O-linked oligosaccharides (O-glycans) are the primary components of the intestinal mucus layer that overlies the gastrointestinal epithelium. This layer is a dense, carbohydrate-rich matrix that consists primarily of mucins containing multiple serine and threonine residues, which have been modified by O-glycans and which account for 80% of the mucin mass (1–6). The mucus layer and epithelial cells comprise an intestinal barrier that protects epithelial and intestinal mucosal immune cells from potentially harmful luminal microflora and food components (3–6) and participates in bacterial colonization (7). The role of intestinal epithelial cells in maintaining barrier function and in the pathogenesis of several common intestinal diseases, such as inflammatory bowel disease (IBD) and colorectal cancer, has been well studied (8–15). However, the physiological and pathological significance of the mucus layer has been less explored.

Increased susceptibility to colitis and colorectal tumors in mice lacking core 3–derived O-glycans

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Altered intestinal O-glycan expression has been observed in patients with ulcerative colitis and colorectal cancer, but the role of this alteration in the etiology of these diseases is unknown. O-glycans in mucin core proteins are the predominant components of the intestinal mucus, which comprises part of the intestinal mucosal barrier. Core 3–derived O-glycans, which are one of the major types of O-glycans, are primarily expressed in the colon. To investigate the biological function of core 3–derived O-glycans, we engineered mice lacking core 3 β1,3-N-acetylglucosaminyltransferase (C3GnT), an enzyme predicted to be important in the synthesis of core 3–derived O-glycans. Disruption of the C3GnT gene eliminated core 3–derived O-glycans. C3GnT-deficient mice displayed a discrete, colon-specific reduction in Muc2 protein and increased permeability of the intestinal barrier. Moreover, these mice were highly susceptible to experimental triggers of colitis and colorectal adenocarcinoma. These data reveal a requirement for core 3–derived O-glycans in resistance to colonic disease.

O-glycans containing GalNAc in α-linkage to serine or threonine residues occur on many membrane and secreted proteins, particularly mucins (1, 2). O-glycans have two main core structures, referred to as core 1– and core 3–derived O-glycans (Fig. 1 A). The biosynthesis of these core structures is controlled by specific glycosyltransferases. IBD is generally recognized as an immune-mediated disorder resulting from an abnormal interaction between colonic microflora and mucosal immune cells in a genetically susceptible host (14, 16). How this interaction develops is not well understood. A deterioration of the mucus layer of the colon is prominent in patients with ulcerative colitis, which is a common form of IBD (3–6). Altered intestinal O-glycan expression appears early in the pathogenesis of ulcerative colitis (6). Interestingly, similarly altered O-glycans are seen in >90% of colorectal cancers, which have a close association with ulcerative colitis (6, 17, 18). Whether or not this abnormal O-glycan expression contributes to the etiology of these diseases is unknown. O-glycans containing GalNAc in α-linkage to serine or threonine residues occur on many membrane and secreted proteins, particularly mucins (1, 2). O-glycans have two main core structures, referred to as core 1– and core 3–derived O-glycans (Fig. 1 A). The biosynthesis of these core structures is controlled by specific glycosyltransferases.
Core 3 β1,3-N-acetylglucosaminyltransferase (C3GnT) activity is enriched in mucin-secreting epithelial tissues, such as gastrointestinal tract, as measured by enzymatic activity assays in tissue lysates (1, 2, 19, 20). The enzyme transfers GlcNAc from UDP-GlcNAc to GalNAcα1-Ser/Thr (Tn antigen) to form the core 3 O-glycan (GlcNAcβ1,3GalNAcα1-Ser/Thr), which can be further modified to form more complex structures, such as core 4 O-glycans (Fig. 1 A). Recently, human core C3GnT (also known as β3Gn-T6 or core 3 synthase) was identified (19, 20). In vitro biochemical analysis suggests that C3GnT is the only enzyme responsible for the biosynthesis of core 3 O-glycans (19, 20).

We hypothesized that core 3–derived O-glycans are a key constituent of the intestinal mucus layer and are important for intestinal barrier function, and that the alteration of core 3–derived O-glycan expression plays a role in the pathogenesis of common intestinal diseases, such as colitis and intestinal tumors. To test these hypotheses, we created mice lacking core 3–derived O-glycans by targeted deletion of the C3GnT gene (C3GnT−/−). We found that deletion of the C3GnT gene eliminated core 3–derived O-glycans and significantly reduced total intestinal glycans. Furthermore, C3GnT−/− mice exhibited an increased susceptibility to experimental colitis and colorectal adenocarcinoma. The results presented in this study indicate that core 3–derived O-glycans are key components of intestinal mucin, and that defects in their expression may be associated with the pathogenesis of colonic disease.

**RESULTS**

**Molecular characterization of the murine C3GnT gene**

A *Mus musculus* chromosome 7 genomic contig (NT_039433) that contains the C3GnT gene and a 2,370-bp, full-length murine cDNA sequence (XM_195661) were identified by BLASTN searches using the published human C3GnT nucleotide sequence (19). The analyses indicated that murine C3GnT is a type II membrane protein with 68% identity to the human C3GnT (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20061929/DC1). Southern blot analysis (unpublished data) confirmed that the murine C3GnT gene has two exons. Exon 1 comprises 36 bp that encode a 5′-untranslated region. Exon 2 comprises 2,318 bp encoding the ATG translational start site, cytoplasmic domain, transmembrane domain, stem region, and the catalytic domain of C3GnT. RT-PCR of RNA extracted from different mouse tissues revealed that murine C3GnT mRNA is predominantly expressed in colonic tissue (Fig. S2 A). The ileum and the salivary glands also expressed low levels of C3GnT transcripts, whereas the rest of the analyzed tissues had no C3GnT expression. To determine if the identified C3GnT gene confers β1,3-N-acetylglucosaminyltransferase activity, we cloned the C3GnT cDNA into a baculovirus expression system (21). Enzymatic analysis indicated that recombinant murine C3GnT exhibited β1,3-N-acetylglucosaminyltransferase activity, which transfers GlcNAc from UDP-GlcNAc to GalNAcα1-R, thus confirming the identity of the cloned murine C3GnT gene (Fig. S2, B and C).

**Generation of C3GnT−/− mice**

To study the biological function of C3GnT, we generated mice lacking the C3GnT gene by targeted homologous recombination in mouse embryonic stem cells (Fig. 1, B and C) using previously described methods (22, 23). RT-PCR and enzymatic assays confirmed that C3GnT mRNA and C3GnT enzyme activity in tissue extracts were eliminated in C3GnT−/− mice (Fig. 1, D and E). To characterize the tissue expression pattern of endogenous C3GnT, we engineered a LacZ reporter gene immediately downstream of the C3GnT ATG translational start site. LacZ staining of different C3GnT−/− tissues confirmed that expression of C3GnT was restricted to colonic tissues (Fig. 1 F and not depicted). Although RT-PCR

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**Figure 1. Generation of C3GnT−/− mice.** (A) The scheme shows the two major O-glycan branching pathways. C3GnT refers to C3GnT. Arrowheads show the possible pathways for further branching, elongation, fucosylation, sialylation, and sulfation. (B and C) Strategy to generate mice lacking core 3–derived O-glycans by targeting the C3GnT gene, and Southern blot genotyping using EcoRV restriction enzyme digestion. (D) RT-PCR confirmed the deletion of the C3GnT gene product. GAPDH was used as an amplification control. (E) C3GnT enzymatic activity in C3GnT+/+ and C3GnT−/− tissues. Error bars represent the mean ± the SD. n = 3. (F) LacZ staining of C3GnT+/+ and C3GnT−/− colonic tissues. Bar, 100 μm.
detected lower levels of endogenous C3GnT mRNA in the small intestine and salivary glands, lacZ staining was not detected in these tissues (unpublished data), which likely reflected sensitivity differences between the two assays.

C3GnT−/− mice developed normally, and both sexes were fertile in a specific pathogen-free barrier facility. Inter-crosses between heterozygotes yielded normal-sized litters with Mendelian inheritance. C3GnT−/− mice had normal peripheral blood counts (unpublished data). Gross morphology and histological examinations of 6–20-wk-old mice revealed no observable differences between wild-type litters (C3GnT+/+) and C3GnT−/− mice in major organs, including heart, liver, salivary glands, stomach, jejunum, ileum, colon, spleen, thymus, and lymph nodes (Fig. S3 and Fig. S4 A, available at http://www.jem.org/cgi/content/full/jem.20061929/DC1; and not depicted). Flow cytometry of C3GnT−/− peripheral blood cells and splenocytes revealed no substantial abnormalities in the absolute number and the ratios of CD3+ T cells (including CD4+, CD8+, and innate-like invariant NKT cells) and of CD19+ B cells (including innate-like marginal zone B cells; Fig. S4 B and not depicted). This suggests that the deficiency of core 3–derived O-glycans does not affect the development of immune cells.

Disruption of the C3GnT gene eliminates core 3–derived O-glycans and exposes the Tn antigen in murine colon

We used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to define the O-glycan structures in C3GnT+/+ and C3GnT−/− colons (24). Consistent with previous data, both core 1– and core 3–derived O-glycans (core 3 O-glycans, fucosylated core 3 O-glycans, and core 4 O-glycans) were dominant structures expressed in intestinal tissues. However, enzymatic desialylation revealed the deletion of the C3GnT gene would expose Tn antigen in intestinal tissues. As predicted, immunochemical staining of mouse colon tissue sections with an anti-Tn mAb labeled colonic tissue, indicating that the C3GnT−/− mice (Fig. 2, C and D). Consistent with those of C3GnT+/+ mice (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20061929/DC1). However, both PAS staining and Alcian blue staining were quantified by Photoshop software based on six sections of three independent mice from each group. Error bars represent the mean ± the SD. Bars: (B) 50 μm; (C) 100 μm.

We then used periodic acid-Schiff’s reagent (PAS) and Alcian blue to determine if the deficiency of core 3–derived O-glycans affects the expression of intestinal glycans. PAS stains neutral carbohydrates, whereas Alcian blue recognizes acidic carbohydrates that may represent siaIylated, fucosylated, or sulfated sugars. PAS and Alcian blue staining remained unchanged in the jejunum and the ileum of both C3GnT+/+ and C3GnT−/− mice (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20061929/DC1). However, both PAS staining and Alcian blue staining were significantly reduced in the colons of C3GnT−/− mice compared with those of C3GnT+/+ mice (Fig. 2, C and D). Consistent with the RT-PCR and lacZ staining data, this result demonstrates that core 3–derived O-glycans are predominantly expressed in mouse colon.
Deficiency of C3GnT results in reduced Muc2 expression in colon and impaired mucosal integrity

To investigate if a deficiency of core 3 O-glycosylation affects the expression of intestinal mucins, we first screened the mRNA levels of the major intestinal mucins by semiquantitative RT-PCR. We observed no significant differences between C3GnT\(^{+/+}\) and C3GnT\(^{-/-}\) colons in mRNA levels of Muc2, Muc3, Muc13, and Muc5ac (Fig. S7 A, available at http://www.jem.org/cgi/content/full/jem.20061929/DC1). However, probing with a glycosylation-independent polyclonal antibody revealed a substantial reduction of Muc2 protein, which is the predominant component of the intestinal mucous layer (3–6, 18, 25) in C3GnT\(^{-/-}\) colonic tissue (Fig. 3, A and B). We questioned whether the decreased expression of Muc2 was caused by a reduced number of goblet cells. However, immunohistochemical analysis of C3GnT\(^{+/+}\) and C3GnT\(^{-/-}\) colon tissues revealed similar expression of intestinal trefoil factor (ITF), which is a nonmucin protein and a product of fully differentiated goblet cells (Fig. 3, A and B) (26). In addition, Alcian blue staining and transmission electron microscopic imaging revealed no considerable morphological difference between C3GnT\(^{+/+}\) and C3GnT\(^{-/-}\) goblet cells (Fig. S7, B and C). Thus, goblet cells are preserved in the C3GnT\(^{-/-}\) colon, but goblet cell production of Muc2 is reduced. We were unable to examine protein expression of other, less-expressed, intestinal mucins because of the lack of glycosylation-independent antibodies to these murine glycoproteins.

To investigate if the loss of core 3–derived O-glycans affects mucosal integrity, we fed C3GnT\(^{+/+}\) and C3GnT\(^{-/-}\) mice with FITC-dextran by gavage, and 4 h later we measured their serum levels of FITC-dextran to evaluate intestinal permeability. Serum levels of FITC-dextran in C3GnT\(^{-/-}\) mice were significantly higher than in C3GnT\(^{+/+}\) mice (Fig. 3 C). Fluorescent microscopic analysis of the C3GnT\(^{+/+}\) and C3GnT\(^{-/-}\) intestinal cryosections revealed no fluorescent infiltration of FITC-dextran into both C3GnT\(^{+/+}\) and C3GnT\(^{-/-}\) small intestinal villi. In contrast, a higher level of fluorescent intensity was observed in C3GnT\(^{-/-}\) colonic tissues compared with that in C3GnT\(^{+/+}\) tissues (Fig. 3 D). Collectively, these data indicate that colonic mucosal integrity is impaired in C3GnT\(^{-/-}\) mice.

We reasoned that the deficiency of core 3 O-glycans and the impairment in mucosal integrity might result in an alteration of mucosa-associated bacteria in C3GnT\(^{-/-}\) mice. We used 16S bacterial ribosomal DNA (rDNA)–based real-time PCR analysis to address this question. Two sets of universal 16S rDNA primers (16S1 and 16S2) reproducibly detected a significant increase in 16S rDNA in C3GnT\(^{+/+}\) colonic tissues in comparison to that in C3GnT\(^{-/-}\) colonic tissues (Fig. 3 E). The increase is not likely caused by variations in luminal bacterial flora because the amount of 16S rDNA in C3GnT\(^{+/+}\) and C3GnT\(^{-/-}\) fecal material was similar (Fig. 3 E). Mesenteric nodes had weak, yet detectable, 16S rDNA signal, but there was no difference between C3GnT\(^{+/+}\) and C3GnT\(^{-/-}\) groups (unpublished data). Mesenteric bacterial translocation is associated with trafficking of bacteria-bearing dendritic cells from the mucosa, typically after Toll-like receptor sensing or other immune activation (27, 28). Thus, no detectable increase in bacterial translocation in C3GnT\(^{-/-}\) mesenteric nodes is consistent with the lack of spontaneous colonic inflammation in C3GnT\(^{-/-}\) mice.

C3GnT\(^{-/-}\) mice are highly susceptible to dextran sodium sulfate (DSS)–induced colitis

DSS is a chemical commonly used to induce experimental colitis (29). We examined the pathological consequence of
the deficiency of core 3 O-glycosylation using this model. We first challenged 6-wk-old C3GnT+/+ and C3GnT−/− males with 2.5% DSS in drinking water for 14 d to determine its lethality rate. We found that 9/10 C3GnT+/+ mice survived the treatment, whereas all C3GnT−/− mice died at the end of the 14-d treatment (Fig. 4 A). We then treated 6-wk-old C3GnT+/+ and C3GnT−/− males with 2% DSS in drinking water for 7 d, followed by 4 d of water without DSS. These experimental conditions induced colitis in both C3GnT+/+ and C3GnT−/− mice. However, the colitis in the C3GnT−/− mice was markedly more severe, with greater weight loss, diarrhea, fecal bleeding, and shortened colon length (Fig. 4, B–D, and Fig. S8, available at http://www.jem.org/cgi/content/full/jem.20061929/DC1). The inflammation was restricted to the colon, especially in the distal colonic region, whereas the small intestine was not substantially affected (Fig. S3). Histological examination showed that both C3GnT+/+ and C3GnT−/− colonic tissues appeared normal before DSS treatment. However, after 2% DSS treatment, C3GnT−/− mice had more severe crypt destruction, larger areas of epithelial ulceration, erosions, and massive inflammatory cell infiltration into the mucosal tissue (Fig. 4, E and F).

To characterize the infiltrating inflammatory cells associated with DSS-induced colitis, we probed C3GnT+/+ and C3GnT−/− colonic tissues with anti-CD3 antibody to identify T lymphocytes and anti-F4/80 antibody to identify monocytes/macrophages. Compared with C3GnT+/+ mice, there was a dramatic increase of both T cells and monocytes/macrophages in the lamina propria of C3GnT−/− mice (Fig. 5, A and B).

To further understand the immunological features of DSS-induced colitis in C3GnT−/− mice, we evaluated intracellular production of the cytokines IL-2, IL-17, IFN-γ, and TNF-α in splenocytes (30). Compared with DSS-treated C3GnT+/+ mice, splenic CD4 T cells from DSS-treated C3GnT−/− mice exhibited modestly elevated levels of IL-2, IFN-γ, and TNF-α, suggesting that effector activation of CD4+ T cells associated with DSS-induced mucosal inflammation is greater in C3GnT−/− mice (Fig. 5 C).

C3GnT−/− mice are highly susceptible to DSS-induced colitis. We speculated that C3GnT−/− intestinal mucosal lymphocytes might be activated because of an impaired mucosal barrier function, even in the absence of DSS challenge. We thus examined TNF-α, IFN-γ, IL-17, and IL-6 expression in intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) from colons of C3GnT+/+ and C3GnT−/− mice without DSS treatment. Notably, the expression of the proinflammatory cytokines TNF-α, IFN-γ, and IL-17 by CD3+ IELs and LPLs was significantly increased in C3GnT−/− mice as compared with C3GnT+/+ mice (Fig. 5 C). This suggests an inflammation-prone status in intestines of C3GnT−/− mice. Interestingly, IL-6 expression was increased in the IELs of C3GnT−/− mice (Fig. 5 D), which may contribute to the increased susceptibility to colorectal tumor in the mice (31).

Accelerated colorectal tumorigenesis in C3GnT−/− mice after azoxymethane (AOM) and DSS treatment

Patients with ulcerative colitis exhibit increased risk of colorectal cancer (32, 33). Abnormal O-glycans such as the Tn antigen are expressed in >90% of colorectal cancers, but are absent in normal colonic mucosa (6, 17). Thus, we explored whether mice lacking core 3 O-glycans are susceptible to colorectal tumorigenesis. We used the well-established AOM/DSS model (34). 12 or 20 wk after AOM/DSS treatment, C3GnT−/− colon and rectum exhibited a fivefold increase in tumor volume (Fig. 6, A and B). Occasionally, small adenomas were found in the proximal colon, but no tumors were observed in the cecum, ileum, and jejunum of C3GnT+/+ or C3GnT−/− mice (unpublished data). Histologically, most lesions were composed of proliferating adenomatous columnar epithelium with prominent architectural and cytological atypia. The changes were consistent with tubular adenoma featuring extensive high-grade dysplasia (intramucosal carcinoma). In C3GnT−/− mice, 20 wk after treatment,
some of the adenomas progressed to adenocarcinomas that invaded muscularis mucosae and submucosa (Fig. 6 C).

To investigate the cellular mechanisms by which a deficiency of core 3–derived O-glycans affects colorectal tumorigenesis, we used bromodeoxyuridine (BrdU) metabolic labeling to examine the rate of colonic epithelial proliferation (25). 3 h after BrdU intraperitoneal injection, the number of BrdU-positive colonic epithelial cells per crypt in C3GnT−/− mice was significantly greater than those in C3GnT+/+ mice with or without AOM/DSS treatment (Fig. 6 D). These results indicate that the absence of core 3–derived O-glycans increases colonic epithelial cell proliferation in C3GnT−/− mice.

Wnt signaling is one of the key signaling pathways in colorectal cancer development in humans (35). Western blot analysis showed that the expression of β-catenin and TCF4, which are components of the Wnt pathway, was significantly greater in tumor and nontumor colon tissue extracts from C3GnT−/− mice (Fig. 6 E). C3GnT−/− tissues also had much more cyclin D1 and c-myc, which are downstream targets of β-catenin and TCF-4. Immunohistochemistry revealed strong nuclear translocation of β-catenin in C3GnT−/− tumor tissues (Fig. 6 F). These data suggest that the molecular mechanism of tumorigenesis in C3GnT−/− mice is similar to that in humans.

**DISCUSSION**

To investigate the function of core 3–derived O-glycans, we generated mice with a targeted deletion of the C3GnT gene. Disruption of the gene eliminated core 3–derived O-glycans in the mouse colon, resulting in reduced expression of Muc2 and increased intestinal barrier permeability. Furthermore, C3GnT−/− mice exhibited increased susceptibility to DSS-induced colitis and AOM/DSS-induced colorectal adenocarcinomas. These data demonstrate the importance of core 3–derived O-glycans in intestinal function.
Factors controlling O-glycan structure in the colon

Unlike core 1–derived O-glycans that are present in most tissues (1, 22), core 3–derived O-glycans are expressed predominantly in colonic epithelial cells (demonstrated here by lacZ reporter staining in \( \text{C3GnT}^{-/-} \) mice), implying an important function in colon. Our MALDI-TOF-MS analyses indicated that disruption of the \( \text{C3GnT} \) gene eliminated all core 3–derived O-glycans, including core 4, and other core 3–derived O-glycan variants. These data provide definitive evidence that \( \text{C3GnT} \) gene encodes all measurable C3GnT activity. This distinguishes C3GnT from many glycosyltransferases that usually exist as multigene families with related structures and functions (1, 2). Interestingly, the common core 1 O-glycan Galβ1,3GalNAcα-O-Ser/Thr is generated by the activity of the core 1 β1,3-galactosyltransferase (T-synthase), which, like C3GnT, is also encoded by a single gene (36).

Tn antigen (GalNAcα-O-Ser/Thr), which is the only structure common to all mucin-type O-glycans, is normally hidden because it is a substrate for different types of additional glycosylation. These include sialylation to form the sialyl-Tn antigen or extensions to form core 1– or core 3–derived O-glycans (Fig. 1 A). As expected, we found that deficiency of C3GnT resulted in the exposure of Tn antigen. Desialylation did not enhance Tn expression, indicating that sialic acid did not cap most of the exposed Tn antigens to form the sialyl-Tn antigen. Furthermore, HPLC and MALDI-TOF-MS analyses demonstrated that the absence of core 3–derived O-glycans did not cause compensatory increase in the biosynthesis of core 1–derived O-glycans (Fig. 2 A and not depicted). These data are consistent with our previous observation that the Tn antigen, which was exposed by global targeted deletion of core 1–derived O-glycans, was not modified by sialylation during mouse embryonic development (22).

Mouse colon expresses enzymes to generate both core 1– and core 3–derived O-glycans, and expresses \( \alpha_2,6 \)-sialyltransferase that modifies the Tn antigen (1, 2, 19, 22). Therefore, our data argue against the idea that the formation of core 1 and core 3 O-glycans may compete because both types of O-glycans share Tn antigen as a substrate, and that exposed Tn antigens are always modified by sialylation. Such notions have been suggested as potential mechanisms for the aberrant expression of some cancer cell-related O-glycan antigens (20). Our data suggest that substrate specificities of the glycosyltransferases for core 1 or core 3 O-glycans may compete because both types of O-glycans share Tn antigen as a substrate, and that exposed Tn antigens are always modified by sialylation. Such notions have been suggested as potential mechanisms for the aberrant expression of some cancer cell-related O-glycan antigens (20). Our data suggest that substrate specificities of the glycosyltransferases for core 1 or core 3 O-glycans may compete because both types of O-glycans share Tn antigen as a substrate, and that exposed Tn antigens are always modified by sialylation. Such notions have been suggested as potential mechanisms for the aberrant expression of some cancer cell-related O-glycan antigens (20). Our data suggest that substrate specificities of the glycosyltransferases for core 1 or core 3 O-glycans may compete because both types of O-glycans share Tn antigen as a substrate, and that exposed Tn antigens are always modified by sialylation. Such notions have been suggested as potential mechanisms for the aberrant expression of some cancer cell-related O-glycan antigens (20). Our data suggest that substrate specificities of the glycosyltransferases for core 1 or core 3 O-glycans may compete because both types of O-glycans share Tn antigen as a substrate, and that exposed Tn antigens are always modified by sialylation. Such notions have been suggested as potential mechanisms for the aberrant expression of some cancer cell-related O-glycan antigens (20). Our data suggest that substrate specificities of the glycosyltransferases for core 1 or core 3 O-glycans may compete because both types of O-glycans share Tn antigen as a substrate, and that exposed Tn antigens are always modified by sialylation. Such notions have been suggested as potential mechanisms for the aberrant expression of some cancer cell-related O-glycan antigens (20). Our data suggest that substrate specificities of the glycosyltransferases for core 1 or core 3 O-glycans may compete because both types of O-glycans share Tn antigen as a substrate, and that exposed Tn antigens are always modified by sialylation. Such notions have been suggested as potential mechanisms for the aberrant expression of some cancer cell-related O-glycan antigens (20).
Muc2ous colitis in such as resident microbiota, contribute to the colitis phenotype of mice from spontaneous colitis. However, it should also be noted that dysfunction of the intestinal epithelial barrier is the primary cause of SAMP ileitis susceptibility (45). Our results underscore an etiologic role of intestinal barrier function in the pathogenesis of IBD.

Enhanced *Escherichia coli* adherence and invasion has been found in Crohn’s disease and colon cancer, presumably because of altered mucosal glycosylation (46). We found that bacterial 16S rDNA was also increased in *C3GnT−/−* colonic issues, suggesting that lack of core 3–derived O-glycans causes either an enhanced adherence of bacteria to colonic mucosa or an increase in translocation of bacteria into colonic mucosa. Notably, the expression of several proinflammatory cytokines was increased in *C3GnT−/−* mucosal lymphocytes from mice that did not receive DSS. This result indicates that deficiency of core 3–derived O-glycans may result in abnormal interactions of intestinal microorganisms and/or their products with mucosal immune cells, may perturb mucosal immune homeostasis, and may thus render *C3GnT−/−* mice more susceptible to DSS challenge. Moreover, the inflammatory products of these cells have been mechanistically linked to increased epithelial permeability through activation of epithelial myosin light chain kinase (47, 48). Accordingly, conditions that reduce barrier integrity and increase inflammation may promote susceptibility to colitis and inflammation-dependent epithelial neoplasia.

The immunologic response to DSS injury in *C3GnT−/−* mice included modest TH1 skewing (IL-2, IFN-γ, and TNF-α), but progressed to neither TH17 nor TH2 immune colitis. This distinguishes *C3GnT−/−* mice from models of TH1 or TH17 colitis (42, 49, 50). In this respect, *Muc2−/−* and *C3GnT−/−* mice may represent different facets of the inflammatory response. The relatively modest immune inflammatory components in *Muc2−/−* and *C3GnT−/−* mice suggest that deficiencies in mucus layer integrity alone are insufficient to drive immune colitis, without the contribution of additional traits affecting innate immune function or immunoregulation (42).

Glycosylation regulates lymphocyte trafficking, and mice transgenic for human α1,2-fucosyltransferase exhibit colitis as a result of defective T cell development resulting from altered lymphocyte glycosylation (51). However, it is unlikely that the *C3GnT−/−* phenotypes are caused by abnormal glycosylation pathways are complex, and they are sometimes promoted by enzymes or factors that function indirectly (1, 21).
of lymphocytes because core 3–derived O-glycans are not expressed in hematopoietic cells (19). In addition, the immune organs of thymus and spleen of C3GnT−/− mice were normal, and C3GnT−/− peripheral blood had a normal distribution of various lymphocyte subsets. These data provide no evidence that core 3–derived O-glycans cause abnormal systemic immune development.

Core 3 O-glycans and carcinogenesis
The biologic mechanisms linking mucin abnormalities with colitis and carcinogenesis are not well understood. Altered core 3 O-glycosylation may impair intestinal mucus integrity, allowing abnormal interaction of intestinal luminal contents and commensal microflora with mucosal immune cells. Increased intestinal permeability observed in C3GnT−/− mice supports this possibility. In addition, loss of core 3–derived O-glycans may cause an alteration in the variety and/or the density of intestinal commensal microflora. Thus, compromised intestinal homeostasis may result in chronic activation of mucosal immune cells in C3GnT−/− mice, even without challenge. Elevated expression of TNF-α, IFN-γ, and IL-6 in the C3GnT−/− IELs and LPLs supports this hypothesis. In addition, the increases in lamina propria CD4⁺ T cells and macrophages and in splenocyte cytokine production suggest that the immune-mediated response to intestinal injury is increased after DSS challenge in C3GnT−/− mice. Products of these and other immune cell types can induce abnormal epithelial cell growth and survival that contribute to carcinogenesis (9, 52).

Greater epithelial cell proliferation in C3GnT−/− mice was observed even in the absence of AOM/DSS treatment. Thus, growth and survival characteristics of C3GnT−/− epithelial cells may respond to increased constitutive exposure to luminal contents, altered commensal microbial composition, or the products that they induce in the local resident mucosal cells. This is comparable to mice impaired in epithelial barrier function because of deficiency of the multiple drug resistance type 1 gene, which also display spontaneous colitis and colonic dysplasia, particularly in the presence of intestinal injury or certain bacterial colonists (33, 53–55). Also, down-regulation of C3GnT expression has been observed in colon carcinoma (20), which may indicate that this trait could be a feature of some epithelium at risk for carcinogenesis. The altered expression of β-catenin/TCF-4, c-myc, and cyclin D1 observed during carcinogenesis in C3GnT−/− mice relates its molecular pathogenesis to that commonly observed in human colorectal cancer (35).

In summary, we have discovered that C3GnT−deficient mice lack core 3–derived O-glycans, exhibit impaired Muc2 expression primarily in colonic tissues, and are highly susceptible to chemical-induced colitis and colorectal tumorigenesis. These data underscore an important in vivo role of core 3–derived O-glycans in intestinal function. The C3GnT−/− mice should be useful to dissect how mucus layer integrity can be manipulated to promote or prevent chronic colitis and colorectal carcinogenesis. Because core 1–derived O-glycans are also a major component of intestinal mucus, generation of mice specifically lacking intestinal core 1–derived O-glycans and mice lacking both core 1– and core 3–derived O-glycans will be valuable to evaluate the overall contribution of O-glycans to intestinal function and to pathogenesis of common intestinal diseases.

MATERIALS AND METHODS
Generation of C3GnT−/− mice. Based on published human C3GnT DNA and amino acid sequences, National Center for Biotechnology Information database searches were used to identify the corresponding murine C3GnT gene. We generated conventional C3GnT gene-deficient mice (C3GnT−/−), as illustrated in Fig. 1 B, by targeted homologous recombination in murine C57 embryonic stem cells (129/SvmlJ origin) using previously described methods (22, 23). In the gene-targeting construct, a neomycin selection marker was used to replace the major part of the coding exon 2 of C3GnT gene (Fig. 1 B). Because there is no available antibody or other molecular probe to C3GnT or core 3–O-glycans, a lacZ reporter was genetically integrated immediately after the endogenous C3GnT promoter region to characterize the expression pattern of C3GnT (Fig. 1 B). Geno-types of mice were initially verified by Southern blotting and then routinely determined by PCR of genomic DNA from tail biopsies. RT-PCR was used to measure C3GnT mRNA transcripts.

Unless specified, 6-wk-old C3GnT−/− male mice in the 129/SvmlJ and C57BL/6J mixed background were used for experiments. Wild-type littermates (C3GnT+/+) were used as controls. All mouse experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committees of the Oklahoma Medical Research Foundation and the University of California.

lacZ staining. 5-μm-thick cryosections of different tissues from C3GnT−/− mice were stained for lacZ based on the previously published method (56). In brief, sections on slides were treated with cold 0.2% glutaraldehyde in PBS for 10 min and rinsed three times with lacZ wash buffer (2 mM MgCl₂, 0.01% sodium deoxycholate, and 2.5% Nonidet-P40 in PBS). The slides were stained overnight in 0.5 mg/ml X-gal, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide in lacZ wash buffer at 37°C. After staining, the slides were rinsed with PBS and counterstained with nuclear fast red.

C3GnT activity assay. C3GnT activity was measured using GalNAcα1-O-p-Nitrophenyl (GalNAcα-pNP; Sigma-Aldrich) as an acceptor following the established methods, with minor modifications (22). In brief, the assay was performed in a 50-μl reaction containing 50 mM Tris-HCl, pH 7.0, 2 mM GalNAcα-pNP, 200 μM UDP-[3H]GlcNAc (60,000–90,000 cpm; PerkinElmer Life Science), 20 mM MnCl₂, 0.1% Triton X-100, and 100 μg protein from tissue extracts from C3GnT−/− and C3GnT+/+ mice. The reactions were incubated at 37°C for 1 h and stopped by adding 950 μl cold H₂O. The products were separated from free UDP-[3H]GlcNAc by Sep-Pak (C18) column chromatography and quantified.

Expression of soluble recombinant epitope-tagged C3GnT. The putative catalytic domain of mouse C3GnT was amplified by PCR using mouse colon cDNA as a template and cloned into a baculovirus expression vector that contains DNA sequence encoding a signal peptide and a human protein C epitope, which is recognized by a Ca²⁺–dependent mAb (HPC4), upstream of the inserted C3GnT sequence (21, 22). Sf9 insect cells transfected with the vector and an empty vector were used to produce high-titer viral stocks. High Five insect cells grown in serum-free medium at 27°C were infected by the recombinant viruses for 72 h. The soluble recombinant C3GnT was purified from the conditioned medium using a HPC4-UltraLink resin column. 100 ng of purified, epitope-tagged C3GnT or 20 μl of transfected or mock-transfected media were electrophoresed on a SDS-PAGE
(4–20%), transferred to a nitrocellulose membrane, and detected with the HPC4 antibody.

**Histology.** For histological analysis, organs from C3GnT−/− and C3GnT+/+ mice were fixed in 10% formalin, processed, and embedded in paraffin. 5-μm-thick tissue sections were stained with hematoxylin and eosin. In another experiment, PAS (Sigma-Aldrich) and Alcian blue (Newcomer Supply) were used to stain general intestinal carbohydrate moieties. Transmission electron microscopy was performed as previously described (22).

**Intestinal glycan structure analysis.** The analysis was performed following our published protocol (24). In brief, intestinal mucus, including the epithelial cell layer, was collected by gently scraping the luminal surface of intestines from C3GnT−/− and C3GnT+/+ mice. Samples were dried and glycans were released by ammonia-based β elimination. The glycans were labeled with 2-aminobenzamide (2-AB) and separated by a phase column (4.6 × 250 mm; Zorbax NH2; Agilent Technologies) using HPLC equipped with a fluorescence detector (Ex 330 nm and Em 420 nm). O-glycans labeled with 2-AB were collected from the HPLC and analyzed for structure composition using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Applied Biosystem) in a linear positive mode. All acquired spectra were smoothed by applying a 19-point Savitzky-Golay smoothing routine. The matrix used for the positive mode was 10 mg/ml 2,5-di-hydroxybenzoic acid prepared in 50% acetonitrile and 0.1% trifluoroacetic acid.

**In vivo intestinal permeability.** We used an established method to determine if the loss of O-glycans increases intestinal barrier permeability (57). In brief, C3GnT−/− and C3GnT+/+ mice were administered 200 μl of FITC-dextran (600 mg/kg body weight; 4 kDa; Sigma-Aldrich) by gavage. Blood was collected 4 h later by retroorbital bleeding. The serum concentration of the FITC-dextran was determined using a fluorimeter (PerkinElmer Life Sciences) with an excitation wavelength at 490 nm and an emission wavelength of 530 nm. Serial-diluted FITC-dextran was used to generate a standard curve. In another experiment, mice were treated with FITC-dextran as described above, and cryosections of the small intestines and colons were prepared for fluorescence microscopy.

**Real-time PCR.** Two sets of universal primers specific for the conserved regions of the bacterial 16S rDNA gene were used (58–60). These included 16S1 (forward, 5′-CCATGAGTCGGAAATCCGTAGT-3′; reverse, 5′-ACTCCCATGTTGTAGCCG-3′) and 16S2 (forward, 5′-TCTACGGGAGCAGCTAG-3′; reverse, 5′-GGACTCACCAGGTATCTAAA-TCTGTGTT-3′). DNA extracted from colonic tissues and colonic fecal materials were used as a template. Colonoc tissues were washed carefully before extraction of DNA to remove residual feces. For the quantification of bacterial 16S rDNA, DNA from each mouse fecal sample was quantified, and 2.5 pg DNA was used as a template in each PCR reaction. For the quantification of 16S DNA in colonic tissues, a pair of primers specific to the mouse P-selectin gene (forward, 5′-AGGATAGCGAAATCTGACCTGGTTTGAAC-3′; reverse, 5′-TAGGTCCTTAGGACCTCCTATA-3′) was used in a separate reaction at the endogenous control to normalize the DNA loading between samples. Real-time PCR was performed on an ABI Prism 7000 spectrophuorometric thermal cycler (Applied Biosystems) using SYBR-green as a double-stranded DNA-specific binding dye. The relative amount of 16S rDNA in each sample was estimated using the ΔΔCT method following the manufacturer’s protocol. Each sample was assayed in duplicate.

**Immunohistochemistry.** To characterize intestinal inflammatory cell infiltrates, cryosections of colons from C3GnT+/+ and C3GnT−/− mice were first incubated with a hamster polyclonal antibody to the lymphocyte marker, CD3 (1:50 dilution; BD Biosciences), or a rat mAb to the macrophage marker, F4/80 (1:200 dilution; Accurate Chemical and Scientific Co.) for 1 h at room temperature. The sections were then incubated for 30 min with biotin-conjugated anti-hamster or rat IgG, and subsequently incubated for 30 min with fluorescein isothiocyanate–conjugated avidin D or Texas red avidin D (Vector Laboratories), respectively. The sections were mounted with Vectashield mounting medium (Vector Laboratories) and analyzed by a fluorescent microscope (ECLIPSE E600; Nikon).

For immunohistochemical detection of Mur2, ITF, and β-catenin, deparaffinized sections were boiled for 20 min in 0.1 M citrate buffer, pH 6.0, for epitope retrieval. The sections were then blocked for 20 min using a protein blocking kit (DakoCytomation) for nonspecific antibody binding. Endogenous avidin-binding proteins were blocked using avidin/biotin blocking reagents (Vector Laboratories). Sections were incubated overnight at 4°C with rabbit anti-Muc2 antibody (1:200; Santa Cruz Biotechnologies), with goat anti-ITF antibody (1:200; Santa Cruz Biotechnologies), with mouse anti-β-catenin mAb (1:500; Transduction Laboratories), or with isotype–matched control IgG, respectively. The sections were subsequently incubated with biotinylated anti–rabbit, –goat, or –mouse IgG antibodies (Vector Laboratories) for 45 min, followed by a 20-min incubation with 0.6% H2O2 in methanol to inhibit endogenous peroxidase activity. The sections were finally incubated with horseradish peroxidase (HRP)–streptavidin (Vector Laboratories) for 30 min, and then developed with a diaminobenzidine substrate and counterstained with hematoxylin.

For Tn antigen staining, deparaffinized sections were incubated with or without 0.5 U/ml salidase from *Arthrobacter ureafaciens* (Roche) at 37°C for 3 h. Sections were incubated for 30 min with biotinylated mAb against the Tn antigen (mouse IgG) or with isotype–matched control mouse IgG. Bound antibodies were detected with HRP–conjugated streptavidin (Vector Laboratories).

**Immunobots.** Colon extracts (40 μg of total protein) from C3GnT−/− and C3GnT+/+ mice were resolved by SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked with 5% nonfat dry milk overnight and incubated with a rabbit polyclonal antibody to β-catenin (Lab Vision Corp.), a mouse mAb to TCF-4 (Millipore), a rabbit polyclonal antibody to c-myc (Santa Cruz Biotechnologies), a mouse mAb to cyclin D1 (BD Biosciences), or an anti–β-actin antibody (Affinity BioReagents) for loading control. Binding was detected with HRP–conjugated anti–rabbit or –mouse IgG (Vector Laboratories) using enhanced chemiluminescence (GE Healthcare).

**DSS-induced colitis model.** To determine the lethality of DSS, mice were fed 2.5% DSS (40 kD; MP Biomedicals, Inc.) in drinking water for 14 d. To induce colitis, C3GnT−/− and C3GnT+/− males were treated with 2% DSS dissolved in drinking water for 7 d, followed by 4 d of regular water. Mice were then killed for histological analysis.

Body weight, the presence of occult or gross blood per rectum, stool consistency, and mortality were monitored daily during the course of treatments. The clinical score was assessed based on these measurements and determined according to a published method (61). Histological scoring was performed in a blinded fashion as a combined score of inflammatory cell infiltration and tissue damage (61).

**Intracellular cytokine staining.** Intracellular cytokine staining was performed to assess the level of cytokines produced by the lymphocytes from the spleens of DSS-treated C3GnT−/− and C3GnT+/+, mice as well as intestinal mucosal lymphocytes from C3GnT−/− and C3GnT+/+ mice without DSS treatment.

In brief, splenocytes were isolated from DSS-treated C3GnT+/+ and C3GnT−/− mice after lysis of erythrocytes with ACK lysing Buffer (Cambrex Bio Science). Intestinal IELs and LPLs were isolated from the large intestines of C3GnT+/+ males (129/SvlmJ background), as previously described with minor modifications (62). For intracellular cytokine staining, isolated lymphocytes were activated with Leukocyte Activation Cocktail containing BD GolgiPlug (BD Biosciences) at 5 × 10^6 cells/well in 96-well plates and cultured for 4–5 h in 5% CO2 at 37°C. Activated cells were
harvested for surface and intracellular cytokine staining. Surface staining of the cells was performed by incubating with FITC-ε, APC-ε, and PerCP-conjugated mAbs specific to CD3, CD4, and CD19 for 30 min on ice. After surface staining, the cells were fixed and permeabilized for 20 min at room temperature using BD Cytofix/Cytoperm buffer and washed twice with the BD Perm/Wash buffer (both BD Biosciences). Intracellular cytokine staining was performed by incubating with the PE-conjugated antibodies against murine IL-2, -6, -10, -12, -17, TNF-α, and IFN-γ on ice for 15 min. The cells were washed three times with BD Perm/Wash buffer and analyzed with a FACSCalibur flow cytometer (BD Biosciences).

AOM/DSS-induced mouse colorectal tumorigenesis model. 6-wk-old C3GnT+/− and C3GnT−/− males were given a single intraperitoneal injection of AOM (10 mg/kg body weight; Sigma-Aldrich) (34). Starting 1 wk after injection, mice were fed with 1.75% DSS in drinking water for 7 d and then received no further treatment. Mice were killed for histopathological analysis 12 or 20 wk after the 7-d DSS treatment. The colon was cut open longitudinally and examined for the presence of tumors. The number and size of tumors were recorded. The tumor volumes were determined by measuring the length (l) and calculated volume (V = l/2 × l × w), as previously described (63). Tissues were fixed in 10% formalin and embedded in paraffin for histopathological analysis. Fresh colonic tissue lysates from C3GnT+/− and C3GnT−/− mice with or without AOM/DSS treatments were prepared for Western blot analysis.

In vivo BrdU labeling assay. Mice were injected intraperitoneally with BrdU (20 mg/kg of body weight; Sigma-Aldrich) (25). 3 h after BrdU injection, mice were killed, and colonic tissues were processed for immunohistochemical analysis. Incorporation of BrdU in proliferating epithelial cells was detected with a rat mAb to BrdU (1:100 dilution; Accurate Chemical and Scientific Co.). The number of BrdU-positive cells per crypt column was quantified.

Statistical analysis. Data were analyzed by the Student’s t test. The Wilcoxon signed-rank test was used to compare data that did not satisfy the Student’s t test. P < 0.05 was considered significant.

Online supplemental material. Fig. S1 shows murine C3GnT genomic structure and deduced protein sequence. Fig. S2 shows characterization of murine C3GnT. Fig. S3 presents histology (hematoxylin and eosin stain) of C3GnT+/− and C3GnT−/− small intestines from 6-wk-old mice. Fig. S4 demonstrates that the immune organs and immune cells develop normally in C3GnT−/− mice. Fig. S5 shows MADI-TOF-MS standards of 2-AB–labeled core 3 structures of different intestinal mucins from C3GnT+/− and C3GnT−/− small intestines. Fig. S7 is an analysis of mRNA transcripts of different intestinal mucins from C3GnT+/− and C3GnT−/− goblet cells. Fig. S8 compares colon length of C3GnT+/− and C3GnT−/− mice after DSS-induced colitis. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20061929/DC1.

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