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Differential Recognition of Diet-Derived Neu5Gc-Neoantigens on Glycan Microarrays by Carbohydrate-Specific Pooled Human IgG and IgA Antibodies

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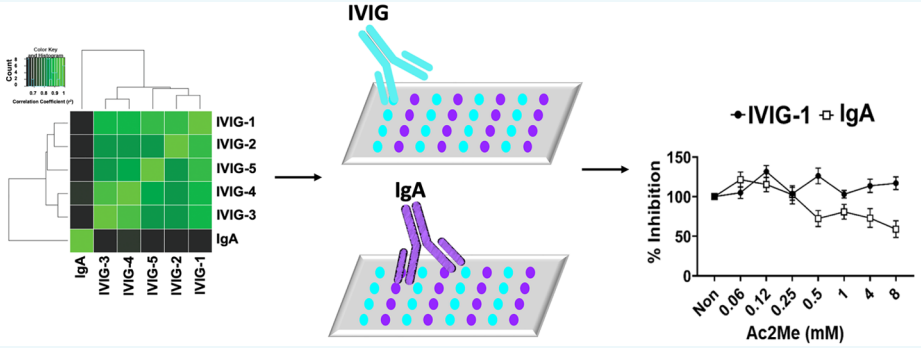
Supporting Information



ABSTRACT: Sialic acids (Sias) cover vertebrate cell surface glycans. *N*-Acetylneuraminic acid (Neu5Ac) and its hydroxylated form *N*-glycolylneuraminic acid (Neu5Gc) are common Sia in mammals. Humans cannot synthesize Neu5Gc but accumulate it on cells through red-meat rich diets, generating numerous immunogenic Neu5Gc-neoantigens. Consequently, humans have diverse anti-Neu5Gc antibodies affecting xenotransplantation, cancer, atherosclerosis, and infertility. Anti-Neu5Gc antibodies circulate as IgG, IgM, and IgA isotypes; however, repertoires of the different isotypes in a large population have not been studied yet. Here, we used glycan microarrays to investigate anti-Neu5Gc IgGs and IgAs in intravenous immunoglobulin (IVIG) or pooled human IgA, respectively. Binding patterns on microarrays fabricated with Neu5Gc- and Neu5Ac-glycans, together with inhibition assays, revealed that different IVIG preparations have highly specific anti-Neu5Gc IgG reactivity with closely related repertoires, while IgAs show cross-reactivity against several Neu5Ac-glycans. Such different anti-Neu5Gc IgG/IgA repertoires in individuals could possibly mediate distinctive effects on human diseases.

INTRODUCTION

Glycans are fundamental building blocks of cells,[1] and their "signature" on proteins, lipids, and surfaces of cells or pathogens has profound biological roles.[2] In vertebrates, cell surface glycans are commonly terminated with sialic acids (Sias) that are acidic carbohydrates with a 9-carbon backbone.[3] The two major Sias in mammals are *N*-acetylneuraminic acid (Neu5Ac) and its hydroxylated form *N*-glycolylneuraminic acid (Neu5Gc), that differ only by a single oxygen atom.[4] Humans lack the *CMAH* gene encoding cytidine 5′-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac) hydroxylase and have no alternative Neu5Gc-synthesis pathway;[5] humans cannot synthesize Neu5Gc. However, Neu5Gc appears at low levels on the surfaces of human epithelia and endothelia cells, and it is especially enriched on carcinoma.[6−8] Neu5Gc can be consumed from dietary mammalian foods,[7,9,10] and although the amounts incorporated are very small, the human immune system recognizes Neu5Gc-glycans as foreign, generating a polyclonal and diverse anti-Neu5Gc antibody response,[7,11−13] even in infants upon exposure to mammalian-derived foods.[14] Sia-containing glycans and glycoconjugates are highly diverse, owing to various modifications of Sia and differences of linkages or the identity of underlying glycans and carrier molecules (proteins, lipids, etc.), as well as their geometric organization, that altogether generate a huge range of diet-derived Neu5Gc-neoantigens.[4,15] As a result, diverse anti-Neu5Gc antibodies are present and circulate in all human sera examined thus far.[7,11−13,16,17]

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Table 1. List of Glycans on Array

ID	type	skeleton	linkage	modification	structure
1	Ac	LacNAc	α3	9OAc	Neu5-9Ac$_2$α2-3Galβ1-4GlcNAcβProNH$_2$
2	Gc	LacNAc	α3	9OAc	Neu5Gc9Acα2-3Galβ1-4GlcNAcβProNH$_2$
3	Ac	LacNAc	α6	9OAc	Neu5,9Ac$_2$α2-6Galβ1-4GlcNAcβProNH$_2$
4	Gc	LacNAc	α6	9OAc	Neu5Gc9Acα2-6Galβ1-4GlcNAcβProNH$_2$
5	Ac	GalNAc	α6	0	Neu5Acα2-6GalNAcαProNH$_2$
6	Gc	GalNAc	α6	0	Neu5Gcα2-6GalNAcαProNH$_2$
7	Ac	Type1	α3	9OAc	Neu5,9Ac$_2$α2-3Galβ1-3GlcNAcβProNH$_2$
9	Ac	Core1	α3	9OAc	Neu5,9Ac$_2$α2-3Galβ1-3GalNAcαProNH$_2$
10	Gc	Core1	α3	9OAc	Neu5Gc9Acα2-3Galβ1-3GalNAcαProNH$_2$
11	Ac	LacNAc	α3	0	Neu5Acα2-3Galβ1-4GlcNAcβProNH$_2$
12	Gc	LacNAc	α3	0	Neu5Gcα2-3Galβ1-4GlcNAcβProNH$_2$
13	Ac	Type1	α3	0	Neu5Acα2-3Galβ1-3GlcNAcβProNH$_2$
14	Gc	Type1	α3	0	Neu5Gcα2-3Galβ1-3GlcNAcβProNH$_2$
15	Ac	Core1	α3	0	Neu5Acα2-3Galβ1-3GalNAcαProNH$_2$
16	Gc	Core1	α3	0	Neu5Gcα2-3Galβ1-3GalNAcαProNH$_2$
17	Ac	LacNAc	α6	0	Neu5Acα2-6Galβ1-4GlcNAcβProNH$_2$
18	Gc	LacNAc	α6	0	Neu5Gcα2-6Galβ1-4GlcNAcβProNH$_2$
19	Ac	lactose	α6	0	Neu5Acα2-6LacβProNH$_2$
20	Gc	lactose	α6	0	Neu5Gcα2-6LacβProNH$_2$
21	Ac	lactose	α3	0	Neu5Acα2-3Galβ1-4GlcβProNH$_2$
22	Gc	lactose	α3	0	Neu5Gcα2-3Galβ1-4GlcβProNH$_2$
23	Ac	GalNAc	α6	9OAc	Neu5,9Ac$_2$α2-6GalNAcαProNH$_2$
24	Gc	GalNAc	α6	9OAc	Neu9Ac5Gcα2-6GalNAcαProNH$_2$
25	Ac	galactose	α3	0	Neu5Acα2-3GalβProNH$_2$
26	Gc	galactose	α3	0	Neu5Gcα2-3GalβProNH$_2$
27	Ac	galactose	α6	0	Neu5Acα2-6GalβProNH$_2$
28	Gc	galactose	α6	0	Neu5Gcα2-6GalβProNH$_2$
29	Ac	galactose	α3	9OAc	Neu5,9Ac$_2$α2-3GalβProNH$_2$
30	Gc	galactose	α3	9OAc	Neu9Ac5Gcα2-3GalβProNH$_2$
31	Ac	galactose	α6	9OAc	Neu5,9Ac$_2$α2-6GalβProNH$_2$
32	Gc	galactose	α6	9OAc	Neu9Ac5Gcα2-6GalβProNH$_2$
33	Ac	Core1	α3	0	Neu5Acα2-3Galβ1-3GalNAcβProNH$_2$
34	Gc	Core1	α3	0	Neu5Gcα2-3Galβ1-3GalNAcβProNH$_2$
35	Ac	Core1	α3	9OAc	Neu5,9Ac$_2$α2-3Galβ1-3GalNAcβProNH$_2$
36	Gc	Core1	α3	9OAc	Neu9Ac5Gcα2-3Galβ1-3GalNAcβProNH$_2$
37	Ac	lactose	α6	9OAc	Neu5,9Ac$_2$α2-6Galβ1-4GlcβProNH$_2$
38	Gc	lactose	α6	9OAc	Neu9Ac5Gcα2-6Galβ1-4GlcβProNH$_2$
39	Ac	lactose	α3	9OAc	Neu5,9Ac$_2$α2-3Galβ1-4GlcβProNH$_2$
40	Gc	lactose	α3	9OAc	Neu9Ac5Gcα2-3Galβ1-4GlcβProNH$_2$
41	Ac-Ac	lactose	α8-α3	0	Neu5Acα2-8Neu5Acα2-3Galβ1-4GlcβProNH$_2$
42	Ac-Ac-Ac	lactose	α8-α3	0	Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3Galβ1-4GlcβProNH$_2$
55	Ac	Lex	α3	0	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβProNH$_2$
56	Gc	Lex	α3	0	Neu5Gcα2-3Galβ1-4(Fucα1-3)GlcNAcβProNH$_2$
57	Ac	6S-Lex	α3	6OSO$_3^-$	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc6S-βProNH$_2$
58	Gc	6S-Lex	α3	6OSO$_3^-$	Neu5Gcα2-3Galβ1-4(Fucα1-3)GlcNAc6S-βProNH$_2$
60	Ac	LNT	α3	0	Neu5Acα2-3Galβ1-3GlcNAcβ1-3LacβProNH$_2$
61	Gc	LNT	α3	0	Neu5Gcα2-3Galβ1-3GlcNAcβ1-3LacβProNH$_2$
62	Ac	6S-LacNAc	α3	6OSO$_3^-$	Neu5Acα2-3Galβ1-4GlcNAc6SβProNH$_2$
63	Gc	6S-LacNAc	α3	6OSO$_3^-$	Neu5Gcα2-3Galβ1-4GlcNAc6SβProNH$_2$
64	Ac-Ac	lactose	α8-α3	0	Neu5Acα2-8Neu5Acα2-3Galβ1-4GlcβPro-HEG-NH$_2$
65	Ac-Ac-Ac	lactose	α8-α3	0	Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3Galβ1-4GlcβPro-HEG-NH$_2$
66	Ac(Ac)	lactose	α3/α6	0	Neu5Acα2-3(Neu5Acα2-6)Galβ1-4GlcβProNH$_2$
67	Gc(Ac)	lactose	α3/α6	0	Neu5Gcα2-3(Neu5Acα-2-6)Galβ1-4GlcβProNH$_2$
68	KDN(Ac)	lactose	α3/α6	0	KDNα2-3(Neu5Acα2-6)Galβ1-4GlcβProNH$_2$
69	Gc-Ac	lactose	α8-α3	0	Neu5Gcα2-8Neu5Acα2-3Galβ1-4GlcβProNH$_2$
70	KDN-Ac	lactose	α8-α3	0	KDNα-2-8Neu5Acα-2-3Galβ1-4GlcβProNH$_2$
71	Ac-KDN	lactose	α8-α6	0	Neu5Acα-2-8KDNα2-6Galβ1-4GlcβProNH$_2$
72	Ac-Gc	lactose	α8-α3	0	Neu5Acα2-8Neu5Gcα2-3Galβ1-4GlcβProNH$_2$
73	Ac-Gc	lactose	α8-α6	0	Neu5Acα2-8Neu5Gcα2-6Galβ1-4GlcβProNH$_2$
74	KDN-Gc	lactose	α8-α3	0	KDNα2-8Neu5Gcα2-3Galβ1-4GlcβProNH$_2$
75	Gc-Gc	lactose	α8-α3	0	Neu5Gcα2-8Neu5Gcα2-3Galβ1-4GlcβProNH$_2$

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Table 1. continued

ID	type	skeleton	linkage	modification	structure
76	Ac-Ac	lactose	α8-α6	0	Neu5Acα2-8Neu5Acα2-6Galβ1-4GlcβProNH$_2$
77	GcMe-Ac	lactose	α8-α3	Me	Neu5GcMeα2-8Neu5Acα2-3Galβ1-4GlcβProNH$_2$

Anti-Neu5Gc antibodies have diverse effects in humans. They are involved in xenotransplantation immunological responses.[4,18−20] In cancer, they have dose-dependent dual effects, facilitating tumor progression via chronic inflammation at a low concentration,[21] but inhibit tumor growth at higher concentrations,[22] and these effects can switch even at 2-fold differences of antibody dose.[23] Anti-Neu5Gc antibodies were recently suggested to be the link between increased cancer risk and red meat consumption.[16,24] Likewise, anti-Neu5Gc antibodies have been suggested to exacerbate vascular inflammatory diseases, such as atherosclerosis.[25] Human infertility could also be mediated by anti-Neu5Gc antibodies in semen and uterine fluids that bind Neu5Gc on sperm and/or endometrium.[26] In addition, Neu5Gc can be found on some glycosylated biotherapeutics, since many of those are produced in nonhuman mammalian cell lines and/or supplemented with animal-derived additives during their production.[27] Treatment with such drugs could alter the repertoire of anti-Neu5Gc antibodies in patients.[28] Furthermore, circulating anti-Neu5Gc antibodies could capture Neu5Gc-glycosylated drugs to generate immune complexes and expedite their clearance.[29] Thus, anti-Neu5Gc antibodies could have detrimental effects on human health. It had previously been shown that different healthy human sera contain anti-Neu5Gc antibodies of IgG, IgM, or IgA isotypes against seven different Neu5Gc-glycans.[12] However, the relationship between the repertoires of anti-Neu5Gc antibodies of different antibody isotypes in a larger cohort had not been studied thus far.

Intravenous immunoglobulin (IVIG) preparations are purified from plasma of thousands of healthy human donors[30] and hence are optimal for investigating antibody repertoires. IVIG preparations are collected and manufactured in several countries around the world, including diverse populations that likely differ in their dietary habits. In addition, manufacturers use slightly different production methods,[30] that overall could influence the pooled antibodies arsenal, diversity, and specificity.

Detection of anti-Neu5Gc antibodies is challenging, not only due to the high diversity of Neu5Gc-glycans but also because of the on-assay glycans presentation, which have led to establishment of different methods for their assessment.[16,31] ELISA assays allow the investigation of individual Neu5Gc-glycan epitopes at a time,[16] while EIA assays with a mixture of Neu5Gc-glycoproteins are commonly used for the detection of overall anti-Neu5Gc reactivity.[16,31−35] In comparison, dedicated sialoglycan microarrays allow high resolution detection of antibody specificity against diverse glycans simultaneously.[36−40] Here, we examined the repertoires of human IgG and IgA in IVIG and serum-derived pooled IgA against glycan microarrays fabricated with a large collection of sialic acid-containing glycans. We compare different preparations and lots of IVIG and pooled IgAs and investigate their binding patterns and specificity against diverse Neu5Gc-glycans and their matched Neu5Ac-glycans. We show that different preparations of IVIG have closely related anti-Neu5Gc IgG repertoires, whereas pooled IgA have cross-reactivity with selected Neu5Ac-glycans.

RESULTS AND DISCUSSION

IVIG and IgA Pool Recognize Diverse Neu5Gc-Glycans. To assess binding repertoires of pooled human serum IgGs or IgAs, the different polyclonal antibody-preparations were screened on glycan microarrays that contain diverse Neu5Gc-glycans and matching Neu5Ac-glycans (Table 1). Matrix analysis revealed high correlation between IVIG-1, IVIG-2, and IVIG-5, slightly less to IVIG-3 and IVIG-4, with least correlation between all IVIG and pooled IgA (Figure 1A).

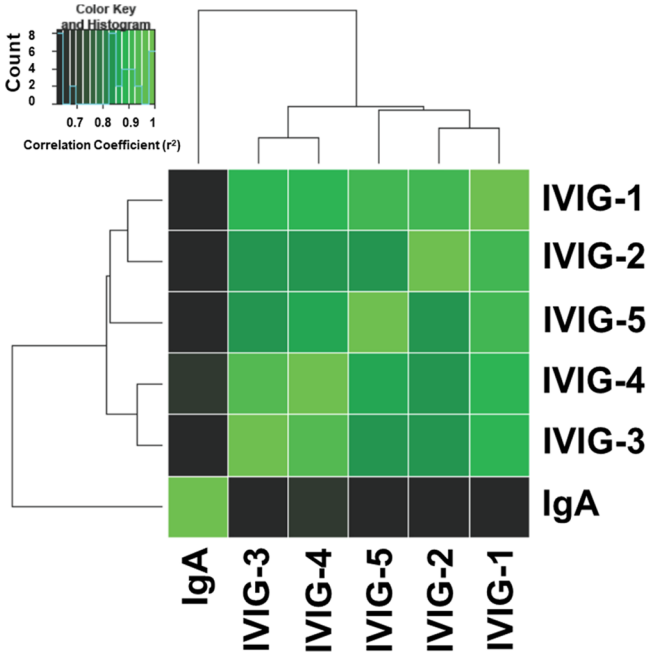

Figure 1. Diverse polyclonal human IgA and IgG preparations show pronounced recognition of Neu5Gc. Dendrogrammed correlation matrix for polyclonal IgA and IgG preparations in color code representation showing isotype-dependent clustering of glycan-recognition.

While there is no current indication that these preparations differ in their clinical efficacy, differences in IVIG repertoires could be affected by the manufacturing processes (as described in drug data sheets) and level of purity. For example, IVIG preparation can differ in purification process, stabilizers, and IgA content (IVIG1-5 IgA reported to be 37 μg/mL, 4 μg/mL, <25 μg/mL, <50 μg/mL, and 1500 μg/mL, respectively). Polyclonal IgA pools were provided by CSL Behring and do not reflect a marketed product. While interesting, we did not have access for pooled human IgM for similar analysis as such preparations are not used clinically.

Further detailed analysis of binding patterns at serial dilutions revealed that all the tested antibodies preparations recognized Sia epitopes with strong preference to Neu5Gc-glycans (Figure 2A-B). In IVIG-1,-5 there were higher intensities against some glycans compared to IVIG-2,-3,-4, although the overall patterns seemed to be similar. While, all IVIG preparations strongly recognized diverse Neu5Gc-glycans

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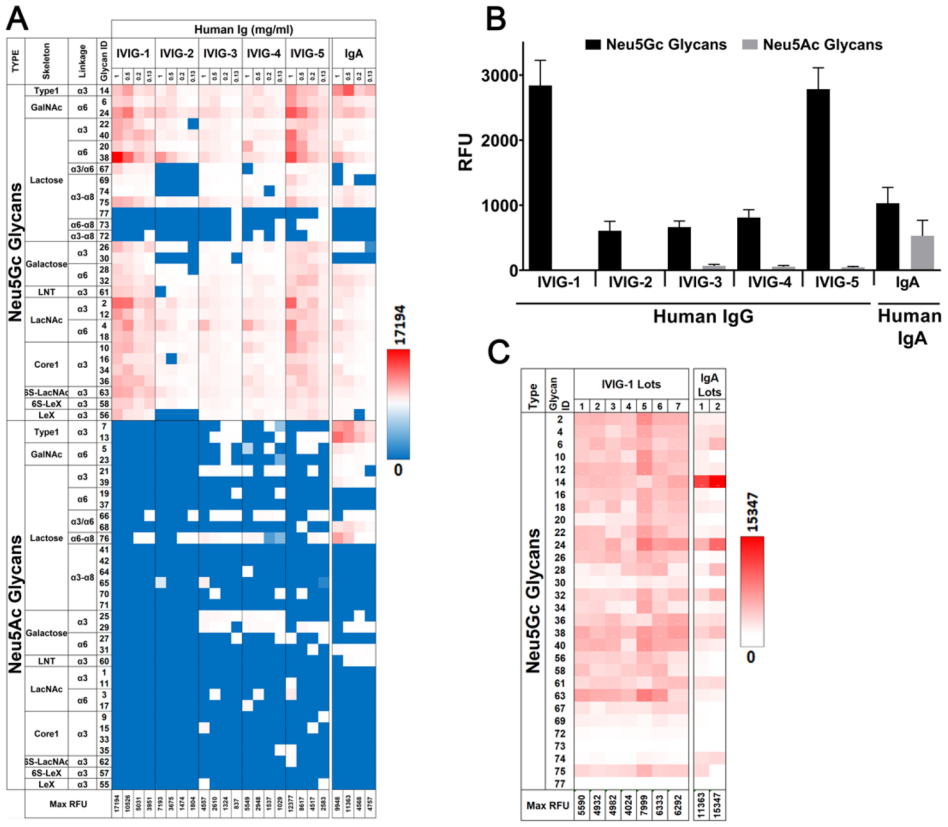

Figure 2. Human IgG and IgA have pronounced recognition of Neu5Gc-glycans with minimal lot-to-lot variability. (A) Each IVIG brand and pooled IgA were examined at serial dilutions of 1, 0.5, 0.25, and 0.125 mg/mL total protein, at 100 μL/array, glycan binding reactivity was detected with Cy3-anti-human IgG (1.2 μg/mL) or Cy3-anti-human IgA (1.6 μg/mL), respectively, and then scanned, and relative fluorescent units (RFUs) were calculated. Binding patterns were examined in accordance with glycans type, skeleton, and linkage, as described in Table 1. (B) Mean RFU of the 4 dilutions of each IVIG brand and pooled IgA against all Neu5Gc-glycans and all Neu5Ac-glycans that are described in A (mean ± sem). (C) Glycan microarray analysis of seven IVIG-1 and two pooled IgA lots was tested at 0.5 mg/mL total protein, 100 μL/array (In IVIG-1, the Pearson correlation coefficient to lot1 was 0.93, 0.92, 0.85, 0.88, 0.9, 0.82, and 0.82 between the two lots of IgA.).

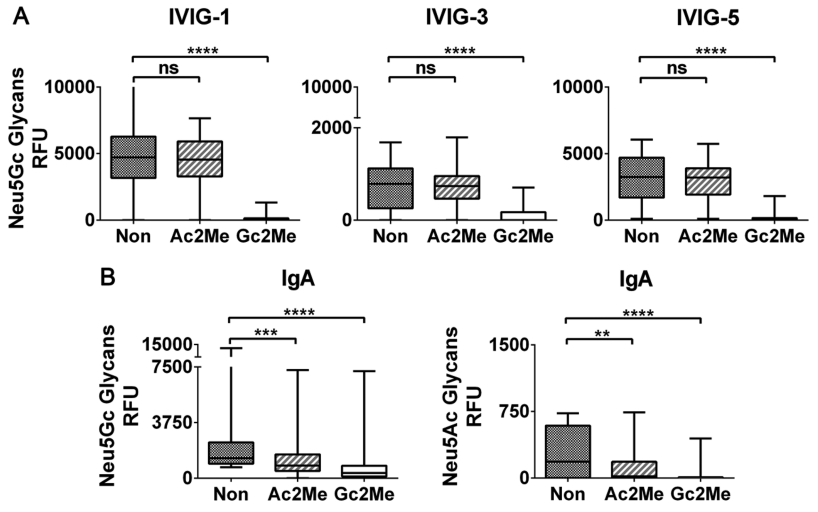

Figure 3. Competitive glycan microarray inhibition assays of human anti-Neu5Gc IgG and IgA. IVIG (A) and pooled IgA (B) were analyzed. IVIG-1, -3, and -5 and pooled IgA were examined at 0.5 mg/mL total protein in PBS/OVA pH 7 or mixed with 2 mM of 2-*O*-methyl-α-Neu5Ac (Ac2Me), 2-*O*-methyl-α-Neu5Gc (Gc2Me) in PBS/OVA pH 7, at 100 μL/tube, and incubated on ice for 2 h. Then, samples were each examined on glycan microarrays, glycan binding reactivity was detected with Cy3-anti-human IgG (1.2 μg/mL) or Cy3-anti-human IgA (1.6 μg/mL), respectively, and then scanned, and relative fluorescent units (RFUs) were calculated. (A) RFU IgG reactivities against all Neu5Gc-glycans in the presence of Ac2Me, Gc2Me, or without inhibitor were calculated (Box and Whiskers showing Min to Max; one-way ANOVA, **** $p < 0.0001$). (B) RFU IgA reactivities against all Neu5Gc-/Neu5Ac-glycans in the presence of Ac2Me, Gc2Me, or without inhibitor were calculated (Box and Whiskers showing Min to Max; one-way ANOVA, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$).

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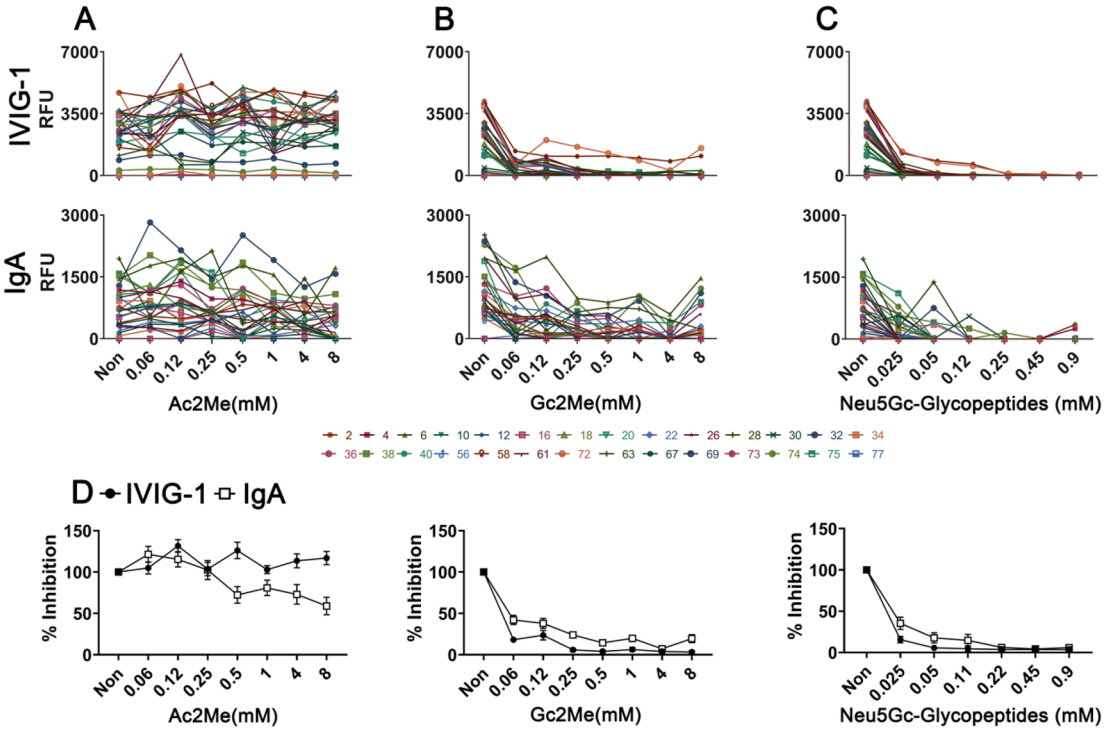

Figure 4. Differential inhibition of anti-Neu5Gc IgG and IgA reactivities. Glycan microarray binding of IVIG or serum IgA against all Neu5Gc-glycans was investigated with serial dilutions of 8 mM to 0.06 mM Ac2Me (A) or Gc2Me (B) or 0.9 mM to 0.025 mM Neu5Gc-glycopeptides, in PBS/OVA pH 7 (C). Inhibitors were serially diluted in PBS buffer (pH 7.0), and IVIG-1 or pooled IgA was added (0.5 mg/mL total protein, PBS/OVA; 100 μL/tube) and then incubated on ice for 2 h. Samples were then each examined on glycan microarrays, and binding was detected with Cy3-anti-human IgG (1.2 μg/mL) or Cy3-anti-human IgA (1.6 μg/mL) and then scanned, and relative fluorescent units (RFUs) were calculated. Statistical analysis was performed with Prism version 8, and outlier values were excluded. (A) Compared to binding with no inhibition, Ac2Me at 4 mM and 8 mM inhibits reactivity of anti-Neu5Gc IgA for each glycan, while IgG reactivity was not inhibited at all (**, $p = 0.0011$; ***, $p = 0.0002$; ns, respectively; two-way ANOVA, Dunnett post-test). (B) Compared to binding with no inhibition, Gc2Me inhibits both anti-Neu5Gc IgA/IgG reactivity (****, $p < 0.0001$; two-way ANOVA, Dunnett post-test). (C) Compared to binding with no inhibition, Neu5Gc-glycopeptides inhibit both anti-Neu5Gc IgA/IgG reactivity (****, $p < 0.0001$; two-way ANOVA, Dunnett post-test). (D) Normalized mean RFU comparing anti-Neu5Gc IVIG-1 and IgA shows that only in IgA Ac2Me inhibits reactivity against Neu5Gc-glycans, while IgG shows no inhibition (two-way ANOVA, Sidak post-test; 4 mM $p = 0.0015$, 8 mM $p < 0.0001$).

with minimal reactivity against Neu5Ac-glycans, pooled IgA also recognized some Neu5Ac-glycans with underlying skeleton structures of Type-1 or GalNAc (ID #5, 7, 13, 23) or lactose (ID #21, 39, 66, 68, 76; Figure 2A). IgA recognition of Neu5Ac-glycans could represent cross-reactivity with Neu5Gc-glycans, especially in light of the fact that they differ only by the additional hydroxyl group. Neu5Ac is a common "self" carbohydrate,[41] hence IgA cross-reactivity with such glycans could result in autoreactivity on cells. Further examination of seven different lots of IVIG-1 and two lots of pooled IgA showed minimal lot-to-lot variability in both IVIG and IgA, with overall similar binding reactivity and intensity (Figure 2C). This analysis revealed that different preparations of IVIG or IgA from a large collection of human donors have similar Neu5Gc-glycans recognition patterns.

Specificity of IVIG and IgA against Neu5Gc-Glycans. To evaluate the specificity against Neu5Gc-glycans, we used competitive glycan microarray assays with free Neu5Ac or Neu5Gc. To better control the efficacy of binding inhibition, we fixed the sialic acids ring structure in the natural Siaα2-linkage in the form of 2-*O*-methyl-α-Neu5Ac (Ac2Me) or 2-*O*-methyl-α-Neu5Gc (Gc2Me), thereby preventing mutarotation to the favorable unnatural β-anomers.[12] Binding patterns of three IVIG preparations and pooled IgA were tested on glycan microarrays, in the presence or absence of Ac2Me or Gc2Me. Binding of all IVIG preparations against Neu5Gc-glycans was strongly inhibited with Gc2Me but not with Ac2Me (Figure 3A). Since Neu5Gc differ from Neu5Ac by a single oxygen atom in its additional hydroxyl at C5, this differential inhibition suggests strong specificity against Neu5Gc-glycans. In contrast, pooled IgA binding to both Neu5Gc-/Neu5Ac-glycans was inhibited with either Gc2Me or Ac2Me (Figure 3B). These observations suggest that human IgA pool contains antibodies that cross-react with the two glycan isoforms. Hence, in normal human serum anti-Neu5Gc IgGs seem to be highly specific, whereas anti-Neu5Gc IgAs show cross-reactivity with related Neu5Ac-glycans. Serum/plasma IgA is the second most abundant isotype after IgG, and although largely considered anti-infectious and anti-inflammatory,[42] some reports also suggest it can be pro-inflammatory.[43] While serum IgA is mostly monomeric, in the mucosa it is more commonly found as polymeric IgA where it is the most privileged isotype.[30,44] Emerging evidence suggests that serum/plasma IgA plays diverse roles in immune functions in health and disease conditions.[43] Hence the cross-reactivity of anti-Neu5Gc IgAs with Neu5Ac-glycans commonly expressed on all human cells could have detrimental immunological effects such as in autoimmune diseases.

To further investigate the differences between reactivities of anti-Neu5Gc IgG/IgA, we tested IVIG and pooled IgA on

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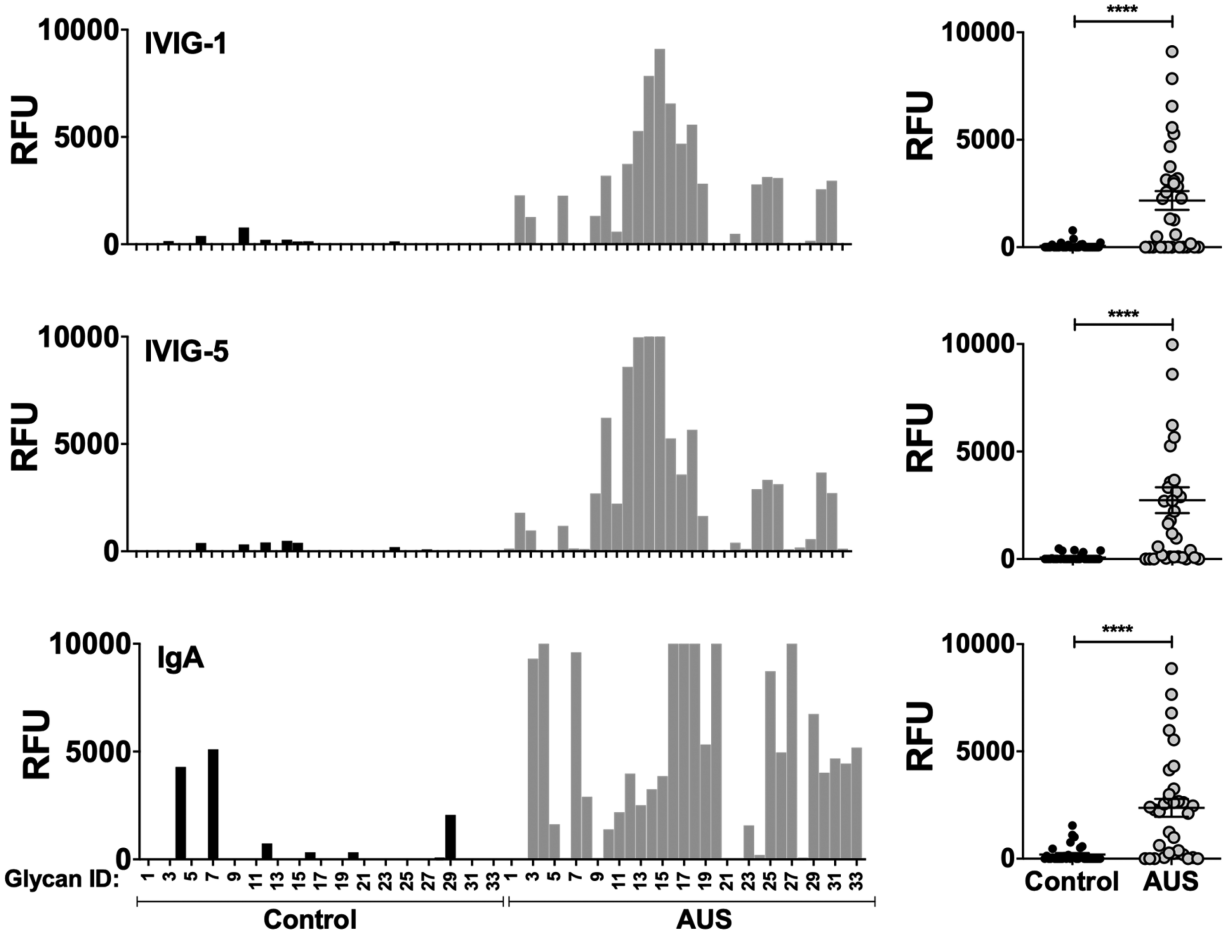

Figure 5. IVIG and pooled IgA contain diverse anti-carbohydrate antibodies. Glycan microarray slides were treated with 50 mU/well AUS sialidase, washed, and then incubated with IVIG-1, IVIG-5, or pooled IgA (0.25 mg/mL at 100 μL/array). Glycan binding reactivity was detected with Cy3-anti-human IgG (1.2 μg/mL) or Cy3-anti-human IgA (1.6 μg/mL), respectively, and then scanned, and relative fluorescent units (RFUs) were calculated and analyzed (scatter plots show mean ± sem; paired *t* test, **** $p < 0.00001$).

glycan microarrays with increasing concentrations of Ac2Me, Gc2Me, or Neu5Gc-glycopeptides. Neu5Gc-glycopeptides were digested from serum sialo-glycoproteins from wild-type C57BL/6 mouse[31] and contain a diverse collection of naturally expressed Neu5Gc-glycan structures; therefore, they could authentically represent the competing entities for circulating anti-Neu5Gc antibodies. Using Ac2Me as a competitive inhibitor, anti-Neu5Gc IgG reactivities were not inhibited even at 8 mM, while anti-Neu5Gc IgA reactivities were inhibited at 4 mM and 8 mM (Figure 4A, D). Using Gc2Me as a competitive inhibitor, both anti-Neu5Gc IgG/IgA reactivities reached >50% inhibition already at 0.06 mM; however, while anti-Neu5Gc IgG reactivities were completely inhibited at 0.25 mM Gc2Me, anti-Neu5Gc IgA reactivities were completely inhibited only at 4 mM (Figure 4B, D). Similar differential IgG/IgA inhibition was observed when Neu5Gc-glycopeptides were used as competitive inhibitors, maximally inhibiting anti-Neu5Gc IgG reactivities at 0.05 mM, while at 0.1 mM for IgA (Figure 4C, D). These results emphasize the differences between serum IgG and IgA recognition of Neu5Gc-glycans and the cross-reactive nature of serum anti-Neu5Gc IgA.

IVIG is widely used for a growing number of clinical conditions,[30] while plasma-derived IgA (pd-IgA; IgAbulin) has been largely examined for prophylaxis and therapy of infectious diseases such as intranasal or oral treatments.[30,45] Thus, plasma-derived IgA currently has a rather limited clinical use, mainly due to difficulties in its large scale production and lack of a clear clinical advantage.[30] In fact, as found in plasma/serum, both IVIG and pooled IgA contain an enormous collection of antibodies against diverse protein- and carbohydrate-antigens.[17,46−48] Here, the specific differences between anti-Neu5Gc IgG and IgA were examined in a system that allows for investigation of recognition of antibodies against unique antigens, even within a large pool of antibodies of diverse specificities. These unique glycan microarrays were printed with a large collection of carbohydrate antigens with terminal Neu5Gc or Neu5Ac. To further demonstrate and emphasize the presence of other anti-carbohydrate antibodies within the pools of IVIG and IgA, we used enzymatic cleavage of sialic acids on the arrays, followed by their binding assays (Figure 5). While there was no (IgG) or low (IgA) recognition of Neu5Ac-glycans against the native sialoglycan microarrays, after the sialidase enzymatic treatment (that peel-off terminal sialic acid moieties from the array-printed glycans), there was a dramatic increase in binding of both IVIG and pooled IgA, representing robust antibodies recognition of the resulting non-sialylated glycans (Figure 5). This increased IgG/IgA binding clearly demonstrates the full capacity of IVIG and pooled IgA to bind multiple carbohydrate antigens. More importantly, it also suggests that within the plasma/serum, antibodies against Neu5Gc/Neu5Ac-glycans compete with all the other anticarbohydrate antibodies. Altogether, these data

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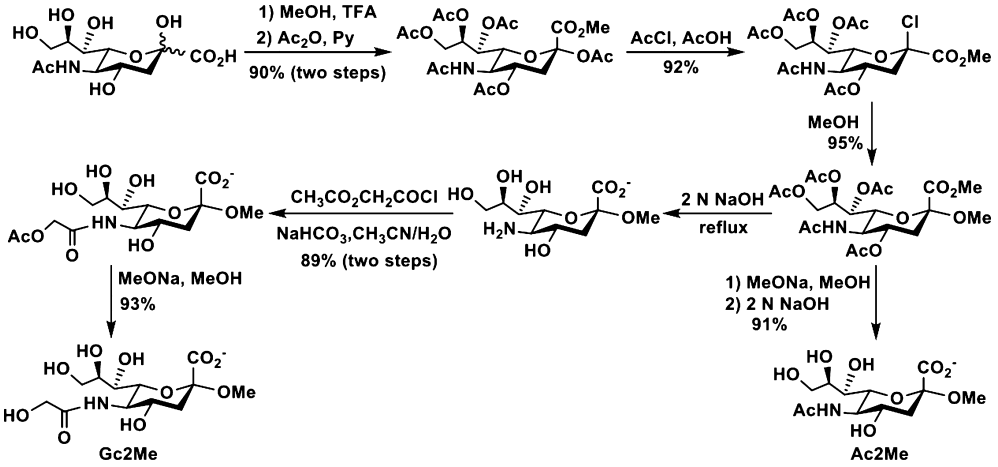

Figure 6. Preparation of 2-*O*-methyl-α-Neu5Gc (Gc2Me) and 2-*O*-methyl-α-Neu5Ac (Ac2Me).

support the notion that sialic acids serve as 'self-associated molecular patterns' or SAMPs,[41] that actually provide a "shield" against a potential attack on self-carbohydrates by circulating antibodies. Hence in that respect, the cross-reactive recognition of "self" Neu5Ac-glycans by IgA could potentially undermine the sialic acids protective defense and represent potential autoreactive detrimental effects, not only by therapeutic pooled IgA but also by circulating IgAs in the blood of individual humans.

CONCLUSIONS

Anti-Neu5Gc antibodies play important biological roles in human health and disease.[4,27,49,50] Sialic acid-focused glycan microarrays provide excellent tools to investigate the full repertoire of such antibodies[36−40] and their isotype relationship. Using sialoglycan-dedicated microarrays, we showed that pooled human IgG or IgA preparations contain diverse anti-Neu5Gc reactivities. Regardless of origin and/or methodology of purification, different IVIG preparations contain highly specific anti-Neu5Gc IgG antibodies with low lot-to-lot variability and high specificity inhibited with Gc2Me or Neu5Gc-glycopeptides but not with Ac2Me. In contrast, pooled IgA samples contain anti-Neu5Gc IgA antibodies that have cross-reactivity with Neu5Ac-glycans and hence may be involved with autoreactivity in some individuals. While IgA is more commonly regarded as anti-inflammatory, it had been suggested that circulating IgA immune complexes could have deleterious roles to the host by sustained activation of IgA receptors.[51] Furthermore, multiplex assays with glycan microarrays suggested that antiglycan IgA repertoires are highly correlated with IgG repertoires within an individual[52] and that measurements of serum anti-glycan antibodies could be significantly influenced by the levels of the different antibody isotypes.[53] Altogether these findings warrant further investigation into the roles of anti-Neu5Gc IgA and their differential effects from anti-Neu5Gc IgG in humans.

MATERIALS AND METHODS

Materials. PBS × 10 was purchased from Hy-laboratories, ethanol amine was purchased from Fisher, and calcium chloride, ovalbumin (Grade V) sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, Tween-20, and Tris/HCl were purchased from Sigma-Aldrich. BCA kit was purchased from Thermo. Antibodies were purchased from Jackson ImmunoResearch: Cy3-anti-human IgA, Cy3-goat-anti-human IgG H+L, Chromopure Whole Human IgG. Five IVIG preparations were examined: GammaGard (Baxter, USA; IVIG-1) was a kind gift from Prof. Adriana Tremolot, Rady Children's Hospital, San Diego. GammaPlex (Bio Products Laboratory UK; IVIG-2), Privigen and Hizentra (both from CSL Behring USA; IVIG-3 and IVIG-4, respectively), and Immunovenin-Intact (BB-NCIPD Bulgaria; IVIG-5) were used. IVIG preparations can differ in purification process, stabilizers, and IgA content (e.g., IgA content is reported to be 37 μg/mL in IVIG-1, 4 μg/mL in IVIG-2, <25 μg/mL in IVIG-3, <50 μg/mL in IVIG-4, and 1500 μg/mL in IVIG-5). Serum-derived pooled IgA preparations were kindly provided by CSL Behring, Bern (Switzerland).

Preparation of 2-*O*-Methyl-α-Neu5Gc (Gc2Me) and 2-*O*-Methyl-α-Neu5Ac (Ac2Me). 2-*O*-Methyl-α-Neu5Ac (Ac2Me) was prepared from commercially available Neu5Ac using reported procedures (Figure 6).[12,54,55] Briefly, Neu5Ac was converted into the corresponding per-*O*-acetylated methyl ester by treatment with trifluoroacetic acid (TFA) in dry methanol followed by reacting with acetic anhydride in dry pyridine. The obtained compound was treated with acetyl chloride in acetic acid (AcCl/HOAc) to produce acetochloroneuraminic methyl ester. The crude product was dissolved in anhydrous methanol, and the mixture was kept at room temperature for 1 h to form the crude acetylated Neu5Ac methyl glycoside. The product was then treated with NaOMe/MeOH and NaOH (2 N), neutralized with H^+ resin, and purified by silica gel chromatography to produce Ac2Me. 2-*O*-Methyl-α-Neu5Gc (Gc2Me) was prepared from acetylated Neu5Ac methyl glycoside, which was treated with NaOH (2 N) and reflux for 8 h to hydrolyze the ester bonds and remove the *N*-acetyl group. Selective acylation of the obtained free amino group using acetoxyacetyl chloride in the presence of $NaHCO_3$ in CH_3CN/H_2O (1:1) followed by de-*O*-acetylation using NaOMe/MeOH produced the desired Gc2Me. The NMR data of both Ac2Me and Gc2Me were consistent with those reported previously.[12]

Sialoglycan Microarray Fabrication. Arrays were fabricated with NanoPrint LM-60 Microarray Printer (Arrayit) on epoxide-derivatized slides (Corning) with 16 subarray blocks on each slide. Glycoconjugates were distributed into one 384-well source plates using 4 replicate wells per sample and 8 μL per well (Version 2.0). Each glycoconjugate was prepared at

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100 μM in an optimized print buffer (300 mM phosphate buffer, pH 8.4). To monitor printing quality, replicate-wells of human IgG (80, 40, 20, 10, 5, and 0.25 ng/μL in PBS with 10% glycerol) and AlexaFlour-555-Hydraside (Invitrogen, at 1 ng/μL in 178 mM phosphate buffer, pH 5.5) were used for each printing run. The arrays were printed with four SMP3 pins (5 μm tip, 0.25 μL sample channel, ~100 μm spot diameter; Arrayit). Each block (subarray) has 18 spots/row, 20 columns with spot to spot spacing of 225 μm. The humidity level in the arraying chamber was maintained at about 70% during printing. Printed slides were left on an arrayer deck overnight, allowing humidity to drop to ambient levels (40–45%). Next, slides were packed, vacuum-sealed, and stored at room temperature (RT) until used.

Sialoglycan Microarray Binding Assay. Slides were developed and analyzed as previously described,[36] with some modifications. Slides were rehydrated with dH_2O and incubated for 30 min in a staining dish with 50 °C prewarmed ethanolamine (0.05 M) in Tris-HCl (0.1 M, pH 9.0) to block the remaining reactive epoxy groups on the slide surface and then washed with 50 °C prewarmed dH_2O. Slides were centrifuged at 200 × g for three min and then fitted with ProPlate Multi-Array 16-well slide module (Invitrogen) to divide into the subarrays (blocks). Slides were washed with PBST (0.1% Tween 20), aspirated, and blocked with 200 μL/subarray of blocking buffer (PBS/OVA, 1% w/v ovalbumin, in PBS, pH 7.3) for 1 h at RT with gentle shaking. Next, the blocking solution was aspirated, and 100 μL/block of IVIG or IgA pools (diluted to 1, 0.5, 0.25, or 0.125 mg/mL total protein in PBS/OVA or as listed in the figure legends) were incubated with gentle shaking for 2 h at RT. Slides were washed three times with PBST and then with PBS for 2 min. Bound antibodies were detected by incubating with secondary antibody diluted in PBS, 200 μL/block at RT for 1 h: Cy3-anti-human IgG (1.2 μg/mL) or Cy3-anti-human IgA (1.6 μg/mL). Slides were washed three times with PBST and then with PBS for 10 min followed by removal from a ProPlate Multi-Array slide module, immediately dipped in a staining dish with dH_2O for 10 min with shaking, and then centrifuged at 200 × g for 3 min. Dry slides were immediately scanned.

Array Slide Processing. Processed slides were scanned and analyzed as described at 10 μm resolution with a Genepix 4000B microarray scanner (Molecular Devices) using 350 gain. Image analysis was carried out with Genepix Pro 6.0 analysis software (Molecular Devices). Spots were defined as circular features with a variable radius as determined by the Genepix scanning software. Local background subtraction was performed.

Preparation of Neu5Gc-Glycopeptides. Neu5Gc-Glycopeptides were prepared by Pronase digestion of wild-type C57BL/6 mouse sera, as described.[31] Briefly, mouse sera (80 mg) were diluted in sterilized digestion buffer (0.1 M Tris-HCl pH 8.0 and 10 mM $CaCl_2$). Simultaneously, filter sterilized Pronase solution (Calbiochem) was prepared at a final concentration of 10 mg/mL in distilled water and incubated at 60 °C for 30 min to remove any contaminating sialidase activities. Serum was digested with 500 μL of sterile Pronase solution at 37 °C, up to 5 days, with daily additions of 250 μL of sterile Pronase. Subsequently, Pronase digest was filtered (Amicon 3 kDa filters; Sigma-Aldrich), the top fraction was collected, and sialic acids content was analyzed and then stored at −20 °C until used.

Competitive Glycan Microarray Inhibition Assays. Slides were developed as described above with the following modification: IVIG or pooled IgA was preincubated with either PBS/OVA, 2-*O*-methyl-α-Neu5Ac (Ac2Me), 2-*O*-methyl-α-Neu5Gc (Gc2Me), or Neu5Gc-glycopeptides, diluted in PBS/OVA buffer (pH 7.0) for 2 h on ice (inhibitor concentrations are described in context). The inhibitor-antibodies complexes were then applied onto the microarray for 2 h, washed, and detected with Cy3-anti-human IgG or IgA secondary antibodies, respectively. Slides were scanned and analyzed using GenePix Pro software as described.

Sialidase Glycan Microarray Assay. Microarray slides were blocked with ethanolamine and washed, as described above, and then treated with *Arthrobacter ureafaciens* Sialidase (AUS) solution (0.25 mU/μL in reaction buffer containing 50 mM sodium acetate pH 5.5, at 100 μL/well) or control treated without sialidase (PBS buffer). The slides were then washed and incubated in blocking buffer, and then IVIG or pooled plasma IgA was applied and analyzed, as described above.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism 8.2 and "R" and described in context in the figure legends.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.9b00273.

Glycan microarray data file (XLSX)

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V.P.-K. designed the experiments, S.L.B.-A. conducted the research with assistance of C.S. and S.V.G. H.Y., X.C., and S.V.G. provided critical reagents. V.P.-K. and S.L.B.-A. wrote the manuscript, and all authors read and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Neu5Gc, *N*-glycolylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; Gc2Me, 2-*O*-methyl-α-Neu5Gc; Ac2Me, 2-*O*-

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methyl-α-Neu5Ac; CMP-Neu5Ac, cytidine 5′-monophosphate-*N*-acetylneuraminic acid; Sia, sialic acid

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