

## Coming together at the hinges: Therapeutic prospects of IgG3

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### ABSTRACT

The human IgG3 subclass is conspicuously absent among the formats for approved monoclonal antibody therapies and Fc fusion protein biologics. Concern about the potential for rapid degradation, reduced plasma half-life, and increased immunogenicity due to marked variation in allotypes has apparently outweighed the potential advantages of IgG3, which include high affinity for activating Fcγ receptors, effective complement fixation, and a long hinge that appears better suited for low abundance targets. This review aims to highlight distinguishing features of IgG3 and to explore its functional role in the immune response. We present studies of natural immunity and recombinant antibody therapies that elucidate key contributions of IgG3 and discuss historical roadblocks that no longer remain clearly relevant. Collectively, this body of evidence motivates thoughtful reconsideration of the clinical advancement of this distinctive antibody subclass for treatment of human diseases.

**Abbreviations:** ADCC - Antibody-Dependent Cell-mediated Cytotoxicity ADE - Antibody-dependent enhancement AID - Activation-Induced Cytidine Deaminase CH - Constant Heavy CHF - Complement factor HCSR - Class Switch Recombination EM - Electron Microscopy Fab - Fragment, antigen binding Fc - Fragment, crystallizable FcRn - Neonatal Fc Receptor FcγR - Fc gamma Receptor HIV - Human Immunodeficiency Virus Ig - Immunoglobulin IgH - Immunoglobulin Heavy chain gene NHP - Non-Human Primate

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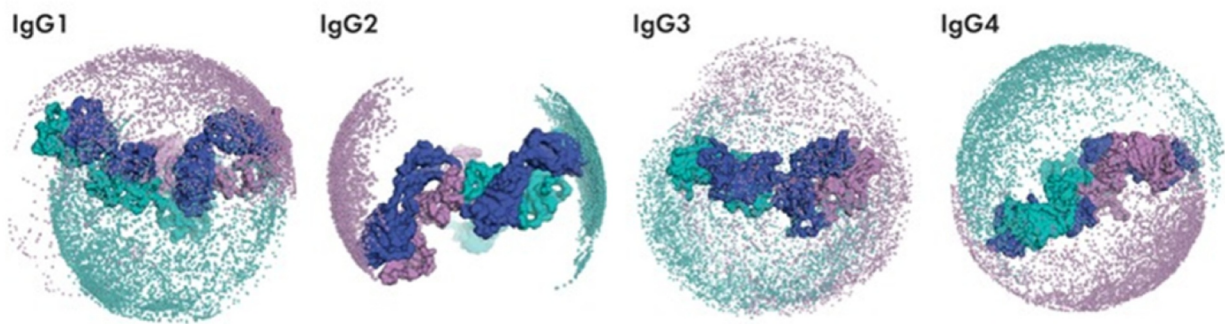
## Introduction

As both understanding of antibody sequence-structure-function relationships and the number of antibody therapeutics in development and clinical use increase each year, it is surprising that the unique attributes of the immunoglobulin gamma 3 (IgG3) subclass have not supported its translation into clinical practice by now. While the absence of IgG3 antibodies for therapy can be attributed to historical concerns about the ability to manufacture IgG3 in large scale as well as its *in vivo* half-life, stability, and immunogenicity, new data suggest that these exclusion criteria should be reconsidered. Here, we summarize the unique structural characteristics of human IgG3, including its extended hinge architecture, which offers both Fab-Fab and Fab-Fc distances and domain flexibilities not observed for other subclasses, and its unique functional attributes, which have yet to be fully leveraged in antibody design. We also explore associations between IgG3 responses in immunization and infection to disease outcomes to elucidate potential immunological significance, and consider subclass switching experiments to provide mechanistic insights into the potential relevance of these associations. We revisit prior studies that have provided inferences to suggest challenges to translational development, and describe recent efforts to stimulate new interest in exploring the range of human IgG diversity for therapeutic purposes.

## Human IgG subclasses: diversity in specificity, structure, and function

The four human IgG subclasses are named according to serum prevalence and exhibit relatively high sequence homology (~95%); however, amino acid variation at key positions results in incredible functional diversity.<sup>1,2</sup> IgG1 and IgG3 subclasses are generally elicited in response to protein antigens associated with viral infections, IgG2 typically targets polysaccharide antigens induced by bacterial infections, and the IgG4 response is associated with repeated exposure to allergens in the absence of an infectious agent.<sup>3-5</sup> Such diversity among target antigens is reflected in functional differences between subclasses. Whereas IgG1 and IgG3 are potent activators of innate immune effector cells, IgG2 and IgG4 possess more subdued effector activity. The existence of these distinct types of IgGs and their association with divergent immunologic stimuli suggests that human subclass diversity results from evolutionary pressures applied over long time periods. However, the immune system also leverages this diversity over short timescales – displaying rapidly divergent activity profiles of isotypes and subclasses when individuals are repeatedly exposed to foreign antigens, in which multiple rounds of class switch recombination (CSR) can occur.

Structurally, the principal differences among the human IgG subclasses relate to their hinge compositions, i.e., the sequence linking the CH1 and CH2 regions that comprise parts of the



	IgG1	IgG2	IgG3	IgG4	Reference
<b>Conformation</b>					
Radius of Gyration	6.00 ± 0.05 nm		4.90 ± 0.05 nm		173
Sedimentation coefficient	6.81 ± 0.10		6.32 ± 0.01		
Rq1, Rq2 (radii of gyration cross section)	2.4 ± 0.1, 1.5 ± 0.1	2.5 ± 0.1, 1.5 ± 0.1	1.8 ± 0.2, 1.4 ± 0.2	2.3 ± 0.1, 1.6 ± 0.2	
<b>Geometry</b>					
Ring dimer (%)	18	4	47	5	23
Mean Fab-Fab angle (°)	117	127	136	128	
Hinge fold flexibility function (Fab-Fab)	±43	±32	±53	±39	
Mean Fab-Fc Angle (°)	107	99	86	98	
Hinge fold flexibility function (Fab-Fc)	±30	±32	±36	±25	
<b>Miscellaneous</b>					
Molecular Mass	146	146	170	146	4
Hinge region (amino acids)	15	12	32/47/62	12	
Interchain disulfide bonds	2	4	5/8/11	2	
Hinge length (Å)			110		24
Total length (Å)			195 ± 10	161 ± 8	174
Known Alleles	14	15	29	4	37

**Figure 1. Flexibility and other features of human IgG subclasses.** **Top:** Representation of the Fab domain flexibility with respect to the hinge regions of the human IgG subclasses in which positions for Fab domains are represented by spheres in purple and teal. Figure adapted from Hansen et al.<sup>6</sup> with permission. **Bottom:** Measured values for various physical attributes of IgG3 compared to other human subclasses.

Fab and Fc domains, respectively (Figure 1). Following reports of full-length human IgG 1, 2, and 4 sequences in the early 1980s,<sup>7</sup> the basis for the most striking distinction of IgG3 was apparent from its sequence, reported a few years later.<sup>8</sup> IgG3 possesses a unique extended hinge, typically composed of quadruplicate proline-rich repeated motifs with extensive interchain disulfide bonds, as well as a slightly extended upper hinge adjoining the CH1 (totaling 62 amino acids), which is encoded by four exons. In contrast, IgG4 has reduced hinge length (12 amino acids, as compared to 15 for IgG1) and can also undergo a process known as Fab arm exchange,<sup>9,10</sup> in which the heavy-chain dimer can dissociate and re-associate in serum. This process leads to functional monovalency in

antigen recognition based on the resultant swapping of Fab specificities. Lastly, IgG2 contains a 12 amino acid hinge with four disulfide bonds. Based on studies of IgG2 disulfide bond isomers,<sup>11,12</sup> it is known that even subtle structural changes in the hinge can affect hydrodynamic radius, antigen binding, ligand blocking, and *in vivo* potency.<sup>12–17</sup>

Collectively, these distinctions among hinges result in a rich structural and conformational landscape (usefully visualized in Hayes et al.<sup>18</sup>). While the hinge contains many constrained proline and disulfide-bonded cysteine residues, its superstructure has been demonstrated to be quite flexible. Only a few antibodies have been crystallized as whole molecules,<sup>19</sup> sometimes requiring perturbation  $\gamma$  correction to gain clarified

density for the hinge, carbohydrate, and solvent-exposed areas. Among IgG subclasses, circular dichroism has shown that the IgG3 hinge has a high degree of secondary structure,<sup>20</sup> and significantly extended Fab–Fc distance. Each core repeat adds an estimated 25 Å between the Fab and Fc.<sup>18,21,22</sup> Electron microscopy (EM), including cryogenic EM, have been used to define IgG hinge flexibility and range of motion<sup>19,23</sup> and enable visualization of multiple conformation states. Aside from Fab–Fc distance, Fab–Fab distance and flexibility, as defined by permissible and mean Fab–Fab angles (Figure 1), are also greater in IgG3 compared to the other IgG subclasses<sup>24</sup> and have been found to directly correlate with upper hinge length.<sup>22</sup> This flexibility results in distinctly different conformational compositions,<sup>6</sup> which could affect epitope accessibility and binding valency.

Such differences in flexibility and reach between Fab arms and the Fc domains among human IgG subclasses suggest that factors beyond intrinsic antigen and Fc receptor binding affinities can affect antibody functionality. Specifically, differences in bivalent antigen-binding capacity have been suggested by a number of studies.<sup>25–27</sup> Toward this end, the most intriguing evidence of the biological consequences of differing hinge composition can be found in the superior HIV-1 neutralization activity of polyclonal, serum-derived bivalent Fab fragments of different subclasses. Whereas equivalent neutralization potency was observed for monovalent IgG1 and IgG3 Fab fragments, suggesting equivalent intrinsic Fab neutralization potency, bivalent IgG3 Fab<sub>2</sub> demonstrated potentiated neutralization relative to IgG1 Fab<sub>2</sub>, pointing toward a key role for distinctions in hinge architecture in activities that do not

require the Fc domain.<sup>28</sup> Notably, multiple broadly neutralizing HIV-specific antibodies were discovered as IgG3s, particularly those recognizing relatively poorly accessible epitopes on the envelope glycoprotein that are proximal to the membrane,<sup>29–31</sup> and subclass switching experiments have demonstrated enhanced neutralization potency of IgG3 forms in a number of cases.<sup>25,32–34</sup> Together, these isotype switching experiments suggest that factors other than Fab affinity contribute to neutralization activity: the unique structure and sequence aspects that differentiate IgG3 from the other subclasses are thought to potentially affect avid viral recognition, a factor shown to be very important to neutralization potency in experiments with non-native linkers between Fab domains and with “unzipped” hinges in which disulfide bond cysteines have been deleted.<sup>33,35</sup>

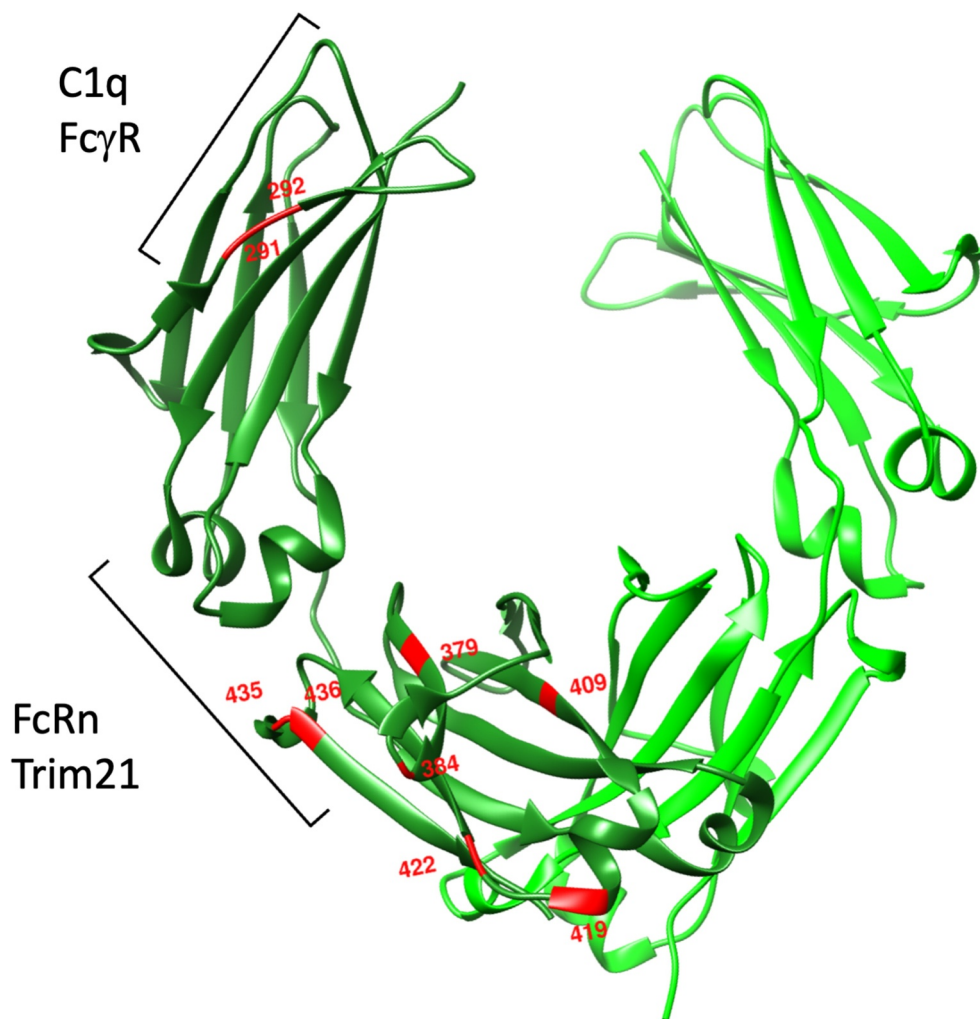
In sum, Fab–Fab and Fab–Fc flexibility along with variable Fab–Fc length distinguish IgG3. Evidence suggests that the IgG3 hinge may allow targeting of antigens or epitopes less suited to ligation by other IgG types, with the potential to more effectively stimulate cellular activity via Fcγ receptors.

### Allotypic IgG3 diversity in humans

With 29 reported allelic variants (Table 1) defined to date, IgG3 is the most polymorphic of the human IgG subclasses (Figure 2).<sup>36,37</sup> Unlike other subclasses that vary principally in terms of isolated amino acid substitutions, IgG3 also has structural allotypes that vary in the number of exon repeats (from 1 to 3) in the core hinge.<sup>38–41</sup> While there is little to suggest direct functional relevance of most allotypic variation

**Table 1. Human IgG3 allotypes.** Individual allelic positions are mapped against IGHG3\*01 sequence by EU position number. The first listed accession number in the IMGT database is listed for each given allelic variant. Although several alleles contain synonymous polymorphisms, only non-synonymous SNPs are listed.

Allele	CH1			Hinge				CH2				CH3+ CHS							Position (EU)	Accession #'s			
	176	192	193	274	291	292	296	309	327	339	379	384	387	392	397	419	435	436					
IGHG3*01	S	S	L	H1+	H2+	H3+	H4	Q	P	R	Y	L	A	T	V	S	P	N	M	Q	R	F	X03604
IGHG3*03				H1+	H3+	H4												V	E			X16110	
IGHG3*04				H1+	H4																		X99549
IGHG3*05				H1+	H2+	H3+	H4																AJ390236
IGHG3*06				H1+	H2+	H3+	H4													K			AJ390237
IGHG3*07				H1+	H2+	H3+	H4													K			AJ390238
IGHG3*08				H1+	H2+	H3+	H4								N								AJ390241
IGHG3*09				H1+	H2+	H3+	H4					V											AJ390242
IGHG3*10				H1+	H2+	H3+	H4																AL122127
IGHG3*11				H1+	H2+	H3+	H4				F												AJ390247
IGHG3*12				H1+	H2+	H4				F													AJ390252
IGHG3*13				H1+	H2+	H3+	H4											K		E			AJ390244
IGHG3*14				H1+	H2+	H3+	H4	L								N						Y	AJ390254
IGHG3*15				H1+	H2+	H3+	H4	L								N		K				Y	AJ390260
IGHG3*16				H1+	H2+	H3+	H4	L										N				Y	AJ390262
IGHG3*17		N	F	H1+	H3+	H4										M			K	V	H	Y	AJ390272
IGHG3*18	Y			H1+	H3+	H4				W						M			K	V	H	Y	AJ390276
IGHG3*19				H1+	H3+	H4				W						M			K	V	H	Y	AJ390279
IGHG3*20				H1+	H2+	H3+	H4	L										N	R			Y	MG920256
IGHG3*21				H1+	H2+	H3+	H4																MG920255
IGHG3*22				H1+	H2+	H3+	H4	L													H	Y	MG920254
IGHG3*23				H1+	H2+	H4				W									K	V	H	Y	MH025837
IGHG3*24				H1+	H2+	H3+	H4											N		K		Y	MG920257
IGHG3*25				H1+	H2+	H3+	H4	K	L													Y	MG920258
IGHG3*26				H1+	H2+	H3+	H4				F		G										MG920259
IGHG3*27				H1+	H2+	H3+	H4	L														Y	MG920260
IGHG3*28				H1+	H2+	H3+	H4				F												MG786813
IGHG3*29				H1+	H2+	H3+	H4	L														Y	MG920261



**Figure 2. Sites of amino acid polymorphisms in IgG3 CH2 and CH3 domains.** Red amino acids indicate sites of amino acid sequence diversity among G3m allotypes. Brackets indicate recognition footprints of various human Fc receptors.

among IgG subclasses,<sup>38</sup> associations between IgG allotypes and a wide variety of infections, malignancies, and autoimmune conditions have been observed.<sup>42–48</sup> In contrast, while IgG3 allotypes have likewise been associated with differences in other aspects of the immune response,<sup>49</sup> both structure and sequence distinctions among allotypes of this subclass are known to affect antibody function.

The best-known example of a highly impactful IgG polymorphism is perhaps the R435H polymorphism observed among several G3m allotypes. This polymorphism alters pH-dependent binding to the neonatal Fc receptor (FcRn), which functions as a mucosal transport and systemic recycling receptor. Whereas allotypes bearing an arginine at this position exhibit rapid clearance with a half-life of approximately 1 week, substitution with histidine results in an extended 21-day IgG1-like half-life.<sup>50</sup> This allotypic variant is quite prevalent in South Asian (10–25%) and African populations (30–60%),<sup>38,51</sup> and has been associated with better transplacental transport of maternal antibody and resulting improvements in neonatal protection from malaria.<sup>51</sup>

Hinge length is a second functional consequence of allotypic variation in IgG3. Polymorphisms in hinge length have been observed primarily in sub-Saharan populations and can occur

concurrently with other point mutations.<sup>4</sup> For example, in the context of an HIV-neutralizing antibody, the IgG3\*17 allotype, with a 47 rather than 62 amino acid-long hinge has been demonstrated to exhibit reduced neutralization potency and effector function.<sup>34</sup> Beyond the shorter hinge, this allotype also has a lysine at position 392, which eliminates a glycosylation motif at a site that has been reported to contribute to molecular stability and affect interactions with FcγRIIIa and antibody-dependent cell-mediated cytotoxicity (ADCC).<sup>34,52,53</sup> Hinge exon deletions that mimic naturally occurring allotypes demonstrate an enhancing effect on complement activation and complement-mediated lysis,<sup>54,55</sup> whereas extensions have been reported to enhance phagocytosis.<sup>56</sup> Collectively, the impact of these genetic differences and potentially others that influence induction, persistence, biodistribution, and the function of IgG3s merits further study in the context of responses to infection or vaccination, as well as in antibody engineering.

### IgG3 Glycosylation

Despite accounting for only 2–3% of the mass of an IgG molecule, glycans affect critical antibody functions such as Fc receptor binding.<sup>57</sup> N-glycosylation of a conserved site in the

CH2 domain is required for antibody binding to Fc receptors, and the specific composition of the glycoform incorporated alters some antibody activities by more than an order of magnitude.<sup>58</sup> While the human subclasses show diverse inherent effector function, the impact of different Fc glycoforms appears to be consistent across subclasses. For example, the enhanced FcγRIII binding imparted by afucosylated glycans is maintained for IgG3.<sup>59</sup> Among naturally derived antibodies, IgG1 and IgG3 N-glycosylation patterns are similar in whole blood as well as among antigen-specific antibody subpopulations,<sup>60</sup> but can differ when produced recombinantly using different cell lines.<sup>61</sup>

In contrast to Fc domain-N-glycosylation, the effect of antibody O-glycosylation on function is less well documented, despite being observed within the hinge region. Only a few antibody classes contain O-glycosylation sites within their hinge regions, including human IgA1,<sup>62</sup> human IgD,<sup>63</sup> human IgG3,<sup>64</sup> and mouse IgG2b.<sup>65</sup> The presence of the O-glycan has been hypothesized to provide proteolytic resistance, but glycosylation sites are only partially occupied in both serum and recombinant IgG3.<sup>64</sup> Whether the presence of O-glycans in the hinge affect structure or function has yet to be thoroughly studied, but may represent another important means to modify antibody bioactivity.

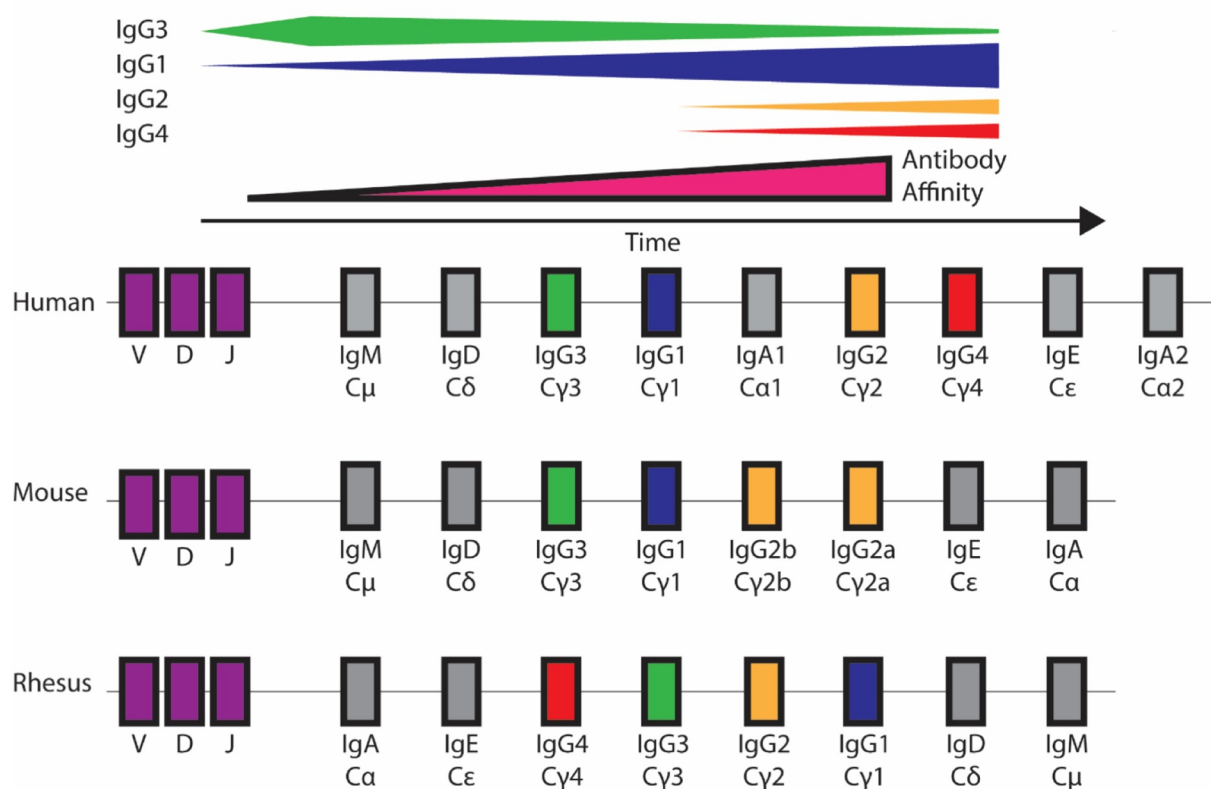
### Subclass and hinge diversity across species

IgG subclasses are not clearly conserved in terms of arrangement, sequence, structure, or activity between humans, non-

human primates (NHP), or mice (Figure 3). In fact, because the subclass naming convention typically follows serum prevalence rather than sequence or functional homology, subclass numbers between species have highly divergent properties. For instance, both mouse IgG2 and IgG3 are capable of activating complement,<sup>66</sup> but mouse IgG2 and its isoforms can additionally activate the immune system through FcγR signaling, and IgG1 lacks or shows dramatically reduced effector function.<sup>67–69</sup> Mice also lack an IgG4 or an analogue, which in humans is capable of undergoing Fab arm exchange. In humans, IgG1 and IgG3 are capable of both potent FcγR signaling as well as complement activation, and IgG2 and IgG4 tend to be more functionally inert. Such broad, overarching differences in IgG subclass characteristics are then compounded by species-specific differences in FcγR properties and expression profiles, which pose substantial challenges when trying to recapitulate the human immune response in animal models.

Activity and receptor affinity differences are more limited among macaque IgG subclasses compared to human and mouse IgG subclasses, whose activity and FcR ligation properties vary by orders of magnitude. In rhesus macaques, all four IgG types have high sequence homology to human IgG1.<sup>70</sup> Neither mice nor NHP possess a subclass with an extended human IgG3-like hinge, and differences in Fab arm exchange of IgG4 also exist between species.<sup>71</sup>

Beyond these fundamental differences in subclass profiles between species, the considerably extended four-exon IgG3



**Figure 3. Human IgG responses over time and IgH locus arrangement in human, mouse, and rhesus.** **Top:** Schematic of subclass composition over time for human IgG. IgG3 appears early and wanes over time. IgG1 additionally increases early and titer remains high. IgG2 and IgG4 appear later in infection. Affinity increases over time during infection due to somatic hypermutation. **Bottom:** IGHC locus arrangement across species. Subclass naming convention is based on serum prevalence rather than genetic similarity.

hinge is a feature that is only currently known to exist in humans (Table 2). Neither rhesus macaques (*Macaca mulatta*), nor chimpanzees (*Pan troglodytes*), nor baboons (*Papio cynocephalus anubis*) have IgGs with extended hinges,<sup>70,72,73</sup> though the chimpanzee IgG3 amino acid hinge, which has only one core hinge region, aligns perfectly with the human IgG3 upper hinge. Among other species, camelid IgG2a contains a 35 amino acid long hinge that has only three inter-chain disulfide bonds, which are situated toward the CH2 domain resulting in a more “open” configuration.<sup>74–76</sup> While the impact of this hinge extension on camelid IgG function is unclear, its existence suggests that length polymorphisms can arise in other species, and may be an example of convergent evolution, as diversification of the Ig locus results in sampling of different sequence-function profiles. Overall, the unique attributes of the human IgG3 hinge in antigen recognition and effector function suggest the existence of a blind spot that may arise from trying to model human responses in animal models.

The IgH locus is variably arranged across species (Figure 3)<sup>77,78</sup> (IMGT Repertoire (IG and T cell receptors) <http://www.imgt.org/IMGTrepertoire/>). Direct comparison across species is much more complex than simply comparing antibodies with similar functions. Certain subclasses share similar effector activity, such as mouse IgG2a and human IgG1, but human Igs do not have the same splicing complexity as mouse. While the exact same process in different species generates similar molecules, each animal has a unique fingerprint to their Ig

repertoires in terms of composition, concentration, and timing of Ig molecules.

### Subclass switching over the course of an immune response

Class switching is a DNA recombination process characterized by double-strand breaks and deletion between “switch” regions driven by activation-induced cytidine deaminase (AID). As a result, while repeated recombination events can occur, they are restricted to downstream subclasses in a process linked to cell division and regulated by cytokines.<sup>79–82</sup> The first IgG subclass in the human IGH locus, IgG3 is associated with potent acute responses, with waning levels often observed over time.<sup>83</sup> Indeed, anti-HIV IgG3 responses peak before 4-weeks post-infection<sup>84</sup> and subsequently decline along with effector function.<sup>85</sup> However, IgG3 responses can be persistently elevated, such as in leprosy<sup>86</sup> and tuberculosis.<sup>87</sup> In contrast, IgG4, the least abundant IgG subclass, is often associated with tolerance and has reduced hinge length and functional monovalency in antigen recognition, as described above. In the context of HIV vaccines, repetitive protein boosting has been associated with increasing prevalence and levels of IgG4 responses,<sup>88–90</sup> a characteristic observed exceedingly rarely in natural infection.<sup>91</sup> Given that sequential switching reactions are associated with greater mutation rates, relatively higher affinity and mutational loads are associated with distal IgG

**Table 2. IgG hinges of various species.** Red lettering indicates disulfide bond positions.

Species	Subclass	Sequence (Upper, Middle)
Human <i>Homo sapiens</i>	IgG1	EPKSCDKTHTCPPCP
	IgG2	ERKCCVECPSP
	IgG3	ELKTPGLDTHHTCPRCPEPSKCDTPPPCPRCPEPSKCDTPPPCPRCPEPSKCDTPPPCPRCP
	IgG4	ESKYGPPCPSP
Chimpanzee <i>Pan troglodytes</i>	IgG1	EPKSCDTTHTCPPCA
	IgG2	ERKCCVECPSP
	IgG3	ELKTPGLDTHHTCPRCPEPSKCDTPPPCPRCP
	IgG4	ESKYGPPCPSP
Western Gorilla <i>Gorilla gorilla</i>	IgG1	EPKSCDTTHTCPPCA
	IgG2	ERKCCVECPSP
	IgG3	ELKTPGLDTHHTCPRCPEPSKCDTSPPCPRCP
	IgG4	ESKYGPPCPSP
Rhesus Macaque <i>Macaca mulatta</i>	IgG1	EIKTCGGGSKPPTCPPCP
	IgG2	GLPCRSTCPPCP
	IgG3	EFTPPCDDTTPPCPPCP
	IgG4	EFTPPCPPCP
Crab-eating Macaque <i>Macaca fascicularis</i>	IgG1	EIKTCGGGSKPPTCPPCP
	IgG2	GLPCRSTCPPCP
	IgG3	EFTPPCDDTTPPCPPCP
	IgG4	EFTPPCPPCP
Pig Tailed Macaque <i>Macaca nemestrina</i>	IgG1	EIKTCGGGSKPPTCPPCP
	IgG2	GRSTCPPCP
	IgG3	EFTPPCDDTTPPCPPCP
	IgG4	EFTPPCPPCP
Yellow Baboon <i>Papio cynocephalus anubis</i>	IgG1	EIKTCGGGSKPPTCPPCT
	IgG2	GHPCRSTCPPCP
	IgG3	EFTPPCDDTTPPCPPCP
House Mouse <i>Mus musculus</i>	IgG2a	EPRGPTIKPCPPCKCP
	IgG2b	EPSPGPISTINPCPPCKECKPCP
	IgG2c	EPRVPITQNPCPPLKECPPCA
	IgG3	EPRIPKSTPPGSSCP
Dromedary <i>Camelus dromedarius</i>	IgG1a	ELKTPQPQSQPECRCPKCP
	IgG2a	EPKIPQPQPKQPQPKQPQPKPEPECTCPKCP
	IgG2c	AHHSEDPSSKCPKCP
	IgG3	GTNEVCKCPKCP
Platypus <i>Ornithorhynchus anatinus</i>	IgG1	EPGPSNPPHCP
	IgG2	GPRFPGNSCP

types.<sup>92–96</sup> Reconstructed histories of CSR among B cell Ig lineages based on variable region sequencing have offered a view of commonly observed patterns of CSR.<sup>97</sup> Among other observations, this work suggests that the proportion of direct switching from IgM or IgD follows position in the IgH locus, with all IgG3s resulting from direct switching, a majority of IgG1s, approximately half of IgG2s, and a minority of IgG4s.<sup>97</sup> Further, consistent with linked induction observed in *in vitro* B cell-stimulation experiments,<sup>98,99</sup> such sequence-based repertoire studies have shown that CSR to IgG3 is preferentially associated with subsequent switching to IgG1.<sup>97</sup>

Interestingly, human IgG subclasses are generally arranged from greater to lesser inflammatory potential (Table 3). This ordering suggests the hypothesis that evolutionary pressures have resulted in an arrangement that supports rapid and potent initial responses that can be dampened by subsequent switching – a scheme that may balance the need for inflammatory acute responses with a means to reduce the consequences of autoimmune antibodies, while still providing long-term protection. Early IgG3 antibodies might be key for enhancing opsonization of pathogenic particles and trafficking to germinal centers, where subsequent B cell somatic hypermutation and affinity maturation feedback loops then allow for the generation of antibodies with greater potency in antigen recognition if not effector function.<sup>100,101</sup> Mature memory B cells tend to skew toward IgG and IgG4 expression, while naïve memory B cells tend toward more IGHM proximal subclasses IgG1 and IgG3.<sup>96</sup> While this locus arrangement – coupled with the low serum proportion of IgG3, its rapid clearance, and waning prevalence after acute responses – may suggest possible downsides to the inflammatory potential of IgG3, there are many human observational studies that suggest its beneficial contributions to protection from infection.

## Natural infection histories

Classically, IgG3 has been grouped with IgG1 in terms of immune function because both bind-activating FcγR well and primarily target protein antigens.<sup>1,102–104</sup> As might be expected given their linked induction in *in vitro* CSR experiments,<sup>98,99</sup> IgG1 and IgG3 responses have been observed to be correlated.<sup>105,106</sup> Between them, however, IgG3 has often been reported to demonstrate enhanced phagocytosis<sup>2,56,107–109</sup> and complement deposition, particularly<sup>108, 109, 110;</sup> in the context of low-density target antigen.<sup>110–112</sup>

**Table 3. FcγR binding affinities of human IgG Subclasses.** Some interactions were not measurable (n.m).

Receptor	Affinities $K_A$ ( $\times 10^5$ $M^{-1}$ )				Reference <sup>1</sup>
	IgG1	IgG2	IgG3	IgG4	
FcγRI	650	n.m.	610	340	
FcγRIIA (H131)	52	4.5	8.9	1.7	
FcγRIIA (R131)	35	1.0	9.1	2.1	
FcγRIIB/C	1.2	0.2	1.7	2	
FcγRIIA (F158)	12.0	0.3	77	2	
FcγRIIA (V158)	20.0	0.7	98	2.5	
FcγRIIB (NA1)	2.0	n.m.	11	n.m.	
FcγRIIB (NA2)	2.2	n.m.	9	n.m.	
FcγRIIB (SH)	2.0	n.m.	11	n.m.	

Consistent with this enhanced functional potency, induction of IgG3 can be a key marker for protection, whereas its absence is associated with infectious disease susceptibility. Adults and children with otherwise normal total IgG levels and functional B cell compartments but IgG3 subclass deficiency tend to present with recurrent upper respiratory tract infections.<sup>113–116</sup> These individuals tend to respond well to intravenous Ig treatment,<sup>117–120</sup> suggesting that their antibody repertoires are relatively functionally naïve toward common pathogens despite multiple encounters. Whether IgG3 is directly responsible for pathogen sequestration or contributes to the development of other protective antibodies is unclear and merits further consideration.

In support of potential mechanistic relevance, IgG3 responses have been associated with better outcomes across diverse infectious pathogens in the setting of both natural infection and vaccination. For instance, IgG3 responses against the HIV V1/V2 loop regions in the RV144 HIV vaccine trial correlated with decreased infection risk.<sup>88,89,121</sup> Early IgG3 responses against chikungunya virus infection are associated with more rapid viral clearance and a reduction in long-term arthralgic sequelae,<sup>122</sup> and in clearance of acute hepatitis C virus infection.<sup>123</sup> A number of studies have reported that IgG3 responses induced by infection are associated with reduced risk of malaria disease. Interestingly, IgG3 responses increase with age, as does clinical immunity.<sup>124</sup> Associations between IgG3 and better outcomes are also observed in children.<sup>125–127</sup> In the context of the IgG3-H435 polymorphism that leads to better transport and recycling by FcRn, IgG3 responses have even been linked to infant protection through enhanced placental transfer of maternal antibody.<sup>51</sup> In contrast, immunity to influenza from vaccination wanes with age,<sup>128</sup> along with a corresponding reduction in the prevalence and magnitude of flu-specific IgG3,<sup>129–131</sup> among many other parameters.

In contrast, in other settings, antibodies can increase infection risk or disease severity by facilitating viral uptake into host cells that express antibody receptors. In these cases, the enhanced ability of IgG3 to ligate antigen and interact with FcγR has the potential to exacerbate disease. As examples, IgG3 antibodies against Ebola, Zika, and enterovirus have been shown to exhibit higher levels of antibody-dependent enhancement (ADE) of infection *in vitro* than other subclasses.<sup>132–134</sup> The most compelling evidence of the relevance of ADE to infection *in vivo* comes from dengue, in which severity of secondary infection is associated with levels and types of virus-specific antibodies present in serum. Subneutralizing quantities,<sup>135</sup> but more concerningly, effective FcγR ligation associated with relatively high IgG1/IgG2 ratios and afucosylation<sup>136,137</sup> have been associated with disease enhancement, though correlation between IgG3 responses and severe disease has not been described.

In summary, it is clear that the potentiated effector function profile of IgG3 can be associated with enhanced protection, suggesting the contributions this unique subclass could make in the context of antibody therapeutics. In cases where IgG3 responses may be causally linked to different infectious disease outcomes, diverse mechanisms are possible. Beyond expected differences in effector function, previous studies have linked

this subclass to potentiated neutralization activity in polyclonal responses<sup>28</sup> and among monoclonal antibodies.<sup>25,32,33,138</sup> Importantly, the neutralization enhancements observed for subclass-switched monoclonal antibodies imply that, beyond the well-known impact of subclass and glycosylation on FcR binding and effector function, structural differences between subclasses can affect other potentially protective antibody activities.

### Lost in translation

Subclass selection represents a modular and predictable means to control antibody function, making it a key aspect of therapeutic antibody development.<sup>139</sup> Despite being perhaps the most functional subclass,<sup>1,53,140,141</sup> there is a surprising lack of IgG3 antibodies among approved therapeutics, as well as a paucity among early clinical and pre-clinical candidates under evaluation. This absence can be attributed to four historical factors: 1) greater allotypic variation among IgG3s, raising concern about potential immunogenicity;<sup>142</sup> 2) the reduced plasma half-life typical of IgG3, increasing the frequency or level of dose required to achieve a given antibody concentration relative to IgG1;<sup>143</sup> 3) the absence of binding to protein A, eliminating this standard approach to antibody purification and requiring alternative and more costly approaches;<sup>144</sup> and 4) the potential of proteolytic susceptibility of the extended hinge, raising concern about *in vivo* stability.<sup>139</sup> However, as discussed below, these perceived challenges now have simple solutions or new data suggest that they may not be sufficiently scientifically backed to cause concern.

As mentioned previously, a single, naturally polymorphic amino acid substitution improves the half-life and mucosal transport properties of IgG3 to those observed for IgG1.<sup>50</sup> Given the success of non-native Fc domains in the clinic, such as those engineered for extended half-life,<sup>145–147</sup> selection of a H435-bearing IgG3 allotype to impart persistent plasma pharmacokinetics seems a simple and low risk means to eliminate what has likely been the single biggest barrier to clinical translation. Allotypic variability has not been associated with anti-drug antibody responses.<sup>148–150</sup> In fact, a number of the polymorphic positions in IgG3 are “isoallotypes”, meaning that these substitutions are present in other subclasses and thus may not have a propensity to be immunogenic.

As to challenges in purification, other purification methods, such as protein G resin<sup>151</sup> and CaptureSelect FcXL affinity matrix are available, and have been used for commercial purification. Alternatively, the use of the same H435 IgG3 allotype that extends half-life also allows for protein A binding,<sup>152</sup> enabling the use of current standard industrial purification methods. Like other subclasses, IgG3 is susceptible to aggregation at low pH.<sup>153</sup> IgG3 potentially has somewhat elevated susceptibility in this regard, including aggregation during expression, as compared to IgG1, but recent work has used sequence swapping to reduce this profile.<sup>154</sup> In terms of thermal stability, IgG3 has been omitted from some studies given its absence from the clinic.<sup>155</sup> However, it is likely that further improvements to expression, purification, and storage schemes will also

provide value and help to smooth the path toward broader *in vivo* and clinical investigation.

Lastly, despite greater sensitivity to enzymatic cleavage *in vitro* in intentional proteolysis experiments,<sup>156–158</sup> hinge cleavage has not been reported to pose a barrier *in vitro* or *in vivo*. Further, as gene therapy tools advance, the prospects for vectored delivery<sup>159,160</sup> of IgG3 could bypass concerns about clearance and stability. Collectively, new evidence and clinical experience suggest that the absence of clinically advanced IgG3 is a historical artifact, and lack of precedent need not be considered a barrier to their future use. Gaining regulatory agency and industry buy-in for development of the first IgG3 monoclonal treatment may be more a problem of changing minds than overcoming scientific barriers.

### Forward toward the clinic: evaluation in animal models and the first human IgG3s tested in humans

A limited number of subclass switching experiments have been carried out that enable comparison of the *in vitro* and *in vivo* activities between subclasses. One study in mice described similar biodistribution but did not test *in vivo* efficacy.<sup>56</sup> In another study, despite lower serum titers, the passive transfer of an HIV envelope glycoprotein variable loop-specific IgG3 antibody was at least equally effective in preventing viral infection as IgG1 in an NHP model of HIV infection.<sup>161</sup> In contrast, in a mouse model of melanoma, IgG3 against the TA99 target antigen was not superior to IgG1 at protecting against metastasis.<sup>162</sup> Though generally supportive of the feasibility to clinically translate IgG3, these case studies do not reflect settings in which IgG3 antibodies were superior in their *in vitro* functions: the TA99-specific IgG3 was not superior to IgG1 *in vitro*, and the HIV envelope variable loop-specific IgG3 showed similar binding activity and phagocytic activity as compared to IgG1.<sup>109</sup> This latter experiment was performed instead to investigate the basis of IgG3 responses to this epitope being correlated with reduced risk of infection in the RV144 vaccine trial.

The strongest evidence in support of enhanced activity of IgG3 *in vivo* comes from the setting of antibodies to pneumococcus. Here, a longer half-life allotype of IgG3 was shown to provide improved protection against pneumococcal pneumonia, as defined by colony-forming units in lung, as compared to IgG1.<sup>50</sup> Other contexts in which IgG3 is clearly more potent than IgG1, such as in the bactericidal activity of antibodies specific to meningococcal factor h binding protein,<sup>163,164</sup> may provide further opportunity to better establish the ability of *in vitro* functional enhancements to improve *in vivo* protective activity. To this end, an anti-Protein A IgG2 antibody fragment isolated from a human donor via phage display was reformatted as an IgG3 and demonstrated strong activity against antibiotic sensitive and methicillin-resistant *Staphylococcus aureus in vitro* and was able to provide 60% protection from fatal *S. aureus* challenge in a mouse model.<sup>165</sup> Other subclasses, however, were not evaluated in comparison. Nonetheless, the promising results of these limited *in vivo* studies and the more prevalent observations from natural infection correlates and *in vitro* studies in which subclass switching from IgG1 to IgG3



has enhanced desirable antibody activities suggest that further investigation *in vivo* is merited.

To our knowledge, two monoclonal IgG3 antibodies have been investigated clinically. A Phase 1/2 clinical trial (NCT02357966) evaluated the Protein A-specific IgG3 monoclonal (omodembamab or 514G3) described above in the setting of *Staphylococcus aureus* bacteremia with promising results. The IgG3 backbone was specifically chosen to avoid Fc interactions with protein A (SpA). Concluded in 2017, this clinical trial found that the IgG3 treatment was well tolerated and generated few severe adverse events.<sup>166</sup> Efficacy data was self-reported by the sponsoring company (Xbiotech Inc.), but as of this time not peer-reviewed. The use of IgG3 to avoid protein A interaction is a good demonstration of utilizing subclass selection for therapeutic design, and its tolerability is encouraging from a safety standpoint.

In the oncology space, one recent study of lung cancer patients found an IgG3 autoantibody against complement factor H (CFH) that was associated with early-stage disease and no evidence of metastasis. Single B cells producing the CFH-specific response were isolated from these patients, the Ig gene sequenced, and a series of preclinical studies then demonstrated tumor cell killing with modulation of the adaptive immune response using the molecule GT103.<sup>167</sup> A recombinant CFH-specific monoclonal antibody, originally identified and formulated as an IgG3, has now been successfully manufactured on a large scale for clinical trial NCT04314089, which was initiated in June 2020. This human-derived antibody will be the first monoclonal therapeutic IgG3 to be tested in humans against cancer and will certainly provide essential information about IgG3 as a viable therapeutic option.

## Beyond natural Ig diversity

Cumulatively, natural histories and subclass switch experiments also point to the potential value of IgG3-inspired engineering of other subclasses. To this end, substantial work has already been invested in innovating on natural forms. Considering developability, IgG3 has been engineered for reduced aggregation and better tolerance of pH stress.<sup>154</sup>

Beyond correction of undesirable physicochemical attributes, chimeric IgG3s have been advanced as molecules with enhanced functionality. For example, such chimeras have shown enhanced complement-dependent cytotoxicity, ADCC function, and target cell depletion *in vivo* in NHP.<sup>53,168</sup> One such molecule is GSK2849330,<sup>169</sup> an anti-HER3 glycoengineered IgG1/IgG3 chimera investigated in two Phase 1 clinical studies to date: NCT02345174, initiated in 2015 and completed in 2016; and NCT01966445, initiated in 2013 and completed in 2017. This molecule was engineered for enhanced ADCC and complement-mediated cytotoxicity by utilizing the IgG3 Fc, although the longer half-life amino acid allotype/point mutation was not used. An imaging study of a radiolabeled form of this antibody showed good tumor uptake,<sup>170</sup> and in a separate study of 29 individuals, one exceptional responder was noted.<sup>171</sup> Accordingly, these and other such strategies may hold further promise in combination with existing glycan

engineering and the wealth and diversity of amino acid point mutations developed to alter FcR and C1q interactions.<sup>172–174</sup>

Based on studies suggesting the significance of the IgG3 hinge to its unique properties, non-native hinges have also been investigated. Constructs exploring alternative hinge topologies have included substitution with traditional, conformationally flexible linkers such as repeating Gly<sub>4</sub>Ser subunits;<sup>175</sup> “unzipped” native hinges, in which cysteines have been eliminated in an effort to increase the span between Fab domains;<sup>33</sup> and hinge-length extension by repeating natural hinge exon subunits an unnatural number of times.<sup>56</sup> These approaches, which can enhance antibody function by orders of magnitude, are thought to work by enhancing binding to low abundance, poorly exposed, or distantly spaced target epitopes. Collectively, these results suggest that beyond translation of natural IgG3 constant domains, antibody engineering based on the unique structural and functional attributes of IgG3 holds promise in improving the potency or activity of IgG1-based therapies.

## Conclusion

In summary, considerable epidemiological data suggest that relative to their serum prevalence, IgG3 antibodies make an important contribution to effective humoral immune responses. Extensive subclass switching experiments *in vitro* point to the potential mechanistic relevance of associations in these cohort studies, with IgG3 often inducing elevated phagocytosis and greater complement activation. Albeit limited, the *in vivo* studies conducted to date comparing IgG3 and IgG1 antibodies suggest that IgG3 results in equivalent or superior outcomes, supporting further exploration of this subclass in early preclinical studies for applications where its structural and functional distinctions may prove advantageous. These applications include those where antibody effector function is desirable, where antigenic epitopes may be sparse or difficult to access, and in the settings in which cohort studies have associated IgG3 responses with improved outcomes. That IgG1 allotypic diversity does not appear to lead to increased immunogenicity risk and non-native Fcs are now commonly employed in the clinic suggest that a high level of concern about elevated anti-drug antibodies against IgG3 is unwarranted. With the ability to extend the half-life, and produce, purify and manufacture IgG3 on a large scale, fundamental arguments against clinical translation of IgG3-based or IgG3-inspired interventions have been successfully resolved. Further experimental data to support IgG3 as a potent therapeutic antibody in cases where enhance effector function and its altered structural characteristics might provide therapeutic advantages, and results from clinical trials to show efficacy could open the door toward additional development of this subclass as an important tool to treat a spectrum of human diseases.

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## Disclosures

EFP is an inventor on a patent/application(s) relating to a novel IgG3, is co-founder, CEO, and a shareholder of Grid Therapeutics, a company conducting a phase I clinical trial of a human IgG3. EFP has in place an approved plan for managing any potential conflicts arising from these interests.

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