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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 transepithelial 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 (sLeα). Analyses of glycosyltransferase expression identified fucosyltransferase 3 (Fut3) as the key enzyme driving sLeα biosynthesis in human intestinal epithelial cells (IECs). Fut3 transfection of sLeα-deficient IECs resulted in robust expression of sLeα. However, this glycan was not expressed on CD44v6 in these transfected IECs; therefore, engagement of sLeα had no effect on PMN TEM across these cells. Analyses of sLeα in human colonic mucosa revealed minimal expression in noninflamed areas, with striking upregulation under colitic conditions that correlated with increased expression of CD44v6. Importantly, intraluminal administration of mAb GM35 blocked PMN TEM and attenuated associated increases in intestinal permeability in a murine intestinal model of inflammation. These findings identify a unique role for protein-specific O-glycosylation in regulating PMN–epithelial interactions at the luminal surface of the intestine. The Journal of Immunology, 2013, 191: 4804–4817.
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 inhibited by NSAIDs. Analyses of sLeA and CD44v6 in human colonic mucosa revealed that sLeA synthesis is α3/4 fucosyltransferase 3 (Fut3)–dependent. Analyses of sLeA and CD44v6 in human colonic mucosa revealed expression that was restricted to regions of active inflammation. Inhibitory effects of mAb GM35 on PMN transmigratory migration (TEM) were confirmed in an in vivo model of intestinal inflammation, where blockade of PMN TEM also prevented increases in intestinal permeability.

Materials and Methods

Cell culture

Cultures of T84 (25), HT29 (26), Caco2 (11), and SKCO15 (27) intestinal epithelial cells (IECs) were grown as previously described.

Abs and reagents

Monoclonal anti-CD44v6 Ab was purchased from R&D Systems (Minneapolis, MN). Monoclonal anti-desmoglein mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-sLeA Ab (NS19-9) was purchased from Dako (Carpinteria, CA). Anti–sialyl Lewis A (anti-sLeA) mAb (CD15a) was purchased from BD Franklin Lakes, NJ). The anti–sialyl Lewis C (anti-sLeC) mAb (Dupon-2) was purchased from Glycotech Corporation (Rockville, MD). Anti-Fut3 mAb was purchased from Abcam (Cambridge, MA). mAb GM35 was isolated as described previously (12). The anti-CD11b/CD18 mAb CBRM1/29 has been described elsewhere (28). Abs against Siglec-5, Siglec-9, and Siglec-14 were purchased from Abcam (Cambridge, MA) and BD Bioscience (San Jose, CA). Protein G-Sepharose beads were purchased from GE Healthcare Life Sciences (Pittsburgh, PA). Zenon Alexa Fluor 488 Mouse IgG1, 568 Mouse IgG1, and 568 Mouse IgG4 labeling kits were purchased from Invitrogen (Carlsbad, CA). BSA, chemotactic peptide IMLF, ABTS, 2-aceamido-2-deoxy-β-D-galactopyranoside, iodoacetamide, guanidinium chloride, streptavidin, DTT, and neuraminidase from Arthrobacter ureafaciens were purchased from Sigma-Aldrich (St. Louis, MO). Tunicamycin was purchased from Calbiochem (La Jolla, CA). Kifunensine was purchased from EMD Chemicals (Newark NJ). N-glycosidase F/PNase-F was purchased from New England Biolabs (Ipswich, MA). Full-length CD44 in a pCMV entry vector plasmid, CD44-specific hairpin RNA (shRNA) constructs, and a Scz construct plasmid in pGFP-V-RS vectors and Fut3 and Scz siRNA duplexes were purchased from Origene (Rockville, MD). Human CD44var6 (CD44v6) instant ELISA kits were purchased from Bender Medsystems (Vienna, Austria). SYBR green supermix was purchased from Bio-Rad (Hercules, CA).

Treatment of human IECs with glycosylation inhibitors

T84 or HT29 IECs were grown in the presence or absence of tunicamycin at 5 μg/ml in complete medium for 24 h at 37˚C to block N-glycosylation. N-glycan extension was removed in incubated cells with 5 mM benzyl GalNAc in complete media for 24–48 h at 37˚C. For removal of sialic acid, T84 and HT29 IEC protein lysates were treated with neuraminidase overnight at 4˚C according to manufacturer’s instructions.

Glycan microarray assay

Abs were submitted for glycan binding analysis to the Consortium for Functional Glycomics (CFG; http://www.functionalglycomics.org), GM35 (cfg_request_2785, pa_v5), at 0.1 μg/ml, and the anti-sLeA Ab NS19-9 (cfg_request_1982, pa_v41) and the anti-sLeC Ab Dupan-2 (cfg_request_2614, pa_v51) at 10 μg/ml. These glycan microarrays contain up to 611 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 fluorescently labeled secondary Abs. An relative fluorescence unit ≥500 was set as a threshold indicative of positive binding.

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 Heps, 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 IMLF. 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 junctions 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 physiologically relevant basolateral-to-apical direction 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 assessed for the 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, preincubated 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). Murine small-intestine and colonic mucosal epithelial lysates were prepared after the serosa and external longitudinal layer of the muscularis propria were stripped away. Isolated mucosal sheets 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 dialyzed...
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 Carboxaphage 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 Carboxaphage cartridge.

**Permeability of glycans**

N- and O-glycans were permeated by sodium hydroxide and iodomethane in DMSO to allow for further structural analysis. Reactions were quenched by double-distilled H2O (ddH2O) after 1 h, and the samples were extracted by chloroform before further washing with ddH2O. The chloroform fraction was gently dried by nitrogen gas and redissolved in a 1:1 mix of methanol and ddH2O. The samples were loaded onto an C-18 cartridge and eluted stepwise with ddH2O and 15, 35, 50, and 75% aqueous acetonitrile. Permeated glycans were usually present in the 35, 50, and 75% acetonitrile fractions.

**Mass spectrometry analysis of glycans**

An Ultraflex-Ⅱ 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 0.5-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 Bio-systems, Foster City, CA). The collision energy was set to 24 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-αLea 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 in 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. Immunofluorescent labeling of IECs was achieved as follows. Non-permeabilized T84 monolayers were fixed using absolute ethanol and subsequently blocked with 3% BSA. Monolayers were then incubated with Zenon Alexa Fluor–labeled primary Abs (10 μg/ml) or with primary Abs and 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). Nuclei were stained with TO-PRO-3 iodide (1:1000 X 5 min, room temperature; 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 on discarded human tissue were carried out in accordance with the Emory University Institutional Review Board approval. All images were captured using an LSM 510 confocal microscope (Carl Zeiss Microimaging, Thornwood, NY) with pan-Neofluor 40X/1.3 oil objective using software supplied by the vendor.

**Transcriptional analysis**

Human IECs were lysed in TRIzol (Invitrogen Life Technologies, San Diego, CA), then subjected to phenol-chloroform extraction 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).

**Marine 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 fMLF or 500 ng of the PMN chemoattractant 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 cytopsin 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 fMLF 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 fMLF 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 TEM, 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 sLea in a static 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...
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 Neu5Ac2-3Galβ1-3(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 sLeα. Further analysis revealed almost equivalent binding of GM35 to sLeα as a component of the extended structures sLeα-sLeβ (Glycan 1) and sLeα-sLeα (Glycan 2), with weaker binding to the sLeα tetrasaccharide alone (Glycan 3; Fig. 1C). In addition to these strong interactions with sLeα-containing glycans, microarray analysis also revealed weaker

![FIGURE 1.](http://www.jimmunol.org/) GM35 binds to the O-linked glycan sLeα. 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 SLε containing N-glycolylneuraminic 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 SLε with both high affinity and specificity, and that GM35 showed no reactivity to glycans on the array terminating in its structural isomer, SLε (Neu5Aca2-3Galβ1-4[Fucα1-3]GlcNAc; Glycan 9; Fig. 1C).

Glycan binding analysis also revealed lower affinity recognition by GM35 of the nonfucosylated SLε precursor, so-called SLε (Neu5Aca2-3Galβ1-3-GlcNAc; Glycans 4, 5, 7, 8; Fig. 1C). mAbs against SLε and SLε are commercially available with anti-SLε 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-SLε mAb NS19-9 (38) and the anti-SLε mAb Dupan-2 (39) were performed. Results confirmed the specific binding of mAb NS19-9 to the tetrasaccharide SLε (Supplemental Fig. 1A). Furthermore, the three SLε-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 SLε variant glycans (Supplemental Fig. 1B) that contained the three SLε constituent sugars in the same conformation as the SLε glycan structures recognized by GM35 (Fig. 1C).

Although SLε 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 carbohydrate epitope by mAb NS19-9 (Fig. 3A, 3B). In addition, CD44v6-specific-sLea to total detected levels of sLea was studied.

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 SLε, as detected by mAb NS19-9. A large SLε 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 SLε is consistent with our prior observations for the GM35 Ag (12). In contrast, SLε 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 SLε (43–45), the relationship between these two entities has not been previously studied.

Next, we examined the functional effects of mAbs specific for either SLε (NS19-9) or SLε (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 Abs (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 but 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 SLε on T84 IECs demonstrated low levels of apical expression of this carbohydrate structure (Fig. 2F), this anti-SLε 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 SLε glycoepitope on PMN TEM-dependent cleavage of CD44v6. ELISA-based detection of CD44v6 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 SLε 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 SLε 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 SLε interferes with the release of epithe- lial CD44v6.

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

NS19-9 was next used to confirm that the SLε 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 SLε carbohydrate epitope by mAb NS19-9 (Fig. 3A, 3B). In addition, pretreatment of IEC protein lysates with neuraminidase ablated binding of the anti-SLε 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 SLε.

Given that GM35 was initially characterized as binding to the transmembrane glycoprotein CD44v6 (12), the relative contribution of CD44-specific-SLε to total detected levels of SLε was next assessed. shRNA-based attenuation of CD44 expression was performed in the readily transfectable, SLε 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 SLε 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 sLe\(\alpha\)-containing glycans on T84 IECs, as well as to identify candidate mechanisms for differential regulation of sLe\(\alpha\) 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 \(\mu\)g/ml NS19-9, 10 \(\mu\)g/ml GM35, or 10 \(\mu\)g/ml noninhibitory IgG1 isotype control mAb before \(1 \times 10^6\) 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 \(\mu\)g/ml NS 19-9 before the addition of \(1 \times 10^6\) 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 \(\mu\)g/ml anti-Occludin mAb and 10 \(\mu\)g/ml Dupan-2, and analyzed by confocal microscopy. Original magnification \(\times 40\). (G) Confluent T84 monolayers were pretreated with 10 \(\mu\)g/ml Dupan-2 or 10 \(\mu\)g/ml noninhibitory IgG isotype control mAb before \(1 \times 10^6\) 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 were quantified by MPO assay. Data are means ± SE (\(n = 3\)). A total of \(1 \times 10^6\) PMNs was added to confluent T84 monolayers treated apically with 10 \(\mu\)g/ml binding control IgG1 (H) or 10 \(\mu\)g/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\textsuperscript{a} and sLe\textsuperscript{c}, our biochemical analyses of the sLe\textsuperscript{a} binding mAbs GM35 and NS19-9, as well as the anti-sLe\textsuperscript{c} mAb CD15s (data not shown), make it likely that the sialyl Lewis structures detected in this study are sLe\textsuperscript{a}. Notably, surface glycan profiling also indicated that the sLe\textsuperscript{c} 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\textsuperscript{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\textsuperscript{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\textsuperscript{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 GM3S (12) and NS19-9 binding (Fig. 2A) to Caco2 IECs, and the positive binding of the anti-sLe<sup>a</sup> mAb CD15s to Caco2 lysates by immunoblot (data not shown), these sialylated Lewis Ag structures detected by MALDI-TOF are most likely to be sLe<sup>a</sup> rather than sLe<sup>e</sup>.

To further characterize the sLe<sup>a</sup>-containing glycans displayed by T84 IECs, MS/MS analysis of the O-glycans of a T84 isolate 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 Man₈GlcNAc₂ to Man₉GlcNAc₂ (m/z 1579.8, 1784.0, 1988.1, 2192.2 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 sLe<sup>a</sup> Ag in T84 IECs is found only on core 2 or extended core 2 O-glycans.

Differential expression of sLe<sup>e</sup> corresponds to differential glycosyltransferase expression

MS analyses revealed global differences in the extent and complexity of glycosylation across IECs, and that sLe<sup>e</sup> is confined to core 2 O-glycans. sLe<sup>e</sup> 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 sLe<sup>e</sup> synthesis (Fig. 4A), including fucosyl and sialyltransferases, was examined. Results demonstrate the expression of any sialyltransferase such as α2,3/α2,6-sialyltransferase 3 (ST3Gal3), which has been shown to be important for sLe<sup>e</sup> synthesis in other cells (46, 47), in addition to changes in Fut3 expression, a 7-fold increase in the expression of α1,3 fucosyltransferase 6 (Fut6) was also observed in T84 relative to Caco2 IECs (p < 0.05), but not relative to SKCO15 cells (Fig. 4B). However, Fut6 has previously been implicated in the generation of sLe<sup>e</sup>, but not sLe<sup>a</sup> (46). No other significant differences were observed for the expression of the additional fucosyltransferases examined (Supplemental Fig. 4C).

The expression levels of ST3Gal sialyltransferases, which catalyze the addition of Neu5Ac to Gal during sLe<sup>e</sup> synthesis, were also examined. Results demonstrate the expression of α2,3 sialyltransferase 3 (ST3Gal3), which has been shown to be important for sLe<sup>e</sup> synthesis in other cells (46), was significantly lower in T84 cells relative to Caco2 and SKCO15 cells. Thus, another sialyltransferase such as α2,5-ST3Gal2 may be involved in sLe<sup>e</sup> biosynthesis in T84 IECs (Fig. 4B). No other significant differences in the expression of ST3Gal sialyltransferases were observed (Supplemental Fig. 4B). Observed differences in ST3Gal3 and Fut3 gene expression were subsequently confirmed at the level of protein expression in that T84 IEC lysates revealed increased expression of Fut3 (Fig. 4C) and lower ST3Gal3 protein expression relative to SKCO15 and Caco-2 IECs.

Fut3 expression drives sLe<sup>e</sup> 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 sLe<sup>e</sup>-deficient SKCO15 cells to determine whether the observed lack of Fut3 expression was truly related to the absence of sLe<sup>e</sup> 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 GM3S (Fig. 5A) and NS19-9 (data not shown), suggesting that Fut3 expression can drive sLe<sup>e</sup> biosynthesis in these IECs. To further demonstrate the importance of Fut3 synthesis for sLe<sup>e</sup> generation 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 sLe<sup>e</sup> expression detected by GM3S or NS19-9 (data not shown), relative to nontransfected HT29 IECs and to cells transfected with Scr siRNA (Fig. 5B).

The effects of mAb GM3S on PMN TEM across Fut3/sLe<sup>e</sup>-expressing SKCO15 IECs were assessed next. Despite successful induction of both Fut3 and sLe<sup>e</sup> expression, mAb GM3S had no effect on PMN trafficking across sLe<sup>e</sup>-expressing SKCO15 cells (Fig. 5C). However, immunofluorescence analysis of the expression of surface epitopes in these transfected IECs revealed little to no colocalization of sLe<sup>e</sup> and CD44v6 (Fig. 5D). This suggests that although Fut3 expression does drive sLe<sup>e</sup> synthesis in SKCO15 IECs, these cells lack other intracellular processes necessary for the posttranslational glycosylation/decoration of CD44v6 with sLe<sup>e</sup>. Thus, GM3S appears to mediate its functional effects on PMN TEM through engagement of the glycan sLe<sup>e</sup> specifically when this glycoepitope is present on the epithelial protein CD44v6.

sLe<sup>e</sup> colocalizes apically with CD44v6 and is upregulated in UC

Further demonstration of the role of sLe<sup>e</sup> decorated CD44v6 in regulating PMN detachment from the apical epithelial surface was assessed through comparison of the cellular localization of sLe<sup>e</sup> relative to its structural isomer sLe<sup>a</sup> and the glycoprotein CD44v6. Confocal microscopic analysis of immunofluorescently stained, nonpermeabilized T84 IEC monolayers revealed that GM3S binds specifically to sLe<sup>a</sup> on apically expressed CD44v6 (Fig. 6A). Furthermore, immunofluorescence and immunohistochemical analyses of colonic mucosa from individuals with UC revealed increased expression of sLe<sup>e</sup> in inflamed tissue versus noninvolved regions from the same patient (Fig. 6B, 6C), indicating that the expression of sLe<sup>e</sup> on intestinal epithelial CD44v6 is physiologically relevant to human intestinal inflammation. Furthermore, immunohistochemical analysis of colonic tissue from individuals with UC revealed GM3S staining of material accumulated within the intestinal crypt, consistent with our in vitro results indicating PMN-dependent shedding of sLe<sup>e</sup>-containing CD44v6 during PMN TEM (Fig. 6C, red arrow). In addition, containing of inflamed UC colonic tissue for CD44v6 and sLe<sup>e</sup> revealed strong colocalization of these molecules (Fig. 6D), demonstrating the localization of the sLe<sup>e</sup> glycan to apically expressed epithelial CD44v6 in inflamed human mucosa.

Intraluminal GM3S attenuates both PMN TEM and associated barrier compromise in murine small intestine

Having previously demonstrated a role for GM3S 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

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**FIGURE 4.** Differential expression of glycosyltransferases in human IECs. (A) Schematic representation of the glycosyltransferases responsible for the biosynthesis of sLeα. (B) RNA was isolated from indicated IECs and analyzed by real-time PCR for levels of expression of ST3Gal3, Fut3, and Fut6. Results are depicted as fold change relative to Caco2 IECs and normalized for expression of the housekeeping gene GAPDH. n = 3–6. Significance was defined at p < 0.05 (*p < 0.05, **p < 0.01). (C) After epithelial cell lysis, equal amounts of protein lysates from indicated IECs were subjected to SDS-PAGE under reducing conditions and immunoblotted with indicated Abs. Data represent n = 3 immunoblots.
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 epithelial surface, 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 CD44 variant glycoproteins 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 glycteope revealed by GM35 as the sialic acid–containing glycan determinant sLea. Lower affinity binding to sLea, the nonfucosylated biosynthetic precursor of sLea, was also detected. However, sLea localized exclusively to N-linked glycans at the apical surface of T84 IECs and an mAb to anti-sLea (Dupan-2) had no functional effect on PMN TEM. Furthermore, expression of sLea 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 5. Fut3 drives sLea expression in SKCO15 IECs, but not on CD44v6. (A) SKCO15 IECs transfected with empty vector or Fut3 containing vector were immunoblotted with indicated Abs. Data represent n = 3 immunoblots. (B) HT29 IECs either nontransfected or transfected with Scr or Fut3 siRNA were lysed and immunoblotted with Abs against Fut3 and sLea. (C) Nontransfected SKCO15 IECs and SKCO15 IECs transfected with Fut3 or an empty vector were grown as confluent inverted monolayers. Monolayers were pretreated apically with 10 μg/ml GM35 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 was quantified by MPO assay. (D) Confluent Fut3 transfected/sLea–expressing SKCO15 monolayers were costained with 10 μg/ml GM35 (red) and 10 μg/ml anti-CD44v6 mAb (green). 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.
FIGURE 6. NS19-9 and GM35 bind to apical sLe\(^a\) 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-sLe\(^a\) 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\(^a\) (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\(^a\) mAb GM35 (brown). (Figure legend continues)
study, we identify a novel role for an sLeα-containing core 2 glycan in PMN–epithelial interactions in the intestine.

In further support of a role for sLeα 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 sLeα 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 sLeα 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 sLeα in mucosal inflammation.

We discovered in our study, an epithelial cell–specific expression pattern for sLeα, with robust expression observed in T84 and HT29, but not Caco2 or SKCO15 IECs. This restricted pattern of expression for sLeα 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 sLeα was exploited to identify specific glycosyltransferases involved in the biosynthesis of sLeα in human IECs. Comparative analysis of galactosyl, sialyl, and fucosyl transferase expression in sLeα-expressing (T84 and HT29) and sLeα nonexpressing (Caco2 and SKCO15) IECs demonstrated numerous differences relevant to the biosynthesis of this inflammation-responsive sLeα 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 sLeα in human IECs, Fut3 predominantly exhibits α1-4 fucosyltransferase activity and has previously been implicated in the synthesis of sLeα 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 sLeα 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 sLeα glycoepitope. However, despite successful forced expression of sLeα 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 sLeα-expressing SKCO15 cells, GM35 was attributed to the observation that although these cells synthesized sLeα, 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 sLeα on CD44v6 to intestinal inflammation. sLeα 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 sLeα or sLeβ) 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 sLeα 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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References