Negative Regulation of T Cell Receptor Signaling by Siglec-7 (p70/AIRM) and Siglec-9*

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Siglec-7 (p70/AIRM) and Siglec-9 are “CD33”-related siglects expressed on natural killer (NK) cells and subsets of peripheral T cells. Like other inhibitory NK cell receptors, they contain immunoglobulin receptor family tyrosine-based inhibitory motifs in their cytoplasmic domains, and Siglec-7 has been demonstrated to negatively regulate NK cell activation. Based on reports of the presence of these siglects on T cells, we sought to determine if they are capable of modulating T cell receptor (TCR) signaling using Jurkat T cells stably and transiently transfected with Siglec-7 or Siglec-9. Following either pervanadate stimulation or TCR engagement, both Siglects exhibited increased tyrosine phosphorylation and recruitment of SHP-1. Effects of Siglec-7- and -9 were also evident in downstream signaling pathways. Both siglects reduced phosphorylation of Tyr319 on ZAP-70, known to play a pivotal role in up-regulation of gene transcription following TCR stimulation. There was also a corresponding decreased transcriptional activity of nuclear factor of activated T cells (NFAT) as determined using a luciferase reporter gene. Like all siglects, Siglec-7 and -9 recognize sialic acid-containing glycans of glycoproteins and glycolipids as ligands. Mutation of the conserved Arg in the ligand binding site of Siglec-7 (Arg24) or Siglec-9 (Arg208) resulted in reduced inhibitory function in the NFAT/luciferase transcription assay, suggesting that ligand binding is required for optimal inhibition of TCR signaling. The combined results demonstrate that both Siglec-7 and Siglec-9 are capable of negative regulation of TCR signaling and that ligand binding is required for optimal activity.

The human siglec family of cell receptors is composed of eleven members of the Ig superfamily, which are functionally related by their ability to bind sialic acid-containing carbohydrates of glycoproteins and glycolipids as ligands (1–3). The Siglects are predominately and differentially expressed on a wide variety of white blood cells (2, 3), the notable exceptions being myelin-associated glycoprotein expressed in glial cells (4, 5) and Siglec-6 expressed in placenta (2, 6). The extracellular region has a variable number of C2-set Ig domains and a single homologous N-terminal “V-set” domain that binds to sialic acids (2). Crystal structure analysis of two Siglects, sialedhesin (Siglec-1) and Siglec-7, have revealed a shallow sialic acid binding pocket with a conserved sequence of six amino acids in the tip of the C-C loop that influences the specificity for binding various sialoside sequences found in nature (7–10). Within this sequence, a conserved arginine residue coordinates with the C-1 hydroxyl group of the sialic acid and is required for binding as evidenced by Arg to Ala mutations that abrogate binding to sialic acid-containing ligands (11, 12).

Another characteristic feature of the siglects is the presence of consensus immunoglobulin receptor family tyrosine-based inhibitory motifs (ITIM) in the cytoplasmic domains of all but sialedhesin (Siglec-1) and myelin-associated glycoprotein (Siglec-4) (2). ITIM motifs are found in an expanding group of immunoglobulin receptor family receptors that negatively regulate immune cell activation, which have been called the inhibitory-receptor superfamily (IRS) by Lanier (13). Members of the IRS exhibit three characteristic properties: (i) they recruit SH2 domain-containing protein tyrosine-based phosphatases such as SHP-1 and/or SHP-2 via a cytoplasmic ITIM motif, (ii) they affect activation receptors in cis, and (iii) require co-ligation with the activation receptors to exert their effect (13). One of the siglects, CD22 (Siglec-2), is well established as a negative regulator of B cell receptor signaling and fulfills all the characteristics of a member of the IRS (13, 14).

Based on the presence of ITIM motifs in the cytoplasmic domains of most siglects, it is generally believed that others will also be shown to be inhibitory receptors (1–3). Direct evidence that this is the case for Siglec-7 has been obtained from several laboratories. Indeed, Siglec-7 was originally cloned as a negative regulator of NK cell cytolyis (15). Recently, Crocker and colleagues (16) demonstrated that NK cells were less able to kill target cells bearing a high affinity Siglec-7 ligand (ganglioside GD3), presumably a result of inhibition of NK activation by recruitment of Siglec-7 to the site of NK cell-target cell contact (16, 17). Antibody cross-linking of Siglec-7 or CD33 (Siglec-3) has also been associated with negative regulation of cell growth by reducing proliferation and inducing apoptosis of CD34-positive hematopoietic precursors and chronic myeloid leukemia cells (18, 19). Similarly, Nutku et al. (20) have shown that antibody ligation of Siglec-8 induces caspase-3-like-dependent apoptosis in eosinophils. Recently, ligation of Siglec-5

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on neutrophils has been shown to augment oxidative burst induced by formylmethionyleucylphenylalanine (21). However, in contrast to the case of CD22 regulation of B cell receptor signaling, the activation receptor modulated by these siglecs in NK cells, eosinophils, and neutrophils has not been identified.

Siglec-7 (p75/AIRM) has been classified as an inhibitory NK cell receptor (NKR) based on its structural homology to other immunoglobulin-like NKRks (22–24), its gene loci (19q13.4–15.4) (25) proximal to other NKR gene families (23), and the presence of ITIM motifs in its cytoplasmic domain (24). Although classic NKRks bind major histocompatibility complex class 1 molecules as ligands, there are a growing number of NKRks like Siglec-7 that bind other ligands or whose ligands are unknown (22). It is noteworthy that Siglec-9 is also expressed on NK cells, although it has not yet been classified as an NKR.

Several NKRks are expressed on subsets of CD8+ T cells with a memory phenotype (26–29) and have been implicated in regulation of T cell receptor (TCR) signaling (30–33). In particular, the immunoglobulin-like NK cell receptor, LIR1/ILT2, has been demonstrated to regulate cytokisine, cytokine secretion, proliferation, and actin cytoskeleton reorganization through negative regulation of the TCR complex (34–37). Because Siglec-7 and Siglec-9 were both detected on subsets of T cells (38, 39), we hypothesized that they may participate in regulation of T cell signaling through the T cell receptor complex as observed for other NKRks. Accordingly, we investigated their ability to regulate TCR activation using Jurkat cell lines expressing Siglec-7 or Siglec-9 and their receptor-binding mutants with Arg to Ala substitutions at residues 124 and 120, respectively. The results show that both Siglec-7 and -9 can negatively regulate TCR activation by recruitment of SHP-1, resulting in reduced transcription of NFAT-mediated gene transcription. Equivalent expression of the receptor-binding mutants had no effect on TCR activation indicating that a functional ligand binding domain is required for optimal activity. The results suggest that these two siglecs may participate in modulating the activation threshold of T cells expressing them.

MATERIALS AND METHODS

Cells, Antibodies, and Reagents—Jurkat cells were routinely cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), and 1 mM sodium pyruvate (Invitrogen). FITC-labeled anti-CD3 (UCHT1), anti-human CD8, and anti-human CD4 TCR mAbs, were purchased from BD Biosciences (San Diego, CA). Red phycoerythrin-conjugated goat anti-mouse IgG mAb was purchased from Jackson ImmunoResearch (West Grove, PA). Anti-Siglec antibodies, and after washing twice with FACS buffer, were stained on glass slides with Vectashield Hard Set Mounting Medium (Vector Laboratories, Burlingame, CA) to reduce photobleaching during observation. In all cases, isotype-matched monoclonal antibodies were used as a negative control.

Expression Constructs—Expression constructs of Siglec-7 and -9 with a FLAG tag epitope at the C terminus were constructed as follows. The entire coding sequences of Siglec-7 and -9 were amplified by PCR using DNA from peripheral blood as a template, with respective sets of specific primers, and KOD polymerase HiFi (Novagen, Milwaukee, WI).

Primary primers were 5′-AAGAGAAAGAGGGCGGACCTTCAACCCCC-AGATATGC-3′ and 5′-GGCGGATCTTGGGGATCTTCGATCT-3′ for Siglec-7, and 5′-AAGAGGAAGAGGGCGGACCTTCAACCCC-ATGC-3′ and 5′-GGCGGATCTTGGGGATCTTCGATCTCGAG-3′ for Siglec-9. The PCR products were inserted into the NotI/BamHI site of the pCMV Tag 4B vector (Stratagene, San Diego, CA) to produce FLAG tag epitope fusion proteins. Mutant constructs with Arg→Gly to Ala substitutions on Siglec-7 and Arg→Gly to Ala substitution on Siglec-9 were also prepared, because these substitutions have already been shown to eliminate sialic acid binding (11, 12). The respective nucleotide substitutions for the Arg to Ala mutation in Siglec-7 and -9 were generated by crossover PCR, and the corresponding expression vectors were constructed as described above. The sequences of all expression constructs were verified by DNA sequencing.

Generation of Stable Expressing Cells—Jurkat cells were washed twice with RPMI medium, and added at a final density of 3 × 10^6/ml to a 0.4-cm gap cuvette (Invitrogen) containing 30 μg of the desired plasmid DNA. Electroporation was then carried out at 250 V, 950 microfarads with the use of a Gene Pulse (Bio-Rad). Cells were immediately transferred to a small tissue culture plate with RPMI containing 5% FCS, viable cells were purified with Histoplaque (Sigma-Aldrich). Following TCR stimulation and incubation for 48 h in RPMI containing 10% FCS, the cells were subjected to FACS analysis, and phosphorylation status was determined by immunoblot.

For NFAT luciferase assays involving transient expression of Siglec-7 or -9, Jurkat cells (1.2 × 10^5) were electroporated with a total of 30 μg of empty vector or Siglec-7 or Siglec-9 expression constructs, 20 μg of NFAT-luciferase reporter constructs (BD Biosciences), and 5 μg of pR-TK control constructs (Promega). For NFAT-luciferase assay using Jurkat cells stably expressing Siglec-7 or Siglec-9, 20 μg of NFAT-luciferase reporter constructs and 5 μg of pR-TK control constructs were electroporated (250 V, 950 microfarads) into 3 × 10^6 cells/ml 0.4-cm gap cuvette of the pCMV Tag 4B vector and plated at 1 × 10^5 cells/ml cell line. Assays were performed in triplicate. After 18-h incubation in RPMI containing 5% FCS, viable cells were purified with Histoplaque and cultured in complete RPMI media for 48 h at 37 °C, 5% CO_2. For NFAT luciferase assay performed with a Dual Luciferase Assay System (Promega), 1 μl of the dual-luciferase reagent was added to 10 μl of each sample according to the supplier’s instructions. Each experiment was repeated three or more times.

Cellular Stimulation, Immunoprecipitation, and Western Blot Analysis—Unless otherwise indicated, Jurkat-derived cell lines (1 × 10^5) were verified by DNA sequencing.
exhibited clear expression of Siglec-7 in donor-specific variation, rather than assay-specific variation. The variation in the expression of the Siglecs in T cells was total cells) as pointed out previously (40). Although no expression of CD3e, αβ, or γδ TCR subunits were added. Representative flow cytometry results of lymphocyte gated cells from three healthy donors are represented. For each donor analysis was conducted two to five times with no significant change in distributions.

were incubated for different time periods with either anti-CD3 (5 µg/ml), PHA (1 mg/ml), or vanadate (200 µM sodium orthovanadate 0.03% H2O2 at 37 °C in 1 ml of RPMI, which inhibits phosphatase activity and increases protein tyrosine phosphorylation). Cells were then lysed at 4 °C in lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and complete Protease Inhibitor Mixture from Roche Applied Science, Indianapolis, IN). After pre-clearing for 1 h at 4 °C with protein G-Sepharose beads (Amersham Biosciences), lysates were subjected to immunoprecipitation with anti-FLAG M2 beads, according to the Sigma manual. Alternatively, cell aliquots were directly lysed in Laemmli sample buffer for subsequent immunoblotting with anti-phospho ZAP70 and anti-ZAP70 antibodies. Precipitates or whole cell lysates were separated by 10% SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes (Amersham Biosciences) and immunoblotted with indicated Abs. Bound Abs were visualized using Western Lightning Chemiluminescence Plus (PerkinElmer Life Sciences).

RESULTS

Expression of Siglec-7 and Siglec-9 by αβ and γδ T Cells—In their initial reports Crocker and colleagues (38, 39) had noted that a majority of cells expressing NK cell markers (CD56 or CD16), and a small subset of CD3-positive peripheral blood leukocytes, expressed Siglec-7 and Siglec-9. However, Vitale et al. (18) found no Siglec-7 expression in CD3-expressing T cells. Because our interest was to investigate the effects of Siglec-7 and -9 on TCR activation, we first confirmed their expression in peripheral blood leukocytes using antibodies to the CD3e, αβ, and γδ TCR subunits. Representative results from three donors are shown in Fig. 1. As observed previously (38, 39), there was clear surface expression of Siglec-7 and Siglec-9 on CD3e, CD3αβ, and γδ TCR subunits. Representative results from three donors are shown in Fig. 1. As observed previously (38, 39), there was clear surface expression of Siglec-7 and Siglec-9 on CD3e chain-positive cells in all three donors. For the three donors, the fraction of CD3αβ-positive cells expressing Siglec-7 and Siglec-9 varied from 3.8 to 10% and from 5.2 to 19%, respectively. Of the Siglec-7αβ-positive cells, greater than 90% expressed the αβ TCR subunits. The population of peripheral blood leukocytes expressing the γδ TCR was small and variable (0.15–1.8% of total cells) as pointed out previously (40). Although no expression of Siglec-9 was observed in the γδ T cells, one donor exhibited clear expression of Siglec-9 in 13% of the γδ T cells. The variation in the expression of the Siglecs in T cells was donor-specific variation, rather than assay-specific variation, because virtually identical results were seen for each donor in two to five separate analyses taken over a span of at least 1 month. Taken together, the results indicate that the majority of CD3-positive peripheral blood leukocytes expressing Siglec-7 or -9 are αβ T lymphocytes, and that the fraction of Siglec-expressing cells differs from donor to donor.

Generation of Stable Siglec-7- and Siglec-9-expressing Jurkat Cells for Analysis of TCR Signaling—Jurkat cells are widely used to study TCR signal transduction. Flow cytometry analysis of Jurkat cells revealed that they do not express either Siglec-7 or Siglec-9 (not shown), making them ideal for the analysis of the affect of these receptors on TCR signaling. Accordingly, we adopted a strategy of comparing TCR signaling of native Jurkat cells with Jurkat cells that are stably or transiently expressing Siglec-7 or Siglec-9. As described in “Materials and Methods,” stable Jurkat cell lines were generated expressing FLAG-tagged Siglec-7 (Sig7) or Siglec-9 (Sig9) or neither siglec (mock-Jurkat; transfected with empty vector). Three independent clonal lines were prepared for each, with Siglec-7 and Siglec-9 expressing cells chosen to have similar levels of siglec expression by flow cytometry (see Fig. 2A for representative Sig-7 and Sig-9 clones). Based on direct comparison with freshly isolated human blood lymphocytes by FACS, the level of expression of the two siglecs on the Jurkat cell lines was intermediate to the expression of Siglec-7 and Siglec-9 on native T lymphocytes (see Fig. 1). For all cell lines, the expression level of the TCR complex was essentially the same as that found on native Jurkat cells (not shown).

Siglec-7 and Siglec-9 Are Partially Co-localized with TCR-CD3 Complex on Jurkat Cells—To evaluate whether the subcellular localization of Siglec-7 and Siglec-9 co-localizes with the TCR-CD3 complex, Sig-7 and Sig-9 expressing Jurkat cells were analyzed for the surface expression of CD3 and the respective siglec by fluorescence microscopy. As shown in Fig. 2B, CD3 and the two siglecs gave a punctate staining pattern reflecting localization in microdomains on the surface of the cell. When merged, partial co-localization of both siglecs with CD3 was observed as evidenced by the yellow and orange staining pattern. Thus, portions of both Siglec-7 and Siglec-9 are in
close physical proximity to CD3/TCR in the membrane. Of the two siglecs, Siglec-9 showed greater co-localization with CD3/H18528 TCR suggesting that the two are differentially localized in more than one microdomain on the surface of the cell (e.g. rafts, clathrin-coated pits, caveolae, microvilli, etc.)

Analysis of the Effects of Siglec-7 and Siglec-9 on TCR Signaling—For analysis of the effects of Siglec-7 and Siglec-9 on TCR signaling, key points in the signaling pathway were chosen for evaluation as summarized in Fig. 3. By analogy with other T cell inhibitory coreceptors with ITIM motifs (e.g. NKR and LIR1/ILT2), Siglec-7 and Siglec-9 would be expected to down-regulate the TCR signaling pathway by recruitment of SHP-1, resulting in de-phosphorylation of the TCR complex and down-regulation of the signaling cascade (15, 41). In Jurkat cells it is well established that TCR stimulation activates Src family kinases, which phosphorylate ITAM motifs (immunoglobulin receptor family tyrosine-based activation motifs) on the cytoplasmic tails of CD3 and ζ chains of the TCR complex. This in turn recruits and activates ZAP70, a Syk kinase, a key step that ultimately leads to the transcription of immune response genes (e.g. interleukin-2) through increased nuclear localization of NFAT transcription factors (42, 43). Accordingly, to investigate the effects of Siglec-7 and Siglec-9 on TCR signaling we have evaluated: 1) recruitment of SHP-1 by Siglec-7 and Siglec-9 following TCR engagement; 2) phosphorylation of ZAP70 at Tyr319; and 3) NFAT-mediated transcription of a luciferase reporter gene (see Fig. 3) (44).

TCR Engagement Enhances Recruitment of SHP-1 by Siglec-7 and Siglec-9—To assess the ability of Siglec-7 and Siglec-9 to recruit SHP-1 when expressed in Jurkat cells, clones stably expressing these siglecs (Fig. 2A) were treated with the phosphatase inhibitor pervanadate to amplify tyrosine phosphorylation and increase the sensitivity of detection. As shown in Fig. 4A, analysis before and after pervanadate treatment revealed a dramatic increase in phosphorylation of both siglecs, with a corresponding increase in recruitment of SHP-1. Although both siglecs were expressed with similar efficiencies, the degree of phosphorylation and recruitment of SHP-1 was much higher with Siglec-9. Thus, the results indicated that both Siglecs exhibited phosphorylation-dependent recruitment of SHP-1, although with different efficiencies.

To determine if increased phosphorylation and recruitment of SHP-1 also occurred following engagement of TCR, cells were subjected to stimulation as described under “Materials and Methods” using either anti-CD3 or PHA, which engages the TCR by binding to N-linked carbohydrate groups (45). Optimal
showed that all were readily detected in Jurkat cells except for SHIP, which is not expressed in this cell line (46).

Reduced Phosphorylation of ZAP-70 by Siglec-7 and -9—To evaluate the effect of Siglec-7 and Siglec-9 on downstream events, phosphorylation of ZAP-70 was examined. Although ZAP-70 is phosphorylated at multiple tyrosines, impairment of Tyr319 phosphorylation is known to result in significant attenuation of the TCR-induced calcium response and NFAT-mediated transcriptional activation (42, 43, 47). Accordingly, the degree of ZAP-70 phosphorylation was evaluated with antibody specific for phosphorylated Tyr319.

Results shown in Fig. 5 evaluate the effect of Siglec-7 or Siglec-9 expression on phosphorylation of ZAP-70 Tyr319 following engagement of TCR with a fixed concentration of anti-CD3 (5 μg/ml). Increased phosphorylation was observed in Jurkat cells (Mock-Jurkat), with maximal increase seen at 3 and 10 min, and elevated levels persisting at 30 min. By comparison, phosphorylation of ZAP-70 was reduced at all time points following TCR engagement in the Siglec-7 (Sig7-Jurkat)- and Siglec-9 (Sig9-Jurkat)-expressing cells. Six independent experiments involving three independently isolated Sig7- and Sig9 clones, three with anti-CD3 activation, and three with PHA activation (1.25 μg/ml), gave essentially the same results. The results demonstrate that Siglec-7 and Siglec-9 can negatively impact TCR signaling at a key downstream step in the activation pathway.

Suppression of TCR-induced Transcriptional Activation by Siglec-7 and -9—To evaluate the effect of Siglec-7 and Siglec-9 on TCR signaling at the transcriptional level, we used a luciferase reporter gene under the control of the cis-acting enhancer element NFAT (NFAT-luciferase). A series of related experiments were performed using cells either stably or transiently expressing Siglec-7 or Siglec-9.

For experiments with stable cell lines, cells were transfected with 20 μg of the pTA-NFAT-luciferase vector 36 h prior to activation with either PHA (1.25 μg/ml) or anti-CD3 (5 μg/ml). Although PHA induced similar levels of Siglec-mediated recruitment of SHP-1 as anti-CD3, it provided a more robust activation of gene transcription. Results in Fig. 6A show representative results of three separate experiments conducted for each condition in triplicate. Incubation of the Mock-Jurkat with PHA induced dose-dependent activation of NFAT-mediated transcription of the luciferase gene, amounting to a 40- to 200-fold increase above basal levels of transcription (Fig. 6A). Relative to Mock-Jurkat cells, dramatic suppression of transcription was seen with the stable cell lines expressing Siglec-7 (Sig7; p < 0.05) or Siglec-9 (Sig9; p < 0.01) at all levels of PHA used for activation. Similarly, with anti-CD3 induction, there was a similar reduction of transcriptional activation in Siglec-7 (p < 0.016)- and Siglec-9 (p < 0.036)-expressing cells (not shown).

Similar experiments were performed in triplicate to confirm these results in the presence of transient expression of the siglec. The vector with the reporter gene (20 μg) was co-transfected with 0, 10, or 30 μg of the expression vector for Siglec-7 or -9, and/or sufficient amounts of the empty vector (pCMV) to keep the total amount constant at 30 μg. Maximal expression of Siglec-7 and Siglec-9 was seen 72 h following transfection and was dependent on the dose of the expression vector used (see Fig. 6B).

Fig. 6C shows representative results of three separate experiments conducted for each condition in triplicate. As observed with the stable cell lines suppression of NFAT transcription was seen with transient transfection of Siglec-7, but only at levels achieved using 30 μg of the expression vector (p < 0.05). More robust suppression was observed with expression of Si-
Fig. 5. Expression of Siglec-7 and -9 reduces phosphorylation of ZAP-70 Tyr319 following TCR engagement.
Stably transfected Jurkat cells were stimulated by anti-CD3 (5 μg/ml) for the time indicated. Cell lysates (10 μg of protein) were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Detection of ZAP-70 (70 kDa) and the phosphorylation status of Tyr319 residue on ZAP-70 were assessed by sequential blotting with specific antibodies.

| Minute after Activation | Mock | Sig7 | Sig9 |
|-------------------------|------|------|------|
| pZAP                    | 0    | 3    | 10   | 30   |
| ZAP                     | 0    | 3    | 10   | 30   |

DISCUSSION

T cell activation following engagement of the TCR is regulated by the degree of phosphorylation of TCR complex, which is subject to both positive and negative regulation by co-receptors. Established negative regulators of T cell signaling include inhibitory NK cell receptors, which recruit phosphatases (e.g. SHP-1) to the TCR complex through cytoplasmic ITIM-like motifs (13, 41). Like the NK cell receptors, CD33-related siglecs (CD33 and Siglecs-5–11) have one or more cytoplasmic ITIM motifs, and their genes are found at the gene locus 19q13.4, a region rich in genes coding for leukocyte-expressed members of the inhibitory-receptor superfamily (2). The fact that Siglec-7 and/or Siglec-9 are expressed on 5–18% of peripheral blood T cells from different donors (Fig. 1) suggested to us that these siglecs could participate in regulating signaling by the T cell receptor complex. In this report we provide direct evidence that Siglec-7 and Siglec-9 can negatively regulate T cell receptor signaling at multiple steps in the signaling pathway. Indeed, with Jurkat cells expressing Siglec-7 or Siglec-9, TCR engagement results in 1) tyrosine phosphorylation of and recruitment of SHP-1 by both siglecs, with corresponding 2) reduced phosphorylation of ZAP-70, and 3) decreased NFAT-dependent activation of gene transcription.

It appears that the ability of Siglec-7 and Siglec-9 to recruit SHP-1 to the TCR complex is responsible for the down-regulation of the signaling pathway in Jurkat cells. Indeed, none of the other phosphatases that bind to ITIM motifs (e.g. SHP-2 or SHIP-2) were detected following immunoprecipitation of Siglec-7 or Siglec-9. Although the mechanism by which Siglec-7 and Siglec-9 co-localize with the TCR remains to be elucidated, it is likely that SHP-1 recruited by them dephosphorylates ZAP-70 and/or the TCR subunits themselves, and as a consequence increases the activation threshold (41).

There were subtle but reproducible differences in the ability of the two siglecs to recruit SHP-1. Although TCR engagement resulted in increased tyrosine phosphorylation and recruitment of SHP-1 by both siglecs, the endogenous level of tyrosine phosphorylation and associated SHP-1 was higher for Siglec-9, resulting in a lesser “fold increase” following activation. In contrast, following pervanadate treatment, recruitment of SHP-1 by Siglec-9 was much higher than by Siglec-7. Siglec-9 also appeared to be more efficient at reducing phosphorylation of ZAP-70 (Tyr319) and NFAT-mediated transcription (Figs. 5 and 6). These differences may be due to a variety of factors, including the observed differences in SHP-1 binding, differences in their relative ability to associate with the TCR complex or differences in their abilities to interact with cis glycoprotein ligands expressed on Jurkat cells that modulate their inhibitory activity.

Optimal inhibition of TCR signaling of both Siglec-7 and Siglec-9 required an active ligand binding domain, because mutation of the conserved Arg in the sialic acid binding site to Ala reduced their efficiency to suppress NFAT-mediated gene transcription. This could reflect loss of favorable cis interactions of these siglecs with one or more sialic acid-containing glycoprotein ligands associated with the TCR. Analogous observations have been made recently for CD22 regulation of B cell signaling (48, 49). Mutation of the conserved Arg to Ala in CD22, or use of small molecule inhibitors of the ligand binding site resulted in decreased SHP-1 recruitment and enhanced activation of B cells, as evidenced by increased calcium influx following B cell receptor ligatation (48, 49). In this regard, it is of interest that these two siglecs exhibit different specificity for sialic acid-containing ligands. For example, Siglec-7 binds with highest affinity to sialosides with the NeuAcα2,8NeuAc linkage, whereas Siglec-9 preferentially recognizes sialosides with the NeuAcα2,3Gal linkages (8, 11, 12, 38, 39, 50). Such differences in specificity could differentially influence their ability to associate with...
the TCR or other cis glycoprotein ligands based on the sialic acid-containing sequences carried on their glycan chains.

Although Siglec-7 has been demonstrated to exhibit properties of an inhibitory receptor in immune cells following antibody cross-linking (15, 18, 19), the demonstration that Siglec-7 and Siglec-9 negatively regulate TCR signaling represents the initial evidence of their inhibitory activity toward a specific activating receptor. The fraction of CD3-positive T cells expressing Siglec-7 and Siglec-9 represented 15–50% or more of all the siglec-expressing cells in the lymphocyte population (Fig. 1). The level of expression of the Siglec-7 and Siglec-9 in native lymphocytes is comparable to or exceeds that expressed in the Sig7-Jurkat and Sig9-Jurkat T cell lines evaluated in this report. Indeed, Siglec-7 is expressed at very high levels in CD3-positive T cells. Thus we suggest that these siglecs participate in regulation of TCR signaling in the CD3-positive cells.

**Fig. 6.** NFAT-mediated trans-activation of gene transcription in Jurkat cells is reduced by the expression of Siglec-7 or -9. To evaluate the effect of siglec expression on TCR-induced nuclear transcription, Jurkat cells and Jurkat cells expressing Siglec-7 or Siglec-9 were transfected with a luciferase reporter gene under the transcriptional control of NFAT element (pNFAT luciferase) prior to activation with PHA (see “Materials and Methods”). A, assessment of Siglec-7 and Siglec-9 on inhibition of TCR-activated NFAT promoter-driven transcription. Jurkat cells and stable cell lines expressing Siglec-7 (Sig7) and Siglec-9 (Sig9) were activated with increasing amount of PHA at 36 h following transfection with NFAT luciferase vector and were assayed in triplicate for luciferase activity using Dual-Luciferase assay system. Results are shown as “fold activation” relative to the level of luciferase in cells incubated with no PHA. The graph shows the average and standard error from three independent experiments. B and C, transient transfection experiments were performed to confirm experiments with stable cell lines. Jurkat cells were transiently co-transfected with the NFAT luciferase vector and an expression vector for Siglec-7 or Siglec-9. The total amounts of the expression vectors were maintained at 30 nM, by combining 0, 10, or 30 nM of a siglec expression vector (pCMV-Sig7 or pCMV-Sig9) with 30, 20, or 0 nM of the empty vector (pCMV), respectively. B, expression of Siglec-7 (left) or Siglec-9 (right) was assessed 72 h after transfection as described in Fig. 2A. C, assessment of Siglec-7 and Siglec-9 on inhibition of TCR-activated NFAT promoter-driven transcription in transient experiments. Following analysis of Siglec expression, cells (10⁶) were incubated for 6 h with 0–1.0 μg/ml PHA-L and were assayed as described for A. For all panels, symbols above the bar indicate the degree of difference between levels of activation in the absence and presence of siglec expression (#, p < 0.05; *, p < 0.01; no symbol, not significant).
that express them. Crocker and colleagues (39) have proposed that the majority of Siglec-7-expressing lymphocytes are CD8-positive memory cells. Thus, based on analogy with expression of other inhibitory NK cell receptors on CD8 memory cells (24, 30, 32, 33, 51), Siglec-7 and Siglec-9 may contribute to setting the activation threshold of T cells, reducing activation-induced cell death and promoting survival of memory T cells.

The only other example of a siglec regulating the activity of a specific receptor is CD22 (Siglec-2) regulation of B cell receptor signaling (14). In contrast to CD22, which is expressed only on B cells (3), both Siglec-7 and Siglec-9 are distributed on a variety of leukocytes in addition to T cells, including NK cells, monocytes, and granulocytes (3, 38, 39). Thus, in addition to regulation of TCR signaling as demonstrated here, it is likely that these two siglec will be found to negatively regulate one or more additional activating receptors present on these other leukocyte cell types.

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Fig. 7. A functional ligand binding domain is required for maximal suppression of TCR signaling. The role of the sialic acid binding domain in regulation of TCR signaling by Siglec-7 and Siglec-9 was investigated using ligand binding mutants missing the conserved Arg in the binding site by substitution of Arg with Ala at amino acids 124 and 120, respectively. These mutants were compared with the native siglecs for their ability to modify NFAT activation in stable cell lines. A, comparison of NFAT-mediated luciferase expression following TCR activation of Jurkat cells stably expressing Siglec-7 (Sig) and the Arg124 to Ala mutant of Siglec-7 (R/A-Sig7) relative to control cells (Mock). B, comparison of NFAT-mediated luciferase expression following TCR activation of Jurkat cells stably expressing Siglec-9 (Sig9) and the Arg120 to Ala mutant of Siglec-9 (R/A-Sig9) relative to no siglec (Mock). Cells were activated with varying concentrations of PHA and assessed for levels of luciferase expression as described in the legend to Fig. 6.
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