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Lectin Galactoside-binding Soluble 3 Binding Protein (LGALS3BP) Is a Tumor-associated Immunomodulatory Ligand for CD33-related Siglecs*

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Heinz Läubli[1], **Frederico Alisson-Silva, Michal A. Stanczak, Shoib S. Siddiqui, Liwen Deng, Andrea Verhagen, Nissi Varki, and Ajit Varki**[2]

From the Departments of Medicine and Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA 92093

Background: Engagement of inhibitory CD33-related Siglecs on immune cells has been shown to influence interactions with cancer cells, including tumor immune evasion.
Results: LGALS3BP binds with high affinity to CD33-related Siglecs and inhibits neutrophil activation.
Conclusion: We identify LGALS3BP as novel, cancer-associated Siglec ligand that can influence neutrophil activation.
Significance: The engagement of inhibitory CD33-related Siglecs by LGALS3BP could support immune evasion of tumor cells.

Lectin galactoside-binding soluble 3 binding protein (LGALS3BP, also called Mac-2 binding protein) is a heavily glycosylated secreted molecule that has been shown previously to be up-regulated in many cancers and has been implicated in tumor metastatic processes, as well as in other cell adhesion and immune functions. The CD33-related subset of sialic acid-binding immunoglobulin-like lectins (Siglecs) consists of immunomodulatory molecules that have recently been associated with the modulation of immune responses to cancer. Because up-regulation of Siglec ligands in cancer tissue has been observed, the characterization of these cancer-associated ligands that bind to inhibitory CD33-related Siglecs could provide novel targets for cancer immunomodulatory therapy. Here we used affinity chromatography of tumor cell extracts to identify LGALS3BP as a novel sialic acid-dependent ligand for human Siglec-9 and for other immunomodulatory Siglecs, such as Siglec-5 and Siglec-10. In contrast, the mouse homolog Siglec-E binds to murine LGALS3BP with lower affinity. LGALS3BP has been observed to be up-regulated in human colorectal and prostate cancer specimens, particularly in the extracellular matrix. Finally, LGALS3BP was able to inhibit neutrophil activation in a sialic acid- and Siglec-dependent manner. These findings suggest a novel immunoinhibitory function for LGALS3BP that might be important for immune evasion of tumor cells during cancer progression.

The immune system evolved to differentiate between "self" and "non-self" to protect organisms from pathogens and maintain homeostasis. Therefore, the immune system also eliminates transformed cancer cells that are recognized as "foreign" by a process called cancer immunosurveillance (1). Although various receptors have been identified that bind to foreign molecules or patterns (2, 3), other receptors have been described that recognize self-associated molecules (such as killer cell Ig-like receptors) (4); or, self-associated molecular patterns (such as Siglecs)[3] (5). Siglecs are a family of immunomodulatory, transmembrane lectins expressed prominently on leukocytes (6–10). Siglecs can be divided into two groups on the basis of their evolutionary conservation (6). Although Siglec-1, Siglec-2, Siglec-4, and Siglec-15 have homologs across species, the CD33-related (CD33r) Siglecs (including Siglec-3 (CD33), Siglec-5 through Siglec-14, and Siglec-16 and Siglec-17 in primates) underwent rapid evolutionary changes, and the CD33rSiglec gene cluster (on chromosome 19q in humans) shows high variability between species (6, 10–12). Primate CD33rSiglecs can be further divided into inhibitory Siglecs, such as Siglec-5, Siglec-7, Siglec-9, and Siglec-10, that transmit inhibitory signals via intracellular immunoreceptor tyrosine-based inhibitory motifs; or, intracellular immunoreceptor tyrosine-based inhibitory motif-like motifs and activating Siglecs, including Siglec-14 and Siglec-16, that activate cells via DAP12 recruitment (6, 8, 10, 11, 13).

Rapid evolution of CD33rSiglecs occurs via multiple genetic mechanisms and is apparently driven not only by the need to maintain self-recognition by innate immune cells but also by the need to escape various types of subversion by pathogens (12). For example, pathogens such as group B streptococci can bind to Siglec-5 and Siglec-9 on myeloid cells and inhibit the antibacterial immune response by virtue of coating themselves with sialic acid or by presenting polypeptide ligands that can bind to Siglecs (14, 15).

It has been shown recently that, during cancer progression, tumor cells up-regulate sialylated Siglec ligands and, similar to

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[1] Present address: Dept. of Medical Oncology and Cancer Immunology Laboratory, University Hospital Basel, Petersgraben 4, 4031 Basel, Switzerland. To whom correspondence may be addressed: E-mail: heinz.laeubli@unibas.ch.
[2] To whom correspondence may be addressed: UC San Diego, La Jolla, CA 92093-0687. Tel.: 858-534-2214; Fax: 858-534-5611; E-mail: a1varki@ucsd.edu.

[3] The abbreviations used are: Siglecs, sialic acid-binding immunoglobulin-like lectins; OSGPase, *O*-sialoglycan-endopeptidase; NK, natural killer.

bacteria, that they can engage Siglecs and inhibit immune cell activation (16, 17). While Jandus *et al.* (16) identified a subset of NK cells that express Siglec-9 and showed that engagement of Siglec-9 led to immune evasion *in vitro*, we found that the expression of Siglec-9 ligands on carcinoma cells can modulate the immune response mediated by myelomonocytic cells (17). Although verification is still ongoing, these data point to a potentially important interaction between NK cells and myelomonocytic cells expressing Siglec-9 and sialylated tumor-associated ligands in human cancer progression.

To better characterize the role of Siglec-9 in cancer, we analyzed sialic acid-dependent ligands expressed on carcinoma cell lines by affinity chromatography and identified the *N*-glycosylated lectin galactoside-binding soluble 3 binding protein (LGALS3BP) as a main ligand. Although LGALS3BP has been found previously to be up-regulated in various cancers and associated with cancer progression and immunomodulation (18–31), the exact mechanism by which this heavily glycosylated molecule influences immune responses to cancer is not completely understood. In this study, we propose a novel mechanism by which LGALS3BP might influence immune cell activation via Siglec engagement.

EXPERIMENTAL PROCEDURES

Lectin Affinity Chromatography for the Identification of Sialic Acid-dependent Siglec-9 Ligands—Siglec-Fc chimeras were produced by transient transfection of HEK293A cells purified with protein A-agarose beads (GE Healthcare) and desialylated with sialidase from *Arthrobacter ureafaciens* prior to use. Tumor cell lines were lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, and 1:200 proteinase inhibitor mixture (Calbiochem), and membranes were isolated by ultracentrifugation after a nuclear spin. Membranes were solubilized in column buffer containing 1% octyl-β-D-glucopyranoside, 20 mM Tris (pH 7.5), and 500 mM NaCl, and membrane-associated ligands were allowed to engage Siglec-Fc chimeras bound to protein A-agarose beads (GE Healthcare) overnight at 4 °C while rotating. Beads were subsequently applied on a minicolumn and washed repeatedly with column buffer. To increase the specificity, the beads were also washed with buffer containing 10 mM lactose in column buffer prior to elution with 3 mM α2-3-Neu5Ac-lactose in column buffer at room temperature over several hours. Elutes were loaded on 10% SDS-PAGE gel and analyzed by silver staining. Samples were also analyzed using electrospray ionization tandem MS after ultra performance liquid chromatography separation by the proteomics core at the University of California, San Diego.

Western Blot Analyses for LGALS3BP—Cells were lysed with radioimmune precipitation assay buffer containing 1:200 proteinase inhibitor and loaded on denaturing, reducing 10% PAGE. Cell culture supernatants were either directly loaded or concentrated via microfiltration. After blotting to PVDF membranes and blocking with Licor blocking buffer, membranes were incubated with the murine monoclonal anti-LGALS3BP antibody SP2 (eBiosciences) at 1 μg/ml overnight at 4 °C. Membranes were subsequently washed with phosphate buffered saline Tween-20 and incubated for 1 h at room temperature with an anti-mouse-IR800 antibody (Licor). Signals were detected with an Odyssey infrared reader (Licor).

Flow Cytometry—For detection of membrane-bound LGALS3BP, the monoclonal mouse SP2 antibody (eBiosciences) or an isotype control (MOPC-2, Sigma) were used. Primary antibodies were stained with a secondary anti-mouse IgG-Alexa Fluor 647 antibody (Invitrogen). Lectin staining was performed with Siglec-Fc chimeras and, subsequently, with anti-human-IgG-PE (BD Biosciences). *O*-sialoglycan-endopeptidase (OSGPase, Cedar Lane) was added to cells to remove mucins as described previously (66). Briefly, cleavage of mucins was done by adding 50 μl of reconstituted OSGPase to 5×10^6 cells in 500 μl of DMEM, and cells were incubated for 1 h at 37 °C. Then the cells were washed and incubated with Siglec-Fc chimeras or anti-LGALS3BP antibody before analysis by flow cytometry.

Knockdown of LGALS3BP—Validated siRNA for LGALS3BP or control siRNA was purchased from Qiagen. Transfection of siRNA was performed by electroporation (Neon, Sigma), and efficiency was tested by cell surface staining of LGALS3BP with the SP2 antibody by flow cytometry.

Transient Transfection of LGALS3BP in 293A Cells—LGALS3BP was cloned from mRNA of HT-29 cells into pcDNA6/myc-His C (Invitrogen). For transient transfection, polyethylenimine was used, and binding to the Siglec-9-Fc chimera was analyzed by flow cytometry.

Immunohistochemistry—Tumor samples from patients with prostate carcinoma and colorectal cancer were received from the NCI National Institutes of Health Cooperative Human Tissue Network. Tumor samples were frozen in O.C.T., and frozen sections were blocked for endogenous biotin and used in immunostain assays with washes between each step. Sections were fixed in 10% neutral buffered formalin and overlaid with the anti-SP2 antibody for detection of LGALS3BP or a polyclonal anti-Siglec-9 antibody (R&D Systems, catalog no. BAF1139), and the binding was detected using alkaline phosphatase-labeled or fluorescein-labeled secondary/tertiary reagents. Photomicrographs and analysis used the Keyence BZ-9000 system.

Binding Analysis of LGALS3BP to CD33rSiglecs—96-well ELISA plates were coated with protein A in bicarbonate buffer at 4 °C overnight. Plates were washed with PBS and blocked in PBS containing 1% BSA for 1 h at room temperature. Subsequently, increasing concentrations of His-tagged, recombinant human or mouse LGALS3BP were added in PBS (both from R&D Systems). LGALS3BP was further labeled with a biotinylated anti-polyHis antibody (Qiagen) and subsequent application of HRP-streptavidin (Jackson ImmunoResearch Laboratory, Inc.). Finally, the quantification of LGALS3BP bound to Siglecs was determined by reaction of orthophenoldiamine with HRP, and absorption was measured at 492 nm.

Analysis of Tumor Cell Interaction with Human Neutrophils—Neutrophils were isolated from freshly drawn blood of healthy volunteers and subsequent separation from peripheral blood mononuclear cells (PBMC) by Ficoll (GE Healthcare). Sedimentation of RBCs with 3% dextran (Sigma) in PBS was followed by lysis with ACK (ammonium-chloride-potassium) buffer (Invitrogen). For the measurement of activation, 5×10^5

neutrophils were added to a 96-well plate after adding Oxy-Burst reagent (Invitrogen). To analyze the effect of LGALS3BP on neutrophil activation, we added human recombinant LGALS3BP (R&D Systems) and LPS under different conditions. Fluorescence was determined after 60 min of incubation at 37 °C.

For analysis of neutrophil-mediated killing, HT-29 tumor cell lines were labeled with calcein-AM dye (Invitrogen) prior to coincubation with freshly isolated neutrophils for 6 h in phenol red-free DMEM containing FCS. Calcein release was measured in the supernatant as a readout of cytotoxicity. LGALS3BP was down-regulated by transfection of specific siRNA, and neutrophil-induced tumor cells apoptosis was measured in cocultures with HT-29. The percentage of CD11b-negative, cleaved caspase 3-positive cells was determined by flow cytometry.

RESULTS

Identification of LGALS3BP as a Sialic Acid-dependent Ligand for Siglec-9—It has been shown recently that ligands for Siglec-9 are strongly up-regulated in the extracellular matrix and on the surface of tumor cells from different carcinomas, including colorectal, prostate, non-small cell lung, breast, and ovarian cancer (16, 17). The engagement of Siglec-9 also influenced the immune response to tumor cells and cancer progression (17). Therefore, analyses of cancer-associated ligands for Siglecs potentially reveal novel targets for immunomodulatory cancer therapy. To analyze whether *O*-glycosylated mucins are ligands for Siglec-9 on LS180 cells, we used OSGPase, an enzyme originally isolated from *Pasteurella hemolytica* that cleaves *O*-glycosylated proteins, mainly mucins, in this context (32). The removal of mucins from LS180 cells by OSGPase reduced the sialic acid-dependent binding of Siglec-9 to these cell lines, as determined by flow cytometry (Fig. 1*A*). This result is in accordance with other studies that identified mucins as Siglec-9 ligands (33, 34).

To further characterize sialic acid-dependent ligands for Siglec-9 on carcinoma cell lines, we performed affinity chromatography with recombinant soluble Siglec-9-Fc immobilized on protein A beads. Detergent-solubilized cell membrane extracts from two different human carcinoma cell lines were used. First, extracts from LS180 colorectal carcinoma cells were passed over a Siglec-9-Fc-immobilized column, which was then washed and eluted with α2–3-Neu5Ac-lactose (3′-sialyllactose). As controls, we used another inhibitory CD33rSiglec, Siglec-6, which presents a much narrower binding spectrum and clearly shows lower binding to LS180 tumor cells in flow cytometry analyses (Fig. 1*B*). The R120K mutant of Siglec-9 that lacks the essential arginine for sialic acid-dependent binding served as an additional negative control. The fractions that were eluted using 3 mM 3′-sialyllactose from Siglec-9-Fc beads were analyzed on a silver-stained SDS-polyacrylamide gel and showed additional bands compared with the same fractions eluted from Siglec-6-Fc and R210KSiglec-9-Fc beads (Fig. 1*C*). The fractions showing additional bands were submitted to proteomic analysis by mass spectrometry, and peptides from LGALS3BP (16 peptides) were present only in fractions from Siglec-9-Fc beads but not from Siglec-6-Fc or R120KSiglec-9-Fc beads. The elution of LGALS3BP from Siglec-9 immobilized beads was confirmed by Western blot analyses using the monoclonal SP2 anti-LGALS3BP antibody (Fig. 1*D*, *first lane*). In addition, the binding of LGALS3BP to the Siglec-9 immobilized beads was confirmed to be sialic acid-dependent, because no binding was observed using R120KSiglec-Fc (Fig. 1*D*, *second lane*). The size of the band was smaller than expected (approximately 52 kDa), most likely because of proteolysis during the process of affinity chromatography (duration of treatment, approximately 48 h). It was also smaller than most of the additional bands seen by the silver staining in Fig. 1*C*, indicating that we lost some differentially bound, highly glycosylated proteins during proteomic analysis, including mucins, probably because of the process of protein digestion and liquid chromatography conditions used for mass spectrometric analysis. The K131Q polymorphism of Siglec-9 that was previously associated with reduced binding to sialylated ligands (17, 35) was also able to bind to LGALS3BP (Fig. 1*D*, *third lane*). Siglec-6 did not bind to the eluted LGALS3BP (Fig. 1*D*, *fourth lane*). Interestingly, LGALS3BP from cell membranes of A549 non-small cell lung cancer cell lines binds to Siglec-9 in a sialic acid-dependent manner (Fig. 1*E*). These results identify LGALS3BP as novel sialic acid-dependent ligand for Siglec-9.

LGALS3BP Presented on the Cell Surface of Carcinoma Cell Lines Is a Siglec-9 Ligand—Although the glycoprotein LGALS3BP is mostly secreted into the extracellular matrix in carcinomas or into the medium supernatants from tumor cell cultures (Fig. 2*A*) (31, 36), it has also been reported previously to be bound to the cell surfaces of different cell lines (31). We confirmed the presence of LGALS3BP on the surfaces of various carcinoma cell lines by flow cytometry (Fig. 2*B*). Not surprisingly, the *N*-glycosylated protein LGALS3BP on the cell surface was not sensitive to treatment with OSGPase (data not shown). LGALS3BP has been found previously to bind galectin-3 on the cell surface (37). Therefore we tested whether the main mechanism of cell surface binding of LGALS3BP is through galectin-3. However, incubation of tumor cells with increasing concentrations of lactose that competes with natural galectin-3 ligands did not alter the presence of LGALS3BP on the surfaces of tumor cells (Fig. 2*C*), a finding that suggests an alternate mechanism of binding of LGALS3BP to the cell surface.

We further analyzed the role of LGALS3BP as a ligand for Siglec-9 on tumor cell lines by siRNA for LGALS3BP. Despite an efficient knockdown (Fig. 3*A*), only some cell lines showed reduced binding to Siglec-9 (Fig. 3*B*), suggesting the presence of additional Siglec ligands. Transfection of *LGALS3BP* into 293A cells led to binding of LGALS3BP antibody (SP2) to the cell surface (Fig. 3*C*) and also increased the binding of Siglec-9-Fc, as determined by flow cytometry (Fig. 3*D*).

LGALS3BP Forms Multimeric Complexes and Is Up-regulated in the Extracellular Matrix of Prostate and Colorectal Cancer—LGALS3BP is a heavily *N*-glycosylated protein that forms multimeric complexes of 1000–1500 kDa in the extracellular matrix (31, 38). Indeed, analysis of the recombinant protein on a native polyacrylamide gel showed a high molecular weight complex compared with what was observed on a reducing SDS-polyacrylamide gel (Fig. 3*E*). LGALS3BP has been reported previously to be up-regulated in various cancers,

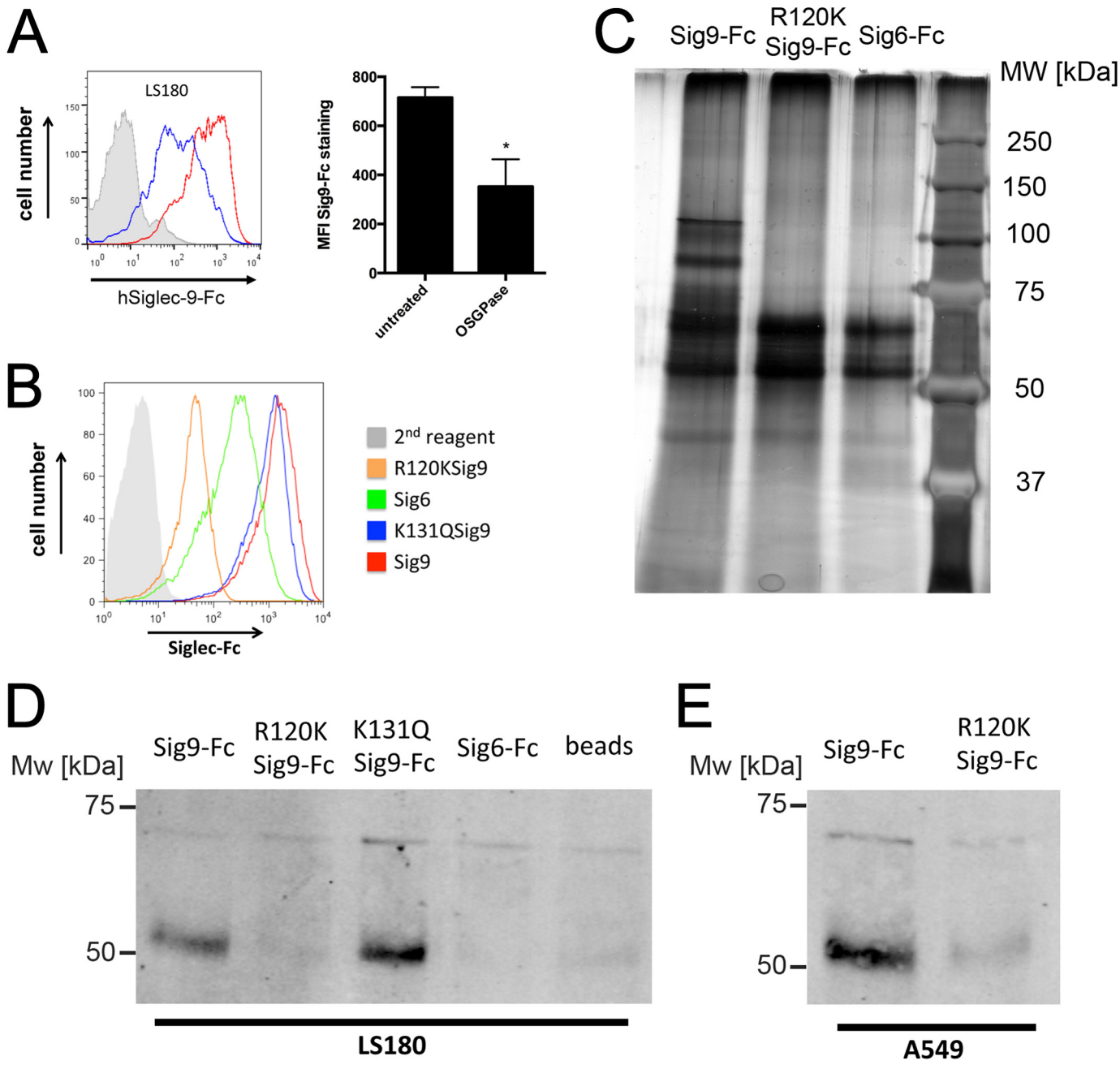

FIGURE 1. **LGALS3BP is a sialic acid-dependent ligand of Siglec-9.** *A*, LS180 cells were assayed for Siglec-9 ligands. *Left panel*, expression before (*red line*) or after treatment with OSGPase (*blue line*) or secondary reagent only (*solid gray area*). *Right panel*, mean fluorescence intensity (*MFI*, $n = 3$) related to Siglec-9-Fc binding to the cell surface of LS180 cells treated with OSGPase or left untreated. *, $p < 0.05$. *B*, representative flow cytometry assessment of binding to LS180 tumor cells by different Siglec-Fc chimeras. *C*, silver-stained polyacrylamide gel of LS180 tumor cell membrane fractions eluted from Siglec-9, R120KSiglec-9, and Siglec-6-Fc's with α2-3-Neu5Ac-lactose (see text for the detailed method). *MW*, molecular weight. *D* and E, Western blot analyses for LGALS3BP of α2-3-Neu5Ac-lactose-eluted fractions from different Siglec-Fcs of membrane preparations of LS180 (*D*) and A549 tumor cells (*E*).

including colorectal cancer (22) and prostate cancer (20). Immunohistochemical analysis of colorectal cancer and prostate cancer specimens showed enhanced expression of LGALS3BP, especially in the extracellular matrix, whereas the adjacent normal colon only showed some expression in goblet cells and in the mucus (Fig. 4*A*). Although some Siglec-9-expressing inflammatory cells were observed to be infiltrating around the LGALS3BP-expressing carcinoma cells (Fig. 4*A*), others were also in areas with less LGALS3BP. Double immunofluorescence staining for LGALS3BP and Siglec-9 of the colorectal cancer samples confirmed the close proximity of the LGALS3BP-positive extracellular matrix and Siglec-9-positive cells (Fig. 4*B*). Similarly, increased levels of LGALS3BP in the extracellular matrix were observed in prostate carcinoma samples (Fig. 4*C*).

LGALS3BP Binds to Siglec-9 and Some Other Inhibitory CD33-related Siglecs—To confirm the binding of Siglec-9 and to test whether other CD33rSiglecs would bind to LGALS3BP, we used recombinant His-tagged LGALS3BP at different concentrations and analyzed its binding to different Siglec-Fcs

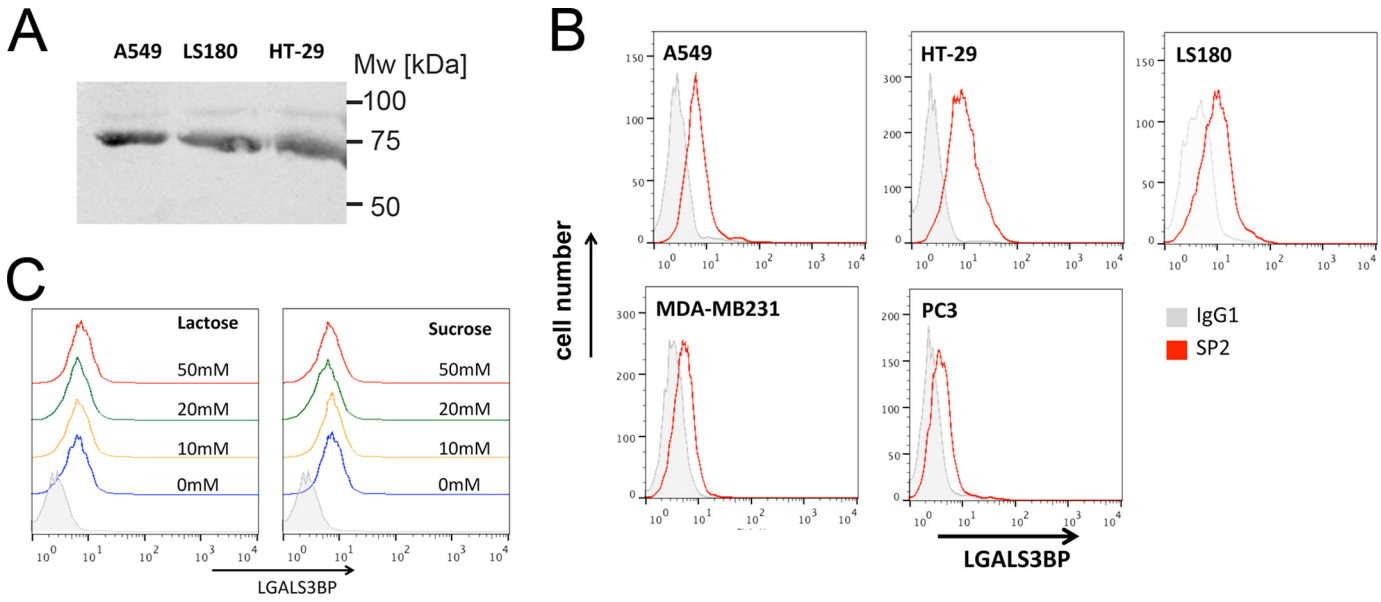

FIGURE 2. **LGALS3BP is a Siglec-9 ligand present on the surfaces of cancer cells.** *A*, immunoblotting for LGALS3BP detection in serum-free supernatant from A549 (non-small cell lung cancer cell line), LS180 and HT-29 (both colorectal cancer cell lines) cultures. *Mw*, molecular weight. *B*, flow cytometry analysis of surface LGALS3BP expression on A549, HT-29, LS180, MDA-MB231 (breast cancer), and PC3 (prostate cancer) cell lines. *C*, treatment of MDA-MB231 cells with ascending concentrations of lactose or sucrose and subsequent analysis of surface LGALS3BP by flow cytometry.

immobilized on ELISA plates. The bound LGALS3BP was detected using an anti-His tag antibody and horseradish peroxidase substrate colorimetric reaction. Although LGALS3BP bound to Siglec-9-Fc at low concentrations, R120KSiglec-9 showed binding at a similar level as control IgG (Fig. 5*A*), indicating a sialic acid-dependent binding as seen before. The previously described K131Q-Siglec-9-Fc that has a reduced binding to sialic acid ligands showed only a slightly diminished binding to human recombinant LGALS3BP (Fig. 5*A*). In addition, we observed high-affinity binding of LGALS3BP to Siglec-5-Fc and to Siglec-10-Fc (Fig. 5, *B–D*), but lower-affinity binding to Siglec-6-Fc, Siglec-7-Fc, and Siglec-11-Fc (Fig. 5, *B* and *D*). We also analyzed the binding of the murine orthologous LGALS3BP (also called Cyp-C associated protein, CyCAP) to the main CD33rSiglec on mouse myeloid cells, Siglec-E. Murine LGALS3BP binds to mouse Siglec-E with a much lower affinity compared with the binding of human LGAS3BP to Siglec-5, Siglec-9, and Siglec-10. A three times higher concentration of the murine LGALS3BP was required to reach a similar absorbance level when comparing the binding affinity of human LGALS3BP to human CD33rSiglecs (Fig. 5*E*).

LGALS3BP Inhibits Neutrophil-mediated Tumor Cell Killing—Recent experiments showed that sialylated tumor cells could inhibit innate immune cell activation and tumor cell killing *in vitro* and *in vivo*, in particular by neutrophils and NK cells (16, 17, 39). Therefore, we wanted to investigate the capability of LGALS3BP to inhibit immune cell activation, in particular neutrophil activation. First, we analyzed the effect of the recombinant LGALS3BP in inhibiting LPS-mediated activation of neutrophils. The addition of LGALS3BP alone was able to inhibit spontaneous production of extracellular reactive oxygen species formation by neutrophils as well as LPS-induced reactive oxygen species production in a Siglec-9-dependent manner because treatment with a Siglec-9 blocking antibody showed a reversal of the effect (Fig. 6, *A* and *B*). This finding indicates that LGALS3BP can inhibit neutrophil activation through interactions with the major inhibitory CD33rSiglec, Siglec-9. To confirm our previous finding that Siglec-9 mediated interactions can inhibit tumor cell killing, two different approaches to test anti-tumor neutrophil activity were performed. First, the cytotoxic effect of neutrophils on tumor cells was measured by the release of calcein from HT-29 cells after 6 h in coculture. In fact, the addition of Siglec-9-blocking antibody induced a higher cytotoxicity of HT-29 cells compared with the isotype control (Fig. 6*C*), in accordance with our previous findings (17). Next, HT-29 cells were transfected with siRNA to reduce LGALS3BP expression (which was shown to decrease binding to Siglec-9, Fig. 3*B*) and incubated with increasing ratios of effector:target cells for 6 h. The reduction of cell surface LGALS3BP significantly increased the percentage of cleaved caspase 3-positive HT-29 cells as a readout of neutrophil induced apoptosis (Fig. 6*D*). These findings indicate that LGALS3BP can have immunomodulatory effects on neutrophils through engagement of the major inhibitory CD33rSiglec, Siglec-9.

DISCUSSION

In this study, we identified LGALS3BP as a cancer-associated ligand that interacts with the inhibitory CD33-related myelomonocytic Siglec-9. Siglec-9 mediated interactions have been described to be immunomodulatory during cancer progression (16, 17). Therefore, Siglec-9 interactions with tumor-associated ligands could become potential targets in therapies to inhibit cancer progression. We identified the heavily *N*-glycosylated protein LGALS3BP as a high-affinity, sialic acid-dependent ligand for Siglec-9. However, sialglyco-microarray analysis shows a broad-spectrum binding pattern for Siglec-9 (12, 40), and it is likely that there is a redundancy of Siglec-9 ligands within cancer tissue. To support this likelihood, we found that efficient knockdown of LGALS3BP reduced binding to some

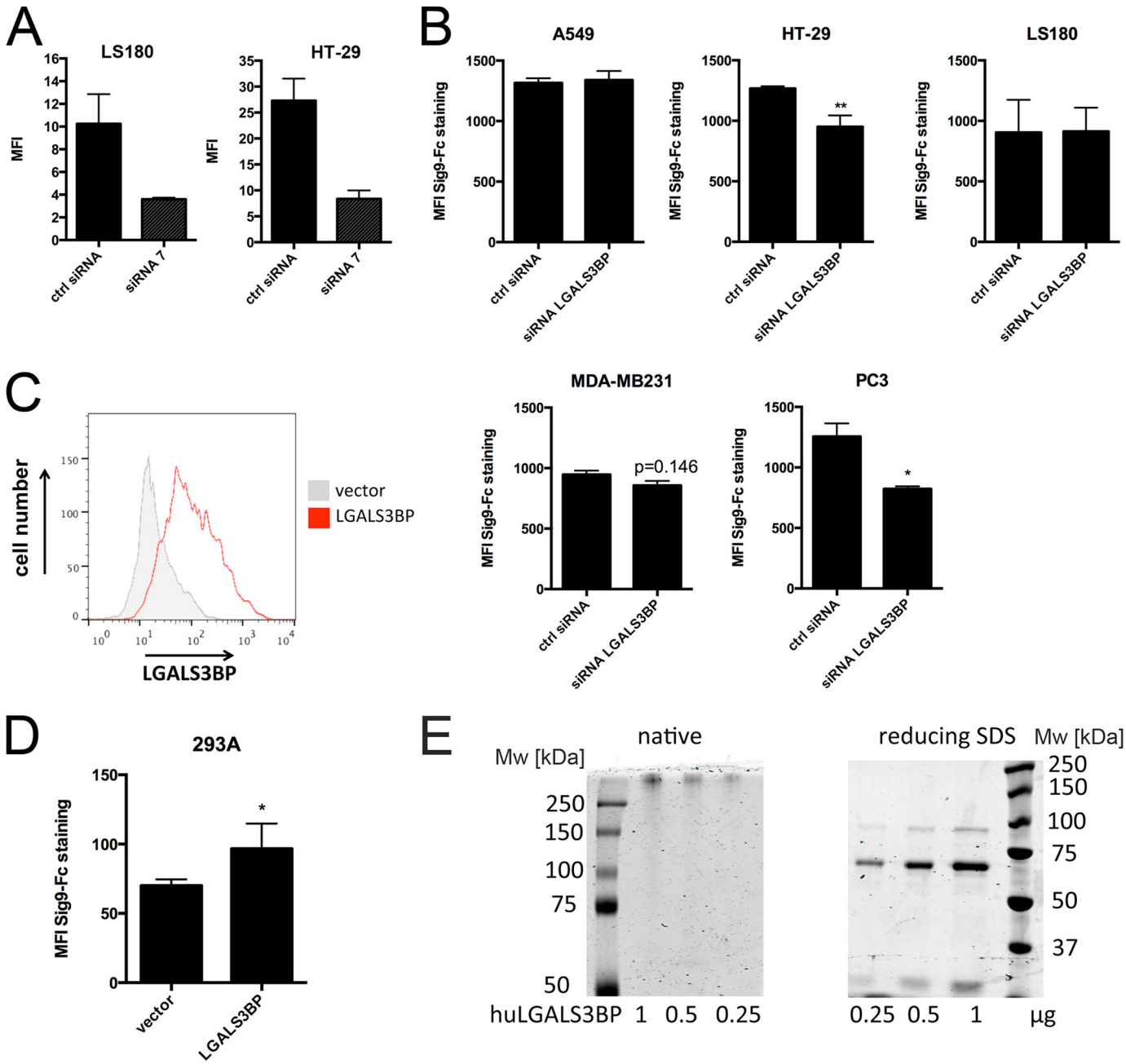

FIGURE 3. **Redundancy of Siglec-9 ligands on carcinoma cell lines.** *A*, inhibition of LGALS3BP expression by siRNA on the cell surface as assessed by flow cytometry on LS180 and HT-29 cells as examples. The reduction of LGALS3BP by siRNA was similar for all cell lines tested. *MFI*, mean fluorescence intensity; *ctrl*, control. *B*, assessment of Siglec-9-Fc binding to carcinoma cell lines after treatment for 24 h with control siRNA or siRNA targeting LGALS3BP by flow cytometry ($n = 3$; mean ± S.D.; *, $p < 0.05$; **, $p < 0.01$ by Student's *t* test). *C*, flow cytometric analysis for LGALS3BP expression of 293A cells transfected with a control vector or with an LGALS3BP-expressing vector. *D*, mean fluorescence intensity analysis of Siglec-9-Fc binding to 293A cells transfected with the LGALS3BP expression vector ($n = 3$; mean ± S.D.; *, $p < 0.05$ by Student's *t* test). *E*, analysis of human recombinant LGALS3BP on a native and reducing polyacrylamide gel. *Mw*, molecular weight.

tumor cell lines. However, other tumor cell lines showed a variable reduction of Siglec-9-Fc chimera binding, pointing to a multiplicity of Siglec-9 ligands on the surfaces of these cell lines.

Several groups have identified other cancer cell-associated ligands for CD33rSiglecs, including mucins such as MUC-1 and MUC-16 (33, 34, 34, 41–44). We also confirmed that tumor cell-associated mucins bind to Siglec-9 by using *O*-sialoglycoprotein-endopeptidase (Fig. 1*A*). Some groups have also found non-sialic acid-dependent, protein-mediated interactions with Siglec-9. Vascular adhesion protein 1 (VAP-1) has been found to bind to Siglec-9 by phage display (45). Prohibitin 1 and 2 have been identified to bind to Siglec-9 in a sialic acid-independent manner, although the essential arginine residue within the carbohydrate recognition domain was needed for the interaction (46). Similar to what is seen with the binding of E-selectin to a broad spectrum of sialyl-Lewis^X ligands (47), several CD33rSiglecs, such as CD33, Siglec-5, Siglec-9 or Siglec-10, also recognize a rather broad spectrum of glycans with different protein backbones (12, 48–51). In contrast to E-selectin, P-selectin is known to have a much narrower spectrum of ligands

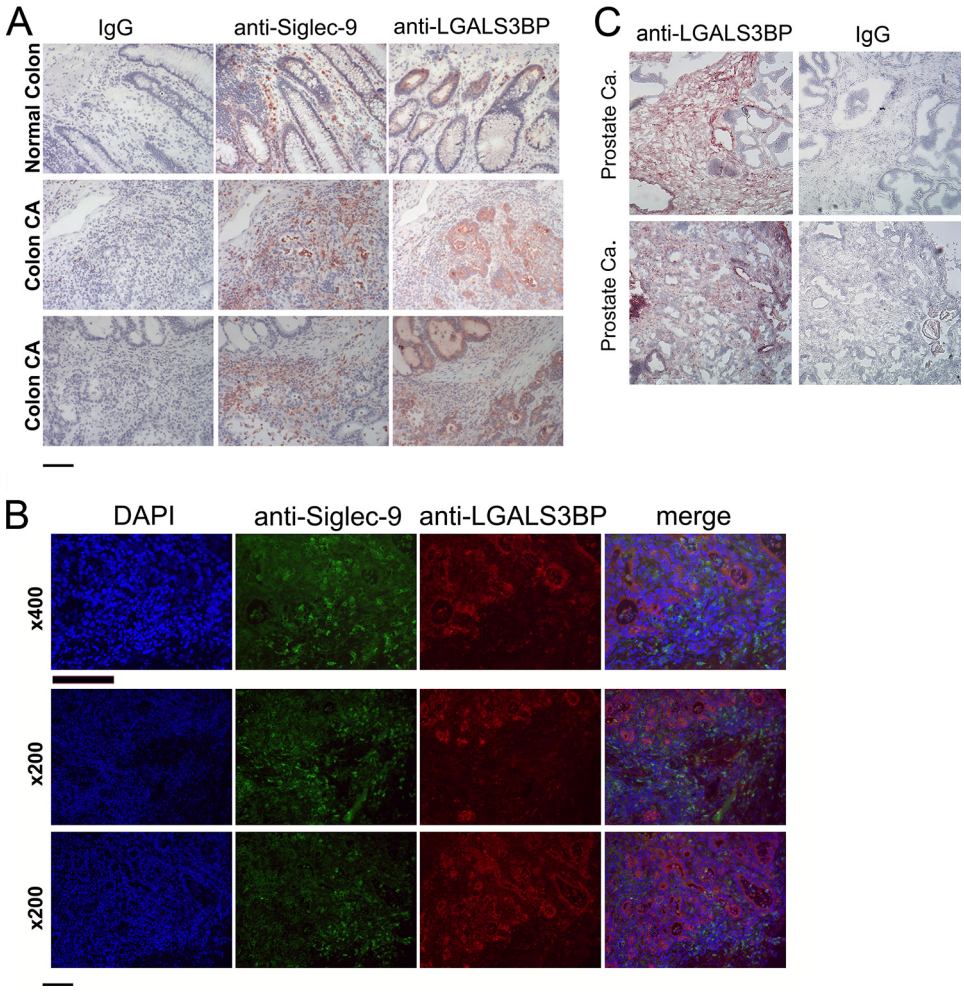

FIGURE 4. **LGALS3BP and Siglec-9 are present in human carcinomas.** *A*, representative immunohistochemical analysis of frozen sections from normal human colon or from colon carcinoma (*CA*) samples for the presence of Siglec-9-positive cells and LGALS3BP (three samples of each were tested). *B*, double immunofluorescence assays for the presence of LGALS3BP and Siglec-9 in colon carcinoma samples. *C*, expression of LGALS3BP in prostate cancer samples. *Scale bars* = 100 μm.

and binds prominently to on ligand, *i.e.* properly posttranslationally modified PSGL-1 (52). Similarly, there are also CD33rSiglecs that recognize a narrow spectrum of ligands, such as Siglec-11 (53).

Indeed, LGALS3BP was also recognized by other inhibitory Siglecs that bind a broad spectrum of ligands, including Siglec-5 and Siglec-10. Therefore, it is possible that LGALS3BP could orchestrate the inhibition of different immune cells. Although Siglec-5 is expressed predominantly on myeloid cells such as neutrophils and monocytes/macrophages, Siglec-10 is also expressed on B cells (6, 10), and recent evidence suggests that Siglec-10 acts with properly glycosylated CD52 to allow binding and inhibition of T cell activation (51). Moreover, Siglec-9 is expressed on a subpopulation of CD8 T cells (54). Therefore, up-regulation of LGALS3BP may directly influence T cell activation against tumors and hamper the generation of an antitumoral Th1 immune response. LGALS3BP bound with a much lower affinity to Siglec-7, which is the main inhibitory CD33rSiglec on NK cells. However, recent experiments described a Siglec-9^{high}, CD56^{dim} population of NK cells that were higher in frequency in cancer patients (16). LGALS3BP could, therefore, also modulate the antitumor NK cell response, as shown for artificial ligands *in vitro* (39). Because the activating Siglec-14 is a paired receptor with Siglec-5 that emerged because of pathogen-host interactions (13), the first two domains used in Siglec-Fc chimeras for our binding assays differ in only one amino acid and bind to the same set of sialic acid-dependent ligands (13). Therefore, LGALS3BP can be considered to bind activating Siglec-14 in a similar manner as it binds to Siglec-5. The LGALS3BP interaction with paired Siglec-5 and Siglec-14 receptors requires further investigation.

LGALS3BP was initially identified as a heavily glycosylated protein in tumor tissue and serum from breast cancer patients (55) and lung cancer cell lines (56). It was later isolated and characterized from the supernatant of tumor cell lines (36), and the same protein was cloned independently as Mac-2 (or Galectin-3) binding protein (38). Although initial studies rapidly associated LGALS3BP with immunomodulatory effects (57, 58), the exact mechanism remained unclear. Recent studies focused more on tumor cells and transmission of signals into the tumor cell (59, 60). For example, binding of LGALS3BP to integrins on tumor cells activate the Akt and Raf-Erk pathways, which was associated with increased survival, proliferation, motility, and migration of cancer cell lines (59). LGALS3BP has

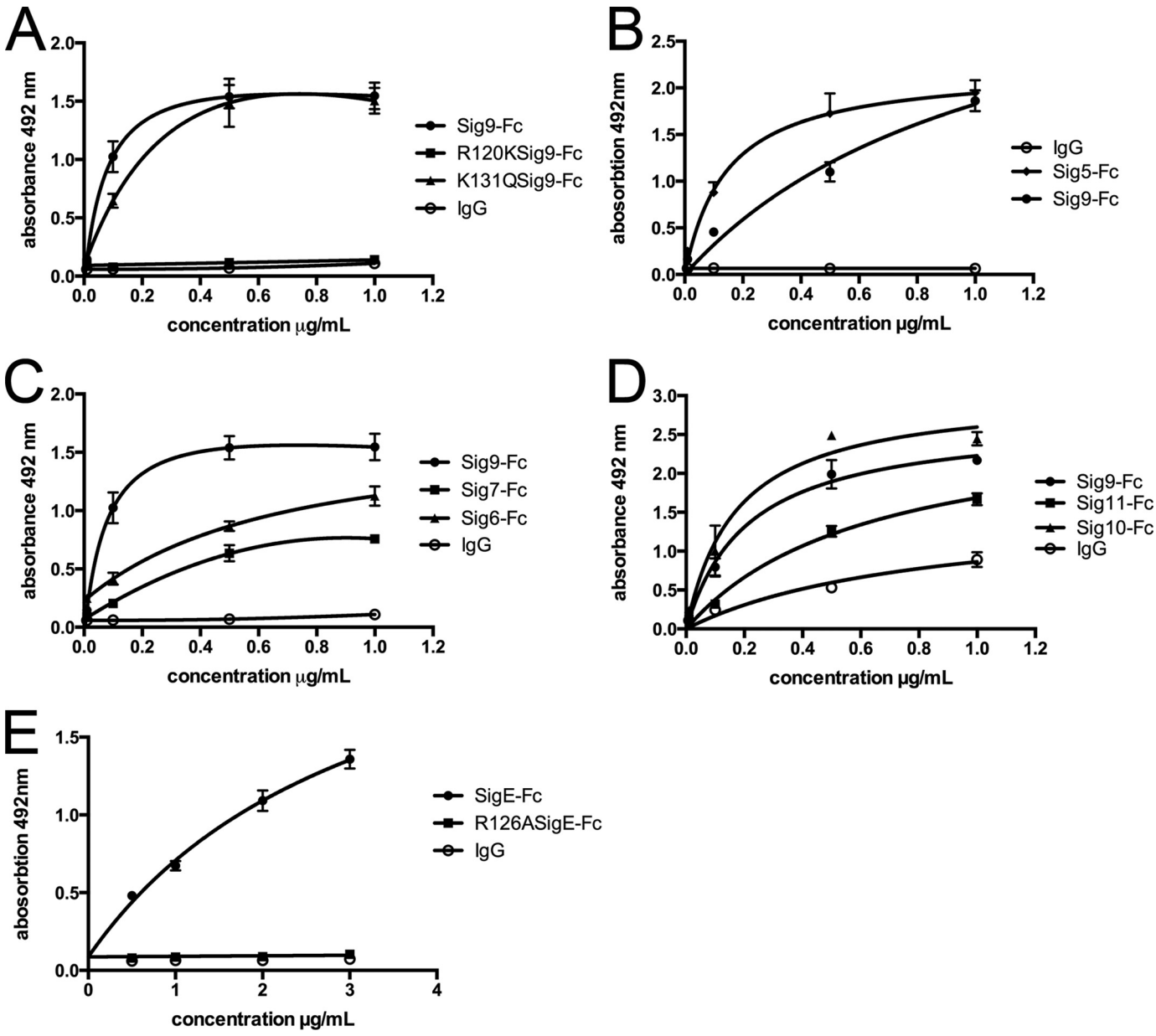

FIGURE 5. **LGALS3BP is a ligands for other inhibitory CD33-related Siglecs.** *A*, binding of recombinant human LGALS3BP to different Siglec-9-Fcs over a dose range. *B–D*, binding of human LGALS3BP to Siglec-5-Fc (*B*), Siglec-6-Fc and Siglec-7-Fc (*C*), and Siglec-10-Fc and Siglec-11-Fc (*D*). *E*, binding of recombinant murine LGALS3BP to Siglec-E-Fc or the arginine mutant chimera R126A-Siglec-E-Fc.

also been shown to induce VEGF expression in cancer cells and is associated with increased angiogenesis (60, 61). Moreover, LGALS3BP has been described to increase IL-6 expression in stromal cells in neuroblastoma models by binding to galectin-3 (62).

Siglecs have one carbohydrate recognition domain, and it is believed that Siglecs transmit intracellular signals when they are clustered by multivalent "*trans*"-ligands (6). Multimeric LGALS3BP complexes would therefore be well suited to engage multiple inhibitory CD33rSiglecs and transmit intracellular signals. Indeed, LGALS3BP inhibits the activation of neutrophils via Siglec-9 engagement (Fig. 6). This indicates that immune cell activation could be modulated via an LGALS3BP-CD33rSiglec pathway during cancer progression and that tumor cells could evade immunosurveillance by up-regulating LGALS3BP. Here we provide preliminary evidence that LGALS3BP can suppress tumor cell killing by neutrophils in part by inhibiting CD33rSiglecs (Fig. 6). However, further investigations are needed to analyze whether other immune cells, such as NK cells, can be inhibited by LGALS3BP.

The murine orthologue of LGALS3BP was initially cloned by a screen for natural ligands of cyclophilin C and termed cyclophilin C-associated protein (63). The LGALS3BP-deficient mice showed an increased reactivity to LPS, which suggests a hyperactive myeloid system (64). In this regard, Siglec-E is expressed on myelomonocytic cells and has also been shown previously to dampen the response to LPS (65). Although murine LGALS3BP binds to Siglec-E (Fig. 5), it happens with a lower affinity than the human LGALS3BP to human inhibitory CD33rSiglecs (Fig. 5). It is therefore unlikely that murine

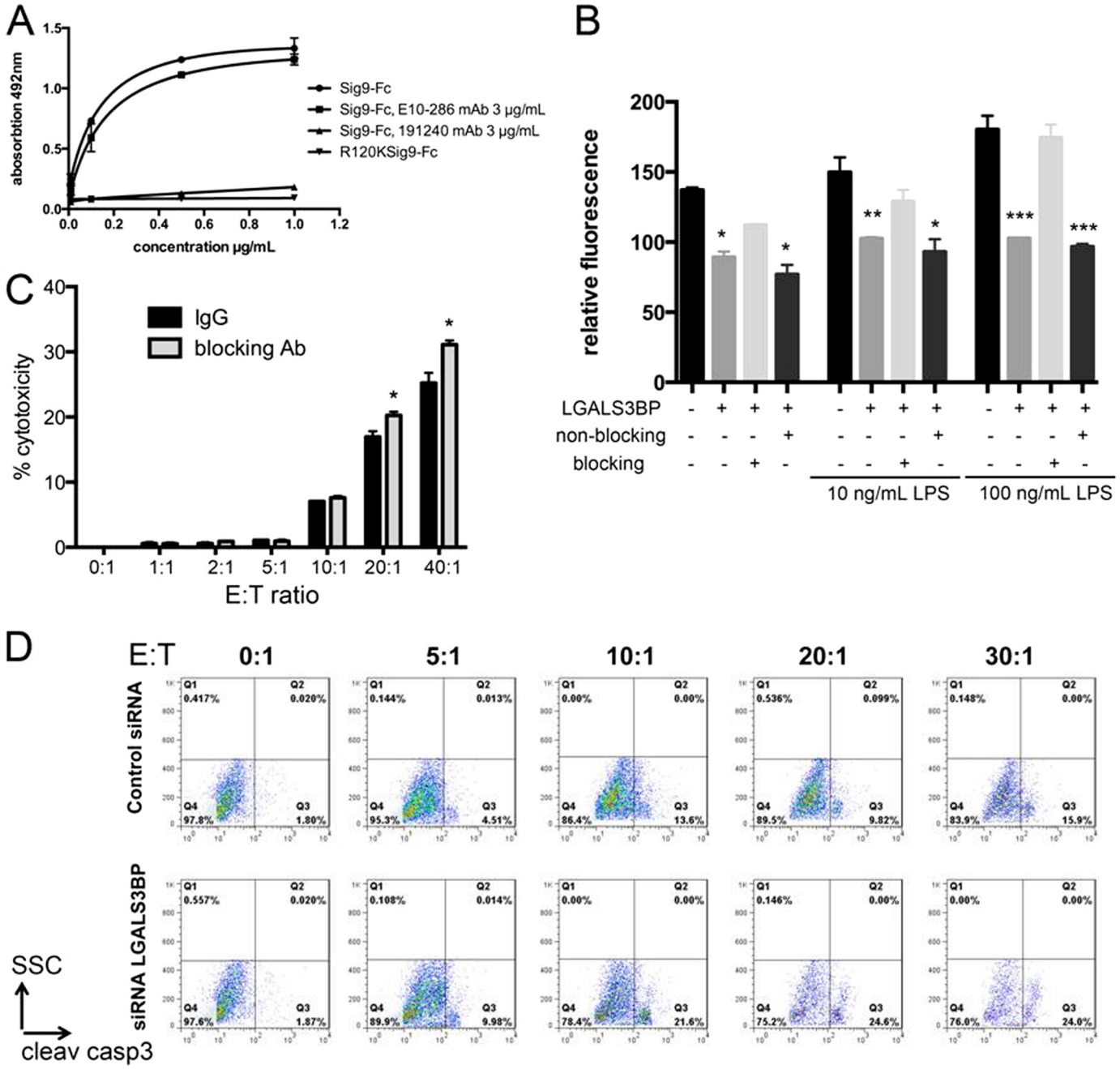

FIGURE 6. **LGALS3BP modulates neutrophil activation via Siglec-9 engagement.** *A*, binding of human recombinant LGALS3BP to Siglec-9-Fc in the presence of blocking monoclonal antibody (*clone 191240*) or non-blocking monoclonal antibody (*clone E10-286*) against human Siglec-9. *B*, analysis of human neutrophil activation by determination of extracellular reactive oxygen production after LPS stimulation and in the absence or presence of multimeric, recombinant LGALS3BP and blocking and non-blocking anti-Siglec-9 antibody ($n = 3$; mean ± S.D.; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by 1-way analysis of variance). *C*, analysis of neutrophil-mediated cytotoxicity against HT-29 tumor cells after 6 h of coculture at various effector to target cell ratios (*E:T*; $n = 3$; mean ± S.D.; *, $p < 0.05$). *Ab*, antibody. *D*, representative flow cytometric analysis of cleaved caspase 3 expression in HT-29 cells treated with control siRNA or siRNA targeting LGALS3BP. The number of apoptotic cells was evaluated by staining for cleaved caspase 3 (*cleav casp3*) in HT-29 cells by gating on CD11b negative cells (tumor cells) 6 h after coculture with freshly isolated human neutrophils at different effector:target ratios. *SSC*, side scatter.

LGALS3BP is a major ligand for Siglec-E. However, further studies are required to investigate other murine Siglecs such as Siglec-F and Siglec-G and their potential to bind LGALS3BP.

Taken together, here we identify a new ligand for CD33rSiglecs that is up-regulated during cancer progression. Our findings address the immunomodulatory effect of LGALS3BP that has been described previously but was poorly understood. Because it has been shown previously that Siglec-9 influences innate immune responses to cancer (16, 17), LGALS3BP binding to Siglec-9 might also modulate tumor immunosurveillance by NK cells and neutrophils. Future studies should investigate the potential role of LGALS3BP or CD33rSiglecs as targets for the treatment of cancer.

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