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

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Chapter 9

General discussion

Gillian Dekkers
The objective of this thesis was to investigate the interactions of IgG with its main effectors complement component 1 (C1q) and Fc gamma receptors (FcγR). This thesis had a special emphasis on the influence of the IgG-Fc-glycans on these interactions and the functional consequences thereof. In the first chapter (Chapter 2), a toolset to create various different glycovariants was established. These methods were then combined in Chapter 3 to create 20 different glycovariants and to conduct functional experiments on both complement and FcγR binding and activation. The results in this chapter form the key to understand the meaning of the basic biological significance and consequences on natural glycan-changes in the human IgG-Fc. The other chapters were devoted to further investigate the evolutionary conservation of the functional change noted upon afucosylation of IgG and the consequences of IgG glycovariation for IVIg therapies. Below, I discuss these aspects in more details.

Glyco-engineering of antibodies; Application and drawbacks

As explained in the introduction (Chapter 1) several methods can be used to change the glycans on a an in-vitro produced glycoprotein such as IgG. Examples of used glyco-engineering strategies are, amongst others: blocking or enhancing substrates, adapting culture conditions, changing glycosylation machinery of the antibody producing cell lines by transient or stable transfection of enzyme encoding genes or knockout of genes, chemoenzymatically changing the glycans by replacing the whole glycan or editing single end groups. Up until now, most research on IgG glyco-engineering has focused on changing a single glycan end group at the time, or requires specialist knowledge and equipment for chemo-enzymatic engineering. Some require enzymatic modification of purified IgG, which can be extremely costly. In Chapter 2 we described the development of glyco-engineering tools to change the glycans of IgG1 at wish by utilizing the machinery of the IgG-producing cell itself. This resulted in the identification of new blocking substrates, transient transfection of glycosyltransferase genes, enhancing substrates and chemo-enzymatic engineering of a single end group for the final toolset. These methods were suitable to be applied alone or in combination, they were used to generate 20 different glycoforms as can be read in Chapter 3.

Our tools were mostly based on transiently changing the cells (HEK Freestyle, in our case) at production of IgG. This allowed us to apply the many combinations, without the need to maintain different cell lines. We aimed to get the most extreme outcomes for our goal to compare functionality of each end group individually and in combination. Potentially, our methods could also be fine-tuned to produce IgG with the concentration of one of the end groups with a specific percentage. For example – by co-transfecting a sub-optimal concentration of enzyme vector compared to the optimal concentration established in Chapter 3, the end group can be changed from anything between what we...
can maximally and minimally achieve.

Our approach could contribute to the development of production lines of glyco-engineered antibodies for therapeutic purposes. Most IgG for therapeutic purposes is produced in mammalian cell lines\textsuperscript{13,14}. Of those, Chinese Hamster Ovary (CHO), or murine NS0 and Sp2/0 cell lines are most often utilized, which also have the potential to add non-human glycan adducts to the N297 glycan, such as alpha gal (Galα 1–3Gal), α-2,3-linked neuraminic (sialic) acid and N-glycolylneuraminic (sialic) acid (Neu5Gc) instead of the human N-acetylneuraminic acid (Neu5Ac)\textsuperscript{15,16}. Alpha gal on therapeutic proteins, including antibodies can induce allergic reactions\textsuperscript{17} and α-2,3-linked neuraminic acid is not naturally found in the human Fc glycan\textsuperscript{18,19}. Our glyco-engineering methods – optimized for human HEK freestyle – could help develop similar methods for these mammalian cell based production lines. The downside of our method is the temporary transfection of glycosyltransferase enzymes, application in production lines would need to seek out more permanent changes to the production cell lines used, to avoid unwanted fluctuations in the glycosylation of the end-product. This is however not a major obstacle.

When we applied our tools for IgG1 production there was no excess of aberrant N-glycans – such as high mannose or hybrid type glycans. One phenomenon we observed was the minor increase of hybrid type glycans when overexpressing GntIII or B4galT1, the enzyme responsible for the bisection of the bi-antennary glycans or galactosylation, respectively, this effect has also been observed previously for GntIII overexpression\textsuperscript{20}. This might be due to a lowered affinity of the already bisected glycan for the enzymes responsible for further processing of the glycan.

The development of our glyco-engineering tools allows for creation of many glycoforms of IgG and potentially also any other N-linked glycoprotein. This expertise aids the research into the function of glycans and glycan composition of our IgG and proteins of interest.

The importance of glycosylation in FcγR-mediated IgG-effector functions

Many situations where antibodies are involved depend on the FcγR mediated effector functions\textsuperscript{21}. From bacterial and viral infections, where antibody-opsonized pathogens are phagocytosed by macrophages (antibody dependent cellular phagocytosis – ADCP) or cancer cells targeted by therapeutic antibodies, which are destroyed by NK- and myeloid cells via antibody dependent cellular cytotoxicity (ADCC) or trogocytosis (where myeloid cells rupture the membrane of target cells). Also allo- and autoimmune diseases where antibodies are often involved, FcγR-mediated mechanisms, sometimes apparently exclusively, are at play\textsuperscript{22}.

Allo-immune diseases arise when an individual has a reaction to a non-self antigen found in other individuals of the same species, for example blood groups or...
histocompatibility antigens (MHC), which can occur after transfusion or during pregnancy. Autoimmunity is the reaction to self-antigens, often complex multifaceted diseases where regulation of the immune response is disturbed (has gone away).

At the start of the research for this thesis, it was already known that afucosylation increases affinity to FcγRIIa\textsuperscript{23,24} and this was confirmed by the results presented in Chapter 3 of this thesis. In this chapter we also found that additional galactosylation of the afucosylated IgG even further increased the affinity for FcγRIII. The knowledge that afucosylation imposes better effector functions has already been put to use to enhance the function of therapeutic antibodies used in cancer treatment\textsuperscript{4,25}. Additional glyco-engineering the galactose end groups could thus even further improve the functionality of these antibodies. The human body also uses this mechanism in natural infections and immune reactions by producing total or antigen specific IgG with low fucosylation and/or changes in the other end groups\textsuperscript{26–30}, with seemingly better effector functions.

For the other glycan end groups, bisection and sialylation, we found some small effects on binding to FcγRIII. Additional sialylation of the galactosylated, afucosylated IgG caused a slight decrease in affinity, which corresponds with other research where the data show similar effects\textsuperscript{31–33}. Interestingly, variation in bisection did not appear to have any effect in the experiments conducted up until now, except for potentially enhancing the changes in binding affinity to FcγRIII due to fucosylation combined with galactosylation and sialylation described above. When the IgG was also high in bisection, we observed a slight trend for even more increased binding to FcγRIIIa upon defucosylation and galactosylation. We also observed a more pronounced negative effect of sialylation for binding to FcγRIII of the highly binding afucosylated, galactosylated IgG. The results of the effect of glycosylation on binding to FcγRIIIa are summarized in table 1.

In the recent year(s), it has become apparent that during viral infections of HIV and dengue fever antigen specific IgG may contain decreased levels of Fc fucosylation\textsuperscript{29,30}. For HIV this was observed especially for those patients who had a longer disease free survival, the so called elite controllers, and this correlated with the degree of antibody mediated cellular viral inhibition (ADCVI) of the patients serum. In dengue a higher degree of afucosylation was more often found in patients with antibody dependent enhancement of disease, which are unwanted side effects of infection\textsuperscript{30}. For these infections, and possibly more viral infections, the enhanced affinity for FcγRIIIa of the afucosylated antigen specific IgG indeed enhances ADCC and ADCVI of the virus and virus infected cells\textsuperscript{34}. For HIV this rationalizes the better clinical outcome for the patients who have more antigen specific antibodies with low fucose, but for dengue it indicates stronger side effects.

Interestingly, the changes in antibody fucosylation and galactosylation of antigen specific IgGs are also found in allo-immune settings. Examples of this are fetal neonatal immune thrombocytopenia (FNAIT) and hemolytic disease of the fetus or newborn (HDFN)\textsuperscript{27,28,35,36}. Both these diseases are in a pregnancy setting where the fetus is positive.
whilst the mother herself is negative for an paternal antigen on platelets or red blood cells (RBC), respectively for FNAIT or HDFN, and thus makes antibodies against the blood cells of the child upon exposure. This can lead to complications and is dangerous for the health of the child and hence it is important to diagnose correctly and timely and also treat accordingly\textsuperscript{37}. In the Netherlands the current way of diagnosis and (prophylactic) treatment differs between cell type and antigen. For HDFN directed against the RhD blood group all pregnant woman are screened for RhD blood group at the beginning of the pregnancy, if found negative she is at risk of developing antibodies. The blood of the mother is used to screen for the RhD type of the fetus in week 27 of the pregnancy (cell-free fetal DNA). When the fetus is RhD positive the mother will be given antenatal prophylactic anti-D treatment\textsuperscript{38–40}.

For FNAIT mainly directed against human platelet antigen 1A (HPA-1A) there is currently no standard screening program. Mothers with previous FNAIT complications will

### Table 1. Direct glycan traits and FcγRIIIa 158V binding

Results based on SPR binding studies of IgG1 glycoforms in Chapter 3 of this thesis. -, +, ++, ++++, ++++ respectively mean 1-2.5 fold, >10-15 fold, >15-20 fold, >20-25 fold and >25 fold increased affinity, when compared to unmodified IgG binding, based on FcγRIIIa 158V binding profile. F0; low fucose, F1; high fucose, G0; no galactose, G1; one galactose, G2; two galactoses, S0; no sialic acid, S1; one sialic acid, S2; two sialic acids, B0; no bisection, B1; high bisection.

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be monitored in follow-up pregnancies. Management varies from monitoring through fetal blood samples and intra-uterine platelet transfusions to maternal IVlg infusions with or without corticosteroid administration39.

The presence and titer of the antibodies do not accurately predict disease, although this correlation is strong enough for anti-RhD antibodies against RBC26. However, the correlation is worse for anti-platelet antibodies41, which is why no screening takes place, and treatment is offered only to females that in a previous pregnancy gave birth to severely thrombocytopenic baby, often with a fatal outcome39. Our, and other groups have found that also the subclass, but more importantly, glycosylation status of these antibodies matters for pathogenicity26,27,35. Screening for glycosylation status is not yet used in diagnosis and therefore the standard test in the Netherlands to evaluate this pathogenicity (for anti RBC reactivity) in the clinic, remains the use of an ADCC, using Fc-receptor expressing monocytes or NK cells to estimate the effector function of the patient’s antibodies42,43. Our group has found that the degree of fucosylation but also galactosylation of these antibodies correlate with enhanced disease severity26–28,35,36. The effector mechanism of these antibodies is thought to take place via FcγR bearing cells in the liver and spleen of the fetus, which target the RBC or platelets44. As shown in this thesis, both the level of fucosylation and galactosylation is also directive for the affinity to FcγRIIIa and therefore might explain the observed correlations of disease severity, probably (partly) mediated through FcγRIIIa28.

Besides during pregnancy, patients can also encounter foreign blood groups upon blood transfusion. This can also cause a similar reaction, producing antibodies against the blood group. And likewise here our group has found aberrant glycosylation patterns of the specific IgG antibodies36,45. The results suggests that there might be a general pattern of exposure that causes the antibody producing B-cell to down regulate its fucosyltransferase or upregulate fucosidases and excrete antibodies without fucose. The mechanism behind this and how it is regulated is still not known. Since we now know much more about the exact binding profile of the various IgG glycoforms to FcγRs and complement (see section below) it would be even more interesting to relate antigen specific single B-cells profiled for glycosylation status, glycosylation machinery enzyme expression and effector functions of its secreted antibodies. This would aid the research field in the development of better vaccines29,30, in better diagnosis of (allo-/auto-) antibody mediated diseases26–28,35,36 and development of therapeautic antibodies with the best effector mechanisms25.

In many autoimmune settings deviant glycosylation patterns of IgG have been detected46,47. This can be either in the total IgG of a patient or in the IgG specific for the disease46,48–51. Most often a decrease in total IgG Fc-galactosylation has been found and associated with disease progression or severity, for instance in Rheumatoid Arthritis (RA)46, Systemic Lupus Erythematosus (SLE)47, or inflammatory bowel disease (IBD)51,52. Total IgG Fc galactosylation increases during pregnancy, in patients with RA this increase is also observed and clearly associated with a remission of disease48,53–55. It has been shown
that FcγRs are important for RA and other autoimmune diseases\textsuperscript{56,57}. The role of these observed IgG glycosylation changes and its influence on disease activity needs to be further elucidated, but based on the data presented in this thesis, and those already found in the literature, it is possible to bring forward several testable hypotheses.

In humans, we know FcγRIIIa is an important activating FcγR, and of all FcγRs, its affinity for IgG is most influenced by changes in IgG Fc glycosylation. We propose a possible model on the involvement of FcγRIIIa in autoimmune diseases presented in Figure 1, where the differences between autoimmune remission and flare are illustrated in the context of changes in total IgG Fc galactosylation. Interestingly, no big changes in IgG Fc fucosylation have been observed in autoimmune diseases, except for RA specific ACPA upon onset of disease\textsuperscript{58}, however, in adults there is always a small percentage of total IgG Fc that is

\begin{figure}
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\caption{The influence of the degree of total IgG galactosylation upon FcγRIIIa occupation and activation in autoimmune remission and flare}

In autoimmune diseases, such as Rheumatoid Arthritis (RA), disease severity is negatively correlated with the degree of galactosylation. Galactosylation of IgG is important for binding to FcγRIIIa, where – in combination with afucosylation – a higher degree of IgG Fc galactosylation increases the affinity. Only \textasciitilde 6\% of normal serum IgGs is afucosylated. During remission (left) the total IgG galactosylation is relatively high, which prevents autoantibodies to engage the FcγRs. During a flare of disease (right) the total IgG galactosylation is low, which reduces the overall threshold of FcγR binding. And thus more pathogenic autoantibodies can bind the FcγRs on effector cells, causing activation (green lightning bolt).
afucosylated\textsuperscript{59}.

In our binding studies we showed, like others, that afucosylated highly galactosylated IgG to have a higher affinity for especially FcγRIIIa\textsuperscript{60,61}. In our cellular studies we also show that afucosylated IgG is able to efficiently induce and block NK cell mediated ADCC via FcγRIIIa binding on these cells. In the context of RA, afucosylated IgG is found to be around \sim 6\% on the glycopeptide level, comparable to healthy subjects. During disease remission, the bulk of antibodies have a normal galactose percentage whilst during a flare the bulk of antibodies is lowered in galactose, including this afucosylated fraction. In both situations the afucosylated IgG have a higher affinity for FcγRIIIa compared to the majority of fucosylated IgG. However, during remission, this fraction has higher levels of galactosylation and has therefore even higher affinity for FcγRIIIa. Under these conditions, immune complexes might be expected to have lower tendency to displace these afucosylated and highly galactosylated IgG from the FcγR, and therefore have less tendency to cause their crosslinking and immune activation, and hence diminished capacity to cause disease (Fig. 1).

We should realize that all these autoimmune diseases are characterized by multifactorial components, ultimately resulting in disease onset and progression. For RA, this includes the acquirement of multiple disease factors such as; infiltration of immune cells into the joint, anti-citrullinated protein antibodies (ACPA), anti-hinge antibodies, rheumatoid factor (IgM based), increased TNF levels, and much more\textsuperscript{56,62}. It is also important to realize that multifactorial aspects of these diseases are also caused, because these are not solely antibody based, but also cell mediated and complement is also often involved.

**The importance of glycosylation in IgG effector functions mediated by complement**

That Fc glycosylation – the presence of the Fc glycan – is important for classical complement activation has been known already for a long time\textsuperscript{63,64}. Whether the composition of and how the Fc glycan then affects this activation has remained an open question. It is known that in RA the involvement of complement is important and the main changes in IgG Fc glycosylation are in the galactose end groups\textsuperscript{46,48}. On the other hand, afucosylation of IgG showed that fucosylation did not enhance or hamper complement mediated cytotoxicity (CDC)\textsuperscript{23,65}. Our results from Chapter 3, where we screened IgG glycovariants for C1q binding and activation, show that galactosylation enhanced C1q binding and increased CDC and that additional sialylation increases this effect on the classical complement activation pathway. Recently, the work of Quast et al.\textsuperscript{66} showed that in antibody models where CDC is the main effector function, an increase in galactose enhances C1q binding and CDC. In their model the addition of sialic acid then hampers or down-modulates this enhanced effect\textsuperscript{66}. As mentioned above our experimental setup did show an enhanced C1q binding and CDC with increasing levels of sialic acid. The difference in effector function could might
depend on the nature of the antigen, effector cells, and/or target cells. This is plausible as hexamerization of IgG on the surface of the target is needed for proper binding and activation of C1q, and the propensity of different antibodies/antigens to do this may vary as they take on different molecular configurations\textsuperscript{67,68}. The results imply that there are also possibilities to enhance IgG complement effector functions using glyco-engineering. Much more research on this mechanism is however required as we do not know how the specificity of the IgG influences this effect or whether these changes are also relevant in a setting where IgG is already enhanced for CDC by protein engineering\textsuperscript{69,70}. This is done by replacing amino acids in the Fc of IgG1, for example in the C1q binding domain, or the domains in the CH3 required for hexamerization. In this way the potency of CDC can be enhanced up to 23 fold\textsuperscript{70,71}. The advantage of solely glyco-engineering is that it is not immunogenic\textsuperscript{72} when administered as therapeutic antibody whilst CDC enhancing mutations in the protein backbone might potentially be immunogenic.

We did not see any effect of altered fucosylation or bisection on the activation of the classical complement pathway, which is in line with previous work\textsuperscript{23,65}. We therefore concluded that only galactosylation and sialylation affect the binding and activation of C1q. Our results on complement activation by IgG1 are summarized in \textbf{Table 2}.

Because in RA higher levels of agalactosylated IgG are present and given the known importance of complement in RA, it was hypothesized that these might activate complement in a different way. Very early on, Malhotra et al. proposed activation of complement by these agalactosylated IgG species via mannose binding lectin (MBL) and the lectin pathway of complement activation\textsuperscript{73}. To our knowledge, this result has never been verified. Our results show that IgG agalactosylation - irrespective of all other glycan end groups - does not induce activation of complement via the lectin pathway, confirming various other studies on this subject\textsuperscript{74,75}.

As discussed above, it has been observed frequently that level of Fc galactosylation of total IgG is lowered and correlated with disease severity in several autoimmune diseases\textsuperscript{46,47,51,52}. As the route of activation is shown not to be via the lectin pathway the theory is still that the involved complement activation occurs via the classical pathway, by binding of antibody (complexes) to C1q.

It may at first seem counterintuitive, agalactosylated IgG being associated with higher pathogenicity whilst having lower potential to activate C1q compared to galactosylated IgG. A possible explanation may be similar to that what we propose for FcyRs, although more speculative. We propose a model of this mechanism illustrated in \textbf{Figure 2}. During remission the total IgG galactosylation is relatively high, whilst during a flare of the autoimmune disease the total IgG galactosylation is relatively low. In the latter case the threshold for activation of C1q might be lowered due to lower steady-state occupancy by the low-galactosylated IgG. If so, then this could allow for relatively increase in activation of complement by pathogenic IgG complexed by its cognate autoantigen.
This model is still very speculative as we do not know the true monomeric binding affinity of IgG or the IgG glycoforms to C1q and therefore what the results of our experiments mean in an *in vivo* setting. This could be experimentally determined, but needs high concentrations of bot IgG and C1q as the monomeric affinity is generally very low.

Eventually, it might be possible to monitor the glycosylation status, especially degree of galactosylation and fucosylation, of the total or disease-specific-antibodies in auto- and allo-immune diseases. This could help to predict or detect an upcoming flare and intervene with immune suppressors in auto-immune diseases or to prevent complications for the mother and fetus in the pregnancy associated diseases.

However, we still do not know whether the changes in IgG glycosylation are causing the, or a response to, the symptoms of these diseases. If they are (partly) causing the pathogenic symptoms, efforts to investigate the prevention of these harmful glycan changes could be undertaken.

### Table 2. Direct glycan traits and complement binding and activation

Results based on complement binding and activation studies of IgG1 glycoforms in Chapter 3 of this thesis.

- -, +, ++, ++++, ++++ respectively mean 0.6-0.8 fold, 0.9-1.1 fold, 1.2-1.4 fold, 1.5-1.7 fold and >1.9 fold increased binding, when compared to unmodified IgG binding, based on the results C1q binding assays. F0; low fucose, F1; high fucose, G0; no galactose, G1; one galactose, G2; two galactoses, S0; no sialic acid, S1; one sialic acid, S2; two sialic acids, B0; no bisection, B1; high bisection.

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However, we still do not know whether the changes in IgG glycosylation are causing the, or a response to, the symptoms of these diseases. If they are (partly) causing the pathogenic symptoms, efforts to investigate the prevention of these harmful glycan changes could be undertaken.
IVIg treatment and dependence on glycoforms

IVIg is composed of the pooled Ig fraction derived from plasma of blood and plasma donations. This product was originally created to treat patients with deficiencies in IgG levels\textsuperscript{76}. Alternatively it was found that high doses of IVIg could relieve symptoms of autoimmune diseases\textsuperscript{77}. Currently IVIg is used in many immune-mediated diseases, such as ITP and Guillain-Barré Syndrome\textsuperscript{78,79}.

There are many theories why and how IVIg should work in autoimmune diseases. These mechanisms can be roughly separated in Fab and Fc mediated function. All possible known working mechanisms have elegantly been reviewed by Schwab and Nimmerjahn\textsuperscript{77}.

Figure 2. A proposed model of the influence of the degree of total IgG galactosylation upon C1q occupation and activation in autoimmune remission and flare

In autoimmune diseases, such as Rheumatoid Arthritis (RA), disease severity is negatively correlated with the degree of galactosylation found in total IgG. Galactosylation of IgG is important for binding to C1q, where a higher degree of IgG Fc galactosylation – and sialylation – increases the affinity. Additionally, C1q requires multimerization/hexamers of IgG for proper activation. During remission (left), the total IgG galactosylation is relatively high, we propose that this might prevent autoantibodies to engage the C1q by preferentially engaging aspecific IgG which is abundantly present. During a flare of disease (right), the total IgG galactosylation is low, which reduces the overall threshold of C1q binding. Additionally, more antigens are possibly captured by IgG, forming complexes. And thus we propose that more (pathogenic auto-)antibodies can bind C1q, possibly causing activation of the classical complement pathway (green lightning bolt).
The research presented in Chapters 4 and 5 of this thesis try to answer some of the open questions.

One of the working mechanisms proposed was that IVIg is able to block the activating receptors on immune effector cells, preventing the auto-antibodies to bind and alleviating the pathogenic effects of these. In Chapter 4 we have looked at the capacity of multiple components of IVIg in a fully human cell based assays to block phagocytosis of opsonized red blood cells, as a model for AIHA. For this research monocyte derived macrophages (M1 and M2, ex vivo GM-CSF and M-CSF matured, respectively) were used as effector cells. The GM-CSF and M-CSF bear a combination of different FcγR. Control experiments showed that activation of GM-CSF was mediated mainly by FcγRI and M-CSF mainly by FcγRIIa. This phagocytosis could be blocked, dose dependently, by adding IVIg in the system. Fractionation of this IVIg in monomers and dimers, the latter having a higher avidity for especially the low FcγR, demonstrated enhanced blocking capacity of the dimeric fraction of IVIg and by artificial dimers. This confirms the proposed working mechanism where IVIg occupies FcγR and thus blocks the pathogenic antibodies. A possible downside of this research is that the concentration of exogenous aspecific IgG was performed at concentration significantly lower (10 µg/ml) than found in serum, which contains roughly 10 mg/ml IgG.

To treat the autoimmune diseases, high dosages (1 g/kg) are required for good efficacy of IVIg treatment. One possible explanation is that only a small fraction if IVIg is responsible for the effector function. This was the basis for another proposed working mechanism of IVIg; by the sialylated fraction of IgG. This was suggested following mouse studies, where IVIg enriched for this fraction showed better efficacy. Mouse specific ICAM-3 grabbing nonintegrin-related 1 (SIGN-R1) or the human dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) is proposed to mediate this effect. In mice, binding of sialylated IgG to this receptor on red pulp macrophages in the spleen induces upregulation of the immunomodulatory FcγRIIb on these cells. Using this fully human system, this hypothesis could not be confirmed in Chapter 4. When aspecific monoclonal IgG with enhanced sialic acid is used as a blocker we do not see a difference compared to non-sialylated IgG. Also, IVIg enriched for sialylation by lectin affinity chromatography, recapitulating the methods used to initially show enhanced effect of sialylated IgG, which mostly enriches for Fab-glycosylated IgG, did not show enhanced blocking capacity in our fully human system. We also did not observe upregulation of the inhibitory FcγRIIb after long term incubation of the macrophages with IVIg. These results are in line with research of other groups, which also show no direct interaction of sialylated IgG with DC-SIGN and those finding that SNA-enriched IVIg does not have enhanced immunomodulatory functions. Our group has also further investigated the possible binding of highly sialylated IgG (75%) to DC-SIGN-expressing cells and have thus far not found any binding (Temming et al. manuscript in preparation).
Whether sialylated IgG in IVIg really mediates the immune modulatory function of IVIg in human autoimmune diseases is thus still unconfirmed.

As IgG1 is not the only subclass present in IVIg, in Chapter 5 we therefore have taken a closer look at the glycosylation, mainly fucosylation, of different IgG subclasses and the influence on effector functions within different cell types. As found in Chapter 3, absence of fucosylation mainly affects the binding to FcγRIIIa, whilst the affinity to the other FcγR was unaffected. We confirmed that this effect was applicable to all subclasses of IgG, with variable increase between subclasses when fucosylated IgG was compared to afucosylated IgG. IgG1 and IgG3 show the a measurable increase in binding upon afucosylation. IgG2 and IgG4 do not bind FcγRIII efficiently, or not at all, and for these subclasses we also observe a (slight) increase in binding, but were not able to quantify the difference.

The abovementioned results of the research on the dimeric fraction of IVIg from Chapter 4 does, however, suggest that IVIg with a higher affinity for the FcγR would have a better FcγR-blocking function. In this reasoning it would be interesting to look if the afucosylated fraction of IVIg with a higher affinity for FcγRIIIa also works better. Unfortunately we are not aware of efficient methods for separation of the Fc-fucosylated and afucosylated IVIg by, for example, lectin columns or modification of the current highly Fc-fucosylated IVIg product by, for example, using fucosydases.

The observed increase in FcγRIII affinity upon IgG subclass afucosylation was then functionally confirmed using an NK cell based ADCC assay, using NK cells which only bear FcγRIIIa, by excluding individuals that possibly express FcγRIIC. We observed an increase in efficacy using either IgG1 or IgG3. While using the similar phagocytosis assay as developed in Chapter 4, did not show an increase in phagocytosis. Neither was afucosylated aspecific IgG better in blocking this assay. In these monocyte derived macrophages – bearing a variety of FcγRs – the fucosylation does not seem to influence the effector function, but this is mostly likely due to the low expression level of FcγRIIIa on these cells, which primarily utilized FcγRI or FcγRIIa to carry out their IgG-mediated effector functions.

Therefore we concluded that the effector cell type in question is crucial when trying to understand the working-mechanisms of IVIg or therapeutic IgG, especially when considering glycovariants as these only target CD16/FcγRIII expressing cells. As not all cells have the same receptor profile, some are not susceptible to glyco-engineering. As for the functionality of IVIg, it is proposed that the red pulp macrophages from the spleen are the main effector cells responsible for phagocytosis of platelets or red blood cells in ITP or AIHA and HDFN, respectively. This cannot be the only mechanism, however, as IVIg treatment is still functional in splenectomized ITP patients, thus arguing that other mechanisms of cell destruction can also occur in these multi-facetted diseases. Neutrophils and NK cells are likely candidates, as they express mainly FcγRIIib or FcγRIIIa respectively, both susceptible to afucosylation which increases their affinity for IgG. The splenic macrophages have a different FcγR expression profile compared to the macrophages used in the phagocytosis...
experiments. FcγRIIIa is the main expressed receptor on splenic macrophages (Bruggeman and Nagelkerke et al., manuscript in preparation). This argues that the response to afucosylated IVlg might work better than normal high fucosylated IVlg, which is supported by the fact that, for example, in FNAIT and ITP red blood cell and platelet destruction, respectively, seem to occur mainly through FcγRIII\textsuperscript{44,89,90}.

The conservation of IgG binding to FcγR – what can we learn from our \textit{in vitro} studies to apply to \textit{in vivo} models

Since the approval of the first therapeutic antibodies, many therapeutic antibody products have been developed and are in development\textsuperscript{13,91,92}. Many different diseases and conditions are targeted with the different therapeutics, from cancer to autoimmune diseases. All have in common that a product needs to be thoroughly pre-clinically tested before being allowed to be administered in humans. Unfortunately, for this purpose still model organisms need to be employed, such as (humanized) mouse models and non-human primate (NHP) models. Once shown functional and not harmful in these models, the manufacturer can start with clinical experiments in humans.

Although currently there are several mouse models which are (partly) humanized for FcγRs, immunoglobulin compartment, or FcRn\textsuperscript{93–96}, but importantly, never in conjunction. In addition, still a lot of research is done in wild type mouse models\textsuperscript{97}. To aid the testing of human IgG in mouse models we have assessed the affinity of the human IgG subclasses to the mouse FcγR family. The results of these screens were shown in Chapter 6 and demonstrate that human IgG is not only capable of binding to the mouse FcγRs but also with similar binding strength as mouse IgG subclasses. This shows the high evolutionary conservation of IgG and FcγR structure between species.

There are a few differences between humans and mice considering IgG subclasses and FcγR structure and expression, as summarized in the introduction Chapter 1. Our binding studies confirm these differences between subclasses, whilst the homologous FcγR; human FcγRIa/mouse FcγRI, human FcγRIIa/mouse FcγRIII, human FcγRIIIa/mouse FcγRIV, have a similar binding pattern to the human IgG subclasses. For instance, IgG2 does not have any affinity for FcγRI of both species and IgG4 binds with highest affinity to FcγRI and low affinity to the other receptors\textsuperscript{98}.

Also, similar to human, for mouse IgG it has been shown that absence of fucose increases the affinity for FcγRIV – the mouse receptor homologous to human FcγRIIIa\textsuperscript{99,100}. This glycan change has also been shown to be functional in enhancing ADCC and phagocytosis \textit{in vitro} and \textit{in vivo} for both species\textsuperscript{23,24,65,99,101–104}. Furthermore, this increase in affinity upon IgG afucosylation has been shown to be conserved even cross-species between afucosylated human IgG binding to mouse FcγRIV (Braster et al. Manuscript submitted). As we have shown in Chapter 3, for human IgG1, not only fucose but also
additional presence or absence galactose influences the affinity for FcγRIII. In Chapter 7 we therefore also screened mouse IgG1 and IgG2a glycovariants with changes in fucose and/or galactose and confirmed the increased affinity of afucosylated IgG for FcγRIV. However, we found no additional effect of IgG2a-galactosylation on affinity to mouse FcγRIV.

We still do not know what additional glycosylation changes, if any – besides fucosylation, galactosylation and sialylation (the latter proposed having a more immune modulatory function in mice, discussed in previous section of this discussion) – and combinations thereof, might mean for mouse FcγR binding and other effector functions. It was shown that different mouse strains vary in glycosylation profiles of the N-glycans of their serum immunoglobulins16,105. Mouse IgG subclass specific screening also showed subtle changes in glycosylation profiles of IgG Fc glycans between the mouse subclasses within one strain16. Additionally there might be a role for the before mentioned non-human glycan adducts present in mouse IgG glycans (Galα 1–3Gal, α-2,3-linked sialic acid and Neu5Gc15, discussed in section “Glyco-engineering of antibodies” of this discussion).

The main message of Chapter 7 is, however, that there is additional functional homology between both species on the IgG/FcγR binding, as the homologous receptors human FcγRIIIa and mouse FcγRIV both contain a conserved glycan in the IgG binding site at position 162106. For humans, it was already shown that this glycan is important for the binding of human FcγRIIIa to IgG and mediates in enhancing the affinity of afucosylated IgG106,107.

In Chapter 7, using constructs to recombinantly produce the FcγR as Fc-Fusion proteins we created wild type and a mutant missing the N162-glycan of the FcγRIII. Screening our glyco-engineered human IgG1 with low fucosylation and/or high galactosylation showed that, compared to unmodified IgG, the affinity to wild type receptor was increased, similar to what we have shown in Chapter 3, but that the affinity to the mutant N162A receptor was not changed compared to unmodified IgG. This confirms the research of Ferrara et al.106, who showed that FcγRIIIa without the N162 glycan could not discriminate between fucosylated and afucosylated IgG.

Currently, our lab is investigating the application of these Fc-FcγRIIIa fusion constructs in a biosensor to determine the degree of IgG Fc fucosylation in a biological sample, such as serum from AIHA patients which might contain low fucosylated platelet specific IgG (unpublished results). The ratio between the affinity for the wild type receptor versus the mutant receptor will change when the sample has a higher or lower fucosylation degree.

For mouse FcγRIV we created identical wild type and mutant receptors, respectively having or lacking the N162 glycan. Screening mouse IgG2a glycovariants confirmed a similar discriminatory role for the glycan in this receptor.

Interestingly, the mutant receptors themselves have a higher affinity for fucosylated IgG compared to wild type receptor. The crystal structure of FcγRIIIa in complex with IgG reveals that the N162 glycan present in FcγRIIIa cause some steric hindrance, which is
relieved upon glycan removal\textsuperscript{107}. Furthermore, FcγRIIIa and FcγRIV contain more glycans on their surface. Ferrara et al. additionally showed that the N-glycan at position 45 is also needed for proper IgG binding\textsuperscript{107}. Additionally, it is known that the composition of these FcγR glycans can vary, depending on the immune cell on which they are expressed\textsuperscript{108,109} or when produced recombinantly\textsuperscript{110}; the cell type and culture conditions. These glycan changes affect the affinity to IgG\textsuperscript{110}. Besides FcγRIII, all other FcγRs are glycoproteins, and the glycans are important for structure and function\textsuperscript{111}. For FcγRIIb we have shown the importance of the presence of a N106 glycan site in FcγRIIb, where a rare in frame deletion resulted in loss of this glycan and the binding of this receptor to IgG\textsuperscript{112}.

This show that it is thus important to consider the glycans and their composition when doing or comparing binding studies of IgGs to FcγRs. Unfortunately there is still much unknown about the exact glycosylation changes, as the methods to study cell type specific FcγR glycosylation are still in development, especially relevant in vivo data is missing, and therefore this portion of this discussion is still very speculative.

**Function of additional glycosylation of immunoglobulin subclasses**

The last chapter of this thesis (Chapter 8) investigates the putative O-glycosylation sites in the hinge of IgG3. This explorative research showed that the repeats within the hinge region of IgG3 contain up to 6 putative O-glycosylation sites, serines and threonines, of which the threonines are partially occupied. The occupation was around 10\% for serum derived IgG3 and 13\% for IgG3 produced in our Hek Freestyle production system. The composition of O-glycans was remarkably stable between indivuals (6 healty tube donors), whilst the N297 glycan showed more individual variation.

The O-glycosylation of the relatively long IgG3 hinge could have several functions as also touched upon in the discussion of Chapter 8. First it could provide stability of this flexible hinge. Second it could aid in the protection of IgG3 against proteolytic digestion by proteases derived from pathogens. The latter has been shown for mouse IgG2b and human IgA, which also have an O-glycosylated hinge, providing such protection\textsuperscript{113,114}. Up until now, we did not engage further studies on the functionality of IgG3 hinge O-glycosylation, which will undoubtedly provide an interesting research line.

**Conclusion**

This thesis we have described the different human and mouse IgG subclasses, their receptors and what we know about IgG Fc glycosylation and functional consequences. We have developed methods to modify the IgG glycan as desired and used this to create different IgG glycoforms of both human and mouse. These glycoforms were assessed in various different settings, from binding to and activation via FcγRs, binding and activation of the classical complement pathway. We concluded that between glycoforms and the
various effector molecules, the strength of effector functions is differential. Fucosylation and galactosylation are important for binding to FcγRs and mainly FcγRIII, galactosylation and sialylation are important for activation of complement.

When used as opsonizing or blocking antibody in FcγR mediated phagocytosis or ADCC we show that the IgG affinity for the main receptors expressed on the effector cells is important. That for the phagocytosis by monocyte derived macrophages, FcγRIa and FcγRIIa are the most important and no glycoforms but only IgG di- or multimers - with higher affinity for these receptors - have increased capacity. Whilst for ADCC by NK cells, with only FcγRIIIa expression, the increased affinity of afucosylated IgG indeed mediates better effector function.

When assessing the affinity of human IgG for mouse FcγR we showed that these bind with similar affinities, confirming evolutionary conservation of these two distinct but functionally linked proteins. We could also partly reproduce the observed functions of IgG glycovariation for mouse IgG and FcγRs. The conserved N162 glycan in human FcγRIIIa and mouse FcγRIV also maintained its function: to discriminate between fucosylated and afucosylated IgG.

Lastly we found additional O-glycosylation sites in the hinge region of IgG3 of which the function still needs to be determined.

All in all, the research described in this thesis shows that IgG glycosylation is not inert. The glycan status and composition of secreted antibodies is functionally important. The results in this thesis aid in diagnosis of antibody mediated diseases as well as the development and improvement of immunoglobulin based therapies.
References


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