Monocyte-Derived Dendritic Cells Function and Differentiation of Tumor-Associated Lewis Glycans Impair the C-Type Lectin DC-SIGN with Colorectal Glycosylation-Dependent Interactions

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Recognizing an invading pathogen or a malignant tumor by cells of the immune system is mediated by receptors on APCs such as dendritic cells (DCs). On DCs two receptor families are involved in the recognition of pathogens and tumors: TLRs, which recognize common pathogen-associated molecular patterns, and C-type lectin receptors, which bind to glycan Ags (1). DCs are specialized APCs that recognize, acquire, process, and present Ags to naive resting T cells for the induction of an Ag-specific immune response (2–4). The induction of an effective response to tumors mainly depends on innate and adaptive immunity coordinated by DCs. DCs are critically important for the induction and maintenance of antitumor immune responses both spontaneously developed and induced as a result of immunotherapy. An inadequate function of the host immune system may render all attempts to use immunotherapy ineffective. It is well known that a defect in the DC system is one of the main factors responsible for tumor escape. Recently accumulated evidence suggests that DC defects in cancer are systemic and based on their abnormal differentiation (5). Importantly, the glycosylation changes associated with cancer include underexpression and/or overexpression of naturally occurring glycans. Often, these epitopes are normally expressed during embryonic and fetal development but are absent in adult tissues (6). Glycosylation signals control critical lymphocyte processes, including lymphocyte homing, thymocyte selection, immune response amplitude, and recognition of pathogens and tumors (7). The roles of glycosylation in these functions are specific, i.e., different functions require specific sugars on specific glycoprotein acceptors (8). Regulated glycosylation of specific acceptor substrates can affect immune functions by creating or masking ligands for endogenous lectins (8). Recently, a great number of diverse transmembrane C-type lectins were identified on DCs that are involved in the recognition of a wide range of carbohydrate structures on tumor-associated Ags. In particular, several C-type lectin receptors such as DC-SIGN, which recognize aberrantly glycosylated forms of Le⁻ᵃ⁻⁹⁻ᵇ⁻ glycans on carcinoembryonic Ag (CEA) and CEA-related cell adhesion molecule 1 (CEACAM1). DC-SIGN ligands containing Le⁻ᵃ⁻⁹⁻ᵇ⁻ glycans are also highly expressed on primary cancer colon epithelia but not on normal colon epithelia, and DC-SIGN is suggested to be involved in the association between DCs and colorectal cancer cells in situ by DC-SIGN recognizing these cancer-related Le glycan ligands. Furthermore, when monocyte-derived DCs (MoDCs) were cocultured with SW1116 cells, LPS-induced immunosuppressive cytokines such as IL-6 and IL-10 were increased. The effects were significantly suppressed by blocking Abs against DC-SIGN. Strikingly, LPS-induced MoDC maturation was inhibited by supernatants of cocultures with SW1116 cells. Our findings imply that colorectal carcinomas affecting DC function and differentiation through interactions between DC-SIGN and colorectal tumor-associated Le glycans may induce generalized failure of a host to mount an effective antitumor response. The Journal of Immunology, 2008, 180: 3347–3356.
lectins expressed by DCs exhibit specificity for Man-containing carbohydrates. However, each C-type lectin may recognize the unique branching and positioning of Man residues on a given pathogen or self-Ag structure (9, 10). Also, oligomerization of C-type lectins influences the affinity and specificity of carbohydrate recognition (11). To date, little is known about the carbohydrate and Ag specificity of C-type lectins expressed on DCs.

Immature monocyte-derived dendritic cells (MoDCs) strongly express DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (12, 13). DC-SIGN is a type II transmembrane C-type lectin with a short amino-terminal cytoplasmic tail and a single carboxyl-terminal carbohydrate recognition domain (CRD). DC-SIGN specifically recognizes glycoconjugates containing mannose (Man), N-acetylgalactosamine (GlcNAc), and fucose (Fuc) on many pathogens and nonsialylated Lewis (Le) α/β (where Le α is Galβ1-3(Fucα1–4)GlcNAc and Le β is Fucα1–2Galβ1–3(Fucα1–4)GlcNAc; Gal, galactose) epitope structures in a Ca2+-dependent manner and has been shown to form tetramers (11). It is specifically expressed on DCs, MoDCs, and specialized macrophages in vitro and also on DC subsets in skin, mucosal tissues, tonsils, lymph nodes, and spleen in vivo (1). DC-SIGN functions as an adhesion receptor and mediates the binding and internalization of pathogens such as viruses (HIV and hepatitis C), bacteria (Mycobacterium), fungi, and parasites (1, 13). Recently, it was reported that immature DCs use DC-SIGN to adhere to colorectal cancer cells through the recognition of Le α and Le β Ags present on carcinoembryonic Ag (CEA) (14).

We recently reported the characterization of oligosaccharide ligands expressed on SW1116, a typical human colorectal carcinoma recognized by mannan-binding protein (MBP), which is a serum C-type lectin and has similar carbohydrate-recognition properties as those of DC-SIGN (15, 16). MBP oligosaccharide ligands have been shown to be large, multiantennary N-glycans carrying a highly fucosylated polylactosamine-type structure on SW1116 carcinoma cells (15) and Le α-Le β-cluster oligosaccharide structures on COLO205 membranes (our unpublished data). Therefore, we concluded that MBP requires clusters of tandem repeats of the Le α/Le β epitopes for colorectal carcinoma recognition (15). As an extension of these studies, we hypothesized that DC-SIGN is also involved in the recognition of the clusters of tandem repeats of the Le α/Le β epitopes on some colorectal carcinomas by DCs. Furthermore, it has been suggested that a dysfunction of DCs induced by a tumor is one of the critical mechanisms for escaping immune surveillance. There are very few examples of similar interactions between DCs and self-molecules, especially abnormal self-molecules such as tumor Ags, and their effects on DC function and the immune response.

In this study, we report that colorectal tumor-associated Le α/Le β glycans are important ligands for DC-SIGN on SW1116 carcinoma cells and primary cancer colon epithelia and that they are required for cellular interactions between DCs and colorectal carcinomas in situ. We showed that DC-SIGN mediates binding to CEA/CEA-related cell adhesion molecule 1 (CEACAM1) derived from colorectal carcinomas through Le α/Le β moieties. In addition, we found that glycosylation-dependent interactions between DC-SIGN and colorectal tumor-associated Le glycans strongly enhanced LPS-induced anti-inflammatory cytokine secretions of IL-6 and IL-10 by MoDCs. Additionally, LPS-induced functional maturation of MoDCs was strikingly inhibited by supernatants of cocultures with SW1116 cells. LPS induces DC functional maturation and cytokine secretion through TLR signaling, suggesting that DC-SIGN, upon the binding of colorectal tumor-associated Le α/Le β glycans, may interfere with TLR-mediated signals.

Materials and Methods

Reagents and Abs

Recombinant human DC-SIGN-Fc (rhDC-SIGN-Fc), anti-human DC-SIGN mAb, and anti-human CEACAM1 polyclonal Ab (pAb) were purchased from R&D Systems. Le α, Le β, and Le α oligosaccharides (where Le α is Galβ1–4(Fucα1–3)GlcNAc and Le β is Fucα1–2Galβ1–4(Fucα1–3)GlcNAc) and anti-Le α mAbs were obtained from Calbiochem. mAbs against Le α/Le β, Le α, and CEA were purchased from Seikagaku Kogyo and Abcam. Anti-human CD83 and CD86 mAbs and human IL-6 and IL-10 ELISA kits were purchased from BD Biosciences. PE-conjugated anti-human IFN-γ, FITC-conjugated anti-human IL-4, and anti-human CD3 (OKT3) mAbs, a human Th1/Th2 ELISA kit, and FITC-conjugated anti-human IL-4 and PE-conjugated anti-human IFN-γ mAbs were obtained from eBioscience. Alexa Fluor 488-conjugated anti-mouse IgG2b, Alexa Fluor 647-conjugated anti-mouse IgG1, and FITC-conjugated anti-mouse IgM secondary Abs were obtained from Molecular Probes and Zymed Laboratories, respectively. UltraFlu LPS from Escherichia coli 0111:B4 and PMA were purchased from Sigma-Aldrich. Anti-human DC-SIGN pAb, anti-Le α mAb, and purified goat IgG were obtained from Santa Cruz Biotechnology. Human IgG (IgG)Fc fragment and anti-human CD28 mAb were obtained from Jackson ImmunoResearch Laboratories and Chemicon, respectively. Tissue cryosection slides prepared from different human normal and malignant colon tissues were purchased from Super Bio Chips. Monosaccharides were obtained from Wako, Nacalai Tesque, and Sigma-Aldrich. All chemicals for gel electrophoresis and Western blotting were purchased from Nakalai Tesque, Atto, Bio-Rad, Pierce, and Zymed Laboratories.

Cell lines and cell culture

Human colon tumor cell lines COLO205 and SW1116 were cultured at 37°C under 5% CO2 in L-15 medium (Sigma-Aldrich) and RPMI 1640 (Nacalai Tesque), respectively, containing 10% FCS, 2 mM glutamine, and 50 μg/ml kanamycin. Human hepatoma cell line HLF and human monocytic leukemia cell line U937 were cultured at 37°C under 5% CO2 in DMEM (Nacalai Tesque) and RPMI 1640 medium, respectively, containing 10% FCS, glutamine, and 50 μg/ml kanamycin. All cell lines were obtained from American Type Culture Collection. DC-SIGN-expressing U937 cells (U937-DC-SIGN) were generated by transfection of the pcDNA3-D-SIGN plasmid with Lipofectamine 2000 reagent (Invitrogen Life Technologies) and then selection was performed in complete medium containing 1 mg/ml G418 (Invitrogen Life Technologies) for stable transfectants. For stimulation, cells were diluted to 1 × 106 cells/ml and then incubated in medium alone or with the specified additions for the times indicated.

Monocyte-derived immature DCs and maturation of immature DCs

Human PBMCs were isolated fromuffy coats of healthy donors by Ficol-Paque Plus (Amersham Biosciences) density gradient centrifugation according to the standard procedure. Monocytes were purified from PBMC by positive selection of CD14+ cells through MACS sorting according to the manufacturer’s instructions (Miltenyi Biotec). Immature MoDCs were derived from the isolated CD14+ monocytes in 10% FCS/RPMI 1640 supplemented with GM-CSF (800 U/ml; PeproTech) and IL-4 (400 U/ml; PeproTech) for 5 days. The cultures were replenished with fresh GM-CSF and IL-4-supplemented medium every 2 days. Mature MoDCs were induced by culturing immature MoDCs with 1 ng/ml LPS (Sigma-Aldrich) in the final 2–3 days. DC maturation was confirmed by expression of CD83, a DC-specific maturation marker on mature but not immature DCs, and by expression of CD86, a costimulatory molecule on mature DCs.

Isolation of naïve CD4+ T cell and intracellular cytokine staining

Naïve CD4+ T cells were isolated from PBMCs of healthy donors by depletion of non-Th cells and memory Th cells with a magnetic cell sorting system according to the manufacturer’s instructions (Miltenyi Biotec). For intracellular cytokine staining, the purified naïve CD4+ T cells were stimulated with anti-CD28 (1 μg/ml) and anti-CD3 (5 μg/ml) Abs followed by relative secondary Abs (5 μg/ml) in the presence of SW1116-MoDC coculture-derived and MoDC culture-derived supernatants for 2 days at 37°C, respectively. The stimulated cells were fixed by 0.25% paraformaldehyde in PBS for 1 h at 4°C, and then permeabilized with 0.2% Tween 20 in PBS for 15 min at 37°C. The fixed and permeabilized cells were blocked with the FACS blocking buffer (1% BSA in PBS), and then stained with both FITC-conjugated anti-human IL-4 (3.75 μg/ml) and PE-conjugated anti-human IFN-γ, stained with AlexFluor 488- and AlexaFluor 647-conjugated anti-human IgG1, and then stained with FITC-conjugated anti-human IL-4 and PE-conjugated anti-human IFN-γ mAbs.
anti-human IFN-γ (1.88 μg/ml) mAbs for 1 h on ice according to the manufacturer’s instructions (eBioscience). The double stained cells were washed twice with FACS buffer, pelleted by centrifugation, and finally suspended in 1 ml of FACS buffer before analysis with a FACScan (BD Biosciences) flow cytometer equipped with the LYSIS II software program.

**Flow cytometry**

Cellular phenotypic analysis was conducted by indirect immunofluorescence staining. HLF, COLO205, SW1116, U937 or U937-DC-SIGN cells were washed once in PBS and then pelleted, and viable cells were resuspended in 1 ml of FACS buffer (PBS containing 2% FCS). To analyze Le glycan expression, the cells were stained with 5 μg/ml anti-Lea, -Leb, -Le, or -Leα mAbs followed by 20 μl of FITC-conjugated relative secondary Abs. To assess the surface expressions of DC-SIGN carbohydrate ligands, the cells were incubated with 5 μg/ml rhDC-SIGN-Fc or theFc portion of hlgG as a control for 1 h in the presence of 10 mM CaCl2 or 20 mM EDTA and then stained with 10 μg/ml anti-DC-SIGN mAb followed by 20 μl of FITC-conjugated secondary Abs. The inhibition experiments on DC-SIGN bind- ing to colorectal carcinomas were performed through incubation with rhDC-SIGN-Fc in the presence of various saccharides. To analyze DC- SIGN expression on U937 and U937-DC-SIGN cell surfaces, the cells were stained with 10 μg/ml anti-DC-SIGN mAb (R&D Systems) or human recombinant DC-SIGN-Fc (0.625 μg/ml; Santa Cruz Biotechnology) followed by Alexa Fluor 488-conjugated secondary Ab in the presence of 10 mM Ca2+. The cells were visualized by laser confocal microscopy (FV1000; Olympus). To monitor cellular interaction between U937- DC-SIGN and immature MoDC and either COLO205 or SW1116 cells, the cells were coincubated for 1 h at 37°C and then costained with 1 μg/ml anti-DC-SIGN or immature MoDC and either COLO205 or SW1116 cells were incubated with rhDC-SIGN-Fc or the Fc portion of hlgG (0.625 μg/ml) and then stained with 1 μg/ml anti-DC-SIGN mAb followed by Alexa Fluor 488-conjugated secondary Ab in the presence of 10 mM CaCl2. The cells were visualized by laser confocal microscopy (FV1000; Olympus).

**Preparation of membrane fractions, purification of glycoproteins, immunoprecipitation, and immunoblotting**

COLO205, SW1116, or HLF cells were homogenized in homogenization buffer (20 mM Tris-HCl (pH 7.5), and protease inhibitor mixture). The homogenate was adjusted with NaCl to 150 mM and then centrifuged at 10,000 x g for 5 min at 4°C to remove cell debris and nuclei. The supernatant was then centrifuged at 150,000 x g for 45 min at 4°C. The resulting total membrane pellet was solubilized with lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Triton X-100, and protease inhibitor mixture) for 1 h on a rotary shaker at 4°C and then centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was used as the cell membrane proteins for ligand purification, immunoprecipitation, and immunoblot samples. To identify the proteins carrying DC-SIGN carbohydrate ligands on colorectal carcinomas, purification of DC-SIGN glycoprotein ligands was performed using DC-SIGN-Fc-protein G beads and hlgG-Fc-protein G beads as a control, and the bound proteins were eluted with EDTA as immunoprecipitation and immunoblot samples. The samples were resolved by electrophoresis on a 5–20% gradient SDS-polyacrylamide gel (Atto) and then transferred to nitrocellulose membranes, followed by immunoblot detection with specific Abs. For visualization, a SuperSignal West Pico chemiluminescent kit (Pierce) was used with HRP-conjugated anti-mouse IgG Abs (Zymed Laboratories) or HRP-conjugated anti-rabbit IgG (Cell Signaling Technology).

**Cytokine production**

For cytokine production, 3 x 10⁵ immature MoDCs were placed into one well of 96-well plate, respectively, and then cultured for 24 h in the presence of IL-6 (500 U/ml), GM-CSF (800 U/ml), and LPS (1 ng/ml) for MoDCs. The effects of SW1116 cells on LPS-induced cytokine production were determined by preincubating the immature MoDCs with goat anti-human DC-SIGN IgG pAb (3.3 μg/ml; Santa Cruz Biotechnology) or purified goat IgG (3.3 μg/ml; Santa Cruz Biotechnology) for 1 h at 37°C, followed by coculturing with 1.2 x 10⁵ SW1116 cells in one well of 96-well plate for 20 h at 37°C. The coculture supernatants were harvested and analyzed for IL-6 and IL-10 production using a commercial ELISA kit from BD Biosciences following the manufacturer’s protocols. The standards and test samples were analyzed on a Wallac 1420 multilabel counter (PerkinElmer) in accordance with the manufacturer’s instructions. All experiments were performed in triplicate and repeated a minimum of three times.

**MoDC functional maturation**

To determine the effects of MoDC-SW1116-cocultured supernatants on LPS-induced functional maturation of MoDCs, 1 x 10⁶ immature MoDCs were incubated with a MoDC-cultured or MoDC-SW1116-cocultured supernatant for 3 days in the presence of IL-4 (500 U/ml), GM-CSF (800 U/ml), and LPS (1 ng/ml). Cultures were replenished with fresh superna tant on the second day. The effects of MoDC functional maturation were determined by cell surface expression of the costimulatory molecules CD86 and CD83 using Alexa-conjugated Abs. The inhibition of MoDC functional maturation as determined with CD86 and CD83, respectively, was measured as follows: (mean fluorescence intensity (MFI) of incubation with MoDC-SW1116-cocultured supernatant/MFI of incubation with MoDC-cultured supernatant) x 100. The MFI values for incubation with MoDC-cultured supernatant were arbitrarily set at 100%. All experiments were performed in triplicate and were repeated a minimum of three times.

**Immunohistochemistry**

The paraffin-embedded cryosections of human normal and malignant colon tissues (Super Bio Chips) were deparaffinized and hydrated with xylene and alcohol, respectively. After blocking with the blocking buffer (0.1% BSA in TBS), the cryosections of human normal and malignant colon tissues were incubated with human recombinant DC-SIGN-Fc (0.625 μg/ml) for 1 h at room temperature in the presence of 5 mM CaCl2. For double immunofluorescence, the cryosections incubated with and without human recombinant DC-SIGN-Fc were stained with primary anti-DC-SIGN (2.5 μg/ml) and anti-Lea or Leb (2.5 μg/ml) mAbs followed by Alexa Fluor 488- and 546-conjugated secondary Abs, respectively. The coexpressions of DC-SIGN ligands or endogenous DC-SIGN with Le glycan were visualized by laser confocal microscopy (FV1000; Olympus).

**Statistical analysis**

The results are expressed as the means ± S.D. of data obtained in three and four experiments performed in duplicate or triplicate. Statistical significance was determined by means of Student’s t-test. p < 0.05 was considered significant.

**Results**

**DC-SIGN binds human colorectal carcinomas in a Ca2+-dependent manner**

To examine whether transmembrane C-type lectin DC-SIGN binds to human colorectal tumor cells, we incubated the colorectal carcinoma cell lines COLO205 and SW1116 and the human hepatoma cell line HLF with a purified chimeric protein consisting of DC-SIGN and the hlgG-Fc domain (DC-SIGN-Fc) in the presence of 10 mM CaCl2 and then analyzed the binding properties by means of confocal microscopy and FACS analysis. As shown in Fig. 1, rhDC-SIGN-Fc but not the purified hlgG-Fc strongly bound to both COLO205 and SW1116 colorectal carcinomas compared with the HLF hepatoma. The Ca2+- dependent EDTA, which removes Ca2+ ions that are essential for carbohydrate binding, completely blocked DC-SIGN binding to the colorectal carcinomas, indicating that the CRD of the lectin is involved in the binding to oligosaccharide ligands on colorectal tumors.

**DC-SIGN recognizes Le ganglios on human colorectal carcinomas**

Recently, it has been reported that Lea and Leb carbohydrates are expressed on some colorectal cancer and breast cancer cell lines, such as SW948 and SKBR3 (14). In this study we show that not only Lea and Leb but also Leα and Leβ glycan epitopes are expressed on the surfaces of the colorectal carcinomas COLO205 and SW1116. As shown in Fig. 2A, both colorectal carcinomas but
not the HLF hepatoma exhibited high levels of four kinds of Le epitopes on the tumor surface, as seen by flow cytometry. These results are consistent with our recent report that clusters of tandem repeats of Le\(^\alpha\)/Le\(^\beta\) epitopes are expressed on SW1116 colorectal carcinoma cells (15). Following up on the DC-SIGN binding and Le glycan expression data described above, we next investigated whether DC-SIGN binding to the surfaces of colorectal carcinomas is dependent upon carbohydrate interaction. Thus, we examined the abilities of four free Le saccharides and several free monosaccharides to competitively block the interaction of DC-SIGN with colorectal tumor-associated Le glycans and that DC-SIGN is the main DC receptor establishing this cell-cell interaction.

**DC-SIGN recognizes tumor-associated Le\(^\alpha\)/Le\(^\beta\) glycans of CEA and CEACAM1 expressed on colorectal carcinomas**

Although the identification of colorectal carcinoma cell surface glycoproteins that bind to DC-SIGN was a critical step for identifying the glycoproteins involved in DC-SIGN-mediated suppression, it is important to note that colorectal carcinoma glycoproteins modified by abnormal glycan may bind to DC-SIGN and not to the regular glycans on normal colorectal cells (14). In addition, binding by DC-SIGN was specific for tumor-associated Le glycans on the colorectal carcinomas, because tumor-associated Le glycans were not observed on the HLF hepatoma. Several research groups have previously and recently reported that ICAM-2, ICAM-3, Mac-1, CEA, CEACAM1, etc. are cellular ligands (12–14).

To identify the glycoproteins carrying DC-SIGN carbohydrate ligands on colorectal carcinomas, we performed immunoblot analysis with Abs against the cellular ligands on the bound proteins of colorectal carcinoma and hepatoma membrane fractions eluted with EDTA from DC-SIGN-Fc-protein G beads and human IgG Fc-protein G beads, respectively. As shown in Fig. 4A, the EDTA-eluted proteins were identical with CEA and CEACAM1 on colorectal carcinoma SW1116 and COLO205 cell membranes but not on the hepatoma HLF cell membrane from DC-SIGN-Fc-protein G beads, which were indeed recognized by DC-SIGN, but none of these proteins were detected on a SW1116 colorectal carcinoma in the EDTA-eluted fractions from IgG Fc-protein G beads. The expression levels of both CEA and CEACAM1 on SW1116 cells were much higher than those on COLO205 cells. CEA is a tumor-associated Ag which is normally expressed during oncofetal development and is overexpressed in nearly all colorectal cancers, 70% of nonsmall-cell lung cancers, and ~50% of breast cancers (17). CEA is a 180-kDa molecular mass glycoprotein member of the Ig supergene family that consists of several structurally related glycoproteins including CEACAM1, which is also expressed on colorectal carcinomas.

Moreover, to determine whether colorectal tumor-associated Le\(^\alpha\) and Le\(^\beta\) glycans on CEA and CEACAM1 mediate interactions with DC-SIGN, we conducted immunoprecipitation with Le\(^\alpha\) and Le\(^\beta\) Abs on the EDTA-eluted proteins described above and then performed immunoblotting of the immunoprecipitates with CEA and CEACAM1 Abs. As shown in Fig. 4B, SW1116 but not COLO205 colorectal carcinomas express high levels of Le\(^\alpha\) and Le\(^\beta\) on both CEA and CEACAM1. The results indicate that DC-SIGN recognizes not only high Man epitopes (11) and Le\(^\alpha\)/Le\(^\beta\) epitopes (16) but also Le\(^\alpha\) and Le\(^\beta\) epitopes and that DC-SIGN mediates interactions with CEA and CEACAM1 through Le\(^\alpha\) and Le\(^\beta\) epitopes on these glycoprotein ligands from some colorectal tumor-associated Le glycans.
colorectal carcinomas such as SW1116 tumors. The other glycoprotein ligands of DC-SIGN on colorectal carcinomas will be identified through mass spectrometry.

Colorectal carcinoma-associated Le glycans modulate immunosuppressive cytokine production by MoDCs through DC-SIGN binding

Monocytes derived from human peripheral blood lymphocytes, widely used as a model for monocyte-immature DC-mature DC differentiation, express very low basal levels of DC-SIGN, and DC-SIGN expression in monocytes is up-regulated by GM-CSF plus IL-4 and down-regulated by LPS as compared with mature MoDCs, which express high levels of CD83 (a mature DC lineage marker) and low levels of DC-SIGN upon LPS induction (Fig. 5A). In contrast to mature DCs that are localized peritumorally, immature DCs are present on tumors such as colorectal carcinomas, suggesting that interactions may occur between immature DCs and colorectal carcinoma cells that modulate DC immunological functions through DC-SIGN-Le glycan binding as described above. To investigate this possibility, we incubated colorectal carcinoma SW1116 cells with immature MoDCs and then visualized cellular interactions via DC-SIGN-Le\textsuperscript{a}/Le\textsuperscript{b} glycan binding. As shown in Fig. 5B, immature MoDCs, but not monocytes (data not shown), strongly interacted with SW1116 cells to form cell-cell clusters through DC-SIGN-Le\textsuperscript{a}/Le\textsuperscript{b} glycan binding as well as DC-SIGN-CEA interaction, as seen on confocal microscopic analysis, suggesting that cellular interactions between DC-SIGN and Le\textsuperscript{a}/Le\textsuperscript{b} glycans occur in a glycosylation-dependent manner on CEA or other glycoproteins.

It has been recently reported that mycobacteria specifically target DC-SIGN through the cell wall component Man-capped lipoolarabinomannan (ManLAM) to impair DC maturation and induce production of the anti-inflammatory cytokine IL-10 (18). We therefore extended our study by investigating the physiological function of DC-SIGN-colorectal tumor-associated Le glycan interactions. To determine whether the cellular interactions influence the suppression of DC functions through cytokine production, we analyzed MoDC secretion of the immunosuppressive cytokines IL-6 and IL-10 after treatment with LPS and coculture with SW1116 carcinomas. SW1116 cells strikingly induced both IL-6

FIGURE 2. Le epitopes on colorectal carcinomas mediate DC-SIGN binding. A, Flow cytometry analysis of the Le epitope expression on colorectal carcinomas. COLO205, SW1116, and HLF cells were incubated with Le-epitope mAb (Le\textsuperscript{a}, Le\textsuperscript{b}, Le\textsuperscript{x}, and Le\textsuperscript{y}), followed by staining with FITC-conjugated secondary Ab. The cells were then analyzed by flow cytometry. Black histograms, Background with secondary Ab only; white histograms, Le-epitope mAb staining, as shown above the panels. B and C, Flow cytometry analysis of the inhibition of DC-SIGN binding to COLO205 (B) and SW1116 (C) by Le saccharides and monosaccharides. COLO205 and SW1116 cells were incubated with rhDC-SIGN-human Fc in the presence of 20 mM Le\textsuperscript{a}, Le\textsuperscript{b}, Le\textsuperscript{x}, and Le\textsuperscript{y} (upper panels), respectively, or 50 mM Man, Gal, Fuc, GlcNAc, and N-acetylgalactosamine (GalNAc) (lower panels), respectively. The cells were incubated with anti-human DC-SIGN mAb followed by FITC-conjugated secondary Ab and then analyzed by flow cytometry. Filled histograms, Background without primary Ab; open histograms, absence of sugar; arrows, presence of a Le saccharide or monosaccharide as shown above the panels.
and IL-10 production by MoDCs when they simultaneously received an LPS activation signal (Fig. 5C). This IL-6 and IL-10 production was significantly inhibited by Abs against DC-SIGN but not by relative goat IgG as a control Ab (Fig. 5, Ca and Cb), which indicates that IL-6 and IL-10 induction is specific for the DC-SIGN-mediated interactions. Taken together, these findings suggest that glycosylation-dependent interactions between DC-SIGN and colorectal carcinoma-associated Le glycans result in down-regulation of DC immunological functions and that LPS induces DC cytokine secretion through TLR signaling, suggesting that DC-SIGN, upon the binding of colorectal tumor-associated Lea/Leb glycans, may interfere with TLR-mediated signals.

A colorectal carcinoma-MoDC coculture-derived supernatant suppresses the functional maturation of MoDCs

As DCs mature and are activated, the expression of certain cell surface proteins such as costimulatory molecules involved in Ag presentation and T cell stimulation changes. Changes in the expression of these cell surface proteins can be used to monitor the maturation and activation state of DCs. Immature DCs are highly efficient as to Ag capture and processing, whereas mature DCs predominantly present Ag. To study the effect of coculture-derived soluble factors on the functional maturation of MoDC, a coculture-derived supernatant was prepared from cocultures of SW1116 carcinoma cells and MoDCs after LPS stimulation. The addition of the SW1116-MoDC coculture-derived supernatant to MoDC cultures led to LPS-induced inhibition of both maturation marker CD83 (Fig. 6, Aa and Ab) and activation marker CD86 (Fig. 6, Ba and Bb) expression compared with a MoDC culture-derived supernatant. Taken together, the results indicate that glycosylation-dependent interactions between DC-SIGN and colorectal carcinoma-associated Le glycans suppress LPS-induced functional maturation of MoDCs, suggesting that DC-SIGN, upon the binding of colorectal tumor-associated Le glycans, may be associated with LPS-induced TLR signal transduction. Our findings demonstrate that a dysfunction of DCs induced by a tumor in a glycosylation-dependent manner is one of the critical mechanisms for escaping immune surveillance.

The colorectal carcinoma-MoDC coculture-derived supernatant may attenuate polarization of T cell differentiation toward a Th1 phenotype

Naive CD4+ T cells can differentiate into either Th1 or Th2 cells with distinct immunological functions. Th1 cells produce mostly
proinflammatory cytokines such as IFN-γ, while Th2 cells produce proallergic cytokines such as IL-4. Next, to determine the effects of the above-described colorectal carcinoma-MoDC coculture-derived supernatant on human T cell polarization and differentiation into Th1 or Th2 phenotypes, purified naive CD4⁺ T cells were isolated from PMBCs of healthy donors and stimulated with both anti-CD3 and anti-CD28 mAbs in the presence of SW1116-MoDC coculture-derived or MoDC culture-derived supernatant. As shown in Fig. 7, the exposure of purified naive CD4⁺ T cells to a SW1116-MoDC coculture-derived supernatant, compared with a MoDC culture-derived supernatant, resulted in a decrease of the percentage of IFN-γ-secreting T cells/Th1 phenotype but no change in the percentage of IL-4-secreting T cells/Th2 phenotype. The results suggest that the colorectal carcinoma-MoDC coculture-derived supernatant may attenuate the Th1-polarizing condition through colorectal carcinoma-induced immunosuppressive cytokines such as IL-6 and IL-10 secreted by MoDCs in our experimental model. Elucidation of the critical mechanism involved in the polarization of T cell differentiation by colorectal carcinoma-MoDC coculture-derived supernatant requires further study.

FIGURE 5. Immunosuppressive cytokine production is regulated by cell-cell communication through the glycosylation-dependent interactions of DC-SIGN with tumor-derived Le⁺/Leᵇ glycans. A, Analysis of monocyte-derived immature MoDCs and maturation of immature MoDCs. Human monocytes were purified from PBMCs by positive selection of CD14⁺ cells using MACS sorting as described under Materials and Methods. Immature MoDCs were derived from isolated CD14⁺ monocytes in medium supplemented with GM-CSF and IL-4 for 5 days. Mature MoDCs were induced by a culture of immature MoDCs with LPS for 2 days. These cells were stained with Abs against the immature DC marker DC-SIGN (upper panels) and the mature DC marker CD83 (lower panels), respectively, and then analyzed by flow cytometry. As a negative control, the autofluorescence of the cells was measured (black histograms). B, Cellular interaction between immature MoDCs and SW1116 cells through DC-SIGN binding to tumor-derived Le⁺/Leᵇ glycans of CEA. Human immature MoDCs were cocultured with SW1116 cells and then costained with Abs against DC-SIGN and Le⁺ (left panels), Leᵇ (middle panels), or CEA (right panels), followed by secondary Abs. The costained cell-cell interactions were visualized by confocal microscopy. C, Cellular glycosylation-dependent interaction increases immunosuppressive cytokine production by MoDCs through DC-SIGN binding. Immature MoDCs (3 × 10⁴) were preincubated with or without anti-DC-SIGN pAb for 1 h at 37°C and then subsequently cocultured with SW1116 cells (1.2 × 10⁵) in the presence of LPS (1 ng/ml) for 20 h at 37°C. The coculture supernatants were harvested and analyzed for IL-6 (a) and IL-10 (b) production, respectively, using commercial ELISA kits following the manufacturer’s protocols. All experiments were performed in triplicate and repeated a minimum of three times.

FIGURE 6. A SW1116 carcinoma-MoDC coculture-derived supernatant suppresses the functional maturation of MoDCs. Immature MoDCs were incubated with a MoDC-cultured or SW1116-MoDC-cocultured supernatant (Sup.) for 3 days in the presence of IL-4, GM-CSF, and LPS (1 ng/ml) as described above, and the cultures were replenished with the fresh supernatant on the second day. The effects of MoDC functional maturation were determined as MoDC-surface expression of CD83 (A) and CD86 (B) analyzed by flow cytometry. The inhibition of MoDC functional maturation for A and B, respectively, was measured as follows: (MFI of incubation with SW1116-MoDC-cocultured supernatant/MFI of incubation with MoDC-cultured supernatant) × 100. The MFI values for incubation with MoDC-cultured supernatant were arbitrarily set at 100%. All experiments were performed in triplicate and were repeated a minimum of three times.
DC-SIGN mediates the interaction of DCs to primary colorectal cancer cells by recognizing colorectal carcinoma-related Le glycan ligands in situ

Recently, van Gisbergen et al. reported that DC-SIGN is involved in the recognition of colorectal cancer cells by DCs through the binding of Le⁺/Le⁰ carbohydrates on CEA (14). The above data and our previous results (15) indicate that Le⁺/Le⁰ glycan expressed on colorectal carcinoma cells are also associated with the interaction of DCs with colorectal cancer cells through DC-SIGN recognition. To investigate whether Le⁺/Le⁰ glycan ligands of DC-SIGN express on primary colorectal cancer tissue, the cryosections of human normal and malignant colon tissue from the same patient were incubated with rhDC-SIGN and then were immunohistochimically examined by double fluorescence staining for DC-SIGN and Le⁺ or Le⁰ glycan. As shown in Fig. 8A, both DC-SIGN ligands and Le⁺/Le⁰ glycan are highly expressed on cancer colon epithelia compared with on normal colon epithelia. Furthermore, DC-SIGN ligands are extremely costained with Le⁺ or Le⁰ glycan on cancer colon epithelium but not on normal colon epithelium (Fig. 8A), suggesting that the increased expression of DC-SIGN ligands on colorectal cancer cells, in addition to the altered glycosylation that results in increased levels of Le⁺ and Le⁰ glycan on colorectal cancer cells, may promote interactions with DC-SIGN. It has been reported that DC-SIGN-positive cells are detected not only within the mucosa of normal colon tissue but also within cancer colon tissue (14, 19). Next, to determine whether DC-SIGN-positive cells may interact with primary colorectal cancer cells in situ, we analyzed the interaction of endogenous DC-SIGN with Le⁺ glycan in cancer colon tissue. As shown in Fig. 8B, DC-SIGN-positive cells within cancer colon tissue are closely

FIGURE 8. Confocal microscopic observation of DCs expressing DC-SIGN and colon epithelia expressing Le⁺/Le⁰ glycan in situ. A, DC-SIGN ligands containing the Le⁺/Le⁰ glycan are highly expressed on primary cancer colon epithelia but not on normal colon epithelia. The deparaffinized cryosections of human normal (a and c) and malignant (b and d) colon tissues from the same patient were incubated with rhDC-SIGN-Fc in the presence of 5 mM Ca²⁺. The cryosections were costained with primary anti-DC-SIGN and anti-Le⁺ or Le⁰ mAbs followed by Alexa Fluor 488- and 546-conjugated relative secondary Abs, respectively. The expressions of DC-SIGN ligands (green) and Le⁺ or Le⁰ glycan (red) were visualized by laser confocal microscopy. The Nomarski images are shown in the left panels. B, DCs expressing DC-SIGN are present within primary cancer colon epithelia. The deparaffinized cryosection of human cancer colon tissue was costained with primary anti-DC-SIGN and anti-Le⁺ mAbs followed by Alexa Fluor 488- and 546-conjugated relative secondary Abs, respectively. The DCs expressing DC-SIGN (green) and colorectal cancer cells expressing Le⁺ glycan (red) were visualized by laser confocal microscopy. The Nomarski image (a) and higher magnification (c) are shown on the left and the right of the double immunofluorescence panel (b), respectively. The arrows in c indicate the association of DC-SIGN-expressing DCs with colorectal cancer cells expressing Le⁺ glycan in situ. Scale bars, 100 μm.
associated with colorectal cancer cells expressing Lea glycans, but the cell-cell association is not found in normal colon tissue (data not shown), suggesting that the association between DC-SIGN-positive cells and colorectal cancer cells may occur in situ by DC-SIGN recognizing colorectal carcinoma-related Le glycan ligands.

Discussion
C-type lectin receptors on APCs are potent Ag-uptake receptors exhibiting specificity for glycan structures. Glycans decorate the surfaces of all mammalian cells including malignant and benign tumors, and the extracellular matrix with which they interact (1). Glycosylation changes during malignant transformation lead to tumor-specific carbohydrate structures that interact with C-type lectins on dendritic cells. The job of decoding glycan information is assigned in part to a great variety of mammalian glycan-binding proteins or lectins (6, 9). Probably one of the most interesting tumor-associated glycan changes is the appearance of Le-type Ags. DC-SIGN not only exhibits high affinity for Man in high Man moieties but also for Fuc in Le glycans (16). Moreover, the most frequently reported tumor-associated changes in glycosylation include an increase in the branching of N-glycans, increases in sia-lylation and polyasialic acid synthesis, the appearance of Le-type Ags in glycoproteins and glycolipids, and the shortening of O-glycan chains (5). In general, all of these changes have been associated with a poor prognosis, as they are linked to the aggressiveness and metastatic capacity of tumors. Good examples of heavily glycosylated tumor Ags are CEA and CEACAM1, which can be secreted or expressed by colon cancer cells (20).

We recently characterized the structures of MBP oligosaccharide ligands expressed on SW1116 tumors, which have been shown to be a novel type of tumor-associated carbohydrate comprising large, multiantennary N-glycans carrying highly fucosylated polyasialic-type structures (15). We demonstrated that, at the nonreducing termini of oligosaccharide ligands, Le°Leb or tandem repeats of the Leb structure prevail, a substantial proportion of which are attached via internal Leb or N-acetyllactosamine units to the trimannosyl core and that the structures characterized are unique and distinct from those of other previously reported tumor-specific carbohydrate Ags (15). Because DC-SIGN exhibits similar carbohydrate recognition specificity to MBP (16), we hypothesized that DC-SIGN may also require clusters of tandem repeats of the Leb/Leb epitopes for colorectal carcinoma recognition like MBP. In this study, we have revealed that both DC-SIGN-transfected monocyte cell lines and primary immature MoDCs bind to colorectal carcinomas through glycosylation-dependent binding of DC-SIGN with tumor-associated Le°Leb glycans on CEA and CEACAM1, which may enable cross-talk between immature DCs and tumors. CEA and CEACAM1 are present on most normal epithelia and retain or exhibit enhanced expression after tumor development. Glycosylation is deregulated in experimental and human carcinomas, resulting in expression of distinct carbohydrate profiles on normal and malignant tissues (21). Although Le glycans may also arise on other proteins during tumor development, we and other groups have shown that DC-SIGN binds to CEA and CEACAM1 only on colorectal carcinomas. Our data here indicate that DC-SIGN mediates binding to CEA/CEACAM1 derived from colorectal carcinomas through Le°Leb moieties (Fig. 4). Additionally, van Gisbergen et al. reported that DC-SIGN is involved in the recognition of colorectal cancer cells by DCs through the binding of Le°Leb carbohydrates on CEA (14).

DC-SIGN-interacting pathogens are thought to modulate DC maturation by interfering with intracellular signaling from TLRs (22). In addition, tumors use a variety of mechanisms to evade detection and elimination by the immune system (19, 23). Maturation of DCs is crucial for their ability to induce adaptive immunity. Although several mediators of DC maturation have been found, their contributions to DC maturation during infection through interactions with tumors are poorly understood. DCs, which play a pivotal role in the development of antitumor immunity, appear susceptible to tumor-mediated immunosuppression (24, 25). However, the molecular mechanism underlying tumor-induced suppression of DC differentiation has not been completely elucidated to date, and the effects of tumors on DC function also remain poorly understood. Immature DCs are highly efficient as to Ag capture and processing, whereas mature DCs predominantly present Ag. Mature DCs are specialized as the native T cell activation necessary for cellular immune responses (26). Immature DCs mature in response to some specific signals such as LPS or inflammatory cytokines (TNF-α and PGE2). We examined the effect of interactions between DC-SIGN and tumor-associated Le glycans on MoDC maturation and found that LPS-induced MoDC activation/maturation was strikingly inhibited upon the addition of MoDC-SW1116 coculture-derived supernatants, as the expression levels of the maturation/activation markers CD83 and CD86 were considerably lower than those of LPS-activated/matured MoDCs (Fig. 6). Previously, it was reported that immature DCs are present intratumorally, whereas mature DCs are located peritumorally (27, 28). Possibly, expression of DC-SIGN specifically on immature DCs enables these cells but not mature DCs to associate with colorectal tumors. The presence of immature DCs with proliferating CD4 and CD8 T cells has been reported (27, 29). The numbers of these mature DCs and tumor-infiltrating lymphocytes correlate with a metastasis-free condition and the survival of colorectal cancer patients (30). This suggests that these mature DCs are able to induce protective T cell responses against the tumor cells.

A variety of cytokines, including IL-6, IL-10, vascular endothelial growth factor (VEGF), and M-CSF, have been shown to affect the maturation of DCs from CD34+ precursors and from MoDCs in vitro (31–33). The failure of the immune system to provide protection against tumor cells is an important immunological problem. It is now evident that inadequate functioning of the host immune system is one of the main mechanisms by which tumors escape from immune control, as well as an important factor that limits the success of cancer immunotherapy. In recent years, it has become increasingly clear that defects in DCs play a crucial role in nonresponsiveness to tumors (5). Our results indicated that when immature MoDCs are cocultured with colorectal carcinomas, the levels of LPS-induced immunosuppressive cytokines IL-6 and IL-10 increase, which inhibits MoDC differentiation and maturation (Fig. 5). These effects are significantly blocked by DC-SIGN Abs, suggesting that the DC-carcinoma interactions are driven by the binding of DC-SIGN to its ligands. In addition, LPS induces DC maturation and cytokine secretion through TLR signaling, suggesting that DC-SIGN, upon the binding of colorectal tumor-associated Le°Leb glycans, interferes with TLR-mediated signals (Fig. 6). In contrast, the colorectal carcinoma-induced immunosuppressive cytokines such as IL-6 and IL-10 secreted by MoDCs may attenuate the Th1-polarizing differentiation in our experimental model (Fig. 7). The future extension of this study is to elucidate the critical mechanism involved in the polarization of T cell differentiation by a colorectal carcinoma-MoDC coculture-derived supernatant. These findings indicate that glycosylation-driven binding of CEA/CEACAM1 and other glycoproteins to DC-SIGN is essential for the interactions of colorectal carcinomas with DCs and that the glycosylation-dependent cellular interactions may result in suppression of DC functions, which may contribute to the survival of colorectal carcinomas. Taken together, our results also provide strong evidence that the production of immunosuppressive
factors in DC-tumor coculture supernatants is one of the mechanisms by which tumors evade immunosurveillance.

In the present work, we also observed that DC-SIGN ligands are highly expressed with Leα or Leβ glycan on cancer colon epithelial but not on normal colon epithelium from the same patient (Fig. 8A), suggesting that the increased expression and the altered glycosylation of DC-SIGN ligands on colorectal cancer cells result in recognition by DC-SIGN. Moreover, we found that DC-SIGN-positive cells within cancer colon tissue are closely associated with Leα glycan-expressing colorectal cancer cells in situ (Fig. 8B), suggesting that the increased expression and the altered glycosylation of DC-SIGN ligands on colorectal cancer cells result in recognition by DC-SIGN. Moreover, we found that DC-SIGN-positive cells within cancer colon tissue are closely associated with Leα glycan-expressing colorectal cancer cells in situ (Fig. 8B), suggesting that the increased expression and the altered glycosylation of DC-SIGN ligands on colorectal cancer cells result in recognition by DC-SIGN. Moreover, we found that DC-SIGN-positive cells within cancer colon tissue are closely associated with Leα glycan-expressing colorectal cancer cells in situ (Fig. 8B), suggesting that the increased expression and the altered glycosylation of DC-SIGN ligands on colorectal cancer cells result in recognition by DC-SIGN.

The evidence presented here suggests that tumor-altered differentiation of DCs as well as accumulation of immature cells with an inhibitory function could impair immune responses. This is relevant because DCs are being evaluated as cellular vaccine adjuvants for the immunotherapy of cancer. However, to date, tumor immunotherapy trials have met limited success for several reasons, including the restricted availability of tumor Ags in a form that renders them immunogenic to host. Therefore, alternative approaches, including improving of APC function, blockade of the tumor-derived inhibitory pathway, and/or optimization of DC function ex vivo, have been evaluated to potentiate the effect of DC-based cancer vaccines. DC-SIGN is not only involved in the recognition of pathogens but also might contribute to the capture and presentation of glycosylated self-antigens. The consequences for antitumor immunity or tolerance induction can be extrapolated from the function of C-type lectins in pathogen recognition and Ag presentation. In addition, in vivo studies on mice recently demonstrated the potency of targeting Ags to C-type lectins on APCs for antitumor vaccination strategies. To date, the exact mechanism of Ag presentation by DC-SIGN in vivo is poorly understood. The data we obtained in this study indicate a balance between the stimulatory effects of IL-6 and IL-10 production and the inhibitory effects of carcinoma tumors or IL-6/IL-10 on DC differentiation and maturation. High concentrations of IL-6 or IL-10 are capable of canceling the inhibitory effects of relatively high concentrations of carcinoma tumors. The present findings may be useful in clinical practice. The ex vivo generation of DCs for therapeutic use in cancer should ideally be performed.

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Disclosures
The authors have no financial conflict of interest.

References