Glycine 236 in the Lower Hinge Region of Human IgG1 Differentiates Fc γR from Complement Effector Function

Maximilian Brinkhaus, Ruben G. J. Douwes, Arthur E. H. Bentlage, A. Robin Temming, Steven W. de Taeye, Matthias Tammes Buirs, Jacoline Gerritsen, Juk Yee Mok, Giso Brasser, Peter C. Ligthart, Wim J. E. van Esch, Peter Verheesens, Hans de Haard, Theo Rispens and Gestur Vidarsson

J Immunol 2020; 205:3456-3467; Prepublished online 13 November 2020;
doi: 10.4049/jimmunol.2000961
http://www.jimmunol.org/content/205/12/3456

Supplementary Material
http://www.jimmunol.org/content/suppl/2020/11/12/jimmunol.2000961.DCSupplemental

References
This article cites 67 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/205/12/3456.full#ref-list-1

Why The JI? Submit online.
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2020 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Abs of the IgG isotype mediate effector functions like Ab-dependent cellular cytotoxicity and Ab-dependent cellular phagocytosis by Fc interactions with FcγRs and complement-dependent cytotoxicity upon IgG-Fc binding to C1q. In this study, we describe the crucial role of the highly conserved dual glycines at position 236–237 in the lower hinge region of human IgG, including the lack of one glycine as found in IgG2. We found several permutations in this region that either silence or largely abrogate FcγR binding and downstream FcγR effector functions, as demonstrated by surface plasmon resonance, Ab-dependent cellular phagocytosis, and Ab-dependent cellular cytotoxicity assays. Although the binding regions of FcγRs and C1q on the IgG-Fc largely overlap, IgG1 with a deletion of G236 only silences FcγR-mediated effector functions without affecting C1q-binding or activation. Several mutations resulted in only residual FcγRI binding with differing affinities that are either complement competent or silenced. Interestingly, we also found that IgG2, naturally only binding FcγRIIa, gains binding to FcγRI and FcγRIIa after insertion of G236, highlighting the crucial importance of G236 in IgG for FcγR interaction. These mutants may become invaluable tools for FcγR-related research as well as for therapeutic purposes in which only complement-mediated functions are required without the involvement of FcγR.

The Journal of Immunology, 2020, 205: 3456–3467.

Glycine 236 in the Lower Hinge Region of Human IgG1 Differentiates FcγR from Complement Effector Function

Maximilian Brinkhaus,* Ruben G. J. Douwe,* Arthur E. H. Bentlage,* A. Robin Temming,* Steven W. de Taeye,**§ Matthias Tammes Buirs,* Jacoline Gerritsen,* Juk Yee Mok,‡ Giso Brasser,‡ Peter C. Ligthart,§ Wim J. E. van Esch,‡ Peter Verheesense,¶ Hans de Haard,¶ Theo Rispens,‡ and Gestur Vidarsson*

Here are four different subclasses of IgG. The isotypes are highly homologous yet play distinct roles in the immune response (1–3). The lower hinge region, directly adjacent to the fragment crystallizable (Fc) region, has been found to be crucial for the interaction of IgG with both FcγRs and C1q. FcγRs mediate immune effector functions such as Ab-dependent cellular phagocytosis and Ab-dependent cellular cytotoxicity (ADCC).
does less so and requires higher concentrations and/or epitope densities but can also effectively activate complement if a target has repetitive epitopes such as found on bacterial polysaccharides (1, 17, 18).

Human IgG is also recognized by the neonatal Fc receptor (FcRn), responsible for salvaging of IgG from lysosomes and therefore for the long half-life of IgG. The same receptor system is also responsible for transcytosis and transplacental transport of IgG from mother to fetus (19–21). We recently reported that this process is negatively affected for IgG2 in comparison with IgG1 by the lack of G236 in IgG2, which may explain the relatively low placental transport rates of IgG2 compared with IgG1. This low transport efficiency could be transferred to IgG1 by deletion of G236 (IgG1ΔG236). Vice versa, insertion of G236 into IgG2 (IgG2+G236) increased placental transport to levels of IgG2 (22, 23). As glycine is devoid of side chains, making it extremely flexible in proteins, we hypothesized that the presence of G236 is important for increased Fab flexibility, which affects transport (22).

G236 is located in the lower hinge region of IgG, which is a region generally important for FcγR and C1q binding. The impact of mutations encompassing the deletion of G236 of IgG1 and the insertion of G236 to IgG2 have been partly studied regarding FcγRI and FcγRIIa binding (17, 24–28). The G236A substitution in IgG1 has been reported to increase binding to FcγRIIa and FcγRIIa-mediated phagocytosis (29). Variants of IgG1 expressing alanine at the adjacent position 237, normally a glycine in all IgG subclasses (G237A), has been reported to have reduced FcγRI affinity and reduced FcγRIIa-mediated ADCC as well as CDC (17). However, these publications only partially distinguished between different FcγR polymorphisms and did not, except for Morgan et al. (17), investigate the impact on complement activation.

Based on our findings of this region on FcRn-mediated transport (22, 23), we revisited these questions and functionally characterized the influence of substitutions and deletion at positions 236 and 237 in IgG1 and IgG2, this time for all FcγRs and their polymorphic variants as well as complement. Thereby, we want to dissect the role of the glycines in the lower hinge on the various Fc-dependent effector functions and identify new Fc-engineered variants with a specific effector function profile.

Materials and Methods

Ethics

Peripheral blood from anonymous, healthy volunteers was obtained with informed, written consent of all subjects in accordance with Dutch regulations. This study was approved by Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki.

Cells

Human embryonic kidney (HEK) 293F cells (Thermo Fisher Scientific) were cultured in FreeStyle 293 Expression Medium (Thermo Fisher Scientific) at 37°C at 5% CO2 and shaking at 125 rpm. RABOS cells were obtained from American Type Culture Collection (ATCC; ATCC CRL-1596) and grown in RPMI 1640 with 10% FCS at 37°C at 5% CO2.

Raji cells were obtained from ATCC (ATCC CCL-86) and grown in RPMI 1640 with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific) at 37°C at 5% CO2.

NK cell isolation was performed as described elsewhere (13). In short, freshly drawn heparinized blood was diluted 1:1 with 1× PBS containing 10% trisodium citrate (TSC) (v/v). Twenty-five milliliters of diluted blood were layered on 15 ml of Ficoll-Paque PLUS (GE Healthcare). Fractions were separated at 400 × g for 30 min at RT without break. Ring fractions of the same donor were combined and washed twice with 1× PBS containing 10% TSC. After counting cells, PBMCs were resuspended in 10% TSC, and the cell concentration was adjusted to 1× PBS containing 1% TSC and 0.5% human serum albumin (Celab; Sanquin) (MACS buffer) and 1 μl of CD56, MACS beads (Miltenyi Biotec) per 1 × 107 cells. After 15 min on ice, cells were washed twice in MACS buffer, applied to an MS Column (Miltenyi Biotec), washed three times, and eluted. Cells were resuspended in IMDM (Life Technologies) containing 10% FCS and incubated at 37°C at 5% CO2.

Polymeronuclear neutrophils (PMNs) were isolated from freshly drawn heparinized blood. Blood was diluted 1:1 with 1× PBS, and 25 ml of diluted blood was layered on 15 ml of Ficoll-Paque PLUS (GE Healthcare). Fractions were separated at 400 × g for 30 min at RT without break. After discarding the top layers, the RBC pellet containing the PMNs was lysed with hypotonic lysis buffer (30) on ice for 15 min. Cells were spun down at 290 × g, the supernatant was carefully discarded, and the pellet was resuspended in 10 ml of lysis buffer. After 5-min incubation on ice, cells were washed with 1× PBS, resuspended in IMDM (Life Technologies) with 10% (v/v) FCS.

To increase FcγRI expression, PMNs were stimulated overnight with 5 μg/ml of FcγRIIa (Sigma-Aldrich) and 10 ng/ml G-CSF (Sigma-Aldrich) at a cell concentration of 5 × 105 cells/ml in IMDM (Life Technologies) with 10% (v/v) FCS. To determine FcγRI expression, PMNs were stained with anti-CD64 biotin (Becton Dickinson) in 1× PBS with 0.1% (m/v) BSA for 30 min on ice, followed by 15 min with streptavidin–allophycocyanin (BioLegend) after washing twice under the same conditions.

Purified RBCs were kindly provided by the Department of Erythrocyte Serology, Sanquin. For RBC biotinylation, 1.4 mg EZ-Link NHS-PEG12-Biotin (Thermo Fisher Scientific) was used to biotinylate 11 × 107 RBCs in a total volume of 250 μl for 15 min. Reaction was quenched by washing cells twice with IMDM (Life Technologies) containing 10% (v/v) FCS.

RBCs were labeled using a PKH26 Red Fluorescent Cell Linker Kit (Thermo Fisher Scientific) following manufacturer’s instructions.

Cloning of Fc variants in anti-biotin and anti-CD20 H chain vectors

Linear DNA strings encoding for different Fc variants, such as IGHG1*03 or IGHG2*01 allotypes (Fig. 1C), were ordered from Integrated DNA Technologies. DNA strings and pcdNA3.1 expression vectors containing the coding sequences for anti-biotin and anti-CD20 H chain variable regions previously generated from (31–33), respectively, were digested with HindIII and NheI FastDigest restriction enzymes (Thermo Fisher Scientific) and 1× FastDigest Green Buffer (Thermo Fisher Scientific) at 37°C for 10 min. The digested H chain vectors were separated on a 1% UltraPure Agarose gel (Thermo Fisher Scientific) with 1:10,000 SYBR Safe (Invitrogen) at 100 V in 1× Tris-acetate-EDTA for 50 min, and the DNA was subsequently purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) according to manufacturer’s instructions. Digested DNA strings were purified directly after digestion using the same kit. DNA concentrations were determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

Ligations were done using a 6× M excess of the insert over the vector with a total amount of 100 μg of DNA in the presence of 400 U of T4 DNA Ligase (New England Biolabs) and 1× T4 DNA Ligase Reaction Buffer (New England Biolabs) in a total volume of 20 μl for 10 min at RT.

Five microliters of the ligation reaction was subsequently added to 50 μl of competent DH5α bacteria (Thermo Fisher Scientific) and incubated on ice for 15 min. Heat shock was performed for 50 s at 42°C followed by 2 min on ice. Cells were plated on Luria–Bertani (LB) agar plates containing 50 μg/ml ampicillin (Thermo Fisher Scientific) and grown overnight at 37°C. Single colonies were picked and grown in 2 ml LB-medium containing 50 μg/ml ampicillin (Thermo Fisher Scientific). Precultures were inoculated to 200 ml LB-medium containing 50 μg/ml ampicillin (Thermo Fisher Scientific) and grown overnight. Plasmids were isolated using a NucleoBond Xtra Maxi Kit (Macherey-Nagel) according to manufacturer’s instructions.

Production of mAbs

HEK 293F cells (Thermo Fisher Scientific) were split 1 d prior to transfection. At the day of transfection, the cell concentration was adjusted to 1 × 107 cells/ml transfection volume in fresh FreeStyle 293 Expression Medium (Thermo Fisher Scientific). The DNA mix contained 31.0, 31.35, and 37.65 μg of pSVLT/p21/p27-mix (34), H chain vector DNA, and L chain vector DNA per 100 ml transfection, respectively. The DNA mix was added to Opti-MEM (Thermo Fisher Scientific) containing 45 μg/ml linear polyethyleneimine HCI MAX (Polysciences), briefly vortexed, and incubated for 10 min at RT. The transfection mixture was added to HEK 293F cultures and incubated at 37°C and 8% CO2, with shaking at 125 rpm. Four hours posttransfection, the cells were washed once with 1× TSC and 0.5% BSA containing 100 μg/ml streptomycin (Thermo Fisher Scientific) and changed to fresh medium. After 6 d, the cells were spun down twice at 3100 × g, and supernatants were collected and filtered with a 0.45-μm syringe filter (Whatman).
Purification of mAbs

Abs were purified from culture supernatants employing an AKTA Prime Plus (GE Healthcare) with a 5-mL Protein G column (GE Healthcare). The ion-exchange gradient elution protocol was used with a flow speed of 5 mL/min, applying the low pH elution buffer (0.08 M citric acid/0.04 M Na2HPO4 (pH 3) for 1 h) to neutralize the 1 mL fractions containing the eluted Abs. Fractions were combined and concentrated using 10 kDa m.w. cutoff Protein Concentrator columns (Thermo Fisher Scientific). Subsequently, the Abs were fractionated using high-performance liquid chromatography–size exclusion chromatography using an AKTA UPC-900, P-9290, and Frac-9 (GE Healthcare) with a Superdex 200 10/500 GL column (GE Healthcare). Fractions of 0.5 mL were collected at a flow speed of 0.5 mL/min. Most of the fractions of each Fc variant of the anti-biotin and anti-CD20 Abs were combined and dialyzed overnight at 4°C to sodium acetate buffer (5 mM pH 4.5) or Ritusinib buffer (35), respectively. On the next day, the Abs were adjusted to a concentration of 1 mg/mL, aliquoted to working stocks of 20 μL, and subsequently stored at −20°C. Once thawed, each aliquot was kept at 4°C and used for a maximum of 1 wk.

SDS-PAGE

Each Ab was tested in SDS-PAGE under reducing and nonreducing conditions. Samples were incubated for 5 min at 70 or 95°C in the presence of 20 mM iodoacetamide (to prevent minor reduction during denaturation) (36) or 0.25% (v/v) 2-ME in NuPage LDS Sample Buffer (Thermo Fisher Scientific) for reduced and nonreduced conditions, respectively. Samples were loaded on a 7.5% Criterion XT Bis-Tris protein precast gel (Bio-Rad Laboratories) and run with MOPS-Tris running buffer (Thermo Fisher Scientific) for 10 min at 150 V followed by 45 min at 120 V. The gel was stained overnight in Blue-Silver solution (10% phosphoric acid, 10% ammonium sulfite, 0.12% Coomassie Blue G-250, Sigma-Aldrich) and 20% methanol (Thermo Fisher Scientific) and thoroughly destained in distilled water.

Opsonization of CD20+ cells

Raji cells were spotted with a 3-fold dilution series of anti-CD20 Ab variants starting at a concentration of 10 μg/mL in RPMI 1640 (Life Technologies) containing 10% (v/v) FCS for 15 min at RT in a 96-well round-bottom plate (Corning Costar). Cells were washed twice with 200 μL of ice-cold 1× PBS containing 0.1% (v/v) BSA (Sigma-Aldrich) and stained with 1/100 mouse anti-human IgG FITC (SouthernBiotech) for 30 min on ice. Cells were again washed twice with 200 μL of ice-cold 1× PBS containing 0.1% (v/v) BSA (Sigma-Aldrich) and measured by FACS.

Surface plasmon resonance affinity measurements to human FcγRs and binding studies to Clq

Surface plasmon resonance (SPR) measurements were performed on an IBIS MX96 (IBIS Technologies) device as described previously (37). All C-terminally biotinylated human (h) FcγRs were spotted using a Continuous Flow Microspotter (Wasatch Microfluidics) onto a single SensEye G streptavidin sensor (SensEye Technologies) in a SensEye G-streptavidin sensor in 3× 1000 nM in 1× PBS containing 0.1% (v/v) BSA (Sigma-Aldrich) and measured by FACS.

Results

Fcγ36 variants have normal integrity and Ag recognition

To investigate the importance of the two glycine residues present at positions 236 and 237, found in human, macaque, and mouse IgG same was done for the biotinylated BSA spots, where the association and dissociation curves of different anti-biotin Abs were subtracted. Calculation of the dissociation constant (Kd) was performed by equi-librium fitting to Rmax = 500, as described earlier (37). Analysis and calculation of all binding data were carried out using Scrubber software version 2 (BioLogic Software) and Excel.

Complement ELISAs

Nunc MaxiSorb flat-bottom 96-well plates (Thermo Fisher Scientific) were coated overnight at 4°C with 10 μg/mL of 10 μg/mL biotin-BSA (Life Technologies) containing 1% (v/v) BSA (Sigma-Aldrich) and 10 μM CaCl2 (Sigma-Aldrich) for 1 h, shaken at 400 rpm at RT. After washing, 100 μL of 1:35 pooled human serum (giving optimal Clq readout in serial dilutions) in 1× Veronal Buffer (12) with 0.1% poloxamer 407, 2 mM MgCl2 (Sigma-Aldrich) and 10 μM CaCl2 (Sigma-Aldrich) was added and incubated for 1 h, shaking at 400 rpm at RT. Plates were washed, and 100 μL of HRP-ylated anti-Clq-2 (38), anti–C3-19 (39), or anti–C4-10 (40) Abs were added and incubated for 1 h, shaking at 400 rpm at RT. After washing, ELISAs were developed with 3,3′,5,5′-tetramethylbenzidine, reactions stopped with 100 μL of 2 M H2SO4, and read out in Biotek Synergy 2 (Biotek Instruments) at 450 nM.

Complement-dependent cytotoxicity

A total of 1.1 × 107 RAMOS cells per well were psonilated with 3-fold dilution series of anti-CD20 Ab variants starting at a concentration of 10 μM in RPMI 1640 (Life Technologies) containing 10% (v/v) FCS for 15 min at RT in a total volume of 80 μL in a 96-well round-bottom plate (Corning Costar). Subsequently, 20 μL of 20% (v/v) pooled serum was added, and the reaction was incubated for 45 min at 37°C at 5% CO2. After stopping the reaction on ice for 2 min, cells were transferred to a 96-well V-bottom plate (Corning Costar) and washed twice with 200 μL of ice-cold 1× PBS containing 0.1% (v/v) BSA (Sigma-Aldrich). Cells were stained with LIVE/DEAD fixable Near-IR Dead Cell Stain (Thermo Fisher Scientific) according to manufacturer’s instructions, washed three times with 200 μL of ice-cold 1× PBS containing 0.1% (v/v) BSA (Sigma-Aldrich), and measured by FACS.

Phagocytosis

For phagocytosis, 1 × 106 biotinylated and PKH26-labeled Abs were opsonized with a 3-fold dilution series of anti-biotin Ab variants starting at a concentration of 3 μg/mL in HEPES buffer in a 96-well V-bottom plate (Corning Costar) for 30 min at RT; shaking at 400 rpm. Afterward, cells were washed with 1% (v/v) FCS containing 0.1% (v/v) BSA (Sigma-Aldrich) and measured by FACS.

ADCC

A total of 1 × 106 biotinylated RBCs were washed twice with 1× PBS, supernatant was discarded, and cells were resuspended in 100 μL of 3μCr (PerkinElmer) for 45 min at 37°C at 5% CO2. Subsequently, cells were washed twice with 1× PBS and resuspended in IMDM (Life Technologies) containing 10% (v/v) FCS. C1r/C1s-labeled RBCs were resuspended in a prepared anti-biotin Ab 5-fold dilution series starting at a concentration of 0.5 μg/mL (PMNs) or 5 μg/mL (NK cells) in IMDM (Life Technologies) containing 10% (v/v) FCS. For the blocking assays, a 3-fold dilution series of IgG1-Fc wild-type (WT) (evitria) in the same medium was added together with the anti-biotin Abs, starting at a concentration of 167 μg/mL NK cells or control-antistimulated neutrophils in IMDM (Life Technologies) with 10% (v/v) FCS were added to opsonized RBCs, reaching a final reaction volume of 100 μL and an E:T ratio of 1:1. The plate was incubated for 45 min at 37°C, shaking at 175 rpm. Cells were washed once with 1× PBS and measured by FACS. The median fluorescent intensity values for PKH26 were determined for the granulocyte population.
(Fig. 1A), except human IgG2 and mouse IgG1, we generated a set of IgG1 Abs with alanine replacements, deletion of one of the glycine residues (ΔG236), IgG2-WT, and two glycine residues (IgG2+G236) (Fig. 1C). This included the IgG1-G236/7A and the double substitution mutant (GAGA). As a negative control, we combined the well-described Fc-silencing LALA-PG mutations with the N297A substitution (41–43) (Fig. 1B).

After cloning, production, and purification, both anti-CD20 and anti-biotin sets of Abs were tested with high-performance liquid chromatography–size exclusion chromatography, and monomeric fractions were collected. All samples revealed the expected bands in SDS-PAGE under reducing and nonreducing conditions as shown in Supplemental Fig. 1A and 1B. Anti-biotin Abs were confirmed to be functional, as demonstrated by affinity measurements to biotin–BSA in SPR (Supplemental Fig. 2A). Anti-CD20 Abs were confirmed to recognize CD20 on Raji cells by FACS. In line with earlier published data, Ab Fc variants within one subclass were found to bind similarly, whereas IgG2 opsonization levels were generally lower (Supplemental Fig. 2B) (44). With this data, taken together with the analytical data, we concluded that all

| Subclass | Species                     | GenBank Code | Position |
|----------|-----------------------------|--------------|----------|
|          |                             | 233 234 235 236 237 238 239 240 |
| IgG1     | homo sapiens                | AXN93653.1   | E L L G G P S V |
| IgG2     | homo sapiens                | AAN76044.1   | P V A - G P S V |
| IgG3     | homo sapiens                | QEV83695.1   | E L L G G P S V |
| IgG4     | homo sapiens                | CAC20457.1   | E F L G G P S V |
| IgG1     | macaca fascicularis         | AGC00820.1   | E L L G G P S V |
| IgG2     | macaca fascicularis         | AGC00821.1   | E L L G G P S V |
| IgG3     | macaca fascicularis         | AGC00822.1   | E L L G G P S V |
| IgG4     | macaca fascicularis         | AGC00823.1   | E L L G G P S V |
| IgG1     | mus musculus                | CAD32497.1   | E - - - V S S V |
| IgG2a    | mus musculus                | AGH20709.1   | N L L G G P S V |
| IgG2b    | mus musculus                | ABQ85916.1   | N L E G G P S V |
| IgG3     | mus musculus                | ADK11691.1   | N I L G G P S V |

FIGURE 1. G236-7 are located in the contact region of IgG1-Fc and FcyRIIIa. (A) Multiple sequence alignment of IgG sequences from human, cynomolgus monkey, and mouse using European Molecular Biology Laboratory - European Bioinformatics Institute Clustal Omega alignment tool (66). (B) Crystal structure of human Fc/FcyRIIIa complex (Protein Data Bank [PDB]: 3SGJ), depicted as surface (left) and zoomed in on the marked region shown as ribbon illustration (right). FcyRIIIa is highlighted in orange. The two half-molecules of the IgG1-Fc are indicated in dark and light gray for chain A and B, respectively. G236 and G237 are highlighted in green in both half-molecules, and P227 is highlighted in blue, indicating the upstream hinge region. Note that the Fc part binds in top-down orientation, requiring flexibility of the Fab arms (data not shown) in a membrane context, which follow further upstream of P227. Substitutions known to abrogate FcyR binding (LALA-N297A-PG) are highlighted in red. The image was rendered using structural data from Ferrara et al. (67) (PDB: 3SGJ) using UCSF Chimera (68). (C) Sequence alignment of human IgG1 and IgG2 variants.
Abs retained their functionality regarding their Ag recognition as well as overall structural integrity.

**FcγR affinity measurements in SPR reveal the importance of G236 for FcγRI binding in human IgG1 and IgG2**

To determine how the amino acid substitutions influence the affinities of the mutant Abs to FcγRs, we employed our IBIS SPR streptavidin array equipped with all FcγR with a C-terminal site-specific biotin tag to guarantee the right orientation. Abs were titrated as analytes, and the *Kₐ* values were calculated on the basis of equilibrium analysis (Fig. 2A). WT-IgG1 and -IgG2, as well as the FcγR-silenced IgG1-LALA-N297A-PG, bound to FcγRs as expected (10, 12, 42, 43). IgG1 exhibited strongest binding to FcγRI, followed by FcγRIIa 158V. IgG2, in contrast, only showed binding to FcγRIIa, with a stronger binding seen for the 131H allotypic variant, as expected (10). The IgG1-LALA-N297A-PG mutant showed no binding to FcγRs (Fig. 2A).

G236A and G237A substitutions in IgG1 did not significantly influence the affinities to FcγRI, but their combination (IgG1-GAGA) showed a significantly lower affinity to FcγRI. IgG1-G236A showed increased binding to both variants of FcγRIIa, which is in line with earlier published data (29). IgG1-G237A and IgG1-GAGA demonstrated no binding to other FcγRs, except for a weak binding to FcγRIIa 131R that was unquantifiable (Fig. 2A). In contrast, deletion of G236 from IgG1 abrogated binding to all FcγRs (Fig. 2), with only a very weak unquantifiable residual binding to FcγRI (Fig. 2A). Consistently, we found the reverse; introducing G236 to IgG2 enabled IgG2 binding to FcγRI (Fig. 2A, 2B). This suggests a direct involvement of G236 in FcγRI interaction, an influence on hinge flexibility, and/or steric hindrance of G236 or adjacent amino acids to be important. Similarly, but to a lesser degree, this mutant also acquired a very weak but detectable binding to FcγRIIa 158V (Fig. 2A). At the same time, IgG2 with G236 added lost binding capacity to FcγRIIa, highlighting the unique relationship of IgG2 with FcγR.

ΔG236, G237A, and GAGA in IgG1 abolish phagocytosis by PMN

Next, we investigated the impact of 236/7 variants on ADCC and phagocytosis, using cell types with different FcγR expression profiles to demonstrate the individual effects on FcγRI-, II-, and III-mediated effector functions.

First, we investigated the impact of 236/7 variants on phagocytosis by PMN. Freshly isolated (unstimulated) PMNs have been described to have high expression of FcγRIIa, whereas FcγRI is generally only expressed after activation [e.g., stimulation with IFN-γ and G-CSF (45, 46)]. Unstimulated PMNs are known to mediate phagocytosis (FcγRIIa and FcγRIIib dependent) (45, 47, 48) and ADCC (FcγRIIa dependent and downregulated by FcγRIIib) (45, 46, 49). No FcγRI expression was found on unstimulated neutrophils, as expected (Fig. 3B). We assessed phagocytosis by unstimulated FcγRIIa 131H/II-H-typed PMNs using biotinylated-, PKH26-labeled RBCs, opsonized with anti-biotin IgG variants. The PKH26 signal of the PMN population was measured by FACS after lysis of extracellular RBC (30) (Fig. 3A, 3C).

The phagocytosis generally followed the affinity trends for FcγRIIa 131H (IgG1-G236A ≥ IgG1-WT = IgG2-WT) (Fig. 3C). Also in line with the observed affinity changes, the deletion of G236 from IgG1, as well as the substitutions G237A and GAGA, strongly reduced phagocytosis in our assay (*p < 0.001*) to a level comparable with IgG1-LALA-N297A-PG (*p < 0.0001*). IgG2+G236 showed also a strong reduction in phagocytic capacity but did mediate phagocytosis at a higher concentration, probably because of residual binding to FcγRIIa 131H, inducing increased avidity (13, 50) (Fig. 2A). The concentration-dependent shift of the PKH26 signal of the PMN population suggests that next to phagocytosis of whole PKH26-labeled RBCs trogocytosis also occurred (Fig. 3A, Supplemental Fig. 3) (45, 51).

IgG1ΔG236 mediates ADCC via FcγRI only at high concentrations, which is abrogated by irrelevant IgG1-Fc

To investigate the role of FcγRI in PMN effector functions, we stimulated PMNs with IFN-γ and G-CSF, resulting in FcγRI up-regulation (45, 46, 52) (Fig. 3B), as our group recently published that both FcγRI and FcγRIIa are crucial for ADCC by stimulated PMNs against opsonized RBCs (A.R. Temming, M.T. Buirs, A.E.H. Bentlage, L.W. Treffers, H. Feringa, S.W. de Taeye, T.W. Kuipers, S.Q. Nagelkerke, G. Brasser, J.Y. Mok, W.J.E. van Esch, T.K. van den Berg, T. Rispen, C.E. van der Schoot, and G. Vidarsson, submitted for publication).

Of the three IgG1-G237A, IgG1-GAGA, and IgG1ΔG236, incapable of mediating phagocytosis, the first two showed highly affinities to FcγRI (Fig. 2). In line with these, this high-affinity variants showed similar PMN-mediated ADCC against IgG-opsonized RBC after FcγRI induction. This was only possible for IgG1ΔG236 at high concentrations (Fig. 3D), likely because of the residual affinity to FcγRI.

We then asked whether the residual activity by IgG1ΔG236 is important under slightly more physiological conditions, as irrelevant IgG is present at very high concentration in blood (>10 mg/ml) (53, 54). We therefore repeated this experiment with a constant concentration of the IgG variants and increasing amounts of IgG-Fc. Under those conditions the activity of IgG1-WT was constant, but that of IgG1ΔG236 was almost fully abrogated (Fig. 3E).

Increased FcγRIIa binding of IgG2+G236 translates to CD56+ NK cell ADCC

To investigate the effects of the mutations on FcγRIIa downstream effector functions, we used NK cells from FcγRIIa 158V-typed donors and biotinylated RBC as targets. In line with the abrogated affinity of all of the variants to FcγRIIa, only IgG1-G236A and IgG2+G236 with strong and residual binding to FcγRIIa, respectively (Fig. 2A), demonstrated NK cell-mediated ADCC, with IgG2+G236 giving severely weakened effector functions. All other mutants were essentially devoid of FcγRIIa-mediated effector functions (Fig. 3F).

CLq binding to IgG is differentially affected by G236-7

We also investigated the impact on the interaction with CLq and subsequent complement activation. For this purpose we set up an SPR measurement to determine CLq binding.

The sensorgrams of CLq binding to anti-biotin Abs bound to biotinylated BSA are shown in Fig. 4A. To quantify differences in avidity, a 1:1 binding model was fitted, acknowledging that this does not reflect the underlying complexities of the multivalent binding process. The corresponding *Kₐ* values are plotted in Fig. 4B. The G236A substitution did not have a significant impact on CLq binding, whereas substituting the adjacent G237A resulted in significantly (*p < 0.05*) lower binding. However, the double GAGA mutations in IgG1 reduced CLq binding strongly (Fig. 4A, 4B). In contrast, no significant changes in affinity for IgG to CLq were observed after either removing G236 from IgG1 or adding it to IgG2 (Fig. 4B). Next, we investigated CLq downstream complement deposition in ELISA-based assays, mimicking the classical complement pathway (7, 14, 55). C4b and C3b deposition were measured after addition of pooled serum to anti-biotin Abs.
deposited in biotin–BSA–coated ELISA plates, in a similar manner as described earlier (12). The slight affinity changes measured by SPR resulted in corresponding reduction in binding of C1q to IgG by the ELISA, which were more pronounced than seen by SPR (Fig. 4B, 4C). IgG2-WT and IgG2+G236’s ability to mediate complement activation in these assays strictly depended on the Ag density (Supplemental Fig. 4). No C1q binding was seen by IgG1-GAGA (Fig. 4C), nor was it able to trigger C3b and C4b deposition (Fig. 4D, 4E). Interestingly, IgG2+G236 seemed to give elevated complement activity by ELISA as compared with WT IgG2 (Fig. 4C–E).

CDC is unchanged in IgG1-DG236 but is abolished by IgG1-GAGA

We then tested the Ab variants for their capacity to mediate CDC. For this we turned to anti-CD20 (rituximab) and CD20+ RAMOS tumor cells. Cells were preopsonized with anti-CD20 Abs and incubated with pooled human serum. IgG1-WT showed

![Figure 2](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org)
FIGURE 3. G236-7 severely affect FcγR-mediated neutrophil phagocytosis and ADCC. (A) FACS plots showing gating on PMN population (left) and decreasing PKH26 signal of PMN population with increasing IgG1-WT concentration (middle), thereby demonstrating phagocytosis of PKH26-labeled RBCs in a concentration-dependent manner. PKH26 signal of PMN populations at concentrations of IgG1-WT of 0.00 μg/ml (dark green) and 3.00 μg/ml (red), as well as the population of PKH26-labeled RBCs without effector cells (violet, right). (B) Example of FcγRI expression of PMNs used for experiments shown in (C)–(E). (C) Normalized results of three independent phagocytosis experiments using freshly isolated, nonstimulated PMNs from FcγRIIa 131H–typed donors (FcγRIIa 131H–mediated phagocytosis). Values were normalized to IgG1-WT. (D) ADCC of biotinylated 51Cr-labeled RBCs by G-CSF/IFN-γ overnight-stimulated PMNs. Efficiency of killing was calculated as percentage of saponin-lysed RBCs, subtracting the spontaneous lysis controls. (E) Blockage of IgG1ΔG236 induced FcγRI-dependent ADCC by IgG1-Fc-WT (mimicking irrelevant IgG1) in comparison with IgG1-WT and IgG1-LALA-N297A-PG. (F) FcγRIIa 158V–dependent ADCC of biotinylated 51Cr-labeled RBCs by CD56+ NK cells from FcγRIIa 158V–typed donors. Data shown are a representative of three independent experiments, unless indicated otherwise. All legends have been ranked according to the response at the highest concentration measured. In (C), the response using IgG1-G237A and IgG1-GAGA overlap completely. IgG1ΔG236 and IgG1-LALA-N297A-PG overlap partially. In (D), the lines of IgG1-G236A, IgG1-G237A, IgG1-GAGA, as well as IgG2-WT and IgG2+G236 largely overlap because of very similar responses. In (E), the lines of IgG1-Fc-WT only and IgG1-LALA-N297A-PG largely overlap without ADCC. Statistical analysis was performed using one-way ANOVA and significant differences are indicated with asterisks. ***p < 0.001, ****p < 0.0001.
concentration-dependent cell lysis, leading to 100% of dead cells for the highest concentration and having a background of below 20% of dead cells without anti-CD20 Abs (Fig. 5). In contrast to what was expected from the ELISA experiments (Fig. 4C–E), IgG2-WT and IgG2+G236 did not exhibit complement-mediated killing in the cellular CDC assay (Fig. 5B), likely because of differences in epitope density and described subclass-specific differences, leading to lower opsonization levels when deposited on CD20+ cells (Supplemental Fig. 2B) (44). The results for the mutated IgG1 variants were in line with C1q binding and results on complement deposition (Fig. 4): We found no significant differences between IgG1-WT and IgG1-G236A or IgG1-G237A and IgG1ΔG236 (Fig. 5B). IgG1-GAGA showed significantly reduced ($p < 0.0001$) capability to mediate cell lysis (Fig. 5B). Only at the highest concentration tested did the IgG1-GAGA show a tendency for nonsignificantly elevated CDC ($p = 0.6727$) compared with

**FIGURE 4.** GAGA mutations in IgG1 abrogate C1q binding and complement deposition. (A) Ab variants were coupled to the sensor at different concentrations. C1q was titrated up to a concentration of 500 nM with an association and a dissociation phase of 300 and 240 s, respectively. Sensorgrams are shown of a representative SPR measurement of three independent experiments of all Ab variants to C1q. (B) Histograms showing $K_D$ values of all Ab variants binding to C1q. For quantification of the differences in avidity, a 1:1 binding model was fitted, acknowledging that this does not represent the underlying complexities of the multivalent binding process. (C) C1q binding. (D) C4b deposition, and (E) C3b deposition measured by ELISA as percentage of IgG1-WT. Statistical analysis was performed using ordinary one-way ANOVA. If not indicated differently, normalized results of three independent experiments were pooled. Legends shown in (C)–(E) have been ranked according to the response at the highest concentration measured. The lines of IgG1-GAGA and IgG1-LALA-N297A-PG overlap without response. In (C), IgG1-WT and IgG1-G236A also largely overlap over the whole concentration range. Significant differences are indicated with asterisks. *$p < 0.05$, ****$p < 0.0001$. 

---

The Journal of Immunology 3463

by guest on July 15, 2021http://www.jimmunol.org/Downloaded from
IgG1-LALA N297A PG (Fig. 5B), likely explained by residual interaction observed with C1q by SPR (Fig. 4A, 4B).

The relative performance of the Fc variants in comparison with WT-IgG in SPR affinity measurements and cell-based functional assays are summarized in Fig. 6, covering both FcγR and complement-related results.

Discussion

FcyRs and C1q bind the lower hinge and upper Fc-CH2 region of IgGs. Mutations within this region may influence the interaction of IgGs with FcγRs and/or C1q and thereby also impact their downstream effector functions. Next to understanding the structural and functional importance of single amino acids, function-driven Fc engineering has become a field of interest in the context of Ab-based therapeutics (5, 56–59).

We recently found that the deletion of G236 from IgG1 (normally absent in germline IgG2) decreased FcRn-dependent placental transport in our model to levels of IgG2, whereas the addition of G236 to IgG2 increased transport to levels of IgG1 (22). We reasoned that G236, given its particular location in the lower hinge region, which is distant from the FcRn-binding site, is an important determinant for Fab flexibility, which may be required for efficient FcRn-mediated transport in the context of vacuolar and, perhaps, tubular transport organelles (22, 60–62). This is supported by findings from Piche-Nicholas et al. (63), providing evidence that charge distribution in the V region has an impact on FcRn-dependent recycling caused by close proximity and potential interaction of Fabs and membrane.

As this region is very close to critical FcγR and C1q binding regions, we revisited earlier published data and systematically characterized mutations in position 236 and 237 in the lower hinge region of IgG1 and IgG2. This region has been subjected to mutational analysis in the context of FcγR and/or C1q binding mainly between the 1990s and the early 2000s (16, 17, 24–28).

The binding of C1q to IgG1 has been described to be mainly dependent on D270, K 322, P329, and P331 (15, 16). Also, the mutation to alanine of the structurally close G237 has been found to reduce complement activation significantly (17). Substitution of L235 to alanine in IgG1 was reported to substantially reduce C1q binding (17). This suggests A235, which is present in germline IgG2, to be responsible for the comparably low C1q binding of IgG2 (1). Our results add to the knowledge about the IgG:C1q interaction by highlighting the structural importance of the combination of G236 and G237 for C1q binding and downstream complement activation, as neither of the single mutations resulted in a significant reduction; only IgG1-GAGA abrogated complement effector functions. Morgan et al. (17) reported that antiHLA-DR-IgG1-G237A exhibits reduced CDC activity, which we find to be only slightly reduced. A reason for this could be differing Ag densities and the nature of the epitopes used in the different systems.

IgG2-WT and IgG2+G236 did not exhibit complement-mediated killing in the CDC assay but were able to induce C3b and C4b depositions for ELISA. This is presumably because of a relatively higher Ag density of our hapten than generally found by CD20 on RAMOS cells as well as lower opsonization levels of anti-CD20.
Abs on CD20+ cells when formatted to IgG2, confirming earlier published data (44). Curiously, apparently the subclasses diverge qualitatively and quantitatively in this respect, as IgG1-GAGA showed no binding to C1q by ELISAs, whereas residual binding was observed by SPR.

Several groups have investigated the influence of mutations in the lower hinge region on FcyR binding (24, 26–28). IgG1ΔG236 and IgG2+G236 were previously found not to bind FcγRI (24). Combinations of mutations, among others encompassing ΔG236 in IgG1, were investigated for their impact on FcγRIIα and FcγRIIB interactions (27, 28). Our results suggest the crucial importance of G236 for FcγR binding of IgG1 and partially contradict earlier published data. IgG1-G236A has been reported to have increased binding to FcγRIIα (29), which we also found in our SPR binding study. The addition of G236 to IgG2 substantially increased FcγRI and FcγRIIα binding, and it increased FcγR-mediated effector functions accordingly, underlining the importance of G236 for FcγR binding in IgG1 and IgG2. This highlights the evolutionary restrictions of IgG2’s interaction with FcγRs in the natural absence of G236, as IgG2 generally only interacts well with FcγRIIα. This interaction is largely lost by insertion of G236. Interestingly, IgG2+G236 also seemed to give elevated complement activity by ELISAs. One could speculate that, next to other acid mutant described with full CDC but abolished FcγR-functions. Lee et al. (64) published a mutant bearing 2 aa substitutions with the same properties for which they confirmed major conformational changes in the Fc region. As G236 is present in the structural hinge of IgG1 and absent in germline IgG2, we assume its deletion is neither likely to have large impacts on the structure nor strongly promote immunogenicity. It could potentially negatively influence Fab flexibility, however, as IgG2-WT with its shorter hinge has been shown to exhibit a smaller angle between Fabs and Fc region in comparison with IgG1-WT (2). Additionally, the deletion of G236 might influence the steric context of the adjacent amino acids. Mutants, being only capable of mediating complement but not FcγR effector function, are interesting in the context of patients carrying low-FcγR-affinity polymorphisms or in diseases in which FcγR-mediated effector functions are undesired in comparison with complement-mediated clearance (64, 65). Enhanced FcγRIII binding by fucosylated Abs has recently been linked to severe disease progression in Ebola and SARS-CoV-2, highlighting the importance of FcγR dead mutations for the development of Ab-based therapies in these diseases (M.D. Larsen, E.L. de Graaf, M.E. Sonneveld, H.R. Plomp, F. Linty, R. Visser, M. Brinkhaus, T. Šustić, S.W. de Taeye, A.E.H. Bentlage, et al., manuscript posted on bioRxiv, DOI: 10.1101/2020.05.18.099507). Taken together, our data provide new insights into which amino acids in the lower hinge region of human IgG differentiate FcγR-mediated from complement-mediated effector functions. Furthermore, it could contribute to revealing how IgG2 has evolved to lack FcγRI and C1q binding and therefore have

![Table](image-url)

**FIGURE 6.** Effect of IgG mutations on FcγR- and C1q-binding in SPR and effector functions. SPR binding data are shown left, effector functions based on cellular assays are shown right. Variants were compared with corresponding IgG1- or IgG2-WT. *Increase seen in sensorgrams, but not determinable, with enhanced effector function [↑↑]. **Decrease in effector function, but abolished in the presence of irrelevant IgG1-Fc [↓↓↓]. The color code for the SPR data is shown at the bottom; effector functions are color coded accordingly.
reduced effector functions. The results also show how this region can be tailored to generate Abs with various degrees of FcγR binding with our without the capability to activate complement.

Acknowledgments
We thank Ninotska Derksen, Gerard van Mierlo, Pleun H De Heer-Ooijevaar, and Mike Brouwer for technical assistance; Vladimir Bobkov, Dr. Madagala Sips, and Prof. Rob Aalberse for fruitful discussions; and the donors for blood donation.

Disclosures
P.V. and H.D.H. were employed by argenx. The work of M.B. is funded by argenx. The work of S.W.D.T. was funded by Genmab. The other authors have no financial conflicts of interest.

References
1. Vidarsson, G., G. Dekkers, and T. Rispens. 2014. IgG subclasses and allotypes: from structure to effector functions. Front. Immunol. 5: 520.
2. Roux, K. H., L. Strelets, and T. E. Michaelsen. 1997. Flexibility of human IgG subclasses. J. Immunol. 159: 3372–3382.
3. de Taeye, S. W., A. E. H. Bentlage, M. M. Meibius, J. I. Meesters, S. Lissenberg-Thunnissen, D. Faulk, T. Sénard, N. Salehi, M. Wahrer, J. Schuurman, et al. 2020. FcγR binding and ADCC activity of human IgG allotypes. Front. Immunol. 11: 740.
4. de Taeye, S. W., T. Rispens, and G. Vidarsson. 2019. The ligands for human IgG and their effector functions. Antibodies (Basel) 8: 30.
5. Bruggeman, C. W., and C. Folman. 2015. Mouse and human Fc receptor functions. Immunol. Rev. 268: 25–51.
6. Gillis, C., A. Gouel-Chéron, F. Jonsson, and P. Bruhrs. 2014. Contribution of human FcyRs to disease with evidence from human polymorphisms and transgenic animal studies. Front. Immunol. 5: 254.
7. Diebold, C. A., F. J. Beurskens, R. N. de Jong, R. I. Koning, K. Stratane, M. A. Lindorfer, M. Voorhorst, D. Ugurlar, S. Rosati, A. J. R. Heck, et al. 2014. Defucosylation of human immunoglobulin G glycans reveals a new function for glycans to modulate effector function. J. Immunol. 193: 1260–1269.
8. Reis, E. S., D. C. Matellos, G. Hajishengallis, and J. L. Dambris. 2019. New insights into the immune functions of complement. Nat. Rev. Immunol. 19: 505–516.
9. Nimmerjahn, F., and J. V. Ravetch. 2008. Fcgamma receptors as regulators of immune responses. Nat. Rev. Immunol. 8: 34–47.
10. Bruhrs, P., B. Iannasci, P. Englund, D. A. Mancardi, N. Fernandez, S. Jorieux, and M. Dairen. 2009. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. Blood 113: 3716–3725.
11. Bruggeman, C. W., G. Dekkers, A. E. H. Bentlage, L. W. Treffers, S. Q. Nagelkerke, C. W. Bruggeman, J. Koirs, P. Lighthart, S. Q. Nagelkerke, J. C. Zimring, et al. 2019. Functional attributes of antibodies, effector cells, and target cells affecting NK cell-mediated antibody-dependent cellular cytotoxicity. J. Immunol. 203: 3126–3135.
12. Parma, J. V., and P. A. Ward. 2011. The neonatal Fc receptor. J. Immunol. 186: 227–235.
13. Michael, T. E., J. S. Lee, D. E. Sletten, K. F. Port, C. N. Ihle, and O. Ihle. 2009. The multiple facets of FcγR in immunity. Immunol. Rev. 286: 252–268.
14. Sarma, J. V., and P. A. Ward. 2011. The complement system. Sci. Prog. 94: 431–458.
15. Bruggeman, C. W., G. Dekkers, A. E. H. Bentlage, L. W. Treffers, M. A. Lindorfer, M. Voorhorst, D. Ugurlar, S. Rosati, A. J. R. Heck, et al. 2014. Defucosylation of human immunoglobulin G glycans reveals a new function for glycans to modulate effector function. J. Immunol. 193: 1260–1269.
16. Reis, E. S., D. C. Matellos, G. Hajishengallis, and J. L. Dambris. 2019. New insights into the immune functions of complement. Nat. Rev. Immunol. 19: 505–516.
17. Timming, A. R., S. W. de Taeye, E. L. de Graaf, L. A. de Neel, G. Dekkers, C. W. Bruggeman, J. Koirs, P. Lighthart, S. Q. Nagelkerke, J. C. Zimring, et al. 2019. Functional attributes of antibodies, effector cells, and target cells affecting NK cell-mediated antibody-dependent cellular cytotoxicity. J. Immunol. 203: 3126–3135.
18. Parma, J. V., and P. A. Ward. 2011. The neonatal Fc receptor. J. Immunol. 186: 227–235.
19. Michael, T. E., J. S. Lee, D. E. Sletten, K. F. Port, C. N. Ihle, and O. Ihle. 2009. The multiple facets of FcγR in immunity. Immunol. Rev. 286: 252–268.
20. Sarma, J. V., and P. A. Ward. 2011. The complement system. Sci. Prog. 94: 431–458.
21. Michael, T. E., J. S. Lee, D. E. Sletten, K. F. Port, C. N. Ihle, and O. Ihle. 2009. The multiple facets of FcγR in immunity. Immunol. Rev. 286: 252–268.
22. Sarma, J. V., and P. A. Ward. 2011. The complement system. Sci. Prog. 94: 431–458.
23. Michael, T. E., J. S. Lee, D. E. Sletten, K. F. Port, C. N. Ihle, and O. Ihle. 2009. The multiple facets of FcγR in immunity. Immunol. Rev. 286: 252–268.
24. Sarma, J. V., and P. A. Ward. 2011. The complement system. Sci. Prog. 94: 431–458.
25. Michael, T. E., J. S. Lee, D. E. Sletten, K. F. Port, C. N. Ihle, and O. Ihle. 2009. The multiple facets of FcγR in immunity. Immunol. Rev. 286: 252–268.
26. Sarma, J. V., and P. A. Ward. 2011. The complement system. Sci. Prog. 94: 431–458.
