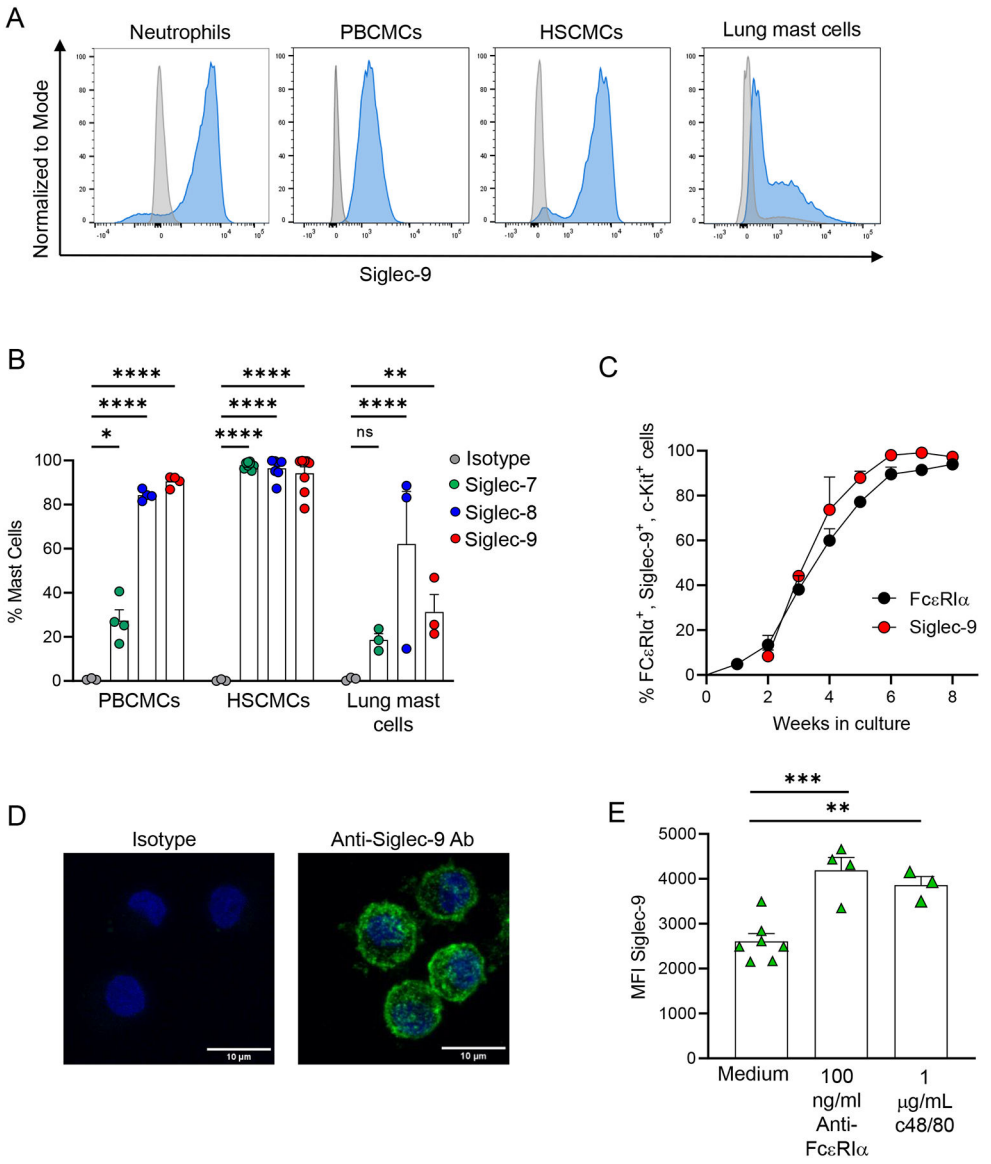

Figure 1. Siglec-9 expression in human primary mast cells.

(A-B) Representative flow cytometry analysis of Siglec-9 surface expression in human neutrophils, human peripheral blood mononuclear cell-derived mast cells (PBCMCs), human skin cultured mast cells (HSCMCs), and human lung mast cells **(A)** and percentage of PBCMCs, HSCMCs and human lung mast cells expressing Siglec-7, Siglec-8, and Siglec-9 **(B). (C)** Kinetics of Siglec-9 surface expression on $CD34^+$ -derived PBCMCs ($n = 8$). **(D)** Representative confocal microscopy images show intracellular and cell surface staining for Siglec-9 in HSCMCs (green fluorescence in right panel). Nuclei were counterstained with DAPI (blue fluorescence). Negative control was performed with isotype control and secondary antibodies (left panel). Scale bar equals 10µm. (E) Mean fluorescence intensity (MFI) of Siglec-9 in HSCMCs stimulated with either an anti-FcεRIα antibody (100 ng/ml), compound 48/80 (c48/80) (1 µg/ml) or maintained in medium alone for 20 min. Flow cytometry data in **A** and confocal images in **D** are representative of 2–6 experiments.

Data in **B, C** and **E** are shown as mean + SEM with circles in **B** (n = 3–8), triangles in **E** (n = 3–6) showing values from individual experiments with cells generated from individual donors. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

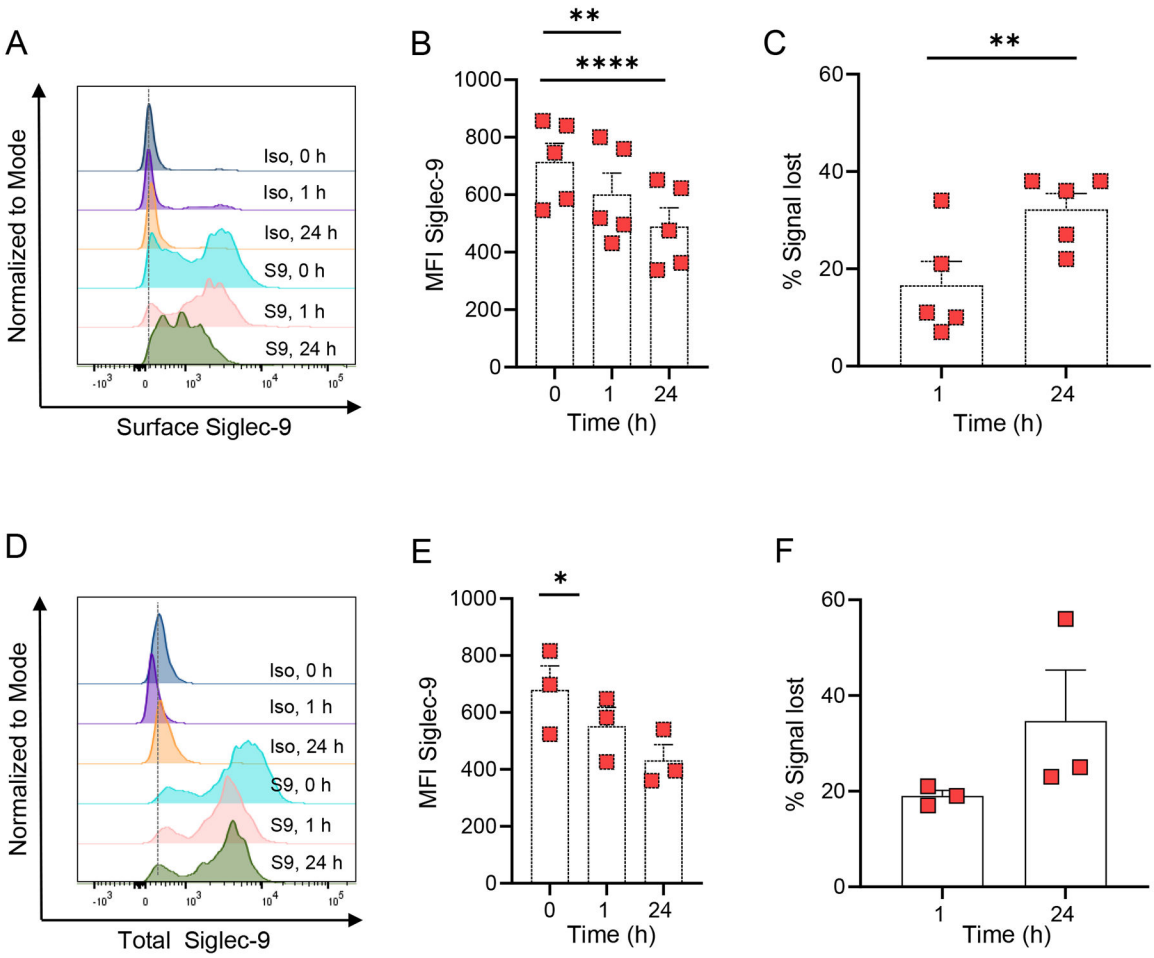

Figure 2. Siglec-9 is internalized following antibody ligation.

(A and **D)** Representative flow cytometry analysis of surface **(A)** and total **(D)** Siglec-9 expression in HSCMCs treated with 5 μg/ml of either isotype control (Iso) or anti-Siglec-9 antibody (S9) for the indicated time points. Mean fluorescence intensity **(B** and **E)** and percentage of signal lost (**C** and **F**) at indicated points for surface (**B** and **C**) and total (**E** and **F**) Siglec-9 expression in HSCMCs treated with 5 μg/ml of either APC-conjugated isotype control or APC-conjugated anti-Siglec-9 antibody (Anti-Sig9) for the indicated time points. The percentage of signal lost was calculated as MFI for Siglec-9 at indicated point minus MFI for Siglec-9 at time 0. Flow cytometry data in **A** and **D** are representative of 3–5 independent experiments. Data in **B** ($n = 5$), **C** ($n = 5$), **E** ($n = 3$) and **F** ($n = 3$) are shown as mean + SEM with squares showing values from individual experiments with cells generated from individual donors. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

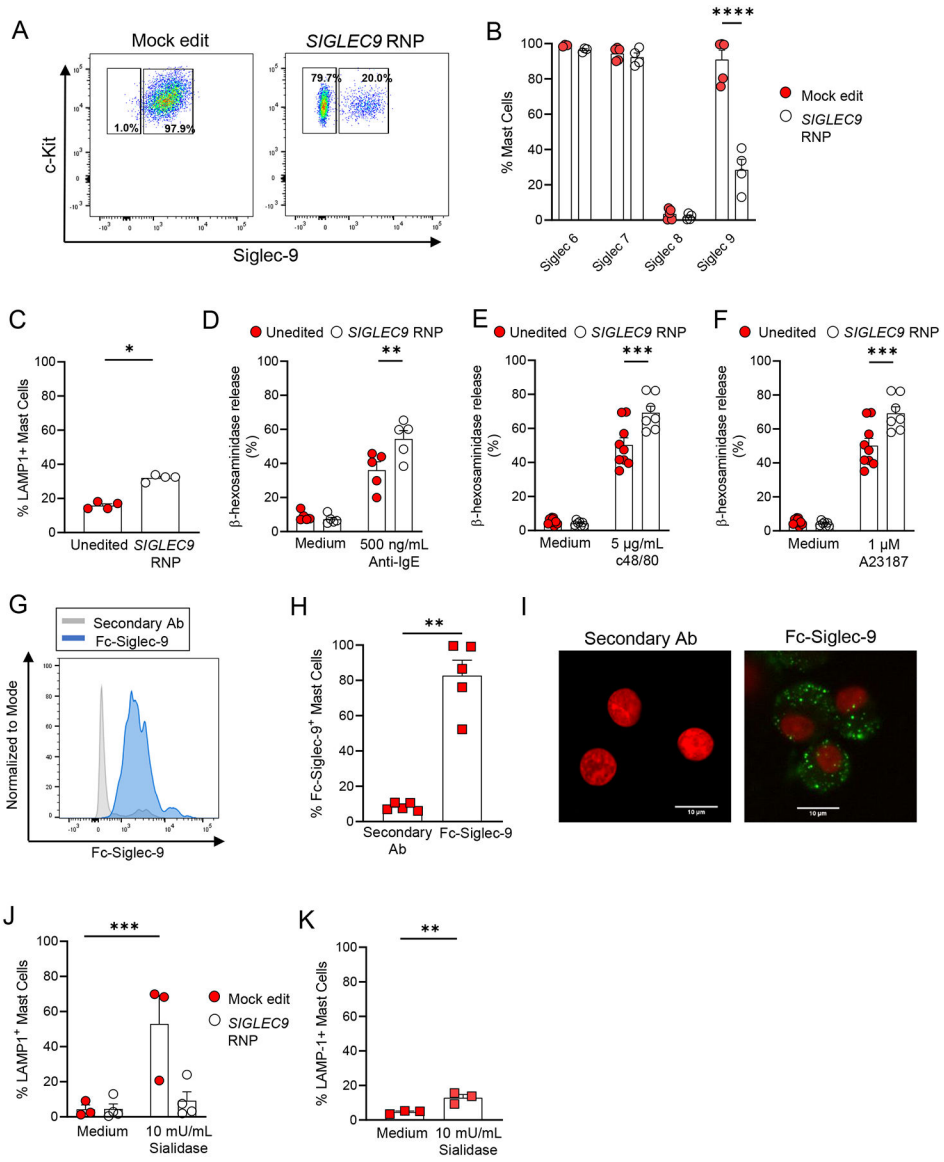

Figure 3. Siglec-9 interactions with sialic acids in *cis* limit mast cell activation.

Representative flow cytometry analysis of Siglec-9 expression of mock- and *SIGLEC9*-edited LAD2 cells. **(B)** Siglec surface expression in mock- and *SIGLEC9*-edited deficient LAD2 cells. **(C)** LAMP-1 surface expression in unedited and *SIGLEC9*-edited deficient LAD2 cells maintained in medium alone. (**D-F**) β-hexosaminidase release by unedited and *SIGLEC9*-edited LAD2 cells upon activation. Unedited and *SIGLEC9*-edited cells were sensitized with IgE (2 μg/ml) overnight and then were challenged with either anti-human IgE (500 ng/ml) (**D**), compound 48/80 (c48/80) (5 μg/ml) (**E**), or calcium ionophore (A23187) (1 μm) (**F**) for 1 h. (**G-I**) Representative flow cytometry analysis of Siglec-9 ligand expression (**G**), percentage (**H**) of HSCMCs expressing Siglec-9 ligands. **(I)** Representative fluorescent microscopy images show cell surface binding of Fc chimera Siglec-9 protein in HSCMCs (green in right panel). Nuclei were counterstained with DAPI (red fluorescence). Negative control was performed with secondary antibodies only (left

panel). Scale bar equals 10μm. (**J-K**) LAMP-1 expression in unedited and *SIGLEC9*-edited LAD2 cells (**J**), and HSCMCs maintained in medium alone or treated with sialidases (10 mU/ml) for 1 h. Flow cytometry data in **A** and **G,** and microscopy images in **I** are representative of 2–3 independent experiments. Data in **B** ($n = 4$), **C** ($n = 4$), **D** ($n = 5$), **E** ($n = 7$), **F** ($n = 5–8$), **H** ($n = 5$), **J** ($n = 3–4$) and **K** ($n = 3$) are shown as mean + SEM. Circles in **B-F** and **J** show values from individual experiments with LAD2 cells. Squares in **H** and **K** show values from individual experiments with cells generated from individual donors. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

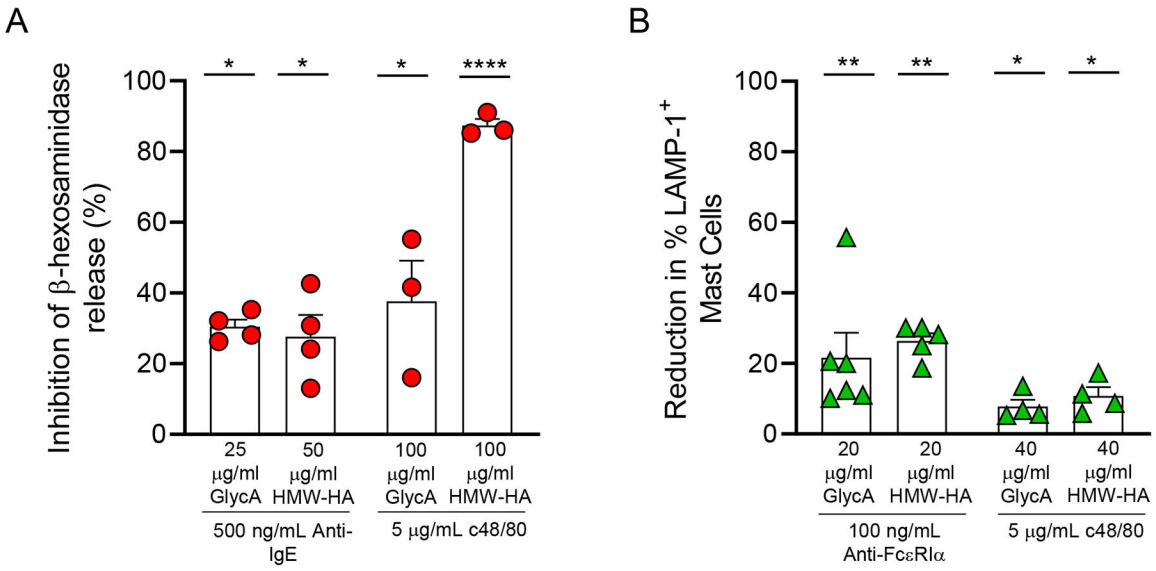

Figure 4. Siglec-9 ligands inhibit mast cell degranulation.

(A-B) Inhibition of β-hexosaminidase release in LAD2 cells **(A)** and reduction in LAMP-1 expression in PBCMCs **(B)** treated with either glycophorin A (GlycA) (25–100 μg/ml) or high molecular weight hyaluronic acid (HMW-HA) (20–100 μg/ml) for 20 min before stimulation. For LAD2 cell IgE-dependent activation, cells were sensitized with IgE (2 μg/ml) overnight and then were challenged with anti-human IgE (500 ng/ml) for 1 h. For PBCMC IgE-dependent activation, cells were treated with anti-FcεRIα antibodies (100 ng/ml) for 20 min. For IgE-independent mast cell activation, LAD2 cells and PBCMCs were stimulated with compound 48/80 (c48/80) (5 μg/ml) for 1 h and 20 min, respectively. Data are shown as mean + SEM. Circles in **A** (n = 3–4) show values from individual experiments with LAD2 cells. Triangles in **B** (n = 4–6) show values from individual experiments with cells generated from individual donors. *P < 0.05, **P < 0.01, ***P < 0.001, ****P <0.0001 vs. cells treated with stimuli alone.

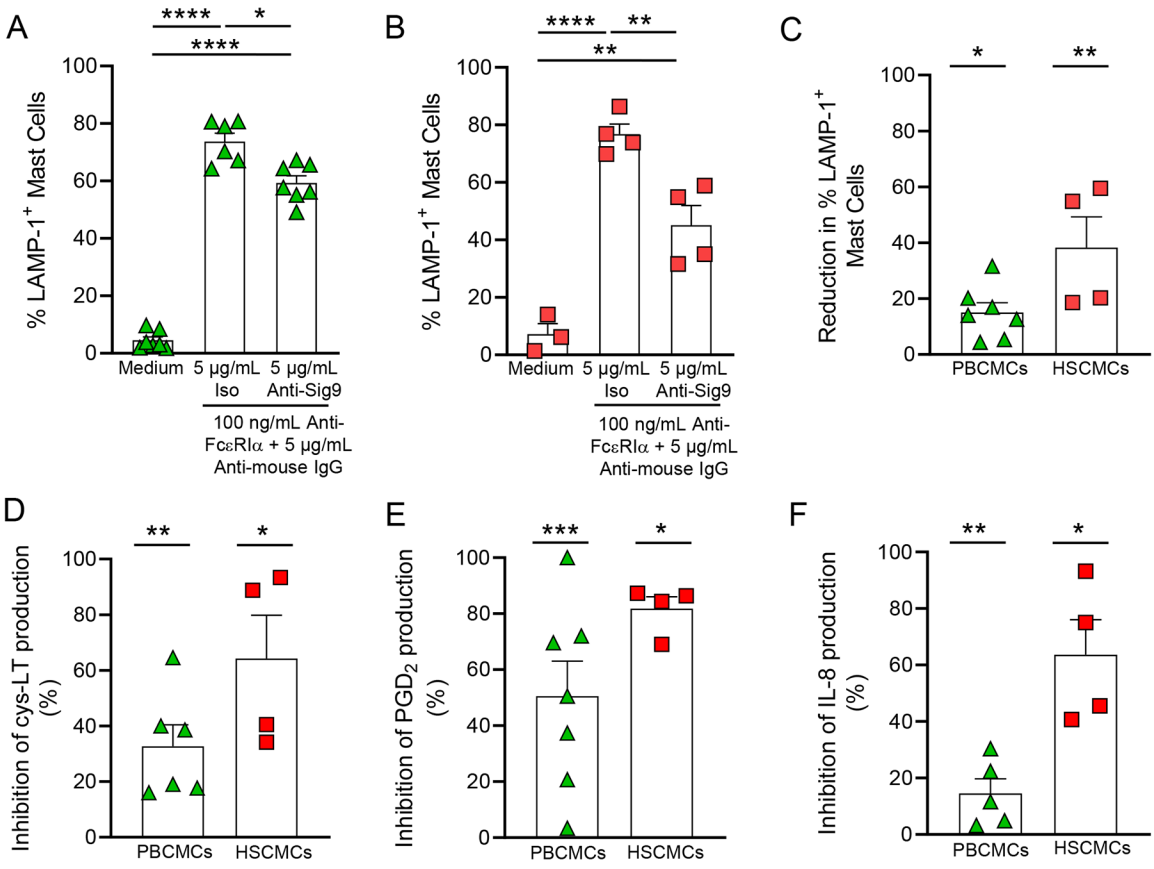

Figure 5. Co-engagement of FcεRI and Siglec-9 inhibits mast cell degranulation, production of arachidonic acid metabolites, and IL-8 release.

(A-F) LAMP-1 expression in PBCMCs **(A)** and HSCMCs **(B),** and reduction in LAMP-1 expression **(C),** cys-LT **(D**), PGD_2 **(E)** and IL-8 production in PBCMCs and HSCMCs maintained in medium alone or incubated with either isotype control or mouse anti-Siglec-9 (5 µg/ml) and stimulated with anti-human FcεRIα (100 ng/ml) and a goat anti-mouse IgG (Fc specific) F(ab')2 fragment antibody (5 µg/ml) to cross-link FcεRIα and Siglec-9. Data are shown as mean + SEM. Triangles in **A** (n = 6–7), **C** (n = 7), **D** (n = 4), **E** (n = 7) and **F** (n = 5), and squares in **B** (n = 3–4) **C** (n = 4), **D** (n = 4), **E** (n = 4) and **F** (n = 4), show values from individual experiments with cells generated from individual donors. In **A** and **B:** *P < 0.05, **P < 0.01, ****P <0.0001. In **C-F:** *P < 0.05, **P < 0.01, ***P <0.001 vs. cells treated with isotype control.