Enhanced Effector Functions Due to Antibody Defucosylation Depend on the Effector Cell Fcγ Receptor Profile

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Abs of the IgG isotype are glycosylated in their Fc domain at a conserved asparagine at position 297. Removal of the core fucose of this glycan greatly increases the affinity for FcγRIII, resulting in enhanced FcγRIII-mediated effector functions. Normal plasma IgG contains ~94% fucosylated Abs, but alloantibodies against, for example, Rhesus D (RhD) and platelet Ags frequently have reduced fucosylation that enhances their pathogenicity. The increased FcγRIII-mediated effector functions have been put to use in various afucosylated therapeutic Abs in anticancer treatment. To test the functional consequences of Ab fucosylation, we produced V-gene–matched recombinant anti-RhD IgG Abs of the four different subclasses (IgG1–4) with and without core fucose (i.e., 20% fucose remaining). Binding to all human FcγR types and their functional isoforms was assessed with surface plasmon resonance. All hypofucosylated anti-RhD IgGs of all IgG subclasses indeed showed enhanced binding affinity for isolated FcγRIII isoforms, without affecting binding affinity to other FcγRs. In contrast, when testing hypofucosylated anti-RhD Abs with FcγRIIa-expressing NK cells, a 12- and 7-fold increased erythrocyte lysis was observed with the IgG1 and IgG3, respectively, but no increase with IgG2 and IgG4 anti-RhD Abs. Notably, none of the hypofucosylated IgGs enhanced effector function of macrophages, which, in contrast to NK cells, express a complex set of FcγRs, including FcγRIIIa. Our data suggest that the beneficial effects of afucosylated biologicals for clinical use can be particularly anticipated when there is a substantial involvement of FcγRIIIa-expressing cells, such as NK cells. The Journal of Immunology, 2017, 199: 204–211.

Immunoglobulin G is the most abundant class of Abs in human plasma, consisting of four subclasses: IgG1, IgG2, IgG3, and IgG4. Because the four subclasses differ in the structure of their constant regions (Fc domain), recognized by FcγRs and complement component C1q, they have different effector functions. This includes Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (1, 2).

The family of FcγRs consists of the high-affinity receptor FcγRI and the low-to-medium affinity receptors FcγRII and FcγRIII. All FcγRs, except for FcγRIIb and FcγRIIIb, are activating receptors through an ITAM. This motif is found either within the cytoplasmic tail of FcγRIIa or in the cytoplasmic tail of the common γ-chain associated with the other activating FcγRs. Alternatively, FcγRIIb contains an ITIM and FcγRIIb lacks an intracellular signaling motif (3, 4). FcγRs bind IgGs of the four subclasses with different affinities. Moreover, polymorphisms in FcγRs influence the binding affinities, most notably for FcγRI (FCGR2A-His131Arg, with higher affinity for the 131His variant) and for FcγRIIa (FCGR3A-Val158Phe, with higher affinity for the 158Val variant). Polymorphisms in FcγRIIIb (e.g., FCGR3B-NA1NA2) do not affect affinity for IgG (3).

All four IgG subclasses contain a conserved asparagine at position 297 to which a glycan is attached. This biantennary glycan greatly increases the affinity for FcγRIII, resulting in enhanced FcγRIII-mediated effector functions. Normal plasma IgG contains ~94% fucosylated Abs, but alloantibodies against, for example, Rhesus D (RhD) and platelet Ags frequently have reduced fucosylation that enhances their pathogenicity. The increased FcγRIII-mediated effector functions have been put to use in various afucosylated therapeutic Abs in anticancer treatment. To test the functional consequences of Ab fucosylation, we produced V-gene–matched recombinant anti-RhD IgG Abs of the four different subclasses (IgG1–4) with and without core fucose (i.e., 20% fucose remaining). Binding to all human FcγR types and their functional isoforms was assessed with surface plasmon resonance. All hypofucosylated anti-RhD IgGs of all IgG subclasses indeed showed enhanced binding affinity for isolated FcγRIII isoforms, without affecting binding affinity to other FcγRs. In contrast, when testing hypofucosylated anti-RhD Abs with FcγRIIa-expressing NK cells, a 12- and 7-fold increased erythrocyte lysis was observed with the IgG1 and IgG3, respectively, but no increase with IgG2 and IgG4 anti-RhD Abs. Notably, none of the hypofucosylated IgGs enhanced effector function of macrophages, which, in contrast to NK cells, express a complex set of FcγRs, including FcγRIIIa. Our data suggest that the beneficial effects of afucosylated biologicals for clinical use can be particularly anticipated when there is a substantial involvement of FcγRIIIa-expressing cells, such as NK cells. The Journal of Immunology, 2017, 199: 204–211.
Ags, as well as in elite controllers of HIV infection (18). The degree of fucosylation correlates with FcγRIIa-mediated ADC蝎 such that Ab afucosylation enhances pathology (14, 19).

With this in mind, it is not surprising that several nonfucosylated therapeutic mAbs have been put to therapeutic use, especially focusing on cancer treatment (4, 20–24). Furthermore, there are indications that glycan composition plays a role in the working mechanisms of other Ig therapies, such as IVIg, a product of IgG pooled from the plasma of thousands of donors. IVIg is used as replacement therapy in primary immunodeficiencies and as an immune-modulating agent in various autoimmune and immune diseases, among which immune thrombocytopenia (ITP) and autoimmune hemolytic anemia (AIHA) (25–30).

Although the modes of action of IVIg as an immune-modulating agent are still largely unresolved (27), one of the proposed working mechanisms is that IVIg saturates FcyRs on splenic macrophages and thereby inhibits the phagocytosis of platelets or RBCs opsonized by antibodies (in case of ITP and AIHA, respectively) (27, 29, 31, 32). Because the degree of Ab fucosylation alters the binding affinity to FcγRII, it may also affect the working mechanisms of IVIg.

To study the functional extent and significance of changes in core fucosylation of human IgG, we generated a series of anti-Rhd mAbs of all four IgG subclasses with and without high levels of fucose. Binding to all human recombinant FcyRs isofoms was assessed using surface plasmon resonance (SPR), and the impact was assessed using primary human NK cells and monocyte-derived macrophages.

Materials and Methods

Human samples

Peripheral blood from healthy volunteers was obtained in heparinized tubes. For NK cell isolation we only used PBMCs from FFGR-genotyped donors that have two copies of FCGR3-158F and do not have a FCGR2C-ORF allele to exclude FcyRIIc as a confounding factor (33). Genotyping was performed as described before (34, 35).

The study was approved by the Medical Ethics Committee of the Academic Medical Center and was performed in accordance with the Declaration of Helsinki.

Ab production

Anti-Rhd H chain variable domain of clone 19A10 (36) was cloned into pEE6.4 (Lanza) expression vector containing either IgG1, 2, 3, or 4 constant domains. IgG constant domains with flanking 3′ NheI and 5′ EcoRI restriction sites were designed and ordered at Mr. Gene and cloned as described previously (37). Anti-Rhd κ L chain was cloned into pEE14.4 (Lanza) expression vector. A combined vector encoding anti-2,4,6-trinitrophenol (TNP) IgG1 H chain and κ L chain was cloned into pEE14.4 vector as previously described (37).

All IgGs were produced by transient transfection of HEK-freestyle cells (Thermo Fisher Scientific) coupled to a maxICw gestic microrTOF (Bruker Daltonics, Bremen, Germany), as described previously (38). The IgG1 glycopeptides (peptide sequence EEQFNSTYR) were eluted first, followed by IgG4 (EEQFNSTYR) and lastly IgG2 (EEQFNSTFR). Tryptic digestion of IgG3 resulted in a glycopeptide bearing a peptide sequence identical to that of IgG4, as well as a misfolded glycopeptide (LREEQFNSTYR).

Using the three-dimensional Max Xtractor software, intensity values were extracted for each peak within a manually specified m/z window and retention time window. The background-subtracted peak intensity of the first three isotopic peaks in both 2+ and 3+ charge state was summed. For the two types of IgG3 glycopeptides, the intensity of both was summed. The values were subsequently normalized by dividing by the total intensity of all glycopeptides, yielding percentage data for each IgG subclass. These data were used to calculate (a)fucosylation levels, that is, the percentage of N-glycans carrying a core fucose.

Human FcγRII constructs

Human FcyR constructs FcRIs (his tag, 10256-H08H100), FcRIIa (131His, biotinylated, 10374-H27H1-B-50 and 131Arg, biotinylated, 10374-H27H8-B-50), FcyRIIb (biotinylated, 10259-H27H8-B-50), and FcγRIIIa (136Phe, biotinylated, 10389-H27H8-B-50, and 136Val, biotinylated, 10389-H27H11-B-50) for SPR analysis were obtained from Sino Biological (Beijing, China). Fusion FcyRIIIB-IgG2-Fc constructs composed of the extracellular domain of the FcγRIIIb of NA1 or NA2 allotype following by an Fc domain were cloned, produced, and site specifically biotinylated as described by (G. Dekkers, L. Trefers, R. Plomp, A.E. Bentlage, M. De Boer, C.A. Koelerman, S.L. Lissenberg-Thunnissen, R. Visser, M. Brouwer, J.Y. Mok, H. Matlung, T.K. van den Berg, W.J. van Esch, T.W. Kuijpers, D. Wouters, T. Rispens, M. Wuhler, and G. Vidarsson, manuscript in preparation).

Biosensor affinity measurements

Using a Continuous Flow Microspotter (Watsoch Microfluidics, Salt Lake City, UT), biotinylated FcyRs were spotted onto a single sensor G– streptavidin sensor (Sens, Enschede, the Netherlands) for binding affinity measurements of each Ab to any of the spotted FcyRs in a parallel manner on the IBIS MX96 (IBIS Technologies, Enschede, the Netherlands) (39). The biotinylated FcyRs were spotted in 3-fold dilutions, ranging from 100 to 3 nM for FcyRIIB and fusion FcyRIIB-IgG2-Fc. All of the other FcyRs were spotted in 3-fold dilutions, ranging from 30 to 1 nM in PBS 0.0075% Tween 80 (Amresco, Solon, OH), pH 7.4. The IgGs were then injected over the IBIS at 2x dilution series starting at 0.98 nM up to 2000 nM in PBS 0.075% Tween 80.

For FcyRII affinity measurements, we used his-tagged FcyRI. Biotinylated anti–his-tagged Ab (GenScript, Piscataway, NJ) was spotted in 3-fold dilutions, ranging from 30 to 1 nM. Prior to the IgG injection, 50 nM his-tagged FcyR was injected. The IgGs were then injected over the IBIS at 3-fold dilution series starting at 0.41 nM up to 100 nM as the highest concentration because of the inherently higher affinity.

Regeneration of the IBIS MX96 was carried out after every sample with acid buffer (10 mM Gly-HCl, 0.075% Tween 80, pH 2.5). Calculation of K_d was done as described previously (40). Analysis and calculation of all binding data were carried out with Scrubber software version 2 (Biologic Software, Campbell, ACT, Australia).

NK cell ADCSc

NK cells were isolated from PBMCs by a CD56 MACs isolation kit (Miltenyi Biotech), according to the manufacturer’s description.

Erythrocytes of RhD-positive donors were isolated and labeled with radiolabeled chromium (19Cr). The cells were then incubated with NK cells for 2 h at 37°C, after which the erythrocytes were opsonized with anti-TNP with or without fucose at a concentration of 10 μg/ml. The amount of Ab deposition was subsequently determined by staining with goat anti-human IgG and analysis by flow cytometry.

Target cell staining and preparation

The amount of Ab deposition on the erythrocyte surface was determined by staining with goat anti-human IgG and analysis by flow cytometry. To control for the amount of Ab deposition on the red cell surface, we varied Ag density by loading the erythrocytes with different concentrations of the TNP target trinitrobenzene sulfonic acid (TNBS) (0.05–5 nM) (Sigma-Aldrich), after which the erythrocytes were opsonized with anti-TNP with or without fucose at a concentration of 10 μg/ml. The amount of Ab deposition was subsequently determined by staining with goat anti-human IgG.

Culture of monocyte-derived macrophages

Monocytes were isolated and cultured into monocyte-derived macrophages, as described previously (41). In short, monocytes were isolated using a CD14 MACs isolation kit and cultured for 9 d in IMDM, supplemented with
with 10% FCS, glutamine, and antibiotics, containing either 10 ng/ml GM-CSF or 50 ng/ml M-CSF.

**Phagocytosis by monocyte-derived macrophages**

Phagocytosis assays with monocyte-derived macrophages were performed as described previously (41). RhD-positive erythrocytes were isolated and stained with CFSE (Life Technologies). The cells were opsonized at an optimal dose of human polyclonal anti-RhD Abs (1.56 IU/ml, Rhedo; Sanquin, Amsterdam, the Netherlands) or anti-RhD mAbs of different subclasses with and without core fucose at 10 μg/ml for 30 min at 37°C, or left unopsonized. In other experiments, the level of Ab deposition was controlled by labeling the RBCs with TNBS and subsequently opsonizing with anti-TNP mAbs, as described above. After washing excess Ab away, the cells were added to the monocyte-derived macrophages in a ratio of 10:1. After incubation, the nonphagocytized RBCs were lysed and the percentage of phagocytosis was determined by flow cytometry.

Phagocytosis of anti-RhD-opsonized erythrocytes was inhibited by adding anti-TNP Abs with or without fucose (as described above), 5 min prior to the addition of erythrocytes, at concentrations of 0.1, 1.0, or 10 μg/ml.

**Flow cytometry**

For determining surface expression on NK cells and monocyte-derived macrophages by flow cytometry, the following Abs were used: PE-Cy7-labeled anti-CD3 (clone SK7; BD Pharmingen), allophycocyanin-labeled anti-CD56 (clone B159; BD Pharmingen), FITC-labeled anti-CD64 (clone 10.1; BD Pharmingen), anti-CD32a (clone IV.3; Stemcell Technologies), anti-CD32b/c (clone 2B6; a gift from MacroGenics, Rockville, MD), and anti-CD16 (clone clone 3G8; BD Pharmingen). Samples were measured by FACSCanto II (BD Biosciences).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 6.07. For comparison of IgG-mediated phagocytosis, a Mann–Whitney U test was used. For comparison of expression levels, cytotoxicity, or blocking studies, testing was performed with unpaired t tests.

**Results**

**Abs with low Fc fucosylation were produced by adding 2FF to the culture medium**

To investigate the effects of Ab fucosylation, we produced IgG against the RhD blood group in the four different subclasses with and without core fucose. We added 2FF, a competitive inhibitor of fucosyl transferase, to the culture medium of the anti-RhD Ab-producing HEK cells. This resulted in a decrease in fucosylation from an average of 90% for all IgG subclasses to 26, 13, 29, and 60% for IgG1, IgG2, IgG3, and IgG4, respectively (Fig. 1). For IgG4, the reduction in fucosylation was consistently less efficient than for the other subclasses.

**Hypofucosylated Abs have a higher binding affinity to FcγRII**

We subsequently determined the binding affinity of the mAbs of different IgG subclasses with high and low fucose for all human FcγRs and their most relevant allotypic variants (FcγRI, FcγRIIα-151His, FcγRIIα-151Arg, FcγRIIβ, FcγRIIα-158Val, FcγRIIα-158Phe, FcγRIIβ-NA1, and FcγRIIα-NA2). We found similar binding affinities for normal (highly fucosylated) IgG as previously reported (Fig. 2) (3). IgG1 and IgG3 bound all FcγR variants, IgG2 only bound FcγRIIα, and IgG4 bound FcγRI. FcγRIα, FcγRIIβ, and FcγRIIIa, but not FcγRIIβ. IgG defucosylation increased the binding affinity to FcγRIIα and FcγRIIβ, with the extent depending on the IgG subclass, but it did not change the binding to any of the other FcγRs (Fig. 2).

We confirmed that the affinity of low-fucosylated IgG1 to FcγRIIα was 5- and 14-fold increased compared with high-fucosylated IgG1 for the FcγRIIα-158Val and the FcγRIIα-158Phe variant (Fig. 2). Similarly for IgG3, an 11- and 22-fold increase in binding upon hypofucosylation of IgG3 was observed for FcγRIIα-158Val and FcγRIIα-158Phe, respectively. Hypofucosylation of IgG1 and IgG3 also increased the binding to FcγRIIβ 7- to 8-fold, irrespective of the NA1/NA2 allotype. The affinity of IgG2 to both allotypes of FcγRIIα became significant and quantifiable upon hypofucosylation, but not to FcγRIIβ. Hypofucosylation of IgG4 also affected its binding to FcγRIIα, which went from observable but not reliably quantifiable to true reproducible binding. Upon hypofucosylation, a significant and reliable binding of IgG4 was also observed to FcγRIIβ of both allotypes (Fig. 2).

**Hypofucosylated IgG1 and IgG3 exert enhanced NK cell effector functions**

Because afucosylated Abs have an increased affinity for FcγRIIα (4, 11, 13), we performed NK cell ADCC assays to investigate the importance of Ab hypofucosylation in a biological FcγRIIα system. We used primary NK cells from donors expressing FcγRIIα as the only IgG receptor on their cell membrane as confirmed by flow cytometry (Fig. 3A).

Although both normally fucosylated and hypofucosylated anti-RhD led to similar deposition on erythrocytes (Fig. 3B), only hypofucosylated Abs led to a strong increase in NK cell ADCC toward erythrocytes (Fig. 3C). The NK cell ADCC of anti-RhD IgG1-opsonized erythrocytes was entirely FcγRIIα-dependent, as anti–FcγRIIα-F(ab′)2 fragments, but not isotype F(ab′)2 fragments, completely blocked ADCC (Fig. 3D). Similar to IgG1 anti-RhD, IgG3 variants induced increased NK cell–mediated ADCC when hypofucosylated, but IgG2 and IgG4 did not (Fig. 3E–G).

Normally, FcγR-expressing cells carry out their function in the presence of competing irrelevant IgG, the concentration of which is increased in patients treated with IVIg, which most likely contributes to the effector mechanism of IVIg. We therefore tested whether hypofucosylation of aspecific IgG blocks NK cell–mediated ADCC more efficiently than does fucosylated IgG, using irrelevant recombinant anti-hapten (TNP) IgG1. Only hypofucosylated IgG1 Abs induced a significant inhibition of anti-RhD IgG1-mediated erythrocyte ADCC (Fig. 3H).

**Hypofucosylated IgGs do not influence macrophage effector functions**

In vivo, FcγR-expressing effector cells other than NK cells are also targeted by IgG-mediated immune responses. We therefore investigated the effect of Ab fucosylation with human macrophages, which express a broader spectrum of FcγRs than NK cells and are considered relevant in immune-mediated clearance reactions by tissue-resident macrophages in liver and spleen.

We compared human GM-CSF– and M-CSF–cultured macrophages, representing the so-called M1 and M2 type of pro- and anti-inflammatory macrophages (41). These macrophages differ in
their relative expression of FcγRI, with GM-CSF–cultured macrophages expressing more of this receptor. The anti-inflammatory M-CSF–cultured macrophages express significantly more FcγRIIa than the GM-CSF–cultured macrophages (Fig. 4A, 4B). Both cell types have considerably less FcγRIIIa expression than NK cells.

GM-CSF–cultured macrophages phagocytize significantly more anti-RhD IgG3-opsonized RBCs than IgG1-opsonized cells; IgG4-opsonized RBCs were phagocytized to an even lower extent, whereas IgG2-opsonized cells were not phagocytized at all. M-CSF–cultured macrophages phagocytized anti-RhD IgG1-, IgG3-, and IgG4-opsonized erythrocytes to a similar extent, whereas IgG2-opsonized cells were again not phagocytized. Upon glycoengineering, all four anti-RhD IgG subclasses generated as hypofucosylated isoforms showed identical opsonizing capacity of the erythrocytes. No increase in phagocytosis was observed upon hypofucosylation of any IgG subclass (Fig. 4C).

To test whether the Ag density and corresponding level of opsonizing Ab binding would contribute to the difference between myeloid and NK cell effector cells, we made use of our mAb against TNP. Erythrocytes were TNP haptenized to different degrees and opsonized with either normally fucosylated or hypofucosylated anti-TNP IgG1 mAb (Fig. 5A). When performing phagocytosis assays with these erythrocytes now expressing a graded level of TNP, we found a strong positive correlation between the level of TNP-labeling, opsonization efficiency, and the percentage of uptake by macrophages (p < 0.0001). Still, we did not observe any difference in erythrocyte phagocytosis with respect to the fucosylation state of the opsonizing mAb (Fig. 5B).

We then tested whether hypofucosylated IgG1 was superior or not in blocking the phagocytosis of Ab-opsonized erythrocytes by macrophages. In this setting we used a clinically applied polyclonal anti-RhD product purified from plasma (RheDQuin; Sanquin) for Ab deposition (Fig. 5C). As expected from previous experiments (41), IgG Abs can block phagocytosis by competing in IgG binding to the FcγRs in a dose-dependent manner. Again under our conditions of macrophage phagocytosis, uptake of anti-RhD-opsonized erythrocytes was blocked irrespective of the fucosylation status of the blocking IgG (Fig. 5D).

Collectively, our data demonstrate that the fucosylation state of IgG greatly impacts the binding affinity to FcγRIIIa, but whether this affinity change results in an enhanced effector function depends on the effector cell type and its level of FcγRIII expression.

Discussion

In the present study we investigated the functional effects of Ab hypofucosylation in different cellular systems. Whereas >90% of IgG normally contains fucose, we reduced this level to <30% upon applying a well-characterized bioengineering method (14, 38). Human NK cells, which express FcγRIIIa as their only IgG receptor, showed increased affinity of hypofucosylated IgG1 and IgG3 for FcγRIIIa, whereas no such increased ADCC was observed with IgG2 and IgG4. Even though a gain of function of
FIGURE 3. Defucosylation of IgG1 and IgG3 significantly affects NK cell–mediated ADCC. (A) Gating strategy for genotype-selected NK cells (dot plots) that express FcγRIIIa, but not other FcγRs (histograms). Relevant isotype control background binding is shown in gray shading. Bar graph represents mean and SEM of $n = 5$. (B) The level of erythrocyte opsonization with 10 µg/ml anti-RhD IgG1 and IgG1 +2FF is similar. Anti-RhD opsonization was visualized with goat anti-human Ig and analyzed by flow cytometry. (C) NK cell ADCC with anti-D IgG1-opsonized erythrocytes. Erythrocytes were labeled with $^{51}$Cr and subsequently opsonized with anti-RhD fucosylated or defucosylated (+2FF) IgG1 or left unopsonized. (D) NK cell ADCC depends on FcγRIIIa. NK cell–mediated ADCC of anti-RhD IgG1-opsonized erythrocytes, either with or without (+2FF) fucose, were compared in the presence of CD16 F(ab')2 to block FcγRIIIa. (E–G) NK cell ADCC with opsonized erythrocytes as in (C) but now with (E) IgG2, (F) IgG3, and (G) IgG4. (H) NK cell ADCC with erythrocytes opsonized with defucosylated IgG1 anti-RhD (10 µg/ml) can only be blocked with defucosylated (+2FF) nonspecific anti-TNP blocking Abs. Only FCGR2/3-genotyped donors not expressing FcγRIIc and homozygous for FCGR3-158F were used. Data represent means and SEM of 6 (B), 10 (C), 4 (D), 9 (E), 6 (F), 6 (G), and 4 (H) experiments. ΔMFI, median fluorescence intensity corrected for the proper isotype control.
FIGURE 4. Monocyte-derived macrophages do not show elevated erythrocyte phagocytosis with defucosylated IgG. (A) FcγR expression levels of FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa on monocyte-derived macrophages, cultured for 9 d with GM-CSF (upper panels) or M-CSF (lower panels). Gray shading represents isotype control background binding to the cells. (B) Bar graphs showing the median fluorescence intensity, corrected for the proper isotype control, of FcγRI (n = 37), FcγRIIa (n = 9), FcγRIIb (n = 22), and FcγRIII (n = 37) of GM-CSF (open bars) and M-CSF (filled bars) cultured macrophages and SEM. For staining of FcγRIIb, only individuals without FCGR2C-ORF alleles were used (33). (C) Phagocytosis of erythrocytes opsonized with Abs of different subclasses with and without (+2FF) fucose (shown on x-axis) by monocyte-derived macrophages cultured with GM-CSF (left) and M-CSF (right). Only individuals not expressing FcγRIIC were used. The percentage of positive macrophages is shown, corrected for unopsonized erythrocytes (n = 10–26). *p < 0.05, **p < 0.01, ****p < 0.0001. ns, not significant.
IgG2 and IgG4 has been previously reported upon fucose removal (1, 42), and was observed in our study with isolated recombinant FcγRs tested in an SPR array, the enhanced binding affinity of nonfucosylated IgG2 and IgG4 must be considered insufficient to exert any biological effect under our conditions. In other cellular systems, hypofucosylated IgG2 and IgG4 may increase ADCC, as shown by Niwa et al. (1), who reported enhanced ADCC of Daudi cells opsonized with anti-CD20.

Besides the increased binding affinity to FcγRIIIa and FcγRIIb upon Ab defucosylation, no effect to other FcγRs is known. There is equivalent binding of defucosylated and fucosylated IgG1 to the neonatal Fc receptor (11), implying that defucosylated Abs have a half-life comparable to fucosylated Abs. Finally, fucose removal also does not change the binding to C1q and have equal complement-dependent cytotoxicity as fucosylated Abs. (11) (G. Dekkers et al., manuscript in preparation).

To compare the enhanced effector function of NK cells with isolated recombinant FcγRs tested in an SPR array, the enhanced binding affinity of nonfucosylated IgG2 and IgG4 must be considered insufficient to exert any biological effect under our conditions. In other cellular systems, hypofucosylated IgG2 and IgG4 may increase ADCC, as shown by Niwa et al. (1), who reported enhanced ADCC of Daudi cells opsonized with anti-CD20.

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Unlike GM-CSF–cultured macrophages, M-CSF–cultured macrophages mostly use FcγRIIa and FcγRI for IgG-mediated phagocytosis of erythrocytes (41). IgG1-, IgG3-, and IgG4-opsonized erythrocytes were phagocytized equally well, whereas IgG2-opsonized cells were again not ingested. This is partly surprising, as IgG2 has reasonable affinity to FcγRIIa (2). IgG1, IgG3, and IgG4 Abs bind FcγRIIa with similar affinity as IgG2, but unlike IgG2, they coengage FcγRI with high affinity, explaining their phagocytosis capacity (3).

Ab hypofucosylation did not affect macrophage effector function. This could be explained by the fact that macrophages express a variety of FcγRs, including FcγRI and FcγRIIa (41). Apparently, the contribution of FcγRIIa to the phagocytosis process is too small to be affected by Ab defucosylation in our phagocytosis assay.

We also tested the impact of IgG fucosylation on macrophage effector function in a different approach, as Ag-specific Abs can also compete for FcγR-binding with the overwhelming IgG concentration found in plasma (41, 43). In this study, we showed that adding an irrelevant IgG mAb to macrophages prior to the addition of anti-RhD–opsonized erythrocytes inhibited phagocytosis in a dose-dependent manner, similar to the addition of IVIg (41). Although the precise working mechanism of IVIg is still debated (32), one hypothesis is that IVIg saturates FcγRs on splenic and/or liver macrophages, hence inhibiting the phagocytosis of platelets or RBCs opsonized by autoantibodies (in the case of ITP and AIHA, respectively) (27, 29, 31, 32). Our studies indicate that irrelevant anti-TNP mAbs inhibit phagocytosis of opsonized red cells by macrophages irrespective of the level of fucosylation. If saturation of FcγRs were indeed the major working mechanism of IVIg, defucosylation alone would not alter the efficacy of IVIg treatment.

Recently, nonfucosylated mAbs have been generated for clinical use, because the increased affinity for FcγRIIa is thought to improve effector functions against cancer (20, 21, 44, 45). In this study, we show that this approach may represent an oversimplified
strategy and will definitely not work in all clinical approaches equally well. We suggest that the clinical efficacy of nonfucosylated Abs will also depend on the cell type predominating in a certain biological effect in vivo. In processes in which NK cells play a predominant role, it would be beneficial to defucosylate therapeutic Abs. However, in a more complex cellular system, where multiple FcγRs contribute to the cellular effector function, no difference may be expected between fucosylated and defucosylated Abs.

In sum, alteration of the 297 Asn glycans of IgG Abs may enhance the clinical response depending on the FcγR expression pattern of the effector cell type that predominates. Alternatively, altered levels of IgG fucosylation may be one of the alterations that could be used to afford enhanced binding activity and effector function beyond the FcγRII expression, processes that we are currently studying in much greater detail by further bioengineering steps.

Disclosures
The authors have no financial conflicts of interest.

References
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1. Niwa, R., A. Natsume, A. Uehara, M. Wakitani, S. Iida, K. Uchida, M. Satoh, and K. Shitara. 2005. IgG subclass-independent improvement of antibody-dependent cellular cytotoxicity by fucose removal from Asn63-linked oligosaccharides. Structure 13: 1043–1055.

2. Vidarsson, G., G. Dekkers, and T. Rispens. 2014. IgG subclasses and allotopes: from structure to effector functions. Front. Immunol. 5: 520.

3. Chisari, F. and J. V. Ravetch. 2008. Anti-inflammatory activities of intravenous immunoglobulin A and IgG. Front. Immunol. 1: 1228–1236.

4. Provan, D., R. Stasi, A. C. Newland, V. Bolton-Maggs, J. B. Bussel, B. H. Chong, D. B. Cines, T. B. Gernsheim, B. Godeau, et al. 2010. International consensus report on the investigation and management of primary immune deficiency in adults. Clin. Immunol. 136: 176–189.

5. Durandy, A., S. V. Kaveri, T. W. Kuijpers, M. Basa, S. Miescher, J. V. Ravetch, and R. Rieben. 2009. Intravenous immunoglobulins—understanding properties and mechanisms. Clin. Exp. Immunol. 158(Suppl. 1): 2–13.

6. Nakagawa, Z., A. T. McClelland, and T. Shinkawa. 2015. Immunomodulation by IV Ig and the role of Fc-gamma receptors: classic mechanisms of action after FcRIIa. Front. Immunol. 6: 674.

7. van der Heijden, J. W., W. K. Reitsma, J. Pasman, J. B. van den Berg, and T. W. Kuijpers. 2014. Phenotypic variation in IgG receptors by nonclassical FcγRIIa alleles. J. Immunol. 192: 1318–1324.

8. Tsang-A-SJoe, M. W., S. Q. Ngaklerg, J. Geissler, M. W. Tanck, C. E. Tacke, J. A. Ellis, W. Zenz, M. B. J. Herden, et al. 2016. Fc-gamma receptor polymorphisms differentially influence susceptibility to systemic lupus erythematosus and lupus nephritis. Rheumatology (Oxford) 55: 939–948.

9. van Buren, W. E., K. Visser, H. Wolbink, and B. van der Schoot. 2009. Production of recombinant IgM from antigen-selected single B cells and restricted usage of Ig-gene segments by antigen-specific B cells. J. Immunol. 182: 299–9–290.

10. Dohmen, S. E., A. Mulder, O. J. Verhagen, C. Eijsink, M. E. Franke-van Dijk, and K. Shitara. 2005. IgG subclass-independent improvement of antibody-dependent cellular toxicity. J. Biol. Chem. 277:6323–6340.

11. Okazaki, A., E. Shoji-Hosaka, K. Nakamura, M. Wakitani, K. Uchida, S. Sakita, K. Tsumoto, I. Kumagai, and K. Shitara. 2004. Fucose depletion from human IgG1 oligosaccharides enhances binding enthalpy and association rate between antibody-bound respiratory syncytial virus particles efficiently primes virus-specific immune responses in mice. J. Virol. 80: 1512–1524.

12. Dohmen, S. E., A. Mulder, O. J. Verhagen, C. Eijsink, M. E. Franke-van Dijk, and K. Shitara. 2005. Specificity and affinity of human FcγRIIIa and anti-CD38 IgG antibodies. J. Proteome Res. 306: 151–160.

13. Nakagawa, Z., A. T. McClelland, and T. Shinkawa. 2015. Immunomodulation by IV Ig and the role of Fc-gamma receptors: classic mechanisms of action after FcRIIa. Front. Immunol. 6: 674.

14. Duvic, M., L. C. Pinter-Brown, F. M. Foss, L. Sokol, J. L. Jorgensen, M. Scho¨ ni, M. Vest, and H. P. Wagner. 1981. High-dose intravenous gammaglobulin for idiopathic thrombocytopenic purpura in childhood. Lancet 1: 1228–1231.

15. Kapur, R., L. Della Valle, M. Sonneveld, A. Hipgrave Ederveen, R. Visser, P. Ligthart, M. de Haas, M. Wurzer, C. E. Tacke, J. V. Ravetch, and G. Vidarsson. 2014. Low anti-RhD IgG-Fc-fucosylation in pregnancy: a new variable predicting RhD status in haemolytic disease of the newborn and newborn jaundice. Blood 124: 3936–4045.

16. Jefferis, R. 2009. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. Trends Pharmacol. Sci. 30: 356–362.

17. Yamane-Ohnuma, N., and M. Satoh. 2009. Production of therapeutic antibodies with controlled fucosylation on the FcγRII expression, processes that we are currently studying in much greater detail by further bioengineering steps.