Early Murine T-lymphocyte Activation Is Accompanied by a Switch from \(N\)-Glycolyl- to \(N\)-Acetyl-neuraminic Acid and Generation of Ligands for Siglec-E\(^*\)\(^5\)

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It is well established that murine T-lymphocyte activation is accompanied by major changes in cell-surface sialylation, potentially influencing interactions with sialic acid-binding immunoglobulin-like lectins (siglec). In the present study, we analyzed early activation of murine CD4+ and CD8+ T-lymphocytes at 24 h. We observed a striking and selective up-regulation in the binding of a recombinant soluble form of siglec-E, an inhibitory siglec, which is expressed on several myeloid cell types including antigen-presenting dendritic cells. In contrast, much lower levels of T cell binding were observed with other siglecs, including sialoadhesin, CD22, and siglec-F and the plant lectins \(N\)\(\text{a}c\)cl\(k\)ia \(a\)mure\(r\)ensis leukagglutinin and \(S\)\(a\)mbucha \(n\)g\(r\)a agglutinin. By mass spectrometry, the sialic acid content of \(N\)\(a\)c\(t\)il\(u\)n\(g\) \(S\)\(i\)g\(l\)ec\(s\) \(s\)\(i\)g\(l\)ec\(t\)\(E\), \(s\)\(i\)g\(l\)ectin \(E\) and \(s\)\(i\)g\(l\)ectin \(F\) and the plant \(M\)\(a\)ack\(k\)ia \(a\)mure\(r\)ensis \(N\)-glycans. Reduced levels of NeuGc on the surface of activated T cells were demonstrated using an antibody specific for NeuGc and the expression of NeuGc on the surface of activated T cells were demonstrated using an antibody specific for NeuGc and the expression levels of the gene encoding NeuAc- to NeuGc-terminating oligosaccharides on cell surface glycoproteins. This is associated with a strong up-regulation of siglec-E ligands, which may be important in promoting cellular interactions between early activated T-lymphocytes and myeloid cells expressing this inhibitory receptor.

T-lymphocytes occupy a central position in the immune system, driving adaptive immunity in response to the specific recognition of antigens on tumors, infected cells and antigen-presenting cells such as dendritic cells and macrophages. Following their maturation in the thymus, T-lymphocytes differentiate into several subsets, each with a distinct effector or regulatory function. These processes are accompanied by alterations in cell-surface glycosylation involving a host of glycosyltransferases and glycosidases. Several groups have reported marked changes in the sialic acid signature of glycoprotein \(N\)- and \(O\)-linked glycans, a process mediated by various sialyltransferases (1–6). These changes influence T-lymphocyte interactions with endogenous mammalian carbohydrate-binding proteins (lectins) such as selectins, galectins, and sialic acid-binding immunoglobulin-like lectins (siglec), which in turn regulate T-lymphocyte functions such as trafficking, intracellular signaling, and apoptosis (7–12).

Siglecs are a family of sialic acid-binding immunoglobulin-like lectins expressed primarily on cells of the hematopoietic and immune systems where they mediate both signaling functions as well as cell-to-cell interactions (13). Siglecs are characterized by their recognition of sialic acids including \(N\)-acetyl-neuraminic acid (NeuAc) and \(N\)-glycolyl-neuraminic acid (NeuGc) in the \(\alpha\)\(2,3\)-, \(\alpha\)\(2,6\)-, and \(\alpha\)\(2,8\)-linkages and display both unique as well as overlapping glycan-specificity profiles. As such, siglecs bind sialylated endogenous ligands both in cis on...
the same cells and in *trans* on other cells, as well as exogenous ligands expressed on several pathogens (14–17).

In humans and mice, most T-lymphocytes do not express any siglec and therefore alterations in T cell sialic acids are more relevant for *trans*-interactions with siglecs expressed on other cells. These could be important for promoting cross-talk between the innate and adaptive immune systems. The macrophage-restricted siglec, sialoadhesin (Sn, siglec-1), is thought to play a role in mediating T cell interactions and shows a preference for α2,3-linked NeuAc, a terminal modification of sialylated O-linked glycans on sialomucins such as its putative T-lymphocyte counter-receptor, CD43 (18). Recent findings support a role for Sn in suppressing regulatory T cell expansion via sialic acid-dependent interactions and exacerbating inflammatory responses in experimental autoimmune encephalomyelitis (19).

CD22 (siglec-2) and most CD33-related siglecs such as murine siglec-E and siglec-F function as inhibitory receptors (20–22), suppressing activation signals by recruiting tyrosine phosphatases SHP-1 and SHP-2 via cytoplasmic immunoreceptor tyrosine-based inhibitory motifs. The B-lymphocyte-restricted receptor CD22 is specific for α2,6-linked sialic acids (23, 24), the recognition of which forms the basis for *cis*-interactions with neighboring CD22 molecules, IgM and CD45 on B-lymphocytes and *trans* interactions with glycoproteins on T cells (25–27). Siglec-F is expressed on eosinophils and alveolar macrophages and in glycan arrays it binds preferentially to sialyllactose presented on polyacrylamide (35). The binding of sialyllactose, a naturally occurring glycolipid, gangliosides, differed from that of siglecs-7 and -9 (36). Regulated expression of siglec-E in myeloid cells like macrophages and dendritic cells may be important for promoting cross-talk between the innate and adaptive immune systems. The macrophage-restricted siglec, sialoadhesin (Sn, siglec-1), is thought to play a role in mediating T cell interactions and shows a preference for α2,3-linked NeuAc, a terminal modification of sialylated O-linked glycans on sialomucins such as its putative T-lymphocyte counter-receptor, CD43 (18). Recent findings support a role for Sn in suppressing regulatory T cell expansion via sialic acid-dependent interactions and exacerbating inflammatory responses in experimental autoimmune encephalomyelitis (19).

**EXPERIMENTAL PROCEDURES**

*Expression and Purification of Recombinant Siglec-Fc Fusion Proteins—* Fusion proteins comprising the Fc portion of human IgG1 and the extracellular region of either siglec-E, siglec-F, CD22, or the first 3 extracellular domains of Sn (Sn1–3) were stably expressed in CHO cells as described previously (28) and their concentrations determined by ELISA. For some experiments including Biacore and affinity isolation, proteins were purified by affinity chromatography on protein G-Sepharose (Sigma Aldrich).

**Up-regulation of Siglec-E Ligands on Activated Murine T Lymphocytes**

**Isolation, Activation, and Culture of Murine Splenic T-lymphocytes—** Spleens were harvested from 4–8 week old C57/B16 mice maintained under specific-pathogen-free conditions. Splenocytes were isolated following red blood cell lysis using standard protocols. For experiments involving the fluorescence-activated cell sorter (FACS) analysis of antibody and lectin binding, splenocytes were activated for 24 h with 100 ng/ml soluble hamster anti-mouse CD3 and anti-mouse CD28 (Invitrogen) and subsequently cultured for up to a total of 5 days in RPMI1640+10% glutamine supplemented with 10% fetal bovine serum, 100 units/100 µg penicillin/streptomycin (GIBCO), 50 µM 2-mercaptoethanol and 20 ng/ml IL-2 (Novartis) at 37 °C, 5% CO₂ in a humidified atmosphere. Activation was confirmed following labelling of naive and activated cells with fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD69 (Invitrogen). For all other analyses, CD4+ and CD8+ T-lymphocytes were isolated using a Pan-T cell isolation kit followed by Automated Magnetic Activated Cell Sorting (AutoMACS) (Miltenyi Biotec). Sorted, pooled T-lymphocytes were then activated for 24 h using 1.0 µg plate-bound hamster anti-mouse CD3 and anti-mouse CD28 in the same medium and under the same conditions as described previously. Activated cells were transferred from antibody-coated to uncoated plates and allowed to grow in the same medium for a further 4 days in the presence of 20 ng/ml IL-2.

**FACS Labeling and Analysis of T-lymphocytes—** Cells obtained as described above were treated with *Vibrio cholerae* sialidase (Sigma Aldrich) in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) for 2 h at 37 °C. Control cells were incubated under the same conditions without enzyme addition. To assess the sensitivity of siglec-E-Fc binding to proteinase K treatment, splenocytes were incubated at 37 °C for 60 min with 0.1 mg/ml proteinase K (Sigma) in DMEM. For labeling with siglec-Fc chimeras, 1 µg/ml serum-free culture medium supernatant containing siglec-E-Fc, siglec-F-Fc, Sn1–3-Fc or CD22-Fc fusion proteins was pre-complexed for 30 min on ice with 1/3000 FITC-conjugated goat anti-human Fc IgG (Sigma Aldrich). Cells were then incubated with pre-complexes for 30 min on ice together with 1/100 R-PE-conjugated rat anti-mouse CD4 and allophycocyanin-conjugated rat anti-mouse CD8 antibodies (Invitrogen). For plant lectin labeling, cells were incubated with 1 µg/ml biotinylated *Maackia amurensis* leukoagglutinin (MAL) or *Sambucus nigra* agglutinin (SNA).
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(VECTOR Labs) followed by a secondary incubation with a 1/200 dilution of FITC-conjugated streptavidin (VECTOR Labs) for a further 30 min. To assess cell surface NeuGc expression, cells were incubated with a 1/500 dilution of chicken anti-NeuGc antibody (Sialix) followed by a secondary incubation with Cy5-conjugated donkey anti-chicken antibody (Jackson Immunoresearch) according to the manufacturer’s instructions. Cells were resuspended in phosphate-buffered saline (10 mM phosphate buffer, pH 7.4, 150 mM NaCl) (PBS) containing 1% bovine serum albumin and 0.1% NaN₃ and events collected using a FACS Calibur (Becton Dickinson). Data were analyzed using FlowJo software (Becton Dickinson).

N-linked Glycan Profiling of Resting, 24-h-activated and 24-h-activated + 4-day-cultured CD4+ and CD8+ T-lymphocytes—Resting and 24-h-activated T cells were purified using a Pan-T cell isolation kit and AutoMACS (Miltenyi Biotec). CD4+ and CD8+ T cells were then purified by FACS using Becton Dickinson FACS Vantage. CD4+ and CD8+ T cells from 24-h-activated and 4-day-cultured T lymphocytes were isolated using CD4+ and CD8+ T cell isolation kits (Miltenyi Biotec). Purity was confirmed by flow cytometry using R-phycocerythrin-conjugated rat anti-mouse CD4 and allophycocyanin-conjugated donkey anti-mouse CD8 antibodies. Between 5 × 10⁶ and 10⁷ purified cells were processed as described previously (38). Briefly, each sample was subjected to homogenization in extraction buffer (25 mM Tris, 150 mM NaCl, 5 mM EDTA, and 1% [3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate, CHAPS at pH 7.4), followed by reduction in 4 M guanidine-HCl (Fierce), carboxymethylation and trypsin digestion. Digested glycoproteins were purified on C₁₈-Sep-Pak columns (Waters Corp, Hertfordshire, UK). N-Glycans were released by PNGase-F (Roche) digestion and sialylococyanin-conjugated rat anti-mouse CD8 antibodies. Between 5 × 10⁶ and 10⁷ purified cells were processed as described previously (38). Briefly, each sample was subjected to homogenization in extraction buffer (25 mM Tris, 150 mM NaCl, 5 mM EDTA, and 1% [3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate, CHAPS at pH 7.4), followed by reduction in 4 M guanidine-HCl (Fierce), carboxymethylation and trypsin digestion. Digested glycoproteins were purified on C₁₈-Sep-Pak columns (Waters Corp, Hertfordshire, UK). N-Glycans were released by PNGase-F (Roche) digestion and then permethylated using the sodium hydroxide procedure. Finally, the permethylated N-glycans were purified on C₁₈-Sep-Pak columns. Methanol-solubilized permethylated samples were premixed with matrix (20 mg/ml 2,5-dihydroxybenzoic acid (DHB) in 70% v/v aqueous methanol), spotted onto a target plate and dried under vacuum. MS data were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer. The collision energy was set to 1 kV, and argon was used as collision gas. The 4700 Calibration standard kit, Calmix (Applied Biosystems), was used as the external calibrant for the MS mode of both instruments and human [Glu1] fibrinopeptide B (Sigma Aldrich) was used as an external calibrant for the MS/MS mode of the MALDI-TOF/TOF instrument. The MS and MS/MS data were processed using Data Explorer 4.9 Software (Applied Biosystems). The spectra were subjected to manual assignment and annotation with the aid of the glyco-bioinformatics tool GlycoWorkBench (39). The proposed assignments for the selected peaks were based on ¹²C isotopic composition together with knowledge of the biosynthetic pathways. The proposed structures were then confirmed by data obtained from MS/MS experiments.

Real-time RT-PCR Analysis of CMAH Gene Expression—Total RNA was isolated from resting (0 h), activated (12 h and 24 h) and 24-h-activated + cultured (48 h total) pooled T-lymphocytes using the RNeasy mini kit (Qiagen) and reverse transcribed using a Quantititect reverse transcription kit (Qiagen). Complementary DNA was analyzed in triplicate by real-time PCR amplification using Power SYBR Green PCR master mix (Applied Biosystems) on StepOne Plus (Applied Biosystems). Melt-curve analysis was performed to ensure mRNA-specific fragments were amplified and data were analyzed using the ΔΔCT method, with 0 h values set as calibrator and expression normalized to GAPDH. Primers: GAPDH-5′ -CAACTCCCATC- TCTTCCACCTTCG-3′, 5′-GTAGGGAGGGCTAGTCATGTT- GGG-3′; CMAH-5′ -GGTGGTCAAGGATGATTGAAACAGA- TG-3′, 5′-CCACCCGGCATGATTGCTTCCTC-3′.

Glycan Array Analyses—Microarrays were composed of lipid-linked oligosaccharide probes: neoglycolipids (NGls), prepared by reductive amination (40) or oxime ligation (36), and glycolipids, mostly representing mammalian type sequences, N-glycans, glycolipids, and the backbones and peripheral regions of O-glycans. These were robotically printed on nitrocellulose-coated glass slides using a non-contact instrument, as described previously (41) ( Supplemental Table S1). For siglec-E-Fc binding, the results of 18 non-sialylated and 83 sialylated oligosaccharide probes at 7 fmol per spot are shown in Fig. 5. For chicken anti-NeuGc and the control antibody (both from Sialix), a different (more recent) version of the microarray was used; results of the 18 non-sialylated and 102 sialylated oligosaccharide probes at 5 fmol per spot are shown in supplemental Table S1.

The microarray binding assays were performed at 19–20 °C. Siglec-E-Fc binding was assayed as described previously (29, 36). In brief, the arrayed slides were blocked for 1 h with 1% w/v BSA (Sigma lot A8577) in Casein Blocker solution (Pierce, Lot GC95833) (casein/BSA). Siglec-E-Fc chimera was pre-complexed with biotinylated anti-human-IgG (Vector) at a 1:3 ratio w/w for 1 h before applying onto the slides at a final concentration of 1.2 µg/ml. Binding was detected with Alexa-Fluor 647-labeled streptavidin (Molecular Probes) at 1 µg/ml in blocker solution. For the analyses of chicken anti-NeuGc and the control chicken antibody, the arrayed slides were blocked for 1 h with fish gelatin blocker (Sialix) at a 1:80 dilution in PBS for 1 h before overlaying and incubating for 90 min with the chicken anti-NeuGc or the control chicken antibody at a 1:500 dilution in the blocking solution. Binding was detected with Cy5-labeled donkey anti-chicken antibody (Jackson Immunoresearch) at a 1:500 dilution in the blocking solution. Microarray data analysis was performed with dedicated software (42).

Surface Plasmon Resonance (SPR) Analysis—Biotinylated glycoconjugates (provided by the Consortium for Functional Glycomics) were dissolved in PBS and immobilized on streptavidin-coated CM5 sensor chips at 25 °C (150–250 response units) (Biacore). Purified siglec-E-Fc, prepared in running buffer (PBS supplemented with 0.005% Tween-20 and 0.1 mg/ml BSA) was injected over all four chip surfaces at 10 µl/minute at 25 °C. M at 25 °C.

Melt-curve analysis was performed to ensure mRNA-specific fragments were amplified and data were analyzed using the ΔΔCT method, with 0 h values set as calibrator and expression normalized to GAPDH. Primers: GAPDH-5′ -CAACTCCCATC- TCTTCCACCTTCG-3′, 5′-GTAGGGAGGGCTAGTCATGTT- GGG-3′; CMAH-5′ -GGTGGTCAAGGATGATTGAAACAGA- TG-3′, 5′-CCACCCGGCATGATTGCTTCCTC-3′.
N-acetyl- and lactosamine-containing glycoconjugates respectively, where sp is a 2-azidoethyl spacer and LC is a long-chain spacer arm of 30.5 Å that reduces steric hindrance (Pierce Protein Research Products).

**Sialidase Treatment, Surface Biotinylation, and Lysis of 24-h-activated T Lymphocytes**—Following 24 h of activation of MACS-purified T-lymphocytes, cells were harvested and treated with *Vibrio cholerae* sialidase as described above or left untreated. Cells were surface-biotinylated with Sulfo-NHS-LC-Biotin (Pierce) in PBS pH 8.0 for 30 min at 4 °C. Unlabeled cells were treated in the same way in the absence of labeling reagent. Cells were then lysed in 20 mM Tris pH 7.4 containing 150 mM NaCl, 1.0 mM CaCl₂, 2.0 mM MgCl₂, 0.3% CHAPS, 1% Nonidet P-40, 400 μM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche) for 30 min at 4 °C. Lysates were centrifuged at 10,000 × g to remove cell debris and nuclei.

**Lectin Precipitations**—Purified siglec-E-Fc was pre-complexed with goat anti-human Fc IgG (Sigma Aldrich) for 60 min at 4 °C following which the pre-complex was coupled noncovalently to nitrocellulose membranes. For streptavidin blots, proteins were separated on 150 mM NaCl, 1.0 mM CaCl₂, 2.0 mM MgCl₂ (TSM), and 0.1% Tween-20 and then incubated with 1:5000 horseradish peroxidase conjugated goat anti-human Fc IgG (Amersham Biosciences-GE).

**T-lymphocyte Activation Leads to an Increase in the Proportion of N-Glycans Containing NeuAc Compared with Those Containing NeuGc**—To obtain insights into glycosylation changes that result in the up-regulation of siglec-E ligands on 24-h-activated T-lymphocytes, glycomic profiling was undertaken. CD4⁺ and CD8⁺ splenic T-lymphocytes were purified and the N-linked glycan profiles of resting non-activated, 24-h-activated and 24-h-activated + 4-day-cultured cells were analyzed using MALDI-TOF MS and MALDI-TOF-TOF MS/MS (Fig. 2). Interestingly, with respect to the sialylated glycans in the N-glycome, activation was accompanied by an increase in the proportion of NeuAc-containing N-linked glycans to NeuGc-containing glycans. For example, it can be seen in Fig. 2 that following activation, the ratio of the NeuAc containing biantennary structures at m/z 2966 and m/z 2996 structures are increased relative to the equivalent NeuGc containing structure at m/z 3026 s in CD4⁺ cells. Direct comparison of the above species in each spectrum, summing the bi-antennary N-linked glycan structures substituted with one or two NeuAc residues (m/z 2966 + m/z 2996) versus the double NeuGc form (m/z 3026), yielded a ratio of ~1:16.2 (NeuAcs:NeuGcs) for the resting CD4⁺ cells, versus ~1:3.8 and ~1:4.4 for the 24-h-activated and 24-h-activated + 4-day-cultured CD4⁺ cells respectively (~4-fold increase on bi-antennary NeuAcs). Similarly, CD8⁺ cells showed an increased proportion of NeuAc structures at m/z 2809 and m/z 2996 (for the m/z 2996; ~1:7.6 and ~1:5 for the 24 h-activated and 24 h-activated + 4 day-cultured CD8⁺ cells, respectively). Activated CD4⁺ and CD8⁺ cells also showed an increased abundance of the GaLa1,3Gal epitope (as shown by an increased abundance of m/z 2809 to m/z 2839, Fig. 2). Structures corresponding to m/z 2809 and m/z 2839 were bi-antennary and terminated with NeuAc or NeuGc respectively as well as the Galα1,3Gal epitope. The N-glycans of 24-h-activated CD4⁺ cells also exhibited a reduction in core-fucosylation (as determined by the ratio of m/z 3026 to m/z 2852) which remained constant after 5 days in culture.

**Reduced Expression of NeuGc on T Cells following Activation Determined with a Specific Antibody**—The increased proportion of NeuAc-terminating saccharides over those terminating with NeuGc seen after T-lymphocyte activation was further investigated using a commercial chicken anti-NeuGc antibody. The specificity of the antibody was validated using a glycan array comprising 102 sialylated and 18 non-sialylated glycoconjugate probes (supplemental Table S1). As expected, the chicken antibody showed strong selectivity for glycossidically...
attached NeuGc with little or no reactivity to NeuAc. An incidental observation was that the antibody showed little or low binding to internally linked NeuGc in GM1 analogs and a preference for NeuGc in the α2,6-linkage over the α2,3-linkage.

When used in flow cytometry with fresh and activated T-lymphocytes, binding of the anti-NeuGc antibody decreased over time and was inversely correlated with binding of siglec-E-Fc (Fig. 3, A and B). Activation was accompanied also by reduced expression of the gene encoding the enzyme CMAH, which is required for conversion of CMP-NeuAc to CMP-NeuGc (43) (Fig. 3C). Taken together, these results demonstrate that early in the activation process, T cells have a reduced expression of the CMAH enzyme and reduced levels of NeuGc at the T-lymphocyte surface.

**Siglec-E-Fc Shows a Clear Preference for NeuAc over NeuGc and Binds a Wide Range of Sialyloligosaccharides**—We next asked if the reduced levels of NeuGc on activated T cells could be correlated with a preference of siglec-E for NeuAc over NeuGc. This was tested by SPR (Biacore) using purified siglec-E-Fc with pairs of biotinylated 3′-sialyllactose and 3′-sialyl-N-acetyllactosamine oligosaccharides, terminating in either NeuAc or NeuGc (Fig. 4). In each case, siglec-E-Fc showed a clear preference for NeuAc over NeuGc, with stronger binding to 3′-sialyllactose than to 3′-sialyl-N-acetyllactosamine.
The glycan binding preference of siglec-E was investigated in more detail using glycan arrays of lipid-linked probes (Fig. 5, supplemental Table S1). As predicted, siglec-E did not bind the non-sialylated oligosaccharides, but gave binding to a broad range of sialylated probes that have $\alpha_2,3$, $\alpha_2,6$, or $\alpha_2,8$ sialyl linkages. Among the probes strongly bound are short sequences (di- and tri-saccharides) terminating with $\alpha_2,3$-linked NeuAc (sequences depicted in Fig. 5B), in the order: Hematoside/GM3 (probes #51, #52), 3$\alpha$-sialyl-$N$-acetyllactosamine (#28), ganglioside GM4 (#50) and 3$\alpha$-sialyllactose (#20). Of note is the stronger binding to 4$\alpha$-O-acetylated 3$\alpha$-sialyllactose (probe #22) than to the non-$\alpha$-O-acetylated analog (#20). This is consistent with a previous observation (36). It is interesting that although GM4 (NeuAc$\alpha_2$-3Gal-ceramide) was bound, little or no binding was observed with ring-opened (DH) NGLs of 3$\alpha$ sialyllactose and 3$\alpha$-sialyl-$N$-acytectumamine (probes #19, #21, #27). This indicates the importance of presentation of NeuAc$\alpha_2$,3Gal- disaccharide for siglec-E binding. The NeuGc analog of GM3 (#53) was less strongly bound than the NeuAc form (probes #51, #52). This is in agreement with the results of the SPR studies (Fig. 4).

With the series of oligosaccharide probes (Fig. 5C), the influence of $\alpha_1,3$-fucosylation of Lewis$^a$ type on siglec-E binding could be evaluated, and it was observed to strongly reduce the intensity of binding (compare probe pairs #33/#37, and #47/#48). As seen with other siglecgs (29), sialation of the sialyl Lewis$^a$ sequence affected the intensity of siglec-E binding: 6$\alpha$-sulfation or 6$\alpha$-sulfation had a dramatic enhancing effect on siglec-E binding (e.g. compare probe #37 with #39 and #41, Fig. 5A). Interestingly, no further increase in binding was observed when both sulfates were present (probe #43, Fig. 5A).

Depicted in Fig. 5D are NeuAc$\alpha_2$,6-related sequences, among which the strongest binding was to sialyl tetrasaccharide LSTb having NeuAc$\alpha_2$,6-internally-linked to GlcNAc (probe #71). When the $\alpha_2$,6-linked NeuAc was at terminal Gal, binding was only observed with the short sequence, 6$\alpha$ sialyllactose (probe #64) but not the long sequences (#72–#79) including sialyl tetrasaccharide LSTc (#72). Siglec-E also bound to sequences containing both $\alpha_2$,3 and $\alpha_2$,6 sialyl linkages (probes #80–#82), and again, the binding to the shorter sequence (probe #80) was stronger than to the longer ones (#81, #82).

Among the sialyl structures that contain $\alpha_2$,8 linkage (Fig. 5D), siglec-E bound polysialic acid series 2- to 11mers (probes #87–#93), showing strongest binding to short di- or tri- saccharides (probes #87, #88). Good binding signals were observed with most gangliosides having $\alpha_2$,8-linked disialyl chains except GD1b (probe #85). The contrast in binding responses of GT1b (#95) and GD1b (#85) highlights the importance of $\alpha_2$,3-linked terminal NeuAc residue for siglec-E binding. There was also a preferential binding to GT1b (#95) over GD1a (#60), and to GD2 (#84) over GM2 (#54), suggesting that the $\alpha_2$,8 disialyl branch may contribute to siglec-E binding.

Siglec-E-Fc Binds Multiple Proteins from 24-h-activated T-lymphocytes in a Sialic Acid-dependent Manner—The glycan binding data showed that siglec-E-Fc bound a wide range of sialylated ligands with a preference for NeuAc over NeuGc. The...
striking up-regulation of siglec-E ligands on activated T cells that are proteinase K- and sialidase-sensitive could therefore be explained by a general increase in NeuAc-containing N- and O-glycans displayed on a wide variety of T cell glycoproteins. It is also possible that a small subset of glycoproteins displaying these ligands could act as dominant counter-receptors. To investigate these possibilities further, siglec-E-Fc pull-down experiments were carried out using lysates prepared from surface biotinylated, 24-h-activated T cells (Fig. 6A). Sialic acid-dependent interactions with siglec-E were determined by pretreating cells with sialidase prior to biotinylation and lysis. This revealed that several cell surface proteins ranging from ~100 to >250 kDa were bound by siglec-E in a sialidase-sensitive manner (Fig. 6A, lanes 3 and 4, arrows). The lower molecular weight bands present in siglec-E pull-downs from untreated and sialidase-treated samples appeared to be present in total cell lysates (Fig. 6A, lanes 1 and 2) and are therefore likely to represent proteins nonspecifically bound. In an alternative approach, we performed siglec-E-Fc overlays on blots of lysates prepared from 24-h-activated T cells, either untreated or sialidase-treated prior to lysis (Fig. 6B). This showed again several discrete proteins recognized by siglec-E in a sialidase-sensitive manner (Fig. 6B, lanes 1 and 2).

To enrich sialylated glycoproteins from T cell lysates prior to blotting and siglec-E-Fc overlays, we precipitated glycoproteins using agarose-coupled MAL and SNA, which have a specificity for α2,3- and α2,6-sialylated glycans, respectively. In both cases, siglec-E-Fc bound to proteins enriched with MAL (Fig. 6B, lanes 3 and 4) and SNA (Fig. 6B, lanes 5 and 6) in a sialidase-sensitive manner (arrows). These included proteins with apparent molecular masses ranging from ~100 to >250 kDa, similar to those observed in the siglec-E pull-down experiments (Fig. 6A) as well as proteins migrating at ~65–72 kDa. Taken together, these data suggested that several glycoproteins on T cells display sialylated ligands recognized by siglec-E-Fc and argue against a small number of dominant counter-receptors. Despite numerous attempts, we were unable to identify by MS the nature of the proteins that were precipitated with siglec-E. Given that CD45 is a major glycosylated protein expressed on activated T cells (5), we asked whether this protein was among those recognized by siglec-E-Fc. To obtain sufficient cells, we used T cells activated for 24 h and expanded by culture for 4 days (Fig. 6C). The results show that siglec-E-Fc can precipitate CD45 in a sialic acid-dependent manner.

**DISCUSSION**

The glycosylation changes that occur during T-lymphocyte differentiation and activation are of biological interest due to their potential role in mediating interactions with lectins expressed by cells of the immune system (7–10, 12). Here we make the novel observation that 24 h after activation of murine CD4+ and CD8+ T-lymphocytes there is a striking up-regulation of ligands for siglec-E, an inhibitory siglec expressed on neutrophils, dendritic cells and activated macrophages (22, 35). However, the absence of parallel up-regulation of ligands at 24 h for SNA and MAL suggests that the increase in siglec-E binding was not simply due to increased levels of α2,3- or α2,6-linked sialic acids recognized by these lectins. MAL and SNA binding to activated T-lymphocytes remained relatively constant from 0 h to 24 h and then increased between 24 h and 48 h. At first sight, these observations may appear incongruous with previous studies showing in some cases that T-lymphocyte activation is accompanied by a decrease in sialylation, especially in core 1-O glycans of CD8+ cells (1–6). However, changes in cell surface sialylation during T cell activation are likely to be microheterogeneous, both at the levels of N- and O-glycans and glycoprotein backbones. For example, Hernandez *et al.* reported that T-cell activation was accompanied by an increase in ST3Gall-mediated sialylation of core 1-O glycans on CD45RB (5) and Comelli *et al.* demonstrated a reduction in sialylated biantennary N-glycans in activated CD4+ and CD8+ murine T-lymphocytes with reduced expression of the ST6Gall gene (6). It should be pointed out that this latter work was
conducted under different experimental conditions from our own. Cells were cultured with a combination of IL-2 and either IL-12 or IL-4, promoting either TH1 or TH2 polarization (6). Furthermore, MALDI-TOF comparisons of N-glycans as well as lectin binding studies were based upon the 72 h culture of cells in IL-2 and IL-12. It is therefore difficult to make direct comparisons with our work, which did not focus upon a 72 h time point. We cannot discount the possibility that the decrease in MAL and SNA binding from 48 h to 120 h is more marked by 72 h.

To gain insights into early changes in glycosylation, we undertook N-glycan profiling analyses. Resting T-lymphocyte profiles were predominantly bi-antennary, core fucosylated N-glycans, capped with NeuGc as observed previously in the glycomic analyses of murine splenic CD4+ and CD8+ T-lymphocytes (6). Comelli et al. (6) reported a dramatic decline in the abundance of NeuGcα2,6Galβ1,4GlcNAc at 72 h following T-lymphocyte activation, the appearance of Galα1,3Gal capping structures in place of NeuGc, and a notable absence of structures terminated with NeuAc. In contrast, we have shown here that early in T-lymphocyte activation there was an increased proportion of NeuAc- versus NeuGc-terminated bi-antennary N-glycans. We did see an increase in Galα1,3Gal capping structures in agreement with the previous glycomic analysis (6).

Because MS-based glycomics analysis shows the relative abundances of glycan structures in total cell lysates, it was important to use an alternative approach to determine if NeuGc levels on the T cell surface were decreased following activation. Using a chicken anti-NeuGc antibody developed previously (44), antibody binding was markedly reduced in activated CD4+ and CD8+ cells. We are confident this reflects a decreased level of NeuGc on the surface of activated T-lymphocytes, as we were able to examine the specificity of this antibody toward sialyl sequences in a glycan array containing 75 oligosaccharides capped in NeuAc and 8 in NeuGc. This screening also revealed that the anti-NeuGc antibody has a preference for glycoconjugates terminating in α2,6-linked NeuGc over α2,3-linked NeuGc and that there is little or no binding to GM1(Gc) sequences where the α2,3-linked NeuGc is internal.

CMP-NeuAc is converted to CMP-NeuGc by the enzyme CMAH prior to transport into the Golgi apparatus where it is transferred to glycan chain acceptors by various sialyltransferases. Although we did not measure levels of CMAH protein, quantitative PCR analysis showed reduced expression of mRNA encoding this enzyme occurring as early as 12 h following T-lymphocyte activation. This suggested that the increased proportion of NeuAc- to NeuGc-terminated N-glycans was most likely due to reduced conversion of NeuAc to NeuGc. Using surface plasmon resonance, we showed that siglec-E-Fc strongly prefers NeuAc over NeuGc containing oligosaccharides and therefore an increased abundance of NeuAc on activated T cells could be a significant factor leading to the increased binding of siglec-E.

Several other siglecs including Sn, MAG (siglec-4), CD22 and Siglec-9 also show a preference for either NeuAc or NeuGc (24, 45, 46). However, NeuGc is largely lacking in humans due to an irreversible 92 bp inactivating frameshift deletion in the human.
CMAH gene. Although NeuGc can be incorporated into some tissues and tumor cells from dietary sources, most human tissues are devoid of an endogenous source of NeuGc (44, 47, 48). Human T cells therefore lack NeuGc and potential up-regulation of siglec ligands in human T cells could not involve the NeuGc to NeuAc switch shown here for murine T cells.

Besides increased NeuAc expression, additional changes in glycan structure and/or presentation on protein carriers could contribute to increased siglec binding to activated T cells in different species. For example, the glycan array data showed a dramatic influence of 6- and 6'-sulfation in enhancing siglec-E binding to the sLe\(^{\alpha}\) sequence, and it was shown previously that activated murine T-lymphocytes up-regulate expression of a candidate sulfotransferase, GlcNAc6ST-1 (6). It is well established that sulfation may act as a positive or negative modulator of the binding of proteins of the innate immune system (49–51) including the siglecs (29): 6'-sulfation of sLe\(^{\alpha}\) structures greatly increased the binding of siglecs-8 and -F whereas 6-sulfation of sLe\(^{\alpha}\) had an enhancing effect on the binding of siglec-9 (29).

Interestingly, either 6- or 6'-sulfation led to greatly increased binding of siglec-E, and the presence of both sulfates did not increase binding further. This is rather different from what is seen with siglec-7 in humans, which binds to both monosulfated analogues but much more strongly to the disulfated (29). Sulfation was not detected with the MALDI-MS profiling technique used in this study and further work is required to measure its levels in glycans.

**FIGURE 5. Carbohydrate microarray analysis of siglec E-Fc.**

A, binding of siglec E-Fc to a glycan array of 101 lipid-linked oligosaccharide probes. The binding signals (fluorescence intensities) shown are the mean values at 7 fmol/spot and are indicated together with the probe sequences in supplemental Table S1. The various types of terminal sialic acid linkage are indicated by the colored panels as defined at the bottom of the panel. B–E, sequences of four groups of oligosaccharide probes marked in the histogram chart are given in panels as cartoon representations. Symbols used for individual monosaccharides are styled after the convention of the Consortium for Functional Glycomics, and are indicated in the key at the bottom of the figure. Circled in dash line (in B–E) are probes that showed little or no binding signals.
following T cell activation and whether it contributes to the increased binding of siglec-E to activated T cells.

Siglec-E is a relatively promiscuous sialic acid binding lectin, binding multiple glycans terminating in α2,3-, α2,6-, and α2,8-linkages. The glycan array data presented here demonstrated that siglec-E shares properties in common with siglecs-7, -8, and -9 and this is consistent with the broad expression of siglec-E on neutrophils, eosinophils, macrophages, and dendritic cells, which also differentially express siglecs-7, -8, and -9 in humans. For example, siglecs-E and -7 both bind well to α2,8-disialyl oligosaccharides and LSTb having internally branched α2,6 sialyl residues. A preferred ligand for siglec-9, GD1α, is also well recognized by siglec-E. Apart from this, siglec-E also showed some unique preferences to glycans in the microarray, such as the strong binding to short α2,3 sialyl sequences, gangliosides GM3 and GM4. The sialic acid specificity of all siglecs resides within the N-terminal V-set domain where a critical salt bridge is formed between the carboxylate of sialic acid and a conserved arginine (Arg126 in siglec-E). In the corresponding region in siglec-9 (52, 53). However, like many lectins, the binding of monovalent siglecs to their monomeric glycan ligands is typically of low affinity in the high micromolar to millimolar range with very fast binding kinetics (54). Stable interactions therefore require high avidity, multimeric complexes. Although siglec-E bound strongly to a broad range of ganglioside sequences in the glycan array, the attenuation of siglec-E binding to protease-treated cells suggests that glycolipids are unlikely to be among the functional ligands on T cells. As short sialyl O-glycans may share motifs with glycolipids, the increased binding of siglec-E to T cells could involve short clustered O-glycan ligands on particular glycoprotein carriers. Candidates include the mucin-like protein CD45, which carries a high density of O-linked glycans, or molecules like CD45 that carry a high number of both O-linked and N-linked glycans (5, 55, 56). These proteins have previously been shown to act as T cell counterreceptors for Sn and CD22, respectively (18, 57). We used both pull-down and blot overlay approaches with and without glycoprotein enrichment to try and identify potential siglec-E glycoprotein counter-receptors on activated T-lymphocytes. These approaches clearly showed that glycoproteins could be recognized in a sialic acid-dependent manner, but a technical problem was high background binding to the affinity matrices used which made it difficult to enrich specific glycoproteins to enable MS-based identification. Nevertheless, it is clear that siglec-E recognizes a large number of glycosylated proteins that include CD45. Their overall patterns of glycosylation are likely to be important for increased T cell binding, rather than up-regulation or altered glycosylation of a small number of dominant glycoprotein counter-receptors.

In conclusion, the present study has provided evidence for a reduced abundance of NeuGc on activated T lymphocytes. This may be at least partially responsible for the increase in siglec-E binding to activated T-lymphocytes. Reduced CMAH expression along with other glycan changes may accompany an earlier expansion phase following T-lymphocyte activation in the presence of abundant immune signals. The significance of siglec-E interactions with sialylated cell surface glycoproteins on activated T-lymphocytes is as yet unclear. Given its role as an inhibitory receptor on myeloid cells, these trans-interactions in the earlier phases of the adaptive immune response may generate inhibitory signals in siglec-E-expressing antigen presenting cells. Alternatively, these interactions may influence T lymphocyte responses. Studies investigating these possibilities involving several models based on siglec-E-deficient mice are currently underway.
Up-regulation of Siglec-E Ligands on Activated Murine T Lymphocytes

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