Colonic Epithelial Cells Express Specific Ligands for Mucosal Macrophage Immunosuppressive Receptors Siglec-7 and -9

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**Supplementary Material**

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Immune cells are known to express specific recognition molecules for cell surface glycans. However, mechanisms involved in glycan-mediated cell–cell interactions in mucosal immunity have largely been left unaccounted for. We found that several glycans preferentially expressed in nonmalignant colonic epithelial cells serve as ligands for siadic acid-binding Ig-like lectins (sigelcs), the immunosuppressive carbohydrate-recognition receptors carried by immune cells. The siglec ligand glycans in normal colonic epithelial cells included disialyl Lewisα, which was found to have binding activity to both siglec-7 and -9, and sialyl 6-sulfo Lewisx, which exhibited significant binding to siglec-7. Expression of these siglec-7/-9 ligands was impaired upon carcinogenesis, and they were replaced by cancer-associated glycans siably Lewisα and siglyx Lewisβ, which have no siglec ligand activity. When we characterized immune cells expressing siglecs in colonic lamina propriae by flow cytometry and confocal microscopy, the majority of colonic stromal immune cells expressing siglec-7/-9 turned out to be resident macrophages characterized by low expression of CD14/CD89 and high expression of CD68/CD163. A minor subpopulation of CD8+ T lymphocytes also expressed siglec-7/-9. Siglec-7/-9 ligation suppressed LPS-induced cyclooxygenase-2 expression and PGE2 production by macrophages. These results suggest that normal glycans of epithelial cells exert a suppressive effect on cyclooxygenase-2 expression by resident macrophages, thus maintaining immunological homeostasis in colonic mucosal membranes. Our results also imply that loss of immunosuppressive glycans by impaired glycosylation during colonic carcinogenesis enhances inflammatory mediator production. The Journal of Immunology, 2012, 188: 4690–4700.
For this purpose, we screened ligand activities of two sets of carbohydrate determinants, which show unique alterations upon malignant transformation. One pair was the set of disialyl Lewis®, preferentially expressed in nonmalignant epithelial cells, and sialyl Lewis®, a well-known cancer-associated determinant (7). The other pair was a combination of sialyl 6-sulfo Lewis®, preferentially expressed in nonmalignant epithelial cells, and a cancer-associated determinant, sialyl Lewis® (12). We also tried to characterize immune cells expressing sigleccs in colonic mucosal membranes and examined the effects of siglec ligation on their functions.

Materials and Methods

Cells and Abs

Anti-disialyl Lewis® (FH7, murine IgG) and anti-disialyl Lewis® (FH9, murine IgG2a) were prepared as described previously (13). The anti-sialyl Lewis® Ab N19-9 (murine IgG1) was obtained from Alexis Biochemicals (Lausen, Switzerland). The Abs G152 (murine IgM), directed to sialyl 6-sulfo Lewis®, and G72 (murine IgM), reactive to both sialyl 6-sulfo LacNAc and sialyl 6-sulfo Lewis®, were prepared as described previously (14). An Ab directed to nonsulfated sialyl Lewis®, CSLEX-1 (murine IgM), was obtained from American Type Culture Collection (Manassas, VA). The stable ST6GalNAc6 transfectant cells were established by the transfection of cDNA for human ST6GalNAc6 to human colon cancer SW1083 cells, as described previously (7). The ECV304 cells stably transfected with FUT7 or GlcNAc6ST-1 cDNA, or cotransfected with both genes, were prepared as described previously (15).

ELISA for binding of recombinant sigleccs to synthetic carbohydrate determinants

In some experiments, the binding specificity of recombinant siglec-7–Fc or siglec-9–Fc was ascertained by ELISA using purified carbohydrate determinants. The synthetic carbohydrate determinants were prepared as described previously (7) and kindly supplied by Professor Makoto Kiso (Gifu University, Gifu, Japan). Recombinant siglec-7–Fc, mutant siglec-7–Fc (R124K), and siglec-9–Fc were prepared as described previously (16). ELISA was performed using synthetic carbohydrate determinants immobilized at the bottom of 96-well culture plates by a standard method described previously (7, 14, 17). GD3 ganglioside (Avanti Polar Lipids) was used as a positive control for the binding of siglec-7–Fc in ELISA assays. Recombinant sigleccs were preincubated with affinity-purified biotinylated rabbit anti-human IgG (Dako, Glostrup, Denmark). For this, recombinant siglecs were preincubated with affinity-purified biotinylated rabbit anti-carbohydrate Abs (20 μg/ml) in 10 mM HEPES. Cells in supernatants were collected and filtered through a 200-μm steel mesh. The cells in suspension were washed twice with PBS, 10 mM HEPES, 0.75 mM EDTA, and 1 mM DTT, and the tissues were minced and incubated for 20 min at 37°C in CMF-HBSS supplemented with antibiotics (100 μg/ml), gentamicin (100 μg/ml), and amphotericin B (1 μg/ml). The 2 × 2-cm pieces of tissue were incubated for 15 min at room temperature in CMF-HBSS supplemented with antibiotics and 1 mM DTT and washed in CMF-HBSS the same way. After two bindings incubations for 20 min at 37°C in CMF-HBSS supplemented with antibiotics, 0.75 mM EDTA, and 1 mM DTT, the tissues were minced and incubated for 15 min at 37°C in HBSS supplemented with antibiotics, 0.1% BSA, 0.5 mg/ml collagenase, 0.4 mg/ml Dispase, 0.01 mg/ml DNase, and 10 mM HEPES. Cells in supernatants were collected and filtered through a 200-μm steel mesh. The cells in suspension were washed twice with CMF-HBSS with antibiotics and centrifuged over a layer of Percoll (100/70) and counted with the Arvo 1420 multilabel counter (Wallac).

Effect of siglecs on LPS-induced production of COX2 and PGE2

The U937 cells transfected with cDNA for siglec-7 (U937/siglec-7 cells) or for siglec-9 (U937/siglec-9 cells), prepared as described previously (7, 21), were cultured for 3 d with 10 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA); after being cultured for one additional day without TPA, they were challenged with 1 μg/ml LPS for 3 h (22–24). The F(ab')2 fragment (10 μg/ml) of anti-siglec-7 Ab or anti-siglec-9 Ab was added to the culture after incubation together with TPA or LPS. The F(ab')2 fragment was prepared by incubating a biotinylated monoclonal anti-siglec-7 Ab or anti-siglec-9 Ab with 20 μg/ml of purified anti-human IgG (Fc-specific) Ab immobilized agarose beads, as described previously (21). Control rat F(ab')2 fragment was obtained from Jackson ImmunoResearch (West Grove, PA). Cells were collected, and total cellular RNA was isolated according to the acid guanidinium thiocyanate-phenol-chloroform extraction method using an isolation kit (Isogen, Tokyo, Japan). First-strand cDNA was prepared using 500 ng total cellular RNA. Synthesis of cDNA was carried out in a 20-μl reaction volume using the SuperScript Preamplification System (Life Technologies BRL, Grand Island, NY), according to the manufacturer’s protocol, with oligo-d(T) as initiation primer. The primers used in RT-PCR analysis for human COX1 was 5'-TGGCGCGAGCTCTCCTGCCTGGCGGCCGCTGCTG-3' and 5'-GCATCAACACAGGCGCTCCCGC-3' and for COX2 were 5'-TCTAAAATGAGATTGTGGGAAAATTGCT-3' and 5'-AGATCATCTGCTCGTAGTTATCG-3', which give a 331-bp product, and for COX3 were 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' and 5'-
CACTGTCTGTAGGCA-3', which give a 489-bp product. Quantitative real-time RT-PCR analyses for COX2 were performed using ABI PRISM 7000 (Applied Biosystems), as described previously (7). The TaqMan probe ID for COX2 was Hs 00153133_m1 (Assays-on-Demand), and TaqMan Endogenous Control (Applied Biosystems) was used for GAPDH. The results were calculated using the comparative Ct method. Relative transcripts were determined by the equation 2-^ΔΔCt. The ΔCt value was determined by subtracting the average GAPDH Ct value from the average target Ct value, and it was used to calculate percentage inhibition of COX2 induction in the LPS-stimulated U937 cells treated with F(ab')2 fragments of anti-siglec-7/-9 Abs. PGE2 in culture supernatant of the cells was evaluated using a Parameter PGE2 assay kit (KGE004; R&D Systems). The culture supernatants were collected for PGE2 determination 2 d after the addition of LPS for the determination of PGE2 production.

Results

Disialyl Lewis^a^ as a ligand for siglec-7/-9

Normal colonic epithelial cells expressed disialyl Lewis^a^, and the expression tended to be decreased in colonic cancer cells. Fig. 1A shows its carbohydrate structure and a representative pattern of its expression in colonic cancer tissues. The loss of disialyl Lewis^a^ determinant is accompanied by the appearance of sialyl Lewis^a^ in cancer cells. We previously described preferential expression of disialyl Lewis^a^ in nonmalignant colonic epithelial cells compared with cancer cells (p < 0.05, n = 6) (25). This was ascertained with a new series of 23 patients in the current study (p = 0.004) (Supplemental Fig. 1), and Fig. 1A shows a typical example. Sialyl Lewis^a^ has a carbohydrate structure quite similar to that of disialyl Lewis^a^; it lacks one of the two sialic acid residues present in disialyl Lewis^a^, and this alteration was proposed to be the result of cancer-associated epigenetic silencing of a gene for an α2,6 sialyltransferase, which is involved in the synthesis of disialyl Lewis^a^ (7).

We previously showed that the disialyl Lewis^a^ determinant serves as a ligand for an immunosuppressive receptor, siglec-7 (7). In this study, we show that the determinant also serves as a ligand for another immunosuppressive receptor, siglec-9. The disialyl Lewis^a^ was found to bind to the recombinant Fc chimera of siglec-9 or siglec-7 in ELISA-based assays using pure synthetic carbohydrate determinants (Fig. 1B). Both siglec-9 and siglec-7 also bound to the pure disialyl Lewis^a^, the carbohydrate determinant lacking the fucose residue, indicating that the fucose residue is not essential for the binding of siglec-7/-9. See Supplemental Fig. 2A for the structure of disialyl Lewis^a^.

We next tried to ascertain whether the binding of siglecs to disialyl Lewis^a^ actually occurred at the cellular level. To see the binding at the cellular level, we generated cells expressing disialyl Lewis^a^ by transfecting a cancer cell line SW1083 with the gene for α2,6 sialyltransferase that synthesizes disialyl Lewis^a^ and screened for the binding of siglecs to the transfectant cells, first by flow cytometry using several recombinant siglec molecules. The results indicated that among the four siglecs of the CD33 family, only siglec-7 and -9 showed binding to the transfectant cells (Fig. 1C). None of the siglecs bound to the parental cancer cells, which express sialyl Lewis^a^, the cancer-associated glycan, but no disialyl Lewis^a^ (see Supplemental Fig. 2B for expression of disialyl Lewis^a^-related glycans in these cells). The binding of siglec-9 to the transfectant cells was inhibited significantly by anti-disialyl Lewis^a^ (FH7) or anti-disialyl Lewis^a^ (FH9) Ab; it was almost completely inhibited by adding both Abs but was not affected by the anti-sialyl Lewis^a^ Ab (N19-9) (Fig. 1C).

Cell–cell interaction mediated by disialyl Lewis^a^ and siglec-7/-9

Interactions of siglecs and their ligands were also found to mediate cell-to-cell adhesion (Fig. 1D). The U937 cells transfected with siglec-7 or -9 adhered to the cells expressing disialyl Lewis^a^; this binding was specifically inhibited by FH7 or more strongly by both FH7 and FH9 Abs, but it was not affected by the anti-sialyl Lewis^a^ Ab (N19-9) (Fig. 1D). These results indicated that disialyl Lewis^a^, the carbohydrate determinant preferentially expressed in nonmalignant colonic epithelial cells, specifically serves as a ligand for siglec-7 and -9, whereas sialyl Lewis^a^, the closely related determinant on cancer cells, does not.

Sialyl 6-sulfo Lewis^x^ as a ligand for siglec-7/-9

There is another set of glycans that shows a similar distribution pattern between benign and malignant colonic epithelial cells; sialyl 6-sulfo Lewis^x^ and sialyl Lewis^x^ (Fig. 2A) shows the carbohydrate structure of sialyl 6-sulfo Lewis^x^, as well as a representative pattern of its expression in colonic cancer tissues. This determinant is preferentially expressed on normal colonic epithelial cells, and its expression is markedly reduced in colonic cancer cells. We previously reported a statistically significant decrease in sialyl 6-sulfo Lewis^x^ in colonic cancers, which was accompanied by an increase in sialyl Lewis^a^ expression (p = 0.007, n = 23) (12). Sialyl Lewis^x^ is closely related structurally to sialyl 6-sulfo Lewis^x^, and it lacks a sulfate residue compared with sialyl 6-sulfo Lewis^x^ (Fig. 2B). Impaired sulfation upon malignant transformation was proposed to be responsible for this shift in glycan expression (12).

The ELISA-based binding assays of siglecs to the pure sialyl 6-sulfo Lewis^x^ determinant were performed by a consortium for functional glycomics; the results are available at http://www.functionalglycomics.org. Siglec-7 was found to be reactive to several glycans having diverse carbohydrate structures, including sialyl 6-sulfo Lewis^x^ which showed the seventh-best binding, Siglec-9 was shown to be the most reactive to sialyl 6-sulfo Lewis^x^ among >200 glycans tested, according to the data posted on the consortium Web site on September 15, 2005. However, the reactivity in ELISA does not always warrant reactivity at the cellular level. Therefore, we tested whether the interaction of sialyl 6-sulfo Lewis^x^ with siglecs is functional at the cellular level by generating cells expressing sialyl 6-sulfo Lewis^x^ and control cells expressing the counterpart glycan, sialyl Lewis^x^.

Our initial screening in flow cytometric analyses of the cells expressing sialyl 6-sulfo Lewis^x^ using recombinant siglecs indicated a strong binding of siglec-7, whereas the binding of siglec-9 was negligible (Fig. 2B). The latter finding was at variance with the consortium results, in which siglec-9 was shown to have a much better binding activity than siglec-7 to sialyl 6-sulfo Lewis^x^ in the cell-binding analysis using immobilized recombinant siglecs (Fig. 2C). Again the strong binding to siglec-7 was detected with the cells expressing sialyl 6-sulfo Lewis^x^, which were obtained by cotransfection of FUT7 and GlcNAc6ST-1 cDNAs. Cells transfected with FUT7 cDNA only, which expressed sialyl Lewis^x^ but not sialyl 6-sulfo Lewis^x^, showed no binding. Unexpectedly, cells transfected with GlcNAc6ST-1 cDNA alone, which expressed sialyl 6-sulfo N-acetyllactosamine (LacNac) but not sialyl 6-sulfo Lewis^x^, showed strong binding comparable to that of the cells cotransfected with FUT7 and GlcNAc6ST-1 cDNAs and expressed both determinants (Fig. 2C; see Supplemental Fig. 2C for glycan expression of the transfectant cells used in these experiments). Because sialyl 6-sulfo LacNc lacks the fucose residue, this suggested that the fucose residue in sialyl 6-sulfo Lewis^x^ is not essential for binding of siglec-7.

Cell–cell interaction mediated by sialyl 6-sulfo Lewis^x^ and siglec-7/-9

In cell–cell adhesion experiments, the U937/siglec-7 cells strongly adhered to cells expressing only sialyl 6-sulfo LacNac (GlcNAc6ST-
FIGURE 1. Siglec ligand activity of disialyl Lewis\(^a\) glycan, which is preferentially expressed on nonmalignant colonic epithelial cells. (A) Structure of disialyl Lewis\(^a\) (upper left panel) and its preferential expression in nonmalignant colonic epithelial cells (upper right panel). The structure of sialyl Lewis\(^a\) determinant and its close association with cancer cells in a consecutive section are also shown (lower panels). Disialyl Lewis\(^a\) determinant was detected by FH7 and sialyl Lewis\(^a\) was detected by N19-9 Abs in immunohistochemistry. Scale bar, 100 \(\mu\)m. (B) Specific binding of recombinant siglec-7/-9 to pure carbohydrate determinants. Binding of recombinant siglec-7/-9-Fc was evaluated by ELISA using immobilized pure synthetic glycolipids bearing disialyl Lewis\(^a\) and disialyl Lewis\(^c\) structure at the bottom of 96-well plates. GD3, the known glycolipid ligand for siglec-7, was used as a positive control for siglec-7. The recombinant siglec-7 mutant-Fc lacking binding activity served as control. (C) Flow cytometric analyses of binding of recombinant siglecs to cells expressing disialyl Lewis\(^a\) (left panel) and its inhibition by specific Abs (right panel). Parental cancer cells SW1083 (parent) expressed sialyl Lewis\(^a\) but not the disialyl Lewis\(^a\) determinant. The cells transfected with ST6GalNAc6 cDNA (ST6-Transfectant) strongly expressed disialyl Lewis\(^a\) and had severely attenuated expression of sialyl Lewis\(^a\), the cancer-associated determinant (Supplemental Fig. 2B). The recombinant siglec-3–Fc and siglec-5–Fc served as negative controls for rIg-Fc molecules instead of siglec-7 mutant-Fc in flow cytometric analyses, because these siglecs do not bind to these cells. Results of flow cytometric analyses of inhibition of recombinant siglec-7/-9 binding to cells expressing disialyl Lewis\(^a\) determinant by specific Abs (right panel). The percentage binding was calculated from mean fluorescence intensities obtained by flow cytometric analyses. The inhibitor Abs used included clone 1-3-A (anti–siglec-9), 13-three dimensional (anti–siglec-7), N19-9 (anti-sialyl Lewis\(^a\)), FH7 (anti-disialyl Lewis\(^a\)), and FH9 (anti-disialyl Lewis\(^c\)). (D) Cell–cell adhesion mediated by siglec-7 or -9 and its specific glycan ligands. Adhesion of U937 cells transfected with siglec-7 (U937/siglec-7) or siglec-9 cDNA (U937/siglec-9) to the parental SW1083 cells (Parent) or ST6GalNAc6-transfectant cells was evaluated by nonstatic cell-adhesion assays. Significant adhesion, observed with the ST6GalNAc6-transfectant cells, was tested for possible inhibition with N19-9, FH7, or FH7 + FH9 Abs. Values are mean \(\pm\) SD from triplicate experiments. The results in (B) and (D) are representative of at least three independent experiments, and those in (C) are representative of two independent experiments. Ca, Cancer cells; N, nonmalignant epithelial cells.
with FUT7 cDNA (FUT7 Transfectant) expressed sialyl Lewisx but not the sialyl 6-sulfo Lewisx determinant. Cells cotransfected with FUT7 and GlcNAc6ST-1 cDNAs (FUT7+6ST Transfectant) strongly expressed sialyl 6-sulfo Lewisx and had severely attenuated expression of sialyl Lewisx, the cancer-associated determinant (Supplemental Fig. 2C). The recombinant siglec-7–Fc served as a negative control instead of siglec-7 mutant-Fc in cell–cell adhesion assays using immobilized recombinant siglec-9 (Fig. 2C) and was not statistically different from the control. (C) Adhesion of cells expressing sialyl 6-sulfo Lewisx to immobilized siglec-7 or -9. Recombinant siglec-7 or -9 was immobilized at the bottom of 24-well plates, and adhesion of ECV304 cells transfected with FUT7 (FUT7 transfectant), GlcNAc6ST-1 (6ST transfectant), or cotransfected with FUT7 and GlcNAc6ST-1 cDNA (FUT7+6ST transfectant) was tested in nonstatic cell-binding assays. In these assays, siglec-7 mutant-Fc was used as a negative control. (D) Cell–cell adhesion mediated by siglec-7 or -9 and its specific glycan ligands. Adhesion of U937 cells transfected with siglec-7 (U937/Siglec-7) or siglec-9 cDNA (U937/Siglec-9) to the ECV304 cells transfected with FUT7 (FUT7 transfectant), GlcNAc6ST-1 (6ST transfectant), or cotransfected with FUT7 and GlcNAc6ST-1 cDNA (FUT7+6ST transfectant) was evaluated by nonstatic cell-adhesion assays. Significant adhesion, observed with the 6ST transfectant cells and FUT7+6ST transfectant, was tested for possible inhibition with anti-glycan Abs CSLEX-1, G72, G152, or with the combination of G72 and G152 Abs. Values are mean ± SD from triplicate experiments in (C) and (D). The results in (B) and (C) are representative of at least three independent experiments, and those in (D) are representative of two independent experiments. Ca, Cancer cells; N, nonmalignant epithelial cells.

FIGURE 2. Siglec ligand activity of sialyl 6-sulfo Lewisx glycan, which is preferentially expressed on nonmalignant colonic epithelial cells. (A) Structure of sialyl 6-sulfo Lewisx determinant (upper left panel) and its preferential expression in nonmalignant colonic epithelial cells (upper right panel). The structure of sialyl Lewisx determinant and its close association with cancer cells in a consecutive section are also shown (lower panels). Sialyl 6-sulfo Lewisx determinant was detected by G152 and sialyl Lewisx was detected by CSLEX-1 Abs in immunohistochemistry. Scale bar, 100 μm. (B) Flow cytometric analyses of binding of recombinant siglecs to cells expressing sialyl 6-sulfo Lewisx. ECV304 cells transfected with FUT7 cDNA (FUT7 Transfectant) expressed sialyl Lewisx but not the sialyl 6-sulfo Lewisx determinant. Cells cotransfected with FUT7 and GlcNAc6ST-1 cDNAs (FUT7+6ST Transfectant) strongly expressed sialyl 6-sulfo Lewisx and had severely attenuated expression of sialyl Lewisx, the cancer-associated determinant (Supplemental Fig. 2C). The recombinant siglec-2–Fc served as a negative control instead of siglec-7 mutant-Fc in flow cytometric analyses, because this siglec does not bind to these cells.
cally significant in cell–cell adhesion assays using U937/siglec-9 cells (Fig. 2D). Other cell lines strongly expressing sialyl 6-sulfo Lewis^x^, such as a subclone of Namalwa cells (18) and SW480 cells transfected with 6-sulfotransferase cDNA (26), also failed to show statistically significant binding to siglec-9 (data not shown).

These results indicated that the sialyl 6-sulfo Lewis^x^ determinant, which is specifically expressed in nonmalignant colonic epithelial cells, served as a ligand for siglec-7 at the cellular level. Another glycan, sialyl 6-sulfo LacNAc, which was previously shown to also be preferentially expressed in nonmalignant epithelial cells (12), was found to serve as a ligand for siglec-7. The possibility that they serve as ligands for siglec-9 cannot be completely excluded, but their ligand activity to siglec-9 at the cellular level seemed to be much weaker than expected from the results of the ELISA-based assays using pure glycans. Interestingly, sialyl Lewis^x^, which is another glycan that is structurally similar and is preferentially expressed in cancer cells, failed to interact with any siglec.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Siglec-7/-9–expressing cells in colonic mucosal membranes. (A) Confocal microscopic observation of cells expressing siglec-7 (upper panel) or siglec-9 (lower panel) in colonic mucosal membranes. Frozen sections of normal colonic tissues were stained with anti–siglec-7 (upper panel) or with anti–siglec-9 (lower panel) in green. A cognate ligand for siglec-7/-9, disialyl Lewis^a^, is stained in red using FH7 Ab. Original magnification ×200. (B) Morphology and distribution of siglec-7/-9–expressing cells in colonic lamina propria. The frozen sections were stained with anti–siglec-7 Ab in green and with anti–siglec-9 Ab in red. Arrowheads indicate siglec-9^+^ cells. Areas of normal colonic epithelial cells are shown by dotted white line and labeled “E” in the merged panel. Original magnification ×100. (C) Flow cytometric analyses of siglec-7/-9^+^ cells in LPMCs. Frequency of siglec-7/-9^+^ cells in CD33^+^ and CD33^−^ fractions in LPMCs, as detected by flow cytometry, and average percentage obtained from six samples (upper panel). Example of flow cytometric analysis of CD33^+^ LPMCs prepared from normal human colon (lower panel). (D) Summary of cell lineage-specific marker analyses of siglec-7/-9–expressing cells in colonic lamina propriae. Average percentage obtained from five samples is shown. CD3 (UCHT1, IgG1), CD4 (RPA-T4, IgG1), CD8 (DK25, IgG1), CD20 (L26, IgG2a), CD56 (B159, IgG1), and CD68 (PG-M1, IgG3) were used to characterize the leukocyte subpopulation in immunohistochemistry. The markers used for evaluation of cell number in each leukocyte subpopulation were CD56 for NK cells, CD8 for killer T cells, and CD68 for monocytes/macrophages. Abs used for leukocytes in peripheral blood are shown in Supplemental Fig. 3. (E) Representative flow cytometric analyses of siglec-7 expression in CD8α^+^ β7-integrin^−^ cells in PBLs. Two-dimensional distribution of CD8 and siglec-7 in gated CD3^+^ cells (left panel, three-color analysis, 2.4% of CD3^+^CD8^α^ cells express siglec-7), two-dimensional distribution of CD8α and siglec-7 in gated CD3^+^CD8^β^ cells (middle panel, four-color analysis, 27.3% of CD3^+^CD8α^+^CD8^β^ cells express siglec-7), and two-dimensional distribution of β7-integrin and siglec-7 in gated CD3^+^CD8^α^ cells (right panel, four-color analysis, 4.2% of CD3^+^CD8^α^β7-integrin^−^ cells express siglec-7). See Table I for statistical analyses.
When the binding of recombinant siglec-7 and -9 was tested by immunohistochemistry using frozen sections, the results indicated that both siglecs preferentially bind to nonmalignant colonic epithelial cells but only weakly to cancer cells (Fig. 3). This confirmed the preferential expression of the ligand glycans for siglec-7 and -9, such as disialyl Lewis^a^ and sialyl 6-sulfo Lewis^a^, in nonmalignant epithelial cells, and its decrease or loss in cancer cells.

Identification of siglec-7/-9–expressing cells in colonic mucosal membranes

To determine the physiological significance of siglec-7/-9 ligand expression in colonic epithelial cells, we examined the characteristics of siglec-7/-9–expressing cells in colonic tissues. Colonic mucosal membranes were found to contain a significant number of siglec-7/-9–expressing cells, some of which were localized in lamina propria very close to colonic epithelial cells expressing their cognate ligands, suggesting the occurrence of possible epithelial–mesenchymal interactions (Fig. 4A). The majority of the cells expressing siglec-7 in colonic lamina propriae had a dendritic appearance (Fig. 4B). Flow cytometry of LPMCs indicated that most siglec-7/-9–expressing cells were CD33^+^, and there were more siglec-7-expressing cells than siglec-9-expressing cells (Fig. 4C). Siglec-7^+^, siglec-9^+^, and siglec-7/-9^+^ siglec-9^+^ cells made up 11.3%, 5.6%, and 2.8%, respectively, of the CD33^+^ cells in the LPMC fraction. Further marker analyses by flow cytometry were hindered because of extensive application of proteases during the course of LPMC preparation; because we noticed that the recovery of siglec-7^+^ cells was not as good as expected, we tried to identify siglec-7/-9^+^–expressing cells in colonic mucosal membranes by direct confocal microscopic observation of the frozen sections.

Results of immunohistochemical staining using cell lineage-specific markers indicated that most siglec-7–expressing cells belonged to the monocyte/macrophage lineage (76%), with a smaller number of CD8^+^ T lymphocytes (19%). NK cells made up <5% of the total siglec-7^+^ cells (Fig. 4D). This was in clear contrast to the siglec-7 distribution in peripheral blood leukocytes of healthy individuals, where the majority (75%) of siglec-7^+^ cells were NK cells, followed by CD8^+^ T lymphocytes (23%). Most siglec-9–expressing cells in the colon also belonged to the monocyte/macrophage lineage (64%), with a smaller number of CD8^+^ T lymphocytes (8%) and other cells (25%, mostly granulocytes). NK cells accounted for <4% of total siglec-9^+^ cells (Fig. 4D). Again, this was in clear contrast to the distribution of siglec-9–expressing cells in peripheral blood leukocytes of healthy individuals, where granulocytes were predominant and accounted for 58% of siglec-9^+^ cells, followed by NK cells (27%).

Closer scrutiny of peripheral lymphocytes indicated that CD8^+^ cells contained significantly more siglec-7^+^ cells than did CD4^+^ cells (p < 0.01), and CD8alpha^+^ cells contained more siglec-7^+^ cells (p < 0.02) (Fig. 4E) and siglec-9^+^ cells (p < 0.05) than did CD8beta^+^ cells (Table I). The Tcrgamma^+^ beta^+^ cell subset also contained a significantly higher number of siglec-7^+^ cells (p < 0.05) (Fig. 4E) and siglec-9^+^ cells (p < 0.05) than did the Tcralpha^+^ beta^+^ cell subset.

### Table I. Siglec-7 and -9-expressing cells in peripheral blood of healthy individuals

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<td>Siglec-7/Tcrdelta^b^-</td>
<td>4.8 ± 3.1</td>
</tr>
<tr>
<td>Siglec-7/CD45RA^-CD3^-</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>Siglec-7/CD45RO^-CD3^-</td>
<td>2.2 ± 1.7</td>
</tr>
<tr>
<td>Siglec-7/CD4^-</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Siglec-7/CD45RA^-CD4^-</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Siglec-7/CD45RO^-CD4^-</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Siglec-7/CD8^-CD3^-</td>
<td>2.6 ± 1.0</td>
</tr>
<tr>
<td>Siglec-7/CD8alpha^-CD3^-</td>
<td>1.1 ± 0.8</td>
</tr>
<tr>
<td>Siglec-7/CD8gamma+CD3^-</td>
<td>14.9 ± 7.7</td>
</tr>
<tr>
<td>Siglec-7/CD45RA^-CD8^-</td>
<td>2.5 ± 1.3</td>
</tr>
<tr>
<td>Siglec-7/CD45RO^-CD8^-</td>
<td>5.3 ± 4.3</td>
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<tr>
<td>Gut-homing cell markers</td>
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<tr>
<td>Siglec-7/B7-integrin^-CD3^-</td>
<td>1.7 ± 0.9</td>
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<tr>
<td>Siglec-7/B7-integrin^-CD4^-</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Siglec-7/B7-integrin^-CD8^-</td>
<td>2.5 ± 1.4</td>
</tr>
<tr>
<td>Siglec-7/CCR9^-CD3^-</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Siglec-7/CCR9^-CD4^-</td>
<td>1.0 ± 0.7</td>
</tr>
</tbody>
</table>

\(^a\)Ratio of siglec-7/-9^+^ cells/each subset (%) was calculated from the results of flow cytometry for at least four healthy individuals.

\(^{b}\)Versus CD16^+^ CD3^- subset.

\(^{c}\)Versus CD56^+^ CD3^- subset.

\(^{d}\)Versus Tcellgamma^b^- subset.

\(^{e}\)Versus CD4^-CD3^- subset.

\(^{f}\)Versus CD8alpha^+^ CD3^- subset.

\(^{g}\)Versus B7-integrin^CD4^- subset.

\(^{h}\)Versus CCR9^- CD3^- subset.
Characterization of siglec-7/-9–expressing macrophages in colonic mucosa

The number of siglec-7+ macrophages was ~4-fold greater than the number of siglec-9+ macrophages in colonic lamina propria (Fig. 5A). Most macrophages were of the siglec-7+/siglec-9− phenotype (78.9 ± 9.7%), and only 11.9 ± 4.6% were siglec-7−/siglec-9+. The population of siglec-7+ siglec-9− cells was minor and accounted for only 9.3 ± 6.2%. This was in clear contrast to the siglec-7−/siglec-9− expressing monocytes in the peripheral blood of healthy individuals, where the majority showed the siglec-7−/siglec-9− phenotype (69.0 ± 11.8%), followed by the siglec-7+ siglec-9− phenotype (31.1 ± 11.8%). Essentially no siglec-7+/siglec-9− cells, which are predominant in colonic mucosa, were observed in peripheral blood monocytes (0.3 ± 0.5%). These results indicated that siglec-7−/siglec-9− expressing macrophages/monocytes in colonic mucosa have characteristics that are quite different from those in peripheral blood monocytes. The predominant macrophages in colonic mucosa were of the siglec-7+/siglec-9− phenotype, and the difference in this subpopulation between the macrophages in colonic mucosa and the monocytes in peripheral blood was statistically significant at p < 0.001 (78.9 versus 0.3%), as ascertained by the unpaired Student t test. In contrast, the predominant phenotype in peripheral blood monocytes was siglec-7+/siglec-9+, and the difference in this subpopulation between the peripheral blood monocytes and macrophages in colonic mucosa was also statistically significant at p < 0.001 (69.0 versus 11.9%). When normal human peripheral monocytes were induced to differentiate into macrophage-like cells by a standard technique using M-CSF, the resulting macrophage-like cells lost their expression of siglec-7− and -9 and became siglec-7−/siglec-9− after long-term culture (Supplemental Fig. 4). This result again suggested that the siglec-expression pattern of the resident macrophages in colonic lamina propria is different from that in macrophages derived from peripheral monocytes.

Further marker analyses revealed that siglec-7+ macrophages/monocytes in colonic mucosa strongly express CD68 and CD163 and only weakly express CD14 and CD89 (Fig. 5B–D). In contrast, the siglec-9− macrophages/monocytes in colonic mucosa tended to express CD14 and CD89 more frequently than did the siglec-7− macrophages/monocytes (Fig. 5B–D). These findings suggested that the siglec-7− cells in colonic mucosa have characteristics similar to so-called “resident macrophages” (27, 28), whereas siglec-9− cells, at least in part, exhibit characteristics more like monocytes and activated macrophages. A significant

FIGURE 5. Characterization of macrophages/monocytes in colonic lamina propria expressing siglec-7 and -9. (A) Distribution of siglec-7−/9− macrophages/monocytes in colonic lamina propria and comparison with that in peripheral blood monocytes. The number of cells positive for either siglec-7− or -9− in CD33+ cells (WM53, IgG1) was taken as 100%. (B) Macrophage/monocyte marker analyses of colonic LPMCs expressing siglec-7 and/or -9. The Abs used were HLA-DR (TAL.1B5, IgG1), CD11c (KB90, IgG1), CD83 (HB15e, IgG1), and CD209 (DC-SIGN, DZX02, IgG2a). (C) Example of confocal microscopy after double staining for anti–siglec-7 (upper panel) or siglec-9 (lower panel) Ab and anti-CD14 Ab, showing that siglec-9− cells frequently express CD14. (D) Example of confocal microscopy after double staining with anti-siglec-7 (upper panel) or siglec-9 (lower panel) Ab and anti-CD163 Ab, showing that siglec-7− cells frequently express CD163. (E) Example of CD209 (DC-SIGN) expression in siglec-7− macrophages/monocytes in lamina propria of colonic mucosal membranes. Arrows indicate macrophages/monocytes expressing both siglec-7 and DC-SIGN. (C–E) Original magnification ×200.
number of siglec-7⁺ cells expressed CD209, known as a marker of dendritic cells (Fig. 5B, 5E).

The stroma of colon cancer tissues also contained a significant number of cells expressing siglec-7 and/or -9. Most of them were macrophages, followed by neutrophils, CD8⁺ T cells, and NK cells, and their relative numbers were not statistically significantly different from those in nonmalignant colonic mucosal membranes. The majority of macrophages were also of the siglec-7⁺ siglec-9⁻ phenotype in the stroma of the colon cancer tissues (data not shown).

Roles of siglec-7-9 in macrophage function

Disialyl Lewis² and sialyl 6-sulfo Lewis², the carbohydrate ligands for immunosuppressive siglec-7/-9, are preferentially expressed in normal colonic epithelial cells, and their expression is lost during colonic carcinogenesis. In this context, we scrutinized the effect of siglec-7/-9 on COX2 expression in macrophages, because the enhanced COX2 expression in stromal cells is implicated in colon carcinogenesis (10, 29). Human monocytoid cells U937 are known to differentiate into macrophage-like cells when cultured with TPA and to produce COX2 upon further LPS stimulation. When U937 cells transfected with siglec-7/-9 were treated with TPA and LPS, ligation of siglec-7/-9 with agonistic Abs significantly suppressed induction of COX2 mRNA (Fig. 6A). The results of quantitative real-time RT-PCR analyses performed in parallel with conventional RT-PCR indicated that ligation of siglec-7 on the U937/siglec-7/-9 cells resulted in suppression of COX2 gene transcription to 48.3 ± 9.1% (mean ± SD, p < 0.05) of control culture with control F(ab)², and ligation of siglec-9 on the U937/ siglec-9 cells suppressed COX2 induction to 22.7 ± 11.5% (p < 0.01). Both siglecs conferred suppressive effects on COX2 transcription, and suppression of COX2 was also reflected in the reduction of its major product PGE₂ (Fig. 6B).

Discussion

Among the glycans in colonic epithelial cells, some are specifically expressed in normal epithelial cells, and some other glycans are preferentially expressed in cancer cells. It is noteworthy that only the glycans that are expressed in normal epithelial cells serve as ligands for siglec-7 and -9, whereas cancer-associated glycans do not, as indicated by the results shown in Figs. 1 and 2. We demonstrated that disialyl Lewis² preferentially expressed in nonmalignant colonic epithelial cells serves as a ligand for siglec-7 and -9, whereas sialyl Lewis² on cancer cells has no binding activity. Further cell-binding experiments indicated that sialyl 6-sulfo Lewis², another glycan preferentially expressed on nonmalignant colonic epithelial cells, also serves as a ligand for siglec-7, whereas sialyl Lewis², the corresponding glycan in cancer cells, does not.

Cancer-associated carbohydrate determinants, such as sialyl Lewis² and sialyl Lewis², are known to serve as ligands for selectins and mediate cancer cell adhesion to vascular beds in the course of hematogenous metastasis (4–6, 20). Expression of these determinants is markedly increased in cancer cells compared with normal epithelial cells. Nonmalignant epithelial cells express disialyl Lewis² and sialyl 6-sulfo Lewis² glycans having more complex structures than sialyl Lewis² (7, 12). Their expression is lost at the early stage of colon carcinogenesis as result of the epigenetic silencing of glycogenes involved in their synthesis (30, 31). Loss of these normal glycans leads to accumulation of less-complex glycans, such as sialyl Lewis² and sialyl Lewis², in cancers. Taken together, our results indicate that colonic epithelial cells lose their siglec ligands upon malignant transformation, and this is associated with the acquisition of selectin ligand glycans.

Our results also indicated that the majority of immune cells expressing siglec-7 and -9 in normal colonic mucosa are macrophages/monocytes. A large number of tissue macrophages bearing siglec-7 was found in colonic lamina propria, and they showed a surface marker pattern similar to that of so-called “res-

FIGURE 6. Inhibition of inflammatory mediator production by human monocytoid U937 cells by siglec-7 or -9. (A) Results of RT-PCR analyses of inhibition of LPS-mediated COX2 induction in U937 cells by ligation of siglec-7 or siglec-9. U937 cells stably transfected with siglec-7 cDNA (upper panel) or with siglec-9 cDNA (middle panel) or parental U937 cells (lower panel) were differentiated into macrophages with TPA and challenged with LPS. The F(ab)² fragment of anti–siglec-7 Ab and/or that of anti–siglec-9 Ab was added to the culture TPA-induced U937 cells with or without LPS for receptor ligation. (B) Results of ELISA analyses of inhibition of LPS-mediated PGE₂ production by U937 cells by ligation of siglec-7 (left panel) or siglec-9 (right panel). F(ab)² fragments were also used to evaluate PGE₂ production by the U937 cells. Values are mean ± SD from quadruplicate experiments. The results in the upper two panels in (A) are representative of at least three independent experiments, and those in the lower panel in (A) and (B) are representative of two independent experiments.
ident macrophages" (27, 28), characterized by low expression of CD14 and CD89, and eventually expressed dendritic cell markers. Most of them predominantly expressed siglec-7, and the expression of siglec-9 was less prominent. This type of siglec-7–dominant macrophage/monocyte was not identified in peripheral blood.

Another notable finding was that a significant population of CD8+ T cells was found to express siglec-7/-9+ in colonic lamina propria, indicating that the distribution pattern of siglec-7/-9 among mucosal immune cells is quite different from that in normal peripheral blood. Human peripheral blood T cells had been known to contain a very small number, if any, of siglec-7/-9+ cells, and the significance of such siglec-7/-9+ CD8+ T cells has largely been overlooked (32). Our detailed analysis of siglec-7/-9+ T cells in normal peripheral blood indicated that siglec-7/-9 are preferentially expressed in gut-associated minor populations, such as TcRγδ cells, CD80+ cells, and the T cells expressing gut-homing receptors β7-integrin and CCR9. These findings imply that siglec-7/-9 is somehow preferentially expressed in gut-associated T cells (33).

Most siglec, including siglec-7/-9, have ITIMs in their cytoplasmic domains, which inhibit immune cell activation by recruiting tyrosine phosphatases SHP-1 and SHP-2. Siglec’s suppression of killing activity and immune mediator production of immune cells is well documented in the literature (2, 8, 34–37). The biological significance of the interaction of resident macrophages/monocytes expressing siglec-7/-9 and their specific glycan ligands on colonic epithelial cells could be the protection of mucosal membrane from excess activation by immune cells. The endogenous glycan ligands on the resident macrophages/monocytes will also play important roles in physiological settings, because cis-ligands are known to inhibit binding of siglec with exogenous glycan ligands (2, 6).

Colonic epithelial cells are rendered increasingly susceptible to cancer progression both in humans and mice by chronic inflammatory stimuli in mucosal stroma (9). In this context, COX2, known to be expressed in stromal cells, such as fibroblasts (10, 11) and macrophages (29), has especially attracted researchers’ attention. COX2 expression in mucosal stromal cells is known to play a crucial role in facilitating colonic tumorigenesis in the APC(MIN) murine model (10, 11). Administration of COX2 inhibitors is known to be effective in preventing colonic carcinogenesis (9). Several intrinsic protective mechanisms for suppressing excessive production of COX2 by resident stromal cells must be operating in normal colonic mucosal membranes. Because only the carbohydrate determinants on normal epithelial cells, but not cancer-associated determinants, served as ligands for siglec, we focused on the effects of siglec ligation on COX2 production of macrophages in the current study.

Experiments using human macrophage cell lines transfected with siglec-7/-9 indicated that the ligation of siglec-7/-9 suppresses LPS-induced COX2 and PGE2 production. These results suggest that normal glycans of colonic epithelial cells exert a suppressive effect on tissue macrophage COX2 expression in colonic mucosa, thus maintaining immunological homeostasis in normal mucosal membranes. These results also imply that the cancer-associated impaired glycosylation of siglec-7 and -9 ligands serves to enhance COX2 production by mucosal macrophages. Because colonic tissues continuously encounter bacterial stimuli from fecal flora, colonic mucosa is equipped with various modules that suppress excess activation of mucosal immune cells and prevent inflammatory damage to the host. The interaction of siglec on mucosal immune cells and their specific glycan ligands on nonmalignant epithelial cells might be one of such homeostatic systems, and its abrogation through cancer-associated abnormal glycosylation provides a plausible mechanism for the progression of colon cancers.

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Disclosures

The authors have no financial conflicts of interests.

References


