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Studies on the Detection, Expression, Glycosylation, Dimerization, and Ligand Binding Properties of Mouse Siglec-E^{*S}

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Edited by Amanda F. Fosang

CD33-related Siglecs are a family of proteins widely expressed on innate immune cells. Binding of sialylated glycans or other ligands triggers signals that inhibit or activate inflammation. Immunomodulation by Siglecs has been extensively studied, but relationships between structure and functions are poorly explored. Here we present new data relating to the structure and function of Siglec-E, the major CD33-related Siglec expressed on mouse neutrophils, monocytes, macrophages, and dendritic cells. We generated nine new rat monoclonal antibodies specific to mouse Siglec-E, with no cross-reactivity to Siglec-F. Although all antibodies detected Siglec-E on transfected human HEK-293T cells, only two reacted with mouse bone marrow neutrophils by flow cytometry and on spleen sections by immunohistochemistry. Moreover, whereas all antibodies recognized Siglec-E-Fc on immunoblots, binding was dependent on intact disulfide bonds and *N*-glycans, and only two antibodies recognized native Siglec-E within spleen lysates. Thus, we further investigated the impact of Siglec-E homodimerization. Homology-based structural modeling predicted a cysteine residue (Cys-298) in position to form a disulfide bridge between two Siglec-E polypeptides. Mutagenesis of Cys-298 confirmed its role in dimerization. In keeping with the high level of 9-*O*-acetylation found in mice, sialoglycan array studies indicate that this modification has complex effects on recognition by Siglec-E, in relationship to the underlying structures. However, we found no differences in phosphorylation or SHP-1 recruitment between dimeric and monomeric Siglec-E expressed on HEK293A cells. Phylogenomic analyses predicted that only some human and mouse Siglecs form disulfide-linked dimers. Notably, Siglec-9,

the functionally equivalent human paralog of Siglec-E, occurs as a monomer.

Sialic acid-binding immunoglobulin-like lectins (Siglecs)⁵ are cell surface receptors of the I-type lectin family (1) that are expressed on innate immune cells and bind to sialylated and non-sialylated ligands (2–4). There are two types of Siglecs, inhibitory and activating, named for their effect on innate immune cells upon ligand binding. Inhibitory Siglecs contain an immunoreceptor tyrosine-based inhibitory motif in the intracellular domain that is phosphorylated upon ligand binding. This typically results in an intracellular signaling cascade that promotes a quiescent state of immune cells. Activating Siglecs carry a positively charged residue in their transmembrane domain and recruit DAP12 adaptor (5), which contains a cytosolic immunoreceptor tyrosine-based activation motif, generating signals that activate the cell (2, 3). An existing conceptual model suggests that activating Siglecs may have evolved to counteract pathogens that exploit inhibitory signals. By binding inhibitory Siglecs, pathogens can suppress the innate immune response. Activating Siglecs have similar binding properties but opposite signaling, and thus may prevent pathogen exploitation (6–8).

Two broad groups of Siglecs are identified based on their patterns of evolution. Sialoadhesin, CD22, myelin-associated glycoprotein (MAG) and Siglec-15 are highly conserved and show few changes among species (2). In contrast, CD33-related Siglecs evolve rapidly in mammals (2, 9). As there are not clear functional orthologs between primates and rodent Siglecs, these receptors are assigned an alphabetic letter in rodents and a number in primates. Siglec-E, the main CD33-related Siglec in mouse, was first discovered by a yeast two-hybrid screen using SHP-1 as a bait (10). Studies using a sheep polyclonal Siglec-E antibody specific for the ectodomain indicated that Siglec-E is expressed on neutrophils, macrophages, monocytes, dendritic cells, and a subset of Natural killer cells (11). The organ with the

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^S This article contains supplemental Table S1.

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⁵ The abbreviations used are: Siglecs, sialic acid-binding immunoglobulin-like lectins; CD, cluster of differentiation; SHP-1, Src homology region 2 domain-containing phosphatase-1; AUS, *Arthrobacter ureafaciens* sialidase; PNGase F, peptide *N*-glycosidase F; IHC, immunohistochemistry; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

Siglec-E Antibodies and Dimerization

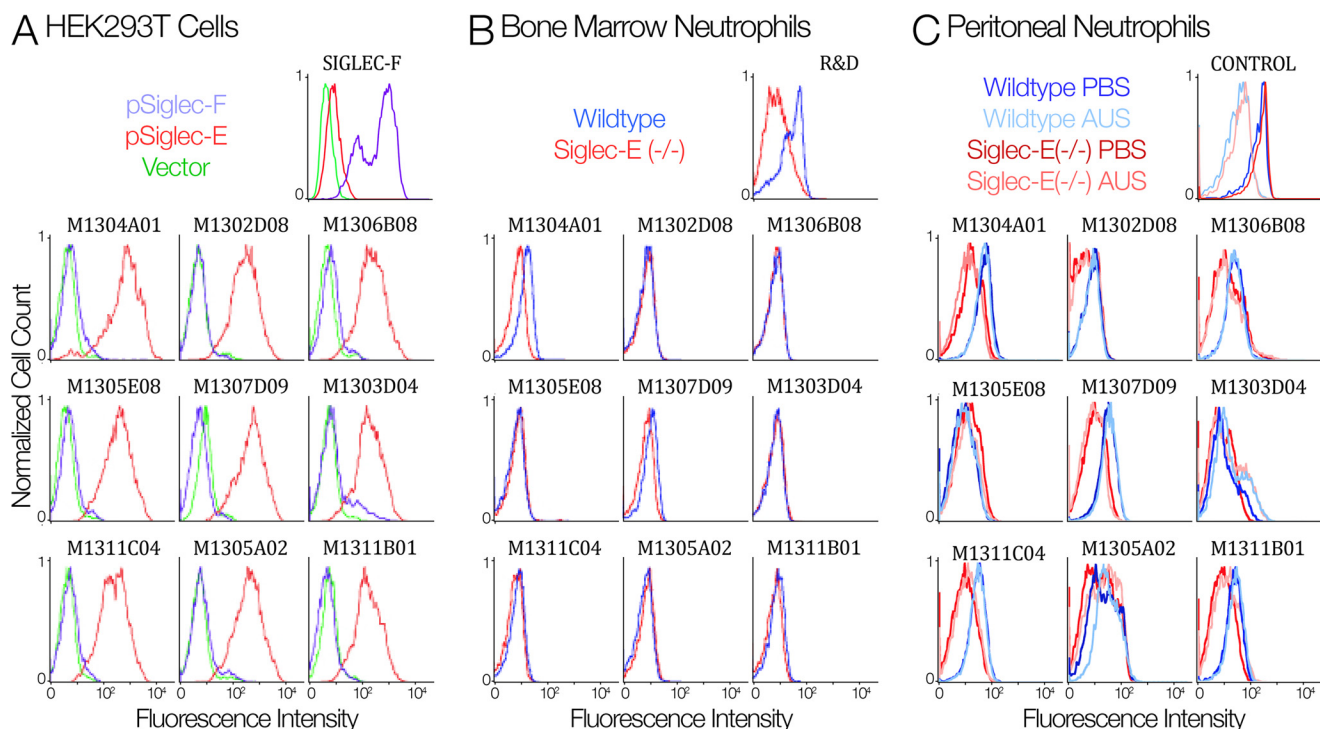


FIGURE 1. Reactivity of monoclonal Siglec-E antibodies to HEK293T cells and *ex vivo* isolated neutrophils. *A*, HEK293T cells transfected with empty vector pcDNA3.1 (green), Siglec-E construct (red), or Siglec-F construct (blue) were stained with anti-Siglec-E antibodies. Flow cytometry was performed to confirm the expression of Siglec-E on 293T cells ($n = 3$). There is no cross-reactivity of these antibodies with Siglec-F transfected cells. Next, HEK293T cells were transfected with plasmids encoding Siglec-F, Siglec-E, or an empty vector control pcDNA3.1. Expression was assessed using Siglec-F antibodies (*A*, *Siglec-F* panel). *B*, bone marrow neutrophils from wild type (blue) or Siglec-E^{-/-} (red) mice were stained with anti-Siglec-E antibodies. Commercially available goat anti-mouse polyclonal R&D antibody was used as positive control. *C*, peritoneal neutrophils were isolated and stained with different Siglec-E antibodies with and without AUS treatment. The desialylation of cells by AUS was confirmed by FACS using Siglec-9-Fc as a probe (*C*, *Control* panel).

highest level of Siglec-E is spleen. Siglec-E consists of 467 amino acids, it has a predicted molecular mass of ~55 kDa and 10 putative *N*-glycosylation sites (10). In SDS-PAGE, its apparent molecular mass is 75–80 kDa under reducing conditions and ~150–200 kDa under non-reducing conditions (10). Therefore, Siglec-E is likely expressed as a disulfide-linked dimer on the cell surface.

Upon lipopolysaccharide (LPS) stimulation, Siglec-E down-regulates Toll-like receptor signaling in a MyD88-dependent manner (12). In a recent study, it was shown that many Siglecs (including Siglec-E) can bind to sialic acids on Toll-like receptors and could potentially act as a brake to slow down or resolve inflammation (13). Removal of sialic acids with sialidase Neu1 can disrupt this interaction, resulting in activation of inflammatory processes. LPS stimulation induces exaggerated recruitment of neutrophils in the lung in Siglec-E knock-out mice (14, 15). In mouse models of bacterial infection (16), tumor growth (17), and sepsis (18), Siglec-E plays a critical role in the pathogenesis of diseases. Siglec-E also modulates oxidative stress that leads to aging (15).

Many of these studies deciphering the role of Siglec-E involved the use of Siglec-E knock-out mouse (14). No blocking/non-blocking antibody pair specific to Siglec-E is available at present. Polyclonal antibodies specific for the ectodomain of Siglec-E were first developed to determine the expression in organs and cells (11). Later, a murine monoclonal antibody was used to detect Siglec-E followed by immunoprecipitation with an anti-phosphotyrosine antibody (4G10) (12). Some commercially available antibodies have been used to detect Siglec-E in

transfected Chinese hamster ovary (CHO) cells (19) and mouse microglia (20). Polyclonal Siglec-E antibody from R&D Systems (goat anti-Siglec-E) has been used in multiple studies (15–17). In a recent study, Siglec-E antibody was used to block the interaction of Siglec-E with sialylated ligands (18). In addition to antibodies that have been described in the literature, the ones that are commercially available include those from LifeSpan Biosciences (goat polyclonal Siglec-E and clone 8D2 rat monoclonal Siglec-E).

In this study, we generated nine new rat monoclonal antibodies against the extracellular domain of mouse Siglec-E. Two of these clones recognized Siglec-E on transfected and primary cells by flow cytometry, Western blot, and immunohistochemistry. Moreover, we characterized two pairs of blocking and non-blocking antibodies that can be used for studying binding of sialylated ligands. Last, we identified the cysteine residue that allows Siglec-E to form a dimer, and studied the effects of dimerization on ligand binding and cell signaling.

Results and Discussion

Reactivity of Monoclonal Antibodies Generated against Siglec-E—Immunization of rats with Siglec-E-Fc generated nine monoclonal antibodies specific for Siglec-E-Fc and did not react with human IgG. Using flow cytometry, human HEK293T cells expressing Siglec-E or Siglec-F were tested for reactivity and specificity for full-length Siglec-E. All newly generated monoclonal antibodies showed binding to the transfected cells expressing Siglec-E but not Siglec-F, thus demonstrating their specificity for Siglec-E (Fig. 1A). We also transfected Siglec-F

into HEK293T cells, and these cells showed binding to Siglec-F when checked with Siglec-F antibody. The non-reactivity of monoclonal Siglec-E antibodies to Siglec-F is thus not due to a problem with the transfection (Fig. 1A, *Siglec-F* panel). However, when these antibodies were tested on primary mouse neutrophils isolated from bone marrow, only clone number M1304A01 showed appreciable binding, and clone M1307D09 showed partial binding (Fig. 1B). Next, we analyzed these antibodies for binding to primary mouse peritoneal neutrophils, and found that four antibodies (clones M1304A01, M1307D09, M1311C04, and M1311B01) bound to these cells (Fig. 1C). We reasoned that the variability in binding might be due to engagement of Siglec-E by cis ligands expressed on the cell surface. However, we did not detect any difference in binding of these antibodies following pre-treatment of the cells with *Arthrobacter ureafaciens* sialidase (AUS), which cleaves sialic acids from the cell surface (Fig. 1C). The desialylation activity of AUS was confirmed by fluorescence-activated cell sorting (FACS) using Siglec-9-Fc as a probe (Fig. 1C, *Control panel*). It remains uncertain why all antibodies were able to detect epitopes on transfected human cells *in vitro* but only a few worked on *ex vivo* isolated mouse cells using flow cytometry. One possibility is that most Siglec-E proteins are present in intracellular compartments rather than on the cell surface. However, flow cytometry on mouse splenocytes with and without permeabilization shows that there is no Siglec-E in an intracellular compartment (Fig. 2B). We next tested these antibodies in immunohistochemistry using spleens of wild type and Siglec-E knock-out mice. The same two clones (M1304A01 and M1307D09) that detected Siglec-E on bone marrow neutrophils stained macrophages in the spleen of wild type animals, but not Siglec-E-null mice (Fig. 2A). Thus epitopes of all nine antibodies are fully accessible when expressed on HEK293T cells, but most are variably masked in *ex vivo* isolated mouse cells, and in spleen sections on immunohistochemistry (IHC).

Characterization of Blocking and Non-blocking Action of Antibodies—We further characterized the ability of these antibodies to block the interaction of Siglec-E-Fc with sialylated ligands. Two of nine antibodies (M1305E08 and M1305A02) blocked this interaction (Fig. 3, A and B). We confirmed the blocking properties of these two antibodies using plates coated with protein A, or with anti-human IgG Fab, to rule out any possibility that protein A directly interacted with Siglec-E antibodies. Two other antibodies (M1304A01 and M1303D04) were found to be non-blocking presumably because their epitopes are outside the sialic acid-recognizing V-set domain of Siglec-E (Fig. 3, A and B). These pairs of blocking and non-blocking antibodies can be used for the *in vitro* receptor blocking experiments, but apparently not for *in vivo* studies.

Complex Interactions of Antibodies in Western blots—Next, we characterized these antibodies using Western blotting analysis of recombinant Siglec-E-Fc or spleen cell lysates. We compared reducing and non-reducing gels to determine whether the epitopes of these antibodies are disulfide bond dependent. Furthermore, PNGase F treatment was used to investigate dependence of antibody binding on *N*-glycans. We found that all nine antibodies detected recombinant Siglec-E-Fc under non-reducing conditions (Fig. 4, 3rd panel). However, only one anti-

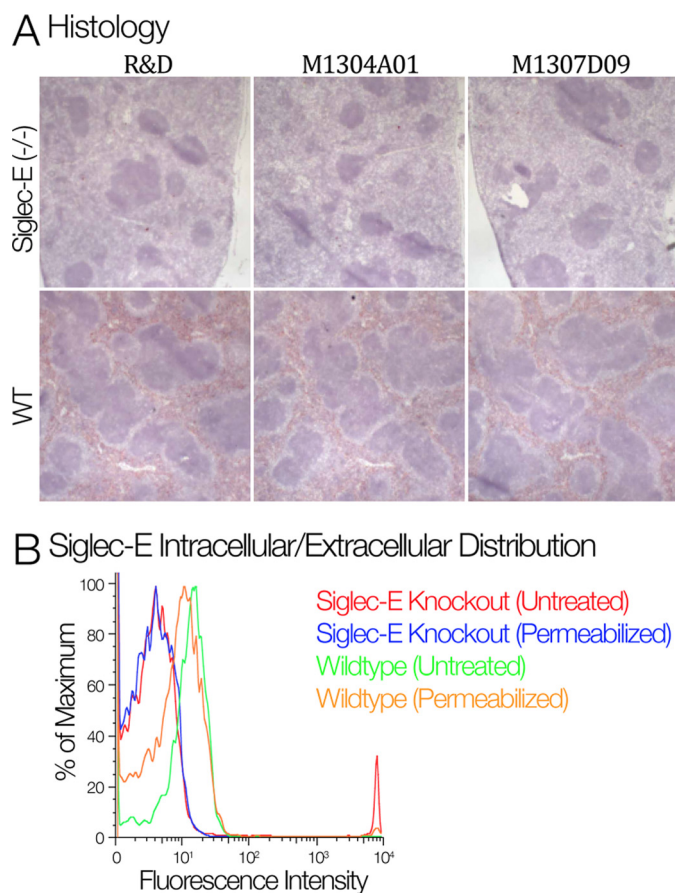
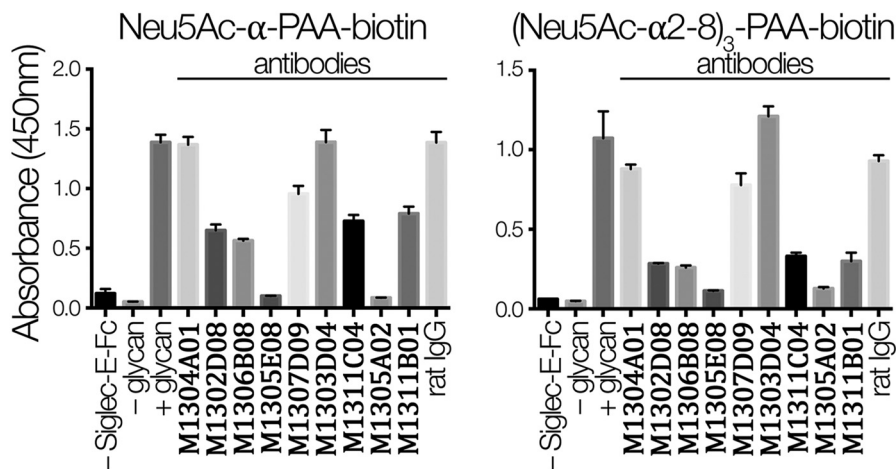


FIGURE 2. Reactivity of Siglec-E antibodies to spleen sections on IHC. A, the Siglec-E antibodies (M1304A01 and M1307D09) were used along with commercially available R&D antibody on spleen sections. B, expression of Siglec-E on the cell surface and intracellular was monitored by flow cytometry of permeabilized or non-permeabilized spleen macrophages. Splenocytes from Siglec-E knock-out mouse were used as a negative control for baseline expression.

body (M1307D09) showed strong binding after disulfide bond reduction, and another antibody (M1304A01) showed weak binding (Fig. 4, *bottom panel*). Pretreatment of Siglec-E-Fc with PNGase F to cleave *N*-glycans showed that most binding was also dependent on *N*-glycans (Fig. 4, *2nd panel*). Notably, the same antibodies (M1307D09 and M1304A01) that detected Siglec-E-Fc on Western blots after disulfide bond reduction also detected it after the PNGase F treatment. The other seven antibodies detected the epitope with relatively weaker binding. The lysates of spleen from WT mice were subjected to Western blotting analyses and only two antibodies (M1304A01 and M1307D09) showed strong binding (Fig. 4, *top panel*). The reason for these variations in antibody binding and their biological relevance remains unknown. Regardless, only two antibodies (M1307D09 and M1304A01) have a cognate epitope that does not depend on species of cell type, denaturation, disulfide bonds, or *N*-glycans. The summary of all results related to Siglec-E antibodies is shown in Table 1.

Cysteine 298 of Siglec-E Is Involved in Dimerization—A poorly explored area of Siglec structural biology is the existence of disulfide-mediated dimerization and oligomerization. We searched the literature and public databases to determine which Siglecs are known to exist as monomers, disulfide-linked

A ELISA | Protein A Coating



B ELISA | Anti Human IgG Fab

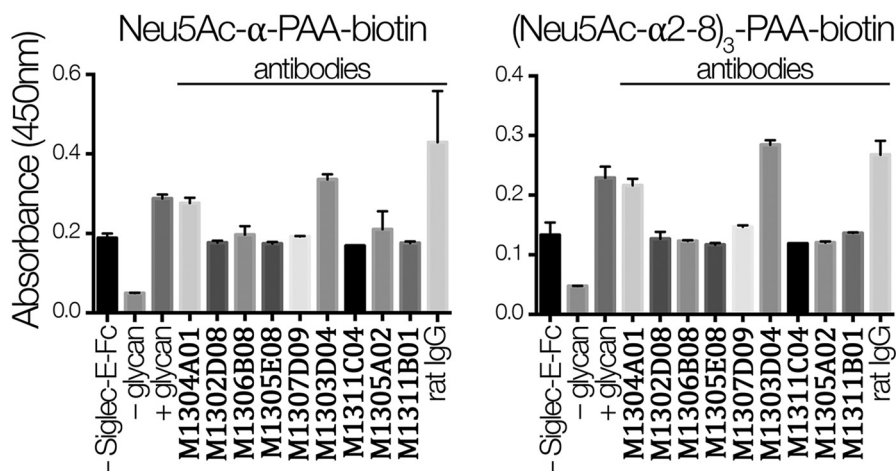


FIGURE 3. **Characterization of Siglec-E antibodies as blocking and non-blocking.** A, the plates were coated with protein A and the interaction of Siglec-E with sialylated ligands was assessed by ELISA in the presence of each monoclonal Siglec-E antibody. B, the ELISA plates were coated with anti-human IgG Fab and the reactivity of all nine monoclonal antibodies with sialylated ligands were tested. The data show mean absorbance at 450 nm and error bars show standard deviation.

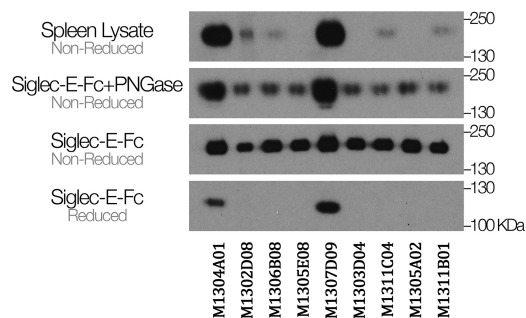


FIGURE 4. **Western blot with Siglec-E antibodies under reducing or non-reducing conditions.** Western blot with nine Siglec-E antibodies using recombinant Siglec-E-Fc under non-reducing conditions, under reducing conditions, non-reducing condition after PNGase-F treatment, and on spleen lysate of WT mice under non-reducing conditions was performed.

dimers, or multimers (Table 2). CD22 is reported to be a multimer (21–23). Siglec-5 (24), Siglec-8 (25), and Siglec-11 (26) are thought to be disulfide-linked dimers. In contrast, myelin-asso-

ciated glycoprotein (27), Siglec-7 (28), and Siglec-9 (this study, see below) exist as monomers. The oligomerization state of many other Siglecs remains unknown (Table 2). Siglec-E is expressed as a disulfide-linked dimer on the cell surface of 293A cells (10). We were interested in finding out which cysteine residue is involved in dimerization, and made a prediction based on three-dimensional structures using homology models and computational docking. Models based on the crystal structure of Siglec-5 (29) show that all six cysteines in V-set and C2set1 domains form disulfide bonds with each other either within a domain or between the domains (Fig. 5A). None of the six cysteines in the first two domains is free to make a disulfide linkage between Siglec-E molecules (Fig. 5A). Models of the C2set2 domain of Siglec-E show that there is one free cysteine group (position Cys-298) that can potentially bond with the cysteine of another Siglec-E molecule (Fig. 5A). We mutated this cysteine to alanine (C298A) in Siglec-E-Fc and released the Fc portion from Siglec-E by Xa protease digestion

TABLE 1
Summary of results with rat anti-mouse Siglec-E antibodies

Clone	Flow cytometry				IHC		Western blot			
	HEK293 transfectant	Bone marrow neutrophil	Thioglycollate-elicited peritoneal Neutrophil	Peritoneal neutrophils treated with sialidase	(spleen)		Reducing (recombinant protein 0.05 µg/ml)	Non-reducing (recombinant protein)	Non-reducing (de-glycosylated recomb protein 1.5 µg/ml)	Spleen non-reducing
M1304A01	Pos ^a	Pos	Pos	Pos	Pos	Weak	Strong + + +	Weak	Pos	None
M1302D08	Pos	Neg	Neg	Neg	Neg	Weak	Strong + +	Very weak	Pos	Weak
M1306B08	Pos	Neg	Neg	Neg	Neg	Neg	Strong + + +	Very weak	Very weak	Weak
M1305E08	Pos	Neg	Neg	Neg	Neg	Neg	Strong + +	Neg	Neg	Strong
M1307D09	Pos	Weak +	Pos	Pos	Pos	Pos	Strong + + +	Weak	Pos	Weak
M1303D04	Pos	Neg	Neg	Neg	Neg	Neg	Strong + +	Very weak	Neg	None
M1311C04	Pos	Neg	Weak	Weak	Neg	Neg	Strong + + +	Very weak	Very weak	None
M1305A02	Pos	Neg	Neg	Neg	Neg	Neg	Strong + + +	Very weak	Neg	Strong
M1311B01	Pos	Neg	Weak	Weak	Neg	Neg	Strong + + +	Very weak	Very weak	Weak

^a Pos, positive; Neg, negative.

of a cleavage site that had been engineered into the original construct. When probed on a non-reducing gel (Fig. 5B) a dimer of WT Siglec-E and monomer of mutant Siglec-E was released by Xa digestion. This confirms that Cys-298 is involved in the dimerization of Siglec-E. Computational docking between two identical Siglec-E C2set2 domains confirms the possibility of a dimer conformation that places these cysteine residues in close proximity with the C2set2 domains oriented in the same direction (Fig. 5C). Thus, we pinpointed a residue involved in the formation of intermolecular disulfide bonds between two molecules of Siglec-E. Interestingly, alignments of Siglec-E (Siglec-7) across mammals show that Cys-298 is present in some species (rodents, carnivores) and absent in others (primates). The cysteine residues that form disulfide linkages within and between domains are broadly conserved (Fig. 5D).

C298A Mutation Marginally Affects Ligand Binding and Signaling—Given that one cysteine mutation converted a dimer of Siglec-E into monomer, we asked what effects this change might have on expression and ligand binding preferences. We expressed WT and mutant (C298A) full-length Siglec-E in HEK293A cells and found no difference in the cell surface expression (Fig. 6B), suggesting that the mutation does not affect protein folding and trafficking. Therefore, we compared the binding properties of WT Siglec-E-Fc with mutated C298A Siglec-E-Fc on an updated version of a previously described sialoglycan array (30), containing multiple sialoglycan targets (supplemental Table S1). The overall binding profiles are comparable (Fig. 6A); however, there are several noteworthy differences in binding. Multimeric interactions with disialyl and trisialyl probes differ markedly in their binding to the WT and mutant Siglec-E-Fc. Given the importance of multimeric interactions in glycan recognition, these differences could induce effects on cellular behavior. Comparison of the binding between glycan array spots with or without sialic acid 9-O-acetylation showed that this modification has complex effects on recognition by Siglec-E, in relationship to the underlying linkages and glycan structures (in some instances, there is enhanced binding, and in others binding is decreased). Given the lability of these ester groups, further studies will be needed to precisely define the details of these effects. Regardless, considering the high level of 9-O-acetylation found in mice and the complex regulation of this modification, it is likely that these differences will be biologically significant.

Next, we asked if there was any difference in expression and cell signaling between monomeric and dimeric Siglec-E when transfected. Expression of both native and mutant Siglec-E was comparable in HEK293A cells (Fig. 6B). We also performed immunoprecipitation of Siglec-E followed by Western blot with anti-phosphotyrosine and SHP-1. No difference was found in the phosphorylation status of Siglec-E between the monomeric and dimeric forms (Fig. 6C). Moreover, the SHP-1 recruitment was also not different between the two forms (Fig. 6C). This could be because the analysis was done in 293A cells but Siglec-E has immunomodulatory functions only in immune cells. Unfortunately, due to the difficulty of obtaining stable expression, we could not do this analysis in macrophage-like THP1 and RAW264.7 cells.

Siglec-E Antibodies and Dimerization

TABLE 2

Dimerization status of human and murine Siglecs and number of cysteines in domains

The number of cysteines was determined by searching through the amino acid sequences on the Uniprot website.

Siglec	Oligomerization	Disulfide linked?	V-set	C2-set 1	C2- set 2	C2- set 3	C2- set 4	References
Sn (1)	Unknown	Unknown	3	3	2	2	2	
CD22 (2)	Multimer	No	3	3	3		4	21–23
CD33 (3)	Dimer	Yes	3	3				
MAG (4)	Monomer	NA	3	3	2	2	4	27
Siglec-5	Dimer (neutrophils, WB)	Yes	3	3	2	5		24
Siglec-6	Unknown	Unknown	3	3	2			
Siglec-7	Monomer (CHO cells)	NA	3	4	2			28
Siglec-8	Dimer (CHO cells)	Yes	3	3	3			25
Siglec-9	Monomer (Our finding)	NA	3	4	3			
Siglec-10	Unknown	Unknown	3	3	2	3		
Siglec-11	Dimer	Yes	3	3	2	2		26
Siglec-12	Unknown	Unknown	4/3	3	2			
Siglec-14	Unknown	Unknown	3	3	2			
Siglec-15	Unknown	Unknown	4	2				
Siglec-16	Unknown	Unknown	3	3	2	3		
Siglec-E	Dimer (293A cells)	Yes	3	3	3			10
Siglec-F	Unknown	Unknown	3	3	2			
Siglec-G	Unknown	Unknown	?	?	?	?		
Siglec-H	Unknown	Unknown	?	?				

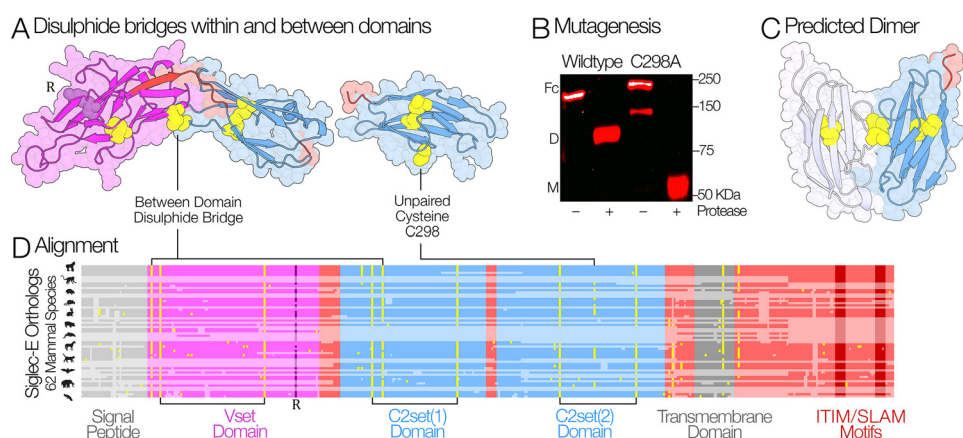


FIGURE 5. Siglec structural models identify cysteines involved in dimerization. *A*, homology models of Siglec-E based on the crystal structure of Siglec-5 (Protein Data Bank code 2ZG2). Cysteines are shown in yellow, the arginine residue (R) that is critical for sialic acid binding is shown in purple. Cysteines form disulfide bridges between the V-set domain (purple) and the first C2set domain (blue) as well as within each of these domains. The C2set2 domain has an unpaired cysteine (C298). *B*, mutagenesis confirms that Cys-298 is involved in dimerization. Siglec-E-Fc was digested with protease Xa to release monomer or dimer of Siglec-E. The released Siglec-E was probed on Western blot under non-reducing conditions. *First lane* is WT Siglec-E-Fc, *second lane* is WT Siglec-E-Fc after protease Xa digestion, *third lane* is an alanine mutant (A298) Siglec-E-Fc, and *fourth lane* is A298 Siglec-E-Fc after protease Xa digestion. *C*, putative spatial conformation of the C2set2 domain dimer in Siglec-E. Computational docking finds a dimer conformation where Cys-298 residues are in position to form a disulfide bridge between molecules. *D*, Multiz alignments of Siglec-E (and putative orthologs) in 62 mammal species; light-shaded parts of the alignment are missing sequence data. Disulfide bridges within and between domains are broadly conserved, but interestingly the Cys-298 position that controls dimerization between molecules is not evolutionarily conserved.

Human Siglec-9 Exists as a Monomer—In humans, Siglec-9 is the functionally equivalent paralog of mouse Siglec-E. We next expressed full-length Siglec-9 in HEK293A cells and probed the cell lysate after running on reducing and non-reducing SDS-PAGE gels. Siglec-9-Fc was used as a positive control for the blot (Fig. 7A). There was no difference in the size of Siglec-9 between reducing and non-reducing gels. We confirmed this finding on the cell lysate of primary neutrophils isolated from healthy blood donors (Fig. 7B). Thus, it was concluded that unlike Siglec-E, its functional equivalent Siglec-9 is a monomer. Additionally, we also used NetNGlyc and NetOGlyc prediction software to check the predicted *N*- and *O*-linked glycosylation sites in mouse Siglec-E (Fig. 8).

Conclusions and Perspectives—In this study, we generated nine monoclonal antibodies against mouse Siglec-E. Two of the antibodies consistently detected epitopes when using different methods of detection: on flow cytometry (*in vitro* and *ex vivo* isolated cells), Western blot (reducing, non-reducing gel and

PNGase F treatment), and IHC. Although we cannot fully explain the difference between these antibodies and the others we generated, we noticed that, the epitopes of other seven antibodies depended on disulfide bonds and *N*-glycans. We also characterized two pairs of blocking and non-blocking antibodies that can be used *in vitro* to assess Siglec-E function in blocking experiments. Further studies are needed to explain why epitopes of these antibodies are fully accessible on *in vitro* transfected cells but lost on *ex vivo* isolated cells.

Regarding the dimerization of Siglec-E, we identified a critical residue (Cys-298) that is important for disulfide-linked dimerization. There was a small difference in ligand binding and cell signaling between the monomeric and dimeric forms of Siglec-E. Dimerization appears to evolve across mammals, and it will be interesting to explore the relevance of dimerization on Siglec function and immune regulation. This study focused on dimerization by disulfide linkage, but we do not rule out the possibility of non-covalent interactions in Siglec dimerization.

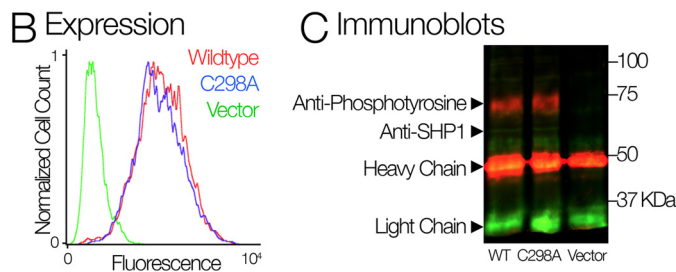
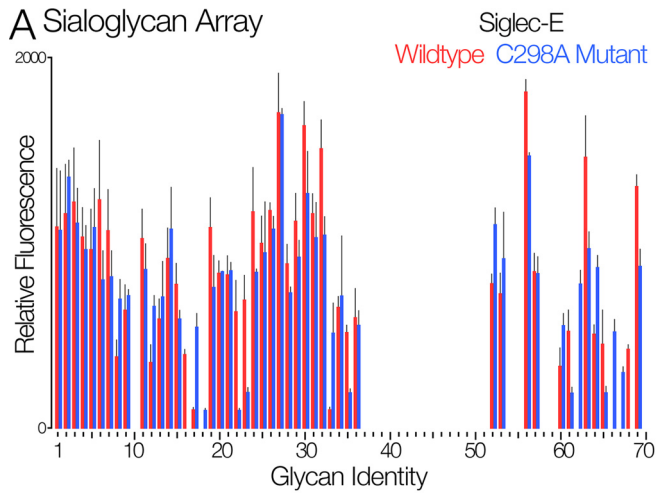


FIGURE 6. Sialoglycan array, immunoprecipitation, and Western blot with WT and mutant Siglec-E to decipher the role in ligand binding and cell signaling. *A*, sialoglycan arrays with WT Siglec-E-Fc and mutant (C298A) Siglec-E-Fc was performed. RFU, relative fluorescence unit. *B*, Siglec-E or mutant Siglec-E (C298A) was transfected in human HEK293A cells and cell surface expression was quantified using FACS. Similar expression of monomeric and dimeric Siglec-E was found on the cell surface. *C*, WT and mutant Siglec-E (C298A) was expressed in HEK293A cells. Siglec-E was immunoprecipitated and probed on Western blots using anti-phosphotyrosine antibody. WT and mutant Siglec-E (C298A) was expressed in HEK293A cells, Siglec-E was immunoprecipitated and probed on Western blot using SHP-1 antibody.

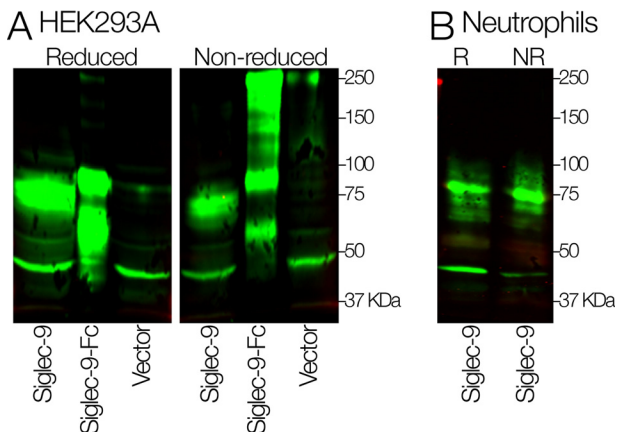


FIGURE 7. Western blot under reduced and non-reduced conditions to determine the dimerization status of Siglec-9. *A*, reducing/non-reducing Western blotting was performed on cell lysate of HEK293A cells expressing Siglec-9. *B*, Siglec-9 exists as a monomer, which was further confirmed with reducing (R)/non-reducing (NR) Western blotting performed on purified human neutrophils.

It is still unclear why some Siglecs exist as monomers whereas others as dimers. It will be interesting to find out the relationship between ligand preference of monomeric and dimeric Siglecs and how this influences their functions as cell surface receptors.

Siglec-E Predicted Glycosylation

QNPQEVFTLNVERKVVVQEGLCVLPVCFNSYLLKRLTDWTDSDPVGFWY	50
REGTDRRKDSIVATNNPIRKAVKETRNRFFLLGDPWRNDCSLNIREIRKK	100
DAGLYFFRLERLGRKTKYNYMWDKMTLVVTALNTNPQILLPETLEAGHPSNL	150
TCSVPWDCGWTAPPIFSWTC	200
TSVSFLSTNTTGS	200
SVLTTITPQPQDHGTNLT	200
CQVTLPGT	250
TVNST	250
RMITIRLNVSYAPKNLTVTIYQGADSVSTILKNGSSLP	250
SEGQSLRLICSTDSYPPANLSWSWDNLTLCPSKLSKPGLELFPVHLKHG	300
GVYTCQAQHALGSQHSLSLSPQSSATLSEMMMG	335

FIGURE 8. Predicted N- and O-linked glycosylation sites in mouse Siglec-E. N-Glycosylation sites (predicted by NetNGlyc) are highlighted in blue and O-glycosylation sites (predicted by NetOGlyc) are highlighted in red.

Experimental Procedures

Purification of Siglec-E-Fc—The DNA sequence corresponding to the extracellular segment of Siglec-E was amplified with primers 5'-ATAACTCGAGCAGAACCCCAAGAGG-GTTTC-3' and 5'-TATTGGATCCTGTGGAGACAGGCTC-AAG-3' from pSPORT6-CMV-Siglec-E (Thermo Scientific clone ID 3662856) and cloned in Signalp IgG Plus A. Plasmid encoding for Siglec-E-Fc C298A was generated by site-directed mutagenesis using primers 5'-GATAACCTGACCCTGGC-CCCATCAAAGTTGTC-3' and 5'-GACAACCTTTGAT-GGGGCCAGGGTCAGGTTATC-3' and a Stratagene Quik-Change II XL Site-directed Mutagenesis Kit. Siglec-E-Fc and mutant Siglec-E-Fc plasmids were transfected into HEK293A cells in serum-free media supplemented with Nutridoma (Roche). Soluble Siglec-E-Fc was purified from cell culture supernatants by affinity chromatography with protein A-Sepharose (GE Healthcare). Proteins were incubated with 25 milliunits of AUS (EY Laboratories) at room temperature for 1 h.

Generation of Rat Antibodies against Siglec-E—Lewis rats were initially immunized with 75 µg of immunogen in Titermax Gold adjuvant (Sigma) followed by three more immunizations with 50 µg of immunogen in Alum adjuvant (Thermo Scientific) and a final boost with 75 µg of immunogen in phosphate-buffered saline (PBS). After fusion of the lymph node B cells with SP2/0 myeloma cells, the hybridomas were screened by enzyme-linked immunoassay (ELISA) on antigen-coated plates. Siglec-E-Fc positive clones that did not show reactivity to human IgG-Fc were subcloned twice and isotyped. Hybridoma clones producing antibodies were harvested and subsequently, monoclonal antibodies were purified by protein G column (Pierce).

Peritoneal Neutrophil Isolation—The peritoneal neutrophils were isolated as described previously by Chen *et al.* (31). Briefly, 1 ml of 3% thioglycollate was injected in the peritoneum of the mice and after 12 h peritoneal exudate was collected using ice-cold PBS. The AUS sialidase treatment of the cells was performed using 25 milliunits of AUS for 15 min at room temperature as described previously (32).

Western Blotting Analysis—For Western blot on recombinant Siglec-E-Fc Chimera (BioLegend, catalogue number 551504), 1.5 to 2.0 µg of recombinant protein was used for gel electrophoresis. For the spleen lysate, 250 to 300 µg of lysate was used. For reducing gels, the samples were mixed with reducing loading buffer (with DTT), and for non-reducing gel

Siglec-E Antibodies and Dimerization

the samples were mixed with non-reducing loading buffer (without DTT). PNGase F (Promega, catalogue number V4831, 40 units) treatment was performed at 37 °C overnight before mixing with the non-reducing loading buffer. For the Western blot on the human neutrophil lysate, 50 ml of human blood was collected from healthy volunteer donors with the informed consent using the protocol approved by IRB, University of California, San Diego, CA. The neutrophils were isolated using polymorphprep solution (Axis-Shield) as described previously (33). The reducing and non-reducing gels were carried out with the neutrophil lysate as described above.

The proteins were resolved in 4–12% BisTris two-dimensional gels (Invitrogen, catalogue number NP0326BOX) and transferred to nitrocellulose membranes at 200 mA for 2 h. The membranes were blocked with 5% milk in Tris-buffered saline and Tween 20 (TBS/T) and were assembled into miniblotted 28 probing sets (Immunetics, Cambridge, MA), which created 20 individual channels onto the membranes. Each rat anti-mouse Siglec-E antibody at 1 µg/ml was added to the individual channel as the primary antibody, and the miniblotter was shaken for 2 h at room temperature. The channels were marked on the membranes before the latter were removed from the miniblotter. The whole membranes were washed 4 times with TBS/T solution and incubated with goat anti-rat IgG secondary antibody conjugated to horseradish peroxidase (BioLegend, catalogue number 405405) for 1 h at room temperature. The membranes were washed, developed with an ECL substrate, and exposed to X-ray film from 2 s to 30 min.

ELISA—High binding flat bottom 96-well plates (Co-star) were coated with protein A (ThermoScientific) or anti-human IgG Fab (Jacksons ImmunoResearch) in 50 mM sodium carbonate/bicarbonate, pH 9.6, overnight at 4 °C. Wells were washed with PBS containing 0.05% Tween 20 (PBST) and incubated with Siglec-E-Fc at room temperature for 1 h. After washing with PBST, Siglec-E antibodies were incubated for 1 h at room temperature. Plates were then washed and incubated with biotinylated sialic acid probes (Glycotect). The biotinylated probes were detected by streptavidin-HRP and TMB substrate (R&D Systems). Absorbance was detected at 450 nm.

Immunohistochemistry—Spleen of WT and Siglec-E^{-/-} mice were isolated and embed in OCT. Five-µm sections were made and used for immunostaining following protocols (mousepheno.ucsd.edu). Briefly, frozen sections were blocked for endogenous peroxidases and biotin and overlaid with Siglec-E antibodies for 1 h at room temperature, in a humid chamber. After washing, sections were overlaid with secondary reagents and then with tertiary reagents, with washes between. Binding was detected using the AEC substrate (Vector Labs), followed by Mayers hematoxylin for nuclear counterstain. The slides were coverslipped using an aqueous mounting medium for viewing, and digital photomicrographs were taken using an Olympus BH2 microscope equipped with Olympus Magnafire camera and arranged for presentation using Adobe photoshop.

Flow Cytometry—HEK293T cells were transfected with a plasmid encoding Siglec-E, Siglec-F, or pcDNA3.1. 48 h after transfection, cells were stained with different Siglec-E antibodies on ice for 30 min. After incubation with primary antibody, cells were washed with PBS and incubated with anti-rat Alexa

Fluor 647 secondary antibody (Invitrogen). Cells were washed after secondary incubation and analyzed by flow cytometry (BD FACSCalibur). For the permeabilization, the FACS, BD cytoperm/cytofix (catalogue number 554714) kit was used. Data were analyzed using FlowJo software.

Glycan Array—The sialoglycan array was performed as described (30). Briefly, slides were blocked with ethanolamine and placed in a microarray hybridization cassette (AH C4X8S, Array, Sunnyvale, CA) to divide them into subarrays. Ovalbumin (1% w/v) in PBS, pH 7.4, was used to block the subarrays. Subsequently, Siglec-E-Fc and mutant Siglec-E-Fc at the concentration of 40 µg/ml was added to each subarray. Samples were incubated with gentle shaking for 2 h at room temperature. The slides were washed, and diluted anti-human IgG-Cy3 antibody in PBS was added to the subarrays. The secondary antibody was incubated at room temperature for 1 h. Slides were then washed, dried, and scanned by Genepix 4000B microarray scanner (Molecular Devices Corp., Union City, CA). Genepix Pro 7.0 analysis software (Molecular Devices Corp.) was used for the analysis.

Immunoprecipitation and Immunoblots—HEK293A cells were transfected with Siglec-E and mutant Siglec-E (C298A). 48 h later, cells were lysed and proteins were incubated overnight with protein G-Dynabeads (Life Technologies) conjugated with anti-Siglec-E antibody (M1307D09). After incubation, beads were washed with TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 8.0) and eluted with 1× Laemmli buffer for 10 min at 100 °C. Proteins were separated on 10% acrylamide gels, transferred on nitrocellulose membrane, and stained with anti-phosphotyrosine (4G10) and SHP-1 (Upstate). To probe the desired protein, secondary antibodies from LI-COR (Lincoln, NE) were used. Odyssey instrument from LI-COR was used to acquire the signal and it was analyzed using Image Studio software (LI-COR).

Homology Modeling of Protein Structures—The three-dimensional structure of mouse Siglec-E was predicted based on an empirical structure of the V-set domain and first C2set domain of Siglec-5 (Protein Data Bank code 2ZG2) (29). Homology models of the V-set and C2set domains were generated in phyre2 (34), and visualized in PyMOL. The potential for cysteine residues to create disulfide bridges between C2set domains was assessed by protein docking using Clus-Pro (35).

Author Contributions—S. Siddiqui and A. Varki largely wrote the manuscript. S. Siddiqui, F. S., and A. V. designed the experiment. S. Siddiqui, F. S., S. Springer, W. J., D. K., J. Z., B. D., Z. K., H. Y., L. D., Y. N.-M., and X. C., N. Varki, and A. Verhagen performed the experiments.

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