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Fc Engineering of Human IgG1 for Altered Binding to the Neonatal Fc Receptor Affects Fc Effector Functions

Algirdas Greveys,† Malin Bern,† Stian Foss,†† Diane Bryant Bratlie,‡ Anders Moen,§ Kristin Stoen Gunnarsen,†† Audun Aase,‡ Terje Einar Michaelsen,‡,§ Inger Sandlie,†† and Jan Terje Andersen†

Engineering of the constant Fc part of monoclonal human IgG1 (hIgG1) Abs is an approach to improve effector functions and clinical efficacy of next-generation IgG1-based therapeutics. A main focus in such development is tailoring of in vivo half-life and transport properties by engineering the pH-dependent interaction between IgG and the neonatal Fc receptor (FcRn), as FcRn is the main homeostatic regulator of hIgG1 half-life. However, whether such engineering affects binding to other Fc-binding molecules, such as the classical FcγRs and complement factor C1q, has not been studied in detail. These effector molecules bind to IgG1 in the lower hinge–C H2 region, structurally distant from the binding site for FcRn at the C H2–CH3 elbow region. However, alterations of the structural composition of the Fc may have long-distance effects. Indeed, in this study we show that Fc engineering of hIgG1 for altered binding to FcRn also influences binding to both the classical FcγRs and complement factor C1q, which ultimately results in alterations of cellular mechanisms such as Ab-dependent cell-mediated cytotoxicity, Ab-dependent cellular phagocytosis, and Ab-dependent complement-mediated cell lysis. Thus, engineering of the FcRn–IgG1 interaction may greatly influence effector functions, which has implications for the therapeutic efficacy and use of Fc-engineered hIgG1 variants. The Journal of Immunology, 2015, 194: 000–000.

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Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; ADCML, Ab-dependent complement-mediated cell lysis; ADCP, Ab-dependent phagocytosis; DSF, differential scanning fluorimetry; FcRn, neonatal Fc receptor; GST, glutathione transferase; HCD, high-energy collisional dissociation; HER2/29E, adherent human embryonic kidney cell line 293E; hFcRn, human neonatal Fc receptor; hIgG1, human IgG1; HN, H433K/N434F; LS/MS/MS, liquid chromatography–tandem mass spectrometry; IHH, E253M/H310A/H435A; MN, M428L/N434S; MS/MS, tandem mass spectrometry; MST, M252Y/S254T/T256E; M252Y/S254T/T256E/H433K/H435F; NIP, 5-iodo-4-hydroxy-3-nitro-phenacetyl; PIBST, PBS containing 0.05% Tween 20; PMN, polymorphonuclear neutrophil; RT, room temperature; SPR, surface plasmon resonance; SRBC, sheep RBC; †t0, melting temperature; TBD, 3′,5′-tetramethylbenzidine; WT, wild-type.

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CD20 expressed on B cells, which is approved for treatment of non–Hodgkin lymphoma, chronic lymphocytic leukemia, vasculitis, and rheumatoid arthritis (1, 2). However, there is a need for improved therapeutic efficacy and patient convenience, which drives the industry toward development of next-generation Abs. Already available Abs as well as new Abs are therefore optimized with regard to improved serum half-life and tissue distribution or modulated effector functions such as Ab-dependent cell-mediated cytotoxicity (ADCC), Ab-dependent cellular phagocytosis (ADCP), and Ab-dependent complement-mediated cell lysis (ADCM) (1). For instance, enhancement of antitumor properties can be achieved by engineering the Fc region for stronger binding to activating FcγRs and C1q, or weaker binding to the inhibitory FcγRIIb to increase ADCC and ADCML effector activity (3–8). Alternatively, decreased ADCC and ADCML may be beneficial in terms of minimizing toxicity in other therapeutic settings (9–13). In either case, engineering is accomplished through alterations of amino acids in the Fc part, either by selection from display libraries of Ab fragments or by structure-guided design. Additionally, tailoring of the antennary glycans attached to the Fc part offers a powerful method to modify binding to the classical FcγRs for enhancement or elimination of ADCC activity (14–16). In general, Fc engineering for desired effector functions may be combined with any specificity of interest.

Most of the Abs approved or in clinical trials are built on the IgG class of Abs (1). In humans, IgG1, IgG2, and IgG4 all have a serum half-life of ∼3 wk, whereas IgG3 has a half-life of 1 wk, comparable with the short half-life of other Ab classes (1–2 d) (17, 18). The long half-life of IgG1 and its ability to bind FcγRs as well as C1q have made it attractive as an Ab therapeutic. The long half-life relies on its interaction with the neonatal Fc receptor (FcRn) (19, 20).

FcRn is widely expressed in different cell types and tissues, as for instance the vascular endothelium that covers the interior of blood and lymphatic vessels and in hematopoietic cells such as dendritic cells, predominantly within endosomal compartments (21–25). Similar to other circulating proteins, IgG is continuously pinocytosed by these
and other cells. Subsequently, the low pH in the endosomes triggers binding to FcRn, followed by recycling to the cell surface and release upon exposure to the nearly neutral pH of the blood (24, 26, 27). Unbound material is directed from endosomes to lysosomes for degradation. Thus, the pH dependence of the interaction with FcRn is essential for efficient receptor-mediated recycling and rescue from degradation, which results in extended circulatory half-life. The serum half-life of therapeutic IgG Abs may be decreased or increased by engineering of the constant Fc domains for altered binding to human FcRn (hfFcRn) (28–35). Such design has great potential for improving pharmacokinetics, as increased serum half-life offers the benefits of greater efficacy, less frequent dosing, and lower cost.

FcRn is an MHC class I-related molecule consisting of a unique transmembrane H chain that is noncovalently associated with β2-microglobulin (36). Site-directed mutagenesis studies and crystal structure analysis of the FcRn/IgG Fc complex show that the Fc amino acid residues at positions 252–256 in the C2 domain and at 310, 433, 434, and 435 in the C3 domain are at the core or in close proximity to the FcRn interaction site, and that the conserved histidine residues H310 and possibly H435 are responsible for the pH dependence (32, 36–38). These and other residues may be targeted by mutagenesis to alter the binding kinetics toward FcRn. A challenge when designing IgGs with extended half-life is to maintain the pH dependence of the FcRn interaction. An IgG engineered to bind FcRn with higher affinity and reduced pH dependence potently inhibits the interaction of FcRn with endogenous IgG and thus rapidly lowers the concentration of circulating IgG, and the mutant itself is also rapidly degraded. This has been demonstrated to be the case for a human IgG1 (hIgG1) mutant with five substitutions in the Fc (M252Y/S254T/T246E/H433K/N434F; MST/HN), denoted as Abs for degradation. However, the mutant has shown promise as an FcRn blocker that accelerates the clearance of pathogenic IgG in mouse models (34, 39, 40).

A handful of hIgG Abs have been developed that show favorable binding kinetics toward hFcRn, improved affinity at acidic pH with no or moderate binding at neutral pH (28–35). One such hlgG1 variant, with two mutations within the C3 domains (M428L/N434S; MN) or moderate binding at neutral pH (28–35). One such hIgG1 variant, pathogenic IgG in mouse models (34, 39, 40).

Materials and Methods

Cell culture

The adherent human embryonic kidney cell line 293E (HEK293E) was obtained from American Type Culture Collection and maintained in DMEM or RPMI 1640 (BioWhittaker) supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 2 mM l-glutamine, 25 µg/ml streptomycin, and 25 U/ml penicillin (all from BioWhittaker). The cells were incubated at 37°C in a humidified 5% CO2, 95% air incubator.

Construction and production of anti–5-iodo-4-hydroxy-3-nitrophenacyl hlgG1 variants

A vector cassette system (pLNOH2-NIPhlgG1-oriP) for subcloning of Fc fragments into the framework encoding WT hlgG1 has previously been described (45, 46). The vector contains the constant H chain gene from hlgG1 with specificity for the hapten 5-iodo-4-hydroxy-3-nitrophenacyl (NIP), and it was used as a template for subcloning of DNA fragments encoding (synthesized by GenScript) a panel of Fc mutants using the restriction sites AgeI and SfiI (C2 mutations) or SfiI and BamHI (C3 mutations): M252Y/S254T/T246E (MST), M252Y/S254T/H433K/N434F (MST/HN), denoted as Abs for degradation. However, the mutant has shown promise as an FcRn blocker that accelerates the clearance of pathogenic IgG in mouse models (34, 39, 40).

A handful of hlgG1 Abs have been developed that show favorable binding kinetics toward hFcRn, improved affinity at acidic pH with no or moderate binding at neutral pH (28–35). One such hlgG1 variant, with two mutations within the C3 domains (M428L/N434S; MN) engineered into the vascular endothelial growth factor–specific Ab bevacizumab (Avastin), has demonstrated 4-fold longer half-life in hFcRn transgenic mice, and 3-fold in cynomolgus monkeys (41). Importantly, extended serum persistence was shown to translate into enhanced antitumor activity in a xenograft study in mice (41). A second hlgG1 variant with anti-lysozyme specificity with two mutations in the C3 domain (H433K/N434F; HN) showed a 4-fold reduced half-life in wild-type (WT) mice, but enhanced FcRn-mediated transport in an ex vivo human placenta model (42). A third example is an hlgG1 variant with anti-respiratory syncytial virus specificity (motavizumab) (Avastin), has demonstrated 4-fold longer half-life in hFcRn transgenic mice, and 3-fold in cynomolgus monkeys (41). Alternatively, it may be desirable to tune the half-life to achieve intermediate or shorter persistence as a means to optimize the target/ontarget localization ratios of Abs used for tumor imaging. This can be accomplished by targeting Fc residues directly involved in binding to FcRn (I253, H310, and H435), and such IgG mutants have been shown to have a range of distinct half-life profiles (43, 44). The positions of the amino acid residues are highlighted in the crystallographic illustrations of hlgG1 Fc shown in Fig. 1.

However, a highly relevant question with regard to such engineering is whether substitutions of residues at or near the FcRn binding site could lead to structural perturbations that affect Fc-mediated effector functions. This is essential, as some Abs require potent effector functions for optimal clinical activity. In this study, we examine how engineering for altered binding to FcRn affects binding to the classical FcγRs and complement factor C1q. We compare the panel of Fc-engineered hlgG1 variants, produced side by side with the same specificity, for binding to human C1q (hC1q) and all human FcγRs (hFcγRs). In most cases, we found binding to be reduced. However, one of the hlgG1 variants, hlgG1-HN, showed improved binding to hC1q. Importantly, the differences in binding translated into reduction of hFcγR- and hC1q-mediated cellular processes such as ADCC, ADCP and ADCML, except in the case of hlgG1-HN. These findings have implications for tailoring of recombinant hlgG1 Abs with extended half-life and desired effector functions.
PBBS containing 4% skin milk (Sigma-Aldrich) for 2 h and washed four times with PBBS containing 0.1% Tween 20 (PBBS; Sigma-Aldrich). Purified anti-NIP hlgG1 variants were diluted in 4% skin milk/PBST and incubated for 1 h at room temperature (RT). The plates were washed four times as described above before alkaline phosphatase–conjugated polyclonal goat anti–hlgG Fc Ab (Sigma-Aldrich), diluted (1:4000) in 4% skin milk/PBST, was added for 1 h at RT. The plates were then washed as above before 100 μl p-nitrophenylphosphate substrate (Sigma-Aldrich) diluted to 10 μg/ml in diethanolamine buffer was added. The absorbance was measured at 405 nm using a Sunrise spectrophotometer (Tecan). Alternatively, a monoclonal HRP-conjugated goat anti-mouse λ chain Ab (SouthernBiotech) was used followed by visualization using the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (Merck), and the reactions were stopped by adding 50 μl HCl. The absorbance was measured at 450 nm using the Sunrise spectrophotometer (Tecan).

ELISA measurements of FcγRs binding

Serial dilution of anti-NIP hlgG1 variants (0.08–10 μg/ml) diluted in 4% skin milk/PBST were added to BSA-NIP–coated plates (2 μg/ml) and incubated for 1 h at RT, prior to washing using PBST (pH 6.0). Then, 10 μg/ml hFcRn was diluted in 4% skin milk/PBST (pH 6.0) and added for 1 h at RT before washing as above. Bound receptor was detected using a biotinylated monoclonal mouse anti-hFcRn Ab (ADM11) (50) followed by alkaline phosphatase–conjugated streptavidin (GE Healthcare). After washing, 100 μl p-nitrophenylphosphate substrate (Sigma-Aldrich) was added to each well. The absorbance was measured at 405 nm using a Sunrise spectrophotometer (Tecan). The same set-up was performed at pH 7.4.

ELISA measurements of FcγRs and C1q binding

Ninety-six–well plates were coated with 100 μl BSA-NIP at 1 μ/ml. After incubation overnight at 4˚C, the plates were blocked with 4% skin milk/PBS for 2 h and washed four times with PBBS. Serial dilution of anti-NIP hlgG1 variants (0.3–1 μg/ml) diluted in 4% skin milk/PBST were added and incubated for 1 h at RT prior to washing using PBST (pH 7.4). Then, 1 μg/ml GST-tagged hFcγRIIa, hFcγRIIb, hFcγRIIIa, and hFcγRIIIb were diluted in 4% skin milk/PBST and incubated for 1 h at RT before being washed as above. Bound receptors were detected using an HRP-conjugated goat anti-GST Ab (GE Healthcare) diluted 1:5000 in 4% skin milk/PBST. After washing, 100 μl TMB substrate (SouthernBiotech) was added to each well and reactions were stopped by adding 50 μl 1 M HCl. The absorbance was measured at 450 nm using a Sunrise spectrophotometer (Tecan). The same set-up (without non-fat dried milk powder) was performed by adding pure hC1q (0.366 μg/ml) (Quidel), serum from a human volunteer (1:200 dilution; Calbiochem) to the captured anti-NIP Abs. Bound hC1q was detected using a rabbit anti-hC1q Ab (Dako) followed by an HRP-conjugated donkey anti-rabbit Ab (GE Healthcare). After washing, 100 μl TMB substrate (SouthernBiotech) was added to each well before complement source (50 μl) were mixed with SRBCs (50 μl), diluted 1:30 in veronal buffer (pH 7.2) and inactivated rabbit anti-human C3c Ab (Heto Maxi dry). Dried samples were dissolved in 20 μl 0.1% formic acid, sonicated for 30 s, and centrifuged for 10 min at 16,100 × g. Subsequently, 10 μl of each sample was transferred to new vials, and reverse phase (C18) nano online liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of proteolytic peptides was performed using a system consisting of two Agilent 1200 HPLC binary pumps (nano and capillary) with an autosampler, column heater, and integrated switching valve. This system was coupled via a nanoelectrospray ion source to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). For the analyses, 6 μl peptide solution was injected into the 5 × 0.3-mm extraction column filled with Zorbax 300SB-C18 of 5-μm (diameter) particle size (Agilent Technologies).

After washing for 5 min with 0.1% formic acid (v/v) and 5% acetonitrile (v/v) at a flow rate of 10 μl/min, the integrated switching valve was activated and peptides were eluted in the back-flush mode from the extraction column onto a 150 × 0.075-mm C18, 3-μm resin column (GlycproSIL C18–80A, Glycromass). The mobile phase consisted of acetonitrile and mass spectrometry–grade water, both containing 0.1% formic acid. Chromatographic separation was achieved using a binary gradient from 5 to 55% of acetonitrile in water for 60 min with a flow rate of 0.2 μl/min. Mass spectra were acquired in the positive ion mode applying a data-dependent automatic switch between survey scan and MS/MS acquisition. Peptide samples were analyzed with a high-energy collisional dissociation (HCD) fragmentation method with normalized collision energy at 25 and 41, acquiring one Orbitrap scan in the mass range of m/z 300–2000 followed by MS/MS of the three most intense ions in the Orbitrap (R7500). The target value in the LTQ-Orbitrap was 1 million for survey scan at a resolution of 30,000 at m/z 400 using lock masses for recalibration to improve the mass accuracy of precursor ions. Ion selection threshold was 500 counts. Selected sequenced ions were dynamically excluded for 180 s. Data analysis was performed on Xcalibur v2.0. MS/MS spectra for all glycopeptides and were extracted by oxonium ion search; 204.086 (N-acetyliminosialic acid) and 366.1388 (N-acetyliminosialic acid/hsx) were used. HCD fragmentation with normalized collision energy at 25 was used to detect the glycan, and the peptide mass was detected for the IgG glycopeptides. Extracted ion chromatogram for target glycopeptides (EEQYNSTYR and the miscleaved TKPREEQYNSTYR with all different glycan masses) were extracted with 10 ppm accuracy and MS/MS spectra were manually verified. HCD fragmentation with normalized collision energy at 41 was used to white the peptide sequence and to verify that the peptide mass corresponded to the correct peptide sequence. The area under the curve for all extracted glycopeptides was calculated and the percentage ratio for each glycoform was determined.

Ab-dependent complement-mediated cell lysis

The ADCC assay was done essentially as described (51). Briefly, target cells were prepared by mixing 10 μl packed sheep RBCs (SRBCs) with 16 μl PBBS and 60 μCi sodium chromate ([51Cr]; PerkinElmer) and incubating for 1 h at 37°C. Subsequently, NIP-conjugated anti-SRBC rabbit Fab fragments (2.5 μg NIP-Fab or 13.3 μg NIP-Fab) were added and incubated for 1 h at 37°C. The sensitized and labeled SRBCs were washed three times and diluted to 2–4 × 107 cells/ml in veronal buffer (pH 7.2; 1% BSA, 0.25 mM CaCl2, and 0.8 mM MgCl2). The assay was performed in round-bottom microtiter plates (Costar). Serial dilutions of anti-NIP IgG1 Abs (900.0–3.7 ng/ml) (50 μl) were mixed with SRBCs (50 μl) and incubated for 10 min at RT before complement source (50 μl), diluted 1:30 in veronal buffer, was added. All mixtures were incubated for 30 min at 37°C and then spun down at 1200 rpm for 3 min before supernatants were collected with harvesting filters/ devices (Skatron). Release of [51Cr] was measured using a gamma counter (Packard). The cytotoxic index (CI %) was calculated using the formula: CI % = [{cpm (test) – cpm (spontaneous)} / {cpm (maximum) – cpm (spontaneous)}] × 100. Maximum [51Cr] release was determined as radioactivity released when the SRBCs were lysed with water containing 0.1% Tween 20. Spontaneous [51Cr] release was measured by incubating SRBCs with complement source.

Ab-dependent cell-mediated cytotoxicity

The ADCC assay was done essentially as previously described (51). Briefly, 50 μl 5-fold dilution of anti-NIP hlgG1 variants (200.0–0.06 ng/ml) and 50 μl haptenated, 51Cr-labeled SRBCs (3 × 106 cells/ml) were added into round-bottom microtiter plates (Costar) and incubated for 10 min at RT before 3 × 106 cells/ml were added. PBMCs were obtained from healthy volunteers.
donors using Lymphoprep (Axis-Shield), and NK cells and monocytes were isolated by negative selection using Dynabeads untouched human NK cells and Dynabeads untouched human monocyte kits (Invitrogen), respectively, following the manufacturer’s instructions. Cells were diluted into DMEM/HEPES/1% FCS. The microtiter plate was spun down at 300 × g for 2 min and incubated for 4 h at 37˚C. The plates were centrifuged for 3 min at 300 × g, and supernatants were collected with harvesting filters/devices (Skatron). Maximum [51Cr] release and spontaneous [51Cr] release were measured as described above.

Ab-dependent cellular phagocytosis

The phagocytosis activity was measured as respiratory burst, as previously described (52). Briefly, NIP-haptenated meningococci killed by ethanol were used as target cells whereas dihydrodihoradamine 123 (Sigma-Aldrich)–primed polymorphonuclear neutrophils (PMNs) from a health donor were used as effector cells. Serial dilutions of anti-NIP IgG1 variants were prepared in DMEM/HESPES/1% FCS (50 μl), mixed with 5 μl meningoocci (106/ml), and incubated for 30 min at 37˚C in microtiter plates. Then, 5 μl hIgG-depleted human serum was added as a complement source and incubated for 10 min at 37˚C. Furthermore, 50 μl 4 × 107 dihydrodihoradamine 123–primed effector cells were added and incubated for 10 min at 37˚C. Flow cytometry was performed gated on the PMNs, and the percentage of respiratory burst was estimated. ADCP was also performed without the complement source.

Protein stability by differential scanning fluorimetry

Protein stability of monomeric IgG1 variants was measured by protein stability by differential scanning fluorimetry (DSF) using a LightCycler RT-PCR instrument (Roche). SYPO Orange (Sigma-Aldrich) was used at a 1:10000 dilution, and the protein concentration was 0.1 mg/ml in a final volume of 25 μl. All samples were run in triplicate in 96-well LightCycler 480 multwell plates. The peaks of excitation and emission of SYPO Orange are 490 and 580 nm, respectively. The 450-nm excitation and 568-nm emission filters on the RT-PCR instrument were used. The RT-PCR instrument was programmed to raise the temperature from 25˚C to 95˚C after a stabilization period of 10 min at 25˚C. Two measurements at each temperature were recorded, and data were collected every 0.5˚C. Data transformation and analysis were performed using the DSF analysis protocol (53).

Statistical analysis

All experiments were repeated at least three times and error bars represent SEM. A one-way ANOVA test (Dunnett test) was used to calculate the statistical significance. A p value < 0.05 was considered significant.

Results

Design of human Fc-engineered IgG1 variants

To construct recombinant IgG variants, we used two plasmids, one encoding the hIgG1 H chain and one encoding the 𝜆 mouse L chain, which together form Abs with specificity for the hapten NIP. In addition to the WT, four Fc-engineered variants were made by substitution of amino acid residues that have previously been shown to modulate binding to FcRn, transplacental transport, and serum half-life, namely hIgG1-HN, hIgG1-MN, hIgG1-MST, and hIgG1-MST/HN (34, 35, 41, 42) (Fig. 1B–D).

The hIgG1 variants were produced by transient transfection of HEK293E cells, and medium fractions were collected every second day for up to 2 wk, pooled, and filtrated prior to purification on a 4-hydroxy-3-nitrophenyl acetyl–coupled column followed by gel filtration. The total concentrations were in the range of 3–0.9 mg/ml culture, where hIgG1-MST/HN was produced with a 3-fold higher yield than that of the others (Fig. 2A). Small fractions of the hIgG1 Abs were run on a nonreducing SDS-PAGE followed by Coomassie staining (Fig. 2B), and the migration profiles for the mutants were found to be similar to those of the WT with major bands at ∼150 kDa. Additionally, ELISA showed that the Abs bound equally well to NIP conjugated to BSA (Fig. 2C). Finally, the stability of the Abs was compared, and the melting temperature (t_m) value for WT anti-NIP hIgG1 was found to be 68˚C, equal to that of the commercial anti-CD20 Ab rituximab, whereas the t_m values for the mutants varied from 61˚C to 67.5˚C (Fig. 2D).

Mapping of glycans attached to the IgG1 variants

To characterize the profile of the glycans attached to N297 on the C2 domain of the hIgG1 variants, we performed LC-MS/MS analysis on glycopeptides after trypsinization. In total, we identified 32 different glycans (Supplemental Table I), where four forms dominated and comprised 77–93% of the total glycan pool, namely 4GlcNac-1Fuc-3Man, 4GlcNac-1Fuc-3Man, 4GlcNac-1Fuc-3Man-1Gal, 4GlcNac-1Fuc-3Man-2Gal, and 2GlcNac-5Man. The relative amount of each glycan was similar, but not identical, between variants and the WT (Supplemental Table I). In particular, hIgG1-WT had 15 and 8% less of the 4GlcNac1Fuc3Man and 4GlcNac1Fuc3Man1Gal forms,

**FIGURE 1.** Crystal structure illustrations of hlgG1 Fc variants. Amino acid residues targeted to modulate binding to hFcRn are highlighted. (A) The key Fc residues involved in binding to FcRn (I253, H310, and H345) at the C12-C13 interface are highlighted in green spheres. (B) M428 and N434 of the C13 domains are highlighted in blue spheres. (C) The Fc residues M252, S254, and T256 of the C22 domains are highlighted in orange spheres. (D) H433 and N434 of the C13 domains are highlighted in red spheres. In all crystal structure illustrations the biantennary glycans attached to the N297 residues of the C22 domains are shown in red. The figures were designed using PyMOL (http://www.pymol.org) with the crystallographic data of hIgG1 (Protein Data Bank accession code 1HZH) (70).
respectively, than did the mutants (Supplemental Table I). Thus, there were some differences in fucosylated oligosaccharides attached to the WT compared with the engineered variants and minor differences between the mutant variants. Because the Abs were produced in the same system side by side, our data suggest that the mutations introduced a slight shift in the profile of glycan attached to position N297.

**Binding of the Fc-engineered hIgG1 to hFcRn**

To investigate how the hIgG1 variants bound soluble hFcRn, we established an ELISA where titrated amounts of the Abs were captured on NIP-conjugated BSA coated in wells followed by addition of hFcRn at pH 6.0 or 7.4. Bound receptor was detected using an mAb (ADM11) directed toward hFcRn, the binding of which does not interfere with IgG binding (50). As expected, hFcRn was shown to bind WT hIgG1 in a strictly pH-dependent manner, binding at pH 6.0 and not binding at pH 7.4, whereas hIgG1-IHH did not bind (Fig. 3). Furthermore, a side-by-side comparison of the four Fc-engineered hIgG1 mutants revealed that they all bound more strongly than did the WT at acidic pH with a hierarchy from strongest to weakest as follows: MST/HN > HN > MN > MST > WT > IHH (Fig. 3A). At neutral pH, detectable binding was measured for MST/HN followed by intermediate binding to HN and MN whereas no binding was detected for the WT, MST, and IHH (Fig. 3B). Thus, the Fc-engineered anti-NIP hIgG1 variants showed a range of distinct FcRn binding properties, in accordance with reported data (34, 35, 41, 42).

**Binding of the Fc-engineered hlgG1 variants to classical hFcγRs**

To assess whether the mutations affected binding to the five classical hFcγRs, ELISA was performed using soluble GST-tagged hFcγRs. Importantly, all recombinant receptors showed expected binding selectivity toward the four hlgG subclasses (Supplemental Fig. 1A–G), and thus they were used for screening of titrated amounts of hlgG1 variants captured on BSA-NIP.

The binding responses obtained toward hFcγRI showed that all variants bound equally as well as did the WT, with the exception of HN and MST/HN, which showed a slight reduction in binding (Fig. 4A). The titrated binding curves (Supplemental Fig. 1H–M) were used to calculate the relative binding response for each of the FcγRs toward the hlgG1 variants, where binding of WT hlgG1 was set to 1.0. Slightly reduced binding (5–20%) was observed between the high-affinity FcγRI and the variants (Fig. 4B). Furthermore, the two allotypes of hFcγRIIa (H/R131) showed reduced binding (20–50%) to the variants, with the most pronounced effect detected being toward MST and MST/HN (Fig. 4C, 4D). Similarly, reduced...
binding (20–90%) was detected toward the two hFcγRIIIa allotypes (FV158), where again the largest negative effect was detected for MST and MST/HN followed by MN and HN (Fig. 4F, 4G). The negative effect of the Fc substitutions was most pronounced for binding to the phenylalanine-containing allotype (F158), and a similar trend was seen as for binding to hFcγRIIib, with 40–90% reduction in binding (Fig. 4H). Furthermore, despite high homology between the hFcγRIIa allotypes and the inhibitory hFcγRIIb, hFcγRIIib showed even poorer binding to MST and MST/HN than did hFcγRIIa. Binding of HN and MN was less affected (Fig. 4E).

Lastly, we selected the H131 allotype of hFcγRIIa to quantify the binding affinity toward the WT and three of the hlgG1 variants (MN, MST, and MST/HN) using SPR (Supplemental Fig. 2). Fitting the equilibrium binding responses to a steady-state affinity model revealed that MST and MST/HN bound with 4- to 5-fold reduced equilibrium binding affinity compared with the WT, whereas MN only showed a slight reduction. In summary, we demonstrate that Fc engineering of hlgG1 for improved FcRn binding may markedly reduce binding to the classical FcγRs.

**ADCP**

To investigate how Fc engineering affected ADCP, meningococci were directly labeled with the hapten NIP followed by addition of titrated amounts of the anti-NIP Abs. Portions of human PMNs from a volunteer were added and phagocytic activity was measured as respiratory burst. The results show that all variants induced ADCP (Fig. 5) with only minor differences between the variants, where MST/HN showed the most pronounced reduction. Furthermore, we investigated how ADCP was affected by the addition of human serum as a complement source, and we found that the phagocytic activity increased by ∼20% for all variants, but most strikingly by a further 20% for IgG1-HN (Fig. 5C, 5D). Thus, Fc engineering of hlgG1 for altered binding to FcRn may have a great impact on complement activation ability.

**ADCC**

Next, an ADCC assay was conducted where 51Cr-labeled SRBCs were coated with rabbit anti-SRBC Fab fragments that were conjugated to the hapten NIP at low or high density, prior to addition of hlgG1 variants and freshly isolated PBMCs. In both cases, ADCC activity was measured as release of [51Cr], and the results obtained show hlgG1-MST to be a rather poor inducer of cytotoxicity, 50% or less efficient than the WT, followed by MN, whereas HN and MST/HN were similar or better than the WT. At low Ag density, the HN variant showed a 2-fold improved ADCC activity over the WT (Fig. 6A–D).

Next, we addressed which effector cells were responsible for the ADCC activities by repeating the assay with NK cells and monocytes isolated by negative selection using magnetic beads. Enrichment of NK cells resulted in overall enhanced ADCC activity at high Ag density, and the relative activity of the variants followed the same trend as that measured using PBMCs, with the exception of MN, which was now equally as good as the WT (Fig. 6E–H). Additionally, the cytotoxicity induced by MST/HN was more pronounced at high Ag density, whereas MN showed improved activity at both low and high Ag densities (Fig. 6E–H). Furthermore, the hlgG1 variants also induced ADCC by monocytes, but not to the same magnitude as that of NK cells at high Ag density (Fig. 6I–L). Interestingly, only minor differences were detected between the hlgG1 variants at high Ag density whereas more pronounced differences were measured at low density (Fig. 6I–L). Specifically, both MN and HN showed 1.5-fold

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**FIGURE 4.** Binding properties of Fc-engineered hlgG1 variants toward classical hFcγRs. ELISA binding of titrated amounts (1000.0–0.5 ng/ml) of WT hlgG1 and the Fc-engineered variants to (A) hFcγRI, and calculation of relative binding of WT hlgG1 and the Fc-engineered variants to (B) hFcγRI, (C) hFcγRIIa-R131, (D) hFcγRIIa-H131, (E) hFcγRIIb, (F) hFcγRIIa-V158, (G) hFcγRIIa-F158, and (H) hFcγRIIib. The experiments were performed at pH 7.4, and obtained data are shown as mean ± SEM of one experiment performed in triplicate (A), and as mean ± SEM of three independent experiments performed in triplicate (B–H). *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA test.
improved ADCC activity over WT, whereas MST and MST/HN showed a reduction of 25 and 50%, respectively (Fig. 6K, 6L).

**Binding of the Fc-engineered hlgG1 variants to hC1q**

Besides FcγR-mediated effector functions, the classical complement cascade is important for humoral immunity. Because the binding sites for the classical FcγRs overlap with that of C1q (3), the key residues involved are again located far from the amino acid substitutions of the Fc-engineered hlgG1 variants. Nevertheless, we measured binding to hC1q by ELISA using two sources of complement, that is, purified hC1q and human serum. Initially, the amount of hC1q present in serum was quantified and found to be 73.2 μg/ml by ELISA (data not shown). Equal amounts of both sources of hC1q were added to titrated amounts of the Abs coated on BSA-NIP.

All hlgG1 variants bound pure hC1q and hC1q in serum with almost identical hierarchies as HN > WT > MN ≥ MST/HN > MST, but the binding responses were stronger when hC1q was provided in serum, and in both cases hlgG1-HN bound more hC1q than did the WT (Fig. 7A, 7B). Notably, the WT performed equally well as MN when pure hC1q was added whereas it bound better in serum (Fig. 7A, 7B). Next, when pure hC1q was added to C1q-depleted human serum, the same hierarchy was measured (Fig. 7C). Furthermore, an even more pronounced effect of the HN mutations was detected when a constant amount of the Abs was directly coated in wells or captured on BSA-NIP followed by adding titrated amounts of hC1q from both sources (Supplemental Fig. 3A–D). Finally, the same hierarchy was found when C3 or C5 deposition was monitored (Fig. 7D, 7E). As controls, we included hlgG1 with a single point mutation (P329A) within the C1q2 domain at the core of the C1q binding site (47), which completely eliminated hC1q binding, as well as hlgG1 with mutation of either of two leucine residues in the lower hinge (L234A and L235A), crucial for FcγR binding (54), which moderately reduced binding (Supplemental Fig. 3E–H).

**ADCML**

To investigate whether the complement binding data would translate into altered ADCML activity, we captured the anti-NIPAbs on 51Cr-labeled SRBCs, again coated with anti-SRBC Fab fragments from rabbit conjugated to NIP at low or high density. After addition of the complement source, release of [51Cr] was measured as ADCML activity, which gave rise to the same hierarchy as that measured in the C1q binding assay (Fig. 8). A similar trend was detected at both Ag densities, whereas the effect of the mutations was most pronounced at low Ag density. In this study, the HN variant showed a 4-fold enhancement of ADCML compared with WT, in stark contrast to the other variants, which induced very little ADCML (Fig. 8C, 8D).

**Discussion**

In the present study, we produced a panel of Fc-engineering hlgG1 variants designed to have increased binding to FcRn, and we investigated how the Fc amino acid substitutions modulate binding to the classical hFcγRs and hC1q, as well as the accompanying effector functions ADCC, ADCP, and ADCML. Such knowledge is of crucial importance prior to therapeutic use of FcRn-engineered Abs.

To compare their binding abilities and cellular effector functions, all hlgG1 variants were produced with the same specificity, side by side.
using the same vector and cell system. The Fc-engineered IgG variants were secreted in similar amounts upon expression in HEK293E cells, except for hIgG1-MST/HN, which was produced in higher yields. This may suggest that the mutations resulted in increased intrinsic protein stability. However, when stability was measured by DSF, a slight reduction in $t_m$ value was observed for MST/HN, which was solely due to the M252Y/S254T/T246E substitutions in the CH2 domains. Additionally, as the nature of the biantennary glycans attached to N297 of Fc plays a major role in modulating binding to the FcγRs, but not FcRn, we mapped the glycans of the hIgG1 variants by LC-MS/MS analysis and found that the dominating forms were present in both the WT and the mutant fractions. Interestingly, a difference in fucosylation was found between the WT and mutant variants, even when mutations are introduced distally from N297. Such an example was also previously described, where a CH3 mutation (Y407E) was introduced into hIgG1 and hIgG4 that caused great alterations of the glycosylation profile of N297 in the C$_4$H2 domains (55).

To assess the binding properties of the produced anti-NIP hIgG1 variants toward hFcyRs, we screened the Abs in ELISA for binding
to GST-tagged hFcγRs. The variants were compared with the WT and showed distinct binding profiles. Whereas binding to the high-affinity FcγRI was only slightly affected, the mutations in all variants had a large impact on binding to the low-affinity receptors, with the MST and MST/HN variants being the most strongly affected (50–90% reduction), with a similar trend for all receptors. The most dramatic effect was detected for the MST variant with substitutions within the CH2 domain. Using SPR, we found that the MST and MST/HN variants bound with a 5-fold weaker affinity toward hFcγRIIa-H131. Interestingly, the inhibitory hFcγRIIb was not affected by the H433K/N434F mutations. Because the glycosylation profiles were similar for the mutant variants, the differences in binding are most likely due to the specific amino acid substitutions. The three mutations within the CH2 domains are in the same domain and in proximity to the binding site for the hFcγRs. Our data indicate that the mutations alter the local structure and/or dynamics of the CH2 domain, which again modulate binding to effector molecules.

Furthermore, when the MST and MST/HN variants were compared with hlgG1-WT in ADCP and ADCC assays, MST showed that both had reduced ability to induce phagocytosis and cytotoxicity. The MST/HN variant showed reduced ability to induce ADCC and ADCC by monocytes at low Ag density, whereas the ADCC inducing activity was enhanced using PBMC or NK cells at both low and high Ag density. The effect seen for MST/HN is probably due to the fact that the HN mutations had a positive effect on ADCC, although in vitro interaction analysis showed that it bound less well to the FcγRIIa allotypes compared with the WT. Furthermore, the HN variant performed overall better than did the WT and remaining variants at high and low Ag density for PBMCs, NK cells, and monocytes.

With regard to ADCC and the effect of the M252Y/S254T/T246E mutations, the result is in line with a previous study that shows this variant, with anti-RSV specificity (MEDI-522), to have reduced binding to hFcγRIIa (F156) and more than 2-fold reduction in ADCC. Interestingly, the ability to induce ADCC was restored by introduction of three additional mutations (S239D/A330L/I332E) (35). These mutations may also compensate for loss of ADCP activity.

The differences in ADCP activity between the hlgG1 variants were minor, except for the HN variant, which showed an enhanced ability to induce phagocytosis at low Ag density. One reason may be due to the fact that FcRn has been shown to take an active part in enhancing processing and elimination of IgG-coated bacteria in neutrophils upon engulfment via cell surface–expressed FcγRs.

FIGURE 7. Binding properties of hlgG1 Fc-engineered variants toward C1q and complement factors. ELISA results showing binding of WT hlgG1 and Fc-engineered variants to (A) pure hC1q, (B) C1q present in human serum, and (C) pure C1q present in C1q-depleted human serum and activation of complement factors (D) C3 and (E) C5. Experiments were performed at pH 7.4 and results are shown as mean ± SEM of one representative experiment out of three.
Furthermore, two recent studies suggest that phagocytosis is affected by the ratio of the hFc\(_{\text{gRIIa}}\) and hFc\(_{\text{gRIIIb}}\) affinities (57, 58). Because the HN and MN variants demonstrate stronger Fc\(_{\text{gRIIIb}}\) binding than do the MST and MST/HN variants, this points to these two having a favorable ratio for phagocytosis.

Classical complement activation was initially studied by measuring binding of the hIgG1 variants to pure hC1q and C1q present in human serum, which revealed that all variants bound with almost similar hierarchy to both hC1q sources. At both conditions, the hIgG1-HN variant showed greatly enhanced hC1q binding, whereas the other mutants bound less efficiently than did the WT. Increased hC1q binding to HN also resulted in higher deposition of C3 and C5, which translated into a 4-fold increased ADCML activity at low Ag density and, additionally, more efficient ADCP in the presence of serum as a complement source. Again, the MST variant was the least efficient. Interestingly, no activity was detected for any variants other than HN at lower Ag density. This finding demonstrates that ADCML activity may well be modulated by mutations in the CH3 domain, structurally distant from the core site for C1q in the hinge and upper part of the CH2 domains. This is in line with our findings that mutating two leucine residues (L234 and L235), which are at the core of the Fc\(_{\text{gRII}}\) site, strongly decreased C1q binding as well as C3 and C5 deposition (62).

Solving of a crystal structure of an hIgG1 Fc fragment containing the MST substitutions has revealed that the mutations do not induce large conformational changes, but instead mediate local alterations of the targeted amino acids such that they favor binding to hFcRn (63). This is confirmed by a recently published cocrystal structure of the Fc fragment in complex with hFcRn, where the T256E mutation creates two additional salt bridges with Gln2 of \(\beta_2\)-microglobulin (63). It now appears that the Fc region of IgG is far more flexible and dynamic than has previously been appreciated. A consequence of this is that changes introduced in one part of the Fc may well have a large impact on the binding properties to Fc effector molecules with distally located binding sites. The consequence of individual mutations is not readily predicted. Thus, as the crystal structure of the MST containing Fc fragment is very similar to that of WT Fc, it is hard to predict why these mutations have such a negative impact on binding of Fc\(_{\text{gRII}}\) and C1q, other than by inducing minor conformational changes or alterations in the dynamics within the C1q2 domains prior to or upon binding.

Regarding the effect of the MN and HN mutations, our data suggest that amino acid substitutions in the CH3 domains induce changes in the Fc that are manifest even at the C1q2 domains. A
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