frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 08 January 2024
DOI 10.3389/fimmu.2023.1304365

OPEN ACCESS

EDITED BY
Yong-Sung Kim,
Ajou University, Republic of Korea

REVIEWED BY
Harald Kolmar,
Darmstadt University of Technology, Germany
William Kelton,
University of Waikato, New Zealand
Anja Lux,
University of Erlangen Nuremberg, Germany

*CORRESPONDENCE
Timon Damelang
tdam@genmab.com

RECEIVED 29 September 2023
ACCEPTED 11 December 2023
PUBLISHED 08 January 2024

CITATION
Damelang T, Brinkhaus M, van Osch TLJ, Schuurman J, Labrijn AF, Rispens T and Vidarsson G (2024) Impact of structural modifications of IgG antibodies on effector functions. *Front. Immunol.* 14:1304365. doi: 10.3389/fimmu.2023.1304365

Impact of structural modifications of IgG antibodies on effector functions

Timon Damelang[1,2,3,4]*, Maximilian Brinkhaus[1,3], Thijs L. J. van Osch[1,3], Janine Schuurman[4], Aran F. Labrijn[4], Theo Rispens[2] and Gestur Vidarsson[1,3]

[1]Sanquin Research, Department of Experimental Immunohematology and Landsteiner Laboratory, Amsterdam, Netherlands, [2]Sanquin Research, Department of Immunopathology, Amsterdam, Netherlands, [3]Department of Biomolecular Mass Spectrometry and Proteomics, Utrecht Institute for Pharmaceutical Sciences and Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, Netherlands, [4]Department of Antibody Research & Technologies', Genmab, Utrecht, Netherlands

Immunoglobulin G (IgG) antibodies are a critical component of the adaptive immune system, binding to and neutralizing pathogens and other foreign substances. Recent advances in molecular antibody biology and structural protein engineering enabled the modification of IgG antibodies to enhance their therapeutic potential. This review summarizes recent progress in both natural and engineered structural modifications of IgG antibodies, including allotypic variation, glycosylation, Fc engineering, and Fc gamma receptor binding optimization. We discuss the functional consequences of these modifications to highlight their potential for therapeutical applications.

KEYWORDS

antibodies, IgG, subclasses, allotypes, glycosylation, FcγR, complement

Introduction

Antibodies, also known as immunoglobulins (Ig), are among the most abundant protein components in the human blood, constituting about 20% of the total protein in plasma by weight. The five major classes of Ig in humans are IgG (70-85%), followed by IgA (5-15%), then IgM (5-10%), with trace amounts of IgD (~0.25%) and IgE (<0.25% of the total serum Igs) (1, 2). These glycoproteins share similar structures and composition (82–96% protein and 4–18% carbohydrate), but differ in size, charge, amino acid sequence and effector function. The most abundant isotype in healthy human serum, IgG, can further be divided into four subclasses: IgG1 (60–70% in plasma), IgG2 (20–30%), IgG3 (5–8%) and IgG4 (1–3%).

IgG antibodies are major effector molecules of the humoral immune system. They provide a link between the adaptive immune system and the effector mechanisms of the innate immune system through high affinity antigen-specific recognition of foreign structures. This can result in either simply blocking the interactions of the foreign

10.3389/fimmu.2023.1304365

molecules with their ligands or in the formation of high avidity interactions with innate molecules, such as complement, and effector cells through Fc gamma receptors (FcγRs), and the neonatal Fc receptor (FcRn) (Figure 1) inducing effector functions (4). Variation in the IgG subclass structures shapes the biological effector activities, in broad terms, IgG1 and IgG3 are more potent in inducing these effector functions, while IgG2 and IgG4 do less so.

Given the extensive scope of the research on IgG antibody modifications, this review focuses only on the major natural and engineered changes, and provides literature references for further reading on variants not covered. We will discuss how they affect structure and functional modalities of IgG themselves and binding to various IgG receptors, introduced below.

Complement

Both IgM and IgG target-bound antibodies can activate the classical complement pathway via the initial binding of hexameric C1q. Of the four IgG subclasses, IgG1 and IgG3 are the most efficient activators of the classical complement pathway; IgG2 and IgG4 (serine in position 331 severely reduces complement binding) require high antigen densities or repeated polysaccharide structures (5, 6). However, given that monomeric IgG has a low binding affinity to the individual globular heads of C1q, it requires multimerization to form a polyvalent, ideally hexameric, high avidity interaction platform for C1q (7–11). The process of multimerization (or hexamerization) is highly dependent on the size of the antigen, expression level, spatial distribution and mobility but also the epitope position, binding angle, hinge length and flexibility (10, 12). Especially the hinge length can influence how and where complement fragments of C4 are deposited (13). These distinctions will be discussed in more detail below.

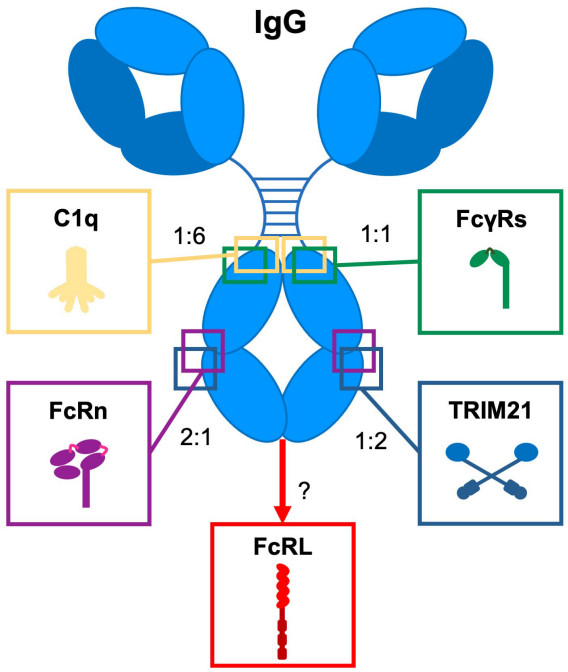

FIGURE 1
Interaction of IgG with Fc effector molecules. Schematic representation of IgG and its Fc-engaging molecules (complement component (C1q), Fc gamma receptors (FcγRs), the neonatal Fc receptor (FcRn), Tripartite motif 21 (TRIM21), and Fc receptor-like (FcRL) molecules through which antibodies exert their biological activity. For each ligand, the binding site on IgG and the stoichiometry of the interaction with IgG is indicated. Adapted from (3).

FcγRs

Humans express five FcγRs: FcγRI, FcγRIIa, FcγRIIb/c, FcγRIIIa, and FcγRIIIb. IgG1 and IgG3 bind with higher affinity than IgG2 and IgG4 to FcγRs on effector cells (Table 1). However, the structural determinants responsible for the subclass-specific affinity variation are still largely unknown, except for binding to FcγRI, which is reduced for IgG4 due to the presence of S331 and F234 in comparison to P331 and L234 in IgG3 (14) and IgG1 (15). In addition, monomeric IgG2 Abs only bind FcγRIIa, most likely due to their short hinges lacking G236 (16). It is important to note that complexed IgG2 was found to bind FcγRIIIa 158V, presumably due to multivalency-induced avidity effects, that might be relevant in (auto) immune responses (17). The interaction of IgG antibodies with FcγRs on effector cells has been recognized as one of the most critical immune response determinants against infections (18–23). Induced effector functions are also influenced by natural (allotypes) and engineered amino acid changes in the Fc region of IgG antibodies, glycosylation profiles, FcγR polymorphisms as well as FcγR expression profiles on different immune cells.

FcRn

IgG antibodies also bind FcRn, which mediates their half-life, placental transport, and bidirectional transportation to mucosal surfaces (24, 25) and orchestrates cellular responses to immune complexes (26–29). The interaction with FcRn is highly pH dependent and only occurs at acidic pH (pH < 6.5) as present in the endosomes. After recycling or transport to the cell surface, the IgG is released again at physiological pH, as present outside of the cells. Naturally occurring or bio-engineered mutations can impact the interaction with FcRn.

Alternative receptors

IgG also bind less studied and underappreciated effector molecules, including the two members of the FcR-like (FcRL) family (FcRL4 and FcRL5) (30, 31), and tripartite motif-containing protein 21 (TRIM21) (32, 33). Although controversial, DC-SIGN has been described to bind to sialylated IgG (34, 35). However, more recent studies found no detectable binding of human IgG Fc to DC-SIGN, indicating that DC-SIGN might not be an IgG receptor after all, at least not in humans (36–38).

10.3389/fimmu.2023.1304365

TABLE 1 Properties of human IgG subclasses.

Structure	IgG1	IgG2	IgG3	IgG4
H chain type	γ1	γ2	γ3	γ4
Molecular mass (kDa)	146	146	170	146
Amino acids in hinge region	15	12	47-62†	12
Disulfide bonds in hinge region	2	4	11	2
Ig H constant gene	IGHG1	IGHG2	IGHG3	IGHG4
Alleles of IGHG	15	18	29	8
N-linked glycans per H chain	1	1	2†	1
O-linked glycans per H chain	0	0	3	0
Biology				
Mean adult serum level (g/L)	6.98	3.8	0.51	0.56
Proportion of total IgG (%)	43-75	16-48	1.7-7.5	0.8-11.7
Half-life (days)	21	21	7/~21†	21
Placental transfer	+++	++	++/+++†	++
Ab targets				
Proteins	++	+/-	++	++*
Polysaccharides	+	++	+	+
Allergens	+	(-)	(-)	++*
Binding capacity				
Complement (C1q)	++	+	+++	+/-
FcγRI	+++	-	+++	++
FcγRIIa	++	(+)/++‡	++	+
FcγRIIb/c	+	+/-	++	++
FcγRIIIa	++‡/+++§	+/-§	++†‡/+++†	-/++§
FcγRIIIb	+‡§	+§	+‡§	+/-§
FcRn (at pH <6.5)	+++	+++	++/+++†	+++
FcRL4	-	-	+	+
FcRL5	++	+	+	++
TRIM21	+++	+++	+++	+++
Binding to Protein A	+++	+++	-/+++†	+++
Binding to Protein G	+++	+++	+++	+++
Effector functions				
Complement (CDC)	++	+†#	+++	-
Phagocytosis (ADCP)	+++	+‡	+++	+
Cytotoxicity (ADCC)	+++	+†‡	+++†	+

† IgG allotype-specific
‡ FcγR allotype-specific
* after repeated exposure
§ depending on IgG fucosylation status
# against repeated/polysaccharide structures

FcRL molecules, which are part of the Ig superfamily and mainly expressed on B cells, interact with IgG antibodies (39). FcRL4 only binds IgG3 and IgG4, while FcRL5 is able to bind all IgG subclasses (30, 40). The exact binding epitope of FcRL5 on IgG has not been described yet, but a complex binding interaction was hypothesized, in which both the Fab- and Fc-fragments of IgG are involved (40).

Human IgG also binds to TRIM21, an intracellular cytosolic IgG receptor and E3-ligase, with nanomolar affinity through its PRYSPRY domain, which is highly conserved (41, 42). TRIM21 is relevant in the context of antibody-dependent intracellular neutralization of viruses and transcriptional activation of several immune regulator genes (33). The neutralization itself activates proteasome-dependent degradation of the antibody based targets that also seem to be activated in mouse tau immunotherapy models (43).

Here we discuss natural and engineered structural modifications of IgG antibodies that can impact the interactions with complement, FcγRs, FcRn and resulting downstream effector functions.

Structure of IgG antibodies

IgG antibodies are composed of two distinct fragments, the antigen-binding Fab and the Fc domain, which interacts with effector molecules of the immune system (Figure 2A). IgG molecules are generally composed of two identical γ H polypeptide chains of 50 kDa and two identical κ or λ L polypeptide chains of 25 kDa, linked via inter-chain disulfide bonds. Each H chain consists of an N-terminal variable domain (V_H) and three constant domains (C_H1, C_H2, C_H3) connected by a flexible stretch of polypeptide chain, known as the hinge region. The encoding human Ig H constant (IGHC) genes are *IGHG3*, *IGHG1*, *IGHG2*, and *IGHG4*, from 5' to 3' in the IGH locus on chromosome 14q32.33 (44). The L chains also consist of an N-terminal variable domain (V_L) and a constant domain (C_L). In association with the V_H and C_H1 domains, the L chains form the Fab regions, allowing the V regions to shape the antigen-binding region (45). Rearrangement of gene segments and somatic mutations generate variations in the amino acid sequence of the N-terminal domains. This results in variable regions, containing six hypervariable loops, known as complementarity determining regions (CDRs) (46), which together form the antigen recognition site (paratrope) in the Fab region. The lower hinge region and the C_H2/C_H3 domains form the Fc region, which interacts with effector molecules and immune cells. The four IgG subclasses show a homology of more than 90% on the amino acid sequence level of their "constant" domains, but are highly divergent in the hinges and upper C_H2 domains. These regions are crucial for binding to FcγR (mainly the

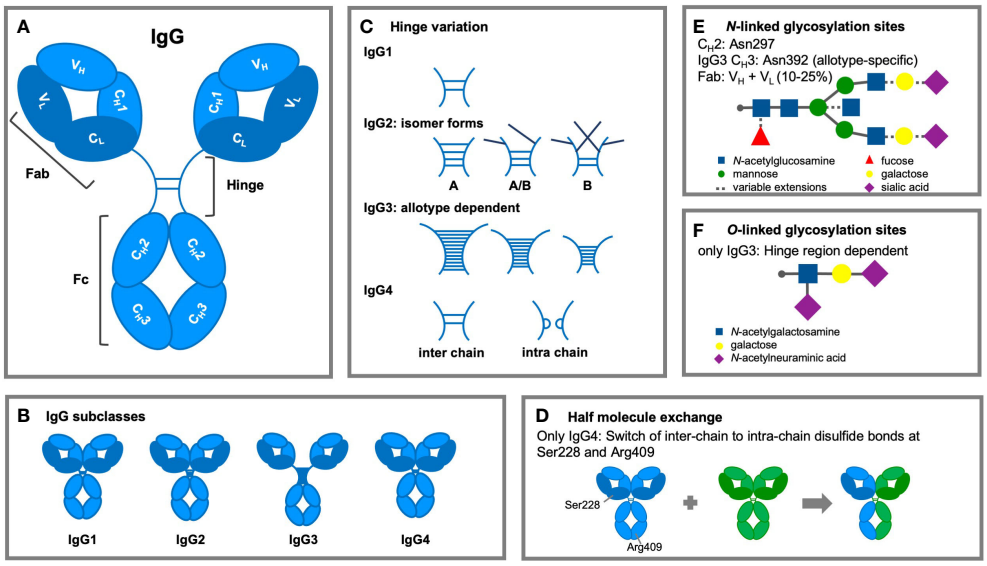

FIGURE 2
Structural modifications of IgG antibodies. **(A)** IgG1-4 antibodies consist of four polypeptide chains, composed of two identical heavy (H; light blue) chains of 50 kDa and two identical light (L; dark blue) chains of 25 KDa, linked together by interchain disulfide bonds. Each H chain consists of an N-terminal variable domain (VH) and three constant domains (CH1, CH2, and CH3). IgG molecules are joined by a flexible stretch of polypeptide chain between CH1 and CH2, known as the hinge region. The VH and CH1 domains and the L chains form the fragment antigen binding (Fab) region. The lower hinge region and the CH2/CH3 domains form the fragment crystalline (Fc) region, which interacts with effector molecules and cells. **(B)** The length (IgG1: 15 amino acid residues, IgG2: 12 residues, IgG3: 32-64 residues, and IgG4: 12 residues) and flexibility of the hinge region varies among the IgG subclasses. **(C)** Subclass differences in hinge flexibility are also impacted by differential number of inter-chain disulfide bonds (IgG3: 11 bonds; IgG1 & IgG4: two bonds; IgG2: four bonds), both IgG2 and IgG4 are found as several isomers. Darker disulfide bonds indicate that they are linked to the light chain due to light chain reshuffling of the C-C bonds. **(D)** IgG4 antibodies can split into two half-molecules (one H chain + one L chain) that can then randomly form complete monovalent-bispecific Abs, which is either termed half molecule exchange or Fab arm exchanged. **(E)** Within the CH2 region is one N-linked glycosylation site containing carbohydrate groups attached to asparagine 297. The highly conserved glycan has a heptasaccharide core and variable extensions, such as fucose, galactose and/or sialic acid (dashed line). Additional N-linked sites have been reported in the antigen-binding region and allotypic variants of IgG3 at position asparagine 392. **(F)** The hinge region of IgG3 exhibits O-linked glycosylation sites.

residues L234, L235, D265 and S298) and C1q, influencing various effector functions such as phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), and complement activation.

Structural variation in the hinge region

The length and flexibility of the hinge region varies among the IgG subclasses (Figure 2B) (47). This affects the conformations of the Fab arms relative to the Fc domain as well as to each other and allows the Fab arms to bind to multiple targets and the Fc to interact independently with effector molecules of the immune system. There are ongoing efforts to determine the diversity of conformational structures and flexibility of the IgG molecules with particle electron tomography (48). This flexibility is strongly affected by the hinge length which varies considerably between the subclasses (IgG1: 15 amino acid residues, IgG2: 12 residues, IgG3: 32-64 residues, and IgG4: 12 residues; Table 1).

The lower hinge region of IgG2 (encoded by the C_H2 exon) lacks one of the double glycines found at position 236-237. This and up to four inter-heavy chain disulfide bridges restrict the flexibility of the IgG2 molecule (16, 49, 50). Similarly, the shorter hinge of IgG4 gives it less flexibility than the one of IgG1 (51). IgG3 has a much longer hinge region than any other IgG subclass (containing up to 62 amino acid residues), but its length is allotype-specific (discussed below). Recently, Bashirova et al. detected a single individual carrying an allele with five *IGHG3* hinge exon (77 amino acid residues) (52).

An IgG3 hinge region can consist of up to 2x21 prolines and 11 disulfide bridges, which results in a poly-proline helix with limited flexibility (51). However, the Fab fragments in an IgG3 molecule are relatively far away from the Fc fragment, giving the entire molecule a greater reach with consequences for downstream effector function, such as ADCC and complement activation (3, 53). The relative flexibility of the Fab arms and the Fc differs following the same order of the hinge length: lgG3 > lgG1 > lgG4 > lgG2 (47).

Inter-chain disulfide bonds

Another structural difference between the human IgG subclasses is the linkage of the H and L chain by disulfide bonds. This bond links the carboxy-terminal cysteine of the L chain to the cysteine at position 220 (in IgG1) or at position 131 (in IgG2, IgG3, and IgG4) in the C_H1 domain. A pair of cysteines in close proximity will form a disulfide bond that fixes and stabilizes the tertiary structure of an IgG molecule, which is essential for the function of the molecule (54). Besides subclass differences in hinge flexibility due to differential number of inter-chain disulfide bonds (IgG3: 11 bonds; IgG1 & IgG4: two bonds; IgG2: four bonds), both IgG2 and IgG4 are found as several isomers, in which the hinge disulfide bonds are differentially interconnected (51) (Figure 2C).

Three main isomeric variants of the IgG2 hinge have been described (IgG2A, B and A/B) (49, 55). These structural isomers were first found in recombinant monoclonal IgG2, however, were later confirmed to be present in serum from healthy and diseases subjects. Their presence is a consequence of alternative disulfide bond formation between the C-terminal cysteine of the LC and a cysteine in the C_H1 domain of the heavy chain (55) and was found to be more prevalent in IgG2 with kappa LCs (56). The IgG2A isomer may confer more flexibility to the Fab arms relative to IgG2B, which can have consequences for downstream effector functions, even though FcγR binding does not seem to be different for the two isomers (57). This seems to strongly affect how IgG2 interacts and cross-links its target, which can lead to superagonistic antibodies when targeting cellular antigens. These superagonistic effects are independent of the Fc (58).

The two isomers of IgG4 differ in the disulfide bonding of hinge cysteines that are classically bonding the two H chains. However, these disulfide bonds are in flux, as they are a subject of reduction and re-oxidation, forming intra-chain disulfide bonds between cysteines found at positions 226 and 229, resulting in non-covalently linked half-molecules in addition to covalently linked inter-chain isomers (59, 60).

IgG4 Fab arm exchange

IgG4 antibodies are unique and dynamic molecules due to their ability to undergo a process called Fab arm exchange (FAE; Figure 2D). *In vivo*, an IgG4 antibody can reassemble after secretion, recombining two halves of two random IgG4 (one H chain and one L chain) to form functionally monovalent-bispecific antibodies (59, 60).

Two amino acids seem required for FAE *in vivo*. One is the serine at position 228 in the core hinge region of IgG4, and the other one is arginine at position 409, which results in weaker C_H3-C_H3 interactions (60, 61). These two amino acids at positions 228 and 409 are unique to IgG4, which might cause a conformational change that could explain the poor FcγR and C1q binding properties of IgG4 (62). Interestingly, the arginine residue at position 409 is polymorphic as the IGHG4*03 allele harbors a lysine at that position (allotypes discussed below). Nevertheless, this allotype, regardless of containing S228 is not prone to FAE, showing that both serine at position 228 and arginine in position 409 are essential (59).

IgG4 antibodies that underwent FAE cannot effectively crosslink the target antigen. This effect in combination with the requirement for repeated antigen exposure, low affinity to activating FcγRs and complement, may contribute to the anti-inflammatory properties of IgG4 (59, 63). In this way, the strong immune effector functions otherwise provided by IgG1, IgG3 and even IgG2, are likely to be toned down after class switching to IgG4, both by the lesser interactions Fc-receptors and complement, but also due to its monovalency. IgG4 is therefore generally regarded as less important subclass in the autoimmune setting. However, IgG4 can still be highly pathogenic, for example in the form of (blocking) autoantibodies in pemphigus (64), primary membranous nephropathy (65, 66), or in IgG4-related disease (67). Elevated levels of serum IgG4 have also been associated with asthma (68) and tissue eosinophilia (69). Even more intriguingly, the monovalency of IgG4 may even be required in myasthenia gravis when targeting

10.3389/fimmu.2023.1304365

muscle-specific kinase (MuSK) where monovalency of IgG4 can be required to block neuromuscular signaling and produce pathogenicity (70–72).

Interindividual variations of IgG antibodies: allotypes

Allotypes describe antibody genetic markers (Gm) on the constant regions of antibodies, likely found across all isotypes and light chains, but most studied for the four IgG subclasses (Figure 3). A large number of polymorphic IgG variants were originally discovered due to serological reactivities to IgG originating from other individuals (alloreactivity) (73), which were used to study population variations (74). However, sequencing efforts of the IgG genes from different ethnic groups revealed that even more polymorphic variants exist, especially for IgG3 (75, 76). Non-synonymous mutations are the basis for differences in primary amino acid sequences between polymorphic variants. Polymorphic variants have been identified on the γ1-4, α2, and ϵ H chains of the subclasses IgG1-4, IgA2 and IgE, and on the κ L chain (Km allotypes) (77–79). Currently, 27 Gm polymorphisms have been identified and categorized in the ImMunoGeneTics information system (IMGT), which correspond to changes in the amino acid sequence of the constant region (encoded by 15 *IGHG1*, 18 *IGHG2*, 29 *IGHG3*, and 8 *IGHG4*). An overview of all the IgG allotypes, which differ in amino acid sequence, is presented in Figure 3. However, numerous additional single mutations do exist on a genetic level, which do not result in amino acid changes and are not shown in the figure (79). Recently, 28 novel variant sites in *IGHG1-3* alleles were discovered in seven genetically isolated Brazilian population (76). In addition, 11 novel alleles and 17 extensions of known IGHG and IGHM alleles were observed in a study using a novel sequencing technique (80). This demonstrates that the diversity of the IGHG genomic region is still far from being fully characterized in whole genome sequencing databases (81).

Haplotypes and non-coding allotype-associated regions

IgG allotypes were found to correlate with plasma IgG levels (82, 83), which has been hypothesized to be the result of variation in the non-coding switch regions or unfavorable RNA transcripts. IgG allotypes can affect class-switching efficiency and thereby serum isotype and subclass concentrations (83–86). The concentration of IgG1 is lower in individuals with the IGHG1*03 allele in combination with IGHG2*02 and any of IGHG3*01/*04/*06/*09/

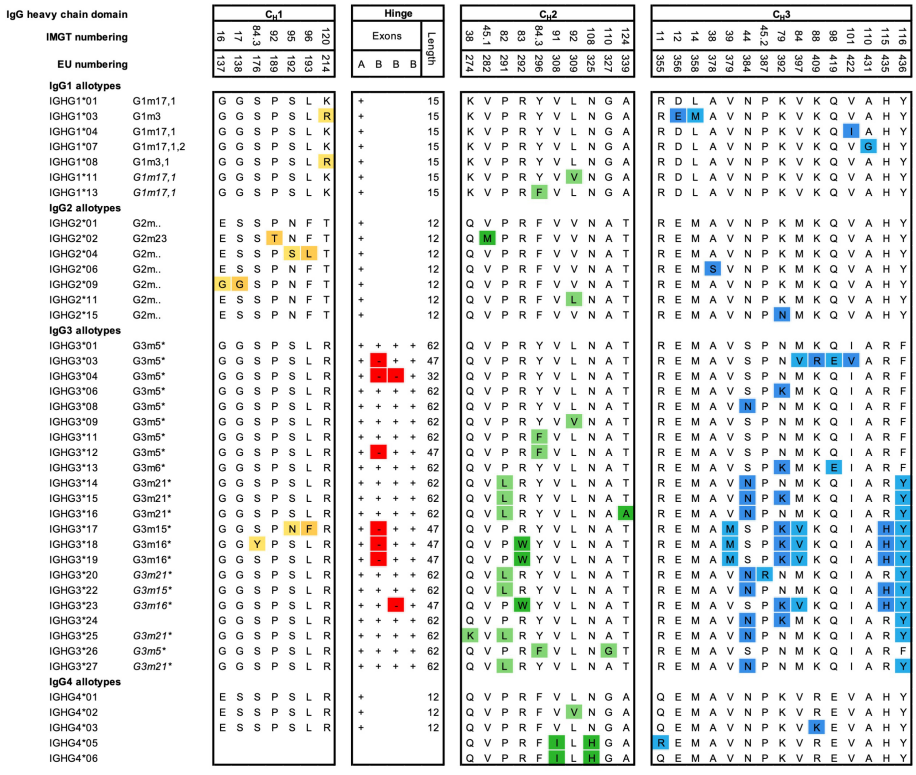

FIGURE 3
Amino acid variation between IgG allotypes. Variation at the amino acid level between IgG allotypes within the IgG1, IgG2, IgG3, and IgG4 subclasses according to the Reference to ImMunoGeneTics information system (IMGT). For each domain, CH1, hinge, CH2 and CH3, amino acid differences between polymorphic variants are indicated. Polymorphisms in the hinge region are identified by the presence or absence of hinge exons **(A, B)** (3). Additional 'silent' mutations exist but are only visible on the genetic level. Historical nomenclature (Gm), based on serology, are included, but indicated in italics for new alleles, that have not been assigned a name to in accordance with Gm system.

*11/*12 as compared with those who lack this combination (82). IgG3 polymorphisms within sterile promoter region that undergo transcription preceding class switching could affect both class-switching efficiency directly and antibody serum levels (83, 85). This could explain why higher lgG3 levels were found in individuals carrying IGHG3*01/*04/*06/*09/*11/*12 compared to IGHG3*14/*15/*16 (82).

IgG1 allotypes have also been associated with changes in antibody subclass distribution, magnitude and functionality in specific responses, such as to HIV envelope glycoprotein vaccination (86) or to opsonized herpes simplex virus-infected fibroblasts (87–89). The IGHG2*02 allele containing a V282M substitution in the C_H2 domain and a P189T substitution in the C_H1 domain seems to grant a functional advantage against bacterial infections, but the mechanism behind this is not understood (90). IgG3 allotypes with longer half-life (discussed below) and good transplacental transport (R435H) (91, 92), although produced at lower levels (83), may provide better protection against infectious diseases (93). Other reported associations between human IgG allotypes and disease conditions include autoimmunity (94, 95) and cancer (96, 97).

Functional consequences due to allotypic diversity

IgG3 is the most polymorphic subclass due to various levels of molecular diversity observed with the *IGHG3* alleles (Figure 3). IgG3 allotypic variations can have structural and functional consequences, such as shorter hinge regions and extended half-life compared to other allotypes (17, 91). The hinge region is encoded by one A exon, and from one to three 15 amino acid long B exons, depending on the G3m alleles, so it can vary from 32 to 62 amino acids and can influence structural conformations (75). The length of the hinge region can influence the capacity of IgG3 allotypes to induce effector functions, such as ADCC, likely through altered proximity at the immunological synapse (3). It has also been demonstrated that IgG3 allotypes with a phenylalanine at position 296 (IGHG3*11 and IGHG3*12), or a tryptophan at position 292 (IGHG3*18 and IGHG3*19) exhibit a lower affinity to FcγRIIIa and ADCC activity. In addition, IgG3 allotypes with a leucine at position 291 (IGHG3*14, IGHG3*15, and IGHG3*16) also showed reduced ability to mediate ADCC, but without apparent changes in affinity to FcγRIIIa (3). The same study also found that IgG3 antibodies with a short hinge, e.g., IGHG3*04 (2 exons), exhibited the strongest ADCC capacity, but this was not reflected by an increased affinity for the receptor FcγRIIIa (3). This reduced hinge length is similarly associated with increased ADCC against HIV infected cell lines (98), but also increased inflammation and death in cerebral malaria (99). This shorter synapse due to the shorter IgG3 hinge seems to reflect on the capacity of anti-CD20 antibodies to increase ADCC against $CD20^+$ tumor cells (100). Curiously, this enhancement of ADCC by shorter distance between effector and target cell was suggested to result in less phagocytosis (98, 100), which may quench antibody inflammatory properties.

The two IgG3 variants IGHG3*01m (GenBank : MK679684) and IGHG3*17, both carry mutations at amino acid positions 419 (glutamic acid) and 392 (lysine) respectively. Although these positions do not define these allotypes, they are implicated in improved binding to FcγRIII and FcγRIIb receptors and enhanced ADCC responses in anti-HIV antibody responses (81).

In addition, polymorphisms in the C_H3 domain affect the C_H3-C_H3 interdomain interactions (60), with potential consequences for both complement activation (60, 101) and aggregation dynamics (102, 103). IgG3 binds with a higher affinity to C1q in comparison to other IgG subclasses (104). While this affinity is believed to be associated with the enhanced flexibility of IgG3 rather than the length of its hinge region (105) under conditions of low antigen density (106). A more recent study showed that antigen density and antibody hinge length play an important role in antibody-mediated CDC. In addition, the study identified that although the differences between IgG1, IgG3 and IgG4 allotypes were minor, the allelic variant IGHG2*06, containing a unique serine at position 378 in the C_H3 domain, showed less efficient complement activation and CDC compared to other IgG2 polymorphisms (107).

IgG3 has a shorter half-life (~ seven days) than the other three subclasses (21 days). This is because FcRn-mediated transport of IgG3 is inhibited in the presence of IgG1 which seems to be a net result of a less compatible pH-dependent binding of IgG3 containing arginine at position 435, a key-interacting site for FcRn binding (91). However, individuals with certain IgG3 allotypes (Figure 3), harbor a histidine at position 435, which makes their IgG3 half-life comparable with that of IgG1 (91). The same rational applies to FcRn placental transport of the different IgG3 variants (92). In addition, amino acid modifications remote from the FcRn binding site can also affect IgG binding to FcRn (108, 109). Specific allotypes also influence the purification of human IgG3 from serum samples, as only allotypes containing a histidine residue at position 435 can be purified by using protein A (110). To purify IgG3 allotypes without a histidine at position 435, protein G can be used (110).

The functional differences of IgG allotypes, including the impact of differences in the hinge domain, on expression levels, half-life, FcγR and FcRn binding (111), complement activation (7, 111, 112), antigen binding, and immunogenicity are still understudied and relatively unknown. Allotypes may also be relevant to understand the pathogenesis of different infectious diseases, and to exploit immune responses to develop novel antibody-based therapeutics or vaccines.

Glycosylation

Just like most proteins, antibodies can be glycosylated as a post-translational modification. The glycosylation, and differences thereof, in the Fc and Fab domains of the antibody has a critical impact on antibody function e.g., complement activation or ADCC, which will be discussed in the paragraphs below. Antibodies can contain O-linked glycans added to serines or threonines, or *N*-linked glycans added to asparagine (N), if so-called NxS/T-motives

are present, which consist of an asparagine, then any amino acid except proline, followed by a serine or threonine. Both O- and *N*-linked glycan additions occur in the lumen of the endoplasmic reticulum or Golgi apparatus (113). The composition of *N*-linked glycans seems to be in part regulated by the type of antigen, a range of B cell stimuli, including stress, disease, cytokine activity, and innate immune signaling receptors (114, 115). Besides V(D)J recombination, somatic hypermutation, and class switch recombination, *N*-linked glycosylation can be considered as an additional mechanism of antibody diversification (116, 117).

N-linked glycosylation of IgG

IgG generally contains a single highly conserved *N*-linked glycan at position 297. The core structure of the IgG *N*-linked glycans comprises *N*-acetylglucosamine (GlcNAc) and mannose residues with possible extensions with galactose, sialic acid, core fucosylation, and bisection of GlcNAc residues (115). Bisection describes an additional GlcNAc branch on the first mannose residue on an *N*-linked glycan (56) (Figure 2E). The relative abundance of the different glycoforms for global IgG can be influenced and altered by multiple factors, including age, pregnancy, sex (118), epigenetics (119), disease state (120–122). The glycans at N297 can influence the quaternary structure of the Fc region and therefore the antibody stability (123, 124). It also has a great impact on the ability of antibodies to bind to FcγRs and complement (125, 126), which consequently modulates effector functions, such as ADCC and complement-dependent cytotoxicity (CDC) (22, 127–133). For instance, the removal of glycans at this position (N297) abrogates binding to all FcγRs and C1q except for the high affinity FcγRI which retains minor binding after deglycosylation (134).

The core fucose affects binding to FcγRIIIa/b (127, 135), with non-fucosylated antibodies binding FcγRIII much stronger than fucosylated antibodies. The absence of fucose translates to an up to 20-fold higher FcγRIIIa affinity (127, 136, 137). This effect can be even further increased to ~40-fold for FcγRIIIa by hyper-galactosylation of afucosylated IgG1 (128). These elevated affinities seem to translate to even higher enhancement of Natural Killer (NK) cell-mediated ADCC (127, 128, 130, 138, 139). The importance of afucosylated IgG-induced immune responses in humans has been identified in various diseases (22, 140–146). Additionally, antigen-specific glycosylation can vary significantly depending on the nature of the antigen as seen in some infections (21, 141, 146, 147), and vaccinations (148–150). Despite its relevance, the context in which afucosylated antibody responses are formed, remains understudied.

Recent data also suggests the functional importance of increased Fc galactosylation and sialylation after both infection (146) and vaccination (148, 150, 151). Whereas galactose has a positive effect on IgG binding to FcγRs especially in combination with afucosylation (152), sialylation seems to have either no or minor negative effects on the binding to FcγR (128, 153). However, human IgG antibodies containing terminal sialic acid on their Fc *N*-glycans have been shown to reduce ADCC (154). In addition, studies on intravenous immunoglobulin (IVIg) activity in models of inflammatory arthritis, immune thrombocytopenia and epidermolysis bullosa acquisita showed that cleavage of the terminal sialic acid residues of the Fc fragment of IVIg can reduce its anti-inflammatory activity (155–157). However, whether this effect is also true in humans, and if it is channeled through DC-SIGN and similar receptors remains highly controversial as discussed above (37, 38).

IgG-Fc galactosylation seems to promote C1q binding and complement activity (133, 158), whereas the effect of IgG-Fc sialylation on this is conflicting, showing either increased, decreased or no binding to C1q due to sialylation (128, 129, 133, 153, 159, 160). The relative abundance of bisected GlcNAc residues seems to have no effect on either FcγR- or complement-mediated activities (128). Other modifications, e.g. carbamylation of IgG antibodies (161), and omitting clipping of C-terminal lysins (162) negatively impact IgG hexamerization as well. However, little is known about the importance of the biological implications of these changes. Interestingly, it has been shown that glycosylation patterns between allotypes within subclasses are quite similar in terms of fucosylation and sialylation, but substantial differences in bisection and galactosylation were observed between IgG3 allotypes (3).

In addition to N297 glycan site in the C_H2 domain, one IgG2 and several IgG3 allotypes also contain an additional site at position 392 in the C_H3 domain (163). Although occupancy of a glycan at 297 is virtually 100%, the frequency and impact on antibody-mediated functions of *N*-linked glycan at the 392 position are unknown.

Fab *N*-linked glycosylation

N-linked glycosylation sites in the V_H and V_L regions have been observed in 10-25% of all serum IgG (117), which can contribute to both antibody stability (164), and modulate antigen binding (116, 165). In comparison to Fc glycans, the IgG variable domain glycans contain low levels of fucose and higher proportions of sialic acids, bisecting GlcNAc and galactose (166). It has been demonstrated that the presence of Fab glycans on human monoclonal antibodies can increase Fab binding affinity up to two-fold in an antigen-dependent manner (116), notably on anti-citrullinated protein antibodies in rheumatoid arthritis (167). In general, IgG4 antibodies have a 2-fold higher propensity to acquire Fab glycans compared to the other IgG subclasses, or similar to what is observed in IgE (116, 117, 168). This indicates a differential selection pressure of *N*-linked glycosylation site acquisition during affinity maturation of B cells, which depends on the frequency of immunization, antigen type, antibody isotype, subclass, and the location within the V region (117, 164, 169).

N-linked glycosylation motifs are generally not encoded by germline V, D or J segments, however potential glycosylation sites, requiring only a single base pair change during affinity maturation to emerge, are present in the CDR loops (116, 170). Recently, *N*-linked Fab glycans have been demonstrated to negatively affect FcRn-mediated binding in cells and therefore also FcRn-mediated placental transport of IgG in humans. The mechanism seems to involve a direct steric hindrance with the bulky

Fab glycan clashing with the cellular membrane, further exaggerating the steric effect imposed by the Fab region on binding to membrane associated FcRn (171, 172). Variable domain glycans are postulated to convey a selective advantage through interaction with lectins and/or microbiota (170). Furthermore, B cell receptors expressing variable domain glycans also stay longer on the B cell surface, enhance B cell activation, and may contribute to the breach of tolerance of autoreactive B cells autoimmune disease (173). Elevated levels of Fab glycosylation have now been reported for several types of autoantibodies (168, 174), perhaps enriched due to the continuous antigen-exposure.

O-linked glycosylation

O-linked glycosylation sites have only been found in the hinge region of IgG3, but not in the other IgG subclasses (175). O-glycans contain a *N*-acetylgalactosamine (GalNAc) and galactose residues, which may be sialylated (Figure 2F). Approximately 10% of IgG3 derived glycopeptides from human polyclonal serum samples contain O-linked glycans (175). Each IgG3 can contain up to three O-linked glycans at threonine (T) residues at triple repeat regions (TH2-7, TH3-7, TH4-7) on each side of the hinge region (163, 175). The IgG3 hinge region has a high degree of surface accessibility (176), which may explain the lower degree of O-linked glycosylation observed in IgG3 allotypes with a shorter hinge region (175). Although the function of O-glycosylation is still unknown, it might aid to protect the antibody from proteolytic cleavage or maintain the extended conformation and flexibility of IgG3 hinge regions.

Antibody protein engineering to impact effector functions

Besides natural relevant amino acid residues, many engineered mutations have been described that can either enhance, reduce, or abolish antibody interactions with complement, FcγRs and FcRn (Table 2).

Mutations to modulate complement binding

Two main ways of influencing complement activation are on the one hand affecting hexamerization, and on the other hand direct impact on C1q binding. Multiple mutations have been described that impact complement activation in either or both ways (Figure 4).

In the work of Strasser et al., the dynamics of IgG hexamer formation was studied by atomic force microscopy using an artificial surface (10, 11). They describe two different pathways to recruit IgG molecules into hexamers: from solution and through lateral diffusion of IgG-bound surface antigens in the cellular membrane (10, 11). At least four C1q globular heads, meaning engagement with four IgG antibodies, seem to be needed for efficient complement activation (9). Extensive binding subsites were found at the C_H2 domains of IgG for the binding of the globular heads of C1q, one at the FG loop at position 325-331 (also important for the binding of FcγRs), one at the BC loop at position 266-272, and one at the DE loop at position 294-300 (9). The amino acid residues within these regions most critical for the binding of C1q are D270, K322, K326, P329, P331 (naturally a serine in IgG4 antibodies), E333 and K334 (183, 186, 233). Targeting these residues with point mutations, such as D270A (5), P329G/A (5, 183) or K322A (183, 233) can abolish C1q binding. Additionally, the G236/7A (GAGA) variant of IgG1 has been found to abrogate binding to C1q and downstream effector functions completely (16). K326W, K326W+E333S (WS), S267E+H268F+S324T (EFT) or S267E+H268F+S324T+G236A+I332E (EFT+AE) can increase C1q binding and CDC activity up to 47-fold (181, 193, 234). Another variant, comprising of the C_H1 and hinge regions of IgG1 along with the Fc portion of IgG3 (1133), exhibited enhanced CDC that exceeded wild-type levels without gaining full recovery by protein A binding (101). Reconstituting the C-terminal part of the (IgG3-derived) C_H3 domain with that of IgG1 again (113F), eliminated the protein A binding deficiency without compromising the enhanced CDC activity (101).

In addition to mutations that directly enhance C1q, amino acids outside this direct region have also been found that promote Fc : Fc interactions (188). Only very few fulfill the needs essential to use this for therapeutic antibody development (no multimer formation in solution and good pharmacokinetic properties). Variants E430G and E345K have been selected and are exploited as HexaBody Technology (188). Several studies have demonstrated that incorporating either one of these single mutations leads to increased complement activation and CDC of numerous cell types (133, 235–240). The single point mutations K439E and S440K can each be used to inhibit Fc : Fc interactions and subsequent complement activation via charge repulsion, which can in turn be overcome by using double mutants or mixtures containing each mutants (7, 133). The use of two variants which complement each other to gain function is the basis of the conceptual idea for the HexElect technology (241), in which hexamerization and subsequent complement activation is made dependent on the expression of two targets on the same cell. Although the hexamerization of IgG antibodies primarily occurs after antigen binding, the triple mutation E345R+E430G+S440Y (RGY) allows hexamerization in solution in a concentration and pH-dependent manner (7, 8, 188, 242).

IgG-Fc substitutions to modulate FcγR binding

Many mutations have been described for the modulation of FcγR binding (Table 2; Figure 5).

IgG2 Abs, which naturally only bind FcγRIIa and lack G236, gain binding to both FcγRI and FcγRIIIa after insertion of glycine at position 236 (16). Likewise, IgG1 antibodies lacking G236 lose

TABLE 2 Amino acid modifications influencing effector molecule binding and function.

Modification or mutation (EU numbering)	Abbreviation	Subclass				Fc effector molecules									Function				Reference
						FcγRI	FcγRIIa		FcγRIIb	FcγRIIIa		FcγRIIIb	FcRn	C1q	ADCC	ADCP	CDC	t1/2	
		IgG1	IgG2	IgG3	IgG4		R131	H131		V158	F158								
E233P		x																	(15)
L234F					x														(14)
G236A	GA	x																	(16, 177)
G237A		x																	(16)
P238D		x																	(178)
S239A		x																	(179)
I253A		x																	(7, 179, 180)
S254A		x																	(179)
D265A		x																	(179)
S267E		x																	(181, 182)
H268F		x																	(181)
D270A		x																	(179, 183)
R292A		x																	(179, 184)
N297(A/Q/G)	NA	x																	(179, 182, 185)
S298N		x																	(179)
K322A		x																	(183)
S324T		x																	(181)
K326W		x																	(186)
A327Q		x																	(179)
L328E		x																	(178)
L328F		x																	(182)
P329A		x																	(179, 183)
P331S				x	x														(14, 187)
I332E		x																	(177, 184)
E333A		x																	(183, 186)

(Continued)

10.3389/fimmu.2023.1304365

TABLE 2 Continued

Modification or mutation (EU numbering)	Abbreviation	Subclass				Fc effector molecules								Function				Reference
						FcγRI	FcγRIIa	FcγRIIb	FcγRIIIa	FcγRIIIb	FcRn	C1q	ADCC	ADCP	CDC	t1/2	
K338A		x																(183)
E345R	Arg345	x																(7)
E380A		x																(179)
E430G		x																(188)
H433A		x																(7)
N434A		x																(7, 189)
N435W		x																(189)
K439E		x																(7)
S440K		x																(7)
C221D/D222C		x																(190)
S228P/L235E					x													(191)
F234A/L235A					x													(192)
L234A/L235A	LALA	x			x													(17)
L234A/L235E		x																(193)
L234A/G237A		x																(194)
G236A/G237A	GAGA	x																(16)
G236N/H268D		x																(195)
G236R/L328R	RR	x																(196, 197)
G236A/I332E	AE	x																(177, 181)
K236W/E333S	KWES	x																(186)
S239D/I332E	DE or SDIE	x																(177, 181, 182, 198)
P247I/A339Q		x																199)
T250Q/M428L	QL				x													(200)
M252Y/T256D	YD	x																(201)
T256D/T307Q	DQ	x																(201)
T256D/T307W	DW	x																(201)

(Continued)

10.3389/fimmu.2023.1304365

TABLE 2 Continued

Modification or mutation (EU numbering)	Abbreviation	Subclass				Fc effector molecules							Function				Reference
						FcγRI	FcγRIIa	FcγRIIb	FcγRIIIa	FcγRIIIb	FcRn	C1q	ADCC	ADCP	CDC	t1/2	
P257I/Q311I	PIQI	x															(202)
S267E/L328F	SE/LF	x															(178, 182, 196)
H268F/S324T	FT or HFST	x															(181)
S298G/T299A	Ga	x															(203)
K326A/E333A		x															(186)
K326M/E333S		x															(186)
K326W/E333S	WS	x															(186)
A330S/P331S		x															(14)
E380A/N434A		x															(179)
M428L/N434S	MN or LS	x															(204)
H433K/N434F	HN or KF	x															(205)
E233P/L234V/L235A		x															(15, 183)
L234A/L235A/K322A		x															(193)
L234F/L235E/K322A		x															(206)
L234F/L235Q/K322Q	FQQ	x															(207, 208)
L234A/L235A/P329G	LALAPG	x															(208)
L234F/L235E/P331S	FES	x															(206, 208)
L234S/L235T/G236R		x															(208)
L234A/L235A/G237A		x															(208)
L234F/L235E/D265A	FEA	x															(209)
L234Y/G236W/S298A	YWA	x															(210)
L235A/G237A/E318A		x															(211)
G236A/S239D/I332E	GASDIE	x															(177, 184)
G236A/A330L/I332E	GAALIE	x															(212)
S239D/S298A/I332E		x															(198)
S239D/A330L/I332E	SDALIE or DLE	x															(198)

(Continued)

10.3389/fimmu.2023.1304365

TABLE 2 Continued

Modification or mutation (EU numbering)	Abbreviation	Subclass				Fc effector molecules								Function				Reference
						FcγRI	FcγRIIa		FcγRIIb	FcγRIIIa		FcγRIIIb	FcRn	C1q	ADCC	ADCP	CDC	t1/2	
T250Q/M428L/N434S	QLS	x																	(204, 213)
M252Y/S254T/T256E	YTE or MST	x																	(214, 215)
I253A/H310A/H435A	IHH	x																	(216)
P257I/M428L/N434S		x																	(204, 213)
V259I/N315D/N434Y	C6A-74	x																	(217, 218)
S267E/H268F/S324T	EFT	x																	(181)
H285D/T307Q/A378V	DQV	x																	(219)
S298A/E333A/K334A	AAA	x																	(179)
T307A/E380A/N434A		x																	(179, 220)
L309D/Q311H/N434S	DHS	x																	(221)
A327G/A330S/P331S		x																	(15)
I332E/M428L/N434S		x																	(198, 204, 213)
E333A/M428L/N434S	ALS	x																	(204, 213)
E345R/E430G/S440Y	RGY	x																	(7)
D376V/M428L/N434S		x																	(202, 204, 213)
E380A/M428L/N434S		x																	(179, 204, 213)
L234A/L235A/N297A/P329G		x																	(16)
L234A/L235A/M428L/N434S		x																	(204, 213)
G236A/S239D/A330L/I332E	GASDALIE	x																	(182, 222, 223)
S239D/H268F/S324T/I332E		x																	(181)
S239D/I332E/M428L/N434S	SDIE LS	x																	(177, 198, 204, 213)
P257I/Q311I/M428L/N434S	PIQI LS	x																	(196, 204, 213)
S267E/L328F/M428L/N434S	SE/LF LS	x																	(204, 213)
H268F/S324T/M428L/N434S	HFST LS	x																	(181; 204; 213)
H268Q/V309L/A330S/P331S	IgG2m4		x																(224)
T307A/E380A/M428L/N434S		x																	(204; 213)

(Continued)

10.3389/fimmu.2023.1304365

TABLE 2 Continued

Modification or mutation (EU numbering)	Abbreviation	Subclass				Fc effector molecules							Function				Reference
						FcγRI	FcγRIIa	FcγRIIb	FcγRIIIa	FcγRIIIb	FcRn	C1q	ADCC	ADCP	CDC	t1/2	
E233P/L234V/L235A/ΔG236/S267K					x												(225)
L235V/F243L/R292P/Y300L/P396L	VPLIL	x															(226, 227)
F243L/R292P/Y300L/V305I/P396L	Variant 18(LPLIL)	x															(226, 228, 229)
G236A/S239D/I332E/M428L/N434S		x															(177, 184, 204, 213)
G236A/S267E/H268F/S324T/I332E	EFT-EA	x															(181, 211)
S239D/S298A/I332E/M428L/N434S		x															(204, 213)
S239D/A330L/I332E/M428L/N434S		x															(184, 198, 204, 213)
M252Y/S254T/T256E/M428L/N434S	YTE LS	x															(204, 213, 214)
M252Y/S254T/T256E/H433K/N434F	YTE-KF or MST/HN	x															(214, 230)
S267E/H268F/S324T/M428L/N434S	SEHFST	x															(181, 204, 213)
N315D/A330V/N361D/A378V/N434Y	T5A-74	x															(217, 218)
E345R/E430G/S440Y/M428L/N434S	RGY LS	x															(7, 204, 213)
G236A/S239D/A330L/I332E/ M428L/N434S		x															(203, 204, 213)
M252Y/ S254T/T256Y + S239D/ A330L/I332E	YTE-SDALIE	x															(214)
E233D/G237D/P238D/H268D/ P271G/A330R	V12	x															(178)
E233P/L234V/L235A/ΔG236 + A327G/ A330S/P331S		x															(15, 183)
L234A/L235A/G237A/P238S/ H268A/ A330S/P331S	IgG2c4d		x														(231, 232)
V234A/G237A/P238S/H268A/V309L/ A330S/P331S	IgG2σ		x														(194)
G236A/S267E/H268F/S324T/I332E/ M428L/N434S	EFT-EA LS	x															(181, 204, 213)

black = blocking, red = decreased, grey = unchanged, blue = increased.

10.3389/fimmu.2023.1304365

binding to all FcγRs (except for reduced binding to FcγRI) (16, 177). Variants of IgG1 expressing alanine instead of glycine at the adjacent position 237 have reduced FcγRI affinity and reduced FcγRIIIa-mediated ADCC (243), which also highlights the overall importance of the double G at position 236-7 in IgG for FcγR interaction. Another mutation of IgG1 in the lower hinge region that reduces binding to FcγRI due to antibodies which blocked the functions of active antibodies is E233P (15).

The G236A mutation in combination with S239D/I332E (DE) mutations in the IgG1 antibody, increases the binding affinity to FcγRI (3-fold), FcγRIIa (70-fold), FcγRIIb (13-fold) and FcγRIIIa (31-fold) (177). Despite the enhanced affinity to the inhibitory FcγRIIb, this triple mutation enhanced ADCP by macrophages (177). The addition of A330L to G236A/S239D/I332E (GASDALIE) resulted in increased affinity to FcγRIIa (25-fold) and FcγRIIIa F158 (30-fold), while FcγRIIb affinity was only slightly increased (182, 223).

The H268F/S324T (FT) double substitution resulted in decreased FcγRI, FcγRIIa-131R and FcγRIIb binding (181). However, the addition of the DE and G236A/I332E (AE) substitutions to the FT variant improved FcγR binding considerably. The combination of FT + DE resulted in enhanced binding to FcγRs, particularly to FcγRIIIa, whereas the combination of FT + AT resulted in selective enhancement for FcγRIIa and FcγRIIIa binding. These variants enhanced effector functions accordingly (ADCC: up to 22-fold; ADCP: up to 4.7-fold). The S267E/H268F + S324T variant (EFT) increased binding to FcγRIIa-R131 and FcγRIIb significantly. Combination with the AE substitution produced a variant (EFT + AE) with increased FcγRIIa affinity and FcγRIIIa binding slightly better than native IgG1 (181). Whereas, the amino acid substitutions S267E/L328F when introduced into IgG1, resulted in increased affinity to FcγRIIb (430-fold) with minimal changes in binding to FcγRI, FcγRIIa-131H and no binding to FcγRIIa-158V (196). The mutation P238D also enhanced binding to FcγRIIb, while either completely abolishing or significantly reducing binding to FcγRI, FcγRIIa-131H and FcγRIIa-158V (178). This mutation (P238D) in combination with five additional amino acid substitutions E233D/G237D/H268D/P271G/A330R (termed V12) enhanced binding to FcγRIIb even more (~217-fold) (178, 211, 244). Other sets of substitutions described to enhance IgG1 binding to FcγRIIb encompass G236N/H268D and G236N/H268D/A330K, which are abbreviated V2 and V3, respectively (195). IgG1 with V12-, V2- or V3- bearing Fcs were found to engage FcγRIIb for its rather recently discovered recycling function (244), and demonstrated efficient soluble target clearance *in vivo* when combined with antigen-sweeping Fabs (195, 244, 245). Strongly reduced binding to FcγRIIa-131R, but also to both FcγRIIb and FcγRIIa-158F, can be achieved by a single mutation D270A. This mutation does not affect binding to either FcγRI, FcγRIIa-131H, or FcRn (179).

Both the combination of S298A, E333A, and K334A mutations (AAA) in an IgG1 antibody in combination with DE improved the affinity to both allotypes of FcγRIIIa (179, 198). However, an increase in binding to the inhibitory FcγRIIb was also observed with the DE double mutant (198). By the addition of a leucine at position 330, S239D/A330L/I332E (DLE), this effect was reversed. The DLE mutations cause an open conformation of the Fc by separating the two C_H2 by introducing additional hydrogen bonds between S239D/I332E in the Fc and lysine at position 158 in the FcγRIIIa (206). In addition, Mimoto et al. showed that antibodies with mutations L234Y, G236W, and S298A (YWA), in one heavy chain and DLE in the other, mediated ADCC of tumor antigen-expressing cells at a higher capacity than antibodies that contained only the YWA or DLE mutations (210). Combinations of F243L, R292P, Y300L, V305I, and P396L (variant 18) mutations had an improved Fc binding to FcγRIIa and FcγRIIIa (10-fold) without increasing binding to the inhibitory FcγRIIb receptor (<2-fold), resulting in >100-fold increase ADCC activity (226, 228, 229). However, this combination of mutations has evolved to L235V, F243L, R292P, Y300L, and P396L in follow-up studies (227, 246).

To reduce or prevent IgG binding to FcγRs, a single mutation of leucine to glutamic acid at position 235 is sufficient for reduced binding (100-fold) to the FcγRs on U937 cells (247). The removal of G236 in IgG1 abrogates binding to all FcγR, with negligible trace binding still detectable for FcγRI and minor effect on C1q binding and complement activity (16). A refined IgG1 variation was found which comprises the combination of L234A and L235A (LALA), which reduced detectable binding to FcγRI, FcγRIIa, and FcγRIIIa, but also complement, significantly (192, 248). The use of LALA appears to be more effective than either L234A or L235A alone and only in combination, low to undetectable binding to the high affinity Fc receptor FcγRI can be achieved (14). Although the LALA mutations do not completely abrogate FcγR binding, several antibodies have by now been approved with this Fc configuration, for example the humanized anti-IL36R and anti-CD3 IgG1-based antibodies spesolimab and teplizumab, respectively (249). The LALA mutations have also provided a foundation for the modification of other mutations. LALA in combination with P331S can eliminate binding to FcγRs completely without disrupting the overall conformation of the Fc (206, 208). Similarly, LALA combined with glycine at position 329 (LALA-PG) was an enhancement over LALA mutations alone as the combination eliminated all Fc-mediated effector functions, including complement (191, 250). The LALA-PG containing bispecific anti-CD20 x CD3 IgG1 glofitamab was approved for the treatment of relapsed or refractory diffuse large B cell lymphoma (251). Moreover, more than ten monoclonal antibodies with the same Fc configuration are currently in clinical development (252). Similarly, the LALA-PG mutations with a N297A substitution, resulting in non-glycosylated Fc, also have no detectable binding to any FcγR (16, 208). On top of that, Engelberts et al. showed that the combination of L234F/L235E/D265A resulted in no detectable binding to FcγRI, and reduced binding to both the low affinity FcγRs and C1q (209, 253). In addition, the mutations G236R/L328R either reduced or completely abrogated binding to the FcγRs (FcγRIIa-131R and FcγRIIa-158F were not assessed) and the S267E substitution reduced binding for all low affinity FcγRs (196). A

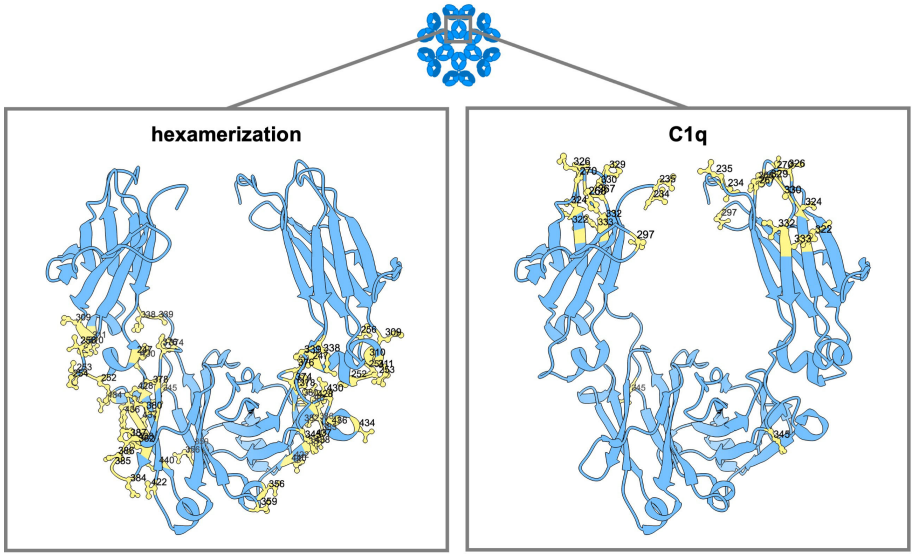

FIGURE 4
Amino acid mutation sites with impact on IgG hexamerization and C1q binding. Ribbon structure of dimeric IgG-Fc regions with highlighted amino acids involved in antibody hexamerization and Fc interaction with complement (C1q).

substitution to lysine at position 267 combined with a series of E233P/L234V/L235A mutations and a deletion of residue G236 showed a lack of binding to all FcγRs (FcγRIIa-158F was not assessed) (225). Together, this points to a crucial role of this lower hinge region to regulate the binding to FcγRs.

Other crucial mutations in various combinations that can eliminate FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa binding include proline 233, alanine 237, and alanine 318 (179, 250). Glycine 237 and glutamic acid 318 are both essential for FcγRII binding (14). Another double mutation is S228P and L235E (SPLE or PE) (254). The SPLE mutations have been introduced into IgG4, although IgG4 has low binding to FcγRs in general due to the phenylalanine at position 234 (255). In addition, it has been shown that S228P stabilizes the structure of IgG4 and prevention of FAE *in vivo*. The Fc variant of IgG2 (referred to as IgG2σ), with V234A/G237A/P238S/H268A/V309L/A330S/P331S substitutions showed no binding to FcγRs and C1q, resulting in the total lack of inducing any immune effector functions (194).

As mentioned before, an alternative strategy to minimize effector functions is to remove the *N*-linked glycosylation site at amino acid residue 297 in the C_H2 domain (256). Point mutations, such as N297A, N297Q and N297G (257), have all been used to this end and N297A and N297G have been incorporated in approved therapeutic mAbs, such as atezolizumab (anti-PD-L1) and mosunetuzumab (anti-CD3xCD20), respectively (258, 259). Interestingly, there have been multiple studies attempting to re-engineer effector function into aglycosylated Fc domains, in order to facilitate the use of prokaryotic expression systems. They showed that engineered deglycosylated Fc variants including substitutions at S298G/T299A (203), N297D/S298T/K326I/A327Y/L328G (DTT-IYG) (260), S298G/T299A/K326I/A327Y/L328G/E382V/N390D/M428L (AglycoT-Fc1004-IYG) (261), and E382V/M428I (262, 263) restore FcγR binding and effector functions.

IgG-Fc substitutions to modulate FcRn binding

Reduced binding to human FcRn was observed, when each of the interface residues I253, H310 and H435 was substituted to alanine (264). Combining the three substitutions I253A, H310A and H435A (IHH) in an IgG1 completely abrogates binding to human FcRn (216). The IHH and the H435A variants are now commonly used as non-FcRn binding variants (216, 265, 266)

Efforts have also been made to influence the IgG-FcRn interaction to increase serum half-life. A study used rationally designed libraries targeting the IgG-FcRn interface around residues 252 to 256 and 433 to 436, which among others identified an IgG1 Fc variant, bearing M252Y, S254T and T256E (YTE) substitutions. This YTE variant exhibited stronger binding to human FcRn (4-fold) at acidic pH without increased binding at neutral pH, and therefore causing increased antibody half-life and serum persistence (214, 215, 267). The YTE-mutated anti-respiratory syncytial virus (RSV) IgG1 nirsevimab has recently been approved as prevention therapy for young children at risk, exhibiting a reported serum half-life of 71 days (268). Following this rational, different variants have been discovered to enhance *in vivo* half-life of IgG antibodies, all based on the same conceptual principle: increasing the binding to FcRn at acidic pH and efficient release at neutral pH. An IgG1 variant with H433K and N434F (KF) substitutions (205) showed enhanced binding to FcRn at endosomal pH (16-fold) and extended half-life in nonhuman primates (NHP) (269). A similar pH-dependent affinity to both human and NHP FcRn was found for the single substitution at position 434 from either asparagine to alanine (~2-fold) or to tryptophan (80-fold), which exhibited prolonged half-life in NHPs compared to wild type IgG (189). Another IgG Fc variant proven to both exhibit 11-fold enhancement in binding affinity to

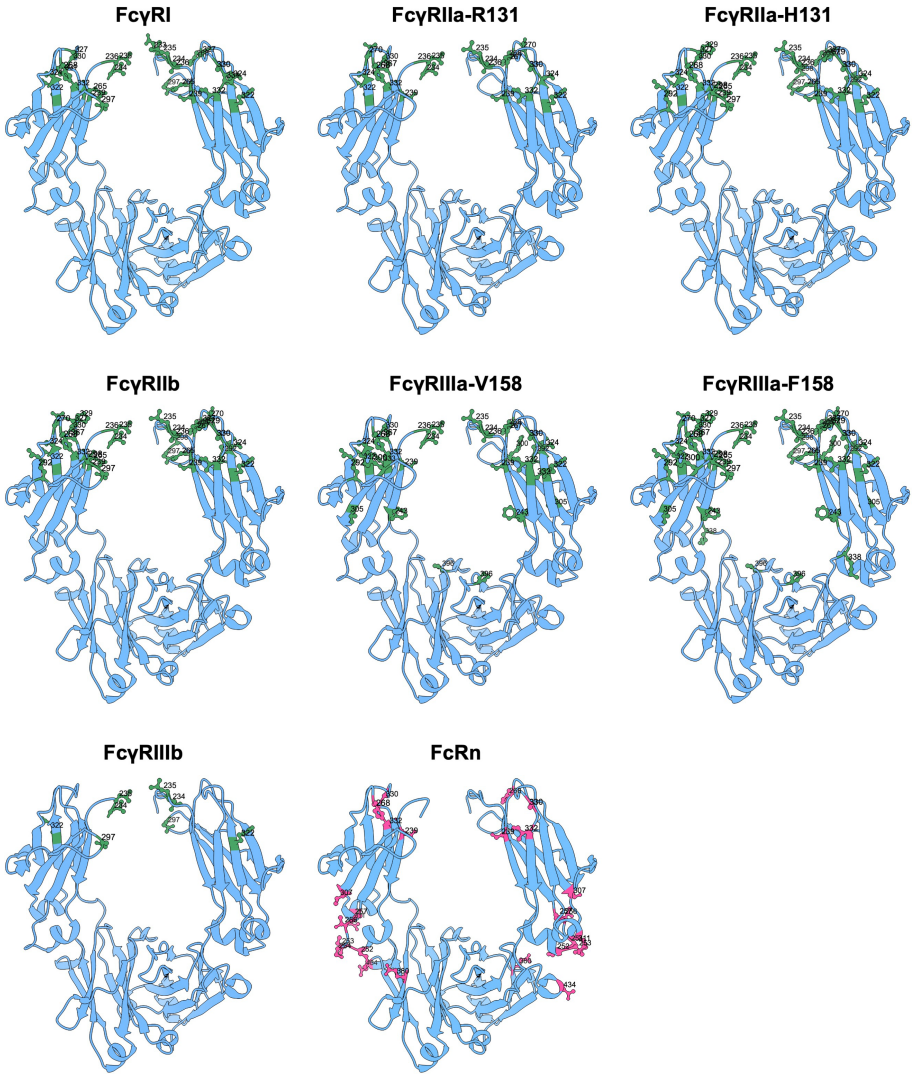

FIGURE 5
Amino acid mutation sites with impact on FcγR and FcRn binding. Ribbon structure of dimeric IgG-Fc regions with highlighted amino acids involved in FcγR and FcRn binding.

FcRn and extend half-life in humans bears M428L and N434S substitutions (LS) (270).

Again, the extended half-life of the LS variant is attributed to the reduced off-rate at pH 6.0 and without increasing binding to FcRn at neutral pH. However, an increased *in vivo* efficacy might depend on the disease model system and target kind, given that the same variant in human FcRn and FcγR transgenic mice did not outperform wild type IgG, despite exhibiting significantly longer half-life (271). Therapeutic antibodies containing an LS variant substituted Fc is are being evaluated in multiple human clinical trials (270). The anti-C5 complement inhibitor ravulizumab was recently approved (272), exhibiting a serum half-life of 56.6 days in patients with generalized myasthenia gravis (273). In addition, The LS variant has also been incorporated in neutralizing monoclonal antibodies (tixagevimab, cilgavimab amubarvimab and romtusevimab) against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (274, 275), all with an extended half-life.

Comparable FcRn binding profiles were described for the single amino acid substitution M428L and the combination of T250Q/M428L, which both achieved significantly slower clearance compared to wild type IgG in rhesus monkeys (276). T250Q/M428L and V308P bearing IgG4s have been found to exhibit prolonged *in vivo* half-life in cynomolgus monkeys (277). Another effort resulted in the identification of three double mutant variants with a half-life comparable to that of the LS variant in both human FcRn transgenic mice and cynomolgus monkeys, comprising either M252Y/T256D (YD), T256D/T307Q (DQ) or T256D/T307W (DW) substitutions (201). Shortly after, the triple mutant L309D, Q311H and N434S (DHS) was described, which was validated in a human FcRn, FcγR and IgG1 transgenic mouse model and even outperformed the YTE and LS variants regarding their half-life (221). However, the prolonged half-life of the YTE (267) and KF (205, 269) variants in combination (YTE-KF or MST-HN) exhibits increased binding to FcRn (20-fold) at both low (pH ≤ 6.5) and neutral pH (278). This YTE-KF variant binds FcRn strong enough to antagonize

the IgG-FcRn interaction and was found to specifically reduce IgG levels *in vivo*. Such an Fc fragment is called an Abdeg (antibody that enhances IgG degradation) (279), which is being clinically investigated under the name efgartigimod for the treatment of IgG-mediated autoimmune diseases and has recently been approved for the treatment of generalized myasthenia gravis (280–282). Other variants with enhanced binding to FcRn at both physiological and acidic pH include M252Y/N286E/N434Y (YEY) and M252Y/V308P/N434Y (YPY) (283), which have been found to utilize FcRn for improved soluble target clearance in NHP when combined with antigen sweeping Fabs (284).

Whereas FcγR binding and *N*-linked glycosylation are generally thought to not affect *in vivo* half-life of IgG (285) and not to affect transport across the placenta (286), a single amino acid deletion at position 294 in IgG1 (ΔE294) was found to result in hypersialylated variants, which exhibited longer half-life than their non-ΔE294 counterparts in human FcRn transgenic mice (218). This was also confirmed for variants bearing half-life extension substitutions found by the same group, such as V259I/N315D/N434Y (C6A-74) and N315D/A330V/N361D/A378V/N434Y (T5A-74) (217, 218). Other than IgG Fc mutations which affect the interaction of IgG with FcRn or FcRn-mediated processes, several groups have found an impact of the Fabs (171), Fab glycans (172), possibly their flexibility is also involved (50), physicochemical properties (109, 287, 288), and antigen-binding on FcRn binding and cellular transport (289, 290).

It is important to highlight that amino acid substitutions, such as LS or YTE, which affect FcRn binding, often also influence the interaction with FcγR (and C1q) and thereby their effector function profile, which is a critical consideration to make depending on therapeutic target and purpose (219, 221, 291).

Outlook: therapeutical antibodies

The number of novel antibody-based molecules undergoing a first regulatory review is at a record level but the number of approved drugs has not kept pace (292). Although we have learned a lot about specific amino acid residues that are required for enhancing or inhibiting antibody-mediated effector functions, the development of antibody-based therapeutics to counteract biological processes may require more than just modifying the sequence of antibody-based molecules to modulate its interaction with the host immune system (293). It has been shown that IgG variants without core fucosylation cause elevated ADCC, through increased FcγRIIIa affinity (56, 127, 128, 146), which has resulted in next-generation glyco-engineered therapeutic antibodies that lack core fucosylation for targeting tumors (292). Some examples include mogamulizumab (KW-0761, AMG-761), which is an afucosylated humanized IgG1 targeting CC chemokine receptor 4 for the treatment of patients with relapsed or refractory adult T-cell lymphoma (294). Another approved afucosulated humanized IgG1 is benralizumab (MEDI-563) which engages IL-5 Receptor α-chain in the treatment of severe eosinophilic asthma (295).

Currently only IgG1, IgG2 and IgG4 antibodies are being considered for therapeutic purposes, but especially IgG3 (most potent subclass in mediating Fc effector functions) and IgG polymorphisms could be utilized to enhance antibody-mediated effector function via to differential binding to endogenous FcγRs and complement proteins (93). Studies have found that monoclonal antibodies with identical variable regions, but different IgG subclasses and allotypes can mediate enhanced Fc effector function, modulated by the hinge length and naturally occurring amino acid substitutions (3, 81, 98). IgG3 is currently not used for any therapeutic antibodies due to its short half-life and large number of alleles which may result in anti-allotypic effects. However, IgG3 variants such as IGHG3*17, *18, and *19 have a half-life equivalent to IgG1 and there is no evidence that allelic mismatch causes any clinical adverse effects (81, 91, 296, 297). Therefore, next generation therapeutic antibodies should consider utilizing IgG3 backbones due to the greater molecular flexibility and stronger affinity to FcγRs and C1q.

Despite being the least abundant IgG in human plasma, IgG4 antibodies, but also mutations such as LALA, are used therapeutically when weak or 'silenced' effector functions are needed. As of 2019, more than 30 biopharmaceuticals with an IgG4-based Fc fragment were approved or were in late-stage clinical development (298). Recently, bispecific monoclonal antibodies have emerged as a growing new class of therapeutics with a wide spread of bispecific antibodies across all stages of clinical trials and platforms (259, 299), some of which were inspired by IgG4 FAE (300–302). Other current areas of development of improved IgG4-based therapeutics focuses on half-life modulation, stability, and inhibiting downstream processes. For example, it has been demonstrated that IgG4 monoclonal antibodies with the S228P/L235E/R409K mutations, not capable of half-molecule exchange, showed less of a tendency to aggregate at low pH than S228P/L235E variants and stabilization of IgG4 antibodies in non-exchanging formats (303). The E357Q/S364K-L368D/K370S variant resulted in a C_H3 region that remained more stable than that of native IgG4 (225). The YTE mutations seem to have the most improved pharmacokinetic properties in combination with reduced effector functions (267, 304).

Although it is possible to learn through natural and engineered Fc modifications, new approaches are needed to consider and screen variants containing multiple mutations, asymmetric binding modes, glycan profiles and effector molecule binding, especially when hexamerization or bispecific antibodies come in play (305, 306). The antibody modifications should be substituted into next generation therapeutic antibodies, but also in monitoring of therapeutically administered IgG (307). Understanding the key determinants that shape antibody-mediated functions is crucial in designing more effective antibody-based therapeutics. Therefore, exploring the associations between IgG allotypes and/or glycan profiles and effector functions, including poorly understood mechanisms such as TRIM21 binding, could open new strategies in the development of therapeutic antibodies. With growing understanding of antibody-mediated immune responses and the constant development of molecular technologies, the future for tailored and effective antibody-based therapeutics seems limitless.

Author contributions

TD: Writing – original draft, Writing – review & editing. MB: Writing – original draft, Writing – review & editing. TvO: Writing – original draft, Writing – review & editing. JS: Writing – review &

editing. AL: Writing – review & editing. TR: Writing – review & editing. GV: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The work of TD was funded by Genmab. The work of MB was funded by argenx.

Conflict of interest

TD, JS, and AL were employed by and/or own warrants and/or stocks in Genmab. The work of MB was funded by argenx. JS and AL are inventors of patents/pending patent applications on technologies and mutations mentioned in this review. GV serves as a consultant for argenx. Genmab and argenx are both biotechnology companies that develop therapeutic antibodies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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