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ORIGINAL ARTICLE

Regulation of mast cells by overlapping but distinct protein interactions of Siglec-6 and Siglec-8

Wouter Korver[1] | Zachary Benet[1] | Alan Wong[1] | Gian Luca Negri[2] | Katherine Chang[1] | Robert Sanchez[1] | John Leung[1] | Naomi De Freitas[1] | Thuy Luu[1] | Julia Schanin[1] | Bradford A. Youngblood[1]

[1]Allakos Inc., San Carlos, California, USA

[2]LM Biostat Consulting Inc., Victoria, British Columbia, Canada

Correspondence
Bradford A. Youngblood, Allakos, Inc. 825 Industrial Road, Suite 500, San Carlos, CA 94070, USA.
Email: byoungblood@allakos.com

Funding information
Allakos, Inc.

Abstract

Background: Sialic acid-binding immunoglobulin-like lectin (Siglec)-6 and Siglec-8 are closely related mast cell (MC) receptors with broad inhibitory activity, but whose functional differences are incompletely understood.

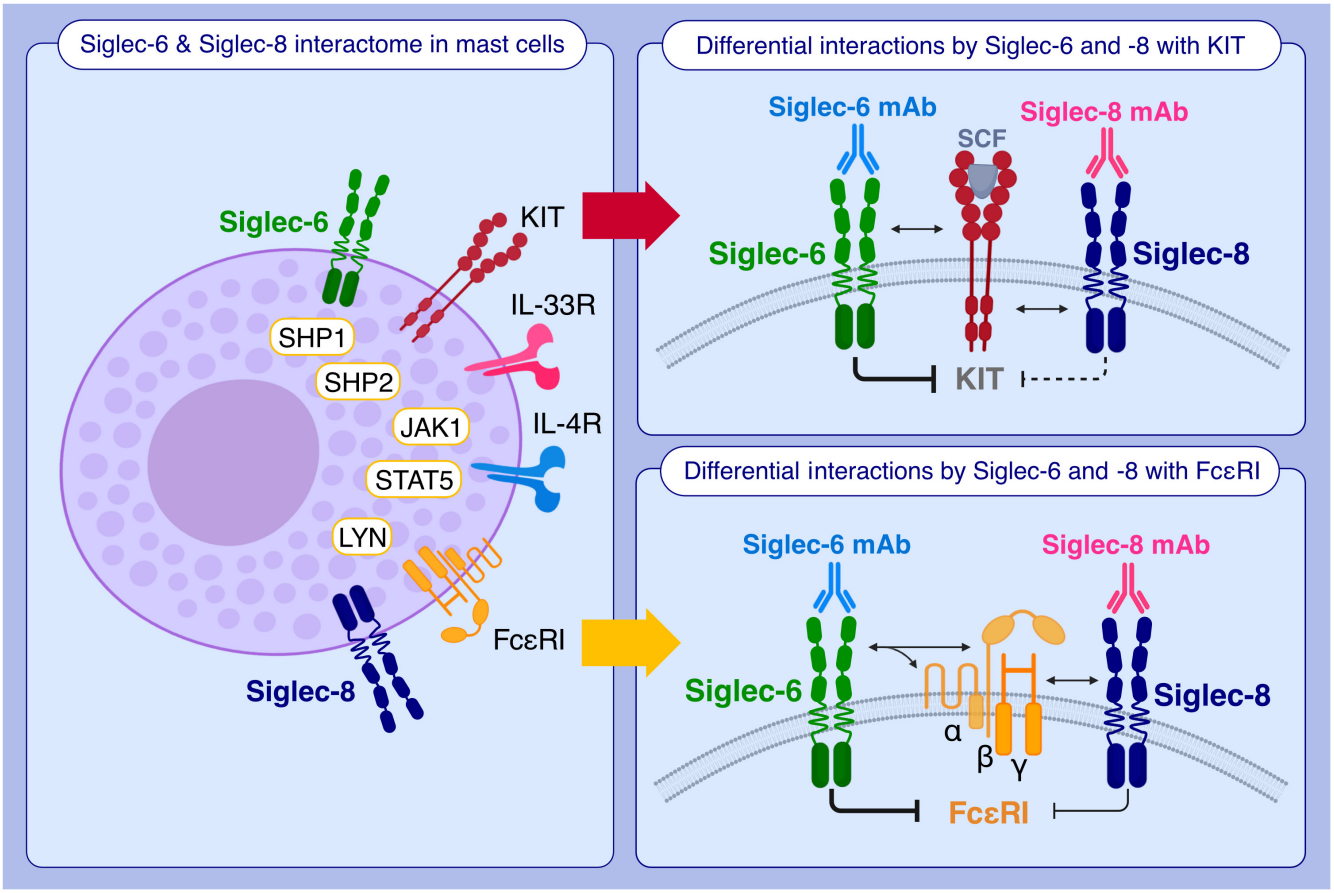
Methods: Proteomic profiling using quantitative mass spectrometry was performed on primary mouse MCs to identify proteins associated with Siglec-6 and Siglec-8. For functional characterization, each receptor was evaluated biochemically and in ex vivo and in vivo inhibition models of IgE and non-IgE-mediated MC activation in Siglec-6- or Siglec-8-expressing transgenic mice.

Results: Siglec-6 and Siglec-8 were found in MCs within large complexes, interacting with 66 and 86 proteins, respectively. Strikingly, Siglec-6 and Siglec-8 interacted with a large cluster of proteins involved in IgE and non-IgE-mediated MC activation, including the high affinity IgE receptor, stem cell factor (SCF) receptor KIT/CD117, IL-4 and IL-33 receptors, and intracellular kinases LYN and JAK1. Protein interaction networks revealed Siglec-6 and Siglec-8 had overlapping yet distinct MC functions, with a potentially broader regulatory role for Siglec-6. Indeed, Siglec-6 preferentially interacted with the mature form of KIT at the cell surface, and treatment with an anti-Siglec-6 antibody significantly inhibited SCF-mediated MC activation more in comparison to targeting Siglec-8.

Conclusion: These data demonstrate a central role for Siglec-6 and Siglec-8 in controlling MC activation through interactions with multiple activating receptors and key signaling molecules. Our findings suggest that Siglec-6 has a role distinct from that of Siglec-8 in regulating MC function and represents a distinct potential therapeutic target in mast cell-driven diseases.

KEYWORDS
interactome, KIT, mast cell, proteomics, Siglec

Abbreviations: FcεRI, Fc epsilon receptor I; ITAM, immunoreceptor tyrosine-based activation motif; JAK1, Janus kinase 1; KIT, proto-oncogene, receptor tyrosine kinase; LYN, tyrosine-protein kinase; mAb, monoclonal antibody; SCF, stem cell factor; SHP, Src homology region 2 domain-containing phosphatase; Siglec, sialic acid-binding immunoglobulin-like lectin; STAT5, signal transducer and activator of transcription 5.



GRAPHICAL ABSTRACT

Siglec family inhibitory receptors interact with multiple activating receptors and signaling molecules in mast cells. Comparative analysis of Siglec-6 and Siglec-8 interactomes suggests distinct roles in mast cell regulation, with some functional overlap. Siglec-6 engagement appears to elicit broader and more complete mast cell inhibition through ITAM and non-ITAM receptors than Siglec-8.

1 | INTRODUCTION

The dynamic nature of the immune system is held in balance by mechanisms that maintain homeostasis amidst constant surveillance, migration, and activation of immune cells.[1] Overstimulation can lead to detrimental immune responses, while underactivity can lead to uncontrolled infections, impaired wound healing, or development of malignancies. Cell surface inhibitory receptors are gatekeepers, or immune checkpoints, that prevent excessive cellular immune responses by attenuating or terminating the activity of activating receptors. These receptors often use tyrosine signaling motifs (e.g., immunoreceptor tyrosine-based inhibitory motif [ITIM], immunoreceptor tyrosine-based switch motif [ITSM]) to deliver inhibitory signals.[2,3] While some inhibitory receptors (e.g., PD-1, CTLA-4, and TIGIT) have been successfully targeted with blocking or antagonizing antibodies in cancer,[4,5] agonistic antibodies are being developed to engage inhibitory receptors to reduce inflammation via native inhibitory pathways.[6]

Sialic acid-binding immunoglobulin-like lectins (Siglecs) are a family of inhibitory receptors primarily expressed on immune cells. Most Siglecs contain intracellular ITIMs that recruit Src Homology 2 (SH2)-containing phosphatases (e.g., SHP-1, SHP-2, and SHIP1) to activate inhibitory signaling pathways. Agonistic Siglec-binding antibodies are broadly inhibitory across multiple immune cells, including B cells, monocytes, and mast cells (MCs),[7] and are currently under clinical investigation for several indications.[8,9]

Siglec-6 and Siglec-8 are closely related molecules almost exclusively expressed on MCs.[10] In previous studies, engagement of Siglec-6 or Siglec-8 with an agonistic monoclonal antibody (mAb) inhibited FcεRI-mediated MC activation in vitro and in vivo.[11,12] Siglec-6 and Siglec-8 also inhibited FcεRI-independent MC activation following stimulation through cytokine receptors and G protein-coupled receptors inter alia.[11,13–15] This FcεRI-independent activation is intriguing because ITIM-containing receptors are mainly thought to recruit immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors, such as FcεRI, to induce MC inhibition, as previously described for Siglec-8.[16] Mechanisms of Siglec-6 and Siglec-8 inhibition have yet to be characterized for non-ITAM signaling, including the stem cell factor (SCF)/KIT (CD117) pathway, which drives MC differentiation, migration, proliferation, and survival.[17]

To understand mechanisms of MC inhibition conferred by these two Siglecs, we used unbiased proteomic profiling to identify cell surface and intracellular proteins that interact with Siglec-6 or Siglec-8 (i.e., "interactomes") in culture-derived, primary MCs. We

investigated differential protein interactions between Siglec-6 and Siglec-8 and how these interactions translate to inhibition of ITAM and non-ITAM-activating receptors. Our findings provide insight into the mechanisms used to mediate broad inhibition of activating pathways within MCs and highlight Siglec-6 and Siglec-8 as distinct targets for silencing aberrant MC activation in allergic and inflammatory diseases.

2 | METHODS

2.1 | Mouse bone marrow mast cell (BMMC) generation and FcεRI-mediated activation

BMMCs were generated from femurs and tibias of Siglec-6 or Siglec-8 C57BL/6 transgenic mice[18,19] and activated in vitro as previously described.[16] BMMCs are referred to as S6-BMMC (from Siglec-6 transgenic mice) and S8-BMMC (from Siglec-8 transgenic mice). See Appendix S1 for details.

2.2 | Human tissue MC isolation and activation

Fresh human lung tissue was procured from healthy donors from the Cooperative Human Tissue Network. Tissues were enzymatically and mechanically dissociated using the gentleMACs™ Dissociator system (Miltenyi Biotec, Germany), per manufacturer's protocol. Cells were washed in phosphate-buffered saline (PBS) and resuspended in RPMI 1640 + 10% low IgG fetal bovine serum (FBS), then plated in 96-well round bottom tissue culture plates at 3000 MC per well and centrifuged for 2 min at 400*g*. To assess the inhibitory activity through Siglecs, cells were resuspended in 10 μg/mL anti-FcεRI (clone CRA-1, Miltenyi Biotec), combined with various concentrations of anti-Siglec-6 (clone AK04, hIgG1, Allakos) and anti-Siglec-8 (2E2, hIgG1, Allakos) or isotype control mouse antibody (MOPC-21, hIgG1, Allakos), at 4°C for 2 min. Cells were washed in PBS, then incubated at 4°C for 2 min in PBS with 20 μg/mL secondary antibody AffiniPure F(ab')2 Fragment goat anti-mouse IgG heavy chain and light chain (H + L) (Jackson Immunoresearch, Cat. #109–006-088). After an additional PBS wash, cells were resuspended and incubated for 20 min at 37°C for MC activation analysis. The percentage of CD63 (clone H5C6, Biolegend) and CD107a (clone H4A3, BD Biosciences) expressing MCs was determined by flow cytometry on a Novocyte Quanteon (Agilent).

2.3 | Immunoprecipitations (IP) for proteomics

Co-IPs on lysates from mouse BMMCs for proteomic analysis were performed using the Dynabeads co-IP kit (Thermo) per manufacturer's instructions. The following antibodies were conjugated to beads (40 μg antibody/7.5 mg beads/IP): rabbit isotype IgG isotype control (Thermo #31235), anti-Siglec-8 rabbit polyclonal Ab (pAb; Thermo, PA5-28846), anti-Siglec-6 rabbit pAb (Thermo, PA5-82377 and H00000946-D01P). 1.5×10^8 BMMC were lysed in extraction buffer B: 1xIP lysis buffer was added from the kit supplemented with 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, and HALT protease inhibitors (Thermo) and samples were eluted in 1 mL of buffer HPH EB (0.5 M NH_4OH, 0.5 mM EDTA). Samples were frozen at −80°C until analysis by liquid chromatography-mass spectrometry (LC–MS).

2.4 | Ethics review

Collection of human tissue was approved by the Vanderbilt University Institutional Review Board (#031078, #010294). Animal experiments were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animal and in compliance with ARRIVE guidelines.

2.5 | Other

Methods for generating transgenic mice, BMMC activation, protein extraction/digestion, MS, gel filtration chromatography, confocal microscopy, transfections/IP/Western blot (WB), PNGase deglycosylation, enzyme-linked immunosorbent assays (ELISA), and proteomic data analysis are in Appendix S1.

3 | RESULTS

3.1 | Siglec-6 and Siglec-8 antibodies induce differential inhibition of FcεRI-activated MCs

Antibodies to Siglec-6 and Siglec-8 inhibit FcεRI-mediated MC activation.[11,12,16,20] Comparison of Siglec-6 and Siglec-8 in human lung tissue showed similar expression levels on MCs (Figure 1A–C). These MCs were activated through FcεRI using an anti-FcεRI antibody (CRA-1), as previously described.[20,21] After determining the maximally MC-activating CRA-1 concentration (Figure S1A), titration of anti-Siglec-6 and anti-Siglec-8 mAbs demonstrated inhibition of FcεRI-mediated degranulation (based on CD63 and CD107a expression) in a concentration-dependent manner (Figure 1D). Interestingly, at higher concentrations, the anti-Siglec-6 mAb induced significantly better inhibition than anti-Siglec-8 (Figure 1D). These data suggest that Siglec-6 and Siglec-8 exhibit different degrees of inhibitory activity in MCs, prompting us to investigate differences in protein–protein interactions, using proteomic profiling.

3.2 | Development of a system to study Siglec-6 and Siglec-8 interactomes

Evaluation of Siglec-6 transgenic mice demonstrated Siglec-6 was highly and selectively expressed on MCs in tissues of the peritoneal

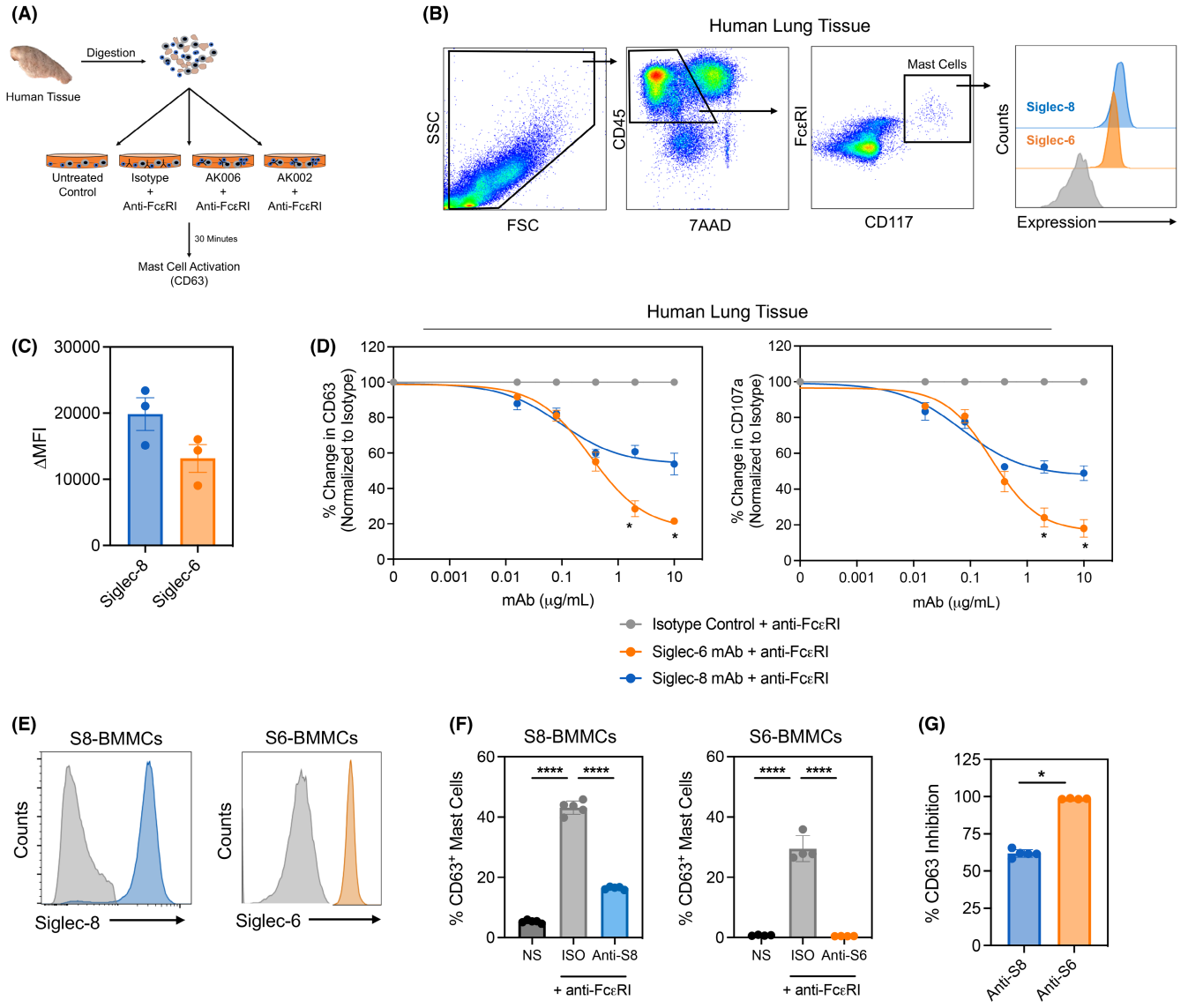


FIGURE 1 Inhibition of MC activation through Siglec-6 and Siglec-8 engagement. (A) Schematic of human tissue MC activation assay. (B) MC gating and Siglec-6/8 expression in human lung tissue by flow cytometry. (C) Expression levels of Siglec-6 and Siglec-8 on lung MC from three donors. Expression is represented as delta median fluorescence intensity (ΔMFI) and was calculated by subtracting the MFI from a fluorescence minus one (FMO) negative control. Data are plotted as mean ± standard deviation. (D) Titration of anti-Siglec-6 and anti-Siglec-8 monoclonal antibodies (mAbs) in combination with anti FcεRI-mediated activation (10 μg/mL), percentage of CD63 (left panel) and CD107a (right panel)-positive MC. (E) Expression of Siglec-8 (blue, left panel), Siglec-6 (orange, right panel) and FMO stain (gray) on S8-BMMC or S6-BMMC, respectively, by flow cytometry. (F) Percentage of CD63-positive S8-BMMC or S6-BMMC: unstimulated (black bars), cross-linked with the anti-FcεRI antibody MAR-1 (250 ng/mL) for 15 min (gray bars) or co-cross-linked with MAR-1 and anti-Siglec-8 mAb (left panel: blue bar) or anti-Siglec-6 mAb (right panel: orange bar). (G) Percent reduction in CD63-positive BMMC after FcεRI-mediated activation in the presence of anti-Siglec-8 (blue) or anti-Siglec-6 (orange) mAbs. NS, unstimulated. Data are plotted as mean ± SD (three independent donors for Panel C and D; 5–6 mice/group for Panel F and G) and are representative of at least two experiments. $^{*}p < 0.05$; $^{****}p < 0.0001$ by one-way ANOVA with Tukey multiple-comparisons test or Mann–Whitney *U* test.

cavity, stomach, and skin, but not on other immune cell populations (Figure S2A–C). Additionally, peritoneal MCs from Siglec-6 transgenic mice underwent IgE-mediated degranulation and were inhibited with an anti-Siglec-6 mAb, demonstrating that MCs and Siglec receptors in the transgenic mice are functional (Figure S2D,E).

S6-BMMC and S8-BMMC expressed FcεRI and KIT, as well as functional human Siglec-6 or Siglec-8, respectively (Figure 1E; Figure S3A–C). These BMMCs could be expanded in long-term cultures in the presence of IL-3 while maintaining their phenotype.[11,16] BMMC activation by anti-mouse FcεRI (MAR-1) was concentration-dependent (Figure S3D), and both anti-Siglec-6 and anti-Siglec-8 mAbs significantly inhibited FcεRI-mediated BMMC degranulation (Figure 1F). Inhibition through Siglec-6 was significantly better than Siglec-8 (Figure 1G), suggesting that the observed differential inhibitory activity between the 2 Siglecs mirrored that observed in human lung MCs described above.

3.3 | Siglec-8 interacts with multiple activating receptors in MCs

To investigate Siglec-8 protein complexes, we evaluated whole cell lysates of S8-BMMC by size exclusion chromatography (SEC) and detected Siglec-8 in complexes of various sizes up to 2000 kDa (Figure S4A), suggestive of an elaborate interactome. To elucidate components of the Siglec-8 interactome, lysates from S8-BMMC were immunoprecipitated with isotype control or an anti-Siglec-8 pAb (Figure 2A). The eluates from IPs from multiple BMMC donors were digested and labeled by Tandem Mass Tag prior to analysis by LC–MS, peptide identification, and quantification (Figure 2B).

We identified 86 intracellular and cell surface proteins in the Siglec-8 co-IPs that were significantly enriched >1.5-fold, compared to the isotype control. Strikingly, the core of the Siglec-8 interactome consisted of receptors critical for MC survival and function, including KIT, the gamma subunit of the high affinity receptor for IgE (FcεRI), the common beta and gamma chain subunits of cytokine receptors required for, among others, IL-2-, IL-3-, IL-5-, and granulocyte-macrophage colony-stimulating factor (GM-CSF)-mediated signaling, plus the IL-4 and IL-33 receptors (Figure 2C). Signaling molecules associated with these activating receptors were also identified as interacting partners with Siglec-8. These include the tyrosine kinase LYN, essential for signaling through FcεRI, and the phosphatases CD45, SHP-1, and SHP-2. Furthermore, JAK1 and STAT5, important signaling molecules downstream of cytokine receptors, were identified in the Siglec-8 interactome (Figure 2C; Table 1). Analysis of Siglec-8 IPs by SEC confirmed the presence of

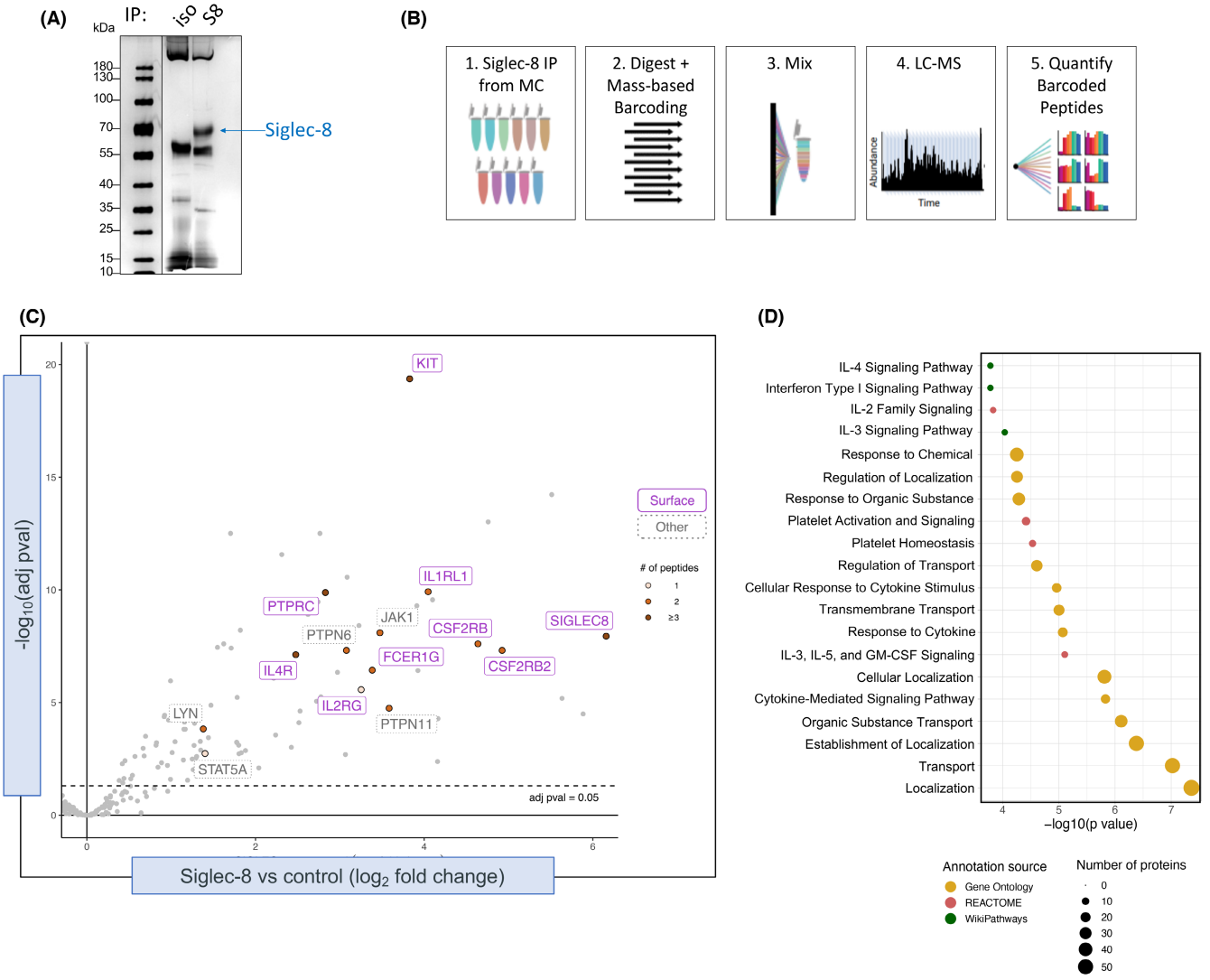


FIGURE 2 Summary of the Siglec-8 interactome. (A) Silver stain gel of representative IPs from S8-BMMCs. Each lane represents about 4×10^6 BMMCs. S8 = anti-Siglec-8 IP, iso = isotype control IP. (B) Schematic of the process for LC–MS. (C) Scatter plot of Log_2 fold-change against statistical significance of Siglec-8 IPs versus isotype control. Highlighted are the key MC signaling proteins listed in Table 1. Predicted membrane/surface proteins in magenta, solid outline; other proteins in gray, dashed outline. (D) Summary of the most significant GO, REAC, and WP processes represented in the Siglec-8 co-immunoprecipitated proteins, ordered by *p*-value. Size of the dot represents the number of proteins associated with each pathway.

TABLE 1 Selected mouse mast cell activating receptors and signaling molecules in Siglec-8 and Siglec-6 interactomes as assessed using transgenic mouse BMMCs.

Symbol	Uniprot	Name	Interactome	Description	Category
FCER1G	P20491	High affinity immunoglobulin epsilon receptor subunit gamma	S8	High affinity receptor for IgE subunit FcεRIγ	MC activating receptor
CSF2RB	P26955	Cytokine receptor common subunit beta/CD131	S8	GM-CSF/IL-3/IL-5 receptor common beta subunit	MC activating receptor
IL2RG	P34902	Cytokine receptor common subunit gamma/CD132	S8	Subunit of IL-2, −4, −7, −9, −15, and −21 receptors	MC activating receptor
PTPRC	P06800	Receptor-type tyrosine-protein phosphatase C/CD45	S8	Transmembrane phosphatase	Signaling molecule
KIT	P05532	Mast/stem cell growth factor receptor KIT/CD117	S6 + S8	Stem cell growth factor (SCF) receptor	MC activating receptor
CSF2RB2	P26954	Interleukin-3 receptor class 2 subunit beta	S6 + S8	GM-CSF/IL-3/IL-5 receptor common beta subunit 2	MC activating receptor
IL1RL1	P14719	Interleukin-33 receptor alpha chain/ST2	S6 + S8	IL-33 receptor subunit	MC activating receptor
IL4RA	P16382	Interleukin-4 receptor subunit alpha/CD124	S6 + S8	IL-4 receptor subunit	MC activating receptor
LYN	P25911	Tyrosine-protein kinase LYN	S6 + S8	Tyrosine kinase essential for FceRI signaling	Signaling molecule
PTPN6	P29351	Tyrosine-protein phosphatase non-receptor type 6/SHP-1	S6 + S8	Intracellular phosphatase	Signaling molecule
PTPN11	P35235	Tyrosine-protein phosphatase non-receptor type 11/SHP-2	S6 + S8	Intracellular phosphatase	Signaling molecule
JAK1	P52332	Tyrosine-protein kinase JAK1	S6 + S8	Intracellular kinase essential for cytokine signaling	Signaling molecule
STAT5A	P42230	Signal transducer and activator of transcription 5A	S6 + S8	Transcription factor, required for FcεRI, KIT, IL-3 signaling	Signaling molecule
FCER1A	P20489	High affinity immunoglobulin epsilon receptor subunit alpha	S6	High affinity receptor for IgE subunit FcεRIα	MC activating receptor
MS4A2	P20490	High affinity immunoglobulin epsilon receptor subunit beta	S6	High affinity receptor for IgE subunit FcεRIβ	MC activating receptor

FcεRIγ in fractions representing high molecular weight (MW) complexes >1000 kDa (Figure S4B).

Enrichment analysis of biological processes, through gene ontology (GO), reactome (REAC), and WikiPathways (WP), confirmed significant enrichment of proteins in pathways related to cytokine signaling within the Siglec-8 interactome (Figure 2D). These data demonstrate an expansive interactome consisting of multiple key activating receptors and signaling molecules, suggesting that Siglec-8 is involved in broad regulation of MC activation at multiple levels.

3.4 | Siglec-6 and Siglec-8 interactomes contain similarities and differences with key proteins that regulate MC activity

Using the same approach, we identified Siglec-6 interacting proteins in S6-BMMCs. Siglec-6 co-IPs from S6-BMMCs revealed a total of 66 proteins that were significantly enriched >1.5-fold relative to the isotype control (Figure 3A and Figure S4C). Most of the same critical MC receptors and signaling molecules identified in the Siglec-8 interactome were identified with Siglec-6, including nine out of 13 critical MC activating receptors and signaling molecules co-immunoprecipitated with Siglec-8 (Table 1). Siglec-6 co-IPs contained the alpha and beta subunits of FcεRI, potentially explaining the more complete inhibition of IgE-mediated activation through Siglec-6 compared to Siglec-8. Although meaningful similarities were found between the Siglec-6 and Siglec-8 interactomes, substantial differences were observed in enrichment analyses. The top 20 pathways enriched in the Siglec-6 interactome included cytokine signaling through IL-3, IL-4, IL-5, and GM-CSF, as was seen with Siglec-8. Additional pathways enriched in Siglec-6 included SCF/KIT, thymic stromal lymphopoietin (TSLP) signaling, pyruvate dehydrogenase, and regulation of metabolic processes (Figure 3B).

To better understand the similarities and differences between Siglec-6- and Siglec-8-interacting proteins, we generated heatmaps to identify common interactors and those specific to Siglec-6 and Siglec-8 (Figure 3C). The Siglec-6 and Siglec-8 co-IPs resulted in 66 and 86 proteins, respectively; 28 overlapped between both interactomes (Figure 3D).

Proteins that were immunoprecipitated specifically with Siglec-6 included different subunits of the FcεRI receptor (FCER1A and MS4A2), protein phosphatase subunits (PPP6R1 and ANKRD44), proteins involved in microtubule dynamics and degranulation (TUBB5 and DOCK2), the receptor for TSLP (CRLF2) and the receptor LILRB4, implicated in inhibition of IgE-mediated MC activation (Figure 3E).[22] Additionally, Siglec-6 specifically interacted with MC metabolism-related proteins, including DLAT, PDHA1, and PDHX.

The interactome specific to Siglec-8 included the cytokine receptor subunits IL2Rγ, the common gamma chain for several interleukins and CSF3RB/CD131, the beta chain required for IL-3, IL-5, and CSF signaling, the inhibitory receptor LILRB3, and a subunit of the heterodimeric TGFβ receptor TGFBR2 (Figure 3F).

3.5 | Siglec-6 and Siglec-8 interaction networks within MCs

Next, we used the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database to link the proteins identified in the interactomes to known and predicted protein–protein interaction networks.[23] Several clusters were identified with representations of proteins from both the Siglec-6 and Siglec-8 interactomes. The largest such shared cluster contained proteins involved in both cytokine signaling and signaling through FcεRI (Figure 4A), followed by proteins involved in protein processing in the endoplasmic reticulum (Figure 4B), nucleocytoplasmic transport (Figure 4C), mitochondrial translocators (Figure 4D), ubiquitin protein ligase machinery (Figure 4E), interferon-induced proteins (Figure 4F), and TGFβ signaling (Figure 4G). Siglec-6-specific clusters included pyruvate dehydrogenases (Figure 4H), the serine–threonine phosphatase complex PPP6 (Figure 4I) and enzymes involved in pyrophosphate metabolism (Figure 4J). No clusters were identified that only contained Siglec-8 specific proteins. Taken together, these data suggest the involvement of Siglec-6 and Siglec-8 in regulating an expansive array of processes critical for MC responses to external stimuli, intracellular signaling, and metabolism, through interaction with a diverse set of proteins, with a potentially broader regulatory role for Siglec-6 than Siglec-8.

3.6 | Siglec-6, but not Siglec-8, interacts with KIT on the MC surface

Due to an unexpectedly large number of non-ITAM receptors found to interact with Siglec-6 and Siglec-8, Siglec-dependent inhibition of the MC growth and survival receptor KIT was further examined. To determine which domains were required for interaction with KIT, plasmids expressing full-length Siglec-6 and Siglec-8 and 3 domain mutants (Figure 5A), as well as full-length KIT, were transiently transfected into BMMCs, and Siglec-KIT interactions were evaluated by subsequent IP/WB. Upon transfecting full-length KIT into BMMC, three KIT species of distinct MW were detected, representing different glycosylation levels (Figure S5).[24] The high MW band represented mature, fully glycosylated KIT, as demonstrated by removing or preventing glycosylation (Figure S5A), and was the only species present at the cell surface, as determined by biotinylation of KIT-expressing cells (Figure S5B).[24] While Siglec-6 interacted with all three species of KIT (Figure 5B: left panels), Siglec-8 only interacted with the two smaller, partially glycosylated, immature forms found intracellularly (Figure 5B: right panels). The Ig-like C-type domain 2 was required for Siglec-8 interaction with KIT, while removal of this domain from Siglec-6 did not affect its interaction with KIT (Figure 5B: lane 3).

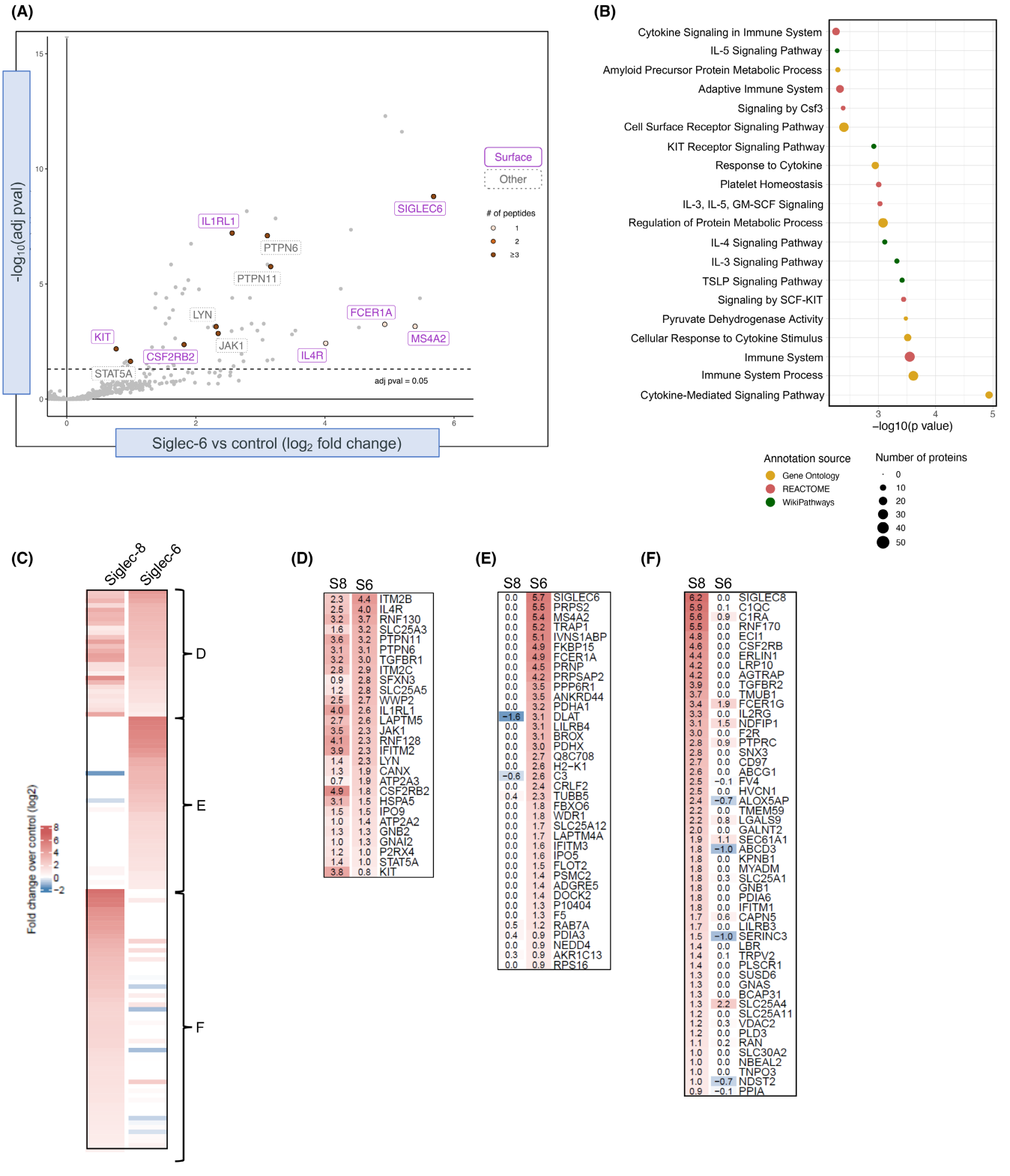


FIGURE 3 Summary of the Siglec-6 interactome and comparison with Siglec-8. (A) Scatter plot of Log_2 fold-change against statistical significance of Siglec-6 IPs versus isotype control. Highlighted are the key MC signaling proteins listed in Table 1. Predicted membrane/surface proteins in magenta, solid outline; other proteins in gray, dashed outline. (B) Summary of the most significant GO, REAC, and WP processes represented in the Siglec-6 co-immunoprecipitated proteins, ordered by *p*-value. Size of the dot represents the number of proteins associated with each pathway. (C) Overview heatmap of all proteins significantly co-immunoprecipitated with either Siglec-8 (left) or Siglec-6 (right), further divided in three sections: (D) Common interactors between Siglec-6 and Siglec-8; (E) Siglec-6 specific; (F) Siglec-8 specific. Ranked top to bottom by Log_2 fold-change for Siglec-6 (D + E) or Siglec-8 (F).

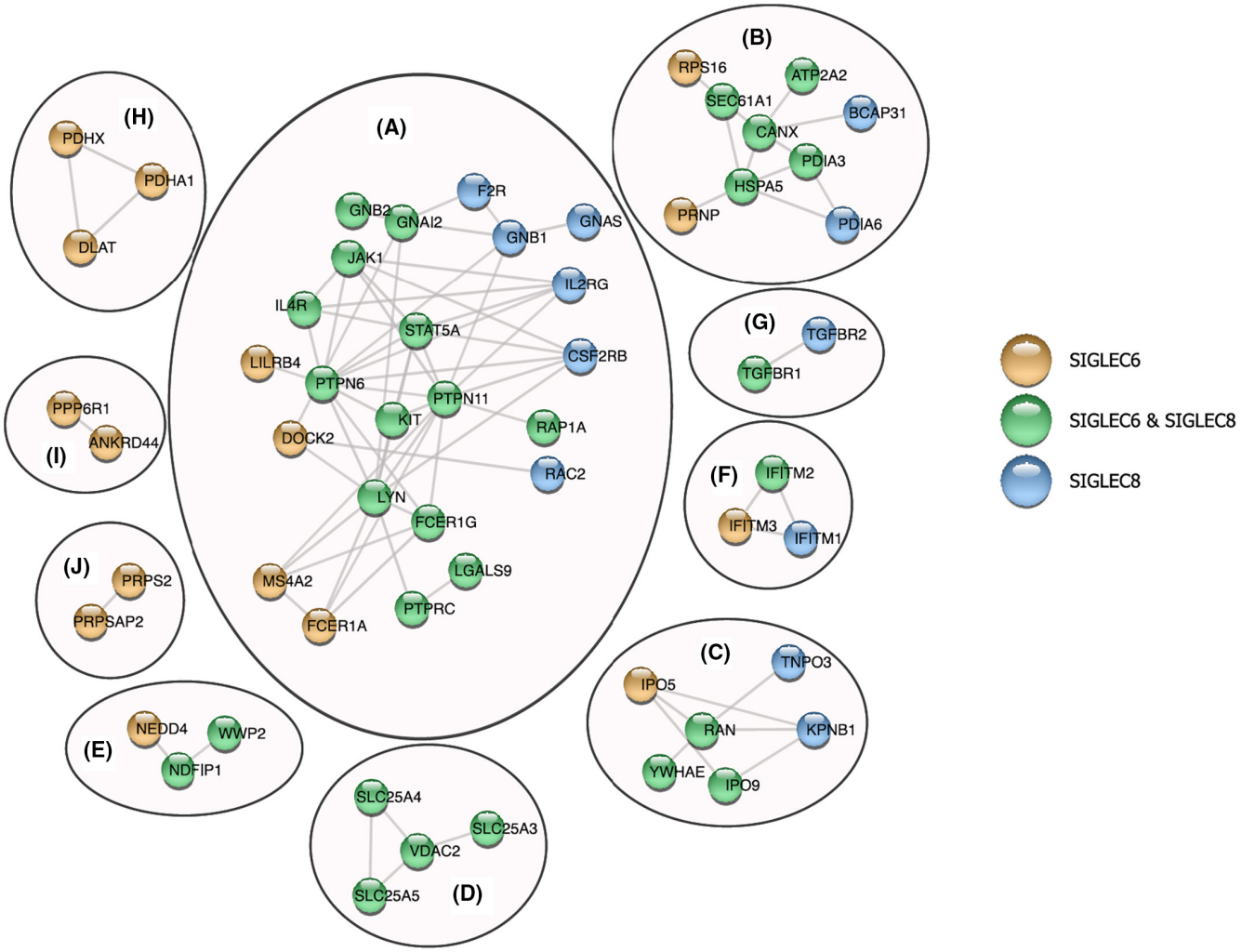


FIGURE 4 STRING analysis of the Siglec-6 and Siglec-8 interactomes. Visual representation of the 10 largest clusters of protein interactions involved in: (A) Cytokine/FcεRI signaling, (B) Protein processing in ER, (C) Nucleocytoplasmic transport, (D) Mitochondrial translocation, (E) Ubiquitin ligase machinery, (F) Interferon-induced proteins, (G) TGFβ signaling, (H) Pyruvate dehydrogenases, (I) PPP6 phosphatase complex, (J) Pyrophosphate metabolism. Proteins specific for the Siglec-6 interactome are depicted in orange, Siglec-8 proteins in blue and common interactors in green.

Siglec/KIT interactions were confirmed by an ELISA-based direct binding assay using both mouse and human recombinant KIT protein, which demonstrated that Siglec-6 binds significantly better to mouse and human KIT than Siglec-8 (Figure 5C,D). However, removal of N-linked oligosaccharides from KIT significantly enhanced Siglec-8 interaction via ELISA (Figure 5E).

3.7 | Siglec-6 inhibits KIT-mediated MC activation

We next asked whether differences in Siglec-6 and Siglec-8 interactions with KIT led to functional differences in SCF-induced signaling in MCs. In vitro MC survival assays with recombinant mouse SCF were established using peritoneal MCs from Siglec-6 and Siglec-8 transgenic mice. Addition of SCF enhanced MC survival and induced downregulation of CD117 in a concentration-dependent manner, as expected (Figure S6A,B,D,E). Treatment with an anti-Siglec-6 or anti-Siglec-8 mAb did not affect SCF-induced MC survival. SCF also induced concentration-dependent MC activation (based on CD63 expression) as previously described.[25] SCF-mediated MC activation was inhibited by an anti-Siglec-6, but not anti-Siglec-8 mAb (Figure S6C,F). These findings suggest Siglec-6, but not Siglec-8 inhibits SCF-mediated MC activation, but not survival.

To evaluate if the in vitro findings translated to an in vivo system, a model of SCF-mediated MC activation was evaluated in Siglec-6 transgenic mice (Figure 6A). Intravenous injection of recombinant mouse SCF induced activation of peritoneal MCs, based on increased CD63 and CD107a expression (Figure 6B,C and Figure S7A,B). Dosing of an anti-Siglec-6 mAb prior to SCF injection in Siglec-6 transgenic mice inhibited SCF-mediated MC activation, based on significant decreases in CD63 and CD107a expression and serum

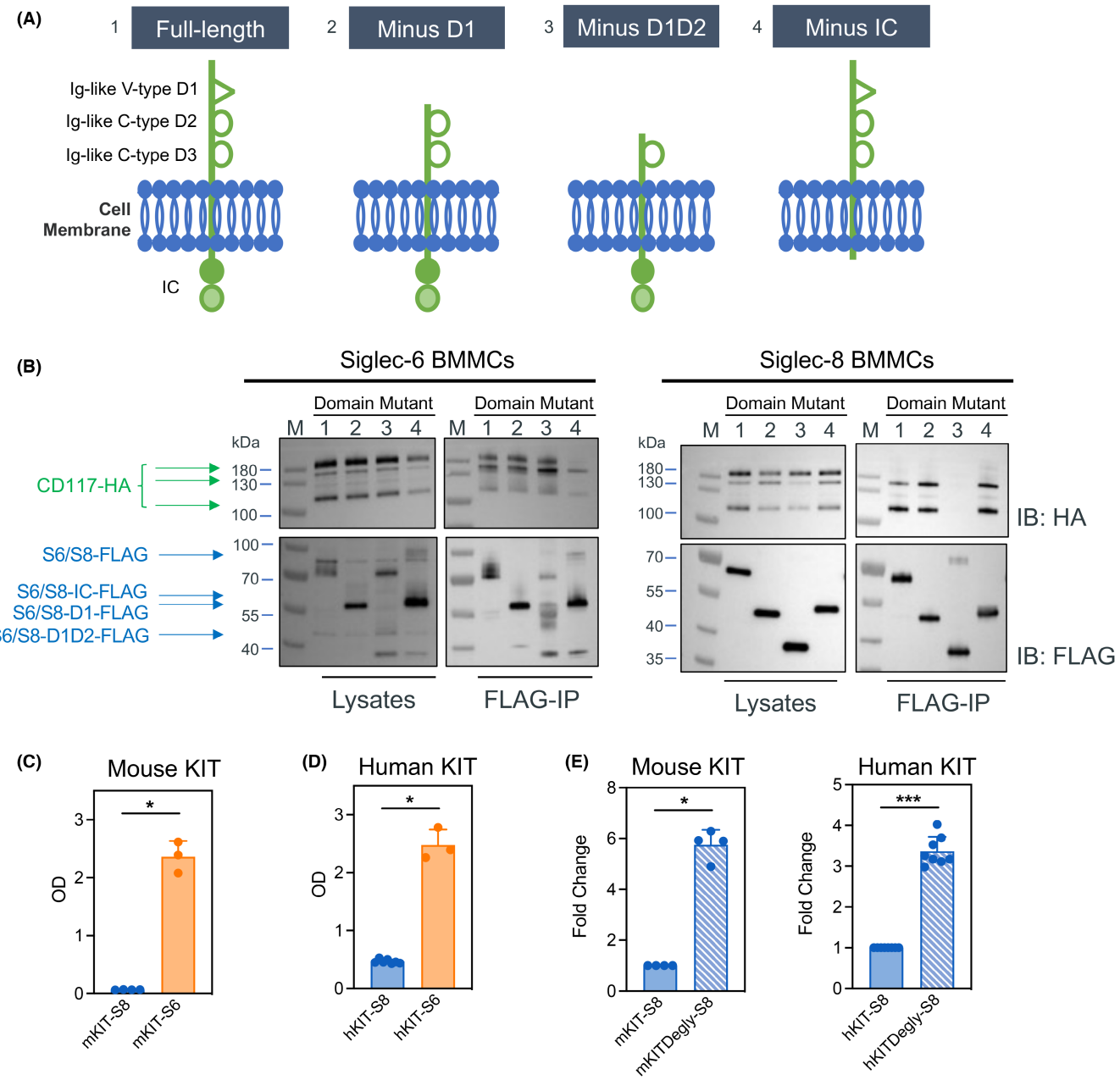


FIGURE 5 Distinctive KIT-Siglec interactions. (A) Graphical representation of full length and domain mutants of Siglec-6/8-FLAG expressed in BMMC. 1 = full-length, 2 = Ig-like V-type (D1) deleted, 3 = D1 and Ig-like C-type (D2) deleted, 4 = intracellular domain deleted. (B) Western blots of lysates and FLAG-IPs from BMMC co-transfected with Siglec-6 (left) and Siglec-8 (right) domain mutants 1–4 in combination with full-length KIT (CD117)-HA. Top panels: immunoblot (IB) with anti-HA antibody. Bottom panels, IB with anti-FLAG. (C) Binding of Siglec-8 (blue) and Siglec-6 (orange) to mouse KIT recombinant protein as determined by ELISA. (D) Binding of Siglec-8 (blue) and Siglec-6 (orange) to human KIT. (E) Fold-change of binding of deglycosylated KIT to Siglec-8 compared to untreated (glycosylated KIT) to Siglec-8. Data are plotted as mean ± SD (3–7 biological replicates for Panel C–E). *$p < 0.05$; ***$p < 0.001$ by Mann–Whitney *U* test.

histamine, as well as significantly reduced infiltration of MCs into the peritoneal cavity (Figure 6B and Figure S7A–D). Anti-Siglec-8 mAb administration prior to SCF injection in Siglec-8 transgenic mice showed trends in inhibiting SCF-mediated MC activation and peritoneal infiltration of MCs but had no effect on serum histamine (Figures 6C and Figure S7B–D). The inhibitory effect of anti-Siglec-6 on SCF-mediated MC activation was greater than anti-Siglec-8 in their respective transgenic mouse models (Figure 6D). These data suggest that an anti-Siglec-6 mAb is a better inhibitor of SCF activation of MCs than anti-Siglec-8.

Lastly, human primary MCs (hMCs) were used to mechanistically investigate Siglec-6 mediated inhibition of SCF activation. Incubation of hMCs with recombinant human SCF induced STAT3 phosphorylation and translocation to the nucleus (Figure S7E), which was strongly attenuated following treatment with an anti-Siglec-6 mAb (Figure 6E,F). SCF also induced phosphorylation of

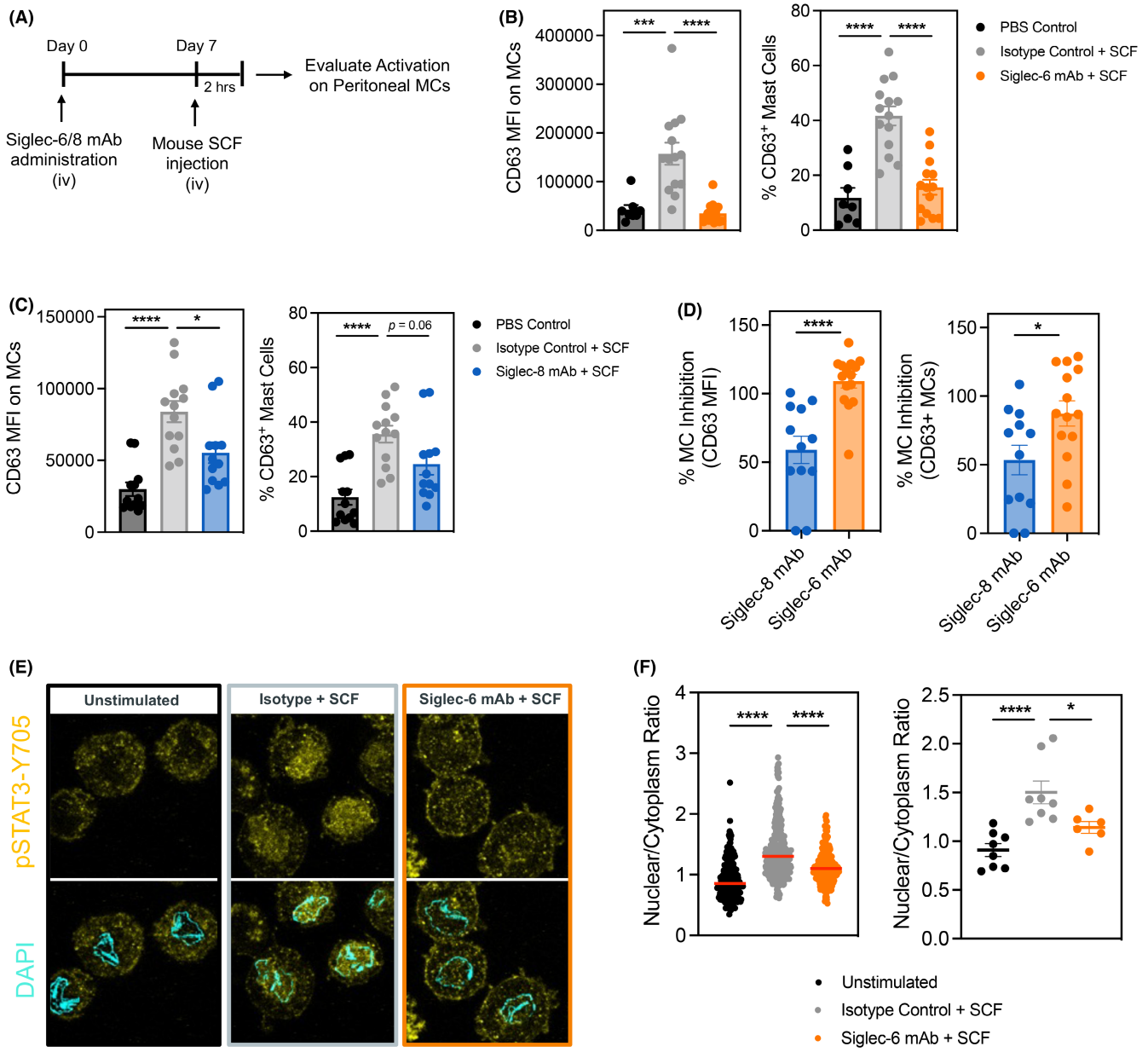


FIGURE 6 Anti-Siglec-6 mAb treatment inhibits SCF-mediated MC activation. (A) Schematic of SCF-mediated MC activation model in Siglec-6 and Siglec-8 transgenic mice (B) CD63 MFI or percentage of CD63+ peritoneal MCs in mice intraperitoneally dosed with 10 mg/kg anti-Siglec-6 mAb (orange) or isotype control (gray) and subsequently intravenously administered 50 μg/kg recombinant mouse SCF or PBS (black). (C) CD63 MFI or percentage of CD63 positive peritoneal MCs in mice intraperitoneally dosed with 10 mg/kg anti-Siglec-8 mAb (blue) or isotype control (gray) and subsequently intravenously administered 50 μg/kg recombinant mouse SCF or PBS (black). (D) Percent inhibition of CD63 MFI or CD63 positive MCs in mice administered SCF and treated with a Siglec-6 (orange) or Siglec-8 (blue) mAb. (E) Representative images of pSTAT3 and DAPI and (F) nuclear to cytoplasm ratio of pSTAT3 in hMCs unstimulated (black) or stimulated with recombinant human SCF in the presence of isotype control (gray) or anti-Siglec-6 mAb (orange). Data are plotted as mean ± SEM (Panel B–D: pooled from two independent experiments with $n = 8$–14 mice/group; Panel F: $n = 2$ independent donors) $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ by one-way ANOVA with Tukey multiple-comparisons test or Mann Whitney *U* test.

p38 (another KIT signaling pathway), but anti-Siglec-6 mAb did not modulate this pathway (Figure S7F), suggesting Siglec-6 does not inhibit all pathways downstream of KIT. These data demonstrate that while engagement of either Siglec can inhibit SCF-mediated activation of MCs, anti-Siglec-6 mAb resulted in a more robust inhibitory effect, possibly explained by the underlying differences in protein interactions described above.

4 | DISCUSSION

Identification of protein–protein interactions is critical to understanding complex cellular functions, providing in-depth knowledge of how cells respond to external signals. Inhibitory receptors are important gatekeepers of activating signals in immune cells and represent an emerging class of therapeutic targets for cancer and

inflammatory diseases. While the function of inhibitory receptors has been widely studied, mechanisms for regulating cellular activation are not well understood. This topic is of great importance since the Siglec-6 and Siglec-8 inhibitory ITIM receptors have shown promise as therapeutic targets for allergic and inflammatory diseases. Antibodies targeting these receptors are in clinical development, with the goal of dampening immune responses in patients with MC-related diseases. Our study examines and compares the inhibitory mechanisms conferred by these two important receptors.

We discovered an extensive array of interacting proteins for both Siglec-6 and Siglec-8 in MCs, suggestive of a broad regulatory role in MC activation and, consistent with previous studies, showing inhibition of both ITAM- and non-ITAM-containing activating receptors.[11,13–15] For example, the interaction between Siglec-8 and IL-1RL1 (IL-33 receptor) aligns with data demonstrating that treatment with an anti-Siglec-8 mAb inhibits IL-33-mediated MC activation.[13] Similarly, anti-Siglec-6 mAb inhibited IgE-mediated IL-4 production, and an anti-Siglec-8 mAb has been shown to reduce TGFβ levels, consistent with its interaction with IL4R and TGFBR2, respectively.[13,20] Additionally, the effects of IL-3, IL-5, and GM-CSF on Siglec-8 signaling have been studied in eosinophils and align with the interaction with CSF2RB.[26] Importantly, the interaction with broad-acting signaling molecules, such as SHP-1/2, STAT5, JAK, and LYN, highlights a potential mechanism for broad inhibition by Siglec-6 and Siglec-8 against different non-ITAM receptors, which are key molecules of cytokine and growth factor signaling cascades. Notably, the majority of the Siglec-6 and Siglec-8 interacting proteins found on the surface of MCs were members of the Ig superfamily, suggestive of an evolutionarily conserved mechanism to regulate cell activation. Although our interactome findings align with previously published functional data obtained from mouse and human mast cells, our methods relied on identifying protein interactions in transgenic Siglec-6 or -8 mice and will need additional validation in human mast cells that endogenously express Siglecs.

We observed interactions between inhibitory and activating membrane receptors in whole cell lysates that were subsequently confirmed using co-IP with different antibodies, transfection of cells with plasmids encoding human receptors, and identification of interactions in large (>1000 kDa) protein complexes. However, we cannot rule out that vesicles or membrane fragments containing multiple receptors were pulled down in the co-IP experiments. Nevertheless, our approach was validated by the presence of multiple intracellular signaling molecules, such as the well-established ITIM motif-binding phosphatases, SHP-1 and SHP-2, and previously reported interactions with activating receptors, such as FcεRI, in the Siglec co-IPs in this study.[16,20] We previously showed that phosphorylation of ITIM motifs in Siglec-8 is required for interaction with SHP-2 and that ITIMs are required for inhibition of FcεRI activation.[16] While we have not investigated ITIM motifs in the context of SCF signaling, the observed direct interactions with signaling molecules, such as members of the JAK/STAT cascade, suggest it is possible that Siglec receptors modulate responses independent of ITIM phosphorylation.

Siglec-6 and Siglec-8 interacted with different subunits of FcεRI, suggesting functional differences. Siglec-6 co-IPs contained alpha and beta subunits of FcεRI, whereas FcεRIγ was the only subunit that interacted significantly with Siglec-8. Our human and mouse MC data suggest more complete inhibition of IgE-mediated MC activation via Siglec-6 than Siglec-8. Notably, the Siglec-6, but not Siglec-8, interactome contained multiple proteins involved in the pyruvate dehydrogenase (PDH) complex (DLAT, PDHA1, and PDHX), a key regulator of the Krebs cycle and mitochondrial dynamics crucial for MC function.[27] Inhibition of PDH compromises MC function, including FcεRI-mediated degranulation and cytokine production.[28] Differences in MC inhibition between Siglec-6 and Siglec-8 might also be influenced by antibody epitope and receptor cluster size, which are important agonistic antibody characteristics for Siglec-6 and other inhibitory receptors.[6,20] Additional studies are needed to elucidate specific mechanisms that contribute to differential FcεRI-inhibition via Siglec-6 and Siglec-8.

While both Siglecs immunoprecipitated KIT, only Siglec-6 interacted with the fully glycosylated, mature cell surface receptor, while Siglec-8 interacted with the less glycosylated intracellular species of KIT. These observations help explain why anti-Siglec-6 mAb treatment resulted in more effective inhibition of SCF-induced activation and the overall broader MC inhibition through Siglec-6. Indeed, profound Siglec-6-mediated inhibition was observed in an in vivo model of KIT activation and in human MCs. Because anti-Siglec-6 mAb administration has previously been shown to reduce MC numbers, we cannot rule out the possibility that both cell depletion and ITIM inhibition contribute to Siglec-6-dependent attenuation of SCF-mediated MC activation. Interestingly, anti-Siglec-6 mAb treatment did not affect MC survival in vitro, but did reduce SCF-mediated activation, suggesting Siglec-6 inhibition is pathway-specific. KIT activation triggers several signaling pathways (e.g., JAK/STAT, RAS/MAPK, and PI3K) that may or may not interface with Siglec-6.[29] Our interactome data showed Siglec-6 interacted with JAK1 and STAT5A, and functionally, anti-Siglec-6 mAb treatment inhibited the translocation of STAT3, but not p38 phosphorylation upon SCF activation, suggesting Siglec-6 regulates specific pathways of SCF-mediated MC activation.

In conclusion, by leveraging the potential of proteomics to discover unexpected and functionally relevant relationships between cell surface receptors and cellular pathways, we identified interactional differences that help explain variations in the mechanistic activity of Siglecs. Our findings suggest Siglec-6 has a distinct role from Siglec-8 in regulating MC function and represents a potential therapeutic target in MC-driven diseases.

AUTHOR CONTRIBUTIONS

WK, ZB, AW, KC, RS, NDF, and TL conducted the experiments; WK, ZB, JL, and JS designed the experiments; GLN analyzed the proteomics data; WK and BAY wrote the paper.

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CONFLICT OF INTEREST STATEMENT

WK, ZB, AW, KC, JL, ND, TL, JS, and BAY are employees of and/or own stock options in Allakos, Inc. GLN is a consultant for Allakos, Inc.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[30] partner repository with the dataset identifier PXD043852.

ORCID

Wouter Korver https://orcid.org/0009-0003-1869-2631
Zachary Benet https://orcid.org/0000-0002-8679-7545
Alan Wong https://orcid.org/0009-0001-9310-4102
Gian Luca Negri https://orcid.org/0000-0001-7722-8888
Katherine Chang https://orcid.org/0009-0003-0390-8479
Robert Sanchez https://orcid.org/0000-0002-0789-1737
John Leung https://orcid.org/0009-0008-4352-8557
Naomi De Freitas https://orcid.org/0000-0001-8697-0451
Thuy Luu https://orcid.org/0000-0003-3068-2121
Julia Schanin https://orcid.org/0009-0000-7422-5403
Bradford A. Youngblood https://orcid.org/0000-0002-2138-2031

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SUPPORTING INFORMATION

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

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