Report

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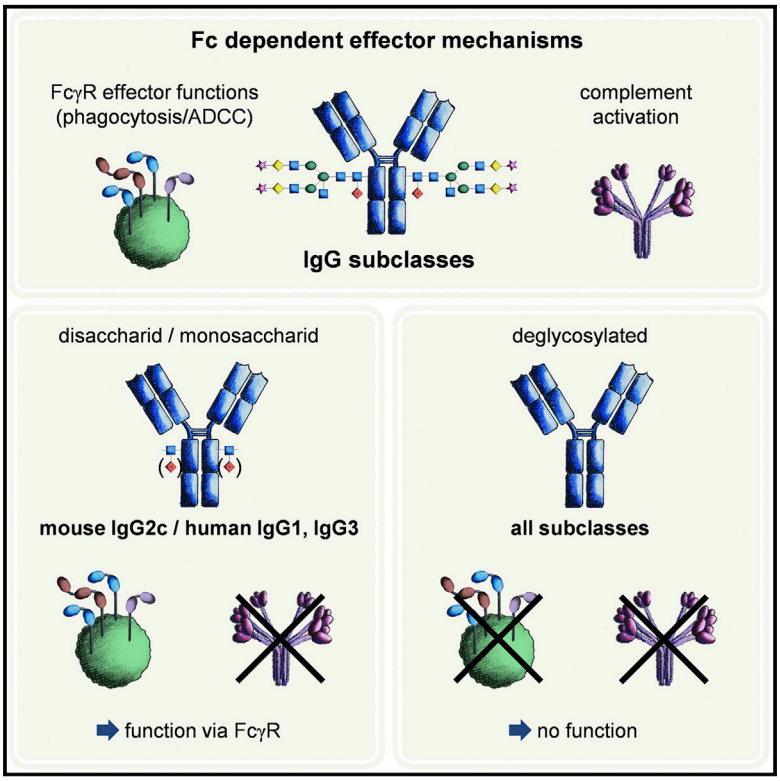
A Monosaccharide Residue Is Sufficient to Maintain Mouse and Human IgG Subclass Activity and Directs IgG Effector Functions to Cellular Fc Receptors

Graphical Abstract



Highlights

- Select IgG subclasses maintain full activity in a monosaccharide variant
- Monosaccharide antibodies mediate effector functions via FcγRs
- Monosaccharide antibodies lose complement activating activity
- Monomeric IgG-FcγR interaction analysis does not predict IgG activity

Authors

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In Brief

Lack of IgG glycosylation is generally thought to impair IgG activity. Kao et al. now show that the most active mouse and human IgG subclasses maintain their in vivo activity even if they only contain a mono- or disaccharide sugar residue but lose their capacity to activate the complement pathway.

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A Monosaccharide Residue Is Sufficient to Maintain Mouse and Human IgG Subclass Activity and Directs IgG Effector Functions to Cellular Fc Receptors

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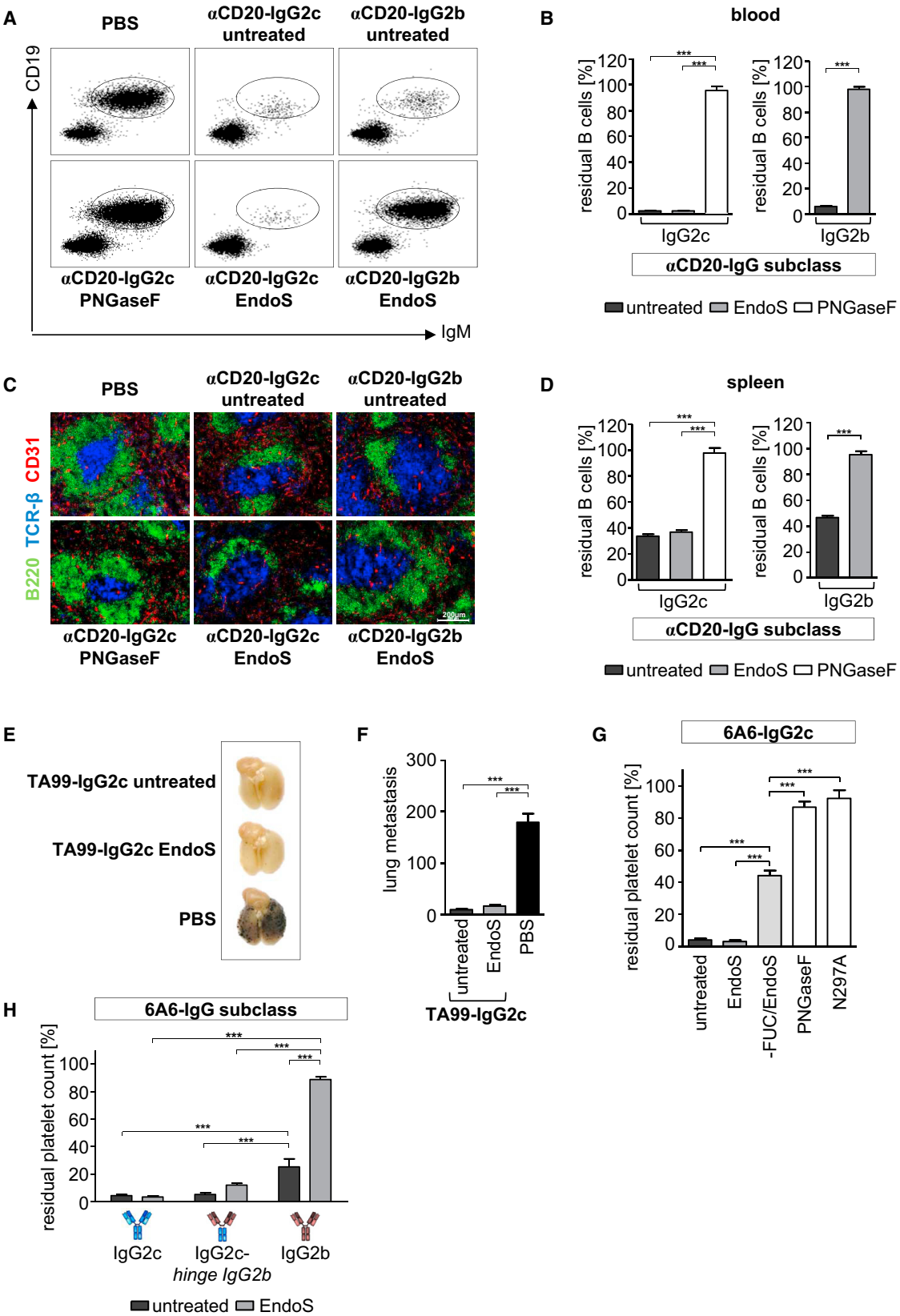
SUMMARY

Immunoglobulin G (IgG) glycosylation modulates antibody activity and represents a major source of heterogeneity within antibody preparations. Depending on their glycosylation pattern, individual IgG glycovariants present in recombinant antibody preparations may trigger effects ranging from enhanced pro-inflammatory activity to increased anti-inflammatory activity. In contrast, reduction of IgG glycosylation beyond the central mannose core is generally believed to result in impaired IgG activity. However, this study reveals that a mono- or disaccharide structure consisting of one N-acetylglucosamine with or without a branching fucose residue is sufficient to retain the activity of the most active human and mouse IgG subclasses in vivo and further directs antibody activity to cellular Fcγ receptors. Notably, the activity of minimally glycosylated antibodies is not predicted by in vitro assays based on a monomeric antibody-Fcγ-receptor interaction analysis, whereas in vitro assay systems using immune complexes are more suitable to predict IgG activity in vivo.

INTRODUCTION

Cytotoxic immunoglobulin G (IgG) antibodies have become an essential component of our armament to treat human malignant and autoimmune disease, and antibodies are under constant development to improve their activity and safety (Lim et al., 2010; Glennie and van de Winkel, 2003; Ruuls et al., 2008). The sugar moiety attached to each of the two IgG heavy chains impacts IgG activity by modulating the binding to classical Fcγ receptors (type I FcRs) and to select members of the C-type lectin family (type II FcRs) (Pincetic et al., 2014; Arnold et al., 2007). For example, afucosylated IgG preparations have a more than 10-fold higher affinity for mouse FcγRIV and human FcγRIIIA, resulting in enhanced cytotoxic activity (Nimmerjahn and Ravetch, 2005; Shinkawa et al., 2003). In contrast, IgG glycovariants rich in terminal sialic acid residues and galactose residues have an anti-inflammatory and immunomodulatory activity by losing affinity for type I FcRs while simultaneously gaining the capacity to bind type II FcRs (Schwab and Nimmerjahn, 2013). Therefore, therapeutic antibodies aiming at an optimal cytotoxic or immunomodulatory activity need to be evaluated for the precise composition of their sugar moiety to prevent unwanted activities. As production cell lines and culture conditions can impact IgG glycosylation, this represents a major quality control issue (Beck et al., 2008). Furthermore, novel approaches aiming at generating antibodies with defined sugar moieties and a minimal level of heterogeneity within the glycan moiety are in the focus of antibody research. An alternative to circumvent this complexity is to generate antibody Fc mutants that no longer require the sugar moiety for their activity (Sazinsky et al., 2008; Jung et al., 2010). A potential problem with this approach may be that these protein-engineered antibodies become immunogenic in patients, limiting their repetitive use. Another promising approach to eliminate the heterogeneity within the sugar moiety would be to generate antibodies with only the minimal level of glycosylation sufficient to maintain IgG activity in vivo. At present, however, the most widely accepted view is that reducing the level of IgG glycosylation beyond the mannose-rich central core impairs or fully abrogates IgG activity (Arnold et al., 2007; Collin et al., 2008). However, there is evidence that at least one mouse IgG subclass may be less prone to inactivation following enzymatic deglycosylation with endoglycosidase S (EndoS), which removes the majority of the IgG sugar moiety by cleaving it after the first N-acetylglucosamine (GlcNAc) residue (Albert et al., 2008; Collin et al., 2008). Moreover, it has been demonstrated that the size of the immune complex generated upon antibody binding to its target cell/molecule can mitigate the requirement for IgG glycosylation, at least in vitro (Lux et al., 2013; Pound et al., 1993). Whether this generally translates to other mouse and human IgG subclasses and is relevant for IgG activity in vivo is not known. To study this, we generated human and mouse IgG subclass glycosylation variants that solely have a mono- or disaccharide sugar moiety instead of their native sugar domain.



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We demonstrate that, quite unexpectedly, mouse IgG2c and human IgG1 and IgG3 subclasses, which represent the most active and therapeutically most widely used IgG subclasses, remained fully functional in this minimally glycosylated form, suggesting that enzymatic processing of heterogeneously glycosylated IgG preparations may represent a strategy to generate well-defined and highly active therapeutic antibody preparations.

RESULTS

A Mono- or Disaccharide Residue Is Sufficient to Maintain Mouse IgG2c Antibody Activity In Vivo

Previous studies provided convincing evidence that removal of the majority of the asparagine 297 (N297)-linked sugar moiety of IgG from different species by EndoS treatment results in impaired antibody activity in vitro and in vivo (Collin et al., 2008). Although, in the majority of these studies, mouse IgG1, IgG2b, and IgG subclasses of other rodents were used, we noted that a mouse IgG2c variant of an autoantibody may remain functional in this minimally glycosylated form (Albert et al., 2008). If generally applicable, then this result would be of great interest, as IgG2c is the most active mouse IgG subclass, due to its capacity to interact with all mouse activating Fcγ receptors (FcγRs), resulting in superior cytotoxic activity (Nimmerjahn and Ravetch, 2006). To investigate whether IgG2c antibodies are generally less prone to being inactivated through deglycosylation, we used three well-established in vivo model systems, including B cell and melanoma cell depletion via CD20- or gp75-specific antibodies, and autoantibody-mediated phagocytosis of platelets via the platelet-specific 6A6 antibody. In all of these model systems, IgG2c antibodies were demonstrated to be the most active IgG subclass and to mediate their activity via the activating Fc receptors FcγRI and IV (Nimmerjahn and Ravetch, 2006). Treatment with EndoS resulted in highly pure antibody preparations that contained only a minimal sugar moiety consisting of a single GlcNAc with or without a branching fucose residue, as determined by mass spectrometry and lectin blot analysis (Figure S1) (Collin et al., 2008). Injecting mice with the EndoS-treated αCD20-IgG2c, but not with the EndoS-treated IgG2b switch variant, resulted in a depletion of B cells in the blood and spleen indistinguishable from the respective fully glycosylated IgG subclasses (Figures 1A–1D). Notably, removal of the complete sugar domain by PNGase F treatment impaired IgG2c activity, establishing that the disaccharide structure is sufficient to retain this IgG subclass in a fully functional state. Similar results were obtained with the EndoS-treated gp75-specific antibody TA99-IgG2c, which was as potent as the fully glycosylated IgG2c variant in depleting melanoma metastasis in the lung of mice injected with B16-F10 melanoma cells, followed by treatment with the differentially glycosylated IgG2c variants (Figures 1E and 1F). Finally, we confirmed these results for the platelet-specific 6A6-IgG2c variant and further demonstrated that this antibody retained more than 50% of its activity, even if only one GlcNAc residue without the branching fucose residue was present (Figures 1G; Figure S1). Again, removal of the sugar residues either by PNGase F treatment or by mutating the N297 acceptor site into an alanine residue abolished the 6A6-IgG2c activity. To investigate whether the hinge region, which is one distinguishing feature between IgG2c and IgG2b, was responsible for this subclass-specific effect, we generated a chimeric antibody consisting of the IgG2b CH1 and hinge domain fused to IgG2c CH2-CH3 domains. As this chimeric antibody remained fully active in its disaccharide glycoform, a major contribution of the IgG2c hinge region seems unlikely (Figure 1H), suggesting that distinctive features of the CH2-CH3 region may be responsible for this effect. Taken together, our analysis revealed that cytotoxic antibodies as well as autoantibodies of the IgG2c subclass retain their in vivo activity, even if they only contain a mono- or disaccharide sugar moiety, whereas they become functionally inactivated if the entire sugar domain is absent.

Immune Complex Binding to FcγRs, Rather Than a Monomeric Antibody-FcγR Interaction Analysis, Predicts Minimally Glycosylated IgG2c Activity In Vivo

To analyze how differential IgG deglycosylation affects the binding to individual FcγRs, we performed surface plasmon resonance (SPR) analysis with immobilized IgG2c antibodies and soluble FcγRs (Figure 2A). In addition, an ELISA-based assay in which monomeric IgG2c antibody preparations are immobilized to plastic wells and soluble FcγR preparations are used to detect bound antibodies was used as an independent experimental approach to study the direct protein-protein interaction of differentially glycosylated antibodies with soluble FcγRs (Figure 2B). Surprisingly, both of these assays demonstrated that EndoS-treated IgG2c antibodies showed a strong reduction in affinity for the activating FcγRs I and IV, in line with data obtained with EndoS-treated human IgG subclasses (Allhorn et al., 2008). The affinity was further diminished if the monosaccharide IgG2c

Figure 1. In Vivo Activity of Minimally Glycosylated IgG Subclasses

(A–D) Shown are representative examples (A and C) and the quantifications (B and D) of the differential capacity of the CD20-specific switch variants IgG2c and IgG2b in their glycosylated (untreated), disaccharide (EndoS), or deglycosylated (PNGase F) form to deplete B cells ($CD19^+IgM^+$) in the peripheral blood (A and B) and spleen (C and D) of C57BL/6 mice 24 hr after antibody administration. PBS-injected mice served as controls, and B cell counts shown in (B) and (D) were normalized to the PBS control group, which was set to 100% (data not shown).

(E and F) Shown are representative lungs (E) and the quantification of lung metastasis at day 11 (F) of mice injected with B16-F10 melanoma cells and treated with the TA99-IgG2c antibody in its fully glycosylated (untreated) or disaccharide form (EndoS). PBS-injected mice served as controls.

(G) Depicted is the platelet-depleting activity of 6A6-IgG2c glycovariants in their fully glycosylated (untreated), disaccharide (EndoS), monosaccharide (-FUC/EndoS), or deglycosylated (PNGase F/N297A) forms in C57BL/6 mice 4 hr after injection of equal antibody amounts.

(H) Residual platelet counts in the peripheral blood of C57BL/6 mice 4 hr after injection of the untreated and EndoS-treated platelet-specific 6A6-IgG2c, 6A6-IgG2b, and the 6A6-IgG2c-*Hinge-IgG2b* variant antibodies.

In (G) and (H), platelet counts after autoantibody injection were normalized to platelet counts before antibody administration, which were set to 100%.

Bar graphs indicate mean values/counts ± SEM of at least two independent experiments with three to five mice per group. Statistical significance was evaluated with an ANOVA and a Bonferroni correction. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

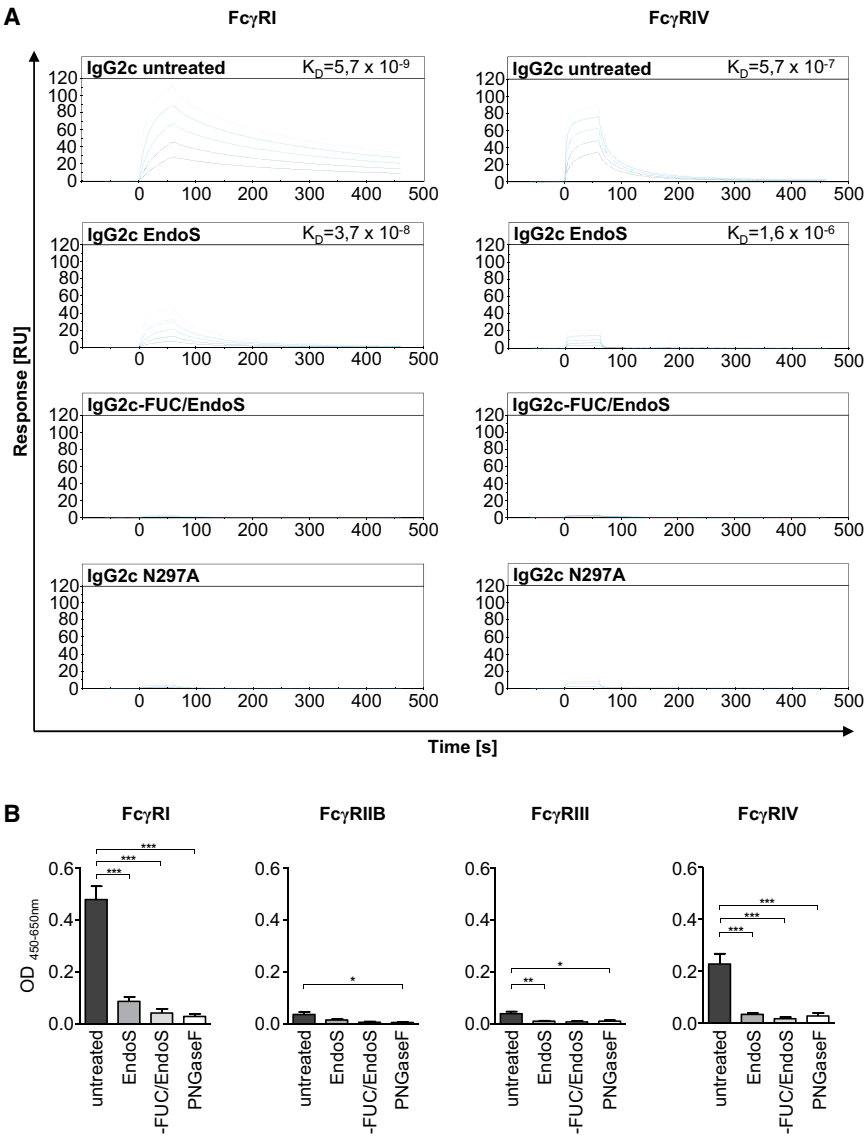


Figure 2. Impact of Minimal IgG2c Glycosylation on the Interaction with Soluble FcγReceptors

(A) SPR sensorgrams showing the binding of five different concentrations of soluble (s) FcγRI and FcγRIV toward immobilized IgG2c glycovariants in their fully glycosylated (untreated), disaccharide (EndoS), monosaccharide (-FUC/EndoS), or aglycosylated (N297A) forms. RU, response units, K_D, dissociation constant.

(B) Shown is the binding of murine soluble FcγRs to immobilized IgG2c glycovariants as determined by ELISA analysis. Bar graphs indicate mean value ± SEM of six independent experiments. Statistical significance was evaluated with an ANOVA and a Bonferroni correction. OD, optical density. *p < 0.05; **p < 0.01; ***p < 0.001.

variant was studied in SPR analysis and was comparable to the loss of affinity for aglycosylated IgG2c in which the N297 residue is replaced with an alanine (N297A). In contrast, analysis of IgG2c immune complex binding to soluble FcγRs (Figure 3A) or FcγR-expressing Chinese hamster ovary (CHO) cells (Figure 3B) revealed a completely different picture. Here, IgG2c binding to FcγRs remained largely unimpaired if they contained only a mono- or disaccharide sugar moiety, consistent with previous in vitro studies investigating human IgG immune complex binding to FcγR-expressing cells (Lux et al., 2013). This effect was especially pronounced for the binding to the high-affinity FcγRI and the medium-affinity FcγRIV. Of note, the afucosylated IgG2c monosaccharide variant showed an increased FcγRIV binding compared to the disaccharide variant in the ELISA-based assay (Figure 3A), consistent with previous studies showing that, in mice, FcγRIV has the ability to recognize afucosylated antibodies with enhanced affinity (Nimmerjahn and Ravetch, 2005). A similar glycosylation-independent binding of IgG2c to FcγRI and FcγRIV became evident in an alternative assay system studying immune complex binding to CHO cells transfected with the individual mouse FcγRs, although, here, no increased binding of afucosylated monosaccharide IgG2c glycovariants to FcγRIV was noted (Figure 3B). Also in line with the in vivo data, IgG1 and IgG2b immune complexes largely lost their capacity to bind to their activating FcγR counterparts upon EndoS and PNGase F treatment (Figure S2).

Minimally Glycosylated Mouse and Human IgG Variants Function via Activating FcγRs and Have a Diminished Binding to C1q

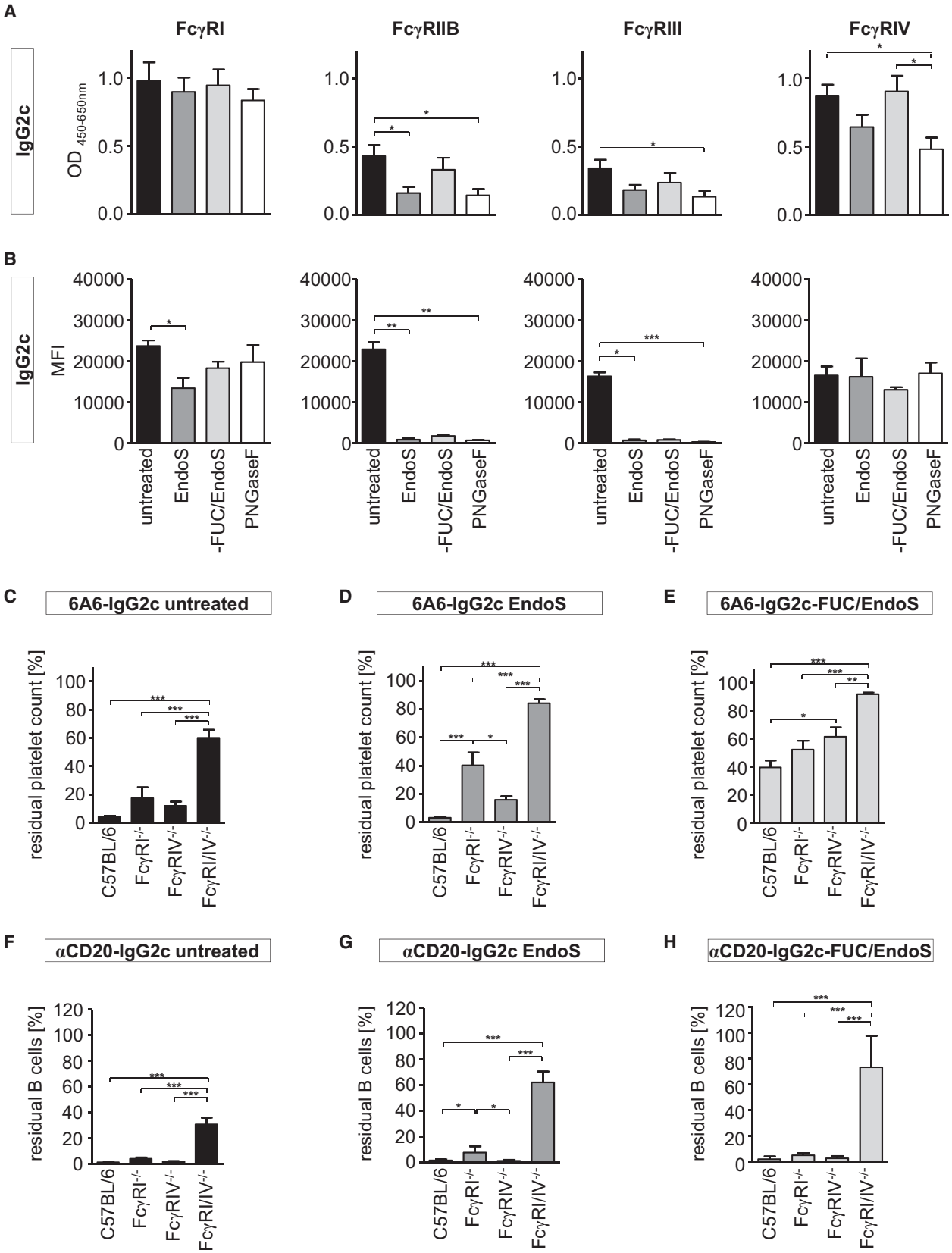
In a final set of experiments, we investigated which activating FcγRs are responsible for the activity of mono- and disaccharide antibodies. As shown in Figure 3C, the activity of the fully glycosylated platelet-specific 6A6-IgG2c antibody was equally dependent on both FcγRI and FcγRIV, confirming previous results (Biburger et al., 2011). The disaccharide variant, however, showed a more pronounced dependence on FcγRI, although FcγRIV was still required for its full functional activity (Figure 3D). In contrast, the activity of the monosaccharide variant became more dependent on FcγRIV but still required FcγRI for maximal triggering of platelet phagocytosis (Figure 3E), consistent with the in vitro immune complex binding data demonstrating enhanced binding of afucosylated IgG2c to this activating FcγR (Figure 3A). Although the B-cell-specific CD20-IgG2c antibody was also largely dependent on FcγRs I and IV in its mono- and disaccharide forms (Figures 3F–3H), some notable differences became obvious. Thus, IgG2c-dependent B cell depletion remained fully active, even if the antibody was in its monosaccharide form (Figure 3H), whereas IgG2c-mediated platelet phagocytosis was

reduced by half compared to the disaccharide variant (Figure 3E). Removing the final GlcNAc residue, however, impaired B cell depletion (Figure 1B). To establish whether this finding is also relevant for human therapeutic antibody preparations used in the clinic, we investigated the impact of EndoS treatment on the activity of rituximab, which is of the human IgG1 subclass and of an IgG3 switch variant in a humanized mouse model. Consistent with the results obtained in our monomeric IgG-FcγR analysis, previous in vitro studies with human IgG subclasses noted a strong reduction in affinity for human FcγRs upon EndoS or endoglycosidase D (EndoD) treatment (Allhorn et al., 2008; Yamaguchi et al., 2006). As shown in Figures 4A and 4B; however, IgG1 and IgG3 disaccharide variants of this antibody fully retained their capacity to deplete human B cells in vivo. In line with the data for the mouse CD20-specific antibody, rituximab-IgG1 also remained active if only one GlcNac residue was present at the N297 residue but lost its cytotoxic effector functions if this final sugar residue was lacking (Figures 4A and 4B). As both IgG-triggered activation of the classical complement pathway and binding to cellular FcγRs may be involved in mediating B cell depletion, we further analyzed the capacity of the human IgG1 and IgG3 antibodies to bind to the complement component C1q (Meyer et al., 2014). Of note, the capacity of both subclasses to bind to C1q was reduced to a level similar to that seen for aglycosylated IgG preparations, regardless of whether they were present in a monomeric or antigen-bound form (Figures 4C and 4D). Given that rituximab has only a limited capacity to kill target cells via complement-mediated cytotoxicity (CDC) in vitro (data not shown), we turned to ofatumumab, a CD20-specific human IgG1 antibody with enhanced CDC activity. Consistent with the previous results, the ofatumumab mediated deposition of C3b, and, ultimately, the lysis of B cells was abrogated if the antibody contained only a disaccharide sugar domain upon EndoS treatment (Figures 4E and 4F), suggesting that mono- or disaccharide antibody preparations, while largely maintaining their FcγR-dependent activities, lose their capacity to trigger a C1q-dependent complement activation.

DISCUSSION

The sugar moiety attached to each of the two heavy chains within the IgG molecule represents a major source of heterogeneity in an antibody preparation, and individual IgG glycoforms have been demonstrated to impact antibody activity. A variety of previous studies have provided conclusive evidence that glycosylation is essential for both the pro- and anti-inflammatory activities of IgG (Pincetic et al., 2014). Thus, an IgG antibody lacking the N297-attached sugar domain is generally considered as a molecule incapable of interacting with cellular FcγRs or the complement pathway (Arnold et al., 2007). Based on this model, enzymes with the capacity to specifically deglycosylate IgG molecules, such as the *Streptococcus pyogenes*-derived enzyme EndoS, which cleaves the sugar moiety after the first GlcNAc residue, have been used very successfully in pre-clinical mouse model systems to suppress the activity of mouse- and other rodent-derived self-reactive antibodies (Albert et al., 2008; Allhorn et al., 2008). Although we could confirm these findings for select IgG subclasses, our study reveals an unexpected general resistance of mouse IgG2c and human IgG1 and IgG3 subclasses to become inactivated upon deglycosylation via EndoS. Thus, a mono- or disaccharide sugar domain was fully sufficient to maintain cytotoxic antibody activity. Of great interest, human IgG1 and mouse IgG2c are the most potent pro-inflammatory and cytotoxic IgG subclasses, and the human IgG1 backbone is used widely in therapeutic antibody preparations. This is of great importance, as it suggests that antibody binding to its cognate antigen and/or avidity effects can overcome the loss in affinity observed in monomeric IgG-FcγR analysis. Fully aglycosylated human IgG1 and IgG3 and mouse IgG2c, however, were no longer active in all of the in vivo model systems, suggesting that a monosaccharide residue can be sufficient to maintain an Fc conformation that allows a productive engagement of cellular FcγRs. Evidence along these lines was provided by nuclear magnetic resonance (NMR) spectroscopy, showing that different chemical shift changes affecting selective amino acids occur in the human IgG1 CH2 domain if it contains a disaccharide, compared to no sugar moiety at all, and by recent structural analysis of IgG in solution (Yamaguchi et al., 2006; Subedi and Barb, 2015). Of interest for clinical applications, our results suggest that mono- or disaccharide antibodies largely mediate their activity via the FcγR system while the activation of the classical complement pathway is impaired. Although we could not directly assess this in vivo, the functional data obtained in our in vitro CDC assay, along with results showing that EndoS-treated red-blood-cell-specific antibodies can no longer mediate complement-dependent phagocytosis of red blood cells in a model of autoimmune hemolytic anemia, strongly support a more general relevance for this observation (Allhorn et al., 2010). Considering the findings of studies suggesting that rituximab-mediated complement activation interferes with an FcγR-dependent NK (natural killer)-cell-mediated lysis of tumor cells and that patients with low levels of C1q respond better to rituximab therapy, mono- or disaccharide IgG1 antibodies may be less prone to complement-dependent inhibition of ADCC reactions (Racila et al., 2008; Wang et al., 2009). Of further interest, the only in vitro assay systems predicting this unchanged activity were immune-complex-based ELISA and fluorescence-activated cell sorting (FACS) analyses but not monomeric IgG-FcγR interaction studies based on SPR or ELISAs. However, even the immune-complex-based assays predicted false-positive results; for example, EndoS-treated IgG2b preparations still interacted with FcγRIV, or aglycosylated IgG2c immune complexes showed an unchanged binding to FcγRI and a substantial residual, or even unchanged, binding to FcγRIV while losing their activity in vivo. A more detailed knowledge about how the size of the immune complex generated in vitro correlates with the size of the immune complex formed with B cells, platelets, or tumor cells in vivo may be essential to generate optimal in vitro assay systems.

With respect to limiting the heterogeneity within therapeutic antibody preparations, our results clearly demonstrate that a minimal level of IgG glycosylation is sufficient for full antibody activity, which may help to generate well-defined therapeutic IgG preparations without unwanted anti-inflammatory or immunomodulatory activities.



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EXPERIMENTAL PROCEDURES

Animals

Female mice at 8–16 weeks of age on the C57BL/6 background obtained from Janvier Labs were used in all experiments. FcγRI-deficient mice (FcγRI$^{-/-}$) and FcγRIV-deficient mice (FcγRIV$^{-/-}$) on the C57BL/6 background were provided by Jeffrey Ravetch (Rockefeller University). FcγRI$^{-/-}$ and FcγRIV$^{-/-}$ were crossed in the lab to obtain FcγRI/FcγRIV$^{-/-}$ double-deficient mice (Biburger et al., 2011). Immunodeficient Rag2/γc$^{-/-}$ mice were provided by Hergen Spits (Academic Medical Center [AMC] Amsterdam). Rag2/γc$^{-/-}$ FcγR$^{-/-}$ mice were generated by crossing Rag2/γc$^{-/-}$ mice with FcγR$^{-/-}$ mice, followed by breeding to a homozygous deletion for all three respective genes (Lux et al., 2014). Mice were kept in the animal facilities of Friedrich-Alexander-University Erlangen-Nürnberg under specific pathogen-free conditions in individually ventilated cages, in accordance with the guidelines of the NIH and the legal requirements of Germany and the United States.

Generation of PBMC Humanized Mice

Adult Rag2/γc$^{-/-}$ FcγR$^{-/-}$ mice were irradiated with 6 Gy and injected intraperitoneally with 5 × 10^6 human peripheral blood mononuclear cells (PBMCs) 6 hr after irradiation as described previously (Lux et al., 2014). PBMCs were isolated by density centrifugation from individual buffy coats. Isolated PBMCs were frozen and stored in liquid nitrogen until further use.

Antibodies and Reagents

The mouse CD20-specific IgG2b/IgG2c, the human CD20-specific rituximab IgG1/IgG3, the platelet-specific 6A6, and the 2,4,6-trinitrophenyl (TNP)-specific 7B4-IgG1/IgG2c/IgG2b antibodies were produced by transient transfection of 293T cells with plasmids encoding for the respective antibody heavy and light chains as described previously (Nimmerjahn and Ravetch, 2005). HEK293S GnTI$^-$ cells were used to produce afucosylated IgG antibodies. The TA99-IgG2c antibody was purchased from BioXCell, and ofatumumab was kindly provided by Paul W.H.I. Parren (Genmab). For all experiments, recombinant EndoS was used, which was provided by Mattias Collin (Lund University). For in vitro digestion, IgG antibodies were incubated with 0.04 μg EndoS/μg IgG in PBS at 37°C for 24 hr. Complete deglycosylation of IgG was achieved by digestion with 80U PNGase F/μg IgG (New England Biolabs) overnight at 37°C in H_2O. The efficiency of EndoS/PNGase F treatment was analyzed by lectin blotting with lens culinaris agglutinin (LCA) as described previously (Albert et al., 2008) and by mass spectrometry analysis. Soluble Flag-tagged Fc receptors were generated by transient transfection of HEK293T cells, and cell culture supernatants were used directly for IgG-binding ELISAs.

Mass Spectrometry Analysis

Glycoproteins (25 μg) were digested overnight with 200 ng of sequencing-grade trypsin at 37°C (Worthington Biochemical). Glycopeptides were purified by reverse-phase solid-phase extraction with Chromabond C18ec beads (Marcherey-Nagel), and purified tryptic digests were analyzed on a nanoACQUITY UPLC system (Waters) coupled to a micrOTOF-Q mass spectrometer (Bruker Daltonics).

Anti-CD20 IgG-Induced B Cell Depletion

25 μg anti-CD20-IgG switch variants were injected intravenously into mice as described previously (Biburger et al., 2011). B cell counts in peripheral blood were analyzed by flow cytometry before and at 6 and 24 hr after antibody injection. The B cell count in spleen was analyzed 24 hr after antibody application. For quantification, B cell counts in animals injected with PBS were set to 100%, and B cell counts in antibody-treated animals are depicted as the fraction of residual B cells compared to the PBS value.

B16-F10 Lung Metastasis Model

Experiments were performed as described previously, with minor modifications (Nimmerjahn and Ravetch, 2005). C57BL/6 mice were injected with 5 × 10^5 B16-F10 melanoma cells intravenously and were injected either with PBS or with 100 μg of TA99-IgG2c glycosylation variants on days 0, 2, 4, 7, and 9 intraperitoneally. At day 11, mice were sacrificed, and metastasis in the lungs were counted.

Platelet Depletion Model

Experiments were performed essentially as described previously (Biburger et al., 2011). In brief, mice were injected intraperitoneally with 4 μg of the recombinant 6A6 antibody isotype switch variants diluted in 250 μl of PBS. Platelet counts before injection and 4 hr after injection were determined by blood collection (50 μl) from retroorbital plexus and measured at a 1:4 dilution in PBS in an Advia 120 hematology system (Siemens). For quantification, platelet counts before autoantibody injection were set to 100%, and the platelet count 4 hr after antibody injection is shown as the fraction of residual platelets compared to this initial value.

Rituximab-IgG-Induced B Cell Depletion in Rag2/γc/FcγR$^{-/-}$

2.5 μg anti-CD20 rituximab-IgG switch variants was injected 18 hr after PBMC transfer into 12- to 14-week-old PBMC-humanized Rag2/γc$^{-/-}$/FcγR$^{-/-}$ mice intraperitoneally. Human B cell counts in the peritoneum were analyzed by flow cytometry 24 hr after antibody injection.

Flow Cytometry

Flow cytometry analysis was conducted on a FACS Canto II (BD Biosciences) with single-cell suspensions of murine peripheral blood and spleen cells and human PBMCs isolated from the peripheral blood of humanized mice and humans (isolated by Ficoll density gradient centrifugation). A detailed description of the method and antibodies used for FACS analysis can be found in the Supplemental Experimental Procedures.

Immunofluorescence Microscopy

5-μm sections of frozen tissue were air dried overnight, followed by fixation in acetone. Slides were stained with anti-B220/CD45R (clone RA3-6B2; BD Biosciences), anti-TCR-β (clone H57-597; Biolegend), and anti-CD31 (clone 390; Biolegend). After incubation, the excess of fluorescent dye was removed by multiple washing steps with PBS. Microscope slides were analyzed on an Axiovert 200 M fluorescence microscope (Carl Zeiss).

SPR Analysis

A Biacore X100 biosensor system was used to assay the interaction of soluble Fcγ receptors I and IV with the indicated glycosylation variants. Antibodies

Figure 3. Impact of Minimal IgG2c Glycosylation on the Interaction of Immune Complexes with Fcγ Receptors in Vitro and In Vivo

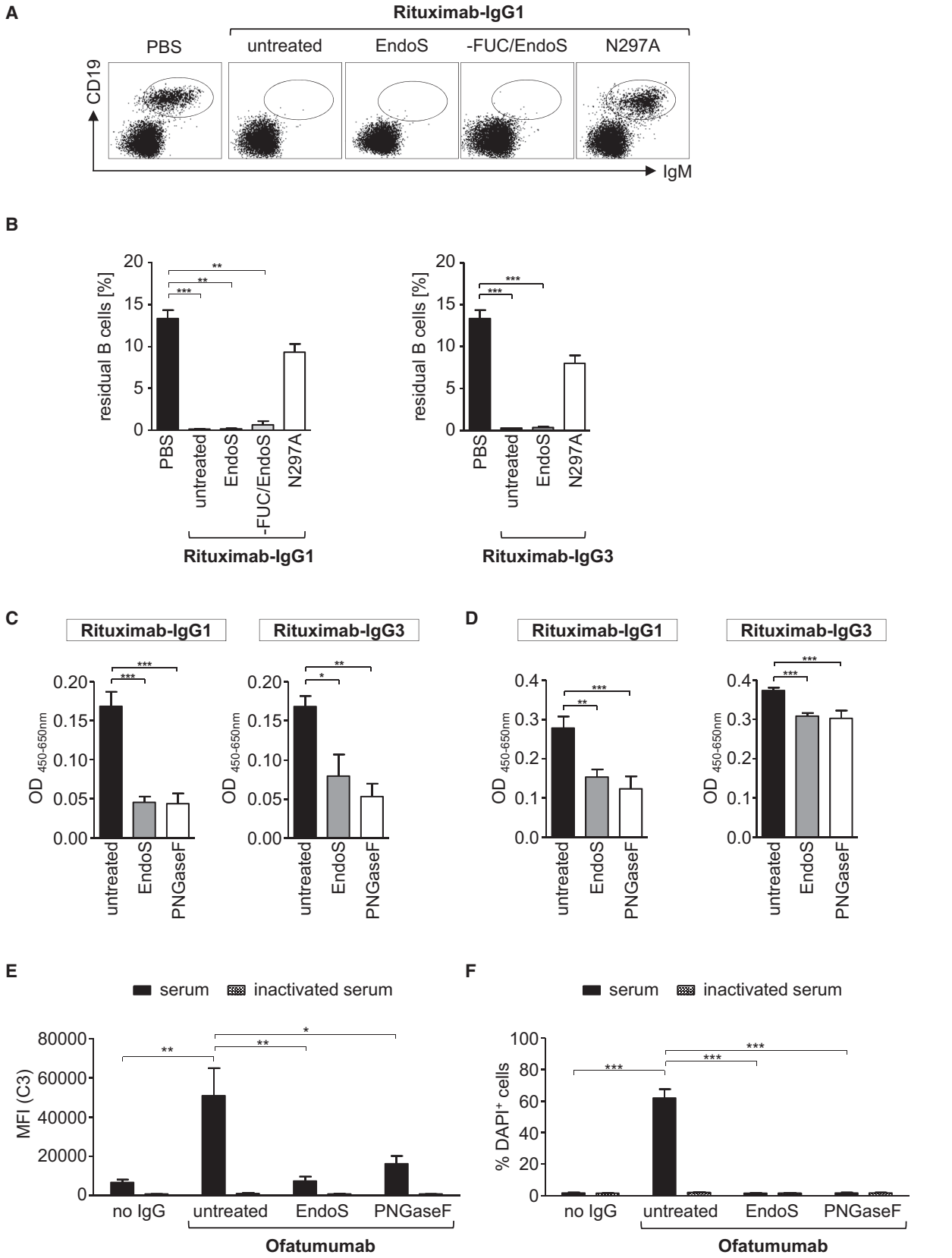
(A) Binding of anti-TNP-IgG2c TNP-BSA immune complexes to immobilized murine soluble FcγRs as measured by ELISA analysis. OD, optical density.

(B) Shown is the binding of murine anti-TNP-IgG2c TNP-BSA immune complexes to CHO cells expressing the indicated mouse Fcγ receptors. MFI, median fluorescent intensity.

(C–E) Depicted are the residual platelet counts in the peripheral blood of C57BL/6, FcγRI$^{-/-}$, FcγRIV$^{-/-}$, and FcγRI/IV$^{-/-}$ mice 4 hr after injection of equal amounts of the fully glycosylated (untreated) (C), disaccharide (EndoS) (D), and monosaccharide (-FUC/EndoS) (E) variants of the 6A6-IgG2c antibody. Platelet counts after autoantibody injection were normalized to platelet counts before antibody administration, which were set to 100%.

(F–H) Shown are the residual counts of CD19$^+$IgM$^+$ blood B cells in C57BL/6, FcγRI$^{-/-}$, FcγRIV$^{-/-}$, and FcγRI/IV$^{-/-}$ mice 24 hr after injection of 25 μg of CD20-specific IgG2c antibody in its fully glycosylated (untreated) (F), disaccharide (EndoS) (G), or monosaccharide (-FUC/EndoS) form (H). B cell counts in the mouse cohorts receiving CD20-specific antibodies were normalized to the B cell counts of the PBS control group, which was set to 100% (data not shown).

Bar graphs indicate mean value ± SEM of six to nine independent experiments. Statistical significance was evaluated with an ANOVA and a Bonferroni correction. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



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were immobilized to one flow cell of a CM5 sensor chip (Biacore) by standard amine coupling, as suggested by the manufacturer. Soluble FcγR receptors were injected at five different concentrations (2-fold dilutions starting from 5 μg/ml for FcγRI and 40 μg/ml for FcγRIV binding) through flow cells at room temperature in HBS-EP running buffer (10 mM HEPES [pH 7.4], 250 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20) at a flow rate of 30 μl/min. Soluble FcγR receptors were injected for 60 s, and dissociation of bound molecules was observed for 10 min. Background binding to control flow cells was subtracted automatically. Affinity constants were derived from sensorgram data using simultaneous fitting to the association and dissociation phases and global fitting to all curves in the set (the 1:1 Langmuir binding model closely fitted the observed sensorgram data and was used in all experiments).

Monomeric FcγR-Antibody Interaction Analysis via ELISA

100 ng 6A6-IgG glycosylation variants were coated on 96-well plates in PBS and incubated with cell culture supernatants of soluble Flag-tagged FcγRs. Binding of FcγRs to immobilized IgG was detected with anti-Flag M2-HRP antibody (Sigma-Aldrich). Soluble FcγR binding to wells without IgG served as a blank/negative control that was subtracted from the sample signal depicted in the figures.

Analysis of IC Binding to Soluble Murine FcγRs

Experiments were conducted as described previously (Nimmerjahn and Ravetch, 2008). A more detailed description can be found in the Supplemental Experimental Procedures.

Analysis of IC Binding to Cellular Murine FcγRs

Immune complexes were incubated with 100,000 CHO cells stably expressing individual murine FcγRs for 1 hr under gentle shaking at 4°C. Bound ICs were detected by flow cytometry on a FACS Canto II, using a PE-conjugated goat anti-mouse IgG $F(ab')_2$ fragment from Dianova. Data were analyzed with FACSDiva software (BD Biosciences).

C1q ELISA

96-well microtiter plates were coated with 100 ng rituximab IgG1/IgG3 glycovariants in 0.05 M carbonate/bicarbonate buffer (pH 9.6) and blocked with 1 × PBS/3% BSA/0.1% gelatin/0.05% Tween 20, followed by incubation with 200 ng native human C1q (AbD Serotec). To analyze C1q binding to antibody-antigen complexes, 96-well microtiter plates were coated with 100 ng TNP-13-BSA in 0.05 M carbonate/bicarbonate buffer (pH 9.6) blocked with 1× PBS/3% BSA/0.1% gelatin/0.05% Tween 20, followed by an incubation with 100 ng of human anti-TNP-IgG (clone 7B4) in 1× PBS/3% BSA/0.1% gelatin/0.05% Tween 20. After incubation with 200 ng native C1q (AbD Serotec), bound C1q was detected with 200 ng of a horseradish-peroxidase (HRP)-conjugated sheep anti-human C1q antibody (AbD Serotec).

CDC Assay

For the CDC assay, 50 μl of the EBV (Epstein-Barr virus)-immortalized human B cell line LCL1.11 (10^6/ml) and 50 μl ofatumumab (20 μg/ml, either untreated, EndoS treated, or PNGase F treated) were incubated for 30 min at 37°C with 5% CO_2 in human serum or heat-inactivated (30 min at 56°C) serum lacking functional complement proteins as a control. Cells were harvested, stained with DAPI to identify dead cells or anti-C3/C3b/iC3b (Cedarlane) to detect complement deposition on the cell surface, and analyzed via FACS analysis (BD Biosciences). Data acquisition and analysis were performed with the FACSDiva software (BD Biosciences).

Statistics

All data are expressed as mean + SEM. Data were analyzed and plotted with GraphPad Prism software (GraphPad Software). In brief, normal distribution was tested with a Kolmogorov-Smirnov test. After passing a normality test, an ANOVA (and subsequent post hoc tests and Bonferroni correction) was used to determine statistical differences between more than two groups. Non-parametric distribution was analyzed using a Kruskal-Wallis test with Dunn's post hoc test. $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.11.027.

AUTHOR CONTRIBUTIONS

D.K., A.L., J.S., and H.D. performed experiments. M.C., A.G., G.L., and J.E. provided essential reagents and techniques. D.K. and F.N. planned experiments and wrote the paper.

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Figure 4. Impact of Minimal Human IgG1 and IgG3 Glycosylation on the Ability to Trigger FcγR- and Complement-Dependent Effector Functions

(A and B) Shown are representative examples (A) and the quantification (B) of B cell counts present in humanized Rag2/γc/FcγR$^{-/-}$ mice 24 hr after injection of 2.5 μg rituximab IgG1 or IgG3 in their fully glycosylated (untreated), disaccharide (EndoS), monosaccharide (-FUC/EndoS), or aglycosylated (N297A) forms.

(C) Depicted is the C1q binding to immobilized rituximab-IgG1 and -IgG3 variants in their fully glycosylated (untreated), disaccharide (EndoS), or deglycosylated (PNGase F) forms as measured by ELISA. OD, optical density.

(D) Shown is the C1q binding to human IgG1 and IgG3 variants of TNP-specific antibodies (clone 7B4) bound to immobilized TNP-BSA in their fully glycosylated (untreated), disaccharide (EndoS), or deglycosylated (PNGase F) forms as measured by ELISA.

(E) Quantification of C3 deposition on the human LCL1.11 B cell line upon addition of fully glycosylated (untreated), disaccharide (EndoS) and deglycosylated (PNGase F) variants of the CD20-specific human IgG1 antibody ofatumumab. C3 deposition was measured by FACS analysis, and the median fluorescent intensity (MFI) is shown. Cells incubated with heat-inactivated serum (no complement activity) and ofatumumab glycosylation variants served as negative controls.

(F) Evaluation of CDC activity of glycosylated (untreated), disaccharide (EndoS), and deglycosylated (PNGase F) glycovariants of ofatumumab directed against the LCL1.11 human B cell line. Complement-dependent lysis of target cells was identified by DAPI staining. Cells incubated with heat-inactivated serum in the presence or absence of ofatumumab served as negative controls.

Bar graphs indicate mean counts/values ± SEM of two to three independent experiments. In all experiments, at least five mice per group were used. Statistical significance was evaluated with an ANOVA and a Bonferroni correction or Kruskal-Wallis test with Dunn's post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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REFERENCES

Albert, H., Collin, M., Dudziak, D., Ravetch, J.V., and Nimmerjahn, F. (2008). In vivo enzymatic modulation of IgG glycosylation inhibits autoimmune disease in an IgG subclass-dependent manner. Proc. Natl. Acad. Sci. USA *105*, 15005–15009.

Allhorn, M., Olin, A.I., Nimmerjahn, F., and Collin, M. (2008). Human IgG/Fc gamma R interactions are modulated by streptococcal IgG glycan hydrolysis. PLoS ONE *3*, e1413.

Allhorn, M., Briceño, J.G., Baudino, L., Lood, C., Olsson, M.L., Izui, S., and Collin, M. (2010). The IgG-specific endoglycosidase EndoS inhibits both cellular and complement-mediated autoimmune hemolysis. Blood *115*, 5080–5088.

Arnold, J.N., Wormald, M.R., Sim, R.B., Rudd, P.M., and Dwek, R.A. (2007). The impact of glycosylation on the biological function and structure of human immunoglobulins. Annu. Rev. Immunol. *25*, 21–50.

Beck, A., Wagner-Rousset, E., Bussat, M.C., Lokteff, M., Klinguer-Hamour, C., Haeuw, J.F., Goetsch, L., Wurch, T., Van Dorsselaer, A., and Corvaïa, N. (2008). Trends in glycosylation, glycoanalysis and glycoengineering of therapeutic antibodies and Fc-fusion proteins. Curr. Pharm. Biotechnol. *9*, 482–501.

Biburger, M., Aschermann, S., Schwab, I., Lux, A., Albert, H., Danzer, H., Woigk, M., Dudziak, D., and Nimmerjahn, F. (2011). Monocyte subsets responsible for immunoglobulin G-dependent effector functions in vivo. Immunity *35*, 932–944.

Collin, M., Shannon, O., and Björck, L. (2008). IgG glycan hydrolysis by a bacterial enzyme as a therapy against autoimmune conditions. Proc. Natl. Acad. Sci. USA *105*, 4265–4270.

Glennie, M.J., and van de Winkel, J.G. (2003). Renaissance of cancer therapeutic antibodies. Drug Discov. Today *8*, 503–510.

Jung, S.T., Reddy, S.T., Kang, T.H., Borrok, M.J., Sandlie, I., Tucker, P.W., and Georgiou, G. (2010). Aglycosylated IgG variants expressed in bacteria that selectively bind FcgammaRI potentiate tumor cell killing by monocyte-dendritic cells. Proc. Natl. Acad. Sci. USA *107*, 604–609.

Lim, S.H., Beers, S.A., French, R.R., Johnson, P.W., Glennie, M.J., and Cragg, M.S. (2010). Anti-CD20 monoclonal antibodies: historical and future perspectives. Haematologica *95*, 135–143.

Lux, A., Yu, X., Scanlan, C.N., and Nimmerjahn, F. (2013). Impact of immune complex size and glycosylation on IgG binding to human FcγRs. J. Immunol. *190*, 4315–4323.

Lux, A., Seeling, M., Baerenwaldt, A., Lehmann, B., Schwab, I., Repp, R., Meidenbauer, N., Mackensen, A., Hartmann, A., Heidkamp, G., et al. (2014). A humanized mouse identifies the bone marrow as a niche with low therapeutic IgG activity. Cell Rep. *7*, 236–248.

Meyer, S., Leusen, J.H., and Boross, P. (2014). Regulation of complement and modulation of its activity in monoclonal antibody therapy of cancer. MAbs *6*, 1133–1144.

Nimmerjahn, F., and Ravetch, J.V. (2005). Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science *310*, 1510–1512.

Nimmerjahn, F., and Ravetch, J.V. (2006). Fcgamma receptors: old friends and new family members. Immunity *24*, 19–28.

Nimmerjahn, F., and Ravetch, J.V. (2008). Analyzing antibody-Fc-receptor interactions. Methods Mol. Biol. *415*, 151–162.

Pincetic, A., Bournazos, S., DiLillo, D.J., Maamary, J., Wang, T.T., Dahan, R., Fiebiger, B.M., and Ravetch, J.V. (2014). Type I and type II Fc receptors regulate innate and adaptive immunity. Nat. Immunol. *15*, 707–716.

Pound, J.D., Lund, J., and Jefferis, R. (1993). Aglycosylated chimaeric human IgG3 can trigger the human phagocyte respiratory burst. Mol. Immunol. *30*, 233–241.

Racila, E., Link, B.K., Weng, W.K., Witzig, T.E., Ansell, S., Maurer, M.J., Huang, J., Dahle, C., Halwani, A., Levy, R., and Weiner, G.J. (2008). A polymorphism in the complement component C1qA correlates with prolonged response following rituximab therapy of follicular lymphoma. Clin. Cancer Res. *14*, 6697–6703.

Ruuls, S.R., Lammerts van Bueren, J.J., van de Winkel, J.G., and Parren, P.W. (2008). Novel human antibody therapeutics: the age of the Umabs. Biotechnol. J. *3*, 1157–1171.

Sazinsky, S.L., Ott, R.G., Silver, N.W., Tidor, B., Ravetch, J.V., and Wittrup, K.D. (2008). Aglycosylated immunoglobulin G1 variants productively engage activating Fc receptors. Proc. Natl. Acad. Sci. USA *105*, 20167–20172.

Schwab, I., and Nimmerjahn, F. (2013). Intravenous immunoglobulin therapy: how does IgG modulate the immune system? Nat. Rev. Immunol. *13*, 176–189.

Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., et al. (2003). The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J. Biol. Chem. *278*, 3466–3473.

Subedi, G.P., and Barb, A.W. (2015). The structural role of antibody N-glycosylation in receptor interactions. Structure *23*, 1573–1583.

Wang, S.Y., Veeramani, S., Racila, E., Cagley, J., Fritzinger, D.C., Vogel, C.W., St John, W., and Weiner, G.J. (2009). Depletion of the C3 component of complement enhances the ability of rituximab-coated target cells to activate human NK cells and improves the efficacy of monoclonal antibody therapy in an in vivo model. Blood *114*, 5322–5330.

Yamaguchi, Y., Nishimura, M., Nagano, M., Yagi, H., Sasakawa, H., Uchida, K., Shitara, K., and Kato, K. (2006). Glycoform-dependent conformational alteration of the Fc region of human immunoglobulin G1 as revealed by NMR spectroscopy. Biochim. Biophys. Acta *1760*, 693–700.