**Title:**

**Impact of post-translational modifications and subclass on IgG activity: From Immunity to Immunotherapy**

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**Abstract**

Humoral immune responses are characterized by complex mixtures of polyclonal antibody species varying in their isotype, target epitope specificity and affinity. Post-translational modifications occurring during antibody production in both the antibody variable as well as constant domain create further complexity and can modulate antigen specificity or antibody Fc-dependent effector functions, respectively. Finally, modifications occurring to the antibody backbone post secretion may further impact antibody activity. An in-depth understanding of how these post-translational modifications impact antibody function, especially in the context of individual antibody isotypes and subclasses, is only starting to emerge. Indeed, only a minute proportion of this natural variability in the humoral immune response is currently reflected in therapeutic antibody preparations. In this review we summarize recent insights into how immunoglobulin G (IgG) subclass and post-translational modifications impact IgG activity and discuss how these insights may be used to optimize therapeutic antibody development.

**Introduction**

For many years, the major decision in developing a therapeutic antibody was whether to choose an antibody format allowing for efficient or limited interaction with cellular and/or humoral effector pathways. With the need to optimize existing cytotoxic antibody activities and the introduction of novel antibody classes, such as immune checkpoint blocking (ICB) or immune stimulatory antibodies (ISA), more complex scenarios had to be considered. Due to the prominent role of immunoglobulin G (IgG) in protective as well as autoreactive immune responses and its near-exclusive use in human therapeutics we will restrict our discussion to this antibody isotype. Indeed, a multitude of studies have highlighted how different IgG subclasses, as well as post-translational modifications, modulate IgG activity. Nevertheless, it is important to note that other antibody isotypes including IgA and IgE, alongside the B cell receptor itself, can be modulated in their activity by post-translational modifications as recently reviewed1, 2, 3.

IgG antibodies are characterized by long serum half-life, the ability to penetrate most tissues, and the capacity to trigger pro- as well as anti-inflammatory effector pathways due to the interaction of the IgG Fc-portion with both cellular and humoral immune system pathways (Fig. 1)4, 5 . This includes binding to the neonatal Fc-receptor (FcRn), responsible for vesicular trafficking and long half-life of most IgG subclasses, specific cell surface Fcgamma receptors (FcRs) as well as cytosolic Fc-receptors such as Trim21, and components of the complement pathway (Fig. 1b) 4, 5, 6. Collectively, the four IgG subclasses in mice and humans show similar distinctive diversity in their capacity to trigger effector functions, such as toxin/virus neutralization, antibody- or complement-dependent cytotoxicity (ADCC or CDC, respectively), phagocytosis (ADCP) or the release of pro-inflammatory mediators7. However, due to historical reasons, their subclass designations do not functionally match between species8. For example, human (h) IgG1 and hIgG3 as well as mouse (m) IgG2a or mIgG2b broadly interact with most activating FcRs and also efficiently activate complement (Fig. 2 and Table 1). In contrast, hIgG2 and hIgG4 and mIgG1 subclasses show more restricted binding to FcRs and complement components. Although this functional diversification is clearly important, it is also redundant to some extent as individuals with immunoglobulin subclass deficiencies, for example lacking IgA1 and all IgG subclasses except IgG3, seem capable of compensating with the remaining antibody repertoire with respect to overall health9, 10.

For cytotoxic antibodies intended to delete unwanted target cells, a common strategy is to have a long half-life, optimal interaction with activating FcRs and/or the complement pathway, while diminishing binding to inhibitory FcRs. The hIgG1 subclass fulfils most of these requirements (Table 1) and so is the frequent choice for this therapeutic antibody class. This “what? Choice?” is despite the fact that several hIgG3 variants display higher activity in all functional categories. hIgG3, however, has more than 15 known allotypic variants found at different frequencies globally, presumably because of evolutionary pressures from different infectious diseases11. Therefore, the relatively less polymorphic nature of IgG1 makes it more suitable for application in humans5, 12.

With the introduction of immunomodulatory antibodies (both ICB and ISA) intended to induce or restrict immune stimulation without causing excessive or off-target immune activation, more complex scenarios have evolved placing other IgG subclasses, such as hIgG2 or hIgG4 center stage. In turn, these requirements have led to new insights into IgG subclass biology, such as the ability for Fab arm exchange and high-affinity binding to FcRI by hIgG4 and the impact of different hIgG2 isoforms on agonistic antibody activity 13, 14. Moreover, accumulating evidence suggests that bone marrow-derived as well as tissue-resident myeloid cells, which express the most diverse sets of IgG receptors, play a major role in IgG-dependent effector functions, relevant for all therapeutic antibody classes 15. This “what” necessitates the adjustment of therapeutic IgG activity to various effector cells expressing different levels of both activating and inhibitory receptor systems 16, 17. Of note, IgG responses occurring in vivo become further diversified by a complex set of post-translational modifications on the background of different IgG subclasses, which provides adjustment of IgG activity. In contrast, current therapeutic antibody preparations are designed – also for regulatory reasons - with the lowest possible level of heterogeneity. Thus, understanding how different IgG subclasses and their many post-translational modifications, occurring before or after IgG secretion, modulate IgG function holds great promise for optimizing therapeutic antibody activity.

**Naturally occurring posttranslational modifications**

All proteins in the human body are subject to post-translational modifications. The impact of post-translational modifications on IgG stability, half-life and function became increasingly clear during the development of therapeutic antibodies (Fig. 1a, c) 18. Changes include oxidations, charge distribution, aspartate isomerization, asparagine deamidation, cysteine introductions, lysine glycation, and introduction of N-linked glycosylation sites19. For reasons of space, we will focus largely on post-translational modifications affecting IgG-effector functions. Here, three major modifications of natural antibodies are relevant for all IgG subclasses and their allotypes5. First, the C-terminal lysine residues in IgG become rapidly clipped from at least human IgG1 and IgG2 after secretion in human plasma by carboxypeptidase N. In the absence of this processing step, IgG’s ability to activate the classical complement pathway is severely diminished because these charged residues interfere with the hexameric assembly of IgG required for optimal C1q binding (Fig. 1c) 20, 21.

Second, involves the gradual accumulation of altered amino acid residues. In particular, this affects the exposed methionine residues at positions 252 and 428 which are localized in the CH2/CH3 interface that is involved in FcRn binding 22. Over time, a slow increase in oxidation occurs which negatively affects the interaction with FcRn, and hence IgG half-life 23 (Fig. 1c). The rate of this conversion is also likely to occur faster in antibodies either produced or accumulating in inflamed areas, as can be the case for autoantibodies or therapeutic antibodies mediating their activity in inflamed tissue environments 24. Moreover, oxidation of tryptophan 277 was suggested to correlate with IgG Fc flexibility which impacts FcRIIIa binding 25. Similarly, deamidation of asparagine 325 in the upper CH2, near the FcR binding region can also affect FcR effector functions negatively 26.

Third, a sugar moiety is added in the endoplasmic reticulum at asparagine 297 (N297) in each of the two IgG heavy chains (Table 2). This glycan is trimmed and then extended in the Golgi, resulting in a biantennary glycan that occupies a large part of the cavity between the two heavy chains comprising the Fc domain (Fig. 1a-c). Its basic core structure consists of two N-acetylglucosamines (GlcNAc), followed by three mannoses forming two antennae, each extended with a GlcNAc residue. These GlcNAc residues can be further extended with a galactose and ultimately a terminal sialic acid. The core structure can also bear a bisecting GlcNAc residue, and generally contains a core fucose. While the biochemical pathway of IgG glycosylation is well understood, the genetic pathways and environmental and developmental cues underlying differential IgG glycosylation are only beginning to emerge 27.

The glycan is important as it interacts directly with the FcR backbone, while also altering the orientation of the two CH2 domains, and potentially their flexibility, to affect FcR binding 28, 29. While C1q binding is also substantially reduced without the N297 glycan, FcRn interactions remain intact and glycan composition does not impact FcRn-mediated half-life or trans-placental transport 30, 31. Interestingly, for specific IgG subclasses, such as mouse IgG2c or human IgG1 a single GlcNac residue with, or without, a branching fucose residue is sufficient for functional interaction of mouse and human IgG with FcRs 32.

Most IgG glycan changes occur within antibody-secreting B cell subsets such as plasmablasts and plasma cells. In line with this notion, antigen-specific IgG glycosylation patterns between different antigens in the same individual can vary enormously and genes associated with differential IgG glycosylation are all expressed in these B cell subsets 33. Similarly, individual mIgG subclasses have subclass specific glycosylation profiles 34, 35, 36. In contrast to exposed sugar moieties, the steric inaccessibility of the IgG-Fc glycan to enzymes after folding may explain the stability of these glycans after secretion (Fig. 1a,b) 37. Of note, however, structural studies of IgG in solution suggest that the IgG sugar domain is highly dynamic and could be accessible for recognition by other proteins38. Whether terminal sialic acid residues may be added after IgG secretion remains controversial 39. However, this plasmablast/plasma cell-extrinsic IgG sialylation process is slow and predominantly affects the exposed F(ab) and not Fc-associated sugars 40. Moreover, sialylation of individual antigen-specific IgG species differs within the same individual in mice and humans and may vary from the sialylation pattern of bulk IgG, suggesting that IgG sialylation is modulated in plasmablasts and plasma cells by external stimuli and occurs pre-secretion 33, 35, 36, 41.

Of note, the differing N-linked glycans in the Fc-domain renders it more variable than initially appreciated. hIgG found in plasma is generally almost fully fucosylated (~94%, except in some immune responses as discussed below), largely devoid of bisecting GlcNAC (10-15%), but highly variable with regard to galactosylation (20-80%) and sialylation (2-10%) 42. Importantly, serum IgG glycosylation is not stable throughout life. For example, IgG galactosylation and sialylation reduces with advancing age, especially during menopause in females 43, 44, 45. In contrast, during pregnancy, IgG galactosylation increases 46. Of those changes, IgG-Fc fucosylation, galactosylation, and sialylation are particularly important for modulating IgG-effector functions (Table 1).

***IgG-Fc-fucosylation – modulator of FcγR activity***

It has been recognized for ~20 years that afucosylated IgG has a strongly enhanced affinity for hFcRIIIa, but not hFcRIIa or hFcRIa, which leads to increased antibody-dependent effector functions 47, 48. Selective enhancement of binding to hFcRIIIa (and the neutrophil-specific GPI-linked hFcRIIIb) is due to the unique presence of an N-linked glycan at position 162 in both hFcRIIIa and hFcRIIIb as well as mFcRIV, the mouse orthologue. The absence of fucose allows a tighter interaction and enhances hIgG affinity 20-40 fold 49, 50. Further increases in affinity can be achieved by increasing galactosylation on afucosylated sugar moieties 37, 48, 51 (Table 1). Depending on the expression level of hFcRIIIa and co-expression of other activating hFcRs, afucosylated IgG variants trigger stronger IgG-dependent effector functions including ADCC and ADCP 47, 52, 53, 54. Of note, immune cell subset differences as well as hFcRIIIa allotype-specific glycosylation variants exist, which can further modulate IgG glycovariant binding and downstream effector functions 55, 56, 57.

Due to the virtual absence of afucosylated IgG in serum, the effect of IgG afucosylation on FcR-mediated activities was largely considered only relevant for IgG engineering to enhance antibodies used in the therapy of cancer or autoimmune disorders. However, more recent insights coming from genome-wide association studies suggest that several SNPs in transcription factors such as the Ikaros zinc finger family (IKZF1 or IKZF3) may impact fucosyltransferase 8 expression in B cells and modulate IgG fucosylation 27. Despite these first insights, a general, mechanistic understanding of how IgG fucosylation and IgG glycosylation are regulated is still largely missing. Moreover, further studies have shown that specific antibody responses can be almost exclusively due to afucosylated IgGs (Fig. 3). A prime example is IgG responses against red blood cell blood group and platelet antigens in pregnancy and during blood transfusion 58, 59, 60. In the setting of fetal anemia or thrombocytopenia, triggered by alloantibodies transferred from mother to fetus, the level of alloantibody afucosylation rather than total alloantibody levels, correlates with disease severity (Fig. 3) 41, 58, 61. More recently, afucosylated IgG responses against HLA have also been found in platelet-transfused patients with refractory thrombocytopenia and after kidney transplantation and associated antibody-mediated rejection 62, 63. These alloimmune responses are all directed against antigens on the surface of cells, mimicking foreign antigens expressed by enveloped viral pathogens and intracellular parasites, such as *Plasmodium falciparum* during its facultative red blood cell stage 33, 64, 65, 66. Afucosylated IgG responses to Dengue virus have been associated with enhancement of infection and progression to more pathogenic disease (Fig. 3) 67, 68. In COVID-19, IgG antibodies promote entry into hFcRIIIa positive cells, but instead of enhancement of infection, they trigger immunopathology via inflammasome activation and pyroptosis (Fig. 3) 69, 70. It is tempting to speculate that this pathway is also excessively activated by afucosylated IgG which occurs during seroconversion in primary infections with SARS-COV-2, and is associated with increased severity, likely through enhanced hFcRIIIa activation 33, 64. Afucosylated IgG responses are not naturally generated against soluble, bacterial or non-enveloped targets, but are generated during hIgG1 and hIgG3 responses to protein antigens 65, 71. Similarly, hIgG2 responses, largely triggered by T-independent polysaccharide antigens, are almost fully fucosylated 72. However, all glycoengineered hIgG subclasses, including hIgG2 and hIgG4, gain appreciable affinity for hFcRIIIa after afucosylation, and, with the exception of hIgG2, also hFcRIIIb, allowing fine-tuning of therapeutic IgG activity by modulating fucosylation 52.

***IgG-Fc-galactosylation – modulator of complement activity***

The hIgG1-Fc galactose content has been demonstrated to affect complement activation. This effect is independent of fucose or bisecting GlcNAc but can be amplified by further sialylation 37, 73. Mechanistically, increased galactosylation enhances C1q binding, subsequent C3b/C4b deposition and activation of the lytic complement pathway (Fig. 14). This effect is not due to enhanced affinity for C1q, but rather to greater Fc-Fc interaction, facilitating hIgG1 hexamer formation on the surface, promoting C1q binding 20, 74, 75. Accordingly, elevated galactosylation of anti-HLA alloantibodies is an important parameter of platelet clearance in platelet-transfused patients with alloantibodies 63, 76. Interestingly, the effect of galactosylation on complement activation seems to be IgG subclass specific as an increase in galactosylation on hIgG2 and hIgG4 antibodies did not increase their ability to fix complement73.

As mentioned earlier, IgG galactosylation is reduced during acute inflammation and with increasing age, which is driven at least in part by changes in sex hormones. Due to the close association with inflammation, agalactosylated hIgG has been assumed to be pro-inflammatory. Initial models suggested this effect might be due to the more exposed mannose core structure in agalactosylated IgG, resulting in enhanced complement activation via the lectin pathway 77. However, this has not been confirmed in vivo in MBL-deficient mice 78. Recent data in COVID-19 patients shows that anti-spike antibodies are highly galactosylated (~80%) at seroconversion in both mild and severe cases, but drop to 30-40% within 2-3 weeks in severely ill patients, mirrored, to a lesser extent, in bulk IgG 33. In light of these data, it remains an open question if agalactosylated IgG is as a consequence or cause of inflammation. Similarly, how the effects of galactosylation on hIgG1 hexamerization and complement activation translate to other subclasses and species is currently unknown. Apart from enhanced C1q binding, hIgG1 galactosylation modestly elevates affinity for hFcRIIa, hFcRIIIa and hFcRIIIb 48, 51, 79, 80, with the strongest effect (~2x) for binding of afucosylated IgG1 to hFcRIIIa 37. Particularly afucosylated highly galactosylated immune complexes activate platelet-hFcRIIa, suggesting that these small affinity changes are functionally relevant, likely through enhanced avidity 81.

**IgG-Fc-sialylation – modulator of anti-inflammatory activity**

Akin to IgG-Fc galactosylation, IgG Fc sialylation is reduced during acute inflammation and aging, which has led to the use of IgG sialylation as a biomarker for inflammatory conditions 82, 83. Highly sialylated IgG antibodies suppress inflammation in various mouse models of allergy and autoantibody-driven inflammation, with supporting evidence in humans 4, 84, 85, 86, 87, 88. Thus, replenishing this IgG glycoform through infusion of pooled serum IgG from healthy donors during intravenous IgG (IVIg) treatment, in diseases where IgG sialylation is reduced, may explain at least part of the therapeutic activity of IVIg 4. The different mIgG subclasses show a marked difference in sialylation, with mIgG2a/c and mIgG2b more sialylated than mIgG1 during steady state, suggesting that either mIgG subclass structure itself determines sialylation and/or that a link between IgG class switching and sialylation exists 34, 35, 36. Supporting a model where external stimuli impact IgG sialylation are studies demonstrating that different immunization protocols lead to altered levels of IgG subclass sialylation; with T cell-independent immunization protocols in particular resulting in higher levels of sialylated IgG species 35, 89. Moreover, vitamin A metabolites and estrogen can induce expression of the sialyltransferase St6Gal1 in B cells, which may explain at least in part why IgG sialylation levels drop in post-menopausal women 90, 91. In contrast, pro-inflammatory cytokines, such as interleukin (IL)23 and Th17-derived cytokines down-modulate St6Gal1 expression, promoting the production of more active, asialylated IgG (auto)antibodies 92. In genome-wide association studies SNPs in the elongation factor 2, expressed in plasma cells, were found to correlate with serum IgG sialylation at least to some extent 27.

Supporting that IgG subclass structure determines sialylation, it is known that exchanging phenylalanine 241 or 243 with alanine in hIgG1 or hIgG3 results in a considerable enhancement of IgG-Fc sialylation 93, 94. Moreover, naturally occurring allotypic variations at position 291 strongly affect IgG galactosylation and thereby sialylation 12, suggesting that IgG structure itself is an important determinant of IgG sialylation. While it has been suggested that high levels of sialylation alter IgG-Fc structure, the uniqueness of these structural alterations to highly sialylated IgG glycoforms has been debated 95, 96. Consistent with an impact of sialylation on IgG function, non-sialylated but not highly sialylated autoantibody immune complexes enhanced osteoclastogenesis 97. More recently, an IgG sialylation-dependent modulation of IgG binding to FcRIIb expressed on endothelial cells was proposed to reverse obesity-induced insulin resistance 98. Interestingly, feeding mice precursors of sialic acid, enhanced IgG sialylation and prevented obesity-induced insulin resistance and inflammatory bone loss, opening new therapeutic avenues to ameliorate autoimmune pathology via a dietary modulation of serum IgG sialylation 97, 98.

In addition, several C-type lectin receptors, such as SIGN-R1, DC-SIGN and DCIR have been reported to be required for the immunomodulatory activity of highly sialylated IgG to suppress inflammation in mouse models of autoimmunity (Fig. 1c) 4. Very recently, sialylated IgG binding to DC-SIGN on Hofbauer cells, a type of fetal macrophage involved in maintaining tolerance at the maternal-fetal interface, was suggested to contribute to immune tolerance induction via NF-kB signaling and IL-10 production, further supporting a general immunomodulatory role of highly sialylated IgG 99. During vaccination, such as against influenza virus, highly sialylated antigen-specific IgG antibodies were shown to feed-back on the B cell response via CD23, which may modulate affinity maturation leading to the optimal generation of broadly protective virus-specific IgG responses 100. To what extent highly sialylated IgG binds directly to receptors such as DC-SIGN or CD23, however, remains a matter of debate 99, 101, 102, 103.

***Fab glycans***

Germline sequences of antibody variable regions do not generally contain N-linked glycosylation sites (NxT/S, where x is any amino acid except proline), and those that do tend not to be glycosylated. However, upon affinity maturation, activation-induced deaminase can introduce these glycosylation sites, particularly as the top loops of the variable regions seem enriched in potential N-linked glycosylation sites after introduction of random mutations 104. Hence, immune responses that undergo many rounds of selection and antigenic stimuli, often have elevated levels of Fab-glycosylation, especially if the Fab-region positively affects antigen binding 104, 105, 106. Fab glycosylation seems most pronounced in hIgG4 (Table 2), the subclass often seen in response to repeated/prolonged antigenic exposures, such as allergens and autoantigens. Fab-associated glycans can also lower the threshold of B cell activation which may contribute to maintenance of autoreactive B cells and disease progression 107, 108. However, Fab-glycans can also affect the biology of IgG by reducing stability and half-life. Fab-glycans can enhance binding to the asialoglycoprotein receptor in liver cells, for example, which can enhance their clearance. Whether Fab-associated glycans enhance or decrease half-life depends on the position of the sugar moiety 106, 109. Although the mechanisms are not entirely clear, Fab glycan size and possibly charge may sterically interfere with IgG binding to the neonatal FcRn leading to reduced serum half-life and diminished transplacental transport 110. In addition, it has been reported in mice, that terminal sialic acid residues in the Fab-region of *Listeria monocytogenes*-specific IgG can be 9-O-acetylated 111. Removing this modification by sialic acid acetylesterases allows recognition of the Fab-associated sugar moiety by CD22, which is essential for affording protection against *Listeria monocytogenes* infections in new-born mice (Fig. 1c). Mechanistically, the interaction of CD22 with Fab-associated sialylated sugar elicits suppression of B cell IL-10 production, affording optimal protection against listeria. In humans, however, this 9-O-acetylation is not evident in plasma IgG, neither the Fc, nor Fab linked N-linked glycans 112.

**The impact of IgG subclass on therapeutic IgG activity**

In this final section, we will briefly highlight how recent insights into the functional diversity of IgG subclasses and posttranslational modifications may improve therapeutic antibody activity. With respect to cytotoxic antibodies, it has been known for >3 decades that the antibody isotype and subclass regulates antibody efficacy 113 and that the differential ability of individual IgG subclasses to interact with activating versus inhibitory FcRs correlates with cytotoxic IgG activity in mice and humans (Fig. 4) 53, 114, 115. Based on key studies identifying IgG1 Fc-domain variants with enhanced binding to selected or all activating FcRs, glyco- or amino acid-engineered second-generation therapeutic antibodies with enhanced cytotoxic activity were introduced into the clinic 18, 116.

With respect to ICBs and ISAs, however, the in vivo pathways underlying antibody activity have turned out to be more complex, requiring a reassessment of the role and function of different IgG subclasses and/or posttranslational modifications 117. A prime example for an ICB target is the programmed death (PD)-1:PDL1 axis, where the object is to block inhibitory PD-1 signaling in effector T cells, and where the isotype and FcR requirements for targeting the receptor versus ligand are clearly different 118. Anti-PD-L1 antibodies are most effective when engaging activating FcR, for example with mIgG2a. Although the molecular basis is not fully resolved, greater anti-tumor efficacy is associated with changes in the myeloid composition of the tumor in an activating FcR-mediated manner. In contrast, PD-1-specific antibodies are most effective in the absence of FcR interactions, which prevents the deletion of tumor-reactive PD-1+ cytotoxic lymphocytes, as well as macrophage-mediated removal of PD-1 antibodies from CD8+ T cells 119, 120. Of note, currently approved PD-1-specific antibodies are on the hIgG4 backbone, which retains appreciable binding for FcRI as a monomeric antibody and multimeric immune complex 116, 121, 122, 123. In pre-clinical models, the use of FcR non-binding variants such as mIgG1-N297A, which prevents post-translational glycosylation of the IgG Fc-domain, or hIgG4-LALA backbones, have resulted in improved immune stimulatory activity (Fig. 4) 120. Indeed, FcR non-binding anti-PD-1 reagents such as Tislelizumab have been developed and entered clinical testing. Similar complexities have appeared during studies investigating the mode of action of CTLA-4-specific ICB antibodies 124. Although initially proposed to solely prevent inhibitory signals in T cells and restore stimulation from costimulatory CD80/CD86 receptors on antigen-presenting cells, subsequent studies demonstrated the importance of regulatory T cell (Treg) depletion in curative anti-tumour activity 125. Isotypes with preferential binding to activating FcRs (including antibodies engineered with enhanced binding to activating FcRs) elicit intra-tumoral Treg cell depletion through macrophages and display greater tumor control 126. In addition, more recent studies indicate that activating FcR engagement also elicits innate immune signaling through type I IFNs evoking immune remodelling in addition to Treg cell deletion127, building on earlier data showing CTLA-4 antibodies engage hFcRIIIA on APCs for optimal responses 128. These studies add weight to the development of anti-CTLA-4 antibodies with greater affinity for activating FcRs through nonfucosylated IgG variants with enhanced binding for hFcRIIIa.

ISAs target receptors on immune cells to elicit their activation and include multiple members of the TNF receptor superfamily (TNFRSF), including CD40, 4-1BB, OX40, CD27, and GITR, as well as members of the immunoglobulin superfamily, such as CD28 and ICOS 117. Most notably, they differ from their cytotoxic/direct targeting counterparts, by being most effectively activated with isotypes that engage the inhibitory FcRIIb 129, 130, 131, 132, 133. Mechanistically, FcRIIb provides additional cross-linking, in trans, independent of inhibitory signaling, to the bivalent antibodies, replicating the interaction with their typically trimeric ligands 134. Although mIgG1 provides optimal stimulation via mFcRIIb in mouse models, no human isotype displays an equivalent FcR binding profile 121, 122. In evaluating human isotypes empirically, White et al discovered that hIgG2 displayed the highest agonistic activity for multiple receptor targets including CD40, 4-1BB, and CD28 135, even converting anti-CD40 antagonists into strong agonists 136. Unexpectedly, this activity was independent of FcRs with multiple anti-TNFR antibodies 136, 137 but directly correlated to receptor clustering 138. However, other studies have indicated that hIgG2 may be further augmented by FcR binding in a context-dependent manner 139, 140. The ability of hIgG2 to deliver agonism relates to its unique hinge 135, 136. It can undergo disulfide-switching as part of a dynamic post-translational process, adopting distinct isoforms, with hIgG2(A) and hIgG2(B) representing the extremes (Fig. 3) 141, 142. In the context of ISAs, hIgG2(B) but not IgG2(A) isoforms are strongly agonistic 135, 136. Building on earlier work 141, 142, agonistic hIgG2(B) variants were shown to be less conformationally diverse, more compact and less flexible as a result of a disulfide cross-over between the opposing heavy and light chains 14. Thus, agonistic activity is likely delivered through the ability to restrict receptor mobility, fostering more efficient receptor clustering and therein activation. hIgG2 was also shown to be more compact than other isotypes with the most flexible isotype, hIgG3, shown to be agonistically inert (Fig. 4) 143.

Although post-translational modifications, and particularly glyco-engineering has been implemented in cytotoxic therapeutic antibodies, these approaches are still relatively rare and under-exploited in ICB and ISA. Similarly, combining post-translational modifications and amino acid sequence modification to modulate Fc-effector function are in their infancy but likely to increase.

**Summary, open questions and outlook**

Recent research has generated fascinating new insights into the diversification and complexity of naturally occurring antibody responses. These results have highlighted that many post-translational modifications of the antibody amino acid backbone act as highly specific adaptions to fine tune IgG activity rather than arbitrary variations occurring during immune responses. Indeed, advanced technologies generating antibodies with specific post-translational modifications have allowed their functional assignment but have also shown that peculiarities of individual monoclonal antibodies may impact these effects. These insights have provided the basis for understanding natural immune responses as well as for optimizing therapeutic antibodies. The same notion applies to understanding IgG subclass activities especially in combination with post-translational modifications, greatly expanding possibilities for further improvement of IgG-based immunotherapy. If vaccine responses could be steered deliberately towards beneficial post-translational modifications, the generation of more effective vaccines for diseases such as those caused by HIV and Plasmodium subspecies may ultimately become possible. However, we are far from understanding how to achieve or prevent certain post-translational modifications, such as IgG fucosylation, which may increase protection against some infectious diseases but exacerbate disease pathology in others (e.g. SARS-CoV-2 and Dengue infections). Moreover, the entire antibody backbone including the antigen-binding domain, play a decisive role in the effect certain post-translational modifications have on antibody function. Thus, continuing to learn from pathogen-specific and autoreactive humoral immune responses and the respective post-translational modifications and IgG subclass responses occurring, at the level of the population and the individual patient, will be key for fully exploiting the post-translational toolbox for improving antibody therapies.

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**Competing interests**

None of the authors declare a competing financial interest.

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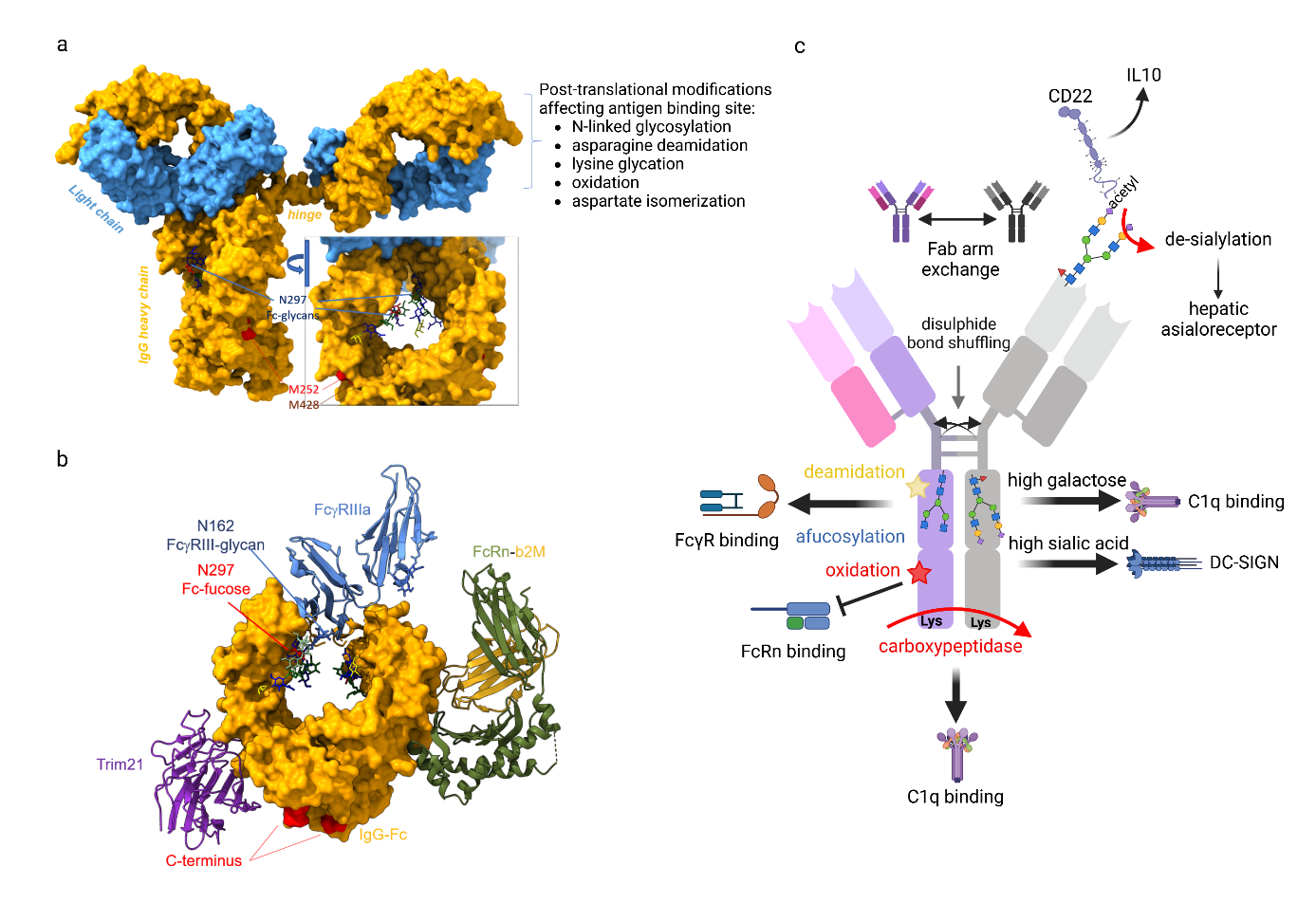
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**Figures:**

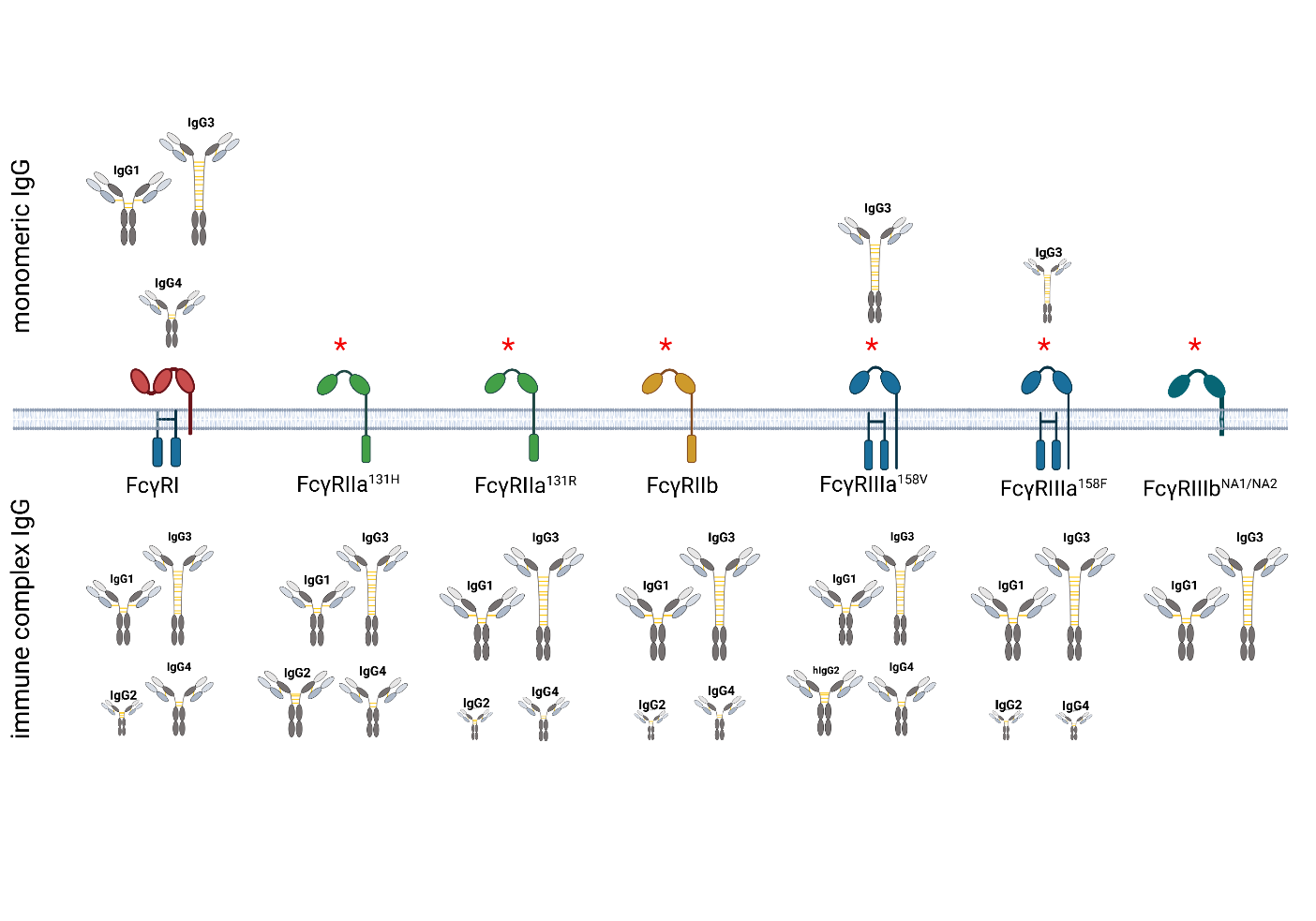
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**Figure 1: IgG structure, interaction partners and impact of post-translational modifications**

a) Shown is the crystal structure of a human IgG1 antibody, depicting the two IgG heavy (yellow) and light (blue) chains as well as the hinge region. The sugar moiety attached to the asparagine 297 (N297) residue in each of the two IgG heavy chains is depicted as a stick model. The inset shows a rotation of the IgG Fc-domain to visualize the sugar domains. In addition, methionine residues 252 and 428 are marked, which become altered by oxidation after IgG secretion into the serum. Post-translational modifications occurring in the F(ab) region are summarized.

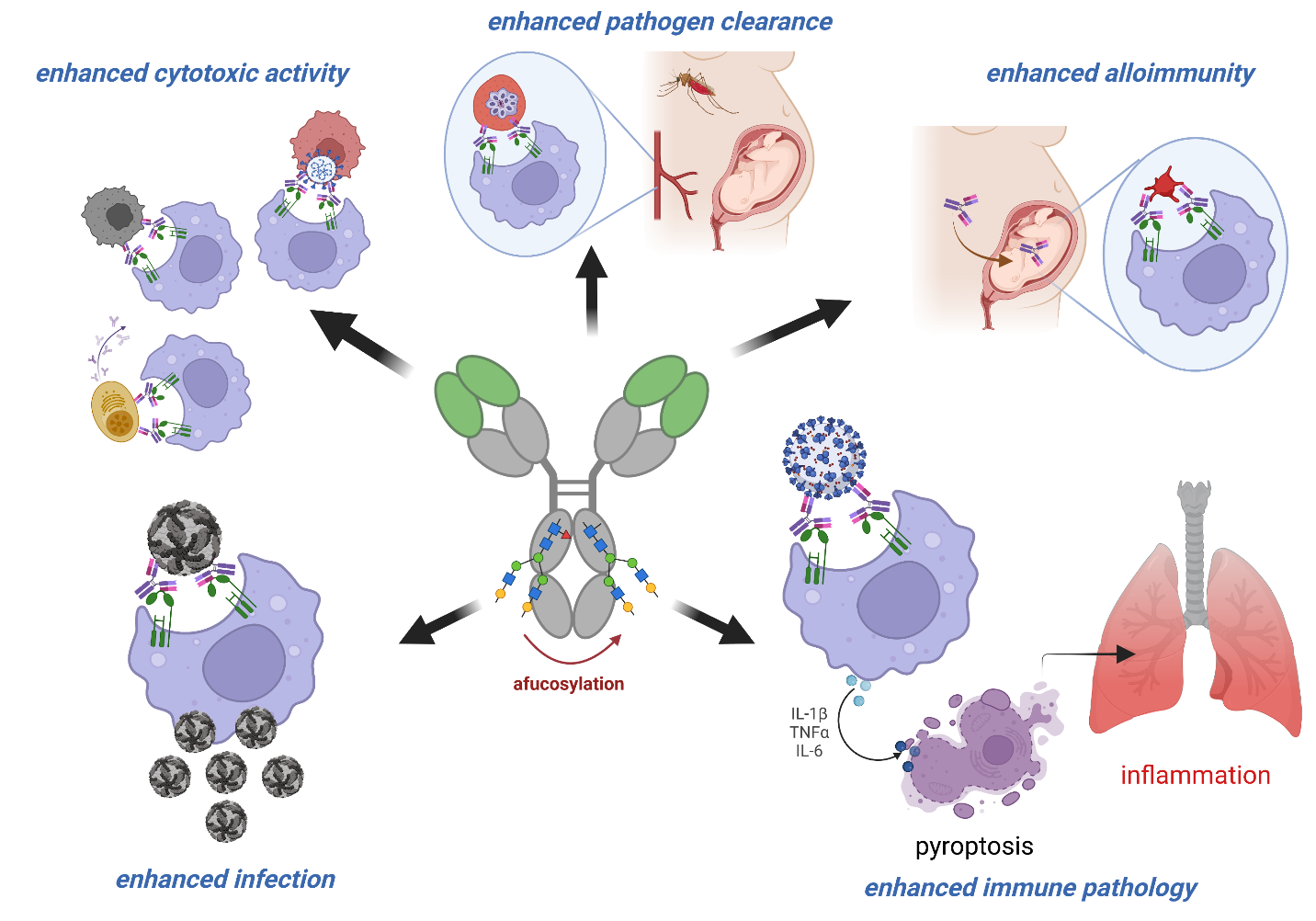
b) Depicted is the crystal structure of hFcRIIIa (3SGJ) aligned with human FcRn, human IgG1-Fc (7Q15), and TRIM21 using ChimeraX v1.5. Sugar domains attached to hFcRIIIa at position N162 as well as the sugar domains attached to the IgG-Fc domains are represented as stick models. The location of the fucose residue in the IgG-Fc sugar domain, causing steric clashes with theh FcRIIIa-162 glycan, is indicated. The lower-IgG1 hinge is represented as an alpha helix swaying away from the viewer and links to the two Fab regions (not shown), and away from the asymmetrical binding pocket of hFcRIIIa. The C terminus (without C-terminal lysine) is shown in red.

c) Schematic representation of an IgG molecule including post-translational modifications and how these modifications affect the binding to effector pathways via FcRs, the neonatal FcRn, DC-SIGN, the complement pathway (C1q) as well as the potential binding to CD22. See text for further details. Figure was created with Biorender.com.

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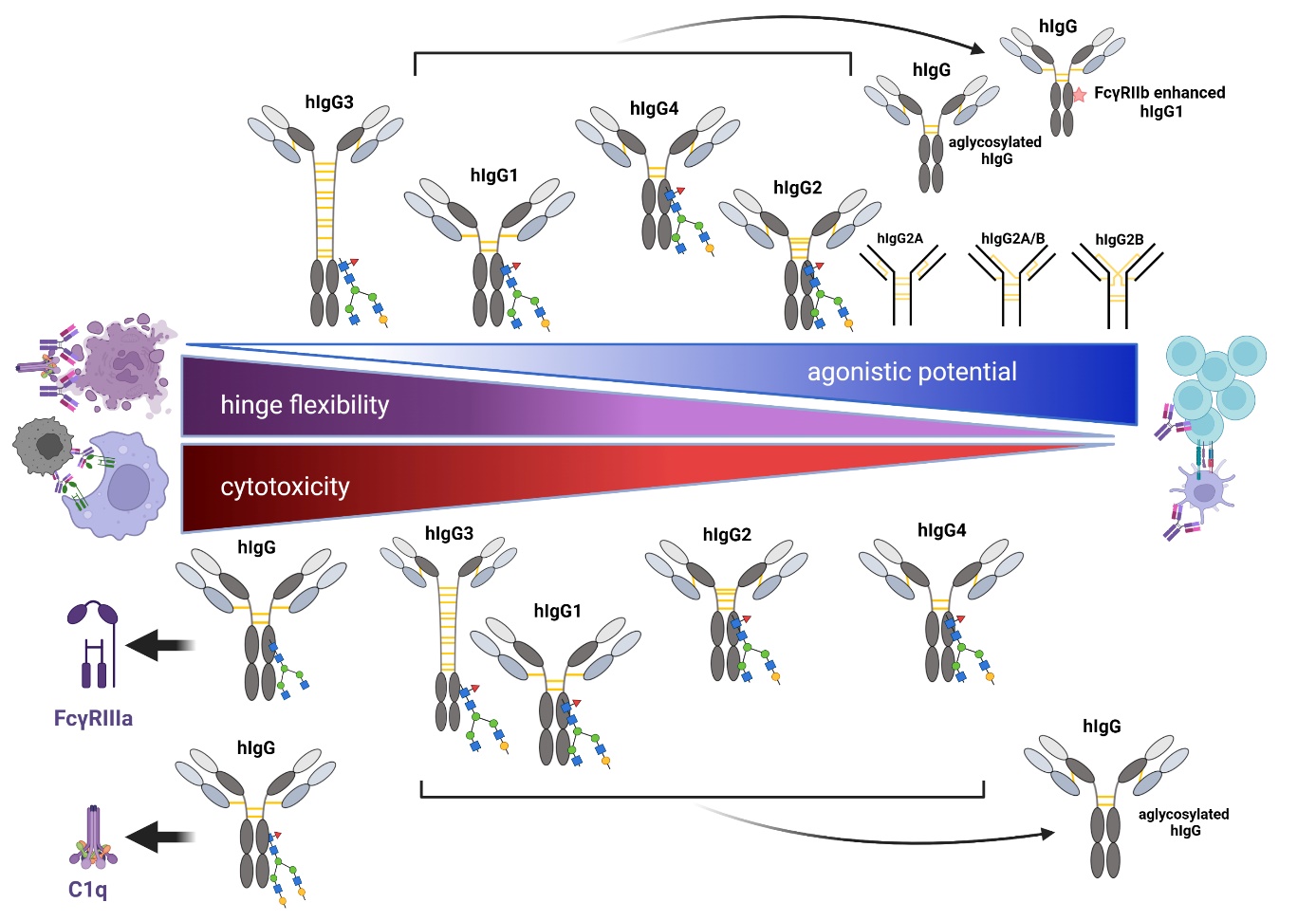
**Figure 2: IgG subclass binding to cellular FcRs in monomeric or immune complex form**

The family of human FcRs consting of one high affinity (FcRI) and several low affinity receptors is depicted. While most FcRs transmit activating signals, FcRIIb (yellow) is an inhibitory FcR. FcRIIIb is a neutrophil-restricted GPI-linked receptor without intrinsic signaling function. Shown is the binding of human monomeric IgG subclasses (upper panel) or human IgG subclasses in the form of immune complexes (lower panel) to the indicated human FcRs expressed on cells 119, 121 (see also Table 1 for affinity data from SPR analysis). The size of the respective IgG subclass indicates a stronger or weaker binding compared to other IgG subclass binding to the indicated FcgR. For FcRIIa, FcRIIIa, and FcRIIIb allelic variants of the respective receptors are indicated. \* indicates that due to the high concentration of IgG subclasses in the serum, IgG1, IgG3, but also IgG2 is found to be associated with low-affinity FcRs. See text and Table 1 for further details. The figure was created with Biorender.com.



**Figure 3: Impact of fucose residues on IgG-dependent effector functions**

Impact of afucosylated IgG glycovariants on the outcome of different antibody mediated immune responses. As shown in the upper left, afucosylated IgG antibodies may strongly increase the activity of cytotoxic antibodies via enhanced affinity for FcRIIIa resulting in a better clearance of target cells including tumor cells, autoreactive cells or virus infected cells. During immune responses against *plasmodium ssp*. infected red blood cells, or alloimmune responses (upper middle and right panels) occurring in pregnant women to paternal blood-groups on red blood cells or platelets, afucoslyated autoantibodies can be formed. In malaria this causes enhanced protection in the infected individual (middle panel), but increased pathology after FcRn-mediated transport of alloantibodies from the mother to the new-born (upper right panel). During certain virus infections, such as Dengue virus or SARS-CoV-2, afucosylated virus specific antibody responses may lead to the enhancement of virus infection and elevated myeloid cell-activation and thereby pathology via increased uptake into hFcRIIIa positive immune cell subsets (lower left). For SARS-CoV-2 this also may induce pyroptosis of target cells, resulting in heightened immune pathology. See text for further details. The figure was created with Biorender.com.



**Figure 4: Impact of IgG subclass and glycosylation on therapeutic IgG activity**

Shown is a schematic overview of how IgG subclass affects the activity of immunomodulatory agonistic (upper panel) or cytotoxic (lower panel) antibodies. Furthermore, the effect of IgG hinge flexibility on immunomodulatory antibody is depicted. Note that all IgG subclasses are glycosylated and that sugar moieties are only indicated if changes in glycosylation are associated with an impact of IgG activity, such as in the case of afucosylated or highly galactosylated sugar moieties. If no specific IgG subclass is mentioned the effect is relevant for all IgG subclasses. See text for further details. The figure was created with Biorender.com.

**TABLE I: Effects of IgG subclass glycan changes on Fc-receptor binding affinity**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | hIgG1 | | | | | hIgG2 | | | | | hIgG3 | | | | | | | hIgG4 | | | | |
|  | Affinity | F\* | B | G | S | Affinity | F | B | G | S | | Affinity | F | B | G | S | Affinity | | F | B | G | S |
| FcγRIa | 65 $ | -† | - | - | - | - | - | ? | ? | ? | | 61 | - | ? | ? | ? | 34 | | - | ? | ? | ? |
| FcγRIIaH131 | 5.2 | - | - | (↑) | - | 0.45 | - | ? | ? | ? | | 0.89 | - | ? | ? | ? | 0.17 | | - | ? | ? | ? |
| FcγRIIaR131 | 3.5 | - | - | (↑) | - | 0.10 | - | ? | ? | ? | | 0.91 | - | ? | ? | ? | 0.21 | | - | ? | ? | ? |
| FcγRIIb/c | 0.12 | - | - | - | - | 0.02 | - | ? | ? | ? | | 0.17 | - | ? | ? | ? | 0.20 | | - | ? | ? | ? |
| FcγRIIIaF158 | 1.2 | ↓Θ 20x | - | ↑ Δ 2x | ↓▪ 2x | 0.03 | ↓ | ? | ? | ? | | 7.7 | ↓ 20x | ? | ? | ? | 0.20 | | ↓ | ? | ? | ? |
| FcγRIIIaV158 | 2.0 | ↓ 10-20x | - | ↑ 2x | ↓▪ 2x | 0.07 | ↓ | ? | ? | ? | | 9.8 | ↓Θ 10x | ? | ? | ? | 0.25 | | ↓ | ? | ? | ? |
| FcγRIIIb | 0.2 | ↓ 20x | - | - | ↓▪ 2x | - | - | ? | ? | ? | | 1.1 | ↓Θ 10x | ? | ? | ? | - | | ↓ | ? | ? | ? |
| C1q | <0.05 | - | - | ↑ | (↑) | (-)🞟 | ? | ? | ? | ? | | <0.05 | - | ? | ? | ? | (-) | | ? | ? | ↑ | ? |

\* F: Fucose; B: Bisection; G: Galactose; S: Sialic acid; $: Association constant (×106 M-1) for monovalent binding given for fucosylated IgG 122 † -: No affinity change; ↓ decreased affinity; ↑ increased affinity, fold change based on 38 generally agreeing with 42, whenevaluated.

Δ: for afucosylated IgG; ▪: for afucosylated bisected IgG; Θ Effect of fucosylation on afucosylated IgG is shown. 🞟Can activate complement at very high epitope densities 5

**TABLE II: Posttranslational modifications of human IgG subclasses**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Subclass** | **hIgG1** | **hIgG2** | **hIgG3** | **hIgG4** |
| **Characteristic** |  |  |  |  |
| Molecular mass (kD) | 146 | 146 | 170 | 146 |
| Amino acids in hinge region | 15 | 12 | 32-62 *a*) | 12 |
| Inter-heavy chain disulfide bonds | 2 | 4 *b*) | 5-11 *a*) | 2 |
| Fab-glycosylation | 11-18% | 10-16% | 11-16% | 40-50% c) |
| Hinge O-linked glycosylation c) | - | - | Yes | - |

1. Depends on allotype5
2. For A/A isomer, depends on isomerization141, 142.
3. 104