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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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Running title: *LGALS3BP is a ligand for Siglecs*

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Background: Engagement of inhibitory CD33-related Siglecs on immune cells was 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: Engagement of inhibitory CD33-related Siglecs by LGALS3BP could support immune evasion of tumor cells.

Abstracts

Lectin galactoside-binding soluble 3 binding protein (LGALS3BP, also called Mac-2 binding protein) is a heavily glycosylated secreted molecule that has previously been shown to be upregulated in many cancers and has been implicated in tumor metastatic processes, and also in other cell adhesion and immune functions. The CD33-related subset of Sialic acid binding immunoglobulin-like lectins (Siglecs) are immunomodulatory molecules that were recently associated with the modulation of immune responses to cancer. Since upregulation 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 -10. In contrast, the mouse homolog Siglec-E binds to murine LGALS3BP with lower affinity. LGALS3BP was observed to be upregulated 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' in order to protect from pathogens and maintain homeostasis. Thus, the immune system also eliminates transformed cancer cells that are recognized as 'foreign' by a process called cancer immunosurveillance (1). While various receptors were identified that bind to foreign molecules or patterns (2, 3), other receptors were described that

recognize self associated molecules such as KIR (killer cell Ig-like receptors) (4) or self associated molecular patterns, such as Siglecs (sialic acid binding immunoglobulin-like lectins) (5). Siglecs are a family of immunomodulatory, transmembrane lectins expressed prominently on leukocytes (6-10). Siglecs can be divided into two groups based on their evolutionary conservation (6). While Siglec-1, -2, -4 and -15 have homologs across species, the CD33-related (CD33r) Siglecs (including Siglec-3 (CD33), Siglec-5-14 and 16-17 in primates) underwent rapid evolutionary changes and the CD33rSiglec gene cluster (on chromosome 19q in humans) show high variability between species (6, 10-12). Primate CD33rSiglecs can be further divided into inhibitory Siglecs such as Siglec-5, -7, -9 and -10 that transmit inhibitory signals via intracellular ITIM (immunoreceptor tyrosine-based inhibitory motif) or ITIM-like motifs and activating Siglecs including Siglec-14 and -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 their virtue of coating themselves with sialic acid, or by presenting polypeptide ligands that can bind to Siglecs (14, 15).

It was recently shown that during cancer progression, tumor cells upregulate sialylated Siglec ligands, and similar to bacteria, can engage Siglecs and inhibit immune cell activation (16, 17). While Jandus *et al*. identified a subset of NK cells that express Siglec-9 and showed that engagement of Siglec-9 led to immune-evasion *in vitro* (16), 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, this data points to a potentially important interaction between NK cells and myelomonocytic cells expressing Siglec-9 and sialylated tumor-associated ligands in human cancer progression.

In order 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 galectoside-binding soluble 3 binding protein (LGALS3BP) as the main ligand. While LGALS3BP was previously found to be upregulated 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 293A HEK cells purified with protein A agarose beads (GE) and desialylated with sialidase from *A. ureafaciens* (AUS) prior to use. Tumor cell lines were lysed in 20 mM Tris, pH7.5, 150 mM NaCl, 1:200 proteinase inhibitor mix (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, 500 mM NaCl and membrane associated ligands allowed to engage Siglec-Fc chimeras bound to protein A agarose beads (GE) overnight at 4°C rotating. Beads were subsequently applied on a mini-column and repeatedly washed with column buffer. In order 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 ESI-Tandem MS after UPLC separation by the proteomics core at University of California, San Diego.

Western blots for LGALS3BP. Cells were lysed with RIPA 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 PBST and incubated for 1 hour at room temperature with an anti-mouse-IR800 antibody (Licor). Signals were detected with an Odysey 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-Alexa647 antibody (Life Technologies). Lectin staining was performed with Siglec-Fc chimeras and subsequently with anti-human-IgG-PE (BD Biosciences). O-sialoglycan-endopeptidase (OSGPase, Cedarlane) was added to cells to remove mucins as previously described (66). Briefly, cleavage of mucins was done by adding 50 µl of reconstituted OSGPase to $5x10^6$ cells in 500 µL DMEM and cells were incubated for 1h at 37°C. Then the cells were washed and incubated with Siglec-Fc chimeras or anti-LGALS3BP antibody before analysis by flow cyotmetry.

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 (Life Technologies). For transient transfection, polyethylenimine (PEI) was used and binding to Siglec-9-Fc chimera was analyzed by flow cytometry.

Immunohistochemistry. Tumor samples from patients with prostate carcinoma and colorectal cancer were received from the National Cancer Institute's 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, overlaid with the anti-SP2 antibody for detection of LGALS3BP or a polyclonal, anti-Siglec-9 antibody (R&D Systems, 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 hour 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). Finally, the quantification of LGALS3BP bound to Siglecs was determined by reaction of ortho-phenoldiamine with HRP and absorption 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 PBMC by Ficoll (GE Healthcare). Sedimentation of RBC with 3% of dextran (Sigma) in PBS was followed by lysis with ACK buffer (Gibco). For the measurement of activation, $5x10^5$ neutrophils were added to a 96 well plate after adding OxyBurst reagent (Invitrogen). To analyze the effect of LGALS3BP on neutrophil activation, we added human recombinant LGALS3BP (R&D Systems) and also LPS in different conditions. Fluorescence was determined after 60 minutes 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 co-incubation with freshly isolated neutrophils for 6 hours in phenol red free DMEM containing FCS. Calcein release was measured in the supernatant as a readout of cytotoxicity. LGALS3BP was downregulated by transfection of specific siRNA and neutrophil induced tumor cells apoptosis was measured in co-cultures with HT-29. The percentage of CD11b negative, cleaved caspase 3 positive cells was determined by flow cytometry.

Results

Identification of LGALS3BP as sialic acid dependent ligand for Siglec-9. It was recently shown that ligands for Siglec-9 are strongly upregulated 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). Engagement of Siglec-9 also influenced the immune response to tumor cells and cancer progression (17). Thus, analysis of cancer-associated ligands for Siglecs potentially reveal novel targets for immunomodulatory cancer therapy. In order to analyze if O-glycosylated mucins are ligands for Siglec-9 on LS180 cells, we used O-sialoglyco-endopeptidase (OSGPase), an enzyme originally isolated from *Pasteurella haemolytica* that cleaves O-glycosylated proteins, mainly mucins in this context (32). Removal of mucins from LS180 cells by OSGPase reduced the sialic acid dependent binding of Siglec-9 to these cell lines determined by flow cytometry (Figure 1A). This result is in accordance with other studies that identified mucins as Siglec-9 ligands (33, 34).

In order 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 cells 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 shows clearly lower binding to LS180 tumor cells in flow cytometry analyses (Figure 1B). 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 to the same fractions eluted from Siglec-6-Fc and R210KSiglec-9-Fc beads (Figure 1C). 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 were confirmed by Western blots using the monoclonal SP2 anti-LGALS3BP antibody (Figure 1D, first lane). In addition, the binding of LGALS3BP to the Siglec-9 immobilized beads was confirmed to be sialic acid dependent, since no binding was observed using the R120KSiglec-Fc (Figure 1D, second lane). The size of the band was smaller than expected (ca. 52 kDa) most likely due to proteolysis during the process of affinity chromatography (duration of treatment ca. 48 hours). It was also smaller than most of the additional bands seen by the silver staining in Figure 1C indicating that we lost some differentially bound, highly glycosylated proteins during proteomic analysis including mucins, probably due to 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 (Figure 1D, third lane). Siglec-6 did not bind to the eluted LGALS3BP (Figure 1E, 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 (Figure 1E). 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. While the glycoprotein LGALS3BP is mostly secreted into the extracellular matrix (ECM) in carcinomas or into the media supernatants from tumor cell cultures (Figure 2A) (31, 36), it was also previously reported to be bound to the cell surface of different cell lines (31). We confirmed the presence of LGALS3BP on the surface of various carcinoma cell lines by flow cytometry (Figure 2B). Not surprisingly, the *N*-glycosylated protein LGALS3BP on the cell surface was not sensitive to treatment with OSGPase (data not shown). LGALS3BP was previously found to bind galectin-3 on the cell surface (37), thus we tested if 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 surface of tumor cells (Figure 2C), 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 (Figure 3A), only some cell lines showed a reduced binding to Siglec-9 (Figure 3B), suggesting the presence of additional Siglec ligands. Transfection of *LGALS3BP* into 293A cells lead to binding of LGALS3BP antibody (SP2) to the cell surface (Figure 3C) and also increased the binding of Siglec-9-Fc, as determined by flow cytometry (Figure 3D).

LGALS3BP forms multimeric complexes and is upregulated 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 to what was observed on a reducing SDS polyacrylamide gel (Figure 3E). LGALS3BP was previously reported to be upregulated in various cancers 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 ECM, while the adjacent normal colon only showed some expression in goblet cells and in the mucus (Figure 4A). While some Siglec-9 expressing inflammatory cells were observed to be infiltrating surrounding the LGALS3BP expressing carcinoma cells (Figure 4A), others also were in areas with less LGALS3BP. Double immunofluorescence staining for LGALS3BP and Siglec-9 of the colorectal cancer samples confirmed the close proximity of LGALS3BP positive ECM and Siglec-9 positive cells (Figure 4B). Similarly, increased levels of LGALS3BP in the ECM were observed in prostate carcinoma samples (Figure 4C).

LGALS3BP binds to Siglec-9 and some other inhibitory CD33-related Siglecs. In order 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-Fc's 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 to a similar level as control IgG (Figure 5A), 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 (Figure 5A). In addition, we observed a high affinity binding of LGALS3BP to Siglec-5-Fc and to Siglec-10-Fc (Figure 5B, C and D), but lower affinity binding to Siglec-6-Fc, Siglec-7-Fc and Siglec-11-Fc (Figure 5B 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 the mouse Siglec-E with much lower affinity when compared to the binding of human LGAS3BP to Siglec-5, -9 and -10. A three times higher concentration of the murine LGALS3BP was required to reach a similar absorbance length when comparing to the binding affinity of human LGALS3BP to the human CD33rSiglecs (Figure 4E). Murine LGALS3BP binds to the mouse Siglec-E with much lower affinity when compared to the binding of human LGAS3BP to Siglec-5, -9 and -10. A three times higher concentration of the murine LGALS3BP was required to reach a similar absorbance length when comparing to the binding affinity of human LGALS3BP to the human CD33rSiglecs.

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). Thus, 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 (ROS) formation by neutrophils as well as LPS induced ROS production in a Siglec-9 dependent manner, as treatment with a Siglec-9 blocking antibody showed a reversal of the effect (Figure 6A and B). This finding indicates that LGALS3BP can inhibit neutrophil activation through interactions with the

major inhibitory CD33rSiglec, Siglec-9. To confirm our previous findings 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 hours in co-culture. In fact, the addition of Siglec-9 blocking antibody induced higher cytotoxicity of HT-29 cells as compared to isotype control (Figure 6C), 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, Figure 3B) and incubated with increasing ratios of effector:target cells for 6 hours. The reduction of cell surface LGALS3BP significantly increased the percentage of cleaved caspase 3 positive cells as a readout of neutrophil induced apoptosis of HT-29 cells (Figure 6E). 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). Thus, 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 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 surface 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-sialoglycoprtein-endopeptidase (Figure 1A). Some groups have also found non-sialic acid dependent, protein-mediated interactions with Siglec-9. While vascular adhesion protein-1 (VAP-1) was found to bind to Siglec-9 by phage display (45), Prohibitin-1 and -2 were 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 or Siglec-10 also recognize a rather broad spectrum of glycans with different protein backbones (12, 48-51). In contrast, P-selectin is known to have a much narrower spectrum of ligands and binds prominently to on ligand, i.e. properly post-translationally 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 inhibition of different immune cells. Whereas Siglec-5 is expressed predominantly on myeloid cells such as neutrophils and monocytes/macrophages, Siglec-10 is expressed also 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). Thus, upregulation of LGALS3BP may directly influence T cell activation against tumors and hamper the generation of a strong anti-tumoral Th1 immune response. LGALS3BP bound with 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 lower in frequency in cancer patients (16). LGALS3BP could therefore also modulate the antitumor NK cell response as shown for artificial ligands *in vitro* (39). Since the activating Siglec-14 is a paired receptor with Siglec-5 that emerged due to pathogen-host interactions (13), the first two domains used in Siglec-Fc chimeras for our binding assays differ only in 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. LGALS3BP interaction with paired Siglec-5 and -14 receptors require further investigation.

LGALS3BP was initially identified as 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). While 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 was also shown to induce vascular growth factor (VEGF) expression in cancer cells and is associated with increased angiogenesis (60, 61). Moreover, LGALS3BP was 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 be therefore well suited to engage multiple, inhibitory CD33rSiglecs and transmit intracellular signals. Indeed, LGALS3BP inhibits the activation of neutrophils via Siglec-9 engagement (Figure 6). This indicates that immune cell activation could be modulated via an LGALS3BP-CD33rSiglec pathway during cancer progression and tumor cells could evade immunosurveillance by upregulating LGALS3BP. Here, we provide preliminary evidence that LGALS3BP can suppress tumor cells killing by neutrophils in part by inhibiting CD33rSiglecs (Figure 6). However, further investigations are needed to analyze if 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 was also previously shown to dampen the response to LPS (65). Although murine LGALS3BP binds to Siglec-E (Figure 5), it happens with lower affinity than the human LGALS3BP to human inhibitory CD33rSiglecs (Figure 5). It is therefore unlikely that murine 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, we here identify a new ligand for CD33rSiglecs that is upregulated during cancer progression. Our findings address the immunomodulatory effect of LGALS3BP that was previously described, but poorly understood. As it was previously shown 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 therapeutic target against cancer.

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Figure Legends

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 (grey solid). Right panel- of Mean fluorescence intensity (MFI, n = 3) related to Siglec-9-Fc binding to the cell surface of LS180 cells treated or not with OSGPase. * $P<0.05$. 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 detailed method). D. and E. Western blot analyses for LGALS3BP of α2-3-Neu5Ac-lactose eluted fractions from different Siglec-Fc's of membrane preparations of LS180 (D) and A549 tumor cells (E).

Figure 2 **LGALS3BP is a Siglec-9 ligand present on the surface 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. B. Flow cytometry analysis of surface LGALS3BP expression on A549, HT-29, LS180, MDA-MB231 (breast cancer), 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.

Figure 3 **Redundancy of Siglec-9 ligands on carcinoma cell lines.** A. Inhibition of LGALS3BP expression by siRNA on the cell surface assessed by flow cytometry on LS180 and HT-29 cells as examples. Reduction of LGALS3BP by siRNA was similar for all cell lines tested (MFI, mean fluorescence intensity). B. Assessment of Siglec-9-Fc binding to carcinoma cell lines after treatment for 24 hours with control siRNA or siRNA targeting LGALS3BP by flow cytometry (N=3, mean ± SD, *$P<0.05$, **$P<0.01$ by Student's *t* test). C. Flow cytometric analysis for LGALS3BP expression of 293A cells transfected with control vector or with LGALS3BP expressing vector. D. MFI analysis of Siglec-9-Fc binding to 293A cells transfected with LGALS3BP expression vector (N=3, mean±SD, *$P<0.05$ by Student's *t* test). E. Analysis of human recombinant LGALS3BP on a native and reducing polyacrylamide gel.

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 samples for the presence of Siglec-9 positive cells and LGALS3BP (three samples of each were tested). B. Double immunofluorescence assays for the presence LGALS3BP and Siglec-9 in colon carcinoma samples. C. Expression of LGALS3BP in prostate cancer samples. Scale bars, 100 microns.

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

Figure 6 **LGALS3BP modulates neutrophil activation via Siglec-9 engagement.** A. Binding of human recombinant LGALS3BP (1μg/mL) to Siglec-9-Fc in the presence of blocking monoclonal antibody (clone 191240, 10μg/mL) or non-blocking monoclonal antibody (clone E10-286, 10μg/mL) 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, blocking and non-blocking anti-Siglec-9 antibody (N=3, mean±SD, *$P<0.05$, **$P<0.01$ by 1-way ANOVA). C. Analysis of neutrophil-mediated cytotoxicity against HT-29 tumor cells after 6 hours of co-culture at various effector to target ratios (E:T, N=3, mean±SD, *$P<0.05$). (**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 in HT-29 cells by gating on CD11b negative (tumor cells) 6 hours after co-culture with freshly isolated human neutrophils at different effector to target ratios (E:T ratios).

Figure 1

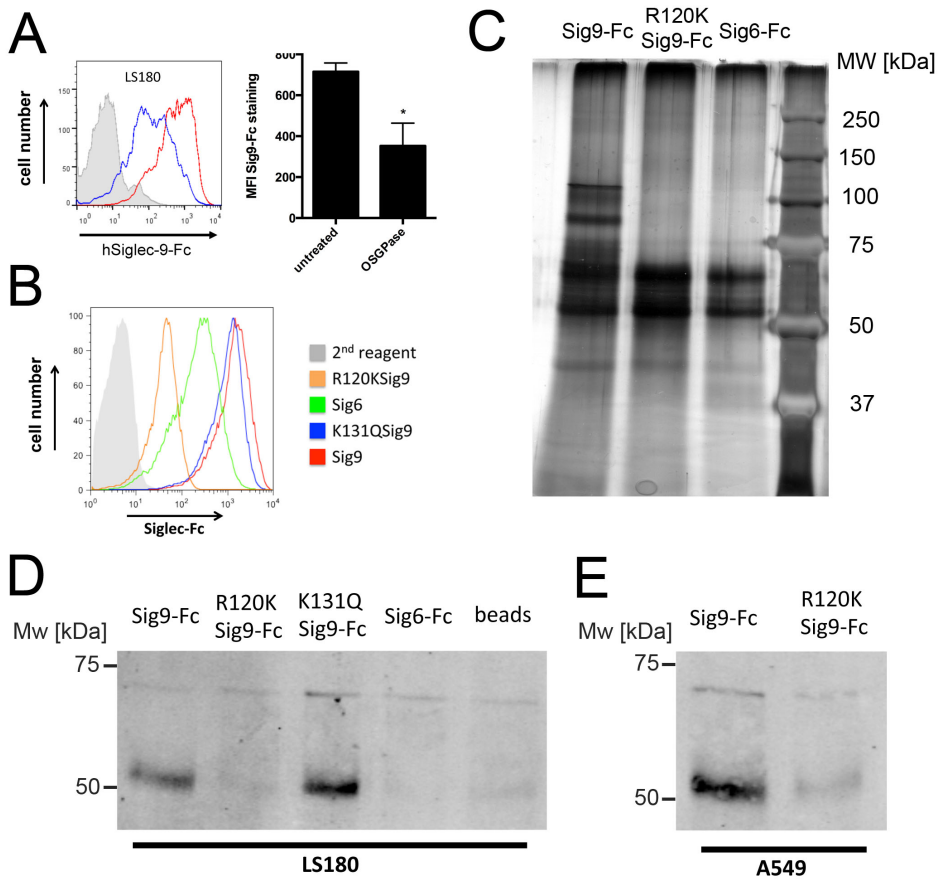

Figure 2

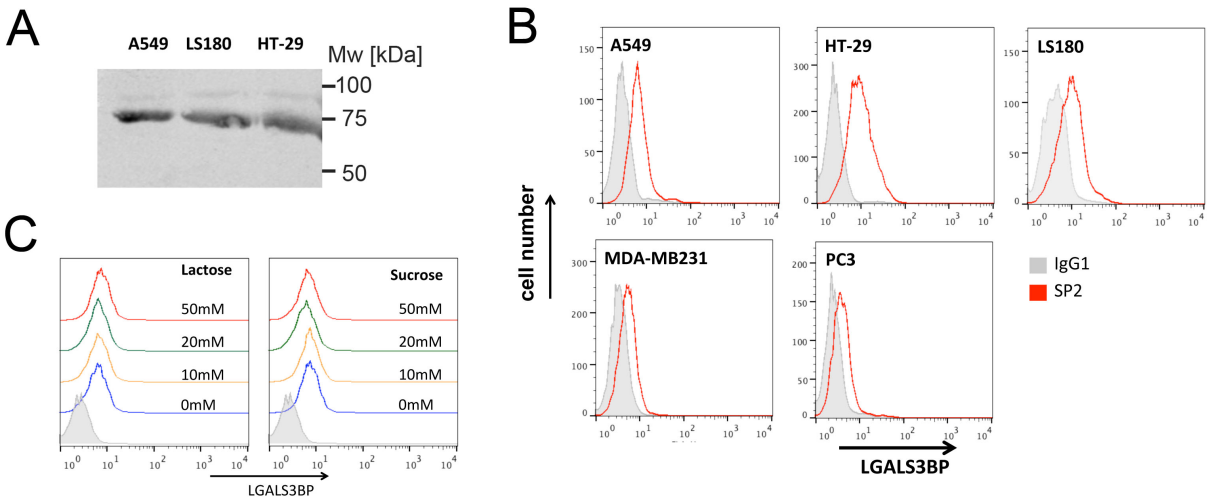

Figure 3

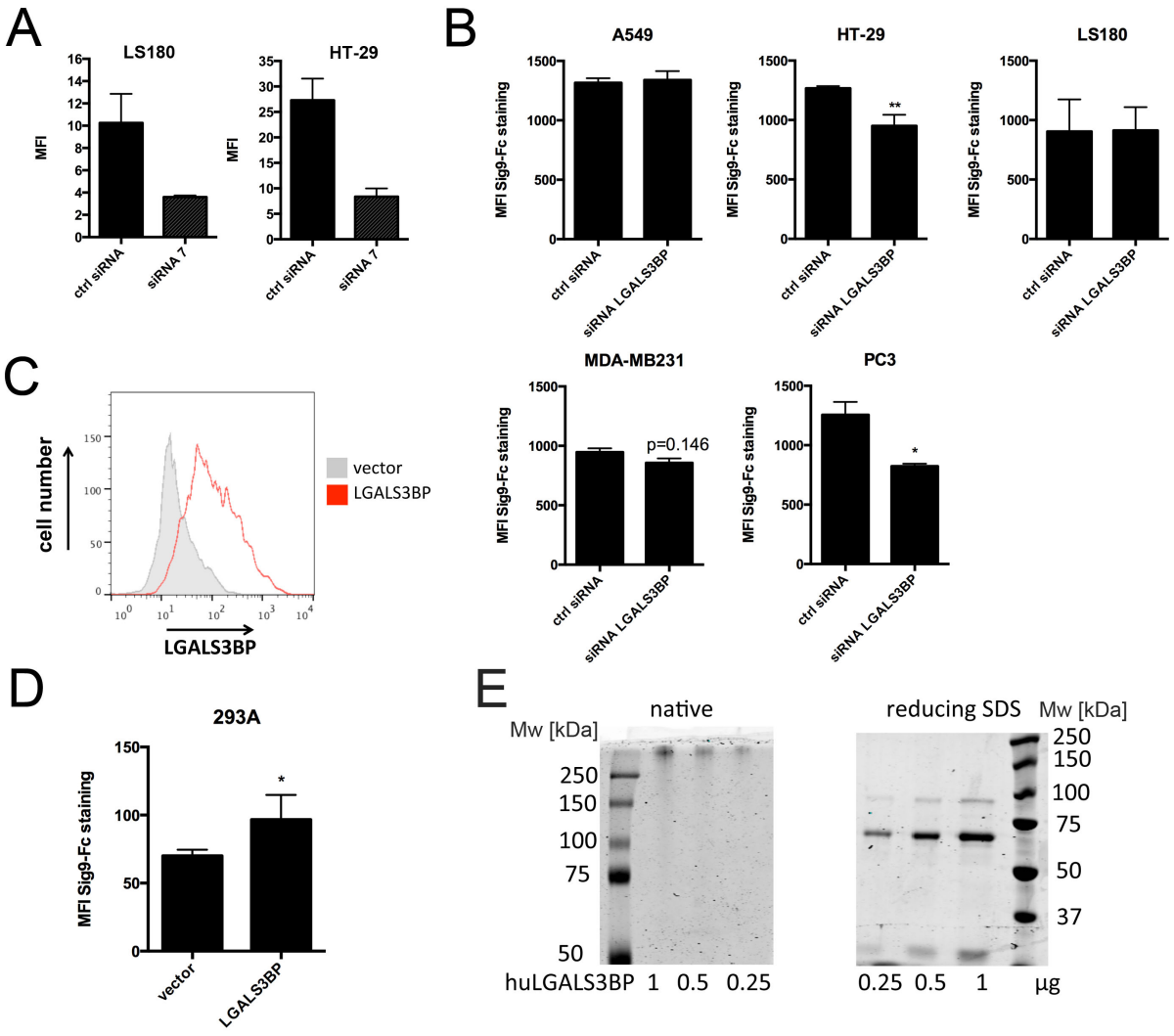

Figure 4

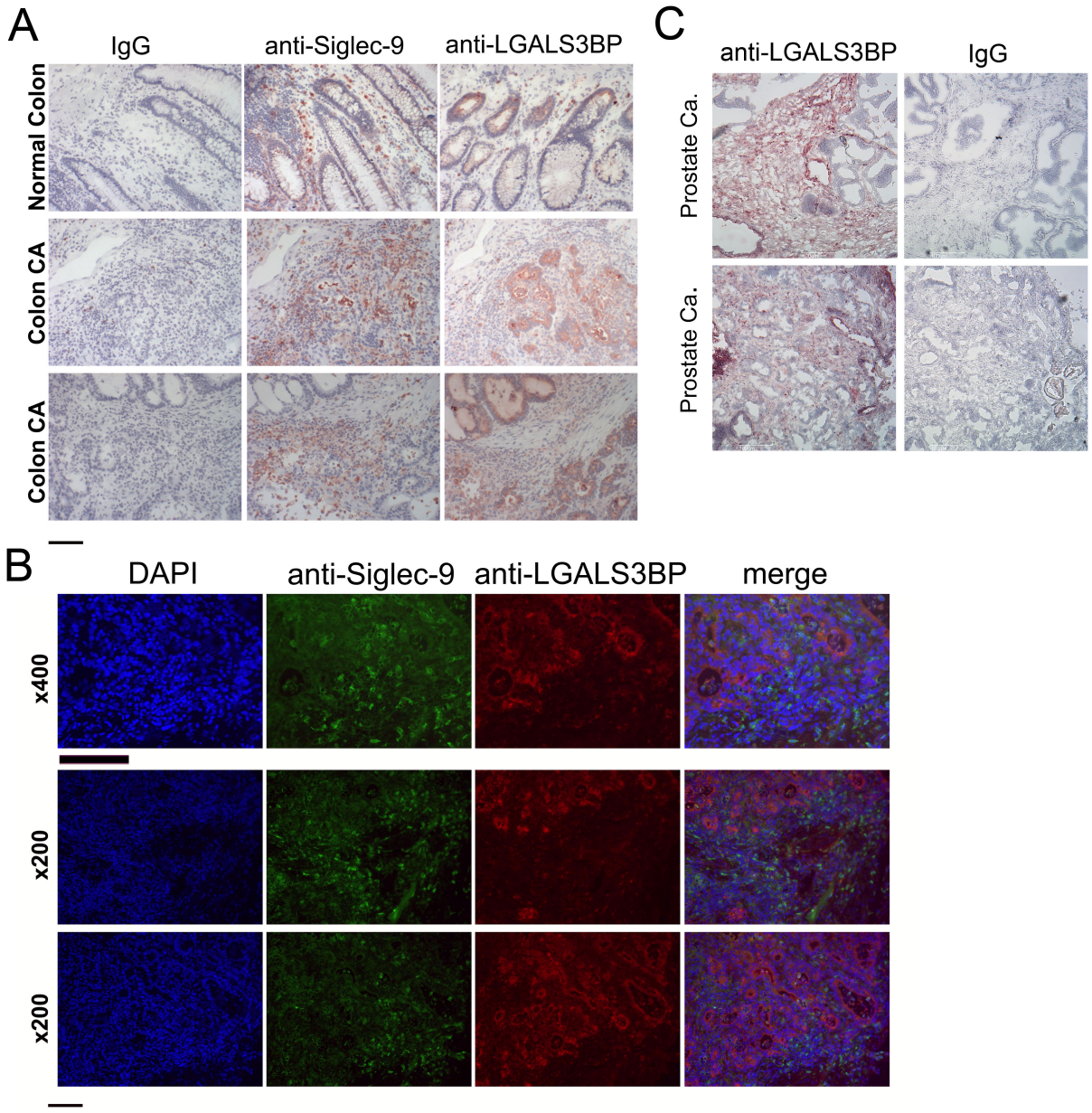

Figure 5

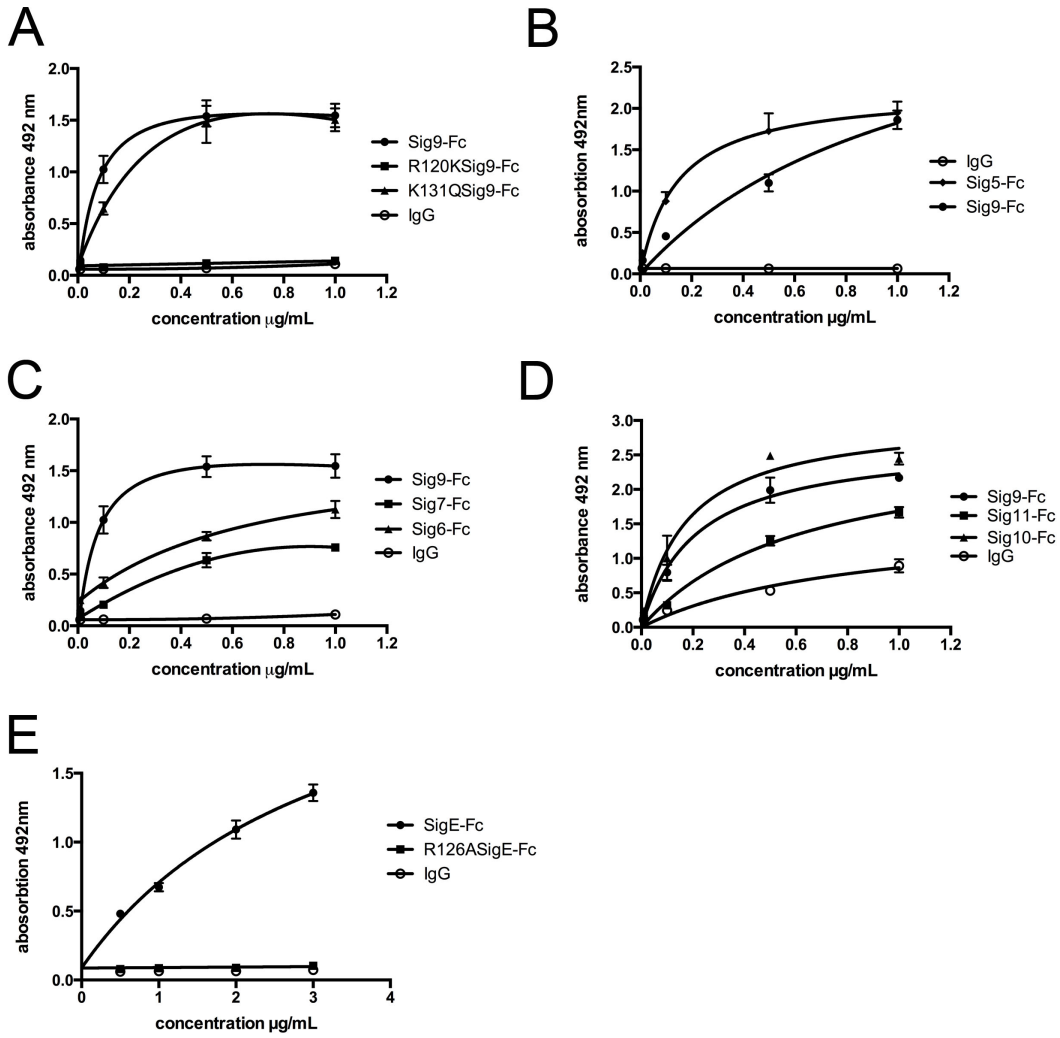

Figure 6