Modulating IgG effector function by Fc glycan engineering

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Contributed by Jeffrey V. Ravetch, February 15, 2017 (sent for review February 9, 2017; reviewed by Robert M. Anthony and Diane Mathis)

IgG antibodies contain a conserved *N*-glycosylation site on the Fc domain to which a complex, biantennary glycan is attached. The fine structures of this glycan modulate antibody effector functions by affecting the binding affinity of the Fc to diverse Fc receptor family members. For example, core fucosylation significantly decreases antibody-dependent cellular cytotoxicity (ADCC), whereas terminal α2,6-sialylation plays a critical role in the anti-inflammatory activity of human i.v. immunoglobulin therapy. The effect of specific combinations of sugars in the glycan on ADCC remains to be further addressed, however. Therefore, we synthesized structurally well-defined homogeneous glycoforms of antibodies with different combinations of fucosylation and sialylation and performed side-by-side in vitro FcγR-binding analyses, cell-based ADCC assays, and in vivo IgG-mediated cellular depletion studies. We found that core fucosylation exerted a significant adverse effect on FcγRIIIA binding, in vitro ADCC, and in vivo IgG-mediated cellular depletion, regardless of sialylation status. In contrast, the effect of sialylation on ADCC was dependent on the status of core fucosylation. Sialylation in the context of core fucosylation significantly decreased ADCC in a cell-based assay and suppressed antibody-mediated cell killing in vivo. In contrast, in the absence of fucosylation, sialylation did not adversely impact ADCC.

Fc receptor | IgG | glycosylation

The humoral immune response is characterized by polyclonality, not only in the diversity of the somatically rearranged, antigen-binding Fab domain of IgG, but also in the IgG constant domain (Fc domain) and the effector molecules with which the Fc interacts upon the formation of an immune complex or the opsonization of a target cell (1). This Fc diversity is mediated by heterogeneity arising from the differences in the amino acid sequences among the four subclasses of human IgG (IgG1, IgG2, IgG3, and IgG4) and from the heterogeneity of the biantennary complex-type *N*-linked glycan attached at Asn297, a site that is conserved in all IgG subclasses and species examined (2). This structural heterogeneity of the Fc domain allows for modulation of its 3D conformation, which results in the selective engagement of particular classes of Fc receptors (FcRs) with distinct effector activities. Thus, for any single Fab, a variety of Fc structures is possible, thereby resulting in the potential for distinct effector responses.

The canonical FcRs (type I FcRs) for human IgG (hFcγRs) include both activating (FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA, and FcγRIIIB) and inhibitory (FcγRIIB) receptors that mediate cytotoxicity/proinflammatory responses and inhibitory responses, respectively. The cellular outcome of IgG interactions with the FcγRs is regulated by the affinity of the IgG Fc for the specific receptor and the expression pattern of those receptors on effector cells (3). Because most effector cells coexpress activating and inhibitory FcγRs, the ratio of the binding affinities of a specific IgG Fc to these receptors determines the outcome of the IgG–FcγR interaction. Thus, IgG modifications that selectively enhance the affinity of IgG for activating FcγRs will augment the biological response, such as antibody-dependent cell-mediated cytotoxicity (ADCC) or cytokine release.

The glycan composition of the IgG Fc domain regulates the differential engagement of FcRs. The N297 biantennary *N*-glycan is composed of a heptasaccharide core, which can be further extended with core fucose (Fuc), terminal galactose (Gal), terminal sialic acid (Sia), and bisecting GlcNAc through selective enzymatic glycosylation reactions (4–6). It is well established that core fucosylation of the Fc *N*-glycan modulates the Fc's affinity for the activating FcγRIIIA. Removal of the core fucose increases the Fc's affinity for FcγRIIIA and thereby augments ADCC in vitro and in vivo (7, 8). On the other hand, terminal α2,6-sialylation has been shown to be critical for the anti-inflammatory activity of i.v. immunoglobulin therapy (9–11). This activity has been demonstrated to be mediated through the interaction of IgG with a different class of Fc receptors, the type II FcRs (11).

Although the effect of Fc sialylation on the anti-inflammatory activity has been characterized (1, 12), how sialylation regulates the Fc's affinity for FcγRs and the ADCC activity is less clear. Whereas previous cell-based assays and animal studies demonstrated that Fc sialylation down-regulates the ability of the Fc to engage FcγR and trigger ADCC activity of antibodies (9, 10, 13), a recent report by Lin et al. (14) has suggested that Fc sialylation does not affect ADCC. However, the conclusion of Lin et al. was supported by their analysis of only nonfucosylated IgG, and the authors presented no data comparing the effect of sialylation in the context of core fucosylation. Furthermore, most biologically relevant human IgGs are core-fucosylated instead of nonfucoslayed glycoforms (15).

Significance

The *N*-glycan composition of the constant (Fc) domain of IgG can modulate antibody effector functions by affecting the ability of the Fc to bind various Fc receptors (FcγRs). Most therapeutic IgG antibodies carry heterogeneous *N*-glycans, which might not be optimal for their therapeutic purpose. To understand the contribution of each *N*-glycan component on antibody effector function, we generated homogeneous IgG1 glycoforms using a chemoenzymatic approach and performed side-by-side in vitro binding, antibody-dependent cell-mediated cytotoxicity (ADCC), and in vivo IgG-mediated cell depletion assays. Our results confirm the dominant positive effect of removing the core fucose on FcγRIIIA binding and ADCC. Our study further reveals that sialylation adversely impacts ADCC in the context of core fucosylation but not in its absence.

Author contributions: T.L., D.J.D., J.V.R., and L.-X.W. designed research; T.L., D.J.D., and S.B. performed research; T.L., D.J.D., and J.P.G. contributed new reagents/analytic tools; T.L., D.J.D., J.V.R., and L.-X.W. analyzed data; and T.L., D.J.D., J.V.R., and L.-X.W. wrote the paper.

Reviewers: R.M.A., Harvard Medical School; and D.M., Harvard Medical School.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1702173114/-/DCSupplemental.

To address this discrepancy and systematically characterize the biological activities of various sialylated/asialylated and fucosylated/afucosylated IgG glycoforms in a controlled manner, we synthesized a series of well-defined and homogeneous IgG isoforms using a chemoenzymatic Fc glycan remodeling approach (16), and performed side-by-side in vitro FcγR-binding analyses, cell-based ADCC assays, and in vivo IgG-mediated cellular depletion studies in mice humanized for the FcγR family members. We found that core fucosylation exerted an adverse effect on FcγRIIIA binding, in vitro ADCC, and in vivo IgG-mediated cellular depletion, regardless of sialylation status. More importantly, we demonstrated that Fc sialylation of IgG antibodies in the context of core fucosylation resulted in a significant decrease in its ADCC activity in cell-based assays and, to a lesser extent, in cellular depletion assays in vivo.

Results

Generation of Well-Defined, Homogenous IgG Glycoforms. Glycoengineering of the widely used anti-CD20 IgG antibody rituximab was performed by a chemoenzymatic glycosylation remodeling method that we have reported previously (16, 17) (Fig. 1). Commercially obtained rituximab with heterogeneous *N*-glycans ($G_{0-2}F$) was deglycosylated with an endoglycosidase (EndoS2) alone to generate (Fucα1,6)GlcNAc-rituximab (**1**), or with endoglycosidase/fucosidase to generate GlcNAc-rituximab (**2**) without core fucose. Then homogeneous glycoforms were generated by a single-step EndoS2 mutant-catalyzed glycosylation with preassembled sialo complex-type (SCT; **3**) or complex-type (CT; **4**) *N*-glycan oxazolines, using **1** or **2** as the acceptor. The four rituximab glycoforms generated were sialylated/core fucosylated (S2G2F; **5**); asialylated/core fucosylated (G2F; **6**); sialylated/afucosylated (S2G2; **7**), and asialylated/afucosylated (G2; **8**). The synthetic IgG glycoforms were assessed by LC-MS analysis and shown to be homogeneous (Figs. S1 and S2). The availability of well-defined, homogeneous glycoforms is important for comparative studies, because those heterogeneous sialylated glycoforms generated by lectin enrichment or incomplete enzymatic transformation and used for previous in vitro or in vivo studies may contain minor but more impactful glycoforms, which could complicate the interpretation of any experimental data.

Characterization of IgG Glycovariant Affinities for FcγRs. Fc interactions with FcγRIIIA, an activating FcγR expressed on the surface of innate immune cells, mediates the killing of Ab-coated target cells in vitro and in vivo (18). Two FcγRIIIA alleles exist in the general population, the higher-affinity V158 allele and the lower-affinity F158 allele. We assessed the binding affinity of our IgG glycoforms to these alleles by both ELISA and surface plasmon resonance (SPR) analysis. On ELISA, the afucosylated glycoforms (S2G2 and G2) showed a higher affinity for FcγRIIIA-V158 compared with the fucosylated glycoforms (S2G2F and G2F) regardless of the sialylation state (EC_{50} = 1.4–1.6 nM vs. 36–55 nM) (Fig. 2*A* and Table 1). In the context of core fucosylation, the sialylated glycoform (S2G2F; showed slightly reduced affinity for FcγRIIIA-V158 compared with the asialylated glycoform (G2F) (EC_{50} = 55 nM vs. 36 nM); however, the difference was not significant. Similar results were obtained with the lower-affinity F158 allele of FcγRIIIA. The SPR binding analysis showed the same trend; regardless of sialylation status, all afucosylated glycoforms showed increased affinity for FcγRIIIA compared with the core-fucosylated glycoforms (Table 2). In the context of core fucosylation, the sialylated glycoforms again showed only a modest reduction in affinity for FcγRIIIA (S2G2F vs. G2F). Furthermore, modification of the sialylation or fucosylation state had only modest effects on interactions with the higher- and lower-affinity alleles of FcγRIIA, as well as the inhibitory FcγRIIB.

Sialylation Reduces the in Vitro ADCC Activity of Core-Fucosylated IgG. We assessed the in vitro ADCC activity of each antibody glycoform in a natural killer (NK) cell activation assay, which is more sensitive and quantitative than a conventional in vitro cell-killing assay (19, 20). This assay quantifies the biological consequences of engaging FcγRIIIA, the sole FcγR expressed by NK cells. We found that both sialylation and core fucosylation regulate ADCC activity in vitro (Fig. 3). Core fucosylation adversely affected ADCC, with both of the afucosylated (S2G2 and G2) glycoforms showing significantly enhanced NK cell activation and CD107a up-regulation compared with the core-fucosylated/asialylated glycoform (G2F). In the presence of core fucosylation, sialylation of the Fc glycan led to a significant decrease in ADCC NK cell activation. Based on EC_{50} values, the sialylated/core-fucosylated glycoform (S2G2F) was >20-fold less active than the corresponding asialylated/core-fucosylated glycoform (G2F); however, sialylation of the afucosylated glycoform did not affect NK cell activation. Thus, the effects of sialylation are dependent on the status of core fucosylation and of consequence only in the context of core fucosylation.

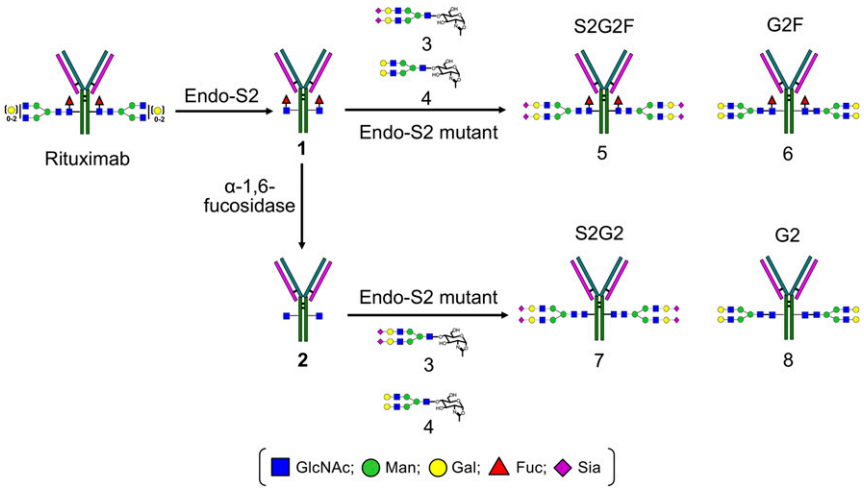

Fig. 1. Synthesis of homogeneous antibody glycoforms via chemoenzymatic glycosylation remodeling of the Fc glycans. Structures of Fc glycans on the synthetic rituximab glycoforms used for the present study: S2G2F, sialylated/core-fucosylated glycoform; G2F, asialylated/core-fucosylated; S2G2, sialylated/afucosylated; G2, asialylated/afucosylated; and $G_{(0-2)}F$, the commercial rituximab with core fucosylation and varied galactosylation.

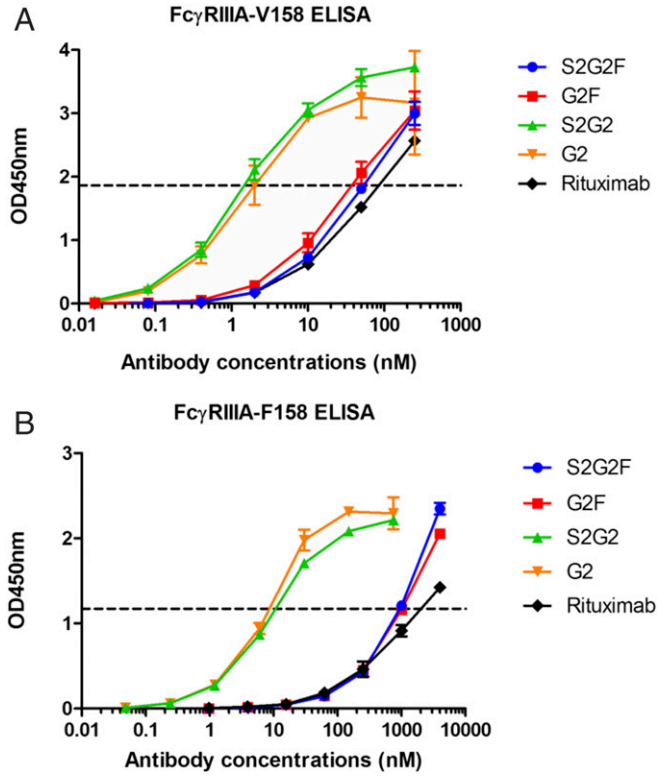

Fig. 2. Binding of rituximab glycovariants to human FcγRIIIA-V158 (*A*) and FcγRIIIA-F158 (*B*). In *A*, FcγRIIIA-V158 (0.5 μg/mL) was coated onto a 96-well plate overnight. Binding was tested with rituximab glycoforms in concentrations ranging from 250 nM to 0.016 nM (fivefold serial dilutions). In *B*, FcγRIIIA-F158 (5 μg/mL) was coated onto a 96-well plate overnight. For afucosylated rituximab glycoforms, binding was tested using concentrations ranging from 750 nM to 0.016 nM (fivefold serial dilutions). For fucosylated rituximab glycoforms, binding was tested using concentrations ranging from 4,000 nM to 0.98 nM (fourfold serial dilutions).

Both Sialylation and Core Fucosylation Regulate IgG-Mediated Cell Killing in Vivo. To further evaluate the impact of *N*-glycan core fucosylation and sialylation on effector function, we tested the ability of rituximab (anti-hCD20) glycoforms to deplete huCD20$^+$ B cells in an FcγR-humanized mouse model. We used mice expressing the full array of human FcγRs on a murine FcγR-deficient background that were crossed to huCD20-Tg mice, to allow targeting of huCD20$^+$ B cells (18, 21). The afucosylated Fc glycovariants (S2G2 and G2) showed significant and similar B220$^+$ B-cell depletion in the blood (Fig. 4), regardless of the sialylation state. These data recapitulate the in vitro binding and ADCC results and confirm that in the absence of core fucose, sialylation has no influence on this effector function. In contrast, the wild-type rituximab and the fucosylated G2F and S2G2F variants did not deplete B cells, suggesting that the core-fucosylated glycoforms had no significant biological impact in this FcγR-humanized in vivo mouse model.

To confirm these in vivo results, we generated glycovariants of a second huIgG antibody specific for a different antigen, mCD4, using the same chemoenzymatic approach, and tested their relative abilities to deplete CD4$^+$ T cells in FcγR-humanized mice. The afucosylated (S2G2 and G2) anti-mCD4 Fc glycovariants significantly and similarly depleted CD4$^+$ T cells in the blood (Fig. 5), regardless of the sialylation state. More interestingly, the sialylated/fucosylated (S2G2F) anti-mCD4 showed consistently less CD4$^+$ T-cell depletion compared with the asialylated/fucosylated (G2F) glycovariant. Although the difference observed was moderate, the result is consistent with the decreased in vitro ADCC activity of the S2G2F glycoform compared with the G2F glycoform. The relatively small difference in the in vivo cellular depletion assay may simply reflect the intrinsically lower sensitivity of the in vivo model, given that none of the fucosylated glycoforms showed >50% cell depletion.

Discussion

In this study, we used well-defined, homogeneous glycoforms to assess the effect of core fucosylation and sialylation on the ability of IgG Fc to interact with FcγRs, mediate in vitro ADCC, and kill antibody-opsonized cells in vivo. The use of well-defined, homogeneous glycoforms provides a major advantage for these comparative studies, given that the heterogeneous glycoforms generated by lectin enrichment or incomplete enzymatic transformation used in previous in vitro or in vivo studies may contain minor, but more impactful contaminating glycoforms, which may complicate the interpretation of results (9, 10, 13, 22–24). We demonstrate that removal of the Fc core fucose moiety could have a profound positive effect on FcγRIIIA binding, in vitro ADCC, and in vivo IgG-mediated cellular depletion, regardless of the sialylation state.

In the context of core fucosylation, the sialylated glycoforms showed only a modest reduction in affinity for FcγRIIIA compared with the asialylated glycoforms. These data confirm the binding results reported by Yu et al. (22), who used mixtures of monosialylated, disialylated, and core-fucosylated Fc glycoforms and demonstrated that the sialylated glycoforms had very similar affinities for FcγRIIIA. However, we provide data showing that in the context of core fucosylation, sialylation significantly decreased the ADCC in a cell-based assay and, to a lesser extent, in animal models, whereas in the absence of the core fucose moiety, sialylation was not much different. The small difference in affinity with FcγRIIIA cannot account for the large difference in ADCC activity observed between the sialylated and asialylated glycoforms in the context of core fucosylation. This discrepancy is likely due to the fact that the setting of the monomeric binding experiments (SPR and ELISA) might not reflect the nature of multivalent interactions (avidity) involved in the Fc–FcγR interaction in the ADCC assay, which may significantly amplify the modest difference observed in the binding affinities.

We also confirmed that core fucosylation profoundly regulates ADCC activity (Fig. 3). Core fucosylation adversely affected ADCC; each of the afucosylated glycoforms showed significantly enhanced ADCC activity compared with the core-fucosylated glycoforms. These results agree with the enhanced FcγRIIIA affinity by our afucosylated glycovariants (Tables 1 and 2), are consistent with all previous reports for afucosylated antibodies (25, 26), and confirm the results of ADCC assays for sialylated/afucosylated antibodies reported recently by Lin et al. (14) and

Table 1. Affinities of rituximab glycoforms for FcγRIIIA (V158 and F158), as determined by ELISA

	EC_{50}, nM (95% CI)				
Glycoform	S2G2F	G2F	S2G2	G2	Rituximab
FcγRIIIA V158	54.50 (35.76–83.06)	35.55 (19.09–66.20)	1.590 (1.294–1.953)	1.414 (0.7310–2.734)	63.11 (49.87–79.86)
FcγRIIIA F158	2,132 (1,452–3,130)	1,582 (1,386–1,805)	9.375 (8.841–9.94)	8.137 (6.591–10.04)	1,259 (555.6–2,854)

Table 2. Binding affinities of glycoengineered rituximab to human FcγRs measured by surface plasma resonance analysis

IgG glycoform	FcγRIIIA V158		FcγRIIIA F158		FcγRIIA R131		FcγRIIA H131		FcγRIIB	
	K_D, M	Fold change	K_D, M	Fold change	K_D, M	Fold change	K_D, M	Fold change	K_D, M	Fold change
Rituximab	1.14×10^{-7}	1.0	2.71×10^{-6}	1.0	9.72×10^{-7}	1.0	4.68×10^{-7}	1.0	1.59×10^{-6}	1.0
S2G2F	1.36×10^{-7}	0.8	2.47×10^{-6}	1.1	7.26×10^{-7}	1.3	3.15×10^{-7}	1.5	1.04×10^{-6}	1.5
G2F	9.53×10^{-8}	1.2	2.58×10^{-6}	1.1	9.28×10^{-7}	1.0	3.87×10^{-7}	1.2	1.43×10^{-6}	1.1
S2G2	1.90×10^{-9}	60	2.09×10^{-8}	130	5.73×10^{-7}	1.7	3.91×10^{-7}	1.2	9.18×10^{-7}	1.7
G2	1.98×10^{-9}	57.6	2.46×10^{-8}	110	5.63×10^{-7}	1.7	4.02×10^{-7}	1.2	9.60×10^{-7}	1.7

Rituximab glycoforms were immobilized on sensor chips via protein A capture. FcγRs were injected as analytes in twofold serial dilutions.

Kurogochi et al. (27). We clearly demonstrate here that in the presence of core fucosylation, sialylation of the Fc glycan leads to further significant decreases in ADCC activity. Thus, the effects of sialylation are dependent on the status of core fucosylation. These results confirm the previous reports by Scallon et al. (13) and Kaneko et al. (9), but are in contrast to a recent report by Thomann et al. (28), which concluded that sialylation did not affect ADCC. However, the so-called disialylated (sialylated/fucosylated) antibodies used by Thomann et al. (28) were actually mixtures of monosialylated and disialylated glycoforms that were contaminated with significant amounts of afucosylated glycoforms.

In contrast, we also observed that in the absence of core fucosylation, both sialylated (S2G2) and asialylated (G2) glycoforms showed similarly enhanced binding to FcγRIIIA, similarly enhanced in vitro ADCC, and similarly enhanced in vivo cell killing compared with the fucosylated glycoforms; there was no apparent difference between the two glycoforms in any of these parameters. In this case, it seems that the adverse effects of sialylation on FcγRIIIA binding and the downstream biological effects were overridden by the predominant beneficial effects contributed by removal of the core fucose. Mechanistically, it is tempting to speculate that the enhanced ability to engage FcγRIIIA stabilizes the Fc's conformation and overcomes the conformational flexibility imposed on the Fc by sialylation. Biophysical and structural studies will be required to address this issue. Regardless, this experimental result is consistent with the recent reports by Lin et al. (14) and Kurogochi et al. (27), although those authors did not test sialylated glycoforms concurrent with core fucosylation. Because core fucosylation is the most common and predominant modification of human IgGs (15), the adverse effects of sialylation on ADCC in the context of core fucosylation, as we demonstrate here, are clearly more biologically relevant for the in vivo activities of natural IgG antibodies.

Furthermore, although it has been previously demonstrated that fucosylated, sialylated Fc efficiently engages type II FcRs (e.g., DC-SIGN and/or other lectins) to drive an anti-inflammatory response (1, 2), the effect of afucosylation on the ability of sialylated Fc to engage type II FcRs remains unknown. Lin et al. (14) implied that sialylated afucosylated IgGs might have similar anti-inflammatory activity as the previously reported sialylated and fucosylated glycoform, but provided no data to support this assumption. Thus, it would be interesting to investigate the effect of Fc sialylation, in the context of afucosylation, on its anti-inflammatory activity using the structurally well-defined glycoforms of IgGs described in this paper.

We used FcγR-humanized mice to assess the effect of various Fc glycoforms in vivo in the context of the human FcγR family members. In both the rituximab (anti-hCD20)-mediated B-cell depletion and anti-mCD4–mediated T-cell depletion tests, the afucosylated glycoforms demonstrated similar and significant depletion of target cells, regardless of sialylation status, confirming our in vitro findings. However, neither the sialylated nor the asialylated glycoform of core-fucosylated rituximab mediated any significant depletion of B cells in vivo in FcγR-humanized mice, whereas core-fucosylated anti-mCD4 mediated a modest depletion of T cells and revealed that the sialylated form (S2G2F) is less effective than the asialylated form (G2F) in mediating cell depletion. The FcγR-humanized mouse model expresses the full array of huFcγRs on a variety of innate immune effector cells, which may have a different effect on the results of in vivo assays compared with the simple in vitro ADCC assay, which measures the effect of engaging a single activating FcγR on a single cell type. Furthermore, this FcγR-humanized

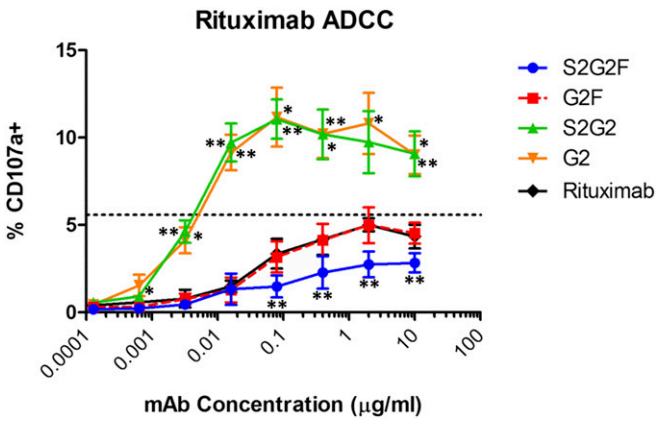

Fig. 3. Effects of core fucosylation and sialylation on in vitro ADCC. Raji cells were coated with the indicated concentrations of each glycovariant, and the cells were washed and incubated at a 1:1 ratio with PBMCs from four different leukocyte donors for 3.5 h before cell surface CD107a expression was assessed on $CD56^+CD3^-$ NK cells by immunofluorescence staining with flow cytometry analysis. Values represent the mean ± SEM frequency of $CD107a^+$ cells among NK cells from cultures with the indicated cells from four individual leukocyte donors. Significant differences between the indicated sample and the corresponding G2F glycovariant sample are shown. $*P < 0.05$; $**P < 0.01$.

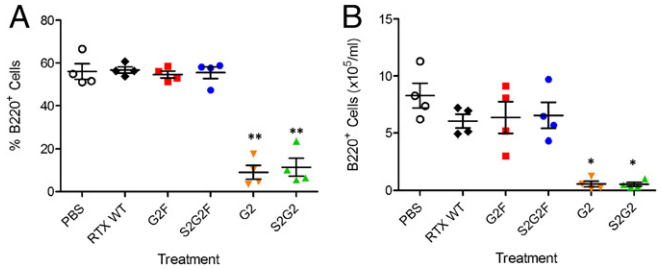

Fig. 4. Effects of core fucosylation and sialylation on in vivo killing of B cells in FcγR-humanized mice. huCD20/FcγR-humanized mice (n = 4 per group) were given 2 mg/kg of the indicated rituximab huIgG1 glycovariant 2 d before analysis of the blood. (*A*) Mean ± SEM frequencies of B220$^+$ cells among live, single lymphocytes. (*B*) Mean ± SEM absolute number of B220$^+$ cells. Significant differences between the indicated sample and the G2F glycovariant sample are shown. *P < 0.05; **P < 0.01.

mouse model expresses the lower-affinity FcγRIIIA-F158 allele, which in our ELISAs demonstrated >10-fold decreased binding affinity for fucosylated IgG compared with the higher-affinity V158 FcγRIIIA allele. Future iterations of this FcγR mouse model will include the higher-affinity V158 allele of FcγRIIIA.

In conclusion, the present study clarifies the effects of Fc sialylation, fucosylation, and their combination on antibody effector functions, and will help guide the development of future antibody-based therapeutics that differentially engage various FcR family members to elicit a diverse array of in vivo effector functions through glycoengineering of the IgG Fc domain.

Materials and Methods

Cell Lines and Mice. Raji cells were maintained in RPMI (Life Technologies) supplemented with 10% (vol/vol) FBS (Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). FcγR-humanized mice, which express all hFcγRs on the FcRα-null C57BL/6 genetic background, have been described previously (21). hCD20-Tg mice, kindly provided by Andrew Chan (Genentech), were crossed onto the FcγR-humanized background. All mice were maintained in a specific pathogen-free facility at The Rockefeller University, and all studies were approved by The Rockefeller University's Institutional Animal Care and Use Committee. Male and female mice 6–10 wk of age were used in all experiments.

Deglycosylation of IgG. To generate (Fucα1,6)GlcNAc-rituximab (**1**), commercial rituximab was incubated with wild-type Endo-S2 (500:1, wt/wt) for 1 h at 37 °C. LC-MS analyses indicated complete cleavage of the *N*-glycans on the heavy chain. The deglycosylated rituximab was purified by protein A chromatography. To generate GlcNAc-rituximab (**2**), the purified (Fucα1,6)GlcNAc-rituximab (**1**) was incubated with the α-fucosidase AlfC from *Lactobacillus casei* (50:1, wt/wt) at 37 °C. After 16 h of incubation, LC-MS monitoring indicated the complete defucosylation of (Fucα1,6)GlcNAc -rituximab (**1**) to give the product, GlcNAc −rituximab (**2**). The defucosylated rituximab was purified by protein A chromatography. LC-MS: calculated for the heavy chain of (Fucα1,6) GlcNAc-rituximab (**1**), M = 49,420 Da; found (*m/z*), 49,412 (deconvolution data); calculated for the heavy chain of GlcNAc−rituximab (**2**) carrying a GlcNAc moiety, M = 49,274 Da; found (*m/z*), 49,265 (deconvolution data).

Enzymatic Glycosylation of (Fucα1,6)GlcNAc-Rituximab to Generate Core-Fucosylated Glycoforms. A solution of 69 μM (Fucα1,6)GlcNAc-rituximab (**1**) and 1.38 mM SCTox (**3**) or CTox (**4**) (20 eq) was incubated with Endo-S2 D184M (0.05 mg/mL) at 30 °C in Tris buffer (0.1 M, pH 7.4) for 15 min. LC-MS analysis indicated completion of the glycosylation reaction. The products S2G2F-rituximab (**5**) and G2F-rituximab (**6**) were purified by protein A chromatography. LC-MS: calculated for the heavy chain of **5**, M = 51,421 Da; found (*m/z*), 51,412 (deconvolution data); calculated for the heavy chain of **6**, M = 50,839 Da; found (*m/z*), 50,830 (deconvolution data).

Enzymatic Glycosylation of GlcNAc-Rituximab to Generate Afucosylated Glycoforms. A solution of 69 μM GlcNAc-rituximab (**2**) and 1.38 mM SCTox (**3**) or CTox (**4**) was incubated with Endo-S2 D184M (0.05 mg/mL) at 30 °C in Tris buffer (0.1 M, pH 7.4) for 30 min. LC-MS analysis indicated completion of the glycosylation reaction. The products S2G2-rituximab (**7**) and G2-rituximab (**8**) were purified using protein A chromatography. LC-MS: calculated for the heavy chain of **7**, M = 51,275 Da; found (*m/z*), 51,266 (deconvolution data); calculated for the heavy chain of **8**, M = 50,693 Da; found (*m/z*), 50,684 (deconvolution data).

Antibodies and Flow Cytometry. Anti-mCD4 antibody (clone GK1.5) with an hIgG1 backbone was generated as described previously (21), and antibody was produced by transient transfection of 293T cells and subsequent protein G purification from culture supernatants, as described previously (29). Fluorescent-conjugated antibodies reactive with huCD56, huCD3, huCD107a, mCD4 (GK1.5), mCD8 (53-6.7), and B220 (RA3-6B2) were obtained from BioLegend. For flow cytometry staining experiments, dead cells were labeled (Fixable Live/Dead Stain; Life Technologies), and cells were incubated with predetermined optimal concentrations of each antibody before undergoing analysis on an LSR-II flow cytometer (BD Biosciences).

ELISA. FcγRIIIA V158 (0.5 μg/mL) or F158 (5 μg/mL; Sino Biological) in PBS buffer (pH 7.4) was coated onto a high-binding 96-well plate (Santa Cruz Biotechnology) overnight at 4 °C. After washing with PBS + Tween 20 (PBST; assay buffer), the plate was blocked with 200 μL of 1% BSA (in PBST) for 2 h. Subsequently, after three washes with PBST, 100-μL serial dilutions of rituximab (commercial rituximab and four synthetic glycoforms) were added to each well, followed by incubation for 1 h. For the V158 ELISA plate, the concentration of each rituximab glycoform ranged from 250 nM to 0.016 nM (fivefold serial dilutions). For the F158 ELISA plate, concentrations of the two afucosylated glycoforms (S2G2 and G2) ranged from 750 nM to 0.016 nM (fivefold serial dilutions), and those of the three glycoforms with core fucose (S2G2F, G2F, and commercial rituximab) ranged from 4,000 nM to 0.98 nM (fourfold serial dilutions). After incubation, the plate was washed five times, followed by incubation with 100 μL of anti-human IgG F(ab')2 HRP (1:5,000 dilution; Invitrogen) for 1 h. Finally, after five more washings, 100 μL of substrate, 3,3′,5,5′-tetramethylbenzidine, was added for signal development. The reaction was stopped by the addition of 100 μL of 2 N sulfuric acid. Absorbance at 450 nm was measured with a Spectra Max M5 microplate reader (Molecular Devices), and data were analyzed using the four-parameter nonlinear regression equation (4PL) for curve fitting.

SPR Assays. Binding between the glycoengineered rituximab glycoforms and FcγRs was measured by SPR using a Biacore T200 instrument (GE Healthcare). Protein A was immobilized at 3,000 resonance units (RU) on a CM5 biosensor chip (GE Healthcare) using amine coupling chemistry at pH 4.5. The experiment was done by capturing each glycoform of rituximab onto the protein A surface as a ligand and flow serial dilutions of FcγRs as analytes. After each cycle, the surface was regenerated by injecting a glycine HCl buffer (10 mM, pH 2.0). The FcγRs tested were FcγRIIIA-V158 and -F158, FcγRIIA-R131 and -H131, and FcγRIIB. Specific experiment conditions for each FcγR differed. For FcγRIIIA-V158, each IgG glycoform was captured at 150 RU. The receptor in 2× serial dilutions (2 μM–7.8 nM for the fucosylated glycoforms and 200 nM–0.78 nM for the afucosylated glycoforms) was injected at 30 μL/min for 180 s, followed by a 300-s dissociation. For FcγRIII-F158, each IgG glycoform was captured at 3,000 RU. The receptor in 2× serial dilutions (4 μM–7.8 nM for the fucosylated glycoforms and 400 nM–0.78 nM for the afucosylated glycoforms) was injected at 30 μL/min for 60 s, followed by a 300-s dissociation. For FcγRIIA-R131, each IgG glycoform was captured at 3,000 RU. The receptor in 2× serial dilutions (4 μM–7.8 nM for all glycoforms) was

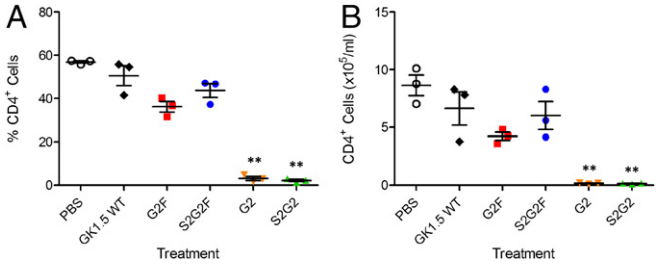

Fig. 5. Effects of core fucosylation and sialylation on in vivo killing of CD4$^+$ T cells in FcγR-humanized mice. FcγR-humanized mice (n = 3 per group) were given 2 mg/kg of the indicated GK1.5 huIgG1 glycovariant 2 d before analysis of the blood. (*A*) Mean ± SEM frequencies of CD4$^+$ cells among live, single lymphocytes from the indicated tissue. (*B*) Mean ± SEM absolute number of CD4$^+$ cells. Significant differences between the indicated sample and the corresponding G2F glycovariant sample are shown. *P < 0.05; **P < 0.01.

injected at 30 μL/min for 60 s, followed by a 300-s dissociation. For FcγRIIA-H131 and FcγRIIB, each IgG glycoform was captured at 500 RU. The receptors in 2× serial dilutions (4 μM–7.8 nM for all glycoforms) were injected at 30 μL/min for 60 s, followed by a 300-s dissociation. The results were fitted to a 1:1 Langmuir binding model in BIA Evaluation software (GE Healthcare) to get kinetic/affinity constants.

In Vitro ADCC NK Cell Activation Assay. The in vitro ADCC NK cell activation assay was modified from a previously described protocol (20, 30). In brief, Raji cells were labeled with various concentrations of rituximab huIgG1 glycovariant antibodies for 20 min, washed twice, and then cultured 1:1 with peripheral blood mononuclear cells (PBMCs) purified from donor leukocytes (obtained from the New York Blood Center) for 3 h in 96-well U-bottom plates. Surface CD107a expression by $CD56^{+}CD3^{-}$ NK cells was assessed by flow cytometry analysis.

In Vivo Depletion Experiments. The FcγR-humanized mice and huCD20/FcγR-humanized mice were given 2 mg/kg of glycovariant mAb i.p., and blood was harvested 2 d later. Red blood cells were lysed hypotonically before flow cytometry analysis.

Statistical Analysis. All statistical differences between the indicated samples were compared using Student's *t* test.

ACKNOWLEDGMENTS. We thank Patrick Smith for excellent technical assistance in carrying out these studies. The research reported in this paper was supported by NIH Grants R01GM096973 (to L.-X.W.) and U19AI111825 and R35CA196620 (to J.V.R.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. Additional support was provided by The Rockefeller University.

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