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α3/4 Fucosyltransferase 3–Dependent Synthesis of Sialyl Lewis A on CD44 Variant Containing Exon 6 Mediates Polymorphonuclear Leukocyte Detachment from Intestinal Epithelium during Transepithelial Migration

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Polymorphonuclear leukocyte (PMN) migration across the intestinal epithelium closely parallels disease symptoms in patients with inflammatory bowel disease. PMN transmigrational migration (TEM) is a multistep process that terminates with PMN detachment from the apical epithelium into the lumen. Using a unique mAb (GM35), we have previously demonstrated that engagement of the CD44 variant containing exon 6 (CD44v6) blocks both PMN detachment and cleavage of CD44v6. In this article, we report that PMN binding to CD44v6 is mediated by protein-specific O-glycosylation with sialyl Lewis A (sLea).

Abbreviations used in this article: CD44v6, CD44 variant containing exon 6; CFG, Consortium for Functional Glycomics; ddH2O, double-distilled H2O; ECD, extracellular domain; Fuc, fucose; Fut3, α3 fucosyltransferase 6; Fut6, α3/4 fucosyltransferase 3; Gal, galactose; GlcNAc, N-acetylgalcosamine; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; MPO, myeloperoxidase; MS, mass spectrometry; m/z, mass to charge ratio; Neu5Ac, N-acetylenuraminic acid; PMN, polymorphonuclear leukocyte; sCD44v6, soluble CD44v6; sLea, sialyl Lewis A; sLeC, sialyl Lewis C; sLex, sialyl Lewis X; sLeX, sialyltransferase 3; TEM, transmigrational migration; UC, ulcerative colitis; WT, wild type.

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differences in glycosylation (23, 24). One such variant is CD44v6, for which we developed a specific mAb (GM35). Using this mAb, we recently described a role for shedding of the ECD of CD44v6 in PMN detachment from the surface of the apical epithelium (12). Despite the highly glycosylated nature of CD44 variant isoforms, the role of specific glycosylation motifs in the function of these important proteins has yet to be characterized.

In this study, we investigated factors regulating PMN release from the apical epithelium. We show that, during inflammation, PMN detachment from the apical surface of the intestinal epithelium is regulated by glycan epitopes present on CD44v6. We demonstrate that the functionally inhibitory effects of the O-glycan–binding mAb GM35 are mediated through sialic acid–dependent binding to sialyl Lewis A (sLeA) specifically expressed on CD44v6, and that sLeA synthesis is regulated by glycan epitopes present on CD44v6.

**PMN isolation**

PMNs were isolated from whole blood obtained from healthy volunteers, with approval from the Emory University Institutional Review Board on human subjects, by using a previously described density gradient centrifugation technique (29). PMNs were resuspended in HBSS with 10 mM Hepes, pH 7.4, and without Ca2+ or Mg2+ at a concentration of 5 × 106 cells/ml. Neutrophils isolated in this way were 97% pure and >95% viable, and were used for transmigration within 2 h of blood draw.

**PMN transmigration assay**

For transmigration experiments, IECs were grown on collagen-coated, permeable, 0.33-cm2 polycarbonate filters (5-μm pore size; Costar) as described previously (9, 25, 30). All epithelial migration experiments were performed in the physiologically relevant basolateral-to-apical direction (i.e., inverted monolayers), in the presence of a chemotactic gradient of 100 nM fMLF. For migration experiments, 1 × 106 PMNs were added to the upper chambers of transwell inserts, and migration was measured at 37°C for indicated times in the presence of 10 μg/ml apically applied GM35, NS19-9, Dupan-2, or isotype control mAb. For analysis of effects of sialic acid-binding Ig-type lectins (Siglecs) on the GM35-mediated blockade of PMN TEM, PMNs were preincubated with 10 μg/ml Abs against Siglec-5, Siglec-9, and Siglec-14 for 20 min before the initiation of PMN TEM. Transmigrated PMNs were quantified by assaying for the PMN azurophilic marker myeloperoxidase (MPO) as described previously (31). PMNs, which migrated through tight junctions, yet remained adherent to the apical surface of the T84 IEC monolayer after basolateral-to-apical migration, were quantified using a previously described procedure (10). In brief, after completion of transmigration, T84 monolayers were removed and transferred to new tissue culture plates containing 1 ml HBSS/well. Plates were spun for 5 min to release PMNs that had migrated through functions yet remained adherent to the apical surface (50 × g, 4°C). Detached PMNs were quantified by MPO assay as described earlier.

**ELISA detection of soluble CD44v6**

PMNs were isolated and stimulated to migrate across confluent T84 monolayers in the presence or absence of apically applied NS19-9 (10 μg/ml), as described earlier. Samples from the apical reservoir were removed at 0, 5, 15, 30, 45, and 60 min and assayed for levels of soluble CD44v6 (sCD44v6) using a CD44v6 ELISA kit according to manufacturer’s instructions. A standard curve was prepared from six standard dilutions of sCD44v6, and levels of sCD44v6 in experimental samples and standards were measured at 450 nm.

**Immunoblotting and immunoprecipitation**

Cell lysates for immunoblotting were prepared with the following lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% TX-100, 1 mM Na3VO4, and 1 mM PMSF) supplemented with 10% mammalian tissue protease inhibitor mixture (Sigma-Aldrich). For immunoprecipitation experiments, precleared cell lysates were incubated with 2 μg relevant mAb for 4 h at 4°C, followed by incubation with protein G-Sepharose beads overnight at 4°C. Washed immunoprecipitates and regular cell lysates were boiled in SDS-PAGE sample buffer under reducing conditions and then subjected to SDS-PAGE followed by transfer to PVDF under standard conditions. Membranes were blocked with 4% milk and incubated with 1 μg/ml of the indicated mAb. Primary Abs were detected using appropriate HRP-linked secondary Abs (Jackson Immunoresearch Laboratories, West Grove, PA). Mouse small-intestine and colonic mucosal epithelial lysates were prepared after the serosa and external longitudinal layer of the muscularis propria were stripped away. Isoelectric focusing gels were solubilized in SDS sample buffer and analyzed for protein expression by immunoblot using indicated Abs. Primary Ab binding was detected with relevant HRP-conjugated secondary Ab (CD44v6) or with HRP-labeled streptavidin for biotinylated GM35.

**Isolation of glycoconjugates**

Human IECs (T84, SKCO15, and Caco2) were processed first by adding 8 M guanidinium HCl in 0.2 M Tris (pH 8.2). Proteins were then denatured by DTT and alkylated by iodoacetamide. Denatured samples were digested using trypsin and the glycopeptide mixture was analyzed using a glycan microarray containing a library of 111 individual structures, representing a library of known natural and synthetic mammalian glycans, in replicates of six. Binding of Abs to specific glycan epitopes was detected using fluorescein-labeled secondary Abs. An relative fluorescence unit ≥500 was set as a threshold indicative of positive binding.
against water overnight before lyophilization was performed. Lyophilized proteins were subsequently digested with trypsin in 50 mM phosphate buffer (pH 8.2) to generate glycopeptides that were susceptible to digestion with N-glycosidase (PNGase F). After PNGase F digestion, released N-glycans were obtained from the run-through of C-18 cartridges and collected by elution followed by subsequent application to a Carboxograph cartridge. Remaining peptides and glycopeptides containing the O-glycans were obtained by elution with 80 and 100% methanol from the C-18 cartridge. O-linked glycans were released by sodium hydroxide–mediated reductive β-elimination (50 mM NaOH, 1 M NaBH4) at 45°C for 16 h. Released O-glycans were obtained in the run-through of C-18 cartridges and collected by elution from a subsequent application to a Carboxograph cartridge.

**Permutation analysis of glycans**

N- and O-glycans were permethylated by sodium hydroxide and iodoacetamide in DMSO to allow for further structural analysis. Reactions were quenched by double-distilled H2O (diH2O) after 1 h, and the samples were extracted by chloroform before further washing with diH2O. The chloroform fraction was gently dried by nitrogen gas and redissolved in a 1:1 mix of methanol and diH2O. The samples were loaded onto a C-18 cartridge and eluted stepwise with diH2O and 15, 35, 50, and 75% aqueous acetonitrile. Permethylated glycans were usually present in the 35, 50, and 75% acetonitrile fractions.

**Mass spectrometry analysis of glycans**

An Ultraflex-III TOF/TOF system (Bruker Daltonics, Fremont, CA) was used for MALDI-TOF mass spectrometry (MS) analysis. Reflective positive modes were used as indicated in the figures. The 2.5-dihydroxybenzoic acid (5 mg/ml in 50% acetonitrile, 0.1% TFA) was freshly prepared to use as the matrix. A 0.05-μl matrix solution was spotted onto an AnchorChip target plate (200 or 400 μm) and air-dried before 0.5 μl sample solution was applied and also allowed to air-dry. The MS/MS data were acquired in positive reflector mode using an Applied Biosystem MALDI-TOF/TOF 4800 plus (Applied Biosystems, Foster City, CA). The collision energy was set to 2 kV, and argon was used as collision gas. Data were acquired using the 4000 Series Explorer Instrument Control Software and were processed using Data Explorer MS processing software (Applied Biosystems). MS/MS spectra were assigned and annotated with the help of the GlycoWorkbench tool from EuroCarbDB (http://www.eurocarbdb.org).

**Flow cytometry and immunostaining**

PMNs were isolated as described earlier and incubated with GM35 (10 μg/ml), NS19-9 (10 μg/ml), anti-SLeα mAb (10 μg/ml), anti-CD44v6 mAb (10 μg/ml), or the anti-CD11b/CD18 mAb CBRM1/29 (10 μg/ml). After washing, PMNs were incubated with a FITC-labeled secondary Ab, fixed with 2% PFA, and analyzed by flow cytometry. Flow cytometric analysis was carried out using a FACScan (Becton Dickinson, Franklin Lakes, NJ) equipped with an argon ion laser tuned at a 488-nm wavelength.

**Immunofluorescence labeling of IECs**

As previously described, fluorescently labeled secondary Abs. After Ab incubations, monolayers were mounted in ProLong antifading embedding solution (Invitrogen Life Technologies, San Diego, CA). Images shown were representative of at least three experiments with multiple images taken per monolayer.

For human tissue staining, frozen sections (6 μm) of discarded resection specimen colonic mucosa from patients with UC were obtained. Inflamed and noninflamed sections of discarded tissue were characterized based on observed extent of disease activity. Tissue was fixed in absolute ethanol, nonspecific protein binding was blocked with 3% BSA, and tissue sections were incubated with primary Abs, washed in HBSS*, and subsequently labeled with appropriate secondary Abs or with Zenon Alexa Fluor–labeled primary Abs (10 μg/ml) or with primary Abs and fluorescein-labeled secondary Abs. After Ab incubations, monolayers were mounted in ProLong antifading embedding solution (Invitrogen Life Technologies, San Diego, CA). For immunohistochemistry, tissue sections were incubated with GM35 at 10 μg/ml followed by hematoxylin and eosin staining to detect primary Ab binding. All procedures were carried out according to the manufacturer’s protocol as described previously (32). RNA was digested with DNase I (Ambion, Austin, TX) to remove contamination with genomic DNA; then cDNA was synthesized by reverse transcription using oligo(dT)12–18 primers and SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY). Real-time PCR was performed using a MyiQ real-time PCR machine and SYBR Green supermix (BioRad, Hercules, CA). Data were analyzed by the ΔΔCt threshold cycle method and normalized to the housekeeping gene GAPDH as previously described (33).

**Murine in vivo PMN trafficking assays**

Male wild type (WT) C57BL/6j mice (Jackson Laboratories) were maintained under specific pathogen-free conditions at Emory University Division of Animal Resources facilities. After overnight fasting, animals aged between 11 and 15 wk were anesthetized by s.c. i.m. injection of a mixture of 100 mg/kg ketamine and 5 mg/kg xylazine. Next, a midline abdominal incision was made and a 4-cm loop of small intestine was exteriorized and ligated at both proximal and distal ends (Fig. 7B). For PMN migration experiments, isolated intestinal loops were injected with 100 μM IMLF or 500 ng of the PMN chemotractant CXCL1 (KC), ± 50 μg indicated mAb, in 200 μl HBSS* followed by loop reinsertion into the peritoneal cavity. The abdomen was then sutured closed, and the animal was monitored for a 90-min incubation. Mice were then euthanized via rapid cervical dislocation, and the abdomen was reopened. Intestinal loops were isolated and lavaged twice with HBSS*. The number of PMNs reaching the intestinal lumen was quantitated based on cytospin analysis and Diff-Quik staining of the lavage fluid (Dade Behring, Newark, DE).

For dextran flux assays, isolated intestinal loops were injected with 100 μM IMLF and indicated Abs (50 μg GM35 or IgG control mAb) before reinsertion into the anesthetized mice for a 60-min incubation. After the initial incubation, FITC-dextran (10 kDa, 1 mg/ml in 200 μl saline) was injected into the intestinal loop, and mice were monitored for an additional 30 min. Passage of dextran out of the intestine into the vasculature was assessed through the measurement of fluorescence in peripheral blood (obtained through cardiac puncture) using the Fluostar Galaxy plate reader (BMG LabTech, Germany). After cardiac puncture, mice were euthanized via rapid cervical dislocation. PMNs were depleted as previously described (34) to assess the role of PMN influx in the observed changes in barrier permeability after introduction of IMLF to the lumen of the murine small intestine. In brief, anti-Ly6G Ab (200 μg/mouse) was injected into the i.p. cavity of mice 24 h before exteriorization of the small intestine loop and dextran flux assay, as described earlier. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Emory University.

**Data analysis**

Statistical differences were determined by two-factor ANOVA using PRISM 5 for Mac OSX version 5.0a 1992–1998, GraphPad software. Values are expressed as the mean and SE from at least three separate experiments.

**Results**

Closely regulated PMN translocation into the intestinal lumen is a crucial part of a successful host response to infection and injury. However, dysregulation of PMN influx into the intestinal tissues is also indicative of inflammatory bowel disease (IBD). In studies to characterize surface molecules that regulate PMN transmigration, we generated a unique mAb (GM35) that blocks PMN trafficking into the intestinal lumen through binding to an epitope on the apically expressed epithelial protein CD44v6 (12). GM35 binds the O-linked glycan SLeα in a sialic acid–dependent fashion

CD44v6 is a high m.w. member of the widely distributed type I transmembrane CD44 family of glycoproteins. Previous reports have indicated that the ECDs of CD44 variant proteins, including CD44v6, are extensively posttranslationally modified to contain N- and O-glycans (13, 21). To determine whether the functional effects of CD44v6 engagement were mediated specifically through glycan binding, we exposed T84 and HT29 IECs, previously shown to express the GM35 epitope, to glycosylation inhibitors before cell lysis and analysis of Ag expression by immunoblot. Pretreatment

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of these IECs with the inhibitor of N-glycosylation, tunicamycin (Fig. 1A), or the inhibitor of complex-type N-glycan formation, kifunensine (data not shown), had no effect on expression of the GM35-binding epitope on CD44v6. In contrast, treatment with benzyl-GalNAc, a small, synthetic sugar analog that competitively inhibits the terminal glycosylation of core 1-O-glycans and subsequent downstream formation of core 2-O-glycans (35), significantly reduced the level of epitope detection by GM35 in T84 and HT29 IECs (Fig. 1B). These findings are consistent with prior reports indicating that the glycans on the extracellular variant exon-encoded regions of CD44 proteins are predominantly O-linked (21).

To both confirm the specificity of GM35 for a carbohydrate ligand and further characterize the glycoepitope recognized by this mAb, we analyzed the affinity of GM35 binding to specific glycan determinants on a glycan microarray developed by the CFG. These glycan arrays contain immobilized glycans, representing a variety of known glycan structures presented on both N- and O-glycan backbones, as described in Materials and Methods. The results show that GM35 binds strongly to each of three glycans containing the tetrasaccharide determinant Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc-R (Fig. 1C). This glycan determinant is composed of four monosaccharides, N-acetyllactosamine acid (Neu5Ac; sialic acid), galactose (Gal), fucose (Fuc), and N-acetylgalactosamine (GlcNAc), and represents the human blood group Ag sLe(a). Further analysis revealed almost equivalent binding of GM35 to sLe(a) as a component of the extended structures sLe(a)-sLex (Glycan 1) and sLe(a)-sLe(a) (Glycan 2), with weaker binding to the sLe(a) tetrasaccharide alone (Glycan 3; Fig. 1C). In addition to these strong interactions with sLe(a)-containing glycans, microarray analysis also revealed weaker

**FIGURE 1.** GM35 binds to the O-linked glycan sLe(a). T84 or HT29 IECs were pretreated with (A) 5 μg/ml tunicamycin or (B) 4 mM benzyl-GalNAc before whole-cell lysis and Western blotting with GM35. Data represent n = 3 immunoblots. (C) The glycans recognized by GM35, including glycan number, glycan structure, glycan name, and relative fluorescence units (RFU) intensity, are depicted with sLex included as a negative binding control. (D) Protein lysates from T84 or HT29 IECs were treated with 50 U neuraminidase before whole-cell lysis and Western blotting with GM35. Data represent n = 3 immunoblots.
binding of GM35 to a variant form of sLeα containing N-glyco-
lyneuraminic acid (Neu5Gc), a derivative of Neu5Ac not normally
synthesized in humans, but which has been reported to be present
in certain cancers (36). It was also interesting to note that GM35
recognized sLeα with both high affinity and specificity, and in that
GM35 showed no reactivity to glycans on the array terminating in its
structural isomer, sLeβ (Neu5Acα2-3Galβ1-4[Neu5Acβ1-3]GlcNAc; Glycan 9; Fig. 1C).

Glycan binding analysis also revealed lower affinity recognition by
GM35 of the nonfucosylated sLeα precursor, so-called sLex (Neu5Acα2-3Galβ1-3-GlcNAc; Glycans 4, 5, 7, 8; Fig. 1C). mAbs against sLeα and sLeβ are commercially available with anti-
sLeα mAbs commonly used to screen for tumors of intestinal, hepatic, and pancreatic origin (37). Therefore, as a positive control for glycan recognition specificity, glycan microarray analyses of the anti-sLeα mAb NS19-9 (38) and the anti-sLeβ mAb Dupan-2 (39) were performed. Results confirmed the specific binding of mAb NS19-9 to the tetrasaccharide sLeα (Supplemental Fig. 1A). Furthermore, the three sLeα-containing glycans recognized with the highest binding by GM35 were identical to those recognized most avidly by mAb NS19-9. In addition, glycan array analyses revealed that mAb Dupan-2 bound specifically to two sLeβ variant glycans (Supplemental Fig. 1B) that contained the three sLeβ constituent sugars in the same conformation as the sLeα glycan structures recognized by GM35 (Fig. 1C).

Although sLeα in its entirety is common to the glycans binding GM35 with highest affinity, all the glycans, even those recognized by GM35 with lower affinity, contain a terminal sialic acid residue linked to Gal by an α-2-3 linkage. To further characterize the GM35 carbohydrate binding interaction, we examined the role of the terminal sugar residue Neu5Ac. Removal of Neu5Ac from IEC protein lysates by neuraminidase treatment resulted in a complete ablation of GM35 binding, suggesting a crucial role for this sugar moiety in the binding of GM35 to its primary target sLeα (Fig. 1D). Having identified the role of (GM35 binding) sialylated epithelial epitopes in PMN TEM, we next wanted to investigate candidates for the PMN sialic acid binding receptor responsible for the GM35-mediated blockade of PMN TEM (12). Therefore, the potential contributions of the known PMN-expressed sialic acid–binding Ig-type lectins (Siglec-5, -9, -14) (40) to the GM35-mediated block of PMN TEM was assessed. Preincubation of PMNs with functionally active Abs against Siglec-5, -9, and -14 (41, 42) had no significant effect on the GM35-mediated block in PMN TEM (Supplemental Fig. 1C). These findings suggest that previously reported PMN-expressed Siglec are unlikely to be the counterligand(s) for the sialylated binding epitope recognized by GM35 on intestinal epithelium.

Anti-sLeα mAb NS19-9, but not anti-sLeβ mAb Dupan-2, inhibits PMN TEM

We have previously demonstrated that the GM35 binding epitope is not expressed on the surface of PMNs (12), and thus its effects on PMN TEM must be mediated through specific interactions with the epithelium. Furthermore, immunoblotting analyses confirmed variations in IEC line expression of sLeα, as detected by mAb NS19-9. A large sLeα glycoprotein of a similar size to the protein recognized by GM35 (12) was detected by mAb NS19-9 in both T84 and HT29 IECs, but not in SKCO15 or Caco2 cells (Fig. 2A). Consistent with these findings, neither NS19-9 nor GM35 had any effect on PMN TEM across Caco2 monolayers (data not shown). This cell-specific pattern of expression for sLeα is consistent with our prior observations for the GM35 Ag (12). In contrast, sLeβ was expressed by T84, HT29, and SKCO15, but not by Caco2 IECs (Fig. 2B). Interestingly, whereas T84 and HT29, but not Caco2, cells are known to independently express CD44v6 and sLeα (43–45), the relationship between these two entities has not been previously studied.

Next, we examined the functional effects of mAbs specific for either sLeα (NS19-9) or sLeβ (Dupan-2) on PMN TEM. Apical treatment of T84 IECs with NS19-9 significantly inhibited PMN TEM, relative either to control IECs treated without Ab (p < 0.01) or to those treated apically with a noninhibitory IgG binding control Ab (p > 0.01; Fig. 2C). Furthermore, the reduction in PMN TEM was associated with a significant increase in the numbers of PMN that had crossed through tight junctions yet remained adherent to the apical surface of the epithelium (Fig. 2D). Thus, the arrest in TEM occurred at the level of detachment from the apical epithelial membrane, as we have previously reported for GM35-mediated blockade of PMN TEM (12). Analysis of the kinetics of the effects of NS19-9 revealed consistent inhibition of migration over a 3-h time course (Fig. 2E). Although immunofluorescent analysis of the subcellular localization of sLeα on T84 IECs demonstrated low levels of apical expression of this carbohydrate structure (Fig. 2F), this anti-sLeα mAb had no effect on PMN TEM across T84 IECs (Fig. 2G), indicating that the carbohydrate determinants that regulate intestinal trafficking of PMN are highly specific.

Given our prior findings that ligation of the GM35 ligand inhibits PMN TEM by blocking shedding of CD44v6, we next explored the effect of NS19-9–dependent masking of the sLeα glycoepitope on PMN TEM by blocking shedding of CD44v6, we next explored the effect of NS19-9–dependent masking of the sLeα glycoepitope on PMN TEM-dependent cleavage of CD44v6. ELISA-based detection of sCD44v6 ECD in the apical supernatants from T84 IECs after PMN TEM (Fig. 2H) confirmed our previous findings that significant levels of CD44v6 were released from T84 IECs between 30 and 60 min of PMN TEM (12). Furthermore, binding of NS19-9 to epithelial sLeα blocked the release of CD44v6 (Fig. 2I). In contrast with the effects seen when NS19-9 is present for the duration of the migration assay, addition of mAbs against sLeα after completion of PMN migration did not prevent the detection of shed CD44v6 (Fig. 2J). These data demonstrate that NS19-9 (and GM35, data not shown) do not prevent binding of released CD44v6 to the capture or detection Abs of the ELISA, but rather that binding of mAbs to sLeα interferes with the release of epithelial CD44v6.

NS19-9 recognizes sLeα displayed by an O-linked glycan on CD44v6

NS19-9 was next used to confirm that the sLeα expressed by T84 and HT29 IECs was displayed on an O-linked carbohydrate backbone. Indeed, treatment of T84 and HT29 IECs with benzyl-GalNAc, but not tunicamycin, reduced recognition of the sLeα carbohydrate epitope by mAb NS19-9 (Fig. 3A, 3B). In addition, pretreatment of IEC protein lysates with neuraminidase ablated binding of the anti-sLeα mAb NS19-9 to its O-linked carbohydrate epitope (Fig. 3C), demonstrating that the negatively charged sialic acid residue is again critical for Ab-mediated recognition of sLeα.

Given that GM35 was initially characterized as binding to the transmembrane glycoprotein CD44v6 (12), the relative contribution of CD44-specific-sLeα to total detected levels of sLeα was next assessed. shRNA-based attenuation of CD44 expression was performed in the readily transfectable, sLeα expressing HT29 IECs. As we have reported previously for the GM35 Ag (12), knockdown of CD44v6 protein expression in HT29 IECs by shRNA plasmids (data not shown) also corresponded to a significant reduction in the expression of sLeα as detected by NS19-9 (Fig. 3D).

We next used glycomic analyses to gain a broader understanding of the global similarities and differences in glycosylation patterns
across IEC lines to further define the structure of sLeα-containing glycans on T84 IECs, as well as to identify candidate mechanisms for differential regulation of sLeα synthesis. Analysis of N- and O-glycans released from T84 IECs using MALDI-TOF MS revealed that O-glycans from T84 IECs have compositions dominated by predicted core 1 (mass to charge ratio \[m/z\] 1256.5 and 895.2) and core 2 structures (\[m/z\] 983.4, 1344.4, and 1705.6; Fig. 3E). Also included among the predicted O-glycans are several higher m.w. extended core 2 structures (\[m/z\] 1879.9, 2329.0, and 2503.1).

**FIGURE 2.** mAb NS19-9, but not Dupan-2, blocks PMN TEM and CD44v6 shedding. Indicated cells were lysed and proteins immunoblotted with NS19-9 (A) or Dupan-2 (B). Confluent T84 monolayers were pretreated with 10 μg/ml NS19-9, 10 μg/ml GM35, or 10 μg/ml noninhibitory IgG1 isotype control mAb before 1 × 10⁶ PMNs were added to the basolateral surface. PMNs were allowed to migrate for 1 h in response to a 100-nM gradient of fMLF. The number of migrated PMNs (C) and the number of PMNs that were adherent to the apical epithelial surface (D) were quantified by MPO assay. Data depict means ± SE (n = 3). (E) Confluent T84 monolayers were treated apically with (closed circle) or without (open circle) 10 μg/ml NS 19-9 before the addition of 1 × 10⁶ PMNs. PMN transmigration was then measured at the indicated time points. Data are mean ± SE (n = 3). (F) Confluent T84 monolayers were costained with 10 μg/ml anti-Occludin mAb and 10 μg/ml Dupan-2, and analyzed by confocal microscopy. Original magnification ×40. (G) Confluent T84 monolayers were pretreated with 10 μg/ml Dupan-2 or 10 μg/ml noninhibitory IgG isotype control mAb before 1 × 10⁶ PMNs were added to the basolateral surface. PMNs were allowed to migrate for 1 h in response to a 100-nM gradient of fMLF. The numbers of migrated PMNs were quantified by MPO assay. Data are means ± SE (n = 3). A total of 1 × 10⁶ PMNs was added to confluent T84 monolayers treated apically with 10 μg/ml binding control IgG1 (H) or 10 μg/ml NS19-9 (I). PMNs were allowed to migrate in the basolateral-to-apical direction in response to a 100-nM gradient of fMLF. At the time points indicated, the T84-containing filters were removed, and the solution from the apical migration reservoir was assayed for sCD44v6 by ELISA. (J) Addition of mAb NS19-9 to the apical migration reservoir after PMN TEM does not prevent binding of released CD44v6 to the capture or detection ELISA mAbs. Significance was defined as p < 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001).
2329.0, and 2503.1). Whereas MS by this approach cannot distinguish between the structural isomers sLe^a and sLe^b, our biochemical analyses of the sLe^a binding mAbs GM35 and NS19-9, as well as the anti-sLe^a mAb CD15s (data not shown), make it likely that the sialyl Lewis structures detected in this study are sLe^a. Notably, surface glycan profiling also indicated that the sLe^a structures on T84 IECs are confined to N-glycans (Fig. 3F).

In contrast with the extensive glycosylation of T84 IECs, the O-glycans released from SKCO15 (Supplemental Fig. 2A, 2B) were much less complex, and Caco2 (Supplemental Fig. 2C, 2D) IECs had decreased structural complexity of both N- and O-glycans. Thus, in addition to differences in sLe^a expression, these IEC lines also revealed more global differences in total N- and O-glycosylation. Furthermore, there were no sialyl Lewis structures of any kind

FIGURE 3. NS19-9 and GM35 bind to O-linked sLe^a. T84 or HT29 IECs were pretreated with (A) 5 μg/ml tunicamycin or (B) 4 mM benzyl-GalNAc before whole-cell lysis and Western blotting with NS19-9. (C) Protein lysates isolated from T84 IECs were treated with neuraminidase before immunoblotting with NS19-9. (D) Representative immunoblot demonstrating that transfection of HT29 IECs with CD44 gene silencing plasmids (shRNA1-3) decreased the expression of sLe^a detected by NS19-9 as compared with scrambled control. Data depict representative results from n = 3 immunoblots. MALDI-TOF/TOF spectrometric analysis of the O-linked (E) and N-linked (F) derivatized and permethylated glycans isolated from T84 IECs.
displayed by either the N- or O-glycans from SKCO15 IECs. MS analysis of the glycans displayed by Caco2 IECs did reveal low levels of expression of a small number of predicted sialyl Lewis structures (m/z 1519, 1880.4). However, given the lack of GM35 (12) and NS19-9 binding (Fig. 2A) to Caco2 IECs, and the positive binding of the anti-sLea mAb CD15s to Caco2 lysates by immunoblot (data not shown), these sialylated Lewis Ag structures detected by MALDI-TOF are most likely to be sLea rather than sLea.

To further characterize the sLea-containing glycans displayed by T84 IECs, MS/MS analysis of the O-glycans was performed through collision-induced dissociation (CID), demonstrating that the six O-linked sialyl Lewis-containing glycans on T84 IECs are core 2 structures (Supplemental Fig. 3A–F). MALDI-TOF MS analysis also revealed that the N-glycans from T84 IECs are mainly composed of high mannose-type glycans, ranging from Man9GlcNAc2 to Man13GlcNAc2 (m/z 1579.8, 1784.0, 1988.1, 2182.2 and 2396.3) with minor biantennary (m/z 2605.6) or triantennary (m/z 3590.0) complex-type structures (m/z 2605.6, 3590.0; Fig. 3F). Importantly, analysis of the N-glycans from T84 IECs revealed no expression of any sialyl Lewis structures. Therefore, the combined results of immunoblot and MS demonstrate that the sLea Ag in T84 IECs is found only on core 2 or extended core 2 O-glycans.

**Differential expression of sLea corresponds to differential glycosyltransferase expression**

MS analyses revealed global differences in the extent and complexity of glycosylation across IECs, and that sLea is confined to core 2 O-glycans. sLea synthesis is also likely to be regulated, at least in part, by enzymes directly implicated in the biosynthesis of the family of sialylated Lewis structures. Therefore, the differential expression of glycosyltransferases with potential roles in sLea synthesis (Fig. 4A), including fusocyl and sialyltransferases, was examined in IEC lines, which either expressed both CD44v6 and sLea (T84 and HT29), expressed sLea or expressed neither CD44v6 nor sLea (Caco2).

**Fut3 expression drives sLea synthesis in CD44v6-expressing cells**

A plasmid containing full-length Fut3, which naturally has both α1,3- and α1,4-fucosyltransferase activities, was transfected into sLea-deficient SKCO15 cells to determine whether the observed lack of Fut3 expression was truly related to the absence of sLea in specific IEC lines. Indeed, transfection with Fut3 resulted in robust expression of this fucosyltransferase relative to SKCO15 cells transfected with an empty vector (Fig. 5A). In addition, overexpression of Fut3 protein correlated with the expression of a large glycoprotein that was detected with GM35 (Fig. 5A) and NS19-9 (data not shown), suggesting that Fut3 expression can drive sLea biosynthesis in these IECs. To further demonstrate the importance of Fut3 synthesis for sLea expression in human IECs, we knocked down expression of Fut3 in HT29 IECs. Transfection with Fut3 siRNA decreased Fut3 protein expression and corresponded to a decrease in sLea expression detected by GM35 or NS19-9 (data not shown), relative to nontransfected HT29 IECs and to cells transfected with Scr siRNA (Fig. 5B).

The effects of mAb GM35 on PMN TEM across Fut3/sLea-expressing SKCO15 IECs were assessed next. Despite successful induction of both Fut3 and sLea expression, mAb GM35 had no effect on PMN trafficking across sLea-expressing SKCO15 cells (Fig. 5C). However, immunofluorescence analysis of the expression of surface epitopes in these transfected IECs revealed little to no colocalization of sLea and CD44v6 (Fig. 5D). This suggests that although Fut3 expression does drive sLea synthesis in SKCO15 IECs, these cells lack other intracellular processes necessary for the posttranslational glycosylation/decoration of CD44v6 with sLea. Thus, GM35 appears to mediate its functional effects on PMN TEM through engagement of the glycan sLea specifically when this glycoepitope is present on the epithelial protein CD44v6.

**sLea colocalizes apically with CD44v6 and is upregulated in UC**

Further demonstration of the role of sLea decorated CD44v6 in regulating PMN detachment from the apical epithelial surface was assessed through comparison of the cellular localization of sLea relative to its structural isomer sLea and the glycoprotein CD44v6. Confocal microscopic analysis of immunofluorescently stained, nonpermeabilized T84 IEC monolayers revealed that GM35 binds specifically to sLea on apically expressed CD44v6 (Fig. 6A). Furthermore, immunofluorescence and immunohistochemical analyses of colonic mucosa from individuals with UC revealed increased expression of sLea in inflamed tissue versus noninvolved regions from the same patient (Fig. 6B, 6C), indicating that the expression of sLea on intestinal epithelial CD44v6 is physiologically relevant to human intestinal inflammation. Furthermore, immunohistochemical analysis of colonic tissue from individuals with UC revealed GM35 staining of material accumulated within the intestinal crypt, consistent with our in vitro results indicating PMN-dependent shedding of sLea-containing CD44v6 during PMN TEM (Fig. 6C, red arrow). In addition, costaining of inflamed UC colonic tissue for CD44v6 and sLea revealed strong colocalization of these molecules (Fig. 6D), demonstrating the localization of the sLea glycan to apically expressed epithelial CD44v6 in inflamed human mucosa.

**Intraluminal GM35 attenuates both PMN TEM and associated barrier compromise in murine small intestine**

Having previously demonstrated a role for GM35 in the prevention of PMN TEM in vitro, we sought to determine whether these findings could be extended into a murine in vivo model of intestinal inflammation. Epithelial cell preparations from small and large
intestines of adult C57BL/6J mice were found to express a glycoprotein recognized by Western blot by GM35 (Fig. 7A). Furthermore, this glycoprotein was of comparable molecular mass with mouse CD44v6 (∼200 kDa). However, in contrast with human tissues, there was robust basal expression of both CD44v6 and the GM35 epitope in murine small and large intestinal epithelia in the absence of exogenous inflammatory stimuli (Fig. 6B, 6C).

The presence of a GM35-binding glycoprotein in murine intestine supported further investigations of the physiologic relevance of the functional effects of GM35 in mouse intestine. Initially, it was verified that injection of exogenous chemoattractant into a closed externalized loop of small intestine from an anesthetized mouse would, within 60 min, result in quantifiable migration of PMNs into the intestinal lumen (model in Fig. 7B, 7C). In the absence of exogenous chemoattractant, PMNs were not detected within the intestinal lumen. However, injection of either fMLF (100 μM) or the murine cytokine KC resulted in a significant influx of PMNs. Importantly, coinjection of GM35 along with fMLF resulted in a significant decrease in the number of PMNs reaching the intestinal lumen, relative to those treated with fMLF and an isotype control mAb (Fig. 7C).

It has previously been shown that PMN infiltration into intestinal tissues disrupts intestinal barrier function (48). Therefore, we examined whether GM35-dependent blockade of PMN TEM would alter barrier function in this model. Isolated small intestinal loops were injected intraluminally with fMLF, in the presence of either GM35 or an isotype-matched IgG control mAb, and PMN migration was allowed to proceed for 60 min. These same loops were then injected with 10 kDa FITC-conjugated dextran and incubated for an additional 30 min. At this time, mice were sacrificed and intestinal permeability was quantified by measurement of fluorescence of the peripheral blood. PMN migration into the intestinal lumen was indeed accompanied by increased intestinal
permeability to 10 kDa FITC dextran (Fig. 7D). Furthermore, this increase in permeability was prevented by either mAb GM35 or by systemic neutrophil depletion, using an antiLy6G mAb (200 μg i.p., 24 h before the experiment; Fig. 7D). Thus, luminal GM35 blocked both chemoattractant-stimulated PMN TEM and associated increases in permeability in murine small intestine.

Discussion

Controlled PMN TEM is an essential component of the innate immune response against invading microorganisms. However, dysregulated influx of PMNs across the intestinal epithelium results in the formation of crypt abscesses and is indicative of IBD. PMN accumulation at epithelial surfaces is also relevant to other inflammatory processes including periodontitis, cystitis, and infectious enterocolitis (49). Therefore, a greater understanding of the mechanisms governing the late stages of PMN TEM, including PMN detachment from the apical epithelium, may provide new and unique therapeutic targets for regulating mucosal inflammation.

Immunoblotting and MS analyses revealed that sLea on CD44v6 is exclusively O-linked and independent of N-glycosylation. These results are consistent with evidence that most variant exons of CD44, including CD44v6, are primarily O-glycosylated (13, 50, 51), with only two alternative exons (CD44v5 and CD44v10) containing NXS/T, the motif for N-glycosylation (13). Furthermore, many of the O-glycans attached to CD44v6 terminate with sialic acid residues (50), consistent with the results of this study revealing that the sialic acid is essential for GM35 ligand binding.

Expansion of these findings using glycan array analysis technology identified the highest affinity glycoprotein recognized by GM35 as the sialic acid–containing glycan determinant sLea. Lower affinity binding to sLe−, the nonfucosylated biosynthetic precursor of sLea, was also detected. However, sLe− localized exclusively to N-linked glycans at the apical surface of T84 IECs and an mAb to anti-sLe− (Dupan-2) had no functional effect on PMN TEM. Furthermore, expression of sLe− has been previously associated primarily with the large transmembrane glycoprotein Muc-1 (52, 53) and not with CD44v6.

The glycans of glycoproteins, including CD44, are known to serve as ligands in recognition systems particularly in inflammation and immunity (54, 55), and glycans displayed by cell-surface glycoproteins have been shown to play an important role in the regulation of leukocyte trafficking (56). Interestingly, leukocyte expressed N-linked glycans are known to act as ligands for E-selectin. In addition, neutrophil core 1–derived O-glycans can also function to bind E-selectin (57). Furthermore, loss of IEC-specific core 1–derived O-glycans is associated with spontaneous colitis in mice (58). In this
FIGURE 6. NS19-9 and GM35 bind to apical sLe\textsuperscript{a} on CD44v6 in inflamed colonic mucosa. Confluent T84 monolayers were costained with 10 μg/ml NS19-9 (green) and 10 μg/ml GM35, 10 μg/ml anti-CD44v6 mAb, or 10 μg/ml anti-sLex mAb (red). Apical protein localization was determined by confocal microscopy analysis. Representative images from \( n = 3 \) experiments are shown en face or in the \( xz \) plane of section. Original magnification ×40 (A). Cryosections of noninflamed colonic mucosa and inflamed sections of colonic mucosa from patients with active UC were examined for localization of sLe\textsuperscript{a} (NS19-9, green) and the epithelial marker Desmoglein 1 (red) as described in Materials and Methods. Original magnification ×40 (B). (C) Immunohistochemical analysis of colonic epithelia from a patient with UC was performed using the anti-sLe\textsuperscript{a} mAb GM35 (brown). (Figure legend continues)
we identify a novel role for an sLea-containing core 2 glycan in PMN–epithelial interactions in the intestine.

In further support of a role for sLea in modulating events at the apical epithelial surface of the intestine during inflammation, this tetrasaccharide determinant, first described by Koprowski et al. (38), has previously been reported to be highly expressed on the surface of established human pancreatic, colon, and gastric cancer cell lines in vitro (59, 60), as well as in human adenocarcinomas of the colon, stomach, gall bladder, and pancreas (38, 61, 62). Whereas sLea itself has previously been reported to act as an important mediator of cell–cell adhesion (63), to our knowledge, our studies of PMN TEM represent the first report of a role for this carbohydrate Ag in the regulation of PMN trafficking. Specifically, we demonstrate that sLea on CD44v6 can be targeted to significantly inhibit PMN TEM through inhibition of cleavage of CD44v6 and the detachment of PMN from the luminal epithelial surface. These findings identify a novel, glycan-dependent mechanism for the regulation of PMN TEM and provide the first evidence, to our knowledge, of a functional role for sLea in mucosal inflammation.

We discovered in our study, an epithelial cell–specific expression pattern for sLea, with robust expression observed in T84 and HT29, but not Caco2 or SKCO15 IECs. This restricted pattern of expression for sLea was further confirmed by MS mapping of the surface glycans expressed by each of the IEC lines examined in this study. This cell-specific expression pattern for sLea was exploited to identify specific fucosyltransferases involved in the biosynthesis of sLea in human IECs. Comparative analysis of galactosyl, sialyl, and fucosyl transferase expression in sLea-expressing (T84 and HT29) and sLea nonexpressing (Caco2 and SKCO15) IECs demonstrated numerous differences relevant to the biosynthesis of this inflammation-responsive sLea determinant.

Most significantly, expression levels of the fucosyltransferases revealed a profound increase in the expression of α3/4 Fut3 in T84 IECs relative to SKCO15 and Caco2 IECs. The absence of α3/4 Fut3 in SKCO15 IECs correlates with the lack of expression of fucosylated Lewis structures observed by MS characterization of the SKCO15 glycan profile. In support of the role of Fut3 in the generation of the GM35 Ag sLea in human IECs, Fut3 predominantly exhibits α1–4 fucosyltransferase activity and has previously been implicated in the synthesis of sLea in other systems (46, 47). Further, it has also been previously reported that α1-3 fucosyltransferases catalyze the final step in the synthesis of a range of glycoconjugates known to be involved in cell adhesion and lymphocyte recirculation (47). Thus, differing levels of sLea synthesis are likely to be due, in part, to the massive differences in Fut3 protein expression seen across IECs. In support of this, transfection of Fut3 into SKCO15 IECs induced a robust expression of the sLea glycoepitope. However, despite successful forced expression of sLea in these Fut3-expressing SKCO15 cells, GM35 did not block PMN TEM across these transfected IECs. This lack of a functional effect of GM35 in these sLea-expressing SKCO15 cells, GM35 did not block PMN TEM across these transfected IECs. This lack of a functional effect of GM35 in these sLea-expressing SKCO15 IECs was attributed to the observation that although these cells synthesized sLea, this glycoepitope was not displayed on CD44v6, further underscoring the importance of the role of this epithelial glycoprotein in regulating PMN TEM.

These findings also emphasize the physiologic relevance of sLea on CD44v6 to intestinal inflammation. sLea is upregulated in concert with CD44v6 in inflamed regions of human colon from patients with UC. Furthermore, luminal injection of GM35 blocks PMN TEM in vivo. This blockade of PMN TEM is associated with preservation of intestinal barrier function, normally compromised by PMN TEM. Therefore, it is highly likely that future identification of both the specific GM35 binding glycan determinant (either sLea or sLea -) and the associated protein ligand in murine intestinal tissues, as well as further characterization of the novel glycan profile of normal and inflamed murine intestinal epithelium, will provide useful targets for future therapeutics in IBD.

Given that expression of both CD44v6 (12) and the sLea determinant are upregulated in the intestinal crypts of patients with UC, this novel glycosylation-dependent interaction is a promising potential target for therapeutic intervention in IBD.
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Disclosures
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