

Sialylated glycoproteins suppress immune cell killing by binding to Siglec-7 and Siglec-9 in prostate cancer

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Abstract

Prostate cancer is the second leading cause of male cancer death in the U.S. Current immune checkpoint inhibitor-based immunotherapies have improved survival for many malignancies; however, they have failed to prolong survival for prostate cancer. Siglecs (sialic acid-binding immunoglobulin-like lectins) are expressed on immune cells and regulate their function. Siglec-7 and Siglec-9 contribute to immune evasion in cancer by interacting with sialic acid-containing glycoprotein ligands on cancer cells. However, the role of Siglec-7/9 receptors and their ligands in prostate cancer remains poorly understood. Here, we find that Siglec-7 and Siglec-9 are associated with poor prognosis in prostate cancer patients, and are highly expressed in myeloid cells, including macrophages, in prostate tumor tissues. Siglecs-7 and -9 ligands were expressed in prostate cancer cells and human prostate tumor tissues. Blocking the interactions between Siglec-7/9 and sialic acids inhibited prostate cancer xenograft growth and increased immune cell infiltration in humanized mice *in vivo*. Using a CRISPRi screen and mass spectrometry, we identified CD59 as a candidate Siglec-9 ligand in prostate cancer. The identification of Siglecs-7 and -9 as potential therapeutic targets, including CD59/Siglec-9 axis, opens up opportunities for immune-based interventions in prostate cancer.

Introduction

Prostate cancer is one of the most prevalent cancers in men worldwide, with a 5-year survival rate of under 30% for men with metastatic disease, despite treatment with androgen deprivation therapies (1-3). While immune checkpoint inhibitor (ICI)-based immunotherapies have quickly become standard of care for several malignancies (4, 5), they have been ineffective in treating prostate cancer, except for a small subset (~1%) with mismatch repair gene mutations (6-9). However, dendritic cell therapies, such as sipuleucel-T, can prolong survival in many advanced prostate cancers and suggest that alternative immune checkpoint pathways could be utilized during disease development and progression (10, 11).

Siglecs, sialic acid-binding immunoglobulin-like lectins, are a family of glycan-binding immunoreceptors that recognize glycans or glycoconjugates bearing the sialic acid monosaccharide(12). The Siglec family is composed of both activating and inhibitory receptors, which each bind discrete sialoglycan ligands. The majority of CD33-related Siglecs function as inhibitory receptors including Siglec-5, Siglec-8, Siglec-7, Siglec-9, and Siglec-10(13-16). Siglec-7 and Siglec-9 are expressed by NK cells, monocytes, dendritic cells (DCs), macrophages, and subsets of T cells in the tumor microenvironment(17-22), and a growing body of evidence implicates these Siglecs as potential immune checkpoints in cancer(23-28).

Aberrant expression of sialic acid-containing glycans (sialoglycans) by cancer cells was initially observed in 1960s (29). More recently, these sialoglycans have been identified as Siglec ligands that can bind directly to Siglec receptors on immune cells in the tumor microenvironment and cause immune suppression (27, 30-32). For example, sialylated glycans on the surface of melanoma, lung and pancreatic cells have been shown to bind to Siglec-7/9 on immune cells to inhibit the immune response (20, 33, 34). Previous work has demonstrated that cancer cell lines

PC3 and DU145 express ligands for Siglecs-7 and -9 (35, 36); however, the role of the Siglecs-7 and -9–sialic acid pathway in prostate cancer remains poorly understood.

To better understand whether Siglecs-7 and 9 could have a role in immune-suppressive pathways in prostate cancer, we analyzed the expression profiles of Siglec isoforms in patient samples and examined their correlation with clinicopathological parameters. We also examined the expression levels of Siglec-7 and Siglec-9 ligands in prostate cancer cells and tumors, and interrogated their functional effects *in vivo*. Finally, we used discovery-based approaches to identify candidate Siglec ligands expressed by prostate cancer cells and evaluated their potential as therapeutic targets.

Results

Elevated Siglec-7/9 expression is correlated with higher recurrence rates in prostate cancer patients.

To determine the association of Siglec-7 and Siglec-9 expression with recurrence free survival (RFS) in PCa, we assessed the association of Siglec expression with clinical outcomes publicly available datasets. In TCGA-PRAD dataset, transcript levels of Siglec-1, -6, -7, -9, -15, and -16 were significantly higher in tumor samples compared to normal tissues (Figure 1A and B and Supplemental Figure S1). Increased expression levels of Siglec-1, Siglec-7, and Siglec-9 were associated with higher cancer grade (Gleason scores) in PCa (Figure 1C, D and Supplemental Figure S2). Moreover, the expression levels of Siglec-7 and Siglec-9 showed a significant association with RFS in 488 PCa patients (Figure 1E). Patients with high expression levels of Siglec-7 and Siglec-9 also exhibited worse RFS outcomes in a cohort of 140 PCa patients in the MSKCC dataset (37) (Figure 1F), suggesting Siglec-7 and Siglec-9 may play a promoting role in PCa progression.

To gain insights into the immune cell populations associated with Siglec expression and clinical outcomes, CIBERSORTx, an analytical tool employing machine learning techniques, was utilized to deconvolute the composition of cell populations based on gene expression data (38). CIBERSORTx analysis of TCGA-PRAD dataset showed that tumor infiltrating immune cells expressed different Siglec profiles in PCa (Supplemental Figure S3). Specifically, dendritic cells (DCs), monocytes, and CD8+ T cells were predominantly associated with the expression of Siglec-7 and Siglec-9, while tumor infiltrating neutrophils expressed high levels of Siglec-9 (Figure 1G and IH). Due to the limitations of immune cell type definitions by the CIBERSORTx algorithm, Siglec-7 and Siglec-9 expression in certain immune cells, including macrophages, could not be

identified. Therefore, we performed a Pearson correlation analysis between Siglec-7 and Siglec-9 expression and a macrophage marker, and found that both are correlated with the macrophage marker CD68 (Figure 1I and 1J). These results suggest that Siglec-7 and Siglec-9 may play an important role in immune cell functions in PCa tumor environment.

High Siglec-7/9 expression is found in myeloid cells in human prostate cancer tumors

To elucidate the Siglec profile of prostate cancers, we analyzed three different stages of PCa, including 5 primary tumors (localized), 14 metastatic hormone sensitive tumors and 6 metastatic castrate resistant tumors by scRNA-seq. The cells were annotated (Figure 2A) using published gene signatures (39, 40). Uniform manifold approximation and projection (UMAP) visualization showed that Siglec-7 and -9 are exclusively expressed on immune cells (Figure 2B and 2C), mainly on myeloid cells including macrophages and myeloid derived suppressor cells (MDSCs) and sparsely expressed on CD4⁺ T and CD8⁺ T cells (Figure 2B-E, and Supplemental Figure S4) in CRPC tumor tissues. Siglec-7 was also highly expressed on DCs and NK cells (Figure 2D). Furthermore, Siglec-7 and Siglec-9 were observed in all stages of prostate cancer (PCa), including localized, hormone-sensitive prostate cancer (HSPC), and castration-resistant prostate cancer (CRPC) (Figures 2F-H, Supplemental Figure S5-6). Additionally, Siglec-10 was highly expressed in macrophages, myeloid-derived suppressor cells (MDSCs), and B cells across all stages of PCa (Figures 2H, supplemental Figure S4-6). To validate the expression of Siglec-7 and Siglec-9 on immune cells in PCa, we performed immunofluorescence and confocal microscopy on PCa metastases in bone tissues and found Siglec-7 and Siglec-9 expression on macrophages, with a colocalization coefficient of approximately 65% with CD68 (Figures 2I and 2J).

Sialylated glycans are detected in prostate cancer cells

To explore the presence of sialic acids on the surface of PCa cells, we conducted a sialic acid fluorometric assay using an enzyme-coupled reaction in which the oxidation of free sialic acid generates an intermediate that reacts with a probe, resulting in the production of a detectable fluorescent product. Cell surface sialic acids were present in all tested PCa cell lines (Figure 3A). Furthermore, treatment with sialidase, an enzyme that cleaves sialic acids, led to a significant reduction in surface sialic acid levels in all PCa cells (Figure 3B-D, Supplemental Figure S7A and B).

To determine whether the sialic acids present on the cell surface of the prostate cancer cells were α 2,3-linked and α 2,6-linked, we utilized SNA (Sambucus nigra agglutinin) to detect α 2,6-linked sialic acids and MALII (Maackia amurensis agglutinin II) to detect α 2,3-linked sialic acids by flow cytometry. All PCa cells stained with both SNA and MALII lectins with higher expression levels of SNA compared to MALII (Figure 3E-F, and Supplemental Figure S7C-F). This suggests that PCa cells are decorated with both α 2,3-linked and α 2,6-linked sialic acids, with the surface sialic acids primarily consisting of α 2,6-linked sialic acids.

We investigated the sialylation status of proteins in prostate cancer tumor tissues and their adjacent normal tissues. Intact glycoproteomics analysis demonstrated that cancer tissues displayed significantly more sialylated spectral counts (~30%) compared to adjacent normal prostatic tissues (~20%) (Figure 3G). Remarkably, more than 85% of the differential glycosylation between PCa and normal prostate were due to increases in sialylation, including complex sialylated, complex fucosylated and sialylated, or both (Figure 3H-I). Gene Ontology (GO) term pathway analysis of these glycosylated proteins including sialylated proteins demonstrated enrichment in pathways associated with immune response/antigen presentation, metabolism, cell adhesion and communication, and others (Figure 3J). Analysis of transcript expression in sialoglycan

biosynthetic genes within tumor tissues from prostate cancer patients reveals upregulation in 7 genes and a corresponding downregulation in 12 genes, as indicated by the tumor/normal ratio (Figure 3K). Across the samples, α -2,3- and α -2,6-sialyltransferases showed substantial modulation across PCa tissues compared to adjacent normal tissues (Figure 3K). K-means clustering based on the correlation between expression of sialoglycan biosynthetic genes and 497 immune genes revealed that the sialoglycan genes could be grouped into three distinct gene sets (Figure S8). Notably, there is a distinct positive correlation with immune-regulated pathways in gene set 1 including ST3GAL1, and ST6GALNAC4, which are positive correlated with immune system processes, specifically highlighting associations with lymphocytes, leukocytes, and T cell activation (Supplemental Figure S8).

Siglecs-7 and -9 bind to ligands on the surface of PCa cells

To determine whether Siglec-7 and Siglec-9 ligands are present on the surface of PCa cells, we employed direct staining using recombinant chimeras of the binding region of Siglec-7 or Siglec-9 molecules fused to human IgG1 Fc domains, referred to as Siglec-7-Fc or Siglec-9-Fc. Flow cytometry analysis revealed high level binding of Siglec-7-Fc to PC3 cells, while 22Rv1 and DU145 cells exhibited moderate Siglec-7-Fc binding levels (Figure 4A and B). Similarly, high level of binding of Siglec-9-Fc was observed in PC3 and DU145 cells, whereas 22Rv1 cells displayed moderate Siglec-9 binding (Figure 4A and C). Conversely, LNCaP and C42B cells showed lower levels of binding of both Siglec-7-Fc and Siglec-9-Fc binding proteins (Figure 4A-B). In general, all PCa cells exhibited higher level binding of Siglec-9-Fc compared to Siglec-7-Fc binding (Figure 4 B and C). Removal of sialic acids from the PCa cell surface by treatment with sialidase resulted in a significant reduction in the binding of Siglec-7-Fc and Siglec-9-Fc, as determined by flow cytometry (Supplemental Figure S9). Confocal fluorescence microscopy

revealed cell surface binding patterns of Siglec-7-Fc and Siglec-9-Fc on PC3 cells that was abolished by treatment with sialidase, confirming the dependence of the staining on sialic acid (Figure 4D). Furthermore, cell surface localization of Siglec-9 ligands was observed in DU145 and LNCaP cells (Supplemental Figure S10). These results demonstrated that Siglec-7 and Siglec-9 bind to sialic acid ligands on the surface of PCa cells.

Siglec-7-Fc and Siglec-9-Fc binding was also tested directly in human PCa tissues. IHC analysis using the recombinant human Siglec-7 and Siglec-9 Fc chimera demonstrated binding localized to the epithelial cells in PCa tissues, while non-cancerous tissues showed either absent or low-level binding of Siglec-7-Fc and Siglec-9-Fc (Figure 4E). We further analyzed Siglec-7 and -9 ligand expression in PCa patient-derived tumor tissues. After digestion of the tissues into single cells by collagenase D, flow cytometry demonstrated that expression levels of Siglec-7 and Siglec-9 ligands were significantly higher in tumor tissues compared to adjacent normal tissues (Figure 4F-I). Notably, binding of Siglec-9-Fc to prostate cancer tissues was found to be particularly elevated in PCa tissues, consistent with that observed in cultured PCa cell lines. Therefore, in both cultured prostate cancer cells and human tissues, sialic acid ligands that bind to Siglecs-7 and -9 are expressed on the cell surface.

Siglecs-7 and -9 blockade inhibits PCa tumor growth in a humanized mouse model

To evaluate the role of Siglec-7 and -9 ligands in vivo, we tested whether blocking anti-Siglec-7 and anti-Siglec-9 mAbs could control the growth of prostate cancer xenografts. We implanted PC3 cells into immune deficient NSG mice and allowed tumors to become established for 1 week. Mice were then injected intraperitoneally with human PBMC, enriched with activated human CD8⁺ T cells. On days 13 and 19, the mice were administered anti-Siglec-7 and anti-Siglec-9 blocking mAbs or IgG isotype control via intraperitoneal injection (Figure 5A). By 25 days, PC3 tumor

volumes were significantly smaller in anti-Siglec7/9 mAb treated group compared to IgG controls (Figure 5B and C). Tumor weight also was significantly lower in the anti-Siglec7/9 mAb treated group compared to IgG controls at the end of the treatment period (Figure 5D). Similar growth inhibition was observed in AR-expressing 22Rv1 cell line xenografts in anti-Siglec-7/9 treated mice compared to IgG isotype controls (Figure 5E-G). When we did not inject mice with PBMC and CD8+ T cell administration, no significant difference in PC3 tumor growth was observed between animals treated with anti-Siglec-7 and -9 mAb and IgG1 isotype treated controls (Figure 4H-J), demonstrating that growth suppression was dependent on activity of the human immune cells. IHC staining of PC3 tumor xenografts treated with anti-Siglec-7/9 mAbs demonstrated higher levels of apoptosis (cleaved caspase 3), decreased proliferation (Ki67) and vascularization (CD31), and increased immune cell infiltrates including CD4+ and CD8+ T cells compared to IgG controls (Figure 4K). Similarly, in the 22Rv1 xenografts, increased apoptosis, and decreased proliferation and vascularization were observed in anti-Siglec-7/9 mAb treated animals compared to isotype controls (Figure 4L). Interestingly, the anti-Siglec-7/9 mAb 22Rv1 tumors showed increased macrophage (CD68) infiltration compared to the IgG control, with fewer T cells infiltrates (Figure 4L). Taken together, these results demonstrate that blocking Siglec-7/9 signaling increases immune responses in prostate cancer.

CD59 is a candidate Siglec-9 ligand in PCa

Since PCa cells exhibit higher binding of Siglec-9 compared to Siglec-7, we sought to identify Siglec-9-binding glycoproteins as candidate ligands. We first performed a CRISPRi screen to in which we FACS sorted cells in which targeted knockdown of proteins substantially diminished Siglec-9-Fc binding. Specifically, we transduced PC3 cells expressing dCas9 with a genome-wide human CRISPRi-v2 library containing 209,070 single guide RNAs that target 18,905 genes (10

sgRNAs/gene) (Figure 6A)(41). Genomic DNA was isolated from the sorted PC3 cells that exhibited reduced binding to Siglec-9 Fc proteins obtained by FACS for subsequent deep sequencing. Notably, CD59, a sialylated glycoprotein, was identified as one of the top candidate ligands for Siglec-9, along with other top candidates such as PARM1, EPHB2, LPL, ISL, and RTBXA2R (Figure 6A).

To further identify Siglec-9 ligands, we used Siglec-9-Fc coupled with protein G beads to immunoprecipitate Siglec-9 ligands from PC3 cell lysates that were untreated or pre-treated with sialidase. Interacting proteins from both sample sets were identified via shotgun proteomics. Proteins that were significantly enriched in the untreated compared to the sialidase treated samples were identified as sialic acid-dependent ligands for Siglec-9. We identified CD47, CD59, CD73 (also known as NT5E), and LGALS3BP as potential candidate cell surface ligands interacting with Siglec-9 (Figure 6B). Once again, CD59 was one of the top candidate ligands identified by LC-MS/MS, implicating CD59 as a potential ligand for Siglec-9.

The scRNA-seq analysis revealed that candidate Siglec-9 ligands CD59, CD47, and LGALS3BP were expressed in tumor cells in metastatic tumor tissues derived from patients with CRPC (Figure 6C). UMAP visualization showed that CD59 was expressed in various types of cells including tumor cells in human localized, metastatic HSPC, and CRPC tumor tissues (Figure 6D and Supplemental Figure S11). CD59 is expressed in cancer cells in localized, HSPC, and CRPC tumor tissues (Figure 6E).

To validate CD59 as a ligand for Siglec-9, we performed western blotting on the Siglec-9-Fc captured proteins from PC3, DU145, and LNCaP prostate cancer cell lines using anti-CD59 antibodies. As depicted in Figure 6F, CD59 protein band was clearly observed in the protein lysates of PCa cells captured using recombinant Siglec-9 Fc protein, while no band was detected in the

isotype IgG Fc pull-down samples. To further validate the binding between Siglec-9 and CD59 on the cell surface, we generated CD59 knockout PC3 cells using the CRISPR-Cas9 system. CD59 protein level was significantly reduced in sgCD59 transduced cells by western blot analysis (Figure 6G). Flow cytometry analysis demonstrated a reduced capacity of Siglec-9 Fc binding in the CD59 knockout PC3 cells compared to the control cells (Figure 6H and I). Moreover, CD8+ T cell cytotoxicity expressed with high level of Siglec-7 and Siglec-9 (Supplemental Figure S12) was enhanced following CD59 knockout in PC3 cells (Figure 5G). These findings were also replicated in other PCa cell lines including DU145 and LNCaP cells (Figure 6K and L), providing further evidence for the role of CD59 as a functional ligand for Siglec-9 in PCa cells. Immunofluorescent staining of PC3 cells with both the Siglec-9-Fc-FITC conjugated antibody and anti-CD59 antibody conjugated with PE confirmed cell surface co-localization of CD59 and Siglec-9 binding (Figure 6M), supporting the notion that CD59 is a potential Siglec-9 ligand in PCa cells.

Discussion

Although prostate cancer has been classified as an “immune cold” malignancy because of very limited immune cell infiltration(42), it was the first cancer for which immunotherapy was FDA approved after demonstration of improved survival of Sipuleucel-T in 2 phase 3 clinical trials (10, 11). In this therapy, leukapheresed peripheral monocytic cells, including antigen presenting cells, are expanded and exposed to the prostate specific glycoprotein prostatic acid phosphatase (PAP) and re-infused into men with metastatic prostate cancer. The finding that a glycoprotein can elicit an immune-response in prostate cancer suggests a latent ability to activate the immune system against prostate cancer through a glycan-mediated immunosuppressive pathway. We propose that substantial changes in sialylation of cell surface proteins in prostate cancer that interact with Siglec receptors on immune cells is one pathway through which glycoproteins

suppress immunity in prostate cancer. We demonstrate the enrichment of sialic acids that can serve as ligands for Siglec-7 and Siglec-9 in prostate cancer cells and human tissues. Our findings reveal that Siglec-7 and Siglec-9 are highly expressed in myeloid cells, including macrophages. Blocking of Siglec-7 and Siglec-9 in prostate cancer xenografts decreases cancer growth and is dependent on the presence of immune cells. Finally, we identify CD59 as a candidate ligand for Siglec-9 in prostate cancer, highlighting a potential target for a novel immunotherapy.

A growing body of evidence implicates the sialic acid/Siglec pathway in immunosuppression in human malignancies (20, 24, 26, 28, 43). Siglec ligands have been identified on many cancer cell types and invariably have been shown to suppress the activation of multiple subsets of immune cells in diverse cancer types (44, 45). As we observed in prostate cancer, blocking of Siglec-7, Siglec-9 or both can result in tumor shrinkage in melanoma and ovarian cancer models (24, 46). In addition, Siglec-9 is detected in many human cancers and high Siglec-9 expression is correlated with short progression free survival(23). For example, Siglecs-7 and -9 are highly expressed on myeloid cells in pancreatic ductal adenocarcinoma (PDAC)(20). Elevated Siglec-9 is reported in PBMC derived CD8+ and tumor infiltrating lymphocytes in patients with non-small cell lung cancer (NSCLC) (34). In prostate cancer, we found that high expression of Siglec 7 and Siglec-9 are associated with poorer progression free survival as well as increased cancer grade, suggesting an immunosuppressive role.

The mechanisms of immunosuppression in cancers via Siglec signaling appear to be diverse and complex. For example, in human leukemia, binding of CD43 to Siglec-7 receptors on NK cells appears to be a critical mechanism of immunosuppression(44). In prostate cancer, our transcriptomic, in vitro and in vivo data implicates myeloid cells including macrophages and myeloid derived suppressor cells which express high Siglec-7 and Siglec-9 in shaping the prostate

cancer immune landscape. In prostate cancer, up-regulation of MYC signaling pathways are some of the most common genetic alterations observed, often in parallel with MYC amplifications (47). We have previously reported that modulation of MYC signaling increases cancer cell sialylation through upregulation of sialyltransferases, particularly ST6GALNAC4(48). In lymphocytic leukemia cell lines, these alterations result in changes in tumor infiltration of NK cells, macrophages, CD4+ T-cells, and CD8+ T cells, and increased CD69 expression on both CD4+ and CD8+ T cells. We observed similar effects following Siglec blockade in our in vivo studies using prostate cancer xenografts and human PBMCs. Additional investigation into the effects of sialic acid/Siglec interactions in prostate cancer will be necessary to elucidate broad immunomodulatory effects of this pathway.

We identify CD59 as a candidate ligand for Siglec-9 in prostate cancer by two independent methods, suggesting it could be a therapeutic target in prostate cancer. Our observation that CD59 acts as an immune-suppressive Siglec-9 ligand agrees with previous observations that protein expression of CD59 in human prostate cancer (49), and other solid tumors including colon cancer (50), breast cancer (51), NLCLC cancer (52), and esophageal squamous cell carcinoma (53) is associated with adverse outcomes. High protein CD59 expression is associated with poor survival outcomes in prostate cancer(49). Whether targeting CD59 would be effective in prostate cancer treatment is uncertain and requires additional investigation. CD59 has several additional functions relevant to immune function including the complement activation cascade where it binds to C8 and C9 to inhibit the formation of membrane attack complex (MAC) pores, conferring cancer cells resistant to complement-mediated cell lysis (54, 55). In addition, it is possible that there are additional Siglec ligands active in prostate cancer immunosuppression. Both the CRISPRi screen and the pull-down experiments showed several additional candidate ligands that could be

functional when CD59 is blocked. Alternatively, these ligands could be active in different contexts, such as on specific immune cells or in particular microenvironments. An alternative therapeutic strategy would be to target Siglec receptors directly, using therapies such as with receptor-blocking antibodies used in this study. In addition, we have developed a bifunctional protein with a ligand that binds cell surface proteins on cancer cells to direct a sialidase specifically to the cancer cells surface and demonstrated efficacy in targeted HER2 in breast cancer(56, 57). A similar therapeutic strategy could be applied to prostate cancer by taking advantage of PSMA or PSCA proteins for specific targeting. Our findings highlight the importance of Siglec-7/9 expression and their interactions with sialic acids on Siglec-7/9 ligands in prostate cancer progression and suggest potential therapeutic targets including CD59/Siglec-9 for immune-based therapeutic interventions.

Methods

The materials, including antibodies and agents, are listed in Table 1 and Supplemental Table S1.

Sex as a biological variable

Since prostate cancer is a male-specific disease, only prostate tumor tissues from men and male mice were used in this study.

Patient samples

Prostate cancer (PCa) tissue samples were obtained from the Stanford Tissue Bank and UCSF Cancer Immunotherapy Program Biobank under an IRB approved protocol and stored at -80 °C prior to analysis. Blood samples from donors who did not have a history of immunologic disorder or had been treated with immunomodulatory drugs within six months were obtained from the Stanford Blood Bank. All patient samples were de-identified for the study.

Cell lines and culture conditions

PC3, DU145, LNCaP, C42B, and 22Rv1 cells, and embryonic kidney 293T cells were obtained from American Type Culture Collection (Manassas, VA). PC3, LNCaP, C42B, and 22Rv1 cells were cultured in RPMI-1640 medium (Invitrogen Inc., CA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (Invitrogen Inc., CA). 293T and DU145 cells were cultured in Dulbecco's modified Eagle medium (Invitrogen Inc., CA) containing 10% FBS and penicillin/streptomycin (100 U/ml). All cells were maintained in a humidified 5% CO₂ incubator at 37 °C and were regularly monitored for mycoplasma contamination and morphological changes.

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using Lymphocyte Separation Medium (Corning). The whole blood was diluted in PBS (1:3) and layered onto 15 mL lymphocyte separation medium, followed by centrifuged at 400 x g for 20 minutes at low speed. The PBMCs were collected from interface layer. The PBMCs were washed with PBS (1X), and collected by centrifuge at 1500 rpm for 10 min.

Tumor digestion

PCa patient tumors were finely minced and immersed in a digestion medium comprising RPMI with 2% fetal bovine serum, collagenase IV (1 mg/mL), and DNase I (100 µg/mL). After incubation at 37 °C with intermittent shaking, the digested tissues were processed through a 100 µm cell strainer to yield a single-cell suspension. The cells were washed using FACS buffer containing 2% fetal calf serum and 2 mM EDTA in PBS and centrifuged at 1500 rpm at 4°C.

Flow cytometry

Siglec-7/9 ligand expression was detected using recombinant chimeras of the Siglec-7 or Siglec-9 binding region fused to human IgG Fc domains (Siglec7/9-Fc). Specifically, Alexa Fluor® 488 anti-human IgG and Siglec-7-Fc or Siglec-9-Fc chimeric protein were diluted to a final concentration of 1 µg/ml in PBS with 0.5% BSA) and incubated on ice for 1 hour to form Siglec-7-Fc/anti-human IgG- Alexa Fluor® 488 or Siglec-9-Fc/anti-human IgG-Alexa Fluor® 488 conjugates. PC3, DU145, LNCaP, C42B and 22Rv1 cells were detached using accutase (Innovative Cell Technologies, Inc). Cells were washed, pelleted and resuspended in the above Siglec-7 or Siglec-9-Fc/anti-human IgG-Alexa Fluor® 488 precomplex solution at a density of 10×10^6 cells/mL. After incubation on ice for 30 minutes, the cells were pelleted by centrifugation at 1500 rpm for 5 min and washed twice and resuspended in PBS containing 2% FBS and 1mM EDTA. Cells treated with sialidase for 30 min in serum free medium were used as a negative

control. Cell surface staining with FITC-conjugated SNA and MAA/MALII were performed similarly except that the cells were stained in SNA-FITC(10 μ g/mL) or MALII (20 μ g/mL) on ice for 1h. The cells were washed and resuspended in PBS containing 2% FBS and 1mM EDTA.

PC3 and DU145 cell sorting was performed using a BD FACSAria II (BD Bioscience) to isolate specific cell populations excluding doublets, cell debris, and dead cells. Fluorescence-minus-one (FMO) samples were utilized to define the gate strategy for positive cells.

Sialic acid assay

The sialic acid levels in PCa cells were quantified using a sialic acid assay kit (ab83375, abcam) according to manufacturer's instructions. Briefly, PC3, DU145, LNCaP, C42B and 22Rv1 cells were detached using cell scrapers, the cells (50 000 cells/well) were transferred into 96-well plates and a working solution (50 μ L), composed of sialic acid converting enzyme, sialic acid development mix and sialic acid probe, was added into plates. After incubating for 30 min at room temperature, the fluorescence signal intensity was measured at 535/537 nm.

Confocal microscopy

PC3, DU145, LNCaP cells were plated onto μ -Slide 8 well glass bottom chamber slides (Ibidi USA) and cultured overnight. The cells were fixed in 4% formaldehyde solution in PBS and treated with buffer or sialidase for 30 min at room temperature. After blocking non-specific binding using anti-human Fc blocking agent in PBS containing 5% goat serum for 1h, cells were incubated with Siglec-7-Fc or Siglec-9-Fc/anti-human IgG R-Phycoerythrin conjugates and imaged using confocal microscopy (ZEISS 880 or ZEISS 980). Colocalization of CD59 with Siglec-9 ligands in PC3 cells was determined by co-staining using Siglec-9-Fc-FITC and anti-CD59-PE antibodies.

Plasmid constructs

The dCas9, and hCRISPRi-v2 libraries originally generated by Dr. Irving Weissman's group were procured from Addgene (41). The GFP plasmid was obtained from Sino Biologicals. The sgRNAs targeting CD59 were obtained from ABM Goods Inc.

Lentiviral production and transduction

All lentiviruses were generated using a third-generation system as described previously (58). Briefly, 293T cells were cultured in the presence of chloroquine diphosphate at a final concentration of 50 μ M for 5 hours. Lentiviral constructs (17 μ g) were mixed with pMDL(11 μ g), VSV-G (6 μ g) and pREV (4 μ g) in HEPES buffered saline with CaCl₂ at a final concentration of 0.5M, and the resulting DNA-calcium phosphate co-precipitate was added onto 293T cells. After incubating overnight, medium was removed and replenished. Two days later, medium containing lentiviruses was collected and concentrated using a PEG-it kit (LV810A-1, System Biosciences).

PCa cells were transduced by lentiviral particles in medium containing polybrene at a concentration of 8 μ g/mL for 3 days. For CRISPRi-v2 library transduction, PC3 cells were first transduced with dCas9 lentiviral particles. Genome wide human CRISPRi-v2 libraries containing with 209,070 single guide RNAs that target 18,905 genes (10 sgRNAs per gene) were then transduced into PC3 cells expressing dCas9. This workflow enabled library coverage of 1000X with 2×10^8 cells.

FACS sorting of cells with low Siglec-9-Fc binding

The CRISPRi-v2 library transduced PC3 cells (2×10^8) were washed, centrifuged and stained with Fixable Viability Dye eFluor™ 450 (65-0863-14, eBioscience), followed by staining with precomplexed Siglec-9-Fc/anti-human IgG-Alexa Fluor® 488 for 30 min on ice. Stained cells were resuspended in FACS buffer consisting of 1 mM EDTA and 2% FBS in PBS and filtered through

a cell strainer (100 μ m). Cells that passed through the strainer were sorted using a BD FACS Aria II with an event rate of 2000-4000 cells/s. Live cells with low fluorescence (defined as ~10% of the parent Siglec-9-Fc-staining) were collected.

Library amplification, sequencing, and data processing

The genomic DNA (gDNA) was isolated from sorted cells expressing low levels of Siglec-9 ligands and unsorted control samples using a NucleoSpin Blood XL kit (740950.50, Macherey-Nagel) following the manufacturer's instructions. Specifically, the harvested unsorted cells or sorted cells were centrifuged at 400 \times g for 5 min and followed by resuspension in PBS at 10 mL/100 million cells. Proteinase K (0.5 mL/100 million cells) and BQ1 buffer (10 mL/100 million cells) were added into resuspended cells. The cells were incubated at 56 $^{\circ}$ C for 1 h and were then cooled to room temperature before adding 100% ethanol buffer (10 mL/100 million cells). DNA was extracted from the lysates using a NucleoSpin[®] Blood column centrifuged at 3000 \times g for 3min. The DNA was washed with BQ2 buffer (10 mL) twice, and the silica membranes were dried by centrifuging at 3000 \times g for 10 min. The DNA was eluted with 70 $^{\circ}$ C BE buffer and centrifuged at 3000 \times g for 5 min (1 mL/column).

The gDNA concentrations were determined using a Nanodrop 2000 instrument (Thermo Scientific), and gDNAs were amplified with TruSeq indices by PCR using NEBNext Ultra II Q5 MasterMix (catalog no. M0544L, New England Biolabs) for 22 cycles at 98 $^{\circ}$ C for 10 s and 65 $^{\circ}$ C for 75 s.

Double SPRI purification using SPRIselect Reagent (Beckman and Coulter) was performed to remove the gDNA and primer background from the PCR by selecting 274 bp fragments (59). Specifically, 0.65X SPRI beads were added into the PCR reaction solution to remove the fragments >300 bp. The suspension was collected, and 1X SPRI beads were added to select

fragments >150 bp. The beads were washed with 80% ethanol, air dried for 10 min, and eluted with EB buffer (20 μ L).

The purified PCR products were sequenced with 10% Phix Spike-in on an Illumina HiSeq4000 by Novogene (41). The FASTQ sequences were demultiplexed and aligned to the reference genome using MAGeCK (60). The sgRNA sequences in samples with low Siglec-9-Fc-FITC staining were compared to unsorted control samples to identify enriched sequences. Genes encoding cell surface (GO:0009986) and glycoprotein were used to select Siglec-9 ligands.

Siglec-9-Fc pulldown and Mass Spectrometry

PC3 cells were harvested using cell scraper and centrifuged at 1500 rpm for 5 min. The cells were washed with PBS (1X) and resuspended in lysis buffer (0.1% NP-40 in PBS with 1x Halt protease inhibitor, ThermoFisher Scientific) at a concentration of 100 mg/ml on ice, followed by sonication using a probe sonicator. The insoluble fraction was pelleted at 20,000 \times g and supernatants were collected to quantify the protein concentration by PierceTM BCA Protein Assay Kit (ThermoFisher Scientific) according to manufacturer's instructions. The samples were diluted in lysis buffer at 1 mg/mL. The samples were incubated with or without 100 mM V. cholerae sialidase for 3 h at 37 $^{\circ}$ C. The Siglec-9-Fc protein (5 μ g) was complexed with Protein G beads (50 μ L) in PBS (250 μ L) for 1 h at room temperature. The Siglec-9-Fc functionalized beads were washed with PBS (1X). The sialidase-treated or untreated lysates were added into Siglec-9-Fc functionalized beads and incubated at 4 $^{\circ}$ C overnight with continuous rotation. The next day, the beads were washed with lysis buffer (2X) and 50 mM (triethyl)ammonium bicarbonate (3X). The proteins were eluted by boiling the beads at 95 $^{\circ}$ C in 50 μ L (triethyl)ammonium bicarbonate with 0.05% Rapigest. DTT was added to the eluate which was then incubated at 60 $^{\circ}$ C for 30 min, followed by alkylation with 10mM iodoacetamide at room temperature for 30 min, digestion by trypsin (1 μ g) in 50 mM

(triethyl)ammonium bicarbonate buffer at 37 °C for overnight, and acidification by formic acid at a final concentration of 2% at 37 °C for 30 min. The samples were dried in a speedvac overnight. The samples were cleaned using a Strata-X column, dried using a speedvac, and resuspended in 10 μ l 0.2% formic acid.

Peptides were separated over a 25 cm Aurora Series Gen2 reverse-phase LC column (75 μ m inner diameter packed with 1.6 μ m FSC C18 particles, Ion Opticks). The mobile phases (A: water with 0.2% formic acid and B: acetonitrile with 0.2% formic acid) were driven and controlled by a Dionex Ultimate 3000 RPLC nano system (Thermo Fisher Scientific). An integrated loading pump was used to load peptides onto a trap column (Acclaim PepMap 100 C18, 5 μ m particles, 20 mm length, Thermo Fisher Scientific) at 5 μ L/minute, which was put in line with the analytical column 5 minutes into the gradient. The gradient was held at 0% B for the first 6 minutes of the analysis, followed by an increase from 0% to 5% B from 6 to 6.5 minutes, an increase from 5 to 22% B from 6.5 to 66.5 minutes, an increase from 22% to 90% from 66.5 to 71 minutes, isocratic flow at 90% B from 71 to 75 minutes, and re-equilibration at 0% B for 15 minutes for a total analysis time of 90 minutes. Eluted peptides were analyzed on an Orbitrap Fusion Tribrid MS system (Thermo Fisher Scientific). Precursors were ionized with a spray voltage held at +2.2 kV relative to ground, the column was held at a constant temperature of 40 °C using a Sonation column oven, and the inlet capillary temperature was held at 275 °C. Survey scans of peptide precursors were collected in the Orbitrap from 350-1350 Th with an AGC target of 1,000,000, a maximum injection time of 50 ms, RF lens at 60%, and a resolution of 60,000 at 200 m/z. Monoisotopic precursor selection was enabled for peptide isotopic distributions, and precursors of $z = 2-5$ were selected for data-dependent MS/MS scans for 2 seconds of cycle time. Dynamic exclusion was set to exclude precursors after being selected once for an exclusion time of 30 seconds with a ± 10

ppm window set around the precursor monoisotope. An isolation window of 1 Th was used to select precursor ions with the quadrupole, and precursors were fragmented using a normalized HCD collision energy of 30. MS/MS scans were collected with an AGC target of 100,000 ions, with a maximum accumulation time of 54 ms and an Orbitrap resolution of 30,000 at 200 m/z. The same method was used for both untreated and sialidase treated samples. Raw data were processed with MaxQuant version 1.6.2.10 (61) and tandem mass spectra were searched with the Andromeda search algorithm (62). 20 ppm, 4.5 ppm, and 20 ppm were used for first search MS1 tolerance, main search MS1 tolerance, and MS2 product ion tolerance, respectively. Oxidized methionine and deamidated asparagine were set as variable modifications, and carbamidomethylation of cysteine was set as a fixed modification. Cleavage specificity was set to Trypsin/P with 2 missed cleavages allowed. Peptide spectral matches (PSMs) were made against a human protein database (reviewed entries only, 20,416 entries total) downloaded from Uniprot. Peptides were filtered to a 1% false discovery rate (FDR) using a target-decoy approach (63), and a 1% protein FDR was applied. Proteins were quantified and normalized using MaxLFQ, and the match between runs feature was enabled. Label free intensity values were log₂ transformed using Perseus version 1.6.2.2.(64), protein groups with signal in all three replicates of at least one condition were kept, and missing values were imputed using default settings of a distribution downshifted by 1.8 with a width of 0.3 sigma.

Siglec-9 Fc pulldowns and western blotting

PC3, DU145 and LNCaP cells were lysed in IP buffer (Thermo Fisher Scientific). Siglec-9 Fc or human-IgG1-Fc (5 µg/sample) were conjugated to Protein G beads (50 µL/sample) at 4°C for 1 h. The conjugated beads were added to the cell lysates and incubated at 4°C for 3 h with gentle shaking. The beads were collected and washed with cell lysis buffer. The bound proteins were

eluted by boiling beads in 1x Laemmli Sample Buffer (Bio-Rad) for 10 minutes prior to loading onto a 4-12% TrisGlycine Gel. Western blotting was performed with an anti-CD59 primary antibody (Sigma) at a 1:1000 dilution in TBS-T overnight at 4 °C. The membrane was incubated with secondary antibody (anti-Rabbit HRP) for 1h at room temperature. Enhanced chemiluminescence (ECL) was used to develop the membrane for imaging.

In vivo analysis of Siglec-7/9 function

NSG (NOD/LtSz-SCID IL-2R γ null) mice were procured from the Jackson Laboratory and housed at the Veterinary Service Center of Stanford University. PC3 and 22Rv1 (1 million cells) were injected subcutaneously into the flank of 8 to 10-week-old male mice at day 0. CD8+ T cells (2 million/mouse) activated by anti-CD3/CD28 beads for 4 days were co-administered with PBMCs (10 million/mouse) on day 7 by intraperitoneal injection. The mice engrafted with tumor cells were randomly assigned into 2 groups. The anti-Siglec-7/9 or isotype control (100 μ g/mouse) were injected intraperitoneally into mice at day 13 and day 19. Tumor size was measured every 3 days using a caliper, and tumor volume was calculated as $V=1/2L \times W^2$. At the end of the experiment, tumor tissues were collected for further analysis.

Staining for Siglec-7 and Siglec-9 ligands on tumor tissue

Tumor were fixed in formalin and sectioned at a thickness of 5 μ m. The tissue slides were incubated in accutase for 10 min at 37 °C for antigen retrieval. The slides were then blocked in a series of blocking buffers: 5% goat serum and 5% BSA in PBS (1 h), avidin blocking buffer (15 min), biotin blocking buffer (15 min), and anti-human Fc blocker in PBS buffer containing 0.5% BSA (1h). Next, the slides were incubated with Siglec-7-Fc or Siglec-9-Fc chimera protein at 4°C overnight, followed by endogenous peroxidase and alkaline phosphatase blocking for 10 min using dual Endogenous Enzyme Block solution (Dako). Finally, the tissue slides were washed and

incubated with anti-human IgG HRP at room temperature for 1h. All other steps are the same as above immunohistochemistry staining.

Transcriptome data analysis

TCGA gene expression data from primary tumors and matched adjacent normal tissues of 52 patients diagnosed with prostate adenocarcinoma (PRAD) were obtained from the Broad Institute. The raw data, comprising 497 primary tumor samples, were processed by normalizing to transcripts per million (tpm) and applying log transformation ($\log_{10}(1 + \text{tpm})$) due to the long-tailed gene expression distributions. Subsequently, log fold changes (lfc) for each gene were calculated as the difference between the log-transformed values of the primary tumor and adjacent normal tissue, $\text{lfc} = \log_{10}(1 + \text{tpm cancer}) - \log_{10}(1 + \text{tpm normal})$. A one-sample t-test was employed to assess the null hypothesis that the log fold change for each gene is zero (`scipy.stats.ttest_1samp`). Multiple hypothesis correction was performed using the Benjamini-Hochberg procedure (alpha threshold of 0.1).

Analysis was performed in python (ver. 3.11.7) with scikit-learn (1.3.2), scipy (ver. 1.11.4), and lifelines (ver. 0.28.0). Code is accessible through github. GO terms annotations are from the basic version of GO ("go-basic.obo" ver. 1.2, 2023-10-09 release) with human annotations from GOA human ("goa_human.gaf", 2023-10-10 release).

To obtain clinical information, we retrieved the TCGA gene expression profile (Prostate Adenocarcinoma, PanCancer Atlas) from 494 primary tumors using cBioPortal (<https://www.cbioportal.org/>). Kaplan-Meier survival analysis was conducted on 488 tumor samples that included clinical survival follow-up data. . The patients were grouped into low/high Siglec-7/9 based on the median expression levels. RNA-sequence data from PRAD TCGA were deconvoluted into individual cell types using CIBERSORTx (38). For each reference dataset, a

signature matrix LM22 containing 547 genes was computed using CIBERSORTx. PRAD TCGA RNA sequencing data were used as an input for deconvolution. CIBERSORTx was operated in "absolute mode" to normalize deconvolution results into percentages.

LC-MS/MS Glycopeptide Analysis

Enriched glycopeptides from PCa and benign adjacent (BA) tissues were analyzed by LC-MSMS using a Dionex UltiMate 3000 RSLCnano system coupled to a LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). A 10 μ L aliquot of the glycopeptide enriched sample was loaded onto a 5 mm Acclaim PepMap 100 C18 trap column (Thermo Fisher Scientific) at a constant flow rate of 5 μ L/min for 10 minutes. Glycopeptides were then separated by reversed phase liquid chromatography on a 25 cm analytical column packed in house with Magic C18, 100 Å packing material (Michrom Bioresources). The chromatographic gradient consisted on holding buffer A (0.1% formic acid in water) for 10 minutes (loading step) at 98% and increasing buffer B (0.1% formic acid in ACN) from 2 % to 35% in 90 minutes. A rapid increase of buffer B to 85% in the next 10 minutes with a 5 minute hold before bringing down buffer B to initial conditions of 2% for column equilibration before the next sample injection. Eluting glycopeptides were ionized using a nanospray Flex ion source voltage with the voltage set to 1.5 kV and the heating capillary temperature set to 200 °C. The mass spectrometry analysis parameters were set as follows: a full MS scan was performed in the FT mass analyzer from 400 to 1800 m/z with a mass resolution of 30,000, and the MS/MS fragmentation was performed using higher-energy collision-induced dissociation (HCD) on the top 10 most abundant precursor ions in the MS1 scan. Dynamic exclusion was enabled, with a 30 second exclusion duration. The following conditions were used for HCD activation: 35 normalized collision energy, 4 m/z isolation width, 0.40 ms activation time, and a minimal precursor signal threshold of 1,000 counts.

Single cell RNA-sequence (scRNA-seq) analysis

Tissues were obtained from patients with localized PCa (NCT03821246), metastatic hormone-sensitive PCa (HSPC) (NCT03007732), and metastatic castration-resistant PCa (CRPC) (NCT03248570), and further processed for scRNA-seq by protocols described previously (39, 40). The raw data from 10x sequencing were processed using the Cell Ranger pipeline (v3, Genome build: GRCh38). The gene expression matrices were subjected to ambient RNAs removal by CellBender (v0.1.0) (65), doublet detection by the package DoubletDetection (10.5281/zenodo.2678041) and then analyzed through the SCANPY pipeline (65) with batch correction by the package Harmony (66). Leiden clustering (default resolution = 1.0), and UMAP plotting were performed for cell clustering. Differential expression analysis identified top-ranked genes upregulated in each individual cluster relative to the combination of all other cells, as determined by the SCANPY function `tl.rank_genes_groups`. Annotation of each unbiased population was achieved through manual inspection of the top-ranked genes of each cluster. The selected gene expressions on UMAPs were performed by SCANPY function `pl.umap`. To calculate gene expression at the sample level, the mean expression of cells in each cell cluster from each sample was calculated and then plotted using GraphPad Prism software.

Statistics

Statistical analyses were performed using one-way analysis of variance (ANOVA) with post hoc Tukey's test for more than two groups. An unpaired or paired Student's t-test (2-tailed) was used for comparison of two groups, as indicated in the figure legends. Survival analysis was conducted using the Kaplan–Meier (log-rank test). Median expression was used as cutoff between high and low Siglec-7 and -9 expressing groups. Data were presented as mean±S.E.M. Statistical

significance was noted as the following: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, non-significant differences were represented as "ns."

Study approval

The use of human specimens received approval from the Institutional Review Board of Stanford University (IRB no. 59488). The use of animals in the study was following the guidelines and regulations set forth by the Institutional Animal Care and Use Committee (IACUC) of Stanford University (12944).

Data availability

CRISPRi screen data are available in Supporting data values. The sc-RNA seq data was deposited into NCBI Gene Expression Omnibus (GEO) with accession number GSE274229. We have used published TCGA datasets, which could be downloaded from Broad Institute and the data with clinical information was obtained from cBioPortal (dataset: "Prostate Adenocarcinoma (TCGA, Firehose Legacy)"). All other data are available in Supporting data values supplemental material.

Author contributions

R.M.W. designed and conducted experiments, analyzed data, and drafted the manuscript, and oversaw the project. J.C.S, Z.F., A. L., N.M.R, S.T, A.B, R.N, X.Z. contributed to the conduct of experiments. G.E.W.M., F.J.G.M. and Z.F. performed bioinformatic analyses of gene expression and proteomic data. H.Z, L.F., S.J.P, E.G.E., C.R.B., and J.D.B designed the studies and oversaw the project. X.Z., J.C.S, N.M.R, S.J.P., H. Z. and J.D.B. revised the manuscript. All authors read and approved the final manuscript.

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Conflict of Interests

R.M.W received a travel award from Bon Opus Biosciences. J.C.S. has received compensation for consulting and SAB membership from Rondo Therapeutics, AbTherx, and Curie Bio, as well as speaker's honoraria from Cullinan Therapeutics and Merck & Co. C.R.B. is a cofounder and Scientific Advisory Board member of Lycia Therapeutics, Palleon Pharmaceuticals, Enable Bioscience, OliLux Bio, InterVenn Bio, Grace Science LLC, Firefly Bio, Redwood Bioscience (a subsidiary of Catalent), Neuravid Therapeutics and ReNagade Bio, and Valora Therapeutic. C.R.B. is a member of the Board of Directors of Alnylam and OmniAb.

References

1. Siegel RL, Miller KD, Wagle NS, and Jemal A. Cancer statistics, 2023. *2023*;73(1):17-48.
2. Lowrance WT, Roth BJ, Kirkby E, Murad MH, and Cookson MS. Castration-Resistant Prostate Cancer: AUA Guideline Amendment 2015. *The Journal of urology*. 2016;195(5):1444-52.
3. Shou J, Zhang Q, Wang S, and Zhang D. The prognosis of different distant metastases pattern in prostate cancer: A population based retrospective study. *The Prostate*. 2018;78(7):491-7.
4. Naimi A, Mohammed RN, Raji A, Chupradit S, Yumashev AV, Suksatan W, et al. Tumor immunotherapies by immune checkpoint inhibitors (ICIs); the pros and cons. *Cell Commun Signal*. 2022;20(1):44.
5. Robert C. A decade of immune-checkpoint inhibitors in cancer therapy. *Nat Commun*. 2020;11(1):3801.
6. Taghizadeh H, Marhold M, Tomasich E, Udovica S, Merchant A, and Krainer M. Immune checkpoint inhibitors in mCRPC - rationales, challenges and perspectives. *Oncoimmunology*. 2019;8(11):e1644109.
7. Rebutzi SE, Rescigno P, Catalano F, Mollica V, Vogl UM, Marandino L, et al. Immune Checkpoint Inhibitors in Advanced Prostate Cancer: Current Data and Future Perspectives. *Cancers (Basel)*. 2022;14(5).
8. Slovin SF. The challenge of checkpoint inhibitors in prostate cancer: drugs for the many but useful to a few. *Immunotherapy*. 2020;12(4):219-21.
9. Wen R, Marti GE, Stark J, Marques FJG, Riley N, Bermudez A, et al. Targeting siglec-7/9 glyco-immune checkpoints in prostate cancer for enhanced immune responses and tumor suppression. *Cancer Res*. 2024;84(6_Supplement):7524-.
10. Small EJ, Schellhammer PF, Higano CS, Redfern CH, Nemunaitis JJ, Valone FH, et al. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2006;24(19):3089-94.
11. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med*. 2010;363(5):411-22.
12. Crocker PR, Paulson JC, and Varki A. Siglecs and their roles in the immune system. *Nat Rev Immunol*. 2007;7(4):255-66.
13. Angata T, Hayakawa T, Yamanaka M, Varki A, and Nakamura M. Discovery of Siglec-14, a novel sialic acid receptor undergoing concerted evolution with Siglec-5 in primates. *FASEB J*. 2006;20(12):1964-73.
14. Carroll DJ, O'Sullivan JA, Nix DB, Cao Y, Tiemeyer M, and Bochner BS. Sialic acid-binding immunoglobulin-like lectin 8 (Siglec-8) is an activating receptor mediating β 2-integrin-dependent function in human eosinophils. *J Allergy Clin Immunol*. 2018;141(6):2196-207.
15. Jiang KY, Qi LL, Kang FB, and Wang L. The intriguing roles of Siglec family members in the tumor microenvironment. *Biomark Res*. 2022;10(1):22.

16. Barkal AA, Brewer RE, Markovic M, Kowarsky M, Barkal SA, Zaro BW, et al. CD24 signalling through macrophage Siglec-10 is a target for cancer immunotherapy. *Nature*. 2019;572(7769):392-6.
17. Belisle JA, Horibata S, Jennifer GA, Petrie S, Kapur A, André S, et al. Identification of Siglec-9 as the receptor for MUC16 on human NK cells, B cells, and monocytes. *Mol Cancer*. 2010;9:118.
18. Haas Q, Boligan KF, Jandus C, Schneider C, Simillion C, Stanczak MA, et al. Siglec-9 Regulates an Effector Memory CD8(+) T-cell Subset That Congregates in the Melanoma Tumor Microenvironment. *Cancer Immunol Res*. 2019;7(5):707-18.
19. Ohta M, Ishida A, Toda M, Akita K, Inoue M, Yamashita K, et al. Immunomodulation of monocyte-derived dendritic cells through ligation of tumor-produced mucins to Siglec-9. *Biochem Biophys Res Commun*. 2010;402(4):663-9.
20. Rodriguez E, Boelaars K, Brown K, Eveline Li RJ, Kruijssen L, Bruijns SCM, et al. Sialic acids in pancreatic cancer cells drive tumour-associated macrophage differentiation via the Siglec receptors Siglec-7 and Siglec-9. *Nat Commun*. 2021;12(1):1270.
21. Zhang JQ, Nicoll G, Jones C, and Crocker PR. Siglec-9, a novel sialic acid binding member of the immunoglobulin superfamily expressed broadly on human blood leukocytes. *J Biol Chem*. 2000;275(29):22121-6.
22. Nicoll G, Ni J, Liu D, Klenerman P, Munday J, Dubock S, et al. Identification and characterization of a novel siglec, siglec-7, expressed by human natural killer cells and monocytes. *J Biol Chem*. 1999;274(48):34089-95.
23. Wu Y, Huang W, Xie Y, Wang C, Luo N, Chen Y, et al. Siglec-9, a Putative Immune Checkpoint Marker for Cancer Progression Across Multiple Cancer Types. *Front Mol Biosci*. 2022;9:743515.
24. Ibarlucea-Benitez I, Weitzenfeld P, Smith P, and Ravetch JV. Siglecs-7/9 function as inhibitory immune checkpoints in vivo and can be targeted to enhance therapeutic antitumor immunity. *Proceedings of the National Academy of Sciences of the United States of America*. 2021;118(26).
25. Wen R, Stark JC, Marti GE, García-Marqués F, Zhao H, Nolley R, et al. Siglec-7/9 are novel immune checkpoints for prostate cancer. *J Immunol*. 2023;210(1_Supplement):89.13-89.13.
26. Mei Y, Wang X, Zhang J, Liu D, He J, Huang C, et al. Siglec-9 acts as an immune-checkpoint molecule on macrophages in glioblastoma, restricting T-cell priming and immunotherapy response. *Nat Cancer*. 2023;4(9):1273-91.
27. Egan H, Treacy O, Lynch K, Leonard NA, O'Malley G, Reidy E, et al. Targeting stromal cell sialylation reverses T cell-mediated immunosuppression in the tumor microenvironment. *Cell Reports*. 2023;42(5).
28. Stanczak MA, Rodrigues Mantuano N, Kirchhammer N, Sanin DE, Jacob F, Coelho R, et al. Targeting cancer glycosylation repolarizes tumor-associated macrophages allowing effective immune checkpoint blockade. *Sci Transl Med*. 2022;14(669):eabj1270.
29. Büll C, den Brok MH, and Adema GJ. Sweet escape: Sialic acids in tumor immune evasion. *Biochim Biophys Acta Rev Cancer*. 2014;1846(1):238-46.
30. Munkley J, and Scott E. Targeting Aberrant Sialylation to Treat Cancer. *Medicines*. 2019;6(4):102.

31. van de Wall S, Santegoets KC, van Houtum EJ, Büll C, and Adema GJ. Sialoglycans and siglecs can shape the tumor immune microenvironment. *Trends Immunol.* 2020;41(4):274-85.
32. Wen R, Zhao H, Zhang D, Chiu C-L, and Brooks JD. Sialylated glycoproteins as biomarkers and drivers of progression in prostate cancer. *Carbohydrate Res.* 2022;519:108598.
33. Jandus C, Boligan KF, Chijioke O, Liu H, Dahlhaus M, Démoulin T, et al. Interactions between Siglec-7/9 receptors and ligands influence NK cell-dependent tumor immunosurveillance. *J Clin Invest.* 2014;124(4):1810-20.
34. Stanczak MA, Siddiqui SS, Trefny MP, Thommen DS, Boligan KF, von Gunten S, et al. Self-associated molecular patterns mediate cancer immune evasion by engaging Siglecs on T cells. *J Clin Invest.* 2018;128(11):4912-23.
35. Meril S, Harush O, Reboh Y, Matikhina T, Barliya T, and Cohen CJ. Targeting glycosylated antigens on cancer cells using siglec - 7/9 - based CAR T - cells. *Mol Carcinogenesis.* 2020.
36. Läubli H, Alisson-Silva F, Stanczak MA, Siddiqui SS, Deng L, Verhagen A, et al. Lectin galactoside-binding soluble 3 binding protein (LGALS3BP) is a tumor-associated immunomodulatory ligand for CD33-related Siglecs. *J Biol Chem.* 2014;289(48):33481-91.
37. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, et al. Integrative Genomic Profiling of Human Prostate Cancer. *Cancer Cell.* 2010;18(1):11-22.
38. Newman AM, Steen CB, Liu CL, Gentles AJ, Chaudhuri AA, Scherer F, et al. Determining cell type abundance and expression from bulk tissues with digital cytometry. *Nat Biotechnol.* 2019;37(7):773-82.
39. He JY, Kim YJ, Mennillo E, Rusu I, Bain J, Rao AA, et al. Dysregulation of CD4(+) and CD8(+) resident memory T, myeloid, and stromal cells in steroid-experienced, checkpoint inhibitor colitis. *Journal for immunotherapy of cancer.* 2024;12(4).
40. Zheng GXY, Terry JM, Belgrader P, Ryvkin P, Bent ZW, Wilson R, et al. Massively parallel digital transcriptional profiling of single cells. *Nature Communications.* 2017;8(1):14049.
41. Horlbeck MA, Gilbert LA, Villalta JE, Adamson B, Pak RA, Chen Y, et al. Compact and highly active next-generation libraries for CRISPR-mediated gene repression and activation. *Elife.* 2016;5.
42. Majidpoor J, and Mortezaee K. The efficacy of PD-1/PD-L1 blockade in cold cancers and future perspectives. *Clinical immunology (Orlando, Fla).* 2021;226:108707.
43. Schmassmann P, Roux J, Buck A, Tatari N, Hogan S, Wang J, et al. Targeting the Siglec–Sialic acid axis promotes antitumor immune responses in preclinical models of glioblastoma. *Sci Transl Med.* 2023;15(705):eadf5302.
44. Wisnovsky S, Möckl L, Malaker SA, Pedram K, Hess GT, Riley NM, et al. Genome-wide CRISPR screens reveal a specific ligand for the glycan-binding immune checkpoint receptor Siglec-7. *Proceedings of the National Academy of Sciences of the United States of America.* 2021;118(5):e2015024118.
45. Gonzalez-Gil A, Li TA, Kim J, and Schnaar RL. Human sialoglycan ligands for immune inhibitory Siglecs. *Mol Asp Med.* 2023;90:101110.

46. Jarvis GA, Li J, Hakulinen J, Brady KA, Nordling S, Dahiya R, et al. Expression and function of the complement membrane attack complex inhibitor protectin (CD59) in human prostate cancer. *Int J Cancer*. 1997;71(6):1049-55.
47. Koh CM, Bieberich CJ, Dang CV, Nelson WG, Yegnasubramanian S, and De Marzo AM. MYC and Prostate Cancer. *Genes & cancer*. 2010;1(6):617-28.
48. Smith BAH, Deutzmann A, Correa KM, Delaveris CS, Dhanasekaran R, Dove CG, et al. MYC-driven synthesis of Siglec ligands is a glycoimmune checkpoint. *Proceedings of the National Academy of Sciences of the United States of America*. 2023;120(11):e2215376120.
49. Xu C, Jung M, Burkhardt M, Stephan C, Schnorr D, Loening S, et al. Increased CD59 protein expression predicts a PSA relapse in patients after radical prostatectomy. *The Prostate*. 2005;62(3):224-32.
50. Shang Y, Chai N, Gu Y, Ding L, Yang Y, Zhou J, et al. Systematic Immunohistochemical Analysis of the Expression of CD46, CD55, and CD59 in Colon Cancer. *Arch Pathol Lab Med*. 2014;138(7):910-9.
51. Ouyang Q, Zhang L, Jiang Y, Ni X, Chen S, Ye F, et al. The membrane complement regulatory protein CD59 promotes tumor growth and predicts poor prognosis in breast cancer. *International journal of oncology*. 2016;48(5):2015-24.
52. Li B, Lin H, Fan J, Lan J, Zhong Y, Yang Y, et al. CD59 is overexpressed in human lung cancer and regulates apoptosis of human lung cancer cells. *International journal of oncology*. 2013;43(3):850-8.
53. Zhou Y, Chu L, Wang Q, Dai W, Zhang X, Chen J, et al. CD59 is a potential biomarker of esophageal squamous cell carcinoma radioresistance by affecting DNA repair. *Cell Death & Disease*. 2018;9(9):887.
54. Davies A, and Lachmann P. In: Delves PJ ed. *Encyclopedia of Immunology (Second Edition)*. Oxford: Elsevier; 1998:497-500.
55. Sugita Y, and Masuho Y. CD59: its role in complement regulation and potential for therapeutic use. *Immunotechnology : an international journal of immunological engineering*. 1995;1(3-4):157-68.
56. Xiao H, Woods EC, Vukojcic P, and Bertozzi CR. Precision glycoalkyl editing as a strategy for cancer immunotherapy. *Proceedings of the National Academy of Sciences of the United States of America*. 2016;113(37):10304-9.
57. Gray MA, Stanczak MA, Mantuano NR, Xiao H, Pijnenborg JFA, Malaker SA, et al. Targeted glycan degradation potentiates the anticancer immune response in vivo. *Nat Chem Biol*. 2020;16(12):1376-84.
58. Wen RM, Qiu Z, Marti GEW, Peterson EE, Marques FJG, Bermudez A, et al. AZGP1 deficiency promotes angiogenesis in prostate cancer. *Journal of translational medicine*. 2024;22(1):383.
59. Fisher S, Barry A, Abreu J, Minie B, Nolan J, Delorey TM, et al. A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. *Genome Biol*. 2011;12(1):1-15.
60. Li W, Xu H, Xiao T, Cong L, Love MI, Zhang F, et al. MAGeCK enables robust identification of essential genes from genome-scale CRISPR/Cas9 knockout screens. *Genome Biol*. 2014;15(12):554.
61. Tyanova S, Temu T, and Cox J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc*. 2016;11(12):2301-19.

62. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, and Mann M. Andromeda: A Peptide Search Engine Integrated into the MaxQuant Environment. *J Proteome Res.* 2011;10(4):1794-805.
63. Elias JE, and Gygi SP. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat Methods.* 2007;4(3):207-14.
64. Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods.* 2016;13(9):731-40.
65. Fleming SJ, Chaffin MD, Arduini A, Akkad A-D, Banks E, Marioni JC, et al. Unsupervised removal of systematic background noise from droplet-based single-cell experiments using CellBender. *Nature Methods.* 2023;20(9):1323-35.
66. Korsunsky I, Millard N, Fan J, Slowikowski K, Zhang F, Wei K, et al. Fast, sensitive and accurate integration of single-cell data with Harmony. *Nature Methods.* 2019;16(12):1289-96.

Figure 1: High Siglec-7/9 expression is correlated with worse clinical outcome in patients with prostate cancer. Violin plots showing that (A) Siglec-7 and (B) Siglec-9 mRNA expression are significantly higher in tumor tissues (n=497) than normal tissues (n=53). Data were analyzed by unpaired student t-test. (C) Siglec-7, and (D) Siglec-9 are correlated with Gleason Score. Data were analyzed by one-way ANOVA with post hoc Tukey's test and presented as mean+S.E.M. (E) High Siglec-7 and Siglec-9 expression is correlated with worse survival in patients with prostate cancer in the PRAD-TCGA database (n=488). (F) High Siglec-7 and Siglec-9 expression are correlated with worse survival in patients with prostate cancer in MSKCC database (n=140). Survival analysis was conducted using the Kaplan–Meier (log-rank test). Median expression was used as cutoff between high and low Siglec-7 and -9 expressing groups. (G) Siglec-7, and Siglec-9 are highly expressed in monocyte, dendritic cells, and CD8+ T cells identified by CIBERSORTx from the PRAD-TCGA database. (H) Scatter plot of RNA expression of Siglec-7 and Siglec-9 compared with CD68 in prostate tumor tissues (n = 494), according to TCGA data. Siglec-7, and Siglec-9 are correlated with macrophage marker CD68. Data were analyzed by Pearson's correlation. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

Figure 2 Siglec-7 and Siglec-9 are co-expressed on myeloid cells in human prostate tumors by single-cell RNA sequencing (scRNA-seq). (A) Uniform manifold approximation and projection (UMAP) plot showing the distribution of cell types. (B) UMAP profiles highlighting Siglec-7 expression, and (C) Siglec-9 expression in immune cells of castration-resistant prostate cancer (CRPC) tumor tissues (n=6). (D) Siglec-7 is predominantly expressed in dendritic cells (DCs), macrophages, myeloid-derived suppressor cells (MDSCs), and natural killer (NK) cells in CRPC. (E) Siglec-9 is primarily expressed in macrophages and MDSCs in CRPC specimens. (F) Siglec-7 and (G) Siglec-9 are expressed in macrophages in human tumor tissues from patients with

localized PCa (n=5), metastatic hormone-sensitive prostate cancer (HSPC) (n=14), and metastatic CRPC (n=6). (H) Dot plot illustrates fractional profiles of Siglec expression in immune cells across localized prostate cancer (n=5), metastatic hormone-sensitive prostate cancer (HSPC, n=14), and metastatic CRPC tumor tissues (n=6). (I) Confocal microscopy images of a prostate cancer bone metastasis showing the co-expression of Siglec-7 and Siglec-9 on macrophages. (J) Colocalization coefficient of Siglec-7 or Siglec-9 with the macrophage marker CD68, observed at 40X magnification (n=7).

Figure 3: Sialic acid is expressed on the surface of PCa cells. (A) surface sialic acid is detected in tested prostate cancer cell lines. Sialidase treatment reduces the surface sialic acid levels in (B) PC3, (C) DU145, (D) LNCaP. (E) The expression levels of α 2,6-linked and α 2,3-linked sialic acids in PC3 cells were analyzed by flow cytometry using Sambucus nigra agglutinin (SNA) and Maackia amurensis agglutinin II (MALII) lectins, respectively. (F) Quantification was assessed using mean fluorescence intensity (MFI) for SNA and MALII staining on prostate cancer cells. (G) Sialylated spectral counts vs total spectral counts of prostate cancer tumor tissues and adjacent normal tissues by mass spectrometry. (H) Summary of glycosylation changes by glycosite in prostate cancer compared to normal prostate samples. (I) Schematics of glycan structures. The schematic diagram was created with Biorender.com. (J) GO term pathway analysis of sialylated proteins in prostate cancer tumor tissues and adjacent normal tissues by mass spectrometry show enrichment in immune response/antigen presentation pathways. Protein names displayed on the right side with the glycosites in parentheses. (K) Analysis of transcript data from PRAD-TCGA database of sialyglycan gene expression demonstrates 7 significantly upregulated genes and 12 downregulated genes in cancer samples compared to non-cancerous prostate tissues. Data were

analyzed by unpaired Student's t-test and presented as mean \pm S.E.M., ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

Figure 4: Siglec-7 ligand (Siglec-7L) and Siglec-9 ligand (Siglec-9L) are expressed in prostate cancer cells. (A) The expression levels of Siglec-7L and Siglec-9L, and (B-C) their corresponding quantification by mean fluorescence intensity by flow cytometry. (D) Confocal microscopy analysis of PC3 prostate cancer cells showing cell surface-associated expression patterns of Siglec-7 and Siglec-9. Treatment with sialidase abolishes the binding of Siglec-7-Fc and Siglec-9-Fc proteins visualized by confocal microscopy, indicating dependence on sialic acid. (E) Representative immunohistochemistry analysis of Siglec-7 and Siglec-9 ligand expression in prostate cancer tumor tissues. High expression levels of Siglec-7 and Siglec-9 ligands are observed in tumor tissues from prostate cancer patients, while normal tissue samples show either absence or low expression levels. (F) Representative Siglec-7 and Siglec-9L expression profile on tumor cells derived from patient tumor tissues by flow cytometry, (G) Expression of Siglec-9L expression profile in all cells from tumor tissue and adjacent normal tissues (n=6). Quantification of (H) Siglec-7L and (I) Siglec-9L in PCa patient samples suggesting significant higher expression levels of Siglec-7L and Siglec-9L in tumor tissues compared to adjacent normal tissues. Data were analyzed paired Student's t-test and presented as mean \pm S.E.M., ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

Figure 5: Siglec-7/9 receptor blockade restricts prostate cancer tumor growth in humanized mouse models. (A) Schematic diagram depicting the implantation of prostate cancer (PCa) cells into NSG mice, followed by injection of PBMC and CD8⁺ T cell mixture, and subsequent administration of anti-Siglec-7 and anti-Siglec-9 monoclonal antibodies (mAbs) or IgG antibodies on specific days. (B) Representative images of PC3 tumors upon treatment with anti-Siglec-7/9 antibodies (n=12)

compared to IgG control (n=12). (C) PC3 tumor growth curve, and corresponding (D) tumor weight. (D) Representative images of 22Rv1 tumors upon treatment with anti-Siglec-7/9 antibodies (n=8) compared to IgG control (n=10). (E) 22Rv1 tumor growth curve, and corresponding (F) tumor weight. (H) Schematic diagram depicting control experiment without PBMC and CD8⁺ T cell injection. Control experiment no difference in (I) PC3 tumor growth and (J) tumor weight between anti-Siglec-7/9 antibody (n=8) and IgG1 isotype control treatment (n=10). (K) Immunohistochemical (IHC) staining of PC3 tumors treated with anti-Siglec-7/9 antibodies showing increased apoptosis (cleaved caspase 3), decreased proliferation (Ki67), decreased vascularization (CD31), and increased immune cell infiltration, including CD4⁺ and CD8⁺ T cells, compared to IgG1 control. (L) IHC staining of 22Rv1 tumors treated with anti-Siglec-7/9 antibodies showing increased apoptosis (cleaved caspase 3), decreased proliferation (Ki67), decreased vascularization (CD31), increased macrophage (CD68) infiltration, and enhanced CD8⁺ T cell numbers compared to IgG1 control. Data were analyzed by unpaired Student's t-test and presented as mean \pm S.E.M., ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ***, $P \leq 0.0001$.

Figure 6: CD59 is a Siglec-9 ligand in prostate cancer. (A) Experimental setup and CRISPRi screen results for identifying Siglec-9 ligands using CRISPR screen. PC3 cells expressing dCas9 were infected with a genome-wide CRISPRi-v2 library, and cells with reduced binding to recombinant Siglec-9 Fc proteins were sorted for deep sequencing (n=3). The schematic diagram was created with Biorender.com. (B) Identification of sialic acid-dependent Siglec-9 ligands through LC-mass spectrometry (MS)/MS pull down analysis by Siglec-9 Fc chimera protein (n=3). The schematic diagram was created with Biorender.com. (C) Analysis of transcript levels of Siglec-9 ligands by sc-RNA sequencing. CD59 and candidate ligands identified by CRISPRi and

MS expressed in metastatic epithelial cells from patients with CRPC. (D) UMAP profile shows the distribution of CD59 in different cell types in human metastatic CRPC tumor tissues, refer to Figure 2A for cell type distribution. (E) CD59 is expressed in cancer epithelial cells from patients with localized, HSPC, and CRPC. (F) Western blotting validation of CD59 as a Siglec-9 ligand using recombinant Siglec-9 Fc protein blotting pull-down blotting. (G) Western blot analysis of CD59 knockout PC3 cells using the CRISPR-Cas9 system. (H) Flow cytometry analysis and (I) corresponding quantification demonstrating reduced binding capacity of Siglec-9 Fc in CD59 knockout PC3 cells compared to control cells. Grey: IgG Fc, black: control cells, red: sgCD59-T1, green: sgCD59-T2. (J) Enhanced cytotoxicity of CD8+ T cells were observed in CD59 knockout PC3 cells, and (K) DU145 cells, and (L) LNCaP. (M) Confocal microscopy showing substantial colocalization between Siglec-9 ligands and CD59 on PC3 cells. Data were analyzed by one-way ANOVA with with post hoc Tukey's test and presented as mean \pm S.E.M., ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ***, $P \leq 0.0001$.

Figure 1

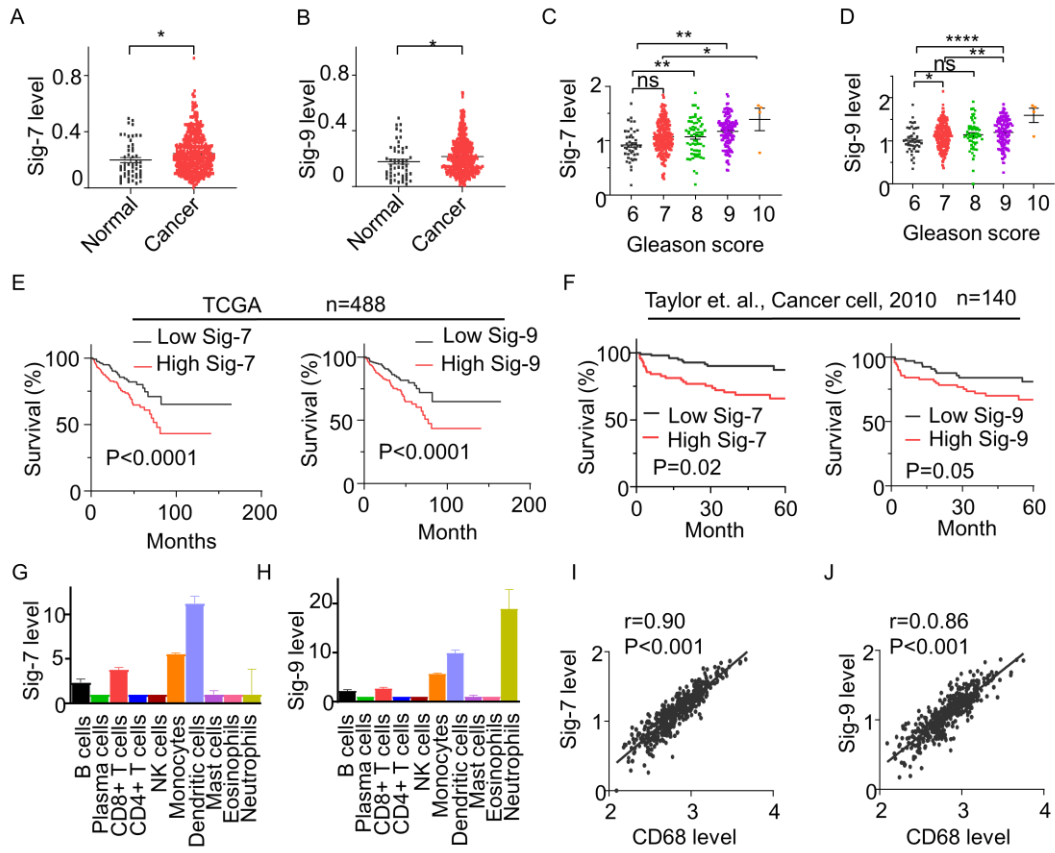


Figure 2

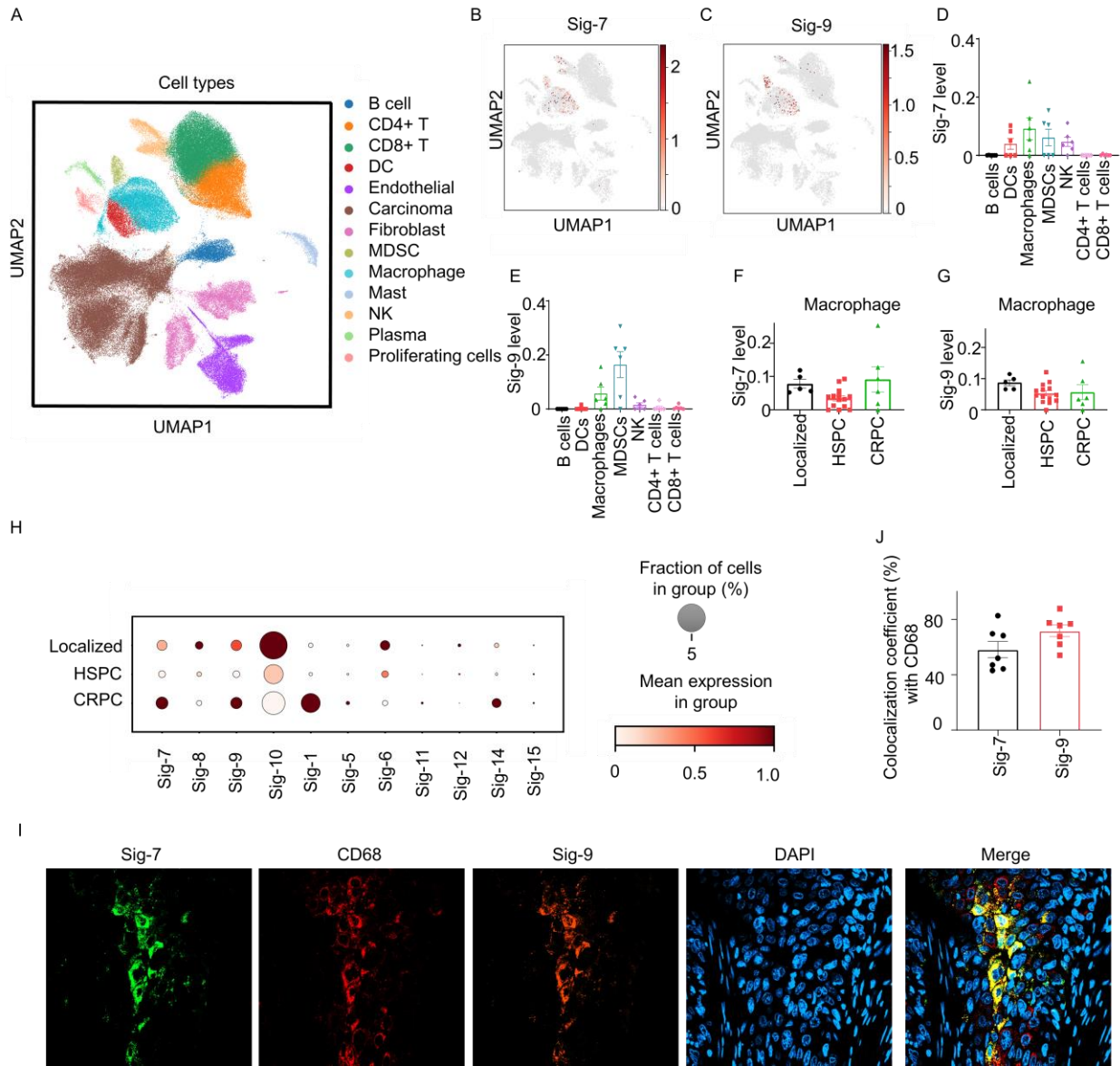


Figure 3

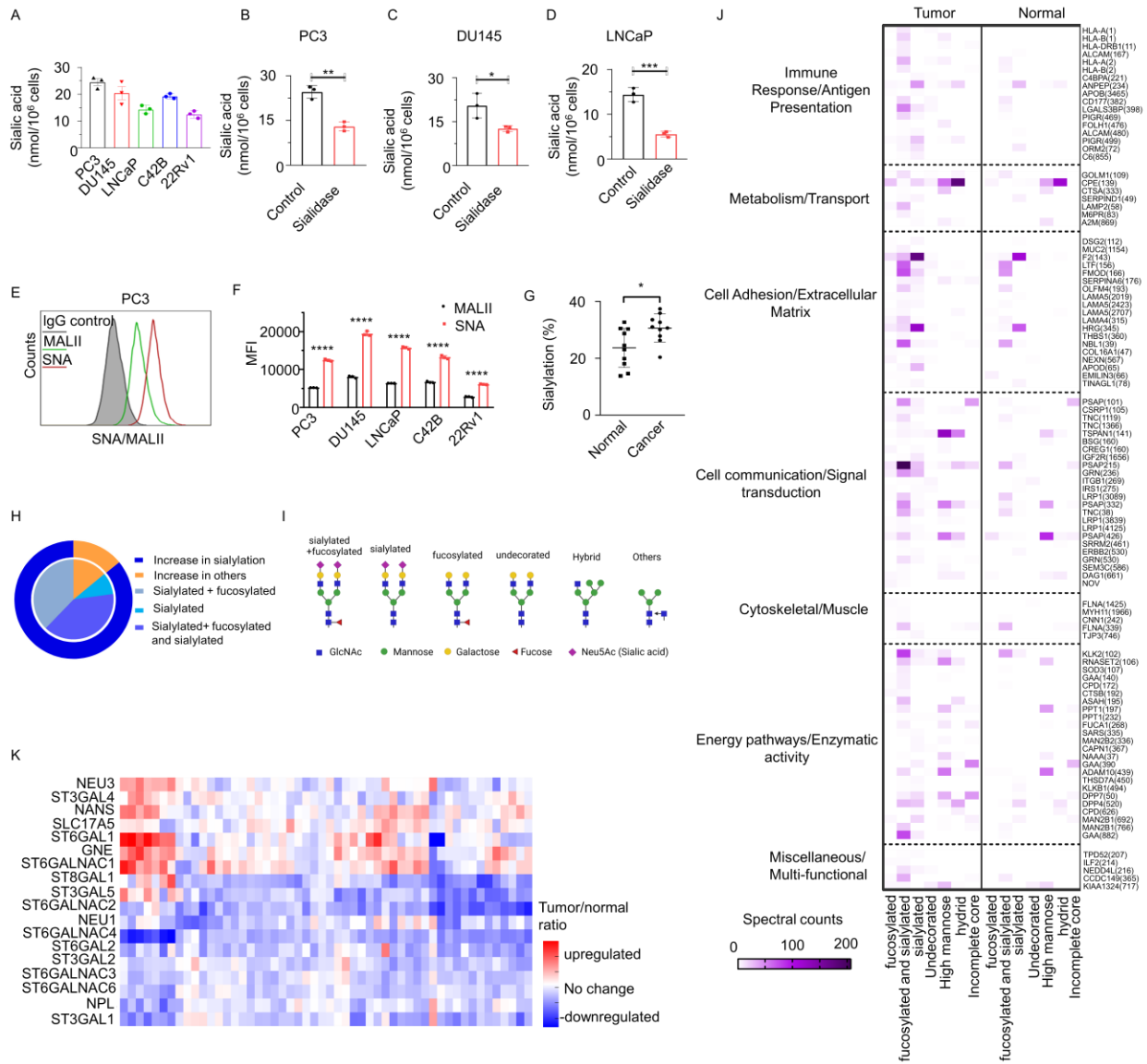


Figure 4

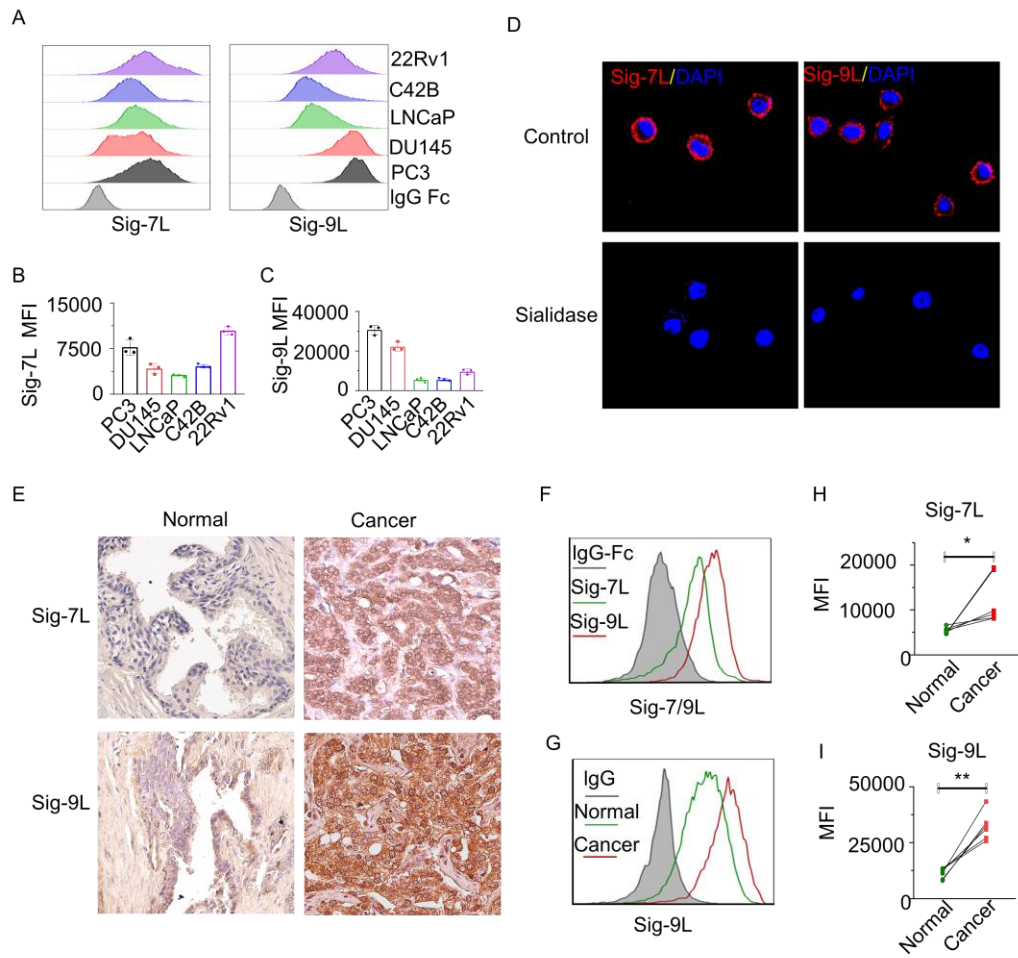


Figure 5

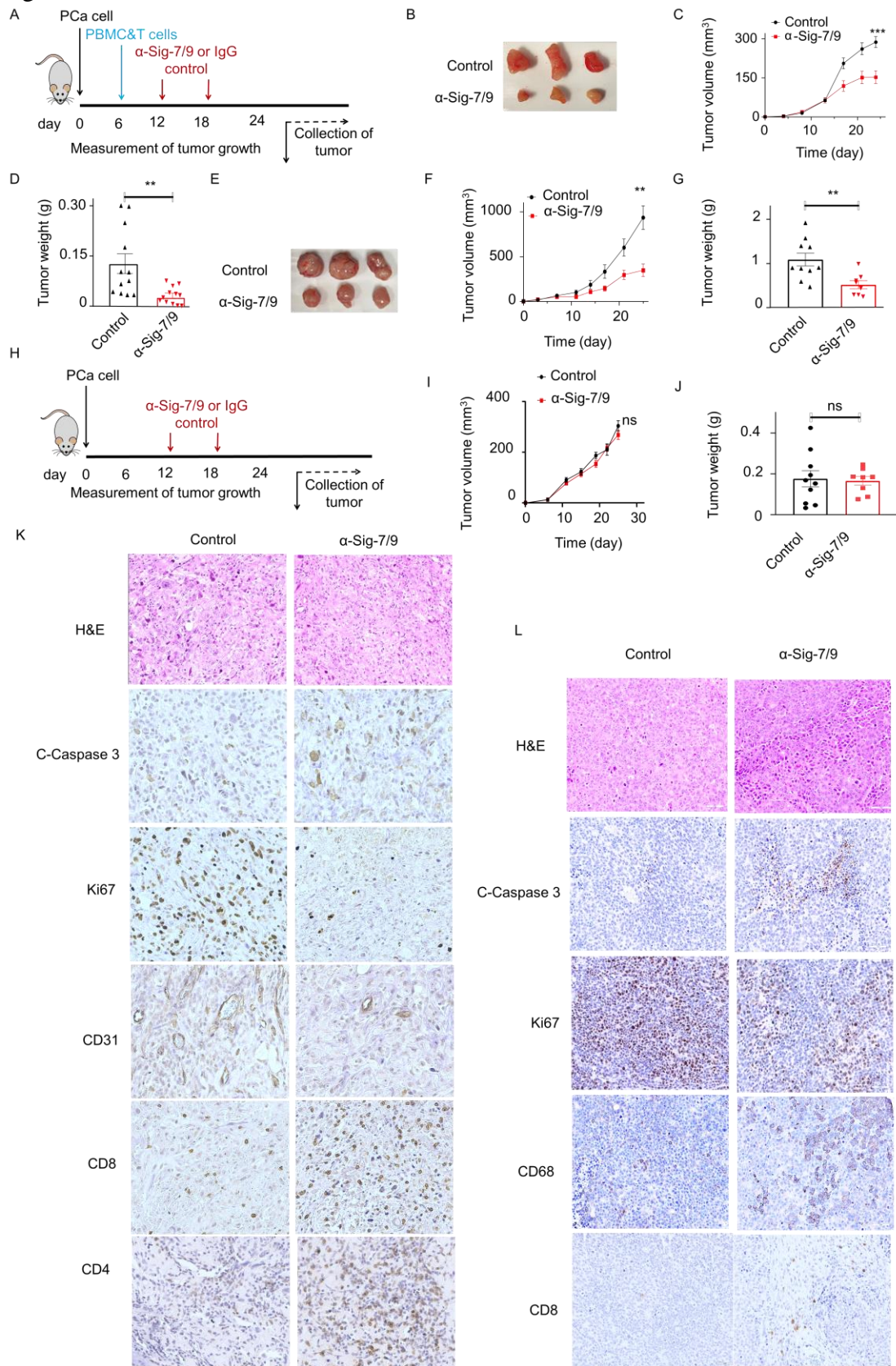


Figure 6

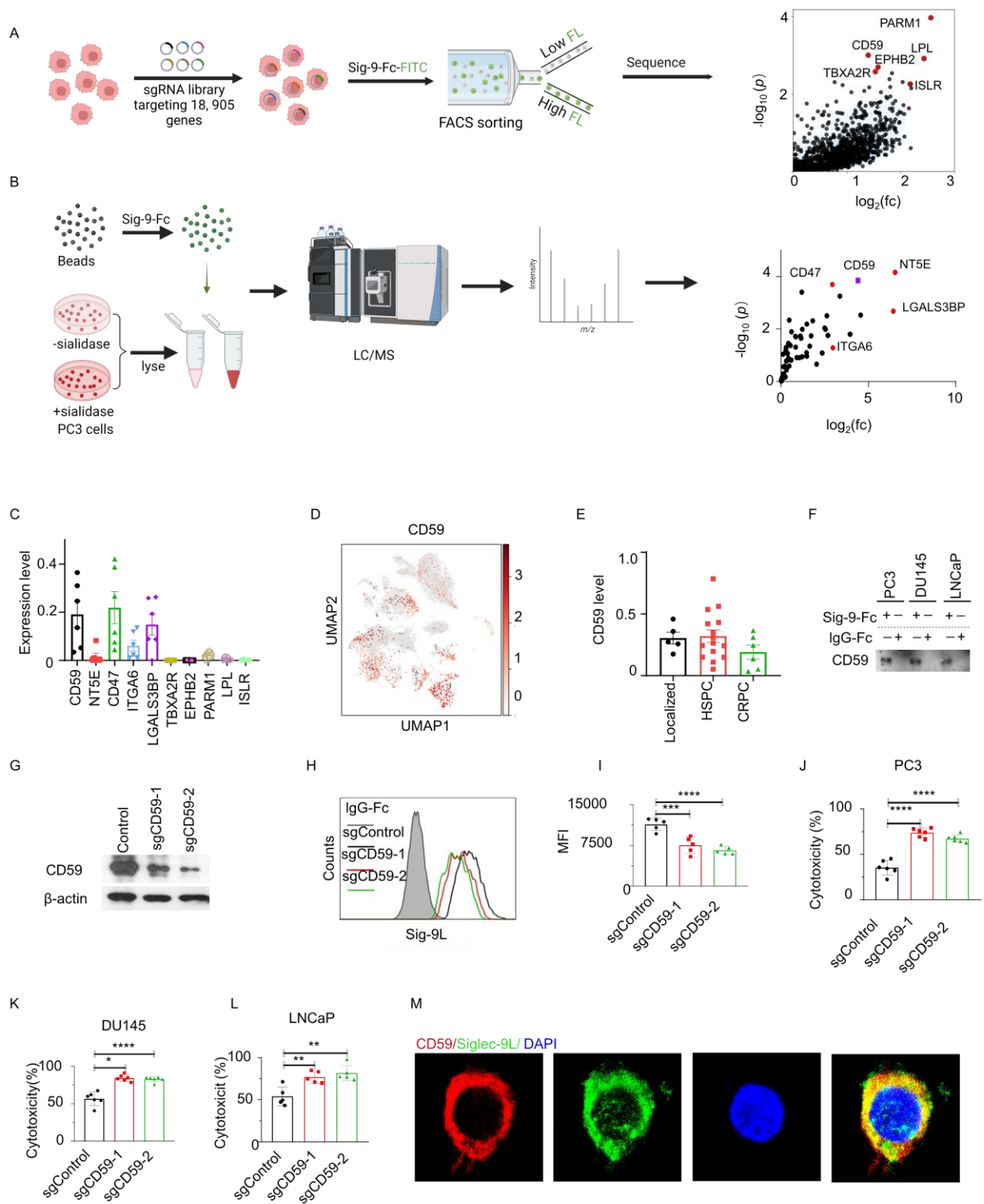


Table 1 A summary of antibodies used in this study

Agents/chemicals	Catalog no	Company
Ig G control antibody (in vivo)	1-001-A	R&D Systems
Anti-Siglec-7 antibody (in vivo)	MAB1138	R&D Systems
Anti-Siglec-9 antibody (in vivo)	MAB1139	R&D Systems
Anti-CD4 antibody	48774S	Cell Signaling Technology
Anti-CD8 antibody	853365	Cell Signaling Technology
Anti-CD31 antibody	ab182981	Abcam
Anti-Cleaved caspase 3 antibody	9661S	Cell Signaling Technology
Anti-Ki67 antibody	9449S	Cell Signaling Technology
Sambucus nigra agglutinin - FITC	SKU#: 21761051-1	Glycomatrix
Maackia amurensis agglutinin II - FITC	SKU#: 21511103-1	Glycomatrix
Avid/Biotin Blocking kit	SP-2001	Vector Laborateries
ABC-HRP Reagent	PK-7100	Vector Laborateries
Anti-Human IgG (Fab') ₂ (blocking)	ab98531	Abcam
Anti-CD59 antibody	HPA026494	Sigma-Aldrich
anti-rat IgG HRP	7077S	Cell Signaling Technology
anti-mouse IgG HRP	7076S	Cell Signaling Technology
anti-rabbit IgG HRP	7074S	Cell Signaling Technology
Siglec-7 Fc chimera protein	1138-SL-050	R&D Systems
Siglec-9 Fc chimera protein	1139-SL-050	R&D Systems
Anti-Siglec-7 antibody (IF)	ab111619	Abcam
Anti-Siglec-9 antibody (IF)	Ab197981	Abcam
Anti-CD68 antibody	PA5-109344	ThermoFisher Scientific
Anti-CD59 antibody (IF)	ab133707	Abcam
Anti-rabbit AlexFluor488	ab150077	Abcam
Alexa Fluor® 555 Conjugation Kit (Fast) - Lightning-Link®	ab269820	Abcam
Alexa Fluor® 647 Conjugation Kit (Fast) - Lightning-Link®	ab269823	Abcam
anti-human IgG-Alexa Fluor® 488 (flow cytometry)	109-545-098	Jackson ImmunorResearch Inc
anti-Human IgG R-Phycoerythrin (confocal microscope)	109-116-170	Jackson ImmunorResearch Inc