Self-associated molecular patterns mediate cancer immune evasion by engaging Siglecs on T cells

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First-generation immune checkpoint inhibitors, including anti-CTLA-4 and anti-programmed death 1 (anti-PD-1) antibodies, have led to major clinical progress, yet resistance frequently leads to treatment failure. Thus, new targets acting on T cells are needed. CD33-related sialic acid-binding immunoglobulin-like lectins (Siglecs) are pattern-recognition immune receptors binding to a range of sialoglycan ligands, which appear to function as self-associated molecular patterns (SAMPs) that suppress autoimmune responses. Siglecs are expressed at very low levels on normal T cells, and these receptors were not until recently considered as interesting targets on T cells for cancer immunotherapy. Here, we show an upregulation of Siglecs, including Siglec-9, on tumor-infiltrating T cells from non-small cell lung cancer (NSCLC), colorectal, and ovarian cancer patients. Siglec pathway in vitro and in vivo resulted in increased anticancer immunity. T cell expression of Siglec-9 in NSCLC patients correlated with reduced survival, and Siglec-9 polymorphisms showed association with the risk of developing lung and colorectal cancer. Our data identify the sialoglycan-SAMP/Siglec pathway as a potential target for improving T cell activation for immunotherapy.

Introduction

The landscape of oncological practice has substantially changed with the introduction of immune checkpoint inhibitors that target inhibitory receptors on the surface of CD8⁺ T cells (1). In particular, blocking of the inhibitory receptor programmed death 1 (PD-1) or its ligand PD-L1 has been shown to induce durable responses across many different cancer types, including non-small cell lung cancer (NSCLC), melanoma, squamous carcinoma of the head and neck, kidney cancer, and ovarian cancer (1, 2). However, most patients have primary resistance to therapeutic PD-1 or PD-L1 blockade or develop secondary resistance (2). Thus, identification of additional pathways mediating suppression of tumor-specific T cells is clearly needed to increase therapeutic efficacy of PD-1- or PD-L1-targeting strategies.

Pattern recognition receptors (PRRs), such as Toll-like receptors or nucleotide-binding oligomerization domain-like (NODlike) receptors, detect pathogens or danger signals, and engage-

Authorship note: MAS and SSS contributed equally to this work. Conflict of interest: The authors have declared that no conflict of interest exists. License: Copyright 2018, American Society for Clinical Investigation. Submitted: February 19, 2018; Accepted: August 16, 2018. Reference information: J Clin Invest. 2018;128(11):4912–4923. https://doi.org/10.1172/JCl120612. ment of these PRRs by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) activates the respective immune cells (3-6). Receptors that bind to PAMPs and DAMPs have also been implicated in cancer progression and anticancer immunity (6, 7). In addition to these well-described pathways, sialoglycans have been shown to serve as a self-associated molecular patterns (SAMPs) by binding to CD33-related sialic acid-binding immunoglobulin-like lectins (CD33rSiglecs) (8, 9), a family of receptors on immune cells containing intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM) (10-13). Unlike the conserved Siglecs (Siglec-1, Siglec-2, Siglec-4, and Siglec-15), CD33rSiglecs, including Siglec-3 (CD33), Siglec-5, Siglec-6, Siglec-7, Siglec-8, Siglec-9, Siglec-10, Siglec-11, Siglec-14, and Siglec-16, have evolved rapidly due to multiple selection forces, including evolutionary pressure by pathogens that can mimic sialoglycan-SAMPs (Sia-SAMPs) and bind to inhibitory CD33rSiglecs to escape innate immune control (14-18). The different Siglecs have different binding preferences for sialoglycan ligands (9). While Siglec-8 has a more defined spectrum of ligands, which include 6'-sulfo-sialyl Lewis x (19, 20), Siglec-9 has a much broader binding spectrum of sialylated ligands (9, 21). Siglec-9 is therefore a typical PRR for Sia-SAMPs.

Recent evidence suggests that the upregulation of Sia-SAMPs can facilitate evasion of immune control during cancer progression

by engaging inhibitory CD33rSiglecs, such as Siglec-7 and Siglec-9 (21-26). Engagement of Siglec-7 and Siglec-9 inhibits NK cellmediated tumor cell killing in vitro (22, 23). Siglec-9 and its murine functional paralog Siglec-E have also been implicated in myeloid cell-mediated cancer progression (21, 24). Binding of the cancerassociated sialylated glycoform of MUC1 to Siglec-9 on macrophages can induce a tumor-associated macrophage (TAM) phenotype that can promote cancer progression and immune evasion (25).

In chronic infections and cancer, expression of checkpoint molecules, including PD-1, CTLA-4, TIM-3, and LAG-3, is observed on T cells and is associated with a state of T cell dysfunction termed T cell exhaustion (27). Chronic infections, such as HIV, have also been shown to upregulate inhibitory CD33r-Siglecs on peripheral CD4⁺ T cells, which suggests that inhibitory Siglec receptors might be involved in T cell immunoregulation (28). In contrast, previous analyses have found very low expression of CD33rSiglecs on peripheral T cells of healthy humans (29) and mice (30). We therefore sought to characterize Siglec expression on tumor-infiltrating T cells and to test the functional role of Siglecs as new potential immune checkpoints that are regulated by glycans within the tumor microenvironment.

Results

Siglec-9 is upregulated on tumor-infiltrating T cells. We analyzed the expression of inhibitory CD33rSiglecs on immune cells in primary NSCLC samples (Supplemental Figure 1A; supplemental material available online with this article; https://doi. org/10.1172/JCI120612DS1). As previously described, CD33r-Siglecs, including Siglec-7 and Siglec-9, were expressed on NK cells and Siglec-9 was also expressed on myeloid cells (Supplemental Figure 1B). Several inhibitory CD33rSiglecs, including Siglec-3 (CD33), Siglec-5, Siglec-7, Siglec-10, and Siglec-9, were expressed on a significant proportion of tumor-infiltrating lymphocytes (TILs), including CD4+ and CD8+ T cells (Figure 1, A-G, and Supplemental Figure 1, C-F). Siglec-9 was the most prominently and consistently expressed CD33rSiglec on TILs across patients (Figure 1G). Lower levels of Siglec-9 were observed on peripheral blood T cells from healthy donors (Figure 1, A-C) or in spleens from patients undergoing splenectomy for nonmalignant disease (Supplemental Figure 1G). There were a few healthy donors that had a larger population of Siglec-9-expressing CD8+ T cells (Figure 1C). In order to further understand the heterogeneity of Siglec-9 expression, we further stained for Siglec-9 in an additional and better-characterized population of 49 healthy donors (Supplemental Figure 1H). The distribution of Siglec-9 on CD8⁺ T cells was similar in both cohorts, and no correlation with age could be seen (Supplemental Figure 1I). No significant difference was seen between sexes (9% in men, 7.2% in women Sig9⁺CD8⁺ T cells of total CD8⁺ T cells). We found an inverse correlation of Sig9+CD8+ with naive CCR7+CD45RA+ T cells (Supplemental Figure 1J). Immunostaining of intratumoral leukocytes showed that Siglec-9 was typically expressed on small lymphocytes and macrophages (Figure 1E). Double staining showed that Siglec-9-positive TILs in NSCLC samples also expressed CD3 (Figure 1F). We also found an upregulation of Siglec-9 on colorectal cancer (CRC) and epithelial ovarian cancer (EOC) TILs or pleural effusions (Supplemental Figure 1K).

There was a significant increase of Sia-SAMPs (Siglec-7 and Siglec-9 ligands) in lung carcinomas compared with healthy lung tissue as well as in acute and chronic inflammatory diseases, as assessed with Siglec-Fc proteins (Figure 1, H and I). Ligands were strongly present on cytokeratin-positive cells, suggesting they were in a trans position, and a trend toward an increase in ligands at higher stages was observed (Supplemental Figure 1L). As Siglec-9 was the most prominent and consistent inhibitory CD33rSiglec upregulated on TILs across different patients, we focused our further analysis on Siglec-9.

Characterization of Siglec-9-expressing intratumoral CD8⁺ T cells. We aimed to further characterize the Sig9+CD8+ TILs in samples from NSCLC patients by multicolor flow cytometry. Sig9+CD8+ TILs coexpressed several other inhibitory receptors, including PD-1 in particular and also TIM-3 and LAG-3 (Figure 2, A-D, and Supplemental Figure 2, A-D). Most Sig9+CD8+ TILs were found within the population with the highest PD-1 expression (PD-1^{hi}, Supplemental Figure 2A). However, not all the PD-1^{hi}CD8⁺ T cells expressed Siglec-9 (Supplemental Figure 2B), suggesting that Sig9*CD8* TILs are a subpopulation of tumor-specific PD-1hi TILs. Sig9+CD8+ T cells also expressed high levels of the transcription factor Eomesodermin (Eomeshi) and low levels of T-bet (T-bet¹⁰, Supplemental Figure 2E). In general, Sig9⁺CD8⁺ TILs had more inhibitory receptors upregulated and coexpressed than Sig9 CD8+ TILs from NSCLC patients (Figure 2E). Several costimulatory receptors were also enriched on Sig9+CD8+ TILs as compared with Sig9⁻CD8⁺ TILs (Supplemental Figure 2, F-J). RNA sequencing (RNA-seq) revealed that several genes were differentially regulated between Sig9+CD8+ TILs and Sig9-CD8+ TILs (Supplemental Figure 2K). The 3 main genes upregulated in Sig9+CD8+ TILs were SPP1 (osteopontin), Ki67, and KLF4 (Figure 2F). We also looked for the expression of genes involved in the generation of Sia-SAMPs. Expression of the rate-limiting enzyme for sialic acid biosynthesis, UDP-N-acetylglucosamine-2-epimerase (GNE), was higher in Sig9⁺CD8⁺ TILs than in Sig9⁻CD8⁺ TILs (Supplemental Figure 2L). Consequently, an upregulation of sialoglycans, as shown by increased staining with Sambucus nigra lectin (SNA) (Supplemental Figure 2M), and an upregulation of cis ligands on TILs, as shown by increased staining with Siglec-9-Fc protein, occurred (Supplemental Figure 2N). Protein levels of osteopontin (SPP1) (Supplemental Figure 2, O and P) and Ki-67 (Supplemental Figure 2Q) were also significantly higher in Sig9⁺CD8⁺ TILs than in Sig9⁻CD8⁺ TILs (Supplemental Figure 3 shows the gating strategy for sorting of primary T cells from tumor samples).

Sig9⁺ TILs are a distinct subset within the CD8⁺ T cell population. We further tested to determine whether Siglecs are upregulated by activation of T cells. While Siglec-5 expression increased significantly on polyclonally activated CD8⁺ T cells from healthy donors, Siglec-9 was only slightly upregulated and Siglec-7 expression was unchanged (Supplemental Figure 4A). Antigen-specific stimulation of T cell clones with reactivity against peptides from influenza antigens, CMV antigens, and melanA led to a slight upregulation of Siglec-9 (Supplemental Figure 4B). Sig9⁺CD8⁺ TILs from NSCLC samples activated with anti-CD3/anti-CD28 antibodies expressed significantly higher surface levels of CD25 (Figure 3A), CD69 (Figure 3B), and the newly identified activation marker Siglec-5 (Supplemental Figure 4C) compared with similarly acti-



Siglec-9 expression on CD8⁺ T cells in PBMCs from healthy donors (left panel), PBMCs from NSCLC patients (middle panel), and TILs from a matched NSCLC patient (right panel). (**B** and **C**) Quantification of Siglec-9 expression on CD4⁺ (**B**) and CD8⁺ (**C**) TILs from NSCLC patients (PBMCs, n = 36; NSCLC, n = 41; mean \pm SD). Statistical analysis by unpaired Student's t test. (**D**) Paired analysis of CD8⁺ T cells from the peripheral blood and tumors of NSCLC patients (n = 20). Statistical analysis by paired Student's t test. (**E**) Immunohistochemical staining of Siglec-9–positive cells in NSCLC sections. Scale bars: 50 μ m. (**F**) Representative immunofluorescence analysis of CD3 and Siglec-9 double-positive cells and Siglec-9 staining or SNA staining and Siglec-9 staining (right panel). Scale bars: 30 μ m (left panel); 50 μ m (right panel).(**G**) Heatmap of expression analysis of different Siglecs in NSCLC samples on CD4⁺ and CD8⁺ TILs. (**H** and **I**) Immunofluorescence study in paraffin-embedded tissue microarrays using recombinant Siglec-9–Fc (human lgG1) fusion protein coupled to secondary PE-conjugated (Fab')2 goat anti-human Fc antibody. Representative images (**H**) and Siglec ligands quantification summary (**I**) are shown. Original magnification, ×400. Scale bars: 50 μ m. Fluorescence values were normalized against an lgG1 isotype control. Lung tissue, n = 5; adjacent lung tissue, n = 9; squamous cell carcinoma (SCC), n = 20; adenocarcinoma, n = 20; small cell lung cancer (SCLC), n = 10; broncho-alveolar carcinoma (BAC), n = 10; atypical carcinoid, n = 5. Data are shown as mean \pm SEM. Statistical analysis performed by 1-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001.



Figure 2. Sig9*CD8* TILs coexpress inhibitory receptors. (**A** and **B**) Expression of PD-1 in primary NSCLC samples on Sig9*CD8* (Sig9*) or Sig9*CD8* (Sig9*) TILs (**A**, n = 44) and representative FACS analysis (**B**). Statistical analysis by paired Student's *t* test. (**C** and **D**) Expression of TIM-3 (**C**, n = 71) and LAG-3 (**D**, n = 18) on Sig9*CD8* or Sig9*CD8* TILs from NSCLC samples. Statistical analysis by paired Student's *t* test. (**E**) Analysis of the number of coexpressed inhibitory receptors on Sig9*CD8* or Sig9*CD8* TILs. (**F**) Volcano plot of RNA-seq on sorted TILs according to their Siglec-9 expression. The 3 significantly differentially expressed genes were MIK67 (Ki67), KLF4, and SPP1. *P < 0.05; ***P < 0.001. Data are presented as mean ± SD.

vated Sig9⁻CD8⁺ TILs. Similar results were seen for polyclonally activated peripheral Sig9+CD8+ cells from healthy donors (Supplemental Figure 4, D-G). CD8⁺ T cells from NSCLC samples were clearly dysfunctional or exhausted, as demonstrated by reduced production of cytokines such as IFN- γ and TNF- α compared with that of CD8+ T cells from healthy donor peripheral blood mononuclear cells (PBMCs) (Figure 3, C and D). However, restimulation of TILs from NSCLC patients with anti-CD3/anti-CD28 antibodies showed that Sig9+CD8+ TILs were much easier to restimulate and secrete at higher levels of multiple cytokines, including IFN- γ , than Sig9⁻CD8⁺ TILs (Figure 3, C and D, Supplemental Figure 5, A-N). In addition, Sig9⁺CD8⁺ cells expressed a distinct pattern of chemokine receptors, including CXCR3, CXCR5, CCR4, CCR6, and CX3CR1 (Supplemental Figure 5, O-T). A recent study also found a subpopulation of PD-1^{hi}CD8⁺ TILs with a distinct, higher functionality (31); this subpopulation was associated with an increased expression of CD5 and a lower expression of CD38 and CD101 (31). We therefore stained for these markers and found an enrichment of CD5 and Ki67 expression (Figure 3, E and F) on Sig9⁺ cells in the PD-1^{hi} gate as well as a reduced presence of CD38⁺ and CD101⁺ (Figure 3G, Supplemental Figure 6). Our data provide evidence that intratumoral Sig9⁺CD8⁺ cells are a distinct subpopulation of tumor-specific CD8⁺ TILs.

Sia-SAMP/Siglec-9 interaction is a target for improving T cell activation. Next, we wanted to explore the functional implication of Siglec expression on T cells. We used the high-affinity CD33rSiglec ligand LGALS3BP, which is upregulated in NSCLC (24). LGALS3BP inhibited CD8⁺ T cell activation in a sialic aciddependent fashion (Figure 4A, Supplemental Figure 7, A and B). We further generated cell lines that expressed lower surface levels of Sia-SAMPs by knocking out GNE. GNE-deficient A549 lung adenocarcinoma cells showed lower binding to Siglec-9 compared

with WT A549 cells (Figure 4B). Feeding GNE-KO A549 cells with N-acetylneuraminic acid (Neu5Ac) to metabolically bypass the mutation led to a recovery of ligands on the cell surface and binding to Siglec-9 (Figure 4B). CD8+ T cell-mediated tumor cell killing was tested by coincubation of T cells from healthy donors and different A549 tumor cell variants in the presence of a CD3/ EpCAM T cell-bispecific (TCB) antibody (catumaxomab). T cellmediated killing of GNE-KO A549 cells and desialylated A549 cells was higher compared with that of WT A549 cells and GNE-KO A549 cells fed with Neu5Ac (Figure 4C). Similar results were obtained with catumaxomab when using HT29 tumor cells (Supplemental Figure 7C and Figure 4D) and TILs obtained from primary NSCLC samples together with A549 and HT29 cells (Figure 4, E and F). Experiments with the anti-CD3/CD19 TCB antibody blinatumomab and CD19⁺ Ramos cells (Figure 4G and Supplemental Figure 7D) or primary CLL samples (Figure 4H) showed similar results. Sig9+CD8+ T cells from NSCLC TILs were significantly more reactive toward GNE-KO A549 cells than Sig9⁻ TILs, with the difference being sialic acid dependent (Figure 4I). Similarly, Sig9⁺CD8⁺ T cells induced more apoptosis in HT-29 and Ramos cells, respectively, than Sig9⁻CD8⁺ T cells (Supplemental Figure 7, E and F).

To test the effect of Siglec-9 blockade on T cell activation in vitro, we used a Siglec-9 antibody and the previously described staphylococcal enterotoxin B (SEB) test (32). Two full IgG Siglec-9 antibodies (191240 and E10-286) inhibited T cell activation and therefore were agonistic (Supplemental Figure 7, G and H). Moreover, Siglec-9 antibodies led to a dose-dependent inhibition of T cell activation tested by upregulation of the activation markers CD25 (Figure 4J) and CD69 (Supplemental Figure 7I). Addition of the Siglec-9 antibody (clone 191240) inhibited IL-2 production and CD69 expression on the surface of TILs upon SEB stimulation of



Figure 3. Sig9*CD8* TILs are a distinct subset within intratumoral CD8* T cells. (**A** and **B**) Upregulation of the activation markers CD25 (**A**) and CD69 (**B**) on Sig9⁻CD8* or Sig9* TILs sorted from primary NSCLC samples and activated with anti-CD3/28 antibodies for 48 hours (n = 9). Statistical analysis by paired Student's t test. (**C**) ELISA analysis of IFN- γ in the supernatant of sorted Sig9⁻CD8* T cells or Sig9*CD8* T cells (n = 3-7, independent patients). Cells were sorted from PBMCs of healthy donors or primary NSCLC samples (TILs). Act, activated. Supernatants from 50,000 cells were analyzed. (**D**) Analysis of TNF- α in the supernatant of sorted Sig9⁻CD8* cells from healthy donors or NSCLC patient samples (n = 3-7, independent donors/patients). Statistical analysis performed by 1-way ANOVA. (**E**) Expression level of CD5 in the CD8*PD-1^{hi} population on Sig9⁻ TILs and Sig9* TILs (n = 9). (**F**) Percentage of Sig9⁻CD8* TILs or Sig9*CD8* TILs in primary NSCLC samples that express Ki67 within the PD-1^{hi} population (n = 9). (**G**) Frequency of CD38^{hi}CD101^{hi} cells on Sig9⁻ and Sig9*CD8*PD-1^{hi} TILs determined by flow cytometric analysis (n = 13). Statistical analysis by paired Student's t test. *P < 0.05; **P < 0.01; ***P < 0.001. Data are presented as mean \pm SD.

primary NSCLC samples (Figure 4, K and L). We hypothesized that bivalent binding and subsequent dimerization of Siglec-9 might be responsible for the agonistic effect on Siglec-9 by the clone 191240. Indeed, monovalent carbohydrate-recognition domainblocking (CRD-blocking) Fab fragments from clone 191240 (Supplemental Figure 7J) significantly increased IL-2 (Figure 4K), but not CD69 (Figure 4L), production upon SEB stimulation. These findings demonstrate that T cell activation in NSCLC tumors can be increased by targeting Sia-SAMPs/Siglec-9 interactions.

Siglec-E is expressed on TILs in mice. We further investigated whether the murine functionally equivalent paralog (Siglec-E) was upregulated on TILs in mouse models. We found a significant upregulation of Siglec-E on murine CD8+ TILs when compared with splenocytes from control mice and tumor-bearing mice in the MC38 subcutaneous tumor model (Figure 5A) and other models (LLC, B16, and EMT6 subcutaneous tumor models; Supplemental Figure 8A). While other inhibitory CD33rSiglecs, including Siglec-F and Siglec-G, were also upregulated on the surface of murine TILs, Siglec-E was the most prominently upregulated inhibitory Siglec tested (Supplemental Figure 8B). We further analyzed the phenotype of SigE+CD8+ murine TILs. The proliferation marker Ki67, the activation markers granzyme B and CD69, and the activating receptor CD27 (Figure 5B, and Supplemental Figure 8, C-E) were all significantly increased on murine SigE⁺CD8⁺ TILs compared with SigE-CD8+ TILs, similarly to what is seen in human TIL samples. The expression and number of murine inhibitory receptors, including PD-1, were also higher on SigE+CD8+ TILs than on SigE⁻CD8⁺ TILs (Figure 5, C–F, and Supplemental Figure 8, F and G). SigE⁺CD8⁺ TILs were predominantly terminally differentiated Eomes^{hi}, Tbet¹⁰, and CD127⁺ T cells (Supplemental Figure 8H). Sorted murine SigE⁺CD8⁺ TILs again showed a stronger reactivation than SigE⁻CD8⁺ TILs, as shown by upregulation of the activation markers CD25 and CD69 (Figure 5G). Taken together, these findings suggest that SigE⁺CD8⁺ TILs in mice are phenotypically similar to Sig9⁺CD8⁺ TILs in humans.

Sia-SAMPs mediate immune escape in vivo. To further investigate the role of Sia-SAMPs in immune evasion, we generated GNE-deficient MC38 cells and compared their subcutaneous growth with that of WT MC38 tumor cells. GNE-KO MC38 tumors showed a clear delay in tumor growth compared with WT MC38 tumors injected subcutaneously in C57BL/6 mice (Figure 5H). Similar observations were made for GNE-KO and WT EMT6 tumors injected subcutaneously into BALB/c mice (Figure 5I). The GNE-KO cell lines showed no growth differences or survival differences in vitro (Supplemental Figure 8I). The frequencies of CD3⁺, CD8⁺, and CD4⁺ T cells in leukocyte infiltrates were significantly higher in GNE-KO MC38 tumors compared with WT MC38 tumors (Figure 5, J and K, and Supplemental Figure 8J), whereas there was no difference in the infiltration of regulatory T cells (Supplemental Figure 8J). GNE-KO tumor infiltrates also contained more classical dendritic cells (CD11c+MHCII+ cells within CD45+ cells) than WT MC38 tumors, whereas other myeloid cell types did not differ significantly (Supplemental Figure 8J). In order to analyze the role of adaptive immunity, we repeated the experiment in



Figure 4. Sia-SAMPs inhibit T cell-mediated tumor cell killing in vitro. (**A**) Inhibition of T cell activation by LGALS3BP in a dose-dependent manner measured by intracellular IFN- γ by flow cytometry. CD8⁺ T cells from healthy donors were activated with anti-CD3 and anti-CD28 antibodies in the presence of increasing amounts of LGALS3BP (μ g/ml, *n* = 3). (**B**) Representative histograms of binding of Sig9-Fc to A549 WT cells, enzymatically desialylated A549 cells (desia), GNE-deficient A549 cells (GNE-KO), and GNE-KO A549 cells refed with 10 mM Neu5Ac. (**C**) Percentage of cleaved caspase-3-positive (clCasp3⁺) WT A549 cells, desialylated A549 cells, GNE-KO A549 cells, or GNE-KO A549 cells fed with Neu5Ac (refed) after incubation with CD8⁺ T cells and catumaxomab (*n* = 10). (**D**) Apoptosis of WT, desialylated, GNE-KO, and refed GNE-KO HT-29 cells measured by upregulation of cleaved caspase-3 in tumor cells (*n* = 6). (**E** and **F**) clCaps3⁺ A549 (e, *n* = 11) or HT-29 (F, *n* = 11) tumor cells after coincubation with TILs from NSCLC or CRC samples. (**G**) CD8⁺ T cells were sorted according to their Siglec-9 expression and incubated with either WT or GNE-KO A549 cells (*n* = 4). (**H**) CD19⁺ RAMOS cells incubated with CD8⁺ T cells from healthy donors in the presence of CD3 and CD19 bispecific antibody blinatumomab (*n* = 7). (**I**) GNE-KO RAMOS cells incubated with CD8⁺ T cells from patients with chronic lymphocytic leukemia (*n* = 3). (**J**) Activation measured by CD25 on CD8⁺ T cells treated with anti-CD3 and anti-CD28 antibodies in the presence of anti-Siglec-9 antibody (clone 191240, *g*/ml, *n* = 4). (**K**) Relative IL-2 production of NSCLC primary tumor samples incubated with SEB and Siglec-9-blocking antibody and the Fab fragments (clone 191240, *n* = 5). (**L**) Measurement of CD69 upregulation on CD8⁺ TILs from NSCLC patients upon incubation with SEB in the presence of anti-bodies or Fab fragments (*n* = 5). Statistical analyses in this figure were performed by 1-way ANOVA. Data are pres

mice deficient for the adaptive immune system (NOD scid gamma [NSG] mice). Both MC38 and EMT6 GNE-KO cell lines showed no growth difference when compared with WT cell lines in NSG mice (Supplemental Figure 8K). From these findings, we concluded the sialylation directly affects tumor growth and also the antitumor immune response by enhancing the adaptive immune response to cancer (Supplemental Figure 9 shows the gating strategy for determining Siglec-E expression on tumor-infiltrating T cells).

Expression of Siglec receptors on T cells directly modulate antitumor immunity. To further investigate the role of Sia-SAMPs and their interactions with CD33rSiglecs, we used a human Siglec-9 transgenic mouse (HS9) that allows selective expression of Siglec-9 in cells producing Cre recombinase (21). Previous analysis demonstrated that human Siglec-9 binds to ligands on murine tumor cells (21). HS9 mice were crossed with CD4-Cre mice to express human Siglec-9 in T cells. These mice expressed Siglec-9

in both CD4⁺ and CD8⁺ T cells (Supplemental Figure 10A). The growth of syngeneic MC38 tumor cells was accelerated in mice expressing Siglec-9 in T cells, and the tumors grew larger compared with those in littermate control mice (Figure 6A). This finding supports the hypothesis that inhibitory Siglecs on T cells can mediate immune evasion. We also used the recently developed Siglec-E16 (SigE16) transgenic mice (18). These mice express a chimeric Siglec receptor with the extracellular domain of Siglec-E and the transmembrane and intracellular domain of the activating human Siglec-16 receptor under the Siglec-E promoter (18). No major differences were observed between the frequency of naive and memory T cells in different genetic animal models (Supplemental Figure 10B). Siglec-E expression in WT and SigE16 mice was similar (Supplemental Figure 10C). Compared with those in WT littermate control mice, subcutaneous syngeneic MC38 tumors grew more slowly and remained smaller in homozygous



Figure 5. Sialylated SAMPs enhance immune escape and tumor growth in vivo. (**A**) Siglec-E expression was determined by flow cytometry on control splenocytes, splenocytes from tumor-bearing mice, and CD8⁺ TILs from subcutaneous MC38 tumors (n = 25-28). Statistical analysis performed by 1-way ANOVA. (**B**) Expression of intracellular Ki67 was examined by flow cytometry on SigE⁻CD8⁺ and SigE⁺CD8⁺ TILs (n = 18). Statistical analysis by paired Student's *t* test. (**C**–**E**) Frequencies of inhibitory immune receptor expression on SigE⁻CD8⁺ and SigE⁺CD8⁺ TILs from MC38 tumors, as studied by flow cytometry. PD-1 (**C**, n = 16), TIM-3 (**D**, n = 18), and LAG-3 (**E**, n = 7) were analyzed. Statistical analysis by paired Student's *t* test. (**F**) Number of coexpressed inhibitory receptors on SigE⁻CD8⁺ or SigE⁺CD8⁺ TILs. (**G**) Upregulation of CD25⁺CD69⁺ upon restimulation of sorted SigE⁻CD8⁺ and SigE⁺CD8⁺ and SigE⁺CD

SigE16 mice (Figure 6B). MC38 tumors had an increased induction of antitumor antibodies (Supplemental Figure 10D). Depletion experiments demonstrated the dependence of the effect on both CD4⁺ and CD8⁺ T cells and directly showed that the genetic reversal of an inhibitory into an activating Siglec receptor can also influence T cell activation (Figure 6, C and D). We also used an adoptive transfer model to study the role of Siglec-E on T cells. We transferred OT-I T cells expressing either WT Siglec-E or activating SigE16 into WT mice with OVA-expressing MC38-OVA tumors and measured the growth of tumors. MC38-OVA tumors in mice transferred with OT-I from SigE16 mice had significantly reduced tumor growth (Figure 6E). In addition, OT-I T cells expressing SigE16 had increased proliferation (Supplemental Figure 10E). In addition, a trend toward increased infiltration was also seen in mice adoptively transferred with SigE16-expressing OT-I T cells (Supplemental Figure 10F). To determine the effect of sialoglycans in trans position on the growth difference in SigE16 mice, we used MC38 WT and GNE-KO cells. The previous growth difference could no longer be seen in the SigE16 background (Supplemental Figure 10G). This finding suggests that the effect seen in SigE16 mice is mediated by interactions of trans ligands with activating SigE16 receptor.

Sig9⁺ TILs and Siglec-9 polymorphisms are associated with clinical end points. We further analyzed the correlation of Siglec expression on T cells with clinical parameters. Patients with a high frequency of Sig9+ TILs (>30% of CD8+ T cells) had significantly worse overall survival (Figure 6F). The significance was slightly reduced in a multivariate analysis taking age and also stage into account (P = 0.0668 with a hazard ratio of 14.6 by a Cox proportional hazard regression analysis). Recently, polymorphisms of Siglec-9 were associated with pulmonary diseases (33). We studied to determine whether the risk of developing lung cancer was associated with the rs2075803 and rs2258983 polymorphisms in the TRICL4 cohort (34). The risk of developing squamous cell lung cancer was significantly reduced in the presence of these polymorphisms (Table 1). We also examined the polymorphisms rs2075803 and rs2258983 in 3,795 cases of CRC and 3,044 controls to estimate the relative risk of developing CRC. Individuals



Figure 6. Engagement of inhibitory Siglecs on T cells mediates immune escape. (**A**) Subcutaneous growth curves of MC38 tumors in littermate Siglec-9 transgenic control mice (HS9) or Siglec-9 transgenic mice crossed to CD4-Cre mice (HS9, CD4Cre) (n = 8-10). The experiment was repeated 3 times. Statistical analysis by 2-way ANOVA. (**B**) Tumor volumes after 21 days of subcutaneous MC38 tumors in littermate control mice (E/E) or homozygous (E16/E16) mice that express the chimeric receptor SigE16 (n = 7). Statistical analysis performed by 1-way ANOVA. (**C**) Subcutaneous growth curves of MC38 tumors in E16 mice or littermate control mice after CD4 and CD8 cell depletion by antibodies (n = 7-8). The experiment was repeated 2 times. Statistical analysis by 2-way ANOVA. (**D**) MC38 tumor volumes after 21 days in E16 mice and littermate control mice and independent depletion of CD4⁺ and CD8⁺ T cells. (**E**) Tumor volume of MC38-OVA tumors after adoptive transfer of OVA-specific OT-I CD8⁺ T cells from WT or SigE16 (E16) mice. Statistical analysis performed by 1-way ANOVA. *P < 0.05; **P < 0.01. Data are presented as mean ± SD. (**F**) Survival analysis of NSCLC patients with low (n = 18) and high percentage (above 30%, n = 11) of Siglec-9 expression on their CD8⁺ T cells. Differences were analyzed by Wilcoxon's test. A multivariate analysis of the 2 groups for age and stage showed slightly reduced significance, with a P value of 0.0669 (multivariate, univariate analysis P = 0.0097) and a hazard ratio of 14.6.

who were homozygous A for the rs2075803 SNP and homozygous C for the rs2258983 SNP had a significantly reduced risk of developing CRC (Supplemental Table 1).

Discussion

Recent studies have shown that inhibitory CD33rSiglecs can modulate the interactions of immune cells with tumor cells by sialic acid-dependent mechanisms (21-23, 25, 26), raising the possibility that these Siglecs could be used for therapeutic checkpoint inhibition, analogous to the remarkable recent therapeutics directed against PD-1 or CTLA-4 (2). We and others have shown that T cells from healthy humans express low levels of CD33related Siglecs (29, 35). However, we show here that expression of the inhibitory Siglec-9 is clearly increased on CD4⁺ and CD8⁺ TILs compared with peripheral T cells from healthy donors. We also observed the presence of Siglec ligands in both cis and trans positions. In addition, we confirmed previous observations that have shown a small population of peripheral T cells with Siglec-7 and Siglec-9 expression (35). Properly glycosylated CD52 has previously been identified as a ligand for Siglec-10 on human T cells (36). Furthermore, inhibitory mouse Siglec-G (often referred to as the murine paralog of human Siglec-10) was found to inhibit DAMP-associated T cell activation (37). These investigations demonstrate that Siglecs can dampen T cell responses in the context of general inflammation.

How Siglec-9 is upregulated on TILs and how Siglecs are regulated in T cells, including intracellular signaling, remain unclear. We observed only a slight upregulation of Siglec-9 upon polyclonal and antigen-specific stimulation of healthy T cells. Other immune checkpoint receptors are upregulated through repetitive antigenic stimulation or the tumor microenvironment, including inhibitory cytokines (27). We have seen a similar effect in several mouse models, indicating that the mechanism for Siglec upregulation in T cells appears to be conserved across species, even though Siglec receptors have diverged quite a lot between mice and humans. We observed a heterogeneity of the frequency in Sig9+CD8+ T cells in the peripheral blood of healthy donors and an inverse correlation with the frequency of naive CD8+ T cells. This finding suggests that healthy individuals with more circulating peripheral memory T cells also have a higher number of Sig9⁺CD8⁺ T cells. Until now, mainly Siglec-9-expressing NK cells and myelomonocytic cells have been associated with immune modulation in cancer. Myeloid cells were more reactive in a mouse model of Siglec-E deficiency, and increased immunosurveillance was observed in these mice (21). Engagement of Siglec-9 on monocytes/macrophages by a cancer-associated, sialylated glycoform of MUC1 showed polarization toward immune-suppressive TAMs with upregulation of PD-L1 (25). Two analyses examined the function of inhibitory Siglec-7 and Siglec-9 in NK cell-mediated tumor cell killing (22, 23). Other immune receptors, including PD-1, are also expressed

	rs Number	Reference allele	Effect allele	OR	95% CI (L)	95% CI (U)	P value
NSCLC	rs2075803	А	G	0.998	0.963	1.035	0.92
NSCLC	rs2258983	C	А	0.998	0.962	1.035	0.91
Adenocarcinoma	rs2075803	А	G	1.03	0.974	1.09	0.29
Adenocarcinoma	rs2258983	C	А	1.036	0.978	1.097	0.23
Squamous	rs2075803	А	G	0.939	0.888	0.993	0.027
Squamous	rs2258983	C	А	0.936	0.884	0.991	0.023

Table 1. Risk for lung cancer depending on Siglec-9 polymorphisms

95%CI (L), 95% lower CI; 95%CI (U), 95% upper CI. P value by Cox proportional regression analysis, multivariate analysis for age, sex, and top significant principal components from previous studies.

on myeloid cells and NK cells within the tumor (38, 39). Further experiments are needed to understand the functional relevance of these immune-modulatory receptors, including Siglec-9 or PD-1, on cell types other than T cells. However, it is likely that CD8⁺ T cells continue to play central roles in cancer immunotherapy, and we define Siglec-9 on CD8⁺ TILs as a potential therapeutic target for cancer immunotherapy.

Further characterization of Sig9+CD8+ TILs revealed that these cells coexpress several inhibitory receptors, including PD-1, TIM-3, Lag3, and others. Our analysis suggests that Sig9+CD8+ TILs are a subpopulation of CD8+PD-1hi TILs, which are often tumor-specific (40). Coexpression of multiple inhibitory receptors on T cells and also PD-1^{hi} TILs was associated with an exhaustion phenotype in cancer, including in patients with NSCLC (27, 41). Indeed, functional analysis of TILs from NSCLC patients showed a significantly reduced capacity to produce cytokines and chemokines (Figure 3). However, Sig9+CD8+ T cells from NSCLC patients were easier to reactivate than Sig9⁻CD8⁺ T cells despite the increased expression of PD-1 and other inhibitory immune receptors on Sig9+ TILs. This finding suggests that Sig9+CD8+ TILs belong to a specific subtype within PD-1hi TILs. A recent analysis of chromatin states in murine and human TILs from melanoma and NSCLC patients demonstrated a subpopulation within PD-1hi TILs that had a relatively higher expression of CD5 and lower expression of CD38 and CD101 and that showed a greater potential for reprogrammability and reactivation (31). Our findings showed an enrichment of high CD5 expression and low CD38 and CD101 expression on Sig9+CD8+ TILs in the PD-1hi gate. This finding could be interpreted as an overlap of Sig9⁺CD8⁺ TILs with this previously described tumor-specific TIL subpopulation that could be potentially reactivated by immunotherapy. Other studies have also shown that, not only exhausted TILs coexpress a high frequency of inhibitory receptors, but also activated TILs (42). This could mean that the Sig9⁺CD8⁺ TILs are in a higher activation state that could potentially be uncovered by blocking these different inhibitory receptors (43).

Our analysis also includes functional studies both in vitro with human tissue and in vivo in different mouse models to demonstrate the relevance of inhibitory Siglecs on TILs and their interactions with cancer-associated Sia-SAMPs. The previous studies have provided evidence that Sia-SAMP/Siglec interactions can inhibit immune cell activation in in vitro models (21–23, 25). The use of bivalent anti-Siglec-9 antibodies (clones 191240 and E10-286) led to an inhibition of T cell activation, which might be due

to the activation of Siglec-9 signaling by dimerization. In contrast, the use of monovalent Fab fragments of the antibody clone 191240, which inhibits Sia-SAMP/Siglec-9 interactions by hampering ligand binding to the CRD, led to increased T cell activation. This result suggests that the Sia-SAMP/Siglec-9 interaction can be targeted for the enhancement of TIL activation in lung tumors. Functional relevance is further supported by the analysis of a Siglec-9 polymorphism in cancer patients. This shows an association of hypomorphic alleles with the risk of developing squamous NSCLC. In addition, the same alleles were also associated with the risk for CRC. These polymorphisms were previously associated with an increased frequency of pulmonary emphysema, exacerbations of chronic obstructive pulmonary disease, and a hyperactive immune response of myeloid cells (33). While the function of these polymorphisms needs to be tested in T cells, their linkage to disease outcome suggests a role for Siglec-9 in the development of lung and CRC.

Our studies also show that the inhibitory Siglec-E receptor, the functional paralog of Siglec-9 and Sia-SAMPs in the tumor, influences the T cell-mediated immune response to tumors in mouse models. Reduction of Sia-SAMPs by using GNE-KO tumor cell lines led to a reduced growth of tumors. In particular, the use of the more antigenic cell line EMT6-HER2 showed rejection of the tumors in some mice when hyposialylated GNE-KO tumor cells were used. The KO of GNE in those cell lines most likely induces additional changes in glycosylation besides reducing the level of sialylation. An upregulation of polylactosamine could change the interaction with other immune-modulatory lectins, such as galectins (44). Also, during the growth of tumors in mice, the cells could potentially acquire sialic acids from the host. Nevertheless, we observed a relevant growth difference in 2 different models. Previous experiments with B16 melanoma cell lines have also shown similar results, although different approaches were used for the reduction of tumor sialylation (45). Another analysis with hyposialylated methylcholanthrene-induced (MCA-induced) tumors has shown growth inhibition of subcutaneous tumors (46). Overexpression of inhibitory Siglec-9 on CD4⁺ and CD8⁺ T cells led to increased tumor growth, which indicates a functional role of inhibitory Siglecs on T cells. Exchange of inhibitory Siglec-E with activating SigE16 (extracellular Siglec-E domain with transcellular activating human Siglec-16) showed T cell-dependent growth inhibition. In an adoptive transfer model, we demonstrated that Siglec-E on T cells is functionally involved, although the model we used, with OVA-specific OT-I T cells, has certain limitations and OVA is not directly comparable to naturally occurring tumor antigens.

Cancer immunotherapy and, in particular, checkpoint blockade with inhibitors of the PD-1/PD-L1/L2 axis and CTLA-4 are now routinely used in daily oncological practice (1, 47). However, only a minority of cancer patients show objective responses under checkpoint blockade and only a few develop long-term remissions. Thus, combination therapies are a promising approach to improving response rates of immunotherapies and outcomes for patients (48, 49). Our data suggest that targeting the Sia-SAMP/Siglec-9 pathway could improve antitumor immunity and define this pathway as an inhibitory immune checkpoint for T cell activation.

Methods

Patients and sample preparation. Surgical specimens were mechanically dissociated, digested with accutase (PAA Laboratories), collagenase IV (Worthington), hyaluronidase (MilliporeSigma), and DNAse type I (MilliporeSigma), filtered, washed, and frozen for future use. Singlecell suspensions were prepared. Human PBMCs were isolated by density gradient centrifugation using Histopaque-1077 (MilliporeSigma) from buffy coats obtained from healthy blood donors (Blood Bank, University Hospital Basel). Single-cell suspensions and PBMCs were frozen for later use in liquid nitrogen.

Multicolor flow cytometry. For multicolor flow cytometry, dead cells were stained using LIVE/DEAD Fixable Blue Dye (Invitrogen) and various panels of antibodies. Doublets were excluded in all analyses. Corresponding isotype antibodies or fluorescence-minus-one (FMO) samples were used as a control, in particular for the Siglec staining. All tumor samples were analyzed with a Fortessa LSR II Flow Cytometer (BD Biosciences). For infiltration analysis, mice were euthanized and tumors were mechanically dissociated and digested as described for the human sample preparation.

Staining for Siglec-7 and Siglec-9 ligands on tumor microarray. Tissue microarrays from US Biomax were used for screening of Siglec-9 ligand expression in lung cancer samples. For immunofluorescence staining, recombinant human Siglec-hFcs (R&D Systems) were mixed with PE-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories) for 1 hour at 4°C prior to use. For the specific visualization of cancer cells, Anti-Multi-Cytokeratin (Leica Biosystems) staining was performed and visualized with a goat anti-mouse IgG coupled to Alexa Fluor 488 (Life Technologies). The general fluorescence per sample was measured in a ScanRI Microarray Scanner (PerkinElmer) and processed with ImageJ software, version 1.48 (NIH). Pictures were taken using a fluorescence microscope with original magnification of ×400 (Zeiss). Fluorescence was quantified using ImageJ software and normalized to background staining (secondary antibody only).

In vitro T cell activation and inhibition. Murine and human T cells were polyclonally stimulated with anti-CD3 and anti-CD28 antibodies (BioLegend). Anti-CD3 antibodies ($0.5 \mu g/ml$) were either coated overnight at 4°C for human T cells or added for mouse T cells. Anti-CD28 was added at 1 $\mu g/ml$. For inhibition assays with sialylated LGALS3BP, LGALS3BP was coated overnight as well. Activation of T cells was determined by staining for activation and proliferation markers by flow cytometry or measuring cytokines in supernatants by ELISA. For the SEB assay, primary NSCLC samples were incubated at 200,000 cells/well with SEB (10 ng/ml, MilliporeSigma) for 48 hours in the presence of full-length or digested Fab fragments of the 191240

anti–Siglec-9 antibody (R&D Systems). T cell activation was assessed by FACS staining, and IL-2 secretion was measured in the supernatant by ELISA (BioLegend).

RNA-seq and analysis. CD8+ TILs were sorted from frozen NSCLC samples by FACS according to their Siglec-9 expression. Gates used were as follows: lymphocytes, singlets, DAPI-CD3+, and CD8+CD4-. RNA was isolated and the library prepared by Microsynth AG. Next-generation sequencing of the library and data analysis were performed by Microsynth AG. Count data from RNA-seq samples were analyzed using the edgeR Bioconductor package in R. Filtered genes, expressed at more than 1 count per million (cpm) in at least 3 samples, were analyzed using the QLF functions with batch correction for patients. All genes were ranked according to their F statistics comparing Siglec-9-positive and -negative samples. A weighted gene set enrichment analysis (GSEA) was performed using this preranked list using the GSEA java application (http://www.broad.mit.edu/gsea/). Box plots comparing expression levels between Siglec-9-positive and -negative cells were performed in R using logarithmically transformed cpm values. All original microarray data were deposited in the NCBI's Gene Expression Omnibus database (GEO GSE115305).

GNE-deficient cells and in vitro tumor cell killing with CD3/EpCAM TCB or CD3/CD19 TCB. Human and murine GNE-deficient tumor cell lines were created using CRISPR/CAS9. Guide RNAs were designed online using e-crisp.org, synthesized by Microsynth AG, and cloned into the pX458 vector (Addgene). A549, HT-29, and RAMOS cell lines were bought from ATCC. After transient transfection in tumor cells, single-cell sorting and subsequent screening for cell-surface sialylation were performed. Multiple GNE-deficient clones were pooled in order to avoid clonal selection when comparing with WT cell lines.

GNE-deficient cell lines and cell lines were refed with 5 mM Neu5Ac for 24 hours. Coincubation with magnetically isolated CD8⁺ T cells from healthy donors or tumor samples in the presence of catumaxomab (anti-CD3/anti-EpCAM; Fresnius) or blinatumomab, (anti-CD3/anti-CD19; Amgen) was performed at a concentration of 10 ng/ml. Alternatively, the respective cell lines were stained with carboxyfluorescein succinimidyl ester (CSFE) (Sigma-Aldrich) and spiked into full-tumor digests obtained from primary NSCLC or CRC samples. Tumor cell killing was analyzed by FACS staining for cleaved caspase-3 and by staining with the LIVE/DEAD Viability/Cytotoxicity Kit (Thermo Fisher Scientific). Tumor cells were gated by size and expression of EpCAM or CD19, respectively, as well as by negative gating using CD3, CD8, and CD4.

Genetic mouse models for in vivo analysis of Siglec function on T cells. Siglec-9 transgenic mice were described previously (21). Siglec-9 transgenic mice (B6.Cg-Tg[CAG-Siglec-9]1Avrk) were crossed with CD4-Cre mice (B6Tg[CD4-cre]1Cwi) to obtain a T cell-specific overexpression of inhibitory human Siglec-9 in the C57BL/6 background. Transgenic mice expressing SigE16 chimeric Siglec-E with transmembrane and intracellular domain of activating human Siglec-16 (B6.Cg-Siglece < tm4E16Avrk>) were also described previously (18). Tumor cell lines were injected into 8- to 12-week-old female mice, and tumor growth was monitored as described above.

SigE16 was crossed into OT-I transgenic mice. For the adoptive transfer of OVA-specific OT-I cells from WT or SigE16 mice, T cells were harvested from splenocytes and injected into WT C57BL/6 mice bearing subcutaneous MC38-OVA tumors. T cells were followed for proliferation by labeling with Cell Trace Violet (CTV) (Thermo Fish-

er Scientific). Tumor growth was measured, and cell numbers were determined by counting beads and flow cytometry.

Association study of Siglec-9 polymorphism. The association analysis of Siglec-9 polymorphisms in lung cancer was studied on the TRILC cohort (34). In total, 11,348 patients with lung cancer and 15,861 controls were studied for the rs2075803 and the rs2258983 SNPs. An association test was performed after adjusting for age, sex, and top principal components. Then we conducted a fixed effects meta-analysis with an inverse variance-weighted average method to combine the summary data from each association test. Meta-analysis was performed using SAS version 9.4 (SAS Institute Inc.). For the analysis of an association of Siglec-9 polymorphisms with the risk of CRC, SNP genotypes for rs2075803 and rs2258983 among cases and controls from imputed genotype data derived from samples genotyped on the Oncoarray platform and USC Norris Cancer Center were used. Using contingency table analysis (Fisher's test), we calculated odds ratios (OR), 95% CIs, and P values using an additive model and genotype-specific OR.

For additional information, see Supplemental Methods.

Statistics. Statistical significance between 2 groups was determined using 2-tailed Student's *t* tests. Significance among more than 2 conditions was assessed using 1-way ANOVAs with multiple comparisons. For survival curve analysis, 2-way ANOVA was used. Survival analysis was performed by the Gehan-Breslow-Wilcoxon test, and multivariate analysis was done by Cox proportional hazard analysis. *P* < 0.05 was considered statistically significant.

Study approval. The local ethical committee in Basel, Switzerland, approved the sample collection and the use of clinical data (Ethikkomission Nordwest- und Zentralschweiz, EK321/10 and UBE

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15–106). Informed consent was obtained from all patients prior to the sample collection. Mouse experiments were approved by the local committee of Basel Stadt (approval number 2747).

Author contributions

MAS and SSS planned and conducted the experiments and analyzed the data. HL conceived the study and planned the project. MAS, DES, AV, AZ, and HL wrote the manuscript. MPT, DST, KFB, SVG, AT, and FS performed experiments. LT, DL, VHS, MVBB, AE, and MS provided important material for the research. WZ, HJL, YH, and CIA analyzed the Siglec polymorphisms.

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Supplemental Data

Self-associated molecular patterns mediate cancer immune

evasion by engagement of Siglec receptors on T cells

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Supplemental material and methods

Antibodies.

The following antibodies were used for Siglec-9 staining. The blocking clone 191240 was used for flow cytometry staining and blocking experiments (R&D Systems). In some experiments, also the non-blocking monoclonal antibody clone E10-286 was used (BD Biosciences). Fab fragments of the 191240 anti-Siglec-9 antibody were generated using the Pierce Fab Micro Preparation kit (Thermo Scientific). The following antibodies were used for staining of human cells in multicolor flow cytometry analysis: Anti-CD4-BV711 (clone SK3), anti-IFNy-BV421 (4S.B3), anti-PD1-PE-Cy7 (EH12.1), anti-BTLA-PE (J168-540), anti-active caspase 3-PE (C92-605) were purchased from BD; anti-CD45-PerCP Cy 5.5 (2D1), anti-CD3-APC eF780 (SK7), anti-4-1BB-FITC (4B4), anti-CD25-PE (CD25-4E3), anti-IL-2-PE (MQ1-17H12), anti-TNFα-APC (MAb11), anti-Perforin-FITC (dG9), anti-LAG-3-FITC (3DS223H), anti-TIGIT-PE (MBSA43) were purchased from eBioscience; anti-CD8-BV605 (SK1), anti-TIM-3-BV421 (F38-2E2), anti-CD160-eF660 (BY55), anti-CD25-BV421 (BC96), anti-Foxp3-AF647 (259D) were purchased from Biolegend; anti-EPCAM-FITC (HEA-125) was purchased from Miltenyi. For flow cytometry staining of murine cells, the following antibodies were used: anti-CD25 (PC61.5), anti-CD45 (A20), anti-CD3 (145-2C11), anti-CD8a (53-6.7), anti-CD11b (M1/70), anti-CD11c (N418), anti-PD-1 (29F.1A12), anti-Tbet (O4-46) and anti-TIM-3 (RMT3-23). For Mo-MDSC and PMN-MDSC determination, anti-CD11b (M1/70), anti-Ly6C (HK1.4), and anti-Ly6G (1A8) antibodies were used. All FACS antibodies for analysis of murine cells were purchased from Biolegend or BD Biosciences.

Siglec-Fc production.

Siglec-Fcs were produced using previously described Siglec-Fc expression plasmids. Briefly, the three N-terminal domains of Siglec-9 were cloned into a human-Fc fusion vector and mutants generated by site-directed mutagenesis. Plasmids were transfected using polyethylenimine (PEI) into 293T HEK cells, cultured in serum free expression medium and supernatants harvested after 72h. Siglec-Fcs were purified using protein A beads and desialylated on column using *Vibrio Cholerae* neuraminidase (VCN) to minimize *cis*-interactions. After elution and concentration by ultracentrifugation, protein concentration was

determined by BCA measurement and functionality assessed by SDS-PAGE and FACSstaining of tumor cell lines.

Histology and immunofluorescence.

For Siglec staining, frozen sections of tumor samples were prepared with a cryostat. Siglec-9 was stained with a monoclonal antibody (R&D Systems) and detected with a secondary, biotinylated anti-mouse IgG and subsequent incubation with streptavidin-HRP. Mouse tissues and immunofluorescence costaining was also performed on frozen sections. For immunofluorescence, primary antibodies were detected with secondary Cy5 or Alex488-coupled antibodies.

Multiplex cytokine measurements.

Siglec-9 positive and negative TILs were sorted from frozen primary NSCLC samples by flow cytometry as described above. Flat bottom 96 well plates were coated over night with anti-CD3 antibody (0.5 µg/ml), T cells plated at 50 000 cells/well and soluble anti-CD28 antibody added (1 µg/ml). After polyclonal stimulation, supernatants were collected, spun down and kept at -80 °C. Inflammatory chemokines and cytokines were measured by a flow cytometry based bead assay (CBA) that allows measurement of 13 inflammatory chemokines or cytokines simultaneously (both from BioLegend). Binding of cytokines to the beads was measured on a Fortessa LSR II flow cytometer (BD Biosciences).

GNE-deficient tumor cells in vivo.

All mouse experiments were approved by the local ethical committee (Ethical Committee of Basel Stadt). Wildtype mice were bought from Janvier Labs or bred in the animal facility of the Department of Biomedicine at the University of Basel, Switzerland. MC38 were provided by Mark Smyth (Peter MacCallum Cancer Centre, Melbourne, AU), and EMT6 were bought from ATCC. For the transplantable syngeneic MC38 or EMT6 tumor models, 500'000 tumor cells were injected subcutaneously into the right thoracic flank of 8 to 12-week-old female C57BL/6 or BALB/c mice respectively. Perpendicular tumor diameters were measured by caliper, and tumor volume calculated according to the following formula: tumor volume (mm³) = $d^{2*}D/2$, where *D* and *d* are the longest and shortest diameters of the tumor (in millimeters),

respectively. GNE-deficient MC38 or EMT6 cells were generated as described above. Tumor growth was measured three times a week and compared to the growth of WT cell lines. Cell growth in vitro was not different between GNE-KO cells and WT counterparts. Animals whose tumors had reached 1200 mm³ were euthanized, and animals having developed ulcerations removed from the experiment. Proliferation of GNE-KO lines were done by counting with a hemocytometer or a MTT assay. Viability was tested by incubating with trypan blue.



Supplemental Figure 1 Upregulation of CD33-related Siglecs on TILs.

(A) Demonstration of gating for Siglec-9 expression on CD8+ TILs from primary NSCLC samples (FMO, fluorescence minus one control). (B) Percent of cells with expression of Siglec-7 (left panel) or Siglec-9 (right panel) on myeloid cells, NK cells or B cells in NSCLC tumor samples by flow cytometry (HD PBMCs n=6, TILs n=20). NK cells were determined as CD45⁺ CD56⁺ CD3⁻, NKT cells as CD45⁺ CD56⁺ CD3⁺, myeloid cells as CD45⁺ CD11b⁺ cells. Also, a comparison with $CD20^+$ B cells and $CD3^+$ T cells was done. (**C**) Upregulation of inhibitory CD33 (Siglec-3) on CD8⁺ and CD4⁺ T cells in the PBMCs from healthy controls, primary NSCLC, CRC, EOC samples or in ascites (mainly colorectal cancers) and pleural effusions (NSCLC samples, n=3-15). (**D**) Percentage of CD8⁺ and CD4⁺ T cells expressing Siglec-5 from healthy donors and cancer patients determined by flow cytometry (n=5-25). (E) Expression of Siglec-7 on PBMCs and TILs (n=5-25). (F) Expression of Siglec-10 on PBMCs and TILs (n=5-25). (G) CD33-related Siglec expression on CD8⁺ human splenocytes from a patient that underwent splenectomy due to an accident. (H) Additional staining of Siglec-9 on CD8⁺ peripheral T cells in an additional cohort of healthy controls (group 2, *n*=49) and comparison with previous control cohort (group 1, n=36) and TILs from NSCLC samples (n=41, same samples as in Figure 1C). (I) Correlation of the number of Sig9⁺ CD8⁺ T cells in the peripheral blood of healthy donors with age (P=0.8 by linear regression analysis). (J) Correlation of the number of Sig9⁺ CD8⁺ T cells in the peripheral blood of healthy donors with the number of CD45RA⁺ CCR7⁺ CD8⁺ naïve T cells (P=0.017 by linear regression analysis). (K) Siglec-9 expression of CD4⁺ (upper panel) and CD8⁺ (lower panel) TILs from patients with CRC, EOC and pleural effusions (PBMCs n=36, CRC n=10, EOC n=7, pleural effusion n=11). (L) Detection of Siglec-7 (upper panel) or Siglec-9 (lower panel) ligands on an NSCLC tissue microarray (n=40, mean±s.e.m). Siglec-7 or Siglec-9 ligands were upregulated in all NSCLC stages. Statistical analysis was performed by Student's t test for 2 conditions and 1-way-ANOVA for multiple conditions. **P*<0.05, ***P*<0.01, ****P*<0.001. Data presented as mean±s.d. if not otherwise stated.



Supplemental Figure 2 Characterization of Sig9⁺ CD8⁺ TILs.

(A) Percentage of PD-1 high expressing cell determined by FACS on Sig9⁺ CD8⁺ TILs and Sig9⁻ CD8⁺ TILs in NSCLC tumor samples (n=44). (**B**) Fold enrichment of PD-1^{hi} in Sig9⁺ CD8⁺ TIL population or Sig9⁺ in PD-1^{hi} CD8⁺ TIL population. This shows that Sig9⁺ CD8⁺ TILs are a subpopulation of the PD-1^{hi} CD8⁺ TIL population. (**C**) Correlation of Sig9⁺ with PD-1^{hi} percentage of CD8⁺ TILs in NSCLC tumor sample (P<0.001, by linear correlation analysis). (**D**) Correlation of Sig9⁺ with TIM-3⁺ percentage of CD8⁺ TILs in NSCLC tumor sample (P=0.0042, by linear correlation analysis). (**E**) Correlation of EOMES^{hi} Tbet^{lo} cells with Sig9⁺ CD8⁺ TILs (P=0.095, by linear correlation analysis). (**F**-J) Expression of costimulatory receptors CD26 (F, n=6), CD27 (G, n=17), CD28 (H, n=15), CD137 (I, n=10) and ICOS (J, n=23) on Sig9⁻ and Sig9⁺ CD8⁺ TILs from NSCLC samples. (**K**) Heatmap of expression of top 20 differentially expressed genes determined by RNA sequencing of sorted Sig9⁺ CD8⁺ TILs and compared to

Sig9⁻ CD8⁺ TILs (*n*=5 samples were sorted). (L) Expression analysis of mRNA levels by RNA sequencing of GNE in Sig9⁺ and Sig9⁻ TILs (5 samples). (**M**) Staining Sig9⁻ CD8⁺ or Sig9⁺ CD8⁺ TILs by flow cytometry with SNA (*n*=6-7). (**N**) Binding of Siglec-9-Fc to CD8⁺ T cells from healthy donor (HD) PBMCs, PBMCs from NSCLC patients or TILs from NSCLC patients (*n*=6-7). (**O**) Fluorescence staining of osteopontin (OPN) and Siglec-9 on primary NSCLC tumor samples (bars, 30μ m). In the left panel, mononuclear OPN⁺ Sig9⁺ cells T cells can be seen. On the right panel (different sample), tumor cells OPN positive and OPN⁺ Sig9⁺ double positive cells have macrophage morphology. (**P**) Intracellular expression of SPP1 (osteopontin) in Sig9⁻ CD8⁺ TILs or Sig9⁺ CD8⁺ TILs from primary NSCLC samples measured by flow cytometry (*n*=25). (**Q**) Expression of Ki67 (MKI67) in Sig9⁻ CD8⁺ TILs or Sig9⁺ CD8⁺ TILs by flow cytometry (*n*=12). **P*<0.05, ****P*<0.001. Data presented as mean±s.d.



Supplemental Figure 3 Gating strategy for sorting of Sig9⁺ CD8⁺ TILs from NSCLC samples.



Supplemental Figure 4 Functional characterization of Sig9⁺ CD8⁺ TILs.

(**A**) Upregulation of Siglec-5, Siglec-7, Siglec-9 on activated CD8⁺ T cells from healthy donors. Activation was obtained by short-time stimulation with anti-CD3 and anti-CD28 antibodies or PMA/lonomycin (*n*=4). (**B**) Upregulation of Siglec-9 on CD8⁺ T cell clones stimulated with a specific antigen (CMV, influenza and melan A) measured by flow cytometry. Representative images of T cell clones. (**C**) Siglec-5 is used as an additional activation marker on TILs sorted from NSCLC samples according to Siglec-9 expression (*n*=7). (**D**) Upregulation of the activation marker CD25 on the surface of polyclonally stimulated (anti-CD3/28 antibodies) Sig9⁺ and Sig9⁻ CD8⁺ T cells in the peripheral blood of healthy donors. The expression was determined by flow cytometry (*n*=17 different healthy donors). (**E**) CD69 was determined on Sig9⁺ and Sig9⁻ CD8⁺ T cells in the peripheral blood of healthy donors after polyclonal stimulation (*n*=16). (**F**) The newly identified activation marker Siglec-5 was determined on Sig9⁺ and Sig9⁻ CD8⁺ T cells in the peripheral blood of healthy donors after polyclonal stimulation (*n*=6). (**G**) Upregulation of intracellular granzymeB in Sig9⁺ and Sig9⁻ CD8⁺ TILs from NSCLC patients after different durations of stimulation with anti-CD3 and anti-CD28 antibodies (*n*=6). **P*<0.05, ***P*<0.01, ****P*<0.001. Data presented as mean±s.d.



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Supplemental Figure 5 Expression of cytokines of Sig9⁺ CD8⁺ TILs.

(**A-F**) Determination of cytokines in the supernatant of sorted Sig9⁺ and Sig9⁻ CD8⁺ TILs from NSCLC patients by a multiplex bead assay (Biolegend). The secretion was also compared to Sig9⁺ and Sig9⁻ CD8⁺ T cells sorted from PBMCs of healthy donors (n=3-7). (**G-N**) Determination of chemokines in the supernatant of sorted Sig9⁺ and Sig9⁻ CD8⁺ TILs from NSCLC patients and compared to Sig9⁺ and Sig9⁻ CD8⁺ T cells sorted from PBMCs of healthy donors (n=3). (**O-T**) Frequency in percent of chemokine receptor expression on Sig9⁻ CD8⁺ TILs or Sig9⁺ CD8⁺ TILs in primary NSCLC samples determined by flow cytometry (n=10). *P<0.05, **P<0.01, ***P<0.001. Data presented as mean±s.d.



Supplemental Figure 6 Gating strategy for the detection of CD101 and CD38 on Sig9⁺ CD8⁺ TILs from NSCLC samples.



Supplemental Figure 7 Siglec-sialoglycan-SAMP interactions influence T cell effector function.

(A) Expression of CD25 analyzed by flow cytometry on the surface of polyclonal stimulated $CD8^+$ T cells in the presence of the Siglec ligand LGALS3BP over a dose range (*n*=3)

independent donors). (B) Expression of the activation marker CD69 polyclonal stimulated $CD8^{+}$ T cells in the presence of the Siglec ligand LGALS3BP (*n*=3 independent donors). (**C**) Binding of Siglec-9-Fc to different HT-29 cells. Wildtype cells (WT) were compared to desialylated (desia), GNE-deficient (GNE-KO) and GNE-deficient with excess of sialic acid (Neu5Ac) in the medium cells. (**D**) Binding to different CD19⁺ Ramos cells. Wildtype cells (WT) were compared to desialylated (desia), GNE-deficient (GNE-KO) and GNE-deficient with excess of sialic acid (Neu5Ac) in the medium cells. (E) Induction of apoptosis measured by cleaved caspase 3 (clCasp3) in A549 cells incubated with unsorted CD8⁺ TILs, and sorted Sig9⁻ or Sig9⁺ CD8⁺ TILs (n=4-6). (**F**) Induction of apoptosis measured by cleaved caspase 3 (clCasp3) in HT-29 cells incubated with unsorted CD8⁺ TILs, and sorted Sig9⁻ or Sig9⁺ CD8⁺ TILs (n=4). (**G**) Inhibition of CD8⁺ T cell activation by different anti-Siglec antibodies measured by flow cytometry. CD25 was determined on CD8⁺ T cells stimulated by anti-CD3 and anti-CD28 antibodies in the presence of anti-Siglec-9 antibodies (191240, blocking IgG, E10-286, non-blocking IgG), anti-Siglec-5 antibody or LGALS3BP as ligand (n=6). (**H**) Ki67⁺ CD8⁺ T cells polyclonally stimulated in presence of antibodies (n=6). (I) Ki67⁺ CD8⁺ T cells polyclonally activated in presence of different concentrations of anti-Siglec-9 antibody (clone 191240 from R&D Biosciences, n=4). (J) Inhibition of binding of calcein AM-labeled A549 cells to immobilized Sig9-Fc in presence of clone 191240 antibody or isotype control (numbers represent x10³ cells). *P<0.05, **P<0.01, ***P<0.001. Data presented as mean±s.d.





(A) Expression of Siglec-E in percent on CD8⁺ TILs in different tumor models including MC38 (n=28), EMT6 (n=13), LLC1 (n=5), or B16 (n=4). All the analysis afterwards in this figure are from the MC38 model. (B) Expression of Siglec-E (n=28, same analysis as used in A), Siglec-F (n=21) or Siglec-G (n=12) on CD8⁺ TILs in the MC38 subcutaneous tumor model determined by flow cytometry. (**C**-E) Expression of granzyme B (**C**, n=12), CD69 (**D**, n=10) and CD27 (**E**, n=18) on SigE⁻ CD8⁺ and SigE⁺ CD8⁺ TILs. (F) Percent of CTLA-4 expressing cells within the SigE⁻ CD8⁺ and SigE⁺ CD8⁺ TIL population (n=10). (G) Presence of PD^{hi} positive cells in the SigE⁻ CD8⁺ and SigE⁺ CD8⁺ TIL population (n=16). (H) Frequency of different EOMES and

Tbet expressing populations in the SigE⁻ CD8⁺ and SigE⁺ CD8⁺ TIL population (n=10). (I) Frequency of different CD127 and KLRG1 expressing populations in the SigE⁻ CD8⁺ and SigE⁺ CD8⁺ TIL population (n=10). (I) *In vitro* proliferation of GNE-KO MC38 and EMT6 cells compared to wildtype cells. The proliferation was assessed by an MTT assay. Viability of cells was measured by trypan blue exclusion. (J, K) Analysis of immune cell infiltrates by flow cytometry in subcutaneous MC38 tumors (n=7). (L) Subcutaneous tumor growth of MC38 GNE-KO or MC38 wildtype cells and EMT6 GNE-KO or EMT6 wildtype cells in NSG mice (n=6). *P<0.05, **P<0.01, ***P<0.001. Data presented as mean±s.d.



Supplemental Figure 9 Gating strategy for determination of Siglec-E expression on CD8⁺ TILs.



Supplemental Figure 10 Genetic model for Siglec-9 expression in T cells.

(A) Schematic drawing of genetic rearrangement and flow cytometry analysis of splenocytes for Siglec-9 expression in HS9 mice and HS9 mice crossed to CD4-Cre mice (CD4-Cre HS9 mice). (B) Frequency of T cells and memory subsets in spleens from different genetic strains (n=4-6). (C) Frequency of Siglec expression on CD8⁺ TILs in subcutaneous MC38 tumors. Representative FACS plots are shown. (D) Representative example of IgG binding of serum from different tumor-bearing mice to MC38 tumor cells. Binding was assessed by FACS analysis and secondary anti-mouse-IgG staining. (E) Proliferation measured by cell trace violet (CTV) of OT-1 T cells in MC38-OVA tumors after adoptive transfer. There is a higher frequency in the higher divisions (x axis, divisions, n=10). (F) Frequency of OT-1 T cells in MC38 wildtype MC38 cells in SigE16 mice (n=6). *P<0.05. Data presented as mean±s.d.

Supplemental Table 1

rs2075803 alleles and odds ratio (OR) for CRC risk

	Control	Case	OR	<i>P</i> value
GG	935	1205		
AG	1469	1879	0.99 (0.88,1.11)	0.91
AA	640	711	0.86 (0.75,0.99)	0.04
Sum	3044	3795		

rs2258983 alleles and odds ratio (OR) for CRC risk

	Control	Case	OR	P value
AA	958	1237		
CA	1351	1730	0.99(0.89,1.11)	0.89
CC	546	573	0.81(0.70,0.94)	0.005
Sum	2855	3540		