Influence of immunoglobulin G-glycan and subclass variation on antibody effector functions
Dekkers, G.

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IgG4
Chapter 1

**General introduction and scope of the thesis**

Gillian Dekkers

Parts of this introduction have been published as:
“IgG subclasses and allotypes: from structure to effector functions“.
Gestur Vidarsson, Gillian Dekkers and Theo Rispens.
**General introduction and scope of the thesis**

Immunoglobulin G (IgG) is one of the most abundant proteins in human serum, accounting for about 10 to 20% of plasma protein. It is the major class of the five classes of immunoglobulins in humans, IgM, IgD, IgG, IgA, and IgE. These closely related glycoproteins, composed of 82-96% protein and 4-18% carbohydrate, differ in heavy chain structure and have different effector functions. IgG can be further divided into four subclasses, named, in order of decreasing abundance in serum IgG1, IgG2, IgG3 and IgG4. Differences in structure and function of IgG subclasses are summarized in Table 1. Although they are more than 90% identical on the amino acid level, each subclass has a unique profile with respect to antigen binding, immune complex formation, complement activation, triggering of effector cells, half-life and placental transport.

Exposure to different types of antigens leads to marked skewing towards one single or several of the IgG subclasses. Selective subclass deficiencies are usually not detrimental to the individual, but do sometimes lead to enhanced susceptibility towards specific classes of pathogens. This can be caused by rare complete isotype- or subclass deficiency due to deletions in the Ig loci of chromosome 14. More often, one or more of the IgG subclass levels – predominantly IgG2 and/or IgG4 – are below the normal range in healthy individuals which sometimes leads to an impaired response to infections with encapsulated bacteria as will be discussed below. All in all, the acquired variability within the Ig locus seems to have selected for beneficial changes during evolution for optimizing or fine-tuning the antibody-mediated immune response.

**IgG antibody responses**

The route by which an antigen enters our body and its chemical composition steers the (secondary) immune reaction into preferential patterns of class switching. Besides direct B cell triggering by the antigen itself, a number of secondary signals will influence differentiation of the B cell, including recognition by pattern recognition receptors like Toll-Like Receptors and cytokines produced by other lymphocytes and antigen presenting cells. For example, protein antigens usually trigger B cells receiving T cell help through MHC-class II expressed by the B cell. For those antigens, class switching tends to be IgG1 or IgG3, but can also be IgG4 or IgE. On the other hand, in the absence of T cell help polysaccharide antigens may induce class switching to IgG2 in particular. B cells undergoing class switching in a primary or secondary immune reaction can also go through subsequent class switching, but those events are limited by the availability of remaining heavy chain genes, not excised from the genome in previous class switching events. The relatively terminal position of the Cy4 cassette may be one of the reasons why IgG4 responses tend to occur after repeated antigen exposure.
Table 1. Properties of human IgG subclasses

<table>
<thead>
<tr>
<th></th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
<td></td>
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<td></td>
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<td>Molecular mass (kDa)</td>
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<td>Amino acids in hinge region</td>
<td>15</td>
<td>12</td>
<td>62 a)</td>
<td>12</td>
</tr>
<tr>
<td>Inter-heavy chain disulfide bonds</td>
<td>2</td>
<td>4 b)</td>
<td>11 a)</td>
<td>2</td>
</tr>
<tr>
<td>Mean adult serum level (g/l)</td>
<td>6.98</td>
<td>3.8</td>
<td>0.51</td>
<td>0.56</td>
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<td>Relative abundance (%)</td>
<td>60</td>
<td>32</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Half-life (days)</td>
<td>21</td>
<td>21</td>
<td>7/~21 a)</td>
<td>21</td>
</tr>
<tr>
<td>Placental transfer</td>
<td>+++</td>
<td>++</td>
<td>++++/+++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Antibody response to:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proteins</td>
<td>++</td>
<td>+/-</td>
<td>++</td>
<td>++ e)</td>
</tr>
<tr>
<td>polysaccharides</td>
<td>+</td>
<td>+++</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>allergens</td>
<td>+</td>
<td>(-)</td>
<td>(-)</td>
<td>++</td>
</tr>
<tr>
<td><strong>Complement activation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1q binding</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><strong>Fc receptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FcγRI</td>
<td>+++ c)</td>
<td>65 d)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FcγRIIaH131</td>
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<td>5.2</td>
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<tr>
<td>FcγRIIaR131</td>
<td>+++</td>
<td>3.5</td>
<td>+</td>
<td>0.10</td>
</tr>
<tr>
<td>FcγRIIb/c</td>
<td>+</td>
<td>0.12</td>
<td>-</td>
<td>0.02</td>
</tr>
<tr>
<td>FcγRIIaF158</td>
<td>++</td>
<td>1.2</td>
<td>-</td>
<td>0.03</td>
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<tr>
<td>FcγRIIaV158</td>
<td>+++</td>
<td>2.0</td>
<td>+</td>
<td>0.07</td>
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<tr>
<td>FcγRIIib</td>
<td>++++</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FcRn (at pH&lt;6.5)268</td>
<td>+++</td>
<td>1.0</td>
<td>+++</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Notes:
- a) Depends on allotype.
- b) For A/A isomer.
- c) Multivalent binding to transfected cells. Adapted from 189.
- d) Association constant Kₘ (×10⁶ M⁻¹) for monovalent binding 189.
- e) After repeated encounters with protein antigens, often allergens.

**IgG1**

Antibody responses to soluble protein antigens and membrane proteins primarily induce IgG1, but are accompanied with lower levels of the other subclasses, mostly IgG3 or IgG4 8. Because IgG1 is normally the most abundant subclass, a lack of IgG1 seen in a variety of primary and secondary antibody deficiencies, can result in decreased total IgG.
levels (hypogammaglobulinemia). IgG1 deficiencies, sometimes in combination with other IgG-subclass deficiencies, are associated with recurrent infections.

*IgG2*

IgG-antibody responses to bacterial capsular polysaccharide antigens can be almost completely restricted to IgG2, and IgG2 deficiency may result in the virtual absence of IgG anti-carbohydrate antibodies, although these responses can also be compensated for by enhanced levels of other IgG-subclasses, in particularly by elevated IgG1 and IgG3 levels. An increased susceptibility to certain bacterial infections is associated with IgG2 deficiency, suggesting a role of IgG2 in the defense to these pathogens. Low concentrations of IgG2 often occur in association with a deficiency in IgG4 and/or IgA1 and IgA2.

An extensive analysis of anti-carbohydrate reactivities in intravenous immunoglobulin revealed that although IgG2 indeed represents the bulk of the reactivity to many glycans, this is not always the case. IgG1 antibodies have also been reported to prevail against H. Influenza b polysaccharide during natural infections. In healthy individuals with normal immune responses, IgG1 and IgG3 can also be detected, and certainly against protein-conjugated glycans, for example in the reaction to second generation pneumococcal vaccines.

*IgG3*

IgG3 antibodies are particularly effective in the induction of effector functions. Being a potent pro-inflammatory antibody, its shorter half-life may function to limit the potential of excessive inflammatory responses. However, the finding that some individuals bearing the G3m allotypic “s” or “15” marker (i.e., G3m(s)/G3m(15) and G3m(st)/G3m(15,16) allotypes) also have IgG3 with prolonged half-life may challenge that assumption. Curiously, IgG3 levels in these individuals do not seem to be increased, which may be explained by γ3-promotor polymorphisms known to affect the frequency of class switching to IgG3 in G3m(g) allotypes, explaining the low concentration in most G3m(g) homozygous individuals. Viral infections, in general lead to IgG antibodies of the IgG1 and IgG3 subclasses, with IgG3 antibodies appearing first in the course of the infection. IgG3-dominated responses appear to be rare. A curious example are so-called anti-hinge antibodies, which bind to the hinge region of F(\(ab\)’)2 fragments but not intact IgG antibodies. Also, antibodies to P and Pk blood group antigens are largely restricted to IgG3. Responses against other red cell antigens (e.g. RhD) and platelets (e.g. human platelet antigen 1a), as seen in transfusion and in pregnancies, are often dominated by either IgG1, IgG3, or both. Decreased IgG3 levels are frequently associated with other IgG subclass deficiencies.
IgG4

Allergens are often good inducers of IgG1 and IgG4, in addition to IgE. IgG4 antibodies are often formed following repeated or long-term exposure to antigen in a non-infectious setting and may become the dominant subclass. Examples are long-term bee keepers and allergic individuals that underwent immune therapy\textsuperscript{7,28–30}. In immunotherapy, relief of symptoms appears to correlate with IgG4 induction. Switching to IgG4 may be modulated by IL10, linking this subclass to down-regulation of immune responses or tolerance induction\textsuperscript{7,31}. IgG4 may also represent the dominant antibody subclass in immune responses to therapeutic proteins, such as factor VIII and IX\textsuperscript{32–34} and at least some recombinant antibodies such as adalimumab\textsuperscript{35}. Furthermore, helminth or filarial parasite infections may result in the formation of IgG4 antibodies\textsuperscript{36,37}, and high IgG4 titers can be associated with an asymptomatic infection\textsuperscript{38}.

Isolated IgG4 deficiencies are rare; it is uncertain what the possible consequences are. On the other hand, a group of disorders, nowadays referred to as IgG4-related diseases (IgG4RD), are characterized by elevated serum IgG4 concentration and tissue infiltration by IgG4-positive plasma cells and may affect a number of organs\textsuperscript{39,40}. The spectrum of IgG4RD is wide and includes patients with autoimmune pancreatitis (AIP), Mikulicz’s disease, hypophysitis, Riedel thyroiditis, interstitial pneumonitis, interstitial nephritis, prostatitis, lymphadenopathy, retroperitoneal fibrosis, inflammatory aortic aneurysm, and inflammatory pseudotumor\textsuperscript{41}. In AIP patients, elevated serum IgG4 (>1.4 g/L) is observed in 70–80% of the cases, as well as in 10% of pancreatic cancer patients. However, as 5% of the normal population also has elevated IgG4 levels, this makes it only suitable for diagnosis in combination with other features of AIP.

Mouse IgG subclasses

Mice (Mus musculus) express 4 antibody subclasses: Generally believed to be IgG1, IgG2a, IgG2b, and IgG3, as found in Balb/c mice (Fig. 1) (see also section below on structure). However, IgG2a is not expressed by all mouse strains, e.g. C57Bl/6, where IgG2c
is found\(^4\). The reason behind this is probably genetic differences in the same locus, giving rise to allotypes (see below for human IgG). For all mouse subclasses there are reports of allotypic variations, depending on the strain of mice\(^{43-46}\). Strain and age of the mice affect the (relative) serum levels of IgG subclasses\(^{47,48}\). As for human, mouse subclasses differ in, amongst others; structure, half-life, ability to activate complement, affinity for Fc receptors, and response to certain pathogen types. Structural and functional differences of the conventional mouse IgG subclasses are summarized in Table 2. When comparing mouse IgG subclasses to the human subclasses there are some similarities in function, however, this is not fully transferable as some reactions are completely different. Mouse IgG2a is best able to activate complement followed by mIgG2b, mIgG3 and mIgG1\(^{49-52}\).

### Table 2. Properties of mouse IgG subclasses

<table>
<thead>
<tr>
<th></th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG2b</th>
<th>IgG3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
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<td></td>
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<td>Molecular mass (kDa)</td>
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<td>160</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>Amino acids in hinge region(^5)</td>
<td>13</td>
<td>16</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>Inter-heavy chain disulfide bonds</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Mean adult serum level (g/l)(^47)</td>
<td>0.3-5</td>
<td>0.1-4</td>
<td>0.1-5</td>
<td>0.1-0.2</td>
</tr>
<tr>
<td>Relative abundance (%)</td>
<td>46</td>
<td>24</td>
<td>27</td>
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<tr>
<td>Half-life (days)(^47)</td>
<td>8-11</td>
<td>3-12</td>
<td>2.6-3.5</td>
<td>4-8</td>
</tr>
<tr>
<td>Placental transfer</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Antibody response to:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proteins</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>polysaccharides</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>allergens</td>
<td>++</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Complement activation</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>C1q binding</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+/-</td>
</tr>
<tr>
<td><strong>Fc receptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>FcγRI</td>
<td>-</td>
<td>a) b)</td>
<td>++</td>
<td>167</td>
</tr>
<tr>
<td>FcγRIIb</td>
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<td>3.3</td>
<td>++</td>
<td>0.4</td>
</tr>
<tr>
<td>FcγRIII</td>
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<td>0.7</td>
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<tr>
<td>FcγRIV</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>29</td>
</tr>
<tr>
<td>FcRn (at pH&lt;6.5)(^568)</td>
<td>+</td>
<td>80</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Notes:**

- a) Cell binding to mouse macrophages\(^569\).
- b) Association constant K\(_A\) (×10\(^9\) M\(^{-1}\)) for monovalent binding\(^44\).
- c) n.d.; not determined.
whilst human IgG3 and IgG1 are best able to activate complement (for human: see section on complement below). The binding interaction with FcγR of either mouse or human is somewhat harder to compare, as the different receptors do not completely match on orthologous, expression and functional level\textsuperscript{53}. Affinity of mouse IgG subclasses to low affinity FcγR is highest for mouse IgG2a, followed by IgG2b, IgG1 and IgG3, and varies per receptor\textsuperscript{54,55} (for human: see sections on FcγRs below). IgG2a is the subclass that is mainly elicited in response to virus infection and protein antigens\textsuperscript{56}, whilst IgG3 is associated with anti-carbohydrate activity, and IgG1 is associated with binding to mast cells\textsuperscript{57}. Combined, this information allows us to compare murine IgG2a and IgG2b with human IgG1 and IgG3, murine IgG2 with human IgG4, and murine IgG3 with human IgG2.

A clue to the similarities and differences in expression, class switching and function between mouse and human subclasses can be found when comparing the genetic locus of each species for immunoglobulin expression (Fig. 2)\textsuperscript{58}. The genes for the V,D, and J regions are followed by the genes for the several heavy chains. This shows a duplication within the human locus, absent in the mouse locus, resulting in duplications of Ig genes and additional enhancers. Divergent evolution then resulted in loss of certain genes, or pseudogenes, and distinct subclasses for either species\textsuperscript{58}.

**Structure**

Similar to the other immunoglobulin isotypes, the IgG immunoglobulin molecule consists of four polypeptide chains, comprised of two identical 50 kDa γ heavy (H) chains and two identical 25 kDa κ or λ light (L) chains, linked together by inter-chain disulfide bonds. Each heavy chain consists of an N-terminal variable domain (VH) and three constant domains (CH1, CH2, CH3), with an additional ‘hinge region’ between CH1 and CH2 (Fig. 3a). Similarly, the light chains consist of an N-terminal variable domain (VL) and a constant domain (CL). The light chain associates with the VH and CH1 domains to form a Fab arm (‘Fab’ = fragment antigen-binding), and functionally, the V regions interact to form the in antigen binding region – acquired through differential assembly of Variable, Diversity (VH only) and Joining gene segments and inclusion of somatic mutations- although their relative contribution to antigen binding varies greatly\textsuperscript{59–61}. Two heavy chain-light chain heterodimers (HL) combine into a single antibody molecule (H\textsubscript{2}L\textsubscript{2}) via disulfide bonds in the hinge region and non-covalent interactions between the CH3 domains (Fig. 3a). The part of the antibody formed by the lower hinge region and the CH2/CH3 domains is called ‘Fc’ (‘fragment crystalline’).

The global structures of the four human IgG subclasses are very similar (Fig. 3 and 4a), but with important differences between each subclass that affect their binding to accessory molecules and receptors, affecting their functionality (Table 1). The four subclasses show over 90% homology in amino acid sequence, with differences that are not randomly distributed. Much variation is found in the hinge region and N-terminal CH2
domain, whereas fewer amino acid differences are found in the other domains. Of these, least is known about the functional consequences - if any - of structural variations found within the CH1 domain. On the other hand, structural differences in the CH2/CH3 domains, forming the Fc-tail, are relatively well-studied.

The residues most proximal to the hinge region in the CH2 domain of the Fc-part are responsible for effector functions of antibodies as it contains a largely overlapping binding site for C1q (complement) and IgG-Fc receptors (FcγR) on effector cells of the innate immune system.

A highly conserved N-linked glycosylation site at position 297 is located at the interface between the two CH2/CH3 forming the Fc of an IgG molecule (Fig. 3a) which is responsible for subtle but important changes of quaternary structure of the Fc – allowing for a more exposed docking-site for FcγR62. As discussed further below, these glycans also directly participate in the FcγR binding63. These interactions can be modulated through highly specific modifications of the N297 glycan – changes that seem to be regulated during specific immune responses in humans64–70. Although this glycosylation site is often regarded as the only glycosylation site in IgG, the V region of approximately 10-15% of all antibodies is also glycosylated (Fab-glycosylation). These sites most often arise through VDJ-recombination or somatic hypermutation, and this glycosylation has been reported to affect antigen binding characteristics59,71–74 (and van de Bovenkamp et al., manuscript submitted75) and allowing binding to regulatory lectins. This in turn, can also modulate the

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**Figure 2. Schematic map of the mouse and human genetic cluster**

Figure adapted from D’addabbo et al. 201114. This schematic representation of the IgH cluster and closest genes shows the homology between mouse (a) and humans (b). Genes are represented by rectangles and enhancers by ovals. With V, D, J genes, followed by genes for the different antibody classes (μ; IgM, δ; IgD, γ; IgG, α; IgA and ε; IgE) and subclasses. 3′RR: 3′ regulatory region, 20bpr: 20 base pair tandem repeat.
activation-threshold required for B cell stimulation, and has been described as a positive selection signal in certain types of follicular lymphomas\cite{74,76}.

The interface between the CH2-CH3 domains also contains the binding site for the neonatal Fc-receptor (FcRn), responsible for the prolonged half-life of IgG, placental passage, and transport of IgG to and from mucosal surfaces. Little variation exists in this region, with FcRn binding only minimally affected, except for IgG3 as discussed further below.

However, the binding profiles of FcyR and C1q to the different IgG subclasses go hand in hand (Table 1), each IgG subclass having its distinctive pattern that has been investigated in detail. How the differences in the primary sequences and post translational
modifications – glycosylation - of the IgG subclasses (Fc and hinge) lead to variations in tertiary structural elements, thereby critically influence the properties of each subclass, is the subject of the following sections.

**Structural variation in the hinge region**

The hinge region forms a flexible linker between the Fab arms and the Fc part. Length and flexibility of the hinge region varies extensively amongst the IgG subclasses (Fig. 4)\(^7\). This affects the possible conformations of the Fab arms relative to the Fc domain as well as to each other. The hinge exon of IgG1 encompasses 15 amino acids and is very flexible. IgG2 has a shorter hinge than IgG1, with 12 amino acid residues. The lower hinge region of IgG2 (actually encoded by the CH2 region) also has a one amino acid deletion (lacking one of the double glycines found at position 235-6), resulting in IgG2 having the shortest hinge of all the IgG subclasses. In addition, the hinges of IgG2 are even more rigid due to a poly-proline helix, stabilized by up to four (with some exceptions discussed below) extra inter-heavy chain disulfide bridges (Fig. 4). These properties restrict the flexibility of the IgG2 molecule. Similarly, the hinge region of IgG4 also contains 12 amino acids and is thus shorter than that of IgG1. Its flexibility is intermediate between that of IgG1 and IgG2\(^7\). Unlike IgG2 it does encode for the CH2-encoded glycines 235-236 in the lower hinge (Fig. 5a).

IgG3 has a much longer hinge region than any other IgG subclasses or Ig human isotype, i.e. about four times as long as the IgG1 hinge, containing up to 62 amino acids (including 21 prolines and 11 cysteines), forming a poly-proline helix with limited flexibility\(^7\). The exact length of the hinge varies between allotypes of IgG3, which apparently has undergone much more evolutionary radiation than the other subclasses (Fig. 5b-c) as discussed below. In IgG3 the Fab fragments are relatively far away from the Fc fragment, giving the molecule a greater flexibility. This long hinge of IgG3 is a result of duplications of a hinge exon, encoded by one exon in IgG1, IgG2 and IgG4, but up to 4 exons in IgG3. One of those exons is common to all IgG3 allotypes, but it also has 1-3 copies of a homologous second type of IgG3-hinge exon (Fig. 5c). The elongated hinge in IgG3 is also responsible for its higher molecular weight compared to the other subclasses. The difference in hinge flexibility influences the relative orientation and movement of the Fab arms and Fc tail of the IgG antibody.

Binding sites for C1q and/or FcγR may be partially or completely shielded by Fab arms, affecting binding of the IgG to these molecules. The relative flexibility of the Fab arms with respect to the Fc differs between subclasses as follows: IgG3 > IgG1 > IgG4 > IgG2\(^7\), which also reflects the relative binding of these subclasses to FcγR and C1q, although this only partially explains the respective activities of the IgG subclass, as discussed elsewhere in this review. This flexibility also affects antigen-binding capacity and immune complex formation.
Inter-chain disulfide bonds

The four IgG subclasses also differ with respect to the number of inter-heavy chain disulfide bonds in the hinge region (Table 1, Fig. 4a). In addition, both IgG2 and IgG4 are found as several isomers, in which the hinge disulfide bonds are differentially interconnected (see below). Another structural difference between the human IgG subclasses is the linkage of the heavy and light chain by a disulfide bond. This bond links the carboxy-terminal cysteine of the light chain to the cysteine at position 220 (in IgG1) or at position 131 (in IgG2, IgG3 and IgG4) in the CH1 domain (Fig. 4a). These two positions are spatially juxtaposed and the essential structure and function of the molecule appears to be conserved between the two types of linkage between heavy and light chain.

Hinge isomers in IgG2 and IgG4

In IgG2, structural hinge isomers have been observed as a result of alternative formation of disulfide bonds between the cysteines in the hinge region of the heavy chains and those involved in the formation of disulfide bonds between the light and heavy chain (Fig. 4b). These isomers were found particularly in IgG2 antibodies with kappa-light chains, but much less for lambda light chains. The major forms are the classical A form, with four disulfide bridges between the two IgG2 heavy chains, and the B form in which one hinge cysteine forms a disulfide bond with the light chain. However, other configurations exist, as these isoforms apparently form independently of each other, giving rise to A/A, B/B but also A/B isoforms (Fig. 4a-b). Fc receptor binding does not seem to be different for the different isomers. IgG2 has also been reported to form covalent dimers, which might be regarded as an additional isomer.

Two isomers of IgG4 differing in the disulfide bonding of hinge cysteines coexist. The core hinge of IgG is formed by a CXXC motif, also found in redox-reactive proteins such as thioredoxins. Compared to IgG1, with a relatively rigid CPPC motif, intra-chain disulfide bonds are more easily formed between these cysteines found at positions 226 and 229 in IgG4, which possesses a CPSC core hinge (Fig. 4c). The result is an observable amount of non-covalently linked half-molecules (consisting of one heavy and one light chain, HL, as opposed to the classical configuration of H2L2) in addition to covalently linked inter-chain isomers (Fig. 4c). An S228P mutant of IgG4, thus with an IgG1-core hinge, does not form half-molecules which is in agreement with the finding that this species does not occur in IgG1. The process is reversible but depends on redox conditions. Formation of the intra-chain isomer (half-molecules) is an important step in the ‘Fab arm exchange’.

IgG4 Fab arm exchange

In vivo, half-molecules of IgG4 recombine randomly with other half-molecules of IgG4, combining specificities of two IgG4 molecules, effectively resulting in monovalent-bispecific antibodies (Fig. 4c), and is controlled by redox conditions. The unique S228
in the core hinge of IgG4 allows formation of the intra-chain isomer, and R409 (rather than the equivalent lysine in IgG1) results in weaker CH3-CH3 interactions (Fig. 5a)\textsuperscript{93,94}. Both determinants appear to be required to observe Fab arm exchange \textit{in vivo}\textsuperscript{90} and has been observed for the therapeutic IgG4 antibody natalizumab\textsuperscript{95}. The functional consequence of this are at least two fold. The resulting IgG4 antibody cannot effectively crosslink the target antigen. Furthermore, multivalent target binding is not possible for bispecific antibodies,
resulting in a lower avidity, although the affinities of IgG4 antibodies are generally high. In combination with the observations that IgG4 responses seem to dominate after repeated antigen exposure (e.g. bee venom), and because IgG4 has low affinity to activating FcγR while retaining relatively high affinity to the inhibiting FcγRIIb (Table 1), this may serve as an evolutionary way to prevent excessive immune responses against these sterile antigens not posing infectious threats. IgG4 has therefore been characterized as ‘blocking antibody’, especially in the context of allergy, where it may compete with IgE for allergen binding.

**Allotypes**

In addition to isotypic variation, allelic variation is found amongst the IgG subclasses (Fig. 5). These polymorphic epitopes of immunoglobulins that can differ between individuals and ethnic groups were originally discovered on the basis of serological findings, as immunogenic determinants were found on IgG from some individuals but not others. Subsequently, allotypic variations were genetically analyzed, and a number of structural determinants identified. A large number of polymorphisms were found in IgG, a finding made useful for example in paternity testing and forensic medicine before HLA typing became available. Exposure of an individual to a non-self allotype can induce an anti-allotype response, and may occur in transfused individuals and has even been described in a pregnant woman. However, not all variations in IgG amino acid sequence lead to determinants that are immunogenic because some determinants are found in other isotypes, and are therefore called isoallotypic variants (Fig. 5). Other variations in amino acid sequence can be present at sites that are minimally exposed and therefore may not result in determinants that can be serologically discriminated.

Since some allotypes have proven to be immunogenic, they may be relevant to consider when developing therapeutic antibodies. Treatment using therapeutic monoclonal antibodies can in principle also lead to an anti-allotype response. However, to date, little evidence has been found for significant anti-allotype responses, e.g., adalimumab or infliximab. There are no known allotypic variations that result in a functionally different antibody, except for IgG3, where a few isoallotypic variants result in extended half-life (discussed in section on FcRn).

In conclusion, most of the genetic variation in IgG have potential implications far beyond the original serological findings. This can have functional consequences, on expression levels, half-life, FcγR binding (inducing Antibody Dependent Cellular Cytotoxicity (ADCC), and Antibody Dependent Cellular Phagocytosis (ADCP)), tendency to form oligomers and activate complement, and influence on immunogenicity – thus, again, therefore have important consequences for antibody-mediated immunotherapies.
**Glycosylation**

All immunoglobulins isotypes contain a conserved glycan, which is at position N297 of the heavy chains for the IgG subclasses (IgG Fc-glycosylation) ([Fig. 3b](#)). In addition, roughly 10-20% of the Fab have N-glycosylation sites in the binding region. As further discussed below, IgG Fc-glycosylation is important for the effector functions of IgG Fc. Complete removal of the Fc glycan abrogates FcγR and C1q binding and inhibits effector functions of IgG. Furthermore, the composition of the glycan affects the affinity for Fc gamma receptors (FcγR) and complement. This is partly because this composition influences the quaternary structure of the Fc. Interactions of the glycan with the protein backbone stabilize the Fc. Addition of the Fc-glycan gives the IgG-Fc a more open conformation, allowing binding to FcγR. In addition, the glycan is in close proximity to the FcγR itself, contribution to the binding through glycan protein binding. Perhaps even more important is the fact that human FcγRIIIa and FcγRIIIb express a conserved glycan at position 162, entering the Fc-space confining the Fc-glycan, enabling a direct glycan-glycan interaction.

It has been shown that fucosylation reduces the affinity for FcγRIII and galactosylation increases the affinity for C1q, which results in changes in *in vitro* effector functions of antibodies bearing these changes. These changes in glycan composition can also be found on IgG from patients. For example, in fetal neonatal allo-immune thrombocytopenia (FNAIT), where antigen specific IgG with decreased fucosylation is found and associated with disease severity. Or in patients with rheumatoid arthritis (RA), where a decrease of galactosylation of total IgG is found and associated with disease progression. It has also been proposed that the composition of the IgG Fc-glycan is important for the function of intravenous immunoglobulin (IVIg). IVIg is pooled immunoglobulins from blood or serum donors, consisting of mainly IgG, used for the treatment of immune deficiencies and autoimmune diseases such as immune thrombocytopenia (ITP) and Kawasaki disease. The following paragraphs will elaborate on the composition, synthesis and clinical relevance of IgG glycosylation.

**Glycan composition**

The core structure of the IgG N-glycans comprises N-acetylglucosamine (GlcNAc) and mannose residues. This can be further extended with galactose, sialic acid, core fucosylation and bi-sected GlcNAc ([Fig. 3b](#)). Several dozen IgG-Fc glycoforms have been found in healthy human serum, of which only a handful represent the dominant form (fucosylated species in one of the following configuration: without galactose, with one or two galactose residues, or with two galactose and a single sialic acid residue, [Fig. 3b](#)). Between Fab and Fc glycans there are several differences in glycosylation, those most pronounced being markedly increased levels of bisection, galactosylation and sialylation in the Fab glycans (including di-sialylation hardly seen in the Fc), but reduced
**Figure 5. IgG subclasses and IgG allotypes.**

**a)** All differences between the IgG isotypes depicted schematically according to their localization (numbered below a graphical representation of the gene) in the different domains and exons depicted above the sequence. Bold underlined numbers (EU numbering) contain isoallotypic variant at that position. Amino acids depicted in bold varies from the other subclasses, but amino acids depicted in italics are present in two subclasses. The green boxed amino acids numbers are residues involved in binding to C1q, in red amino acids involved in FcγR binding, and in blue residues involved in binding to FcRn. “–” instead of a letter for amino acid stands for the missing G236 residue in IgG2.

**b)** The amino acid variation found within IgG1, b) for amino acid stands for the missing G236 residue in IgG2.
fucosylation (\(\sim94\%\) for Fc, vs \(\sim70\%\) for Fab)\(^{107,120}\). This is partially due to accessibility for glycosyltransferases and glycosidases; Fab sites are generally more accessible compared to the conserved site in the Fc which lies more buried between the two heavy chains (Fig. 3a). In addition, glycosylation levels are also controlled by availability of glycosyltransferases in the B-cells. As mentioned above, in several health and disease settings a shift towards certain Fab- and Fc-glycoforms of antibodies have been reported\(^{64–66,70,122,123}\). This shift may occur through epigenetic influence on expression of glycosyltransferases\(^{124}\) and glycosidases, which is clearly affected by various factors including, age, pregnancy, hormones, cytokines, bacterial DNA and food metabolites\(^{125–127}\). It seems that at immunization, IgG glycosylation is determined by the signals the B-cells receive, dependent on amongst others, the nature of the antigen\(^{67,123,127}\). Additionally the variable glycan patterns can be remembered for much longer, as demonstrated by a stable glycan pattern upon multiple subsequent antigen exposures\(^{68}\). This all gives some insight in the regulation and working mechanism of glycosylation in B-cells, especially that of antibodies, but much is still unknown and therefore more knowledge on this has to be gathered in order to understand the full mechanism.

**IgG glycan biosynthesis**

The N-glycosylation of antibodies follows the classical N-glycosylation pathway\(^{128}\). The glycans of N-linked glycans are attached to the asparagine site in the consensus sequence for N-linked glycans: Asn-X-Ser/Thr, where X can be any amino acid except proline. Glycan synthesis starts at the endoplasmic reticulum (ER) when a lipid linked precursor oligosaccharide is synthesized (Fig. 6)\(^{129}\). In the ER lumen this precursor is transferred to the Asn site of the protein. Further processing of the glycan then takes place in the ER and Golgi apparatus, which includes trimming and remodeling of the glycan. The cell type specific spatial and temporal organization of glycosidases and glycosyltransferase expression in ER and Golgi apparatus regulate the final composition of the glycans. In the right panel in Fig. 6 is indicated which enzymes are expressed in the different compartments of the ER and Golgi network\(^{128–130}\). For IgG, assembly of heavy and light chains takes place early, after initial trimming of glucose and mannose groups by the glucosidases and ER mannosidase I and processing continues when the whole complex is transported to the cis-Golgi\(^{128}\). The diversity of the glycans derives from several factors; involvement of many different enzymes and substrates in different compartments, variable modification of glycan core structure to bi-, tri- and tetra-antennary, competition between enzymes for substrates and
acceptors, accessibility of the enzymes to the glycan, incomplete processing and other post-translational modifications on the same protein\textsuperscript{128}.

Extracellularly, glycosyltransferases and glycosidases are present in circulation, mainly editing the terminal sialic acid groups on glycans\textsuperscript{131}. This has been proven to be a functional mechanism in the sialylation of IgG, found in a study where mice with ST6GalT1 deficient B-cells did contain sialylated IgG. This occurs through liver derived ST6galT1 and platelet derived CMP-sialic acid as sugar donor, which are present in circulation\textsuperscript{131}.

Up until now there has been very little focus on the exact expression pattern of the different glycosyltransferases and glycosidases in antibody secreting B-cells and its effect on antibody glycosylation patterns\textsuperscript{132}. Much more is known about the expression of these enzymes in cell lines, that are used for production of monoclonal antibodies, caused by much higher and easier availability of protein material and cells and also due to the interest and requirement of this knowledge from the pharmaceutical industry.

Considering \textit{in vivo} data, a GWAS on the genome and IgG glycosylation levels showed several loci to correlate with the levels of IgG glycosylation\textsuperscript{133}. However, of the 9 significant loci only 4 were previously found to be directly associated with N-glycosylation (ST6GAL1, B4GALT1, FUT8, and MGAT3) and the others probably are involved in higher levels of regulation. Additionally, a study by Menni et al., showed correlation between epigenetic markers and antibody glycosylation, partially on genes for glycosyltransferases\textsuperscript{124}. cDNA sequence libraries of B-cells or immune cell specific proteomics could aid us in finding clues what expression patterns of the specific glycosyltransferases are in these cells. The questions are which ones and at which level specific glycosyltransferases and glycosidases are expressed in B-cells, including the signaling pathways involved. This would however not tell much yet about the specific glycosylation, as this is not solely dependent on the expression level, but also on the site of expression and availability of substrate, as well as nature of recipient glycoprotein and similarly its location. Correlation of the expression data and the IgG glycosylation levels would therefore be very informative.

A start on this research was made by a few groups. A pilot-study showed a positive correlation with the sialyltransferase/neuraminidase (ST3Gal-1/Neu3) ratios expressed on the surface of B-cells and severity of Systemic Lupus Erythematosus, but did not study if this effect was reached through cell-cell interaction or by glycosylation of secreted IgG\textsuperscript{134}. Additionally, it was not studied if the levels of those enzymes was different in the intracellular compartments of the B-cells as they studied only surface expression, and we know that ST3Gal-1 is not involved in IgG Fc glycosylation, but rather ST6Gal-1 for correct 2,6 sialic acid linkage\textsuperscript{135}.

Our knowledge on how the different glycoforms affect the effector function of IgG is still in its infancy, but some aspect have been becoming increasingly clear as summarized below for each glycan end group (fucosylation, bisection, galactosylation and sialylation).
Fucosylation

It has been known for quite some time that core-fucosylation of the IgG-Fc affects binding to FcyRIIIa, with non-fucosylated antibodies binding FcyRIIIa much stronger. This higher affinity translates into higher ADCC and phagocytosis of targets by these antibodies, and has been put to use in therapeutic antibodies, to increase the efficacy of treatment (reviewed by). More recently it has become clear that this also applies to FcyRIIIb expressed on granulocytes. The molecular nature of this increased affinity is discussed below (section FcyRIIIa). After vaccination, or apparently during most normal immune responses, the IgG-responses in humans are restricted to IgG with core-fucose attached, as seen against soluble proteins during influenza or tetanus toxoid vaccination. This also reflected by the fact that ~94% of IgG-Fc glycopeptides are fucosylated in total glycosylation.
serum. Even more strikingly is the fact that IgG fucosylation is prevented in some immune responses against particulate antigens, e.g. red blood cells and platelets, but also witnessed in response to viral infections such as dengue fever, or in some elite controllers of HIV. Thus apparently, FcγRIII-mediated IgG responses can be fine-tuned through IgG fucosylation towards more pro- or anti-inflammatory effects.

**Bisection**

Slight changes in bisection have been detected for some antigen specific IgG responses. Little is known about the importance of the biological implication of these changes. It has been described that fucosylation and bisection occur in a reciprocal manner, with proximal bisection blocking fucosylation of IgG, making it difficult discriminate the effect of bisection from core fucosylation.

**Galactosylation**

Our group has shown that, in addition to reduced fucosylation, an increase in galactosylation of HPA-1a specific IgG in FNAIT correlates with disease severity. Furthermore, immunization trough natural infection seems to result in a transient increase in galactosylation of antigen-specific IgG in humans, while having no effect on total IgG galactosylation. This could be relevant as galactosylation seems to increase affinity to FcγRIIIa and FcγRIIIb. However, general decrease in galactosylation has been found in several autoimmune-diseases (reviewed in), suggesting agalactosylated IgG to be more pathogenic, or galactosylated IgG to have anti-inflammatory activity. This includes rheumatoid arthritis, a disease which often goes into remission during pregnancy – correlating with the general increase in galactosylation in pregnancy. Besides FcγR mediated effector functions, it is proposed that enhanced galactosylation increases the affinity of IgG for C1q and therefore causes increased classical complement pathway activation.

**Sialylation**

As the terminal – and the only charged – sugar moiety, sialylation has been proposed to have the most effect on the structure of the Fc domain of the antibody, by closing the binding site for activating FcγRs, but opening up a cryptic binding site for DC-SIGN in the CH2-CH3 interface. In proof of this, a structural study with actual comparisons of IgG with or withoutFc-sialylation have confirmed this opening and closing of the CH2-CH3 interface and FcγR binding site. Additionally, sialylation of mouse IgG decreased affinity to mouse FcγR in general, although systematic analysis of the importance of this for human IgG-FcγR binding is lacking and some debate about of human sialylated IgG binds DC-SIGN and the function of this interaction. Binding to DC-SIGN sialylated antibodies has been suggested to have strong immunomodulatory function as described below.
(section DC-SIGN). Increased sialylation of IgG generally follows increased galactosylation as galactosylated IgG is the substrate for sialyltransferases (Fig. 3b)\textsuperscript{64,66,135}. For complement activation it was suggested that additional sialylation abrogates the increased C1q binding of galactosylated IgG\textsuperscript{116}.

**Glyco-engineering efforts**

Since it was found that Fc glycosylation is important for the function of IgG many research has gone into modifying this glycan during or after expression of IgG. There are several approaches which can be used to modify the glycan, the easiest are inhibitors of glycosylation or glycan processing. Examples of these are; tunicamycin, which blocks the transfer of GlcNAc to the DicHitol-PP and thus completely blocks N-glycosylation, kifenusine, blocking α-mannosidase 1, leaving the glycan as high mannose form\textsuperscript{155}, and swainsonine blocking α-mannosidase 2 leaving the glycan as hybrid-type\textsuperscript{111,156}. Addition of specific glycan end groups can also be inhibited by blockers added to the antibody producing cell medium or even added to the diet, examples are; 2-deoxy-fluoro-L-fucose, specifically blocking fucose addition by fucosyltransferase\textsuperscript{64,157} or sialic acid analogues blocking sialic acid addition by sialyltransferase\textsuperscript{156}. Furthermore, changes to the glycan can also be induced by adapting the culture conditions, medium and substrates in the medium of the cell lines producing the glycoproteins\textsuperscript{132}.

Besides changing the environment of the IgG producing cell lines, there have also been efforts to change the cell lines itself. Multiple platforms to produce glyco-engineered IgG have been developed and are currently used by the industry. Mammalian or human cell lines knocked out for fucosyltransferase (FUT8), produce antibodies completely lacking core fucose\textsuperscript{158}, knocked in of GntIII enhances the level of bisection\textsuperscript{159}. More elaborately, some groups have completely engineered the glycosylation pathways in several cell lines to produce any or a specific glycoform at will, in CHO cells\textsuperscript{69}, or in Pichia pastoris (yeast)\textsuperscript{160}, insect cells\textsuperscript{161}, or plants\textsuperscript{162,163}. Additionally, also transient transfection of glycosyltransferases to cell lines is sufficient to enhance certain glycan end groups\textsuperscript{164}.

Lastly, a secure way to engineer the glycosylation is by chemo-enzymatic engineering of the glycan, which is widely used by many groups\textsuperscript{148,150,165–169}. Using glycosidases or glycosyltransferases to respectively remove or add whole glycans or certain glycan end-groups ensures very specific editing of the glycan. The advantage of this latter methods is that it allows for comparison with the source material where only a single glycan trait has changed.

**Effector mechanisms**

**Binding to effector molecules**

Antibodies link the adaptive immune system with the effector mechanisms of the
innate immune system. They form a bridge by combining antigen-binding sites with binding sites for many innate receptors and adaptor molecules. The effector mechanisms that will be triggered vary between the different immunoglobulin subclasses. Typically, IgG1 and IgG3 are potent triggers of effector mechanisms whereas IgG2 and IgG4 will induce more subtle responses, and only in certain cases. However, these antibodies remain capable of neutralizing virus particles and toxins. Below, binding to C1q and Fc receptors is discussed, emphasizing the structural aspects that differ between the subclasses (Table 1).

C1q

Upon binding to target surfaces, IgG, as well as IgM, can activate complement. Complement activation is initiated through binding and subsequent activation of C1q, leading to deposition of C3b to further opsonize the target, but also to the formation of the membrane attack complex, C5-C9, causing disruption of the targeted bilipid membrane\textsuperscript{170}. IgG1 and IgG3 can efficiently trigger this classical route of complement\textsuperscript{171}, but IgG2 and IgG4 do so, much less efficient or only under certain conditions for IgG2. This is due in large part to the reduced binding of C1q to the latter subclasses\textsuperscript{171–173}, although it has also been described that in addition to C1q binding, downstream events of the complement cascade (C4b deposition) are differentially affected by the different IgG subclasses\textsuperscript{171}. Residues in the CH2 region important for C1q binding include L235, D270, K322, P329, and P331\textsuperscript{173–176}. In IgG2, reduced C1q binding appears to be largely caused by residue A235 (which is Leu in other subclasses)\textsuperscript{176}, whereas in IgG4, P331 is – at least in part – responsible for the reduced or absent binding of C1q\textsuperscript{173,174}. Structural determinants in the middle or ‘core’ hinge region (residues 226-230) can influence the binding of C1q\textsuperscript{177}. On the one hand, rigidity in this region contributes favorably to C1q binding, whereas removal of cysteine bonds negatively affects binding. It has also been suggested that the relatively long hinge of IgG3 makes the C1q binding site more accessible resulting in more efficient complement activation\textsuperscript{178,179}. However, IgG3 engineered with a short IgG4 hinge binds C1q efficiently, although complement activation was somewhat reduced\textsuperscript{180}.

Interestingly, IgG has recently been suggested to form hexamers by interactions through the CH2-CH3 interface when opsonized on target surfaces, forming an optimal platform for the hexameric configuration of C1q\textsuperscript{106}. These data are supported by mutation in this interface, e.g. I235 in the CH2 and H433 in the CH3 that individually affect complement activation through C1q\textsuperscript{106}. As mentioned previously, the IgG glycoform, mainly galactosylation and sialylation affect C1q binding\textsuperscript{116}. Whether and how the glycoform of IgG influences this binding or the formation of hexamers has to be investigated in more detail.

Curiously, engineered IgG1/3 hybrids with an IgG1-CH1 and hinge regions were found more potent in complement activation compared to wild-type IgG3, with the largest contribution arising from the CH1 domain swap\textsuperscript{181}. Conversely, the binding of C1q to IgG4 can be influenced by shielding of the potential binding site by Fab arms\textsuperscript{179,182–184}. The orientation
of the Fabs have been modelled to be perpendicular to that of the hexameric platform of IgG on solid surfaces and in solution\textsuperscript{106} and may thereby affect C1q binding, although this needs to be confirmed. IgG4 also results in less complement activation by forming small immune complexes, probably because of their monovalency due to Fab-arm exchange, and in this way can even reduce complement activation by IgG1 antibodies\textsuperscript{185}. Although the short hinge of IgG2 may lead to similar shielding of the potential C1q binding site, a notion that fits with its general poor activation of the classical complement cascade, IgG2 can activate this cascade at high densities of surface antigens, as is the case for polysaccharides – to which IgG2 antibodies tend to form\textsuperscript{18,186,187}. At these high epitope densities, IgG2 may be more likely to efficiently form hexamers, increasing the avidity of this subclass for C1q substantially\textsuperscript{106}.

**Fcγ-Receptors**

FcγR bind to a region partially overlapping the C1q binding site. The binding of IgG to these receptors has been studied in detail. For all FcγR interactions, the stretch of amino acids comprising the N-terminus of the CH2 domains and strands adjacent in the 3 dimensional immunoglobulin fold are important for binding. In general this encompasses amino acids 234-239, 265-269, 297-9 and 327-330\textsuperscript{63,188}. However, each of the IgG subclasses has a unique binding profile to each FcγR (Table 1)\textsuperscript{189,190}.

Humans express 5 different FcγR; FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa, and FcγRIIIb (Fig. 7a). FcγRIIa, FcγRIIIa and FcγRIIIb are present in allotypic variants within the population\textsuperscript{189}. The expression profiles of the different FcγR are highly variable between different immune cells of myeloid and NK cell origin\textsuperscript{191,192}.

For the different IgG subclasses, a major distinction can be made between IgG1/IgG3 that interact efficiently with most FcγR, and IgG2/IgG4, which show reduced affinity to a number of FcγR. Furthermore, monomeric IgG3 binds more efficient than monomeric IgG1 to FcγRIIa, FcγRIIIa, and FcγRIIIb; and binding efficiency of complexed IgG3 to all Fc receptors exceeds that of IgG1\textsuperscript{189}. Structural determinants responsible for the differences in affinities for FcγR between IgG1 and IgG3 are still unknown. Below, structural differences that are known to be responsible for the subclass-specific variations are discussed.

**FcγRI**

Although FcγRI is often referred to as a single entity, FcγRI consists of three homologous genes on the short arm of chromosome one\textsuperscript{193}, and several alternative splice variants have been described\textsuperscript{194}. However, only the existence of the full-length form, FcγRIa, consisting of three extracellular domains has been studied in detail. The gene encoding for the potential FcγRIIb variant, potentially consists of a nearly identical receptor with only the two N-terminal extracellular Ig-domains, but retaining the intracellular cytoplasmic tail, while FcγRIc would also lacks the cytoplasmic tail and transmembrane region, and would
therefore be predicted to represent a secreted form. FcγRIa is expressed on monocytes, macrophages and dendritic cells and binds all human IgG subclasses except IgG2, and unlike the other FcγR, contains its unique third membrane-proximal immunoglobulin domain that is also responsible for its higher affinity to IgG\(^{53,192,195}\). Mutations of IgG1 in the lower hinge to the IgG2 equivalents, in particular E233P, L235A, and G236Delta, abrogate binding\(^{196–200}\). Binding to FcγRI is reduced for IgG4\(^{189}\), and both P331S and L234F are implicated to account for the reduced binding in comparison to IgG3\(^{199}\), but P331 may not be important for binding of IgG1\(^{196,200}\). An IgG3 with a partially deleted hinge was found to have reduced binding to FcγRI and FcγRIIa\(^{201}\).

**FcγRIIa**

FcγRIIa is the most widely expressed FcγR on myeloid cells, has been described as the only FcγR with significant binding to IgG2\(^{189,202–204}\). Binding is more efficient for the H131 (‘low-responder, LR) variant than the R131 (‘high-responder, HR) variant (nomenclature based on differential binding to mouse IgG1 which binds the HR much better)\(^{203}\). Binding affinity varies among subclasses as follows: IgG3>IgG1>IgG4=IgG2. Recently, a crystal structure of the complex of IgG1 Fc with FcγRIIa was published\(^{188}\), and contact residues relating to differences in subclass binding include L234, L235, G236 in the lower hinge, and the structurally adjacent A327. Significantly, the R131-site in FcγRIIa is also in close proximity to the lower hinge in this co-crystal structure. Thus the lowered binding affinity of IgG2 to FcγRIIa, and the differential binding to the HR/LR-form of FcγRIIa, may also be
attributed to differences in the hinge of IgG2.

**FcγRIIb/IIc**

The extracellular domain of the inhibiting FcγRIIb is identical to the activating FcγRIIc that is expressed in ~11% of individuals\(^{205,206}\). Binding to the inhibitory receptor FcγRIIb or FcγRIIc is weak for all subclasses, generally preferring IgG3=IgG1=IgG4>IgG2. Interestingly, dissociation constants for binding of monomeric IgG1 and IgG3 are similar, but immune complexes of IgG3 seem to bind more efficiently compared to IgG1\(^{189}\). Binding to most activating Fc receptors is lower for IgG4 compared to IgG1, but this is not the case for the inhibitory receptor FcγRIIb. This altered balance between binding to activating receptors in comparison to inhibitory receptors may be an important feature of IgG4 that contributes to its low pro-inflammatory capacity.

**FcγRIIIa**

Two allotypic variants of FcγRIIIa exist: F158 and V158. The V158 variant has greater affinity for all subclasses and for IgG3 binding efficiency approaches that of FcγRIa\(^{189}\), with general affinities following IgG3>IgG1>>IgG4>IgG2. Besides changing amino acids 233-236 from IgG1 to the IgG2 equivalents, A327G (Ala present in IgG1 and IgG3, Gly in IgG2 and IgG4) also results in decreased binding\(^{200,207}\). Binding affinity of FcγRIIIa seems to be particularly sensitive to core-fucosylation of the N-linked glycan at N297 of the Fc-tail of IgG, as its binding affinity can be enhanced up to 50 times – with corresponding increase in effector function – if the Fc tail is not fucosylated\(^{160}\). Recent work by Ferrara et al. has pinpointed this interaction to be due to carbohydrate-carbohydrate interactions between the glycan on N297 of the heavy chain and glycosylation of FcγRIIIa at position 162 – a position unique to both FcγRIIIa and FcγRIIIb\(^{113}\).

**FcγRIIIb**

There are also functional allotypic variations of the neutrophil FcγRIIIb, referred to as human neutrophil antigen 1 (NA1/ HNA1a) and (NA2/ HNA1b)\(^{208}\). The FcγRIIIb-NA1 form is capable of better ingestion of IgG1- or IgG3-opsonized particles than FcγRIIIb-NA2\(^{209}\). FcγRIIIb generally binds IgG1 and IgG3 but not IgG2 and IgG4, with IgG3 binding better than IgG1\(^{189}\). A crystal structure of the complex of IgG1 Fc with FcγRIIIb reveals amino acids 234-238 to be important contact residues, and the subclass-specific variation in this area again can explain the lack of binding of IgG2 and IgG4 to this receptor\(^{63,210}\).

**FcRn**

In the 1960s, the existence of a receptor responsible for the unusually long half-life of Immunoglobulin G (IgG) (three weeks, **Table 1**) and efficient transport from mother to young was first proposed by Brambell\(^{211,212}\). This was later confirmed by various groups and
eventually cloned and identified as the neonatal Fc receptor (FcRn)\textsuperscript{213–216}.

Structurally, FcRn, is strikingly similar to MHC-class I molecules\textsuperscript{217,218}. FcRn is co-expressed with the non-glycosylated 12 kDa β2-microglobulin. Human FcRn does not bind its ligand at physiological pH (pH 7.4). Only in the acidic environment of endocytic vacuoles (pH \(\leq 6.5\)) binding to FcRn takes place, where solvent exposed histidine residues in IgG are protonated\textsuperscript{216,219,220}. Histidine residues within the Fc-tail of IgG (CH2-CH3 interface) are critical for high affinity binding to FcRn\textsuperscript{200,218,221}. H435 sits at the heart of this interface, and the lowered affinity of R435-containing allotypes of IgG3 to FcRn explains their shortened half-life and lowered placental transport. (\textbf{Table 1}). Consequently, in individuals containing H435 containing IgG3 allotypes (g3m, 15 or 16), IgG3 has a normal half-life of three weeks and is transported efficiently across the placenta\textsuperscript{19,222}.

That FcRn protects IgG from degradation has been confirmed by mouse models: IgG half live is decreased in FcRn or β2-microglobulin deficient mice\textsuperscript{213–216}. FcRn on both endothelial cells and myeloid cells are mainly responsible for recycling\textsuperscript{223,224}. Also, overexpression of FcRn in transgenic animals results in higher IgG serum levels\textsuperscript{225}. However, FcRn starts its function early in life by transport of IgG – and thereby humoral immunity – across the placenta from mother to young\textsuperscript{215,226–228} and in rodents also after birth by transport from mothers milk in the gut of suckling neonates.

In adult life, FcRn is expressed on many epithelial cells, and continues to function in IgG transport across FcRn-expression epithelial barriers\textsuperscript{229}. FcRn is able (in all species) to bi-directionally transcytose cargo across polarized (both epithelial and endothelial) cells\textsuperscript{230–232}. IgG or IgG-antigen complexes have been described to be transported across mucosal surfaces like the intestinal cavity or respiratory epithelium, and thereby to function in immune surveillance\textsuperscript{228,229,233,234}. With this role in mucosal immunity, it complements sIgA in immunoregulatory function as reviewed in\textsuperscript{235}.

On mucosal cells, FcRn has been found to transport IgG and be involved in antigen sampling\textsuperscript{228,229,234}, and its expression on phagocytic cells\textsuperscript{229,236} has recently been found to enhance phagocytosis capacity of IgG-opsonized particles\textsuperscript{237,238}. On antigen presenting cells, this ingestion of IgG-complexes can lead to enhanced presentation\textsuperscript{239–241}. Similar to phagocytosis responses, the enhanced presentation likely requires the external sensing and cellular activation through FcγR and pattern-recognition receptors, handing the IgG-Antigen cargo over to FcRn at low pH\textsuperscript{238,239,242}. Thus, immunoglobulin activities including extended half-life, transport to young, and antigen sampling seem to be orchestrated through a single receptor, the MHC-class I-like FcRn. In contrast, other effector functions of IgG, such as phagocytosis and antigen-presentation seem to be mediated by both FcRn and classical FcγRs.
Mouse Fc Receptors

Mouse express 4 types of FcγR, FcyRI, FcyRIIb, FcyRIIIa and FcyRIV (Fig. 7b). Even through there is much homology between mouse and human FcγR, there are some differences in expression pattern and function, as reviewed by Pierre Bruhns in53. Mouse FcγRI is expressed on monocytes and dendritic cells and binds mouse IgG2a, and mouse IgG2b only in the case of certain allotypes243,244. Mouse FcγRIIb, similar to humans, the only FcγR in mouse with a ITIM signaling domain binds to all mouse IgG subclasses except IgG3. FcγRIII in sequence and expression pattern most similar to human FcγRIIa, and binds to all mouse IgG subclasses except IgG3. FcγRIV is homologous to human FcγRIIIa, but is not expressed on mouse NK cells and binds IgG2a and IgG2b244. FcRn has similar function in mouse as in humans, the binding strength at pH 6.5 is similar for mouse IgG compared to human, whilst half-life of mouse IgG is shorter (Table 2)245.

Alternative Receptors for IgG

FcRL

Fc receptor-like proteins, consisting of 6 members (FCRL1-6) were originally identified as homologues of FcyR but were for a long time regarded as orphan receptors, mostly expressed on B cells. Recently however, FcRL4 and in particular FcRL5 were found to bind Immunoglobulin as well, with the former recognizing IgA, IgG3 and IgG4, while FcRL5 binds all IgG subclasses similarly well, but not IgA. Both these receptors are expressed on B cells, express an ITIM and are known to down regulate B cells after BCR cross linking through recruitment of SHP-1246,247. Although FcRL5 seems broadly expressed on B cells populations248, FcRL4 is only expressed on sub epithelial tissue B cells249 reportedly of mucosal origin, suggesting that perhaps this receptors is involved in negative feedback inhibition through antigen-specific IgG and IgA, respectively.

TRIM21

Tripartite motif-containing protein 21 (TRIM21) is a cytosolic protein expressed in almost all cell types but highly expressed in immune cells. TRIM21 previously known as an autoantigen involved in several autoimmune diseases, e.g. systemic lupus erythematosus (SLE)250. TRIM21 was found to bind IgG with nanomolar affinity251,252. TRIM21 binds IgG in the Fc domain at the CH2-CH3 interface similar to FcRn and protein A/G, it competes for binding to IgG with protein A/G, and binding is independent of N-glycosylation of the CH2 domain253. Later it was demonstrated that TRIM21 functioned as an immunological sensor, targeting IgG-opsonized virus and bacteria for antibody-dependent intracellular neutralization by the ubiquitin dependent proteosome254–257. It also activates further signaling and innate immunity responses are activated, characterized by the NF-κB, AP-1 and IRF pathways258.
The unique localization of this receptor in the cytoplasm leaves many unanswered questions but simultaneously answering many. It helps to explain how partially opsonized pathogens may still be recognized and neutralized during the early phase of infection, escaping recognition by the complement- and FcγR system. The relative importance of this system is still unknown during secondary infections, but may perhaps be relatively more important at locations where complement and the myeloid system are less prominently present, e.g., at mucosal surfaces of the gut.

**DC-SIGN**

Some of the immunomodulatory activity of intravenous immunoglobulin (IVIg) has been attributed to the fraction of sialylated (SA) IgG\(^{153}\). It was proposed that sialylated IgG glycoforms bind to dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN or SIGN-R1 in mice)\(^{259,260}\). Since these glycoforms of IgG represents only a small fraction (<10%) of all IgG in the blood and IVIg treatment typically requires very high doses, it was suggested that this fraction may be predominantly responsible for the immunomodulatory functions of IVIg. However, the precise mechanisms of this interaction are unknown, and still await confirmation – particularly in the human setting, but also in mice as some of the methods used to enrich IVIG for SA were found to predominantly- if not exclusively- enrich for Fab-associated SA\(^{71,72}\).

It has been hypothesized that DC-SIGN binds to the CH2-CH3 interface of the Fc domain of IgG, owing to the opening up of the site where DC-SIGN binds due to the charged sialic acid. The simultaneous closing of the interaction site for FcγRs, has then proposed to yield an anti-inflammatory IgG\(^{151,152}\). Crosslinking of SIGN-R1 in mice has been described to result in the release of IL-33, which in turn activates basophils to secrete IL-4, upregulating expression of the inhibitory FcγRIIb\(^{259}\). However, recently an alternative receptor, the Dendritic cell Immunoreceptor (DCIR) has been put forward as an alternative candidate mediating the anti-inflammatory effect of sialylated-IgG, inducing upregulation of T regulatory cells and minimizing Ig-complex-mediated airway hyperresponsiveness\(^{261}\). To complicate things even further, another report claims sialic acid contents of IgG not to influence IgG binding to DC-SIGN, but to be rather Fab mediated\(^{154}\).

Furthermore, a number of Siglecs (Siglec-2/CD22, Siglec-8, Siglec-9) have been implicated as ligands for IVlg\(^{262-264}\), although in case of Siglec-9, there is evidence that lectin-specific antibodies in IVIG rather than sialylated antibodies are responsible for binding\(^{265}\).
Scope of the thesis

The composition of the conserved N297 glycan in the IgG Fc domain varies upon different circumstances, like infection status, age or in specific antibodies by the nature of immunization. The bi-antennary glycan contains a constant structure of N-acetylglucosamine (GlcNAc) and mannose groups and can be variably extended with core fucose, bisecting GlcNAc (bisection), on the bisecting arms galactoses and terminally sialic acids. Generally the most prevailing glycoforms are fucosylated and with one or two galactoses (G1 or G2 respectively). However, in the heterogeneous mixture 14 prevailing glycoforms can normally be found in human serum.

The binding of the Fc domain to Fc gamma receptors (FcγR) present on myeloid cells or to C1q of the complement system is important for the effector function of the antibodies. Fc-glycan removal abrogates binding and effector functions. The Fc glycan composition further influences the binding affinity to these receptors, by causing global or subtle changes in the Fc conformation and by direct glycan-glycan interactions as described for the Fc-FcγRIII interaction with the N297 glycan on IgG and N162 glycan on FcγRIII. This interactions seems partly responsible for the decreased affinity of fucosylated IgG to this receptor.

By modifying the composition of the IgG Fc glycan the efficacy of IgG based therapies can be influenced. For example, by afucosylating therapeutic monoclonal antibodies used in cancer treatment, these bind better to the FcγRIII and mediate better ADCC. Other IgG therapies like intravenous immunoglobulin (IVIg), or prophylactic anti rhesus D (RhD) could also be more effective by changing the glycan composition. At the start of this research, besides the effect of fucosylation, not much was known of the effect of the other glycan adducts on IgG function.

To understand the mechanisms behind the before mentioned processes we aimed to determine the function of the different possible IgG glycoforms. We therefore put sustainable effort in creating a glyco-engineering toolbox to change each glycan end group as individually as possible (Chapter 2). We aimed to obtain different IgG glycoform pools to be able to screen the different glycoforms for human FcγR binding, cellular activation via FcγRIIIa, and their ability to bind and activate the complement system (Chapter 3).

In this chapter we sought out to see whether or not only fucosylation but also the other glycan adducts and combinations thereof affect binding to each human FcγR and allotypic variant separately. Also, whether any of the found changes in affinity could be translated in mediation of antibody dependent cellular cytotoxicity (ADCC) of red blood cells by FcγRIIIa bearing NK cells. Additionally, we intended to also screen the IgG glycovariants for C1q binding and activation, in both plate based and cellular assays.

We know that IgG glycan composition is important for the working mechanisms of IVIg as used to mediate auto-immune diseases. Multiple different working mechanisms were proposed, such as the importance of a small fraction of the IgG pool in IVIg, which
could be either the multimeric fraction or specific glycoforms, such as Fab glycosylated IgG or Fc-sialylated IgG. The latter was proposed to induce a immune modulatory effect via binding to DC-SIGN, but was until then not confirmed in a human setting. In Chapter 4, to elucidate these questions around the working mechanisms of IVIg we aimed to assess the blocking capacity of multiple components of IVIg, of which; the multimeric fraction, Fab-glycosylated/sialylated and Fc-sialylated in a human monocyte derived macrophage phagocytosis assay.

As it was shown by previous research that fucosylation enhances the activity of each IgG subclass, we aimed to further investigate this mechanism. By creating afucosylated variants of each human IgG subclass and testing these for binding to all human FcγR and allotypic variants. Furthermore, using both the monocyte derived macrophage phagocytosis assay and NK cell based ADCC assays we hoped to find where the functional differences lied, in respect to effector cell FcγR expression of the different IgG subclasses (Chapter 5).

Furthermore, we were curious to what exactly were the binding affinities of human IgG for the mouse FcγR family. As research into therapeutic antibodies requires validation in mouse models (Chapter 6). In Chapter 7 we have looked at the function of N162 glycan in the FcyRIIIa or FcyRIV mouse homologue and investigated whether the discriminatory function of this glycan is conserved between species. Additionally we aimed to see whether the effects of glycovariation on FcγR binding affinity found in chapter 3 for human IgG affinity are similar for mouse IgG glycovariants.

In the last research chapter we were wondering if the long IgG3 hinge contained glycans as there are putative O-glycosylation sites in the hinge of IgG3 (Chapter 8).

Finally, the work of this thesis was summarized and discussed in relevance to the current literature in the general discussion (Chapter 9).
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