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# Enhanced anti-tumor immunity in ST8Sia6 knockout mice

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## Abstract

Inhibitory receptors have a critical role in the regulation of immunity. Siglecs are a family of primarily inhibitory receptors expressed by immune cells which recognize specific sialic acid modifications on cell surface glycans. Many tumors have increased sialic acid incorporation. Overexpression of the sialyltransferase ST8Sia6 on tumors led to altered immune responses and increased tumor growth. Here, we examine the role of ST8Sia6 on immune cells in regulating anti-tumor immunity. ST8Sia6 knockout mice had an enhanced immune response to tumors. The loss of ST8Sia6 promoted an enhanced intratumoral activation of macrophages and dendritic cells, including upregulation of CD40. Intratumoral Tregs exhibited a more inflammatory phenotype in ST8Sia6 KO mice. Using adoptive transfer studies, the change in Treg phenotype was not cell intrinsic and depended on the loss of ST8Sia6 expression in APCs. Thus, ST8Sia6 generates ligands for Siglecs that dampen anti-tumor immunity.

## Introduction

Sialytransfersases are enzymes that add sialic acids to the termini of either N-linked or O-linked oligosaccharides on glycoproteins or glycolipids. There are six sialyltransferases that generate α2,8-linked sialic acids – ST8Sia1 through ST8Sia6. ST8Sia6 generates disialic acids, specifically on O-linked glycoproteins, by adding a single α2,8-linked sialic acid onto an existing α2,3- or α2,6-linked sialic acid (1). Siglecs are a family of transmembrane proteins that bind and recognize specific sialic acid linkages. Siglecs are generally inhibitory receptors primarily expressed on cells of hematopoietic origin and serve to dampen immune responses (2). Intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs) present in the cytoplasmic tails of many Siglecs recruit SHP1 and SHP2 tyrosine phosphatases, leading to immune inactivation (2). Siglec-E recognizes the modification generated by ST8Sia6 (3) and is expressed specifically on macrophages,

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dendritic cells, neutrophils, and monocytes (4). Although it is known that Siglec-E plays a role in modulating the immune response, it is not known how ST8Sia6 expression in the host influences those immune responses.

ST8Sia6 is expressed by dendritic cells, macrophages, and CD4 T-cells. In dendritic cells cDC2s have substantially higher levels of ST8Sia6 as compared to cDC1s and pDCs (Immgen.org). However, unlike T-cells, macrophages and dendritic cells also express Siglec-E. Ligands generated by sialyltransferases can interact with Siglecs on opposing cells (defined as a trans-interaction) as well as on the same cell (defined as a cis-interaction) (5). Previously, we showed that ST8Sia6 overexpression in cancer cells altered macrophage polarization through engagement of ligands generated by ST8Sia6 with Siglec-E (6). In addition, dendritic cells that uptake sialylated antigen, through interaction with Siglec-E, induce immune tolerance and promote the generation of Tregs (7). However, whether endogenous ST8Sia6 helps to set a tolerance threshold in the immune response to tumors is not known.

In this study, we analyzed the immune response in models of cancer in ST8Sia6 KO mice. We challenged C57Bl/6 and ST8Sia6 KO mice with MC38 and 0B16 tumor lines. Injection of tumors into ST8Sia6 KO mice led to reduced tumor burden, which increased survival, as compared to injection of tumors into C57Bl/6 mice. When examining the tumor microenvironment (TME), we found that intratumoral macrophages and dendritic cells in ST8Sia6 KO mice demonstrated a shift from a suppressive to an activated phenotype as compared to macrophages and dendritic cells in C57Bl/6 mice. Additionally, we found that Tregs from tumors in ST8Sia6 KO mice experienced a shift to an inflammatory phenotype. These changes in tumor growth and Treg phenotypes were found to be due to the loss of ST8Sia6 in innate cells. Thus, loss of ST8Sia6 in mice leads to enhanced immune activation after tumor challenge, indicating that ST8Sia6 normally functions to dampen immune responses *in vivo*.

## Materials and Methods

### Cell lines

The MC38 murine colorectal cancer cell line was received from Dr. Yang-Xin Fu (UT Southwestern Medical Center, Dallas, Texas) (8). The B16 and B16-OVA murine melanoma cell lines were received from Dr. Richard Vile (Mayo Clinic, Rochester, MN). Cell lines were screened for Mycoplasma contamination and authenticated by STR profiling (B16 at ATCC and MC38 at IDEXX). Cell lines were cultured as previously described (6).

### Animals

C57Bl/6 (Stock #000664), OTII (Stock #004194), and Rag1 KO (Stock #002216) mice were obtained from JAX. B6.SJL (Stock #564) were purchased from Charles River. ST8Sia6 KO mice were previously described (3). B6.SJL and OTII mice were interbred to generate OTII CD45.1/CD45.2 mice. Experiments were conducted on both male and female mice between 8 weeks to 4 months of age. Tumor growth and survival experiments were performed with 5 mice per group, and tumor harvest experiments were performed with 4–9 mice per group as

denoted in the figures. All mouse work was performed with approval from the Mayo Clinic Institutional Animal Care and Use Committee.

### Flow cytometry, antibodies, and reagents

Flow cytometry of isolated tumors was performed as described in (6). Fluorescently conjugated flow cytometry antibodies CD45-BV785 (Cat. #103149, BioLegend), CD45.1-BV785 (Cat. #110743, BioLegend), CD11b-FITC (Cat. #101206, BioLegend), CD11c-PE (Cat. #117308, BioLegend), CD11c-BV421 (Cat. #117330, BioLegend), F4/80-PE-Cy7 (Cat. # 123114, BioLegend), Ly6C-PerCP (Cat. #128028, BioLegend), Ly6G-BV510 (Cat. #127633, BioLegend), Ly6G-APC (Cat. #127614, BioLegend), Gr1-PerCP (Cat. #108426, BioLegend), IA/IE (MHC Class II)-BV605 (Cat. #107639, BioLegend), TNFRII-Biotin (Cat. #113403, BioLegend), CD40-PE-Dazzle (Cat. #124630, BioLegend), CD80-BV510 (Cat. #104741, BioLegend), CD86-APC (Cat. #105012, BioLegend), Streptavidin-BV605 (Cat. #405229, BioLegend), TCRβ-PE-Cy7 (Cat. #109222, BioLegend), CD4-PerCP (Cat. #100538, BioLegend), CD8α-FITC (Cat. #100706, BioLegend), CD25-BV421 (Cat. #101923, BioLegend), PD-1-APC (Cat. #135210, BioLegend), CD62L-BV510 (Cat. #104441, BioLegend), CD69-BV605 (Cat. #104530, BioLegend), and recombinant Siglec-E-Fc (Cat. #551506, BioLegend) were purchased from BioLegend. RORγt-PE-CF594 (Cat. #562684, BD Horizon) was purchased from BD Horizon. Fixable viability dye Ghost Red 780 (Cat. #13-0865-T100, Tonbo) and Foxp3-PE (Cat. #50-5773-U100, Tonbo) were purchased from Tonbo Biosciences. Arg-1-APC (Cat. #12-3697-82, Invitrogen) and iNOS-PE (Cat. #17-5920-82, Invitrogen) were purchased from Invitrogen. Anti-human IgG-APC (Cat. #109-135-098, Jackson ImmunoResearch Laboratories) was purchased from Jackson ImmunoResearch Laboratories. Magnetic enrichment and negative selection antibodies CD8-Biotin (Cat. #100704, BioLegend), NK1.1-Biotin (Cat. #108704, BioLegend), B220-Biotin (Cat. #103204, BioLegend), CD11b-Biotin (Cat. #101207, BioLegend), CD19-Biotin (Cat. #115503, BioLegend), CD11c-Biotin (Cat. #117303, BioLegend), Gr-1-Biotin (Cat. #108404, BioLegend), and Ter-119-Biotin (Cat. 116203, BioLegend) were purchased from BioLegend. TCRγδ-Biotin (Cat. #13-5811085, eBioscience) was purchased from eBioscience.

### Tumor models and tumor dissociation

MC38, B16 and B16-OVA were injected into the flanks of mice as described in (6). Tumors were isolated and dissociated as described in (6).

### Isolation and adoptive transfer of T-cells

Spleens and lymph nodes from either C57Bl/6, ST8Sia6 KO, or OTII CD45.1/CD45.2 mice were harvested and dissociated as described above. Samples were pooled and washed with PBS. Samples were resuspended in Robosep Buffer (Cat. #20104, STEMCELL Technologies) at a concentration of $2 \times 10^8$ cells per mL for sorting. Cells were magnetically enriched for $CD4^+$ T-cells using the EasySep Mouse Streptavadin RapidSpheres Isolation Kit (Stem Cell Technologies #19860). Biotin-conjugated antibodies against CD8, TCRγδ, NK1.1, B220, CD11b, CD19, CD11c, Gr-1, and Ter-119 were used to remove all non-$CD4^+$ T-cell populations. The enriched $CD4^+$ T-cells were washed 3 times with PBS and resuspended in 5mLs of PBS. $5 \times 10^6$ cells, in a volume of 100 μLs, were retro-orbitally

injected into B16-OVA tumor bearing C57Bl/6 and ST8Sia6 KO mice. $8 \times 10^6$ cells, in a volume of 100 μLs, were retro-orbitally injected into MC38 tumor bearing Rag1 KO mice.

### Statistics

Data were graphed and analyzed using Prism (GraphPad Software). Tumor growth kinetics were compared using a two-way ANOVA, while survival data was assessed using a Mantel-Cox test. Individual parameter datasets across two groups were compared using an unpaired t-test. Error bars represent SEM.

## Results

### Tumor bearing ST8Sia6 KO mice experience reduced tumor growth and increased survival

Previously, we demonstrated that overexpression of ST8Sia6 in murine cancer cells causes increased tumor growth and reduced survival (6). This change in growth and survival was dependent on Siglec-E expression in the host (6). However, the importance of ST8Sia6 in the host immune response was not addressed. Thus, we sought to determine how ST8Sia6 in the mouse influences the immune system in the context of cancer. C57Bl/6 and ST8Sia6 KO mice were injected subcutaneously with either MC38, B16 or B16-OVA tumor cells. ST8Sia6 KO mice injected with MC38 exhibited reduced tumor growth ($p< 0.0006$) and increased survival ($p=0.0019$) as compared with C57Bl/6 mice (Fig. 1A). Similarly, ST8Sia6 KO mice injected with B16 exhibited reduced tumor growth ($p< 0.0001$) and increased survival ($p=0.0023$) as compared with C57Bl/6 mice (Fig. 1B). In addition, ST8Sia6 KO mice injected with B16-OVA exhibited reduced tumor growth ($p=0.0063$) and increased survival ($p = 0.008$) as compared with C57Bl/6 mice (Fig. 1C). Therefore, tumor growth is reduced in ST8Sia6 KO mice.

### Enhanced immune activation in ST8Sia6 KO mice

To understand how the absence of ST8Sia6 on immune cells alters the immune response, tumor-associated macrophages (TAMs) and dendritic cells (TADCs) were analyzed for expression of proteins associated with immune suppression (TNFRII, Arg-1) and activation (CD40, CD80, CD86). TNFRII has been associated with a tolerogenic or suppressive phenotype in dendritic cells and Tregs, although the exact mechanism leading to this suppression is currently unknown (9). Arg-1 expression in M2-like macrophages suppresses immune responses (10). CD40, CD80, and CD86 are costimulatory molecules that are upregulated on APCs during immune activation (11). These proteins are expressed on APCs and serve to activate T-cells (11). TAMs were further categorized into either classical ($CD11c^-$ $F4/80^+$) or hybrid ($CD11c^+$ $F4/80^+$) macrophages, whereas TADCs were categorized into either classical ($CD11b^-$) dendritic cells or conventional dendritic cells type 2 (cDC2s) ($CD11b^+$ $F4/80^-$). Classical ($CD11b^-$) dendritic cells are comprised of cDC1s and pDCs. Gating trees for quantification of all flow cytometry populations are shown in Supplementary Figures 1 and 2. No differences in the types of immune cells infiltrating the tumors were observed (Fig. S3).

In MC38 and B16, both hybrid macrophages and cDC2s from ST8Sia6 KO mice demonstrated a significant decrease in TNFRII expression and a significant increase in

CD40 as compared to tumors from C57Bl/6 mice (Fig. 2). Also, classical macrophages from ST8Sia6 KO mice also had increased CD40 expression and classical DCs also had decreased TNFRII expression in both the MC38 and B16 models (Fig. S4). In addition, hybrid macrophages from ST8Sia6 KO mice exhibited a decrease in Arg-1 in the MC38 tumor model. cDC2s also demonstrated an increase in CD80 expression in ST8Sia6 KO mice in both the MC38 and B16 tumors. Other changes in expression consistent with increased activation and decreased suppression were also observed, however these were not present in both the MC38 and B16 tumors (Fig. 2 and Fig. S4). Thus, the absence of ST8Sia6 on immune cells altered the phenotype of macrophages and dendritic cells towards promoting immune activation.

## Tregs from tumor bearing ST8Sia6 KO mice exhibit reduced TNFRII and increased RORγt

The activated phenotype present in macrophages and dendritic cells within the tumors from ST8Sia6 KO mice could influence T-cell frequencies. Our previous findings demonstrated that when ST8Sia6 was overexpressed in MC38, Treg proportions increased in a Siglec-E dependent manner (6). However, this change was indirect, as neither CD4 or CD8 T-cells express Siglec-E (6). The alterations in innate cell activation in ST8Sia6 KO mice could lead to changes in Treg frequencies or phenotypes. To investigate this, C57Bl/6 and ST8Sia6 KO mice were either injected with MC38 or B16 tumor cells. Tumors were harvested after 12 days, at a point where there was substantial lymphocyte infiltration. The frequencies of CD4 and CD8 T-cells were unaltered within MC38 or B16 tumors in C57Bl/6 and ST8Sia6 KO mice (Fig. 3A, B). Frequencies of Tregs also remained unchanged, as well as the CD8/CD4 ratios (Fig. 3A, B). CD8/Treg ratios were significantly different only in MC38 tumors (Fig. 3A). However, the phenotype of Tregs did significantly change with regards to expression of TNFRII and RORγt in tumors from ST8Sia6 KO mice compared to C57Bl/6 mice. TNFRII expression is associated with functionally suppressive Tregs (12). RORγt positive Tregs have been associated with an inflammatory phenotype (13). Tregs from MC38 and B16 tumors in ST8Sia6 KO mice exhibited a reduction in TNFRII as compared C57Bl/6 mice (Fig. 3A, B). Additionally, Tregs from MC38 and B16 tumors in ST8Sia6 KO mice exhibited an increase in RORγt as compared C57Bl/6 mice (Fig. 3A, B). Therefore, these changes in expression could indicate a shift to a less suppressive Treg in the absence of ST8Sia6.

## Macrophages and dendritic cells from tumor bearing ST8Sia6 KO mice influenced the change in Treg phenotype

ST8Sia6 is expressed in macrophages and dendritic cells with particularly high expression in cDC2s. T-cells also express ST8Sia6 with high expression in CD4 T-cells (Immgen.org). Macrophages, dendritic cells, and Tregs were all phenotypically altered with the loss of ST8Sia6 in tumor bearing mice. We sought to determine whether the loss of ST8Sia6 expression in myeloid cells was altering T-cell phenotypes or if the loss of ST8Sia6 expression in T-cells was altering myeloid cell phenotypes. To address this question, C57Bl/6 and ST8Sia6 KO mice were both injected with B16-OVA tumor cells. ST8Sia6-sufficient OTII CD45.1/CD45.2 T-cells were isolated and transferred into day 8 tumor bearing mice. Tumors were harvested at day 11 and Treg phenotypes were analyzed. Similar to our previous results, OTII Treg frequencies remained unaffected from tumors present in

either C57Bl/6 or ST8Sia6 KO mice (Fig. 4A). However, OTII Tregs from tumors present in ST8Sia6 KO mice exhibited a reduction in TNFRII and an increase in RORγt as compared to tumors present in C57Bl/6 mice (Fig. 4A). Additionally, tumors weighed significantly less in ST8Sia6 KO mice as compared to C57Bl/6 mice, replicating the tumor growth pattern observed in Figure 1 (Fig. 4A). We then examined whether the effect on Treg phenotype was cell-intrinsic. Rag1 KO mice were injected with MC38 tumors cells along with CD4 and CD8 T-cells isolated from either C57Bl/6 or ST8Sia6 KO mice. Tumors were harvested at day 11 and Treg phenotypes were analyzed. Treg frequencies, CD8/CD4 ratios, and CD8/Treg ratios between C57Bl/6 and ST8Sia6 KO mice remained unchanged (Fig. 4B). However, TNFRII and RORγt frequencies in Tregs were not significantly different between C57Bl/6 and ST8Sia6 KO mice (Fig. 4B). Tumor weights were also not significantly different between either C57Bl/6 and ST8Sia6 KO mice (Fig. 4B). Within the tumor microenvironment, hybrid macrophages, cDC2s, classical macrophages, classical dendritic cells, and Tregs were examined in WT and ST8Sia6 KO mice for changes in the generation of ligands for Siglec-E. In ST8Sia6 KO mice, all of these immune cells exhibited reduced binding to recombinant Siglec-E, indicating decreased generation of ST8Sia6-generated ligands as compared to immune cells from tumors in C57Bl/6 mice (Fig. 4C, D). These results indicated that the loss of ST8Sia6 in APCs was responsible for the reduction in tumor growth and changes in Treg phenotypes, and that loss of ST8Sia6 limited to T-cells did not alter tumor growth.

## Discussion

Our previous findings demonstrated the role of Siglec-E in modulating the immune system when ST8Sia6 was overexpressed in cancer cells (6). However, the role of ST8Sia6 within the host immune system remained unknown. We demonstrate here that the loss of ST8Sia6 in tumor bearing mice reduced tumor growth, increased survival, and altered the immune response as compared to ST8Sia6 sufficient tumor bearing mice. Using MC38, B16, and B16-OVA flank tumor mouse models, tumor growth was decreased in ST8Sia6 KO mice as compared to C57Bl/6 mice. Within the TME of tumors present in ST8Sia6 KO mice compared to tumors present in C57Bl/6 mice, macrophages and dendritic cells altered their phenotype towards enhanced immune activation. Tregs from tumors in ST8Sia6 KO mice also displayed an increase in RORγt and a reduction in TNFRII, suggesting a possible shift to a less suppressive Treg. These alterations in the immune response to tumors were due to the loss of ST8Sia6 in macrophages and dendritic cells and not T-cells. Therefore, the loss of ST8Sia6 in the host induces a shift in myeloid cells to exhibit an inflammatory phenotype, which leads to a reduction in tumor growth and the development of less suppressive Tregs.

Inflammatory responses in APCs, such as macrophages and dendritic cells, play a major role in stimulating T-cells to promote anti-tumor responses (14). We observed increased CD40, which is crucial for anti-tumor responses (15). In contexts outside of cancer, overstimulation of the immune system can lead to autoimmune disorders, such as type 1 diabetes and inflammatory bowel disease (IBD) (16, 17). One consequence of cancer immunotherapies designed to remove barriers of immune suppression is the development of autoimmune disorders. Many studies have demonstrated that subsets of patients receiving cancer immunotherapies develop diabetes or colitis (16, 17). ST8Sia6 mediated sialylation

may serve as a protective barrier by dampening immune responses leading to inflammatory diseases. Previously, we showed that overexpression of ST8Sia6 delayed the onset and frequency of hyperglycemia in mice in the streptozotocin-induced model of diabetes (3). If mice were to lose expression of ST8Sia6 in the NOD model of type 1 diabetes, one could hypothesize that disease progression would increase.

ST8Sia6 produces ligands that bind Siglec-E (3, 6). Tumor-associated immune cells in ST8Sia6 KO mice have reduced binding to recombinant Siglec-E. It has been shown that Siglec-E plays a critical role in regulating immune responses. Loss of Siglec-E in a mouse model of atherosclerosis led to increased disease progression (18). Additionally, loss of Siglec-E in a model of pneumonia resulted in increased survival and bacterial clearance along with an increase in inflammatory cytokine production in bronchioalveolar lavage fluid (19). Removal of Siglec-E would prevent interaction with ST8Sia6 generated ligands. Thus, the recognition of sialylated ligands by Siglec-E may dictate how ST8Sia6 regulates the immune system. By extension, loss of ST8Sia6 in models of atherosclerosis and pneumonia could replicate the results seen with the loss of Siglec-E. Sialic acids generated by sialyltransferases, such as ST8Sia6, can participate in trans- and cis-interactions (5). Since macrophages and dendritic cells contain both ST8Sia6 and Siglec-E, cis-interactions between ST8Sia6 generated ligands and Siglec-E on myeloid cells could serve as a barrier for subsequent immune responses. Therefore, removal of ST8Sia6 would sever this interplay and promote inflammatory responses.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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**Key Points**

- Loss of ST8Sia6 in mice reduces tumor growth
- Loss of ST8Sia6 increases inflammatory factors in macrophages and dendritic cells
- Loss of ST8Sia6 in APCs is responsible for enhanced anti-tumor response

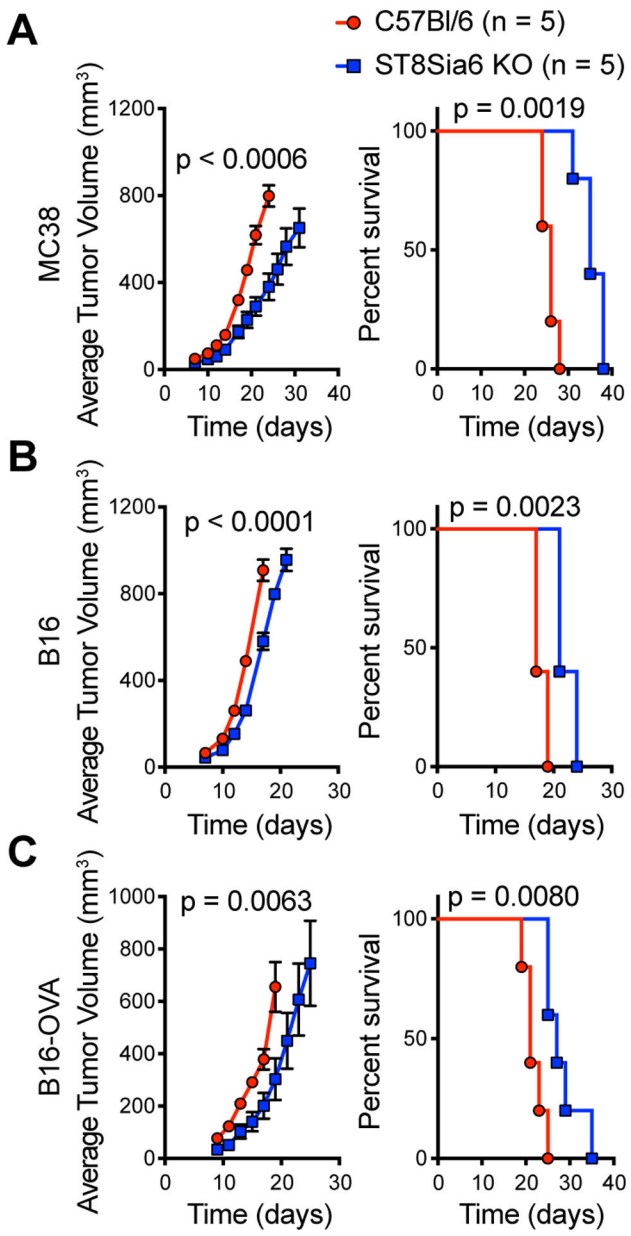


**Fig. 1. Loss of ST8Sia6 in Mice Reduces Subcutaneous Tumor Growth and Increases Survival.**

C57Bl/6 or ST8Sia6 KO mice were injected with (**A**) MC38 or (**B**) B16 or (**C**) B16-OVA murine cancer cells into the right flank. Tumor growth and survival was assessed. 5 mice were analyzed per group. Survival was compared using a Mantel-Cox test. Differences in tumor growth kinetics were analyzed using a two-way ANOVA across all timepoints. P-values were generated when all mice were present within each group.

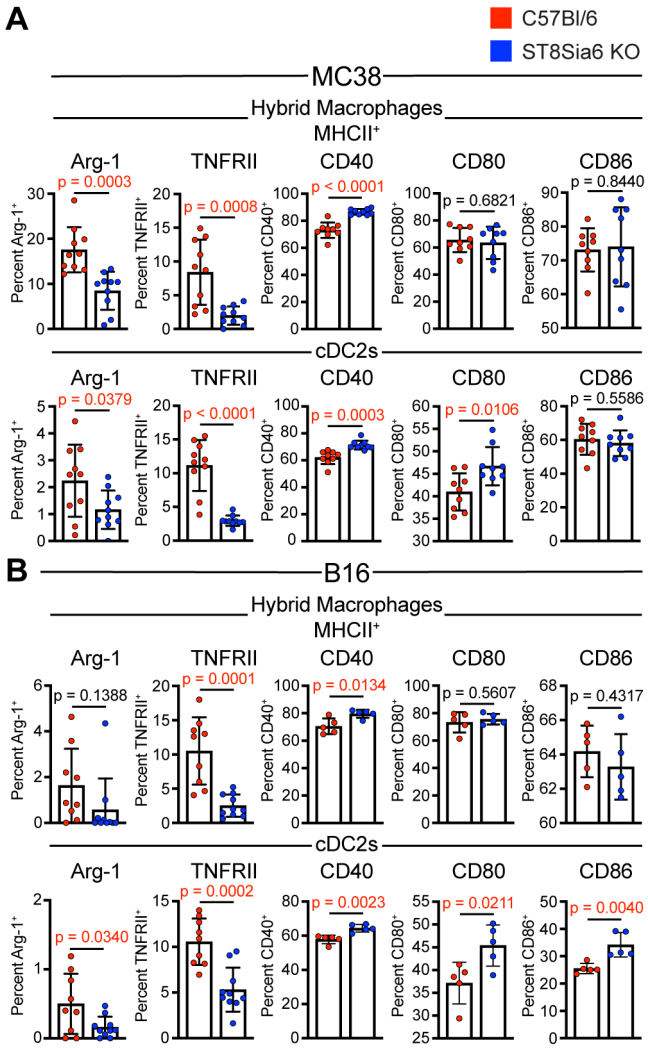


**Fig. 2. Loss of ST8Sia6 in Mice Shifts Myeloid Cells from a Suppressive to Inflammatory Phenotype.**

C57Bl/6 or ST8Sia6 KO mice were injected with either MC38 or B16 murine cancer cells into the right flank. Tumors were harvested at day 7 for myeloid cell analysis using flow cytometry. 5–10 mice were analyzed per group. Tumor associated hybrid macrophages were defined as live singlet $CD45^+$ $CD11b^+$ $CD11c^+$ $Ly6C^-$ $Ly6G^-$ $F4/80^+$ cells or live singlet $CD45^+$ $CD11b^+$ $CD11c^+$ $Gr1^-$ $F4/80^+$ cells. Tumor associated cDC2s were defined live singlet $CD45^+$ $CD11b^+$ $CD11c^+$ $Ly6C^-$ $Ly6G^-$ $F4/80^-$ cells or live singlet $CD45^+$ $CD11b^+$ $CD11c^+$ $Gr1^-$ $F4/80^-$ $MHCII^+$ cells. Myeloid cells from (**A**) MC38 and (**B**) B16 tumors were analyzed for expression of TNFRII, Arg-1, CD40, CD80, and CD86 by flow cytometry. Frequencies of $TNFRII^+$, $Arg\text{-}1^+$, $CD40^+$, $CD80^+$, and $CD86^+$ TAMs and TADCs were quantified. Statistics were performed using an unpaired t-test between groups.

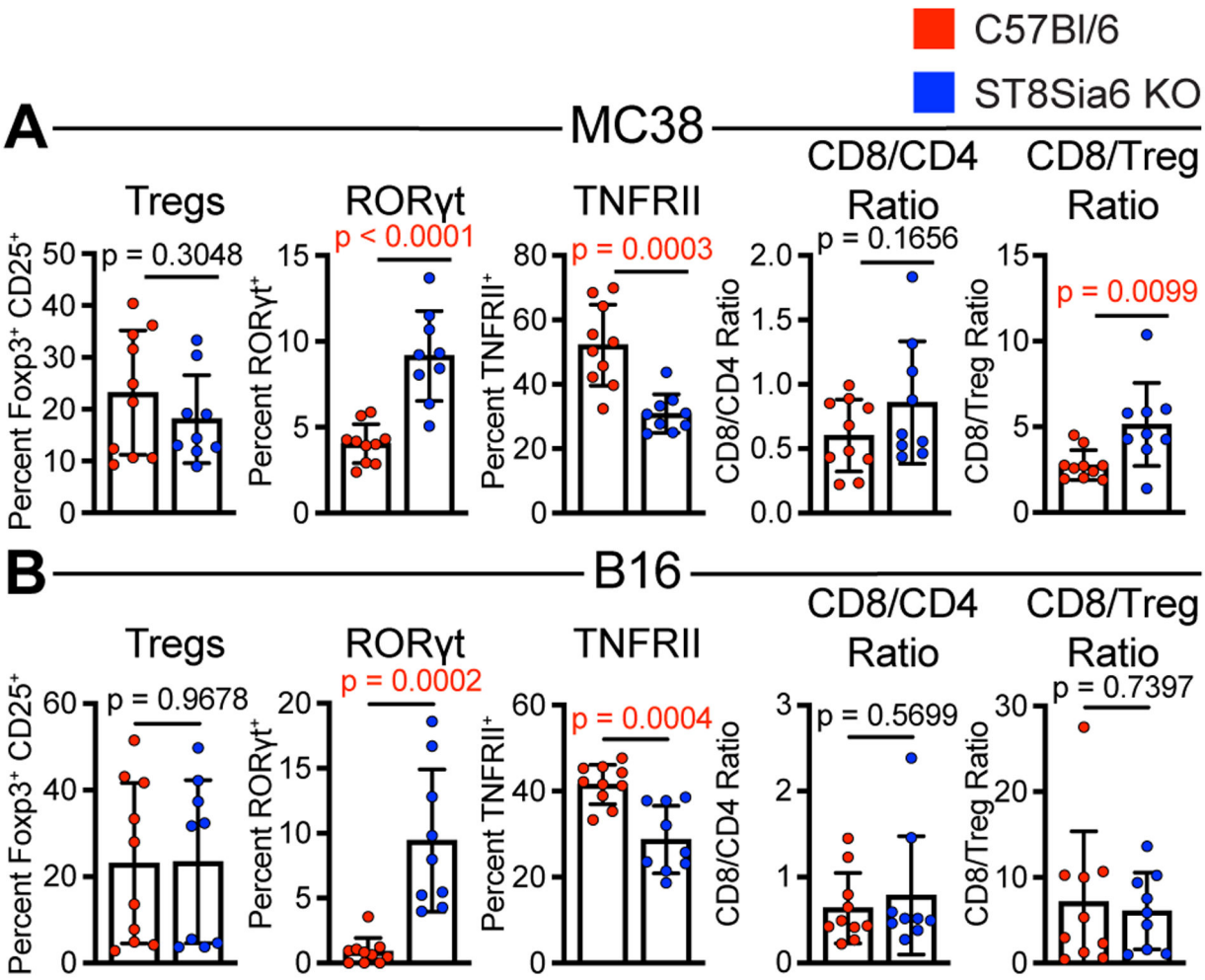


**Fig. 3. Loss of ST8Sia6 in Mice Reduces TNFRII and Increases RORγt Expression on Tregs.**

C57Bl/6 or ST8Sia6 KO mice were injected with either (**A**) MC38 or (**B**) B16 murine cancer cells into the right flank. Tumors were harvested at day 12 for T-cell analysis using flow cytometry. 5 mice were analyzed per group. Tregs were defined as $Foxp3^+$ $CD25^+$ within the $TCR\beta^+$ $CD4^+$ T-cell pool. Tregs were analyzed for expression of TNFRII and RORγt. Frequencies of $CD4^+$ and $CD8^+$ T-cells as well as $Foxp3^+$ $CD25^+$ Tregs were quantified. Frequencies of $TNFRII^+$ and $ROR\gamma t^+$ Tregs were quantified. The relative ratios of intratumoral CD8/CD4 and CD8/Treg numbers were examined. Statistics were performed using an unpaired t-test between groups.

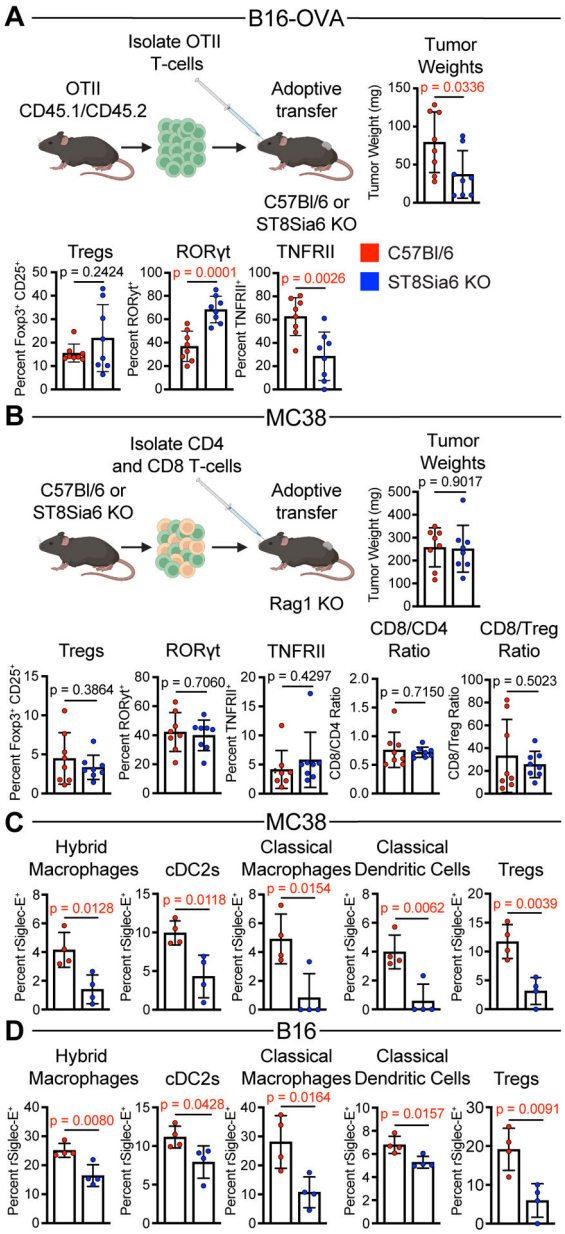


**Fig. 4. Macrophages and Dendritic cells from ST8Sia6 Deficient Mice Influence Treg Phenotypes.**

(**A**) C57Bl/6 or ST8Sia6 KO mice were injected with B16-OVA murine cancer cells into the right flank. At day 8, OTII T-cells were isolated from OTII CD45.1/CD45.2 mice and adoptively transferred into either tumor bearing C57Bl/6 or ST8Sia6 KO mice. Tumors were harvested at day 11 for CD45.1$^+$ OTII T-cell analysis using flow cytometry. 8 mice were analyzed per group. Tregs were defined as Foxp3$^+$ CD25$^+$ within the TCRβ$^+$ CD4$^+$ T-cell pool. Tregs were analyzed for expression of TNFRII and RORγt and frequencies of TNFRII$^+$ and RORγt$^+$ Tregs were quantified. Additionally, tumor weights were calculated from both C57Bl/6 and ST8Sia6 KO mice. (**B**) Rag1 KO mice were injected with MC38 murine cancer cells into the right flank. On the same day of tumor challenge, CD4 and CD8 T-cells from either C57Bl/6 or ST8Sia6 KO mice were adoptively transferred into tumor

bearing Rag1 KO mice. Tumors were harvested at day 11 for T-cell analysis using flow cytometry. 8 mice were harvested per group. Tregs were defined as in (A). Frequencies of $TNFRII^+$ and $ROR\gamma t^+$ Tregs were quantified. The relative ratios of intratumoral CD8/CD4 and CD8/Treg numbers were examined. Additionally, tumor weights were calculated from Rag1 KO mice either adoptively transferred with T-cells from C57Bl/6 or ST8Sia6 KO mice. C57Bl/6 or ST8Sia6 KO mice were injected with either MC38 or B16 murine cancer cells into the right flank. Tumors were harvested at day 7 for myeloid cell or day 12 for T-cell analysis using flow cytometry. 4 mice were analyzed per group. Myeloid cells and T-cells from (**C**) MC38 and (**D**) B16 tumors were probed for ligands using recombinant Siglec-E and analyzed by flow cytometry. Frequencies of recombinant Siglec-E binding positivity in hybrid macrophages, cDC2s, classical macrophages, classical dendritic cells, and Tregs were quantified. Statistics were performed using an unpaired t-test between groups.