

Review

The history of IgG glycosylation and where we are now

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Received 23 July 2019; Revised 15 August 2019; Editorial Decision 16 August 2019; Accepted 16 August 2019

Abstract

IgG glycosylation is currently at the forefront of both immunology and glycobiology, likely due in part to the widespread and growing use of antibodies as drugs. For over four decades, it has been recognized that the conserved N-linked glycan on asparagine 297 found within the second Ig domain of the heavy chain (CH2) that helps to comprise Fc region of IgG plays a special role in IgG structure and function. Changes in galactosylation, fucosylation and sialylation are now well-established factors, which drive differential IgG function, ranging from inhibitory/anti-inflammatory to activating complement and promoting antibody-dependent cellular cytotoxicity. Thus, if we are to truly understand how to design and deploy antibody-based drugs with maximal efficacy and evaluate proper vaccine responses from a protective and functional perspective, a deep understanding of IgG glycosylation is essential. This article is intended to provide a comprehensive review of the IgG glycosylation field and the impact glycans have on IgG function, beginning with the earliest findings over 40 years ago, in order to provide a robust foundation for moving forward.

Key words: glycan, glycosylation, IgG, sialylation

The historical context

The earliest paper clearly linking IgG glycosylation with IgG biology was published in 1976, in which it was reported that four patients with variable agammaglobulinemia could not glycosylate their IgG heavy chains, leading to very low circulatory IgG concentrations (Ciccimarra et al. 1976). Although those findings were later refuted by another study that reported these patients could glycosylate their IgG (Schwaber and Rosen 1984), this initial study appears to have drawn attention to an otherwise overlooked aspect of IgG structure and function.

A thorough review of the literature on IgG glycosylation clearly shows two overlapping eras, with the first beginning in the 1970s. During this period, the focus was primarily on the impact of inflammation and disease on IgG glycosylation, and the functional impact of those changes. Indeed, most of what we know about the proinflammatory glycoforms of IgG came from this era, and thus these studies represent many of the fundamentals we now understand. Not

only does inflammation correlate with decreased galactosylation, increased fucosylation and increased bisecting GlcNAc, but changes in Fc glycans also change both the structural integrity and conformation of the Fc domain, as well as the affinity to Fcγ receptors (FcγRs). As such, it was during this period that the relationship between IgG glycosylation, inflammation and function was largely mapped.

The second era of IgG glycosylation work remains ongoing and can be roughly traced back to a *Science* article in 2006 (Kaneko et al. 2006). In that study, Jeffrey Ravetch's laboratory reported that terminal α2,6-sialylation of IgG N-glycans accounted for the anti-inflammatory activity of high-dose intravenous immunoglobulin (i.e., IgG) therapy for rheumatoid arthritis (RA) (Kaneko et al. 2006). Despite many of the fundamentals on the proinflammatory effects of IgG glycosylation having been worked out prior to this article, it is clear that the discovery of IgG sialylation being a key to the inhibitory effects of IgG has generated much interest. Indeed, approximately one-third of articles on IgG glycosylation date from 1976 to 2006,

a 30-year span, while the other two-thirds have been published since 2006.

In this review, both eras of research are discussed in an effort to provide a detailed summary of where the field was, is and potentially will be, with an eye toward laying the foundation for new paths forward in exploring the potential of using the glycome as the next medicinal frontier.

Antibody structure and function

Antibodies are at the heart of adaptive immunity, biomedical research and modern pharmaceutical development. In vivo, their activity can be broken into a number of categories, which include antigen neutralization, promotion of phagocytosis and microbial killing via opsonization and macrophage activation, fixation of complement and induction of antibody-dependent cellular cytotoxicity. The combination of these activities with a high level of antigen specificity is why IgG is the desired outcome and measure of vaccine efficacy. Antibodies are also ubiquitous laboratory tools found across all biomedical fields through their use in immunohistochemistry, confocal microscopy, western blotting, immunoprecipitations, ELISA and other detection technologies, as well as tools for in vivo cellular depletion, cell sorting and purification. Since the release of the first monoclonal antibody (mAb) drug Orthoclone OKT3 in 1986 (Liu 2014), and the subsequent success of Rituxan (rituximab) released in 1997 (Scott 1998), there has been an explosion of antibodies as drugs both in the clinic and the current pharmaceutical pipeline. The reason for this is manifold, but these drugs are now among the top revenue generators for the industry. For example, Humira, a tumor necrosis factor α (TNF α)-neutralizing antibody therapy for autoimmunity, has earned AbbVie approximately \$115 billion dollars to date (<https://www.axios.com/abbvie-humira-2018-sales-20-billion-e4039176-baeb-44ff-b4fe-1b63005283b9.html>). With this central place in biomedical science, understanding the variables underlying IgG function is not only big business, it is absolutely critical for efficacy.

Conventional immunology states that the function of IgG is driven by the fragment crystallizable (Fc) domain, which is encoded solely by the γ heavy chain, and comprised of two constant Ig domains, CH1 and CH2. While this domain is not responsible for antigen specificity—that is under the purview of the Fab domain, which includes the variable regions—it is the point of contact for all of the Fc γ R molecules. As a result, changes in structure of the Fc domain would likely have a profound impact on receptor interactions. Within CH2, there is a universally conserved site of N-linked glycosylation, asparagine 297 (N297). This site predominantly carries complex-type biantennary glycans with varied amounts of core fucose (Fuc), galactose (Gal), bisecting GlcNAc and terminal N-acetylneuraminic acid (Neu5Ac/sialic acid) residues (Figure 1). Importantly, these glycans fill a large pocket between the two copies of the CH2 domains, as described later and shown in Figure 2.

In the beginning

In 1976, an article appeared in the *Journal of Clinical Investigation* in which four patients with variable agammaglobulinemia were described (Ciccimarra et al. 1976). Their low IgG concentrations were attributed to an inability to glycosylate IgG, as measured by ex vivo culturing primary B cells from each patient in the presence of tritiated mannose and glucosamine. Released IgG was reportedly absent any

carbohydrate label, leading to the conclusion that these patients did not glycosylate IgG at all. Eight years later, it was found that these patients were not deficient in IgG glycosylation (Schwaber and Rosen 1984), but this early study appears to have sparked interest in the role of IgG glycans on function.

Not long after this initial publication, it was reported that tunicamycin, an inhibitor of the oligosaccharyltransferase (OST) responsible for initiating glycosylation on nascent glycoproteins in the endoplasmic reticulum, reduced IgG plasma cell secretion of IgG by 28% and IgM by 81% (Hickman and Kornfeld 1978). Several years later, it was discovered that while tunicamycin eliminated IgG glycosylation, tyrosine sulfation was dramatically increased on the heavy chain, perhaps as a functional compensatory posttranslational mechanism to promote secretion (Bauerle and Huttner 1984).

In the same year, Dr. Thomas Rademacher and team discovered that changes in glycosylation of IgG were associated with rheumatoid and osteoarthritis arthritis (RA and OA, respectively) (Parekh et al. 1985). In a cohort of patients from both Oxford and Tokyo, they reported glycan changes that continue to hold up to modern day scrutiny. Importantly, they did not find novel N-glycans appearing in RA/OA samples, but instead found that the relative abundance of the glycans present changed with disease compared to the healthy controls. The primary difference was a reduction in glycans with galactose and a concomitant increase in glycans terminating in GlcNAc, although reductions in sialylated species were also apparent within the Oxford portion of the cohort (Parekh et al. 1985). Galactosylation changes were also found in juvenile onset RA (Parekh et al. 1988a). These data are the foundation upon which the general, widely accepted and often repeated notion that decreased IgG galactosylation is a sign of inflammation is founded.

Consistent with this report, in 1987, IgG from RA patients was analyzed using lectins. It was found that IgG from RA patients bound more to ConA compared to normal patients, indicating a greater prevalence of hybrid and high mannose N-glycans associated with inflammatory disease (Malaise et al. 1987). In that same year, the galactosyltransferase activity from circulating B cells was found to be significantly reduced in RA patients (Axford et al. 1987). This was later replicated in RA-prone MRL-lpr/lpr mice, in that the galactosyltransferase B4GalT1 was decreased in association with RA development (Axford et al. 1994; Jeddi et al. 1994) and IgG glycans had reduced galactosylation (Bond et al. 1990; Mizuochi et al. 1990).

Another major step forward for the field occurred in 1988. The basic foundations of the study of another RA cohort duplicated prior findings, including the RA association with a relative loss of galactose and gain of terminal GlcNAc, without change in fucosylation (Pekelharing et al. 1988). However, during pregnancy, they found a reversal of this trend, whereby the IgG contained more galactose and sialic acid than IgG from nonpregnant females, while fucose, GlcNAc and total carbohydrate content was unchanged. This is significant because during pregnancy, women with RA often go into remission, and this study was the first to suggest that not only does pregnancy shift IgG glycosylation but that a shift to more galactose and sialic acid may be immunosuppressive, thereby temporarily limiting autoimmunity (Pekelharing et al. 1988). This idea was supported a few years later in a study with RA women and DBA/1 mice (Rook et al. 1991), which revealed loss of galactosylation with disease, reversal of those changes during pregnancy and recurrence after pregnancy. And much like the MRL-lpr/lpr mouse studies that came later (Axford et al. 1994; Jeddi et al. 1994), these glycan alterations were present in DBA/1 mice with collagen-induced arthritis (CIA).

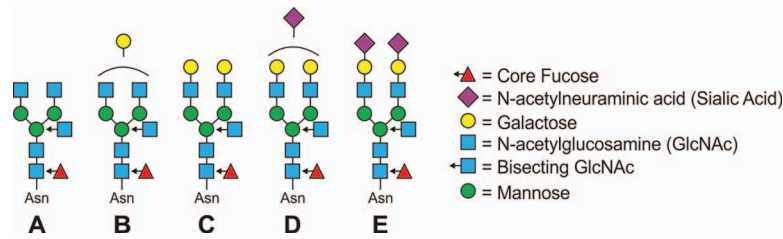


Fig. 1. IgG glycans. Schematic showing common glycoforms found on mouse and human IgG molecules. These are typically biantennary with (A) terminal GlcNAcs, (B) monogalactosylated, (C) digalactosylated, (D) one sialic acid or (E) two sialic acids, and each of these with or without core fucose and bisecting GlcNAc. Other glycoforms are known to exist, but these are the major classes.

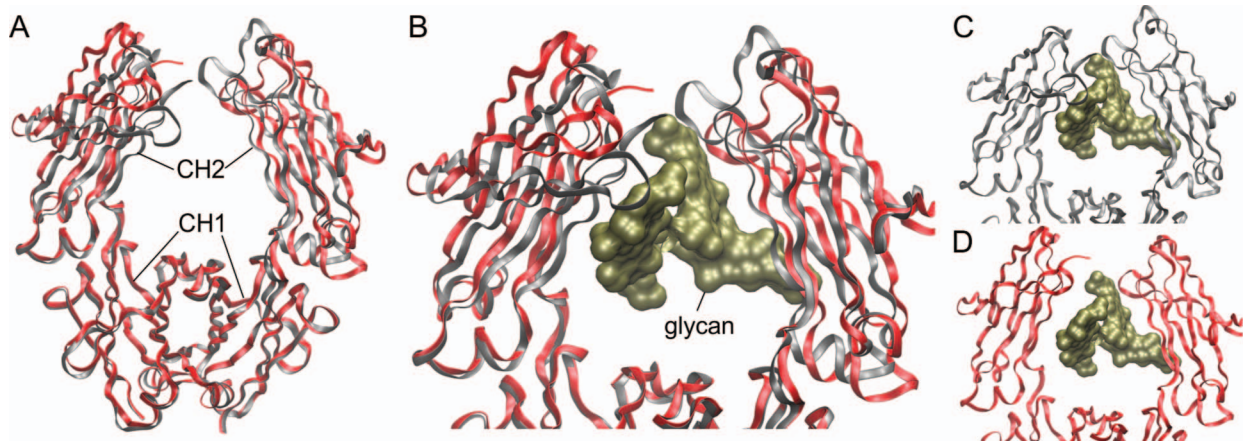


Fig. 2. Structural impact of α 2,6-sialylation on IgG Fc. Crystal structures of a normally glycosylated (nonenriched) wild-type Fc domain [PDB: 4Q7D] (red ribbons) compared to the structure of the same Fc carrying fully sialylated N-glycans [PDB: 4Q6Y] (gray ribbons), demonstrating the difference in conformation. (A) A broad view of the full structure highlights the close similarity of the CH1 domains but the divergence in conformation and position of the CH2 domains. (B) A closer view of the two structures, including a molecular surface rendering of the glycans (gold) from the disialylated Fc structure (gray). (C) The complete structure of the Fc with disialylated glycans. (D) The structure of the wild-type Fc with the glycan from the disialylated Fc structure to illustrate the change in the CH2 domain position relative to the glycan.

Mice with CIA had reduced galactosylation and pregnant females showed increased IgG galactosylation. Remarkably, this increased galactosylation was not observed in pseudo-pregnant mice (Rook et al. 1991). Collectively, these data demonstrate that IgG glycosylation is dynamic and regulated with respect to inflammation and autoimmunity.

While this early work laid the foundation, a focus on RA continues even today. With increasing technological innovations in glycomics and a greater understanding of the immune system, more and more detail has been revealed. For example, using two strains of mice, one susceptible to pristane-induced arthritis (DBA/1) and one resistant (CBA/IgB), it was discovered that both strains had strongly elevated IL-6 and both showed increased agalactosyl IgG (Hitsumoto et al. 1992). These data suggested that IL-6 may be a player in the mechanism leading to the change in glycoforms of IgG. Consistent with this interpretation, the kinetics of IL-6 production parallels the kinetics of agalactosyl IgG release in this system (Thompson et al. 1992).

Large cohort studies have also robustly confirmed that during pregnancy, when many female RA patients enter remission, galactosylation and sialylation are increased and are highly correlated, but then decrease significantly during postpartum relapse (van de Geijn et al. 2009). Interestingly, a study in 1995 showed that while women with RA have increased IgG galactosylation associated with pregnancy-associated remission, the ratio of agalactosyl-IgG to galactosylated IgG was notably higher in the fetus, suggesting that galactosylated

(presumably noninflammatory) IgG is particularly good at crossing the placental barrier (Williams et al. 1995). However, nearly 20 years later, a study comparing fetal and maternal Fc glycans found similar levels of galactosylation, sialylation, bisecting GlcNAc and fucosylation (Einarsdottir et al. 2013). The reason for this discrepancy remains unclear.

In a rather curious study in 1996, IgG glycosylation was monitored in RA patients who fasted for 7–10 days and then followed a strict vegetarian diet for the subsequent 3.5 months. The levels of agalactosylated IgG went down during the fasting, and this was correlated with improved disease presentation (Kjeldsen-Kragh et al. 1996). This observation is interesting because it appears to link the overall metabolic state of the individual to IgG glycosylation.

Finally, it is important to note that it is clear that IgG fucosylation can also change in RA patients. Initially, individuals with juvenile chronic arthritis were studied, and it was found that IgG fucosylation was increased in those with disease (Flogel et al. 1998). This was extended the following year to adult RA patients, showing a 40% increase in IgG fucosylation compared to healthy controls (Gornik et al. 1999).

Galactosylation outside of RA

In 1988, the RA founded observation that IgG losses galactosylation with disease was expanded to include systemic lupus erythematosus (SLE) and Crohn's disease (Tomana et al. 1988). This was the first

time that changes in IgG glycosylation were implicated as a general feature of inflammation, rather than a peculiar change seen only in RA. Since then, a long list of circumstances, be they inflammation, infection, cancer or aging, has been correlated with decreased IgG galactosylation.

From an infection perspective, reduced IgG galactosylation has been found in *Mycobacterium tuberculosis* and leprosy patients (McCulloch et al. 1995). More recently, loss of galactosylated IgG was seen in both HIV⁺ (Moore et al. 2005) and HCV⁺ (Mehta et al. 2008) patients. From the autoimmune and inflammation perspective, IgG α 2,6-sialylation and galactosylation is reduced among anti-PR3 (anti-proteinase 3) antibodies in Wegener's granulomatosis with polyangiitis (GPA) and is correlated with disease severity (Espy et al. 2011), while Lambert-Eaton myasthenic syndrome and myasthenia gravis patients showed reductions in IgG galactosylation without changes in total sialylation (Selman et al. 2011). Nonalcoholic fatty liver disease patients also show similar changes, with decreased IgG galactosylation and increased fucose and bisecting GlcNAc (Zhao et al. 2018).

Cancer is another area in which IgG glycosylation is altered. In prostate cancer, the decrease in IgG galactosylation was found to strongly correlate with serum prostate-specific antigen (PSA) levels (Kanoh et al. 2004). Decreases in IgG galactosylation also occur in multiple myeloma (Aurer et al. 2007), stomach adenocarcinoma (Bones et al. 2010) and gastric cancer (Kodar et al. 2012).

In a study of 151 individuals ranging in age from 1 to 70 years, it was reported that the amount of GlcNAc-terminating IgG glycans decreased from birth to approximately 25 years of age, but then increased slowly thereafter (Parekh et al. 1988b). This change was mirrored in the degree of galactosylation, with increases up to 25 years of age and decreases after. This study was the first to suggest that the changes in galactosylation associated with chronic inflammatory conditions could potentially represent a premature aging of the immune system. In a somewhat more recent study, individuals over 65 years of age were found to have reduced α 2,6-sialylation in IgG1 and IgG2 compared to young (0–19 years old) and adult (20–39 years old) groups (Keusch et al. 1996). Mirroring RA data, the geriatric group also had increased agalactosyl glycoforms in IgG2, 3 and 4 compared to healthy adults. This was supported 2 years later in women, which showed decreased galactosylation with age (Shikata et al. 1998).

The glycan influence on function

Thus far, our discussion of IgG glycosylation has focused upon the early identified changes, particularly galactosylation, seen associated with a variety of biological circumstances. While interesting, these trends leave open the possibility that they are nothing more than the consequence of the biological context and are therefore little more than broad inflammatory biomarkers. However, given the physical placement and conserved nature of the Fc N-glycan at N297, it is not difficult to imagine that changes in the size, composition and charge of the glycans could have a profound impact on structure and function of the Fc domain of IgG and therefore play an active role in determining optimal interaction partners and function.

Credit for the fundamental discovery that glycans actually change IgG function appears to go to Dr. Hans Wigzell. In 1983, Dr. Wigzell published an article in the *PNAS* in which a mAb was functionally compared to a nonglycosylated version of itself (Nose and Wigzell 1983). As with other early work, the nonglycosylated mAb was produced by culturing the hybridoma cells in tunicamycin. They

found no change in antigen or protein A binding properties, but the ability to activate complement, associated with Fc γ R on cells, and the ability to induce antibody-dependent cellular cytotoxicity (ADCC) was lost. In addition, clearance of immune complexes in vivo was significantly reduced. Two years later, it was reported that mAbs known to be suppressive in vivo failed to suppress responses when they lacked glycosylation (Heyman et al. 1985), and that this function was independent of complement (Heyman et al. 1988), suggesting that the glycans can also promote anti-inflammatory functionality via Fc γ R engagement.

Mirroring this initial discovery, most of this early period focused on the impact of completely removing the N-glycans on Fc. For example, human IgG1 and IgG3 lacking N-glycans were found to not bind Fc γ RI and Fc γ RII (Walker et al. 1989). Similarly, rat IgG from tunicamycin-treated hybridomas showed Fc receptor binding deficiencies and reduced transportation from the gut lumen to blood (Peppard et al. 1989), and mutagenesis of N297 in a mouse-human chimeric IgG yielded greater sensitivity to proteases, failed to bind Fc gamma RI, failed to activate complement, and IgG3 showed a shorter serum half-life (Tao and Morrison 1989). Surface plasmon resonance (SPR) studies also confirmed alterations in affinity between deglycosylated Fc and Fc γ Rs (Radaev and Sun 2001).

In more detailed studies in which differential glycoforms, not just the presence or absence of a glycan, were compared, other features began to emerge. IgG lacking either sialic acids or galactoses were reported to have weaker binding to Fc γ R (Adler et al. 1995). In 2001, it was reported that removal of galactose residues on the Fc glycan did not alter binding to Fc γ RIIB, but that removal of the GlcNAcs and mannoses significantly reduced binding (Mimura et al. 2001).

Since many IgG functions, albeit not all, are dependent upon Fc γ R binding and signaling, it is not surprising that these changes in binding affinity translate into functional differences (Nose and Wigzell 1983). IgG secreted from hybridomas treated with the glucosidase inhibitor castanospermine (which limits N-glycan processing to yield predominantly high mannose forms), but not the mannosidase inhibitor swainsonine (which permits N-glycan process into hybrid structures), had enhanced lymphocyte and NK cell-mediated ADCC activity. Yet, ADCC mediated by polymorphonuclear cells (PMNs) was reduced in IgG from swainsonine and tunicamycin, but not castanospermine-treated hybridomas (Rothman et al. 1989). These data suggested that IgG Fc glycans can differentially influence Fc γ R binding, and that the outcome may depend upon which receptor is present.

Recombinant mouse-human chimeric IgG1 produced in CHO Lec1 cells, which lack the ability to process high mannose N-glycans and therefore cannot synthesize complex-type N-glycans (Stanley and Chaney 1985), showed an inability to support complement-mediated hemolysis and was incapable of complement consumption, C1q binding and C1 activation (Wright and Morrison 1994). Moreover, this IgG1 molecule showed reduced Fc γ RI binding affinity and had a shorter half-life in vivo. Interestingly, the clearance enhancement could be overcome by injection with mannan, suggesting that the increased clearance was due to binding the mannosylated N-glycans on the IgG molecule. Then in the following year, exciting findings based on a combination of X-ray and NMR data were published showing that the agalactosylated IgG glycoforms with terminal GlcNAc residues are able to bind the mannose-binding lectin (MBP), and thus selectively activate the lectin-arm of complement (Malhotra et al. 1995).

These findings not only suggested that Fc γ R binding is dependent upon the conformation of the Fc domain, and that the glycans are

important contributors to the structural integrity of IgG, but also revealed a potential mechanistic connection between the loss of galactose and inflammation through differential Fc γ R ligation and stimulation of the MBL/lectin pathway of complement.

Glycans and the Fc domain

With evidence pointing toward a potent structure–function effect of the N297 glycan on IgG, structural biologists began entering the field. It appears that the first structural detail of the Fc domain with the glycans resolved was in 1983 (Sutton and Phillips 1983). In that article, it was reported that the N-glycans at N297 formed a number of noncovalent bonds between amino acids within the CH2 domain and the glycans themselves. The mere fact that the glycans could be resolved at all is testimony to their structural confinement within the Fc domain. Indeed, many years later, it had become quite clear that the structural integrity (Krapp et al. 2003; Yamaguchi et al. 2006) and thermostability (Mimura et al. 2000) of the Fc domain were dependent upon the N-glycans.

Over a decade later, mutagenesis helped to further define the key carbohydrate–protein contacts, which appear to drive conformational states preferred by various Fc γ Rs (Lund et al. 1995). Specifically, mutagenesis of K246, D249 and E258 in human IgG3, all of which form direct bonds with a GlcNAc and Gal on one branch of the glycan (Sutton and Phillips 1983), did not alter Fc γ RI or Fc γ RII binding. In contrast, mutagenesis of D265, which contacts the first GlcNAc within the N-glycan core, abolished binding. These observations were also duplicated in IgG2b (Lund et al. 1995), suggesting a conserved mechanism influencing overall Fc structure.

In a follow-up study, the same group showed that other mutations in the Fc CH2 region resulted in increased galactosylation and sialylation (Lund et al. 1996). As an example, mutagenesis of F243 to alanine allowed for dramatic increases in total sialylation, going from 4% to 73% sialylated. These data suggested that the ability to sialylate may be driven by accessibility of the glycans and the intramolecular interactions within the Fc domain, while also providing a potential way to increase IgG sialylation in antibody-based drugs.

Another study repeated some of these observations by showing that wild-type IgG3 mAb expressed in CHO cells had very little sialic acid (~3%), and that all of it was α 2,3-linked, but mutagenesis of F243 resulted in robust α 2,3-linked sialylation (~53%). The key advance, however, came with recombinant expression of ST6Gal1 in the CHO cells, which converted half of the sialic acid to α 2,6-linkage. Remarkably, the α 2,3-linked sialylated IgG3 had reduced recognition by Fc γ RI and Fc γ RII compared to wild-type IgG3, but the introduction of α 2,6-linked sialic acids fully restored Fc γ R recognition and activation (Jassal et al. 2001). These data demonstrate that α 2,3- and α 2,6-linked sialic acids are not equivalent in terms of Fc structure and must be separated in order to understand the impact of IgG sialylation on function. Moreover, the degree of sialylation appears to involve accessibility to the termini of the glycans, which is directly impacted by the local protein conformation.

Translating the glycomics

If the IgG glycans change under inflammatory and other circumstances, and these changes alter the conformation and function of the Fc domain in a way that has a direct influence over their binding to receptors and immunologic environment, it stands to reason that targeting those glycans should alter the inflammatory

course. In support of such a notion, and using a model of collagen antibody-induced arthritis, it was discovered that normally nonpathogenic autoantibodies could be converted into pathogenic antibodies through selective isolation of the IgG glycoforms lacking galactose (Rademacher et al. 1994), an IgG modification contemporaneously shown to have indistinguishable antigen binding and specificity (Donadel et al. 1994). This 1994 study was the first to directly demonstrate that specific glycoforms are not only associated with disease, but can be directly causative, thereby laying the ground work for things to come.

IgG glycans and antigen exposure/immunization

Inflammatory disease is not the only venue in which IgG is a central player. In an interesting 1998 study, it was found that moving mice reared in specific pathogen-free (SPF) environmental conditions into a more conventional environment elicited not only increases in total IgG but also in the amount of agalactosylated IgG. This suggests that the sudden exposure to new environmental/microbial antigens, potentially during vaccination, causes the same type of inflammatory shift in IgG glycosylation previously described in RA and other diseases (Parekh et al. 1985).

More directly relevant to vaccination, when mice were immunized with BSA, BSA-specific IgG titers dramatically increased, and these antibodies were predominantly agalactosylated (Lastra et al. 1998). During the immunization period, the other IgG (i.e., not BSA-specific) present in the mouse did not shift in glycoform. However, weeks later, the BSA-specific IgG glycoforms shifted to be more like the non-BSA-specific IgG glycans as the animals returned to a steady state weeks later (Lastra et al. 1998). These data demonstrate that antigen-specific IgG glycosylation can differ from the bulk IgG present in circulation during times of strong antigen-driven antibody production, but that this may simply be a function of what is being made at the moment in that environment.

A number of years later, a study was published in which the levels of fucose, galactose, mannose and sialic acids were measured on antigen-specific IgG after immunization and subsequent boosts.

It was reported that with each additional immunization boost, the resulting IgG molecules were increasingly fucosylated, but that the galactose, mannose and α 2,6-linked sialic acid content were unchanged after serial boosting (Guo et al. 2005). It would be interesting to know the fate of these IgG molecules long after the final boost, or how they compared to the bulk IgG over time.

A new era

It should be clear at this point that a plurality of IgG glycosylation data focused on RA, in humans and mouse models, in which alterations in IgG glycans had been consistently seen across time and cohort demographics. These generally included the increase in agalactosylated and fucosylated glycans, as well as changes in the level of bisecting GlcNAc and sialylation. As such, it should not be surprising that the next major step in the field occurred in a mouse model of arthritis—the K/BxN serum-transfer model.

In 2006, the IgG glycosylation story remained focused on galactose and the proinflammatory nature of the agalactosyl glycoforms, but a report in *Science* significantly altered that narrative. In the laboratory of Dr. Jeffrey Ravetch, it was found that α 2,6-sialylation of IgG was responsible for the anti-inflammatory activity of IVIg/IgG in autoimmune disease therapy of the K/BxN model of RA (Kaneko et al. 2006). This was recapitulated 2 years later, with the demon-

stration that the IVIg effect could be duplicated with isolated Fc domains preferentially harboring α 2,6-sialylated glycans (Anthony et al. 2008a). This was the first time the IVIg effect had been shown to work without fully intact IgG, robustly demonstrating independence from antigen binding and immune complex formation.

Although changes in IgG glycosylation associated with disease, function, Fc γ R binding and structure were all reported previously, as reviewed here, these two initial IVIg studies (Anthony et al. 2008b; Kaneko et al. 2006) led to an explosion of work aimed at further understanding the relationship between IgG glycans and human health, as well as how to harness those observations in the design and deployment of antibody-based drugs.

Much of the IVIg data stems from the use of SNA, the *Sambucus nigra* agglutinin lectin, which binds to α 2,6- but not α 2,3-linked sialic acid-containing glycans, to enrich for sialylated IgG. This led to several studies focused upon the IgG glycoforms found in such preparations. In 2009, it was reported that most SNA binding to IgG was due to sialylation of Fab fragment-localized glycans, and that Fc-localized glycans required at least 2 sialic acids in order to associate with SNA (Stadlmann et al. 2009). Another report claimed that SNA enriched specifically for sialylated Fab-glycans, not sialylated Fc glycans (Guhr et al. 2011). However, the isolated Fc fragment study (Anthony et al. 2008a) combined with the observation that only about 30% of IgG molecules carry sites of N-glycosylation in the Fab domain argues that Fc glycan α 2,6-linked sialylation, especially highly sialylated glycoforms, is potentially anti-inflammatory in the K/BxN setting.

Outside of the K/BxN model, however, results have been mixed. IVIg therapy was effective at reducing platelet loss in a model of passive immune thrombocytopenia (PIT), but the SNA-enriched IgG had no protective effect at all, indicating that IgG α 2,6-linked sialylation is not a protective determinant within IVIg in the PIT setting (Guhr et al. 2011). In another murine immune thrombocytopenia (ITP) model, α 2,6-sialylated and desialylated IgG were found to work equally well in ameliorating the disease (Leontyev et al. 2012). IVIg-mediated reduction in autoimmune hemolytic anemia also was found to be dependent upon the galactosylation of IgG1, but not total sialylation (Yamada et al. 2013), and a recent study suggested that the clinical outcome in antibody-mediated transplant rejection patients was functionally independent of IgG α 2,6-sialylation (Barba et al. 2019).

Conversely, in a study focused upon Guillain-Barre syndrome, which is often treated with IVIg, investigators reported that sialylated and galactosylated IgG were better at inhibiting C3 deposition compared to the original IVIg preparation or IgG lacking galactose (Sudo et al. 2014). It was also reported that desialylated immune complexes enhance osteoclastogenesis in RA, whereas sialylated IgG did not (Harre et al. 2015). This was extended in mice receiving N-acetylmannosamine (ManNAc), which is known to drive the production of the nucleotide-sugar CMP-sialic acid required for all sialyltransferases (Gu and Wang 1998). These mice showed increased total IgG sialylation and were less susceptible to inflammatory bone loss (Harre et al. 2015). Finally, in an SLE study, it was found that anti-histone IgG was poorly sialylated, but that the α 2,6-sialylated fraction displayed reduced ability to promote phagocytosis of secondary necrotic cells by monocytes (Magorivska et al. 2016).

It was published in 2014 that increased galactosylation and total sialylation induces more robust antibody-dependent cellular phagocytosis (ADCP) (Chung et al. 2014). Analysis of antituberculosis IgG molecules from latently Mtb-infected individuals showed increased galactosylation and enhanced macrophage activation compared to

actively infected individuals. However, inconsistencies with respect to the impact of sialylation of IgG exist for ADCC activity. In a 2015 PNAS article, it was reported that IgG with enhanced α 2,6-sialylation was optimized for ADCC (Lin et al. 2015). However, later in that same year, it was shown that α 2,6-IgG sialylation reduced complement-mediated cytotoxicity (CDC) (Quast et al. 2015) and that α 2,6-sialylation of IgG1 had no significant impact on ADCC activity (Thomann et al. 2015).

Given the varied outcomes with sialylated IgG, it is not surprising that the mechanism driving immune inhibition remains a debate. Two reports proposed that the mechanism in K/BxN arthritis depends upon the sialylated Fc domain binding to the C-type lectin receptor DC-SIGN (Anthony et al. 2008a, 2011). This ligation was reported to induce the release of IL-33, triggering IL-4 production in basophils and the upregulation of the inhibitory Fc γ RIIB receptor on macrophages (Anthony et al. 2011). However, this mechanism remains somewhat controversial in that the ability of sialylated IgG/Fc to bind DC-SIGN has been directly challenged in two subsequent studies (Temming et al. 2019; Yu et al. 2013). The nature of the discrepancy remains unclear.

Another explanation for the α 2,6-sialylation effect, which is not mutually exclusive with the proposed DC-SIGN pathway, is that α 2,6-sialylation appears to substantially reduce the binding affinity to all of the Fc γ Rs (Anthony et al. 2012). Also, structural studies comparing fully α 2,6-sialylated Fc to singly and asialo-Fc revealed that the conformation of the CH2 domain is significantly different with sialylation (Ahmed et al. 2014) (Figure 2), supporting the differential Fc γ R binding properties. Indeed, Fc sialylation increases circulatory half-life of antibodies (Bas et al. 2019), likely by avoiding ligation by both the asialoglycoprotein receptor (ASGPR) in the liver and the neonatal Fc receptor FcRn.

Despite the progress in the field since 2006, the manner in which α 2,6-sialylated IgG/Fc actively inhibits inflammation remains a topic of great interest and investigation. One reason is that sialylation clearly does not always inhibit inflammation or disease. The effect probably depends upon the context, the underlying disease mechanisms and other metabolic factors that we can only speculate about at the present time.

Core fucose and ADCC

In contrast to the sialylation effect, it is now well established that core fucosylation of IgG N-glycans, in which fucose is added α 1,6 to the protein-adjacent GlcNAc of N-glycans, directly impacts IgG ADCC activity. The initial discovery was the demonstration that IgG molecules lacking fucose had enhanced ADCC via increased Fc γ RIIIA binding and signaling (Okazaki et al. 2004; Shields et al. 2002; Shinkawa et al. 2003). These data are receiving much more attention now that the IgG glycan field has grown.

In 2008, it was shown that low fucose levels on IgG enhanced mononuclear cell (e.g., NK cell)-mediated ADCC (Peipp et al. 2008). However, the same study also showed the opposite for PMN-mediated ADCC. For PMNs, high fucose on IgG enhanced ADCC (Peipp et al. 2008). These data suggest that core fucosylation impacts the association of the Fc domain with various Fc γ Rs differently, raising the possibility of selectively targeting different effector cells for ADCC using engineered antibodies. Indeed, it is clear that decreasing core fucosylation on IgG increases the binding affinity between the Fc domain and Fc γ RIIIA. Comparing the crystal structures of fucosylated vs. nonfucosylated IgG in complex with Fc γ RIIIA revealed that the core fucose causes steric hindrance at the

point of interaction (Mizushima et al. 2011). With these data, there is growing realization that fucose could significantly impact antibody-based cancer therapies, such as rituximab and trastuzumab, which are believed to rely upon ADCC for efficacy (Cartron et al. 2002; Weng and Levy 2003).

A move to high throughput

One of the limitations in the field of glycobiology has been the relative difficulty in determining glycan structure, which limits the number of samples and therefore statistical power associated with any trend being measured. If one desires a high level of detail, mass spectroscopy on many micrograms of isolated material remains the only way forward; however, there have been major steps taken to make hydrophilic interaction liquid chromatography (HILIC), a high-throughput platform to analyze glycans. This is particularly useful with IgG glycans since they tend to be no more than biantennary.

Relying largely on the HILIC approach, there has been a significant investment in Croatia, under the leadership of Gordon Lauc at Genos, to analyze IgG from many thousands of samples in an effort to better define the variability and trends seen with IgG glycosylation. This has generated many publications—too many to adequately review here—aligning glycan structure with variables such as sex, age, disease status and country of origin. For example, a 2011 report using IgG isolated from the plasma of nearly 2300 individuals from either Vis or Korčula (both Croatian Adriatic islands) or the Northern Scottish Orkney Islands was published (Pucic et al. 2011). They reported that disialylated N-glycans represented only about 3% of the total glycans, and that nearly all (96%) of all neutral glycans were core fucosylated. Within this cohort, the number of agalactosylated IgG glycans was approximately 40% of all neutral glycans, with glycans harboring one or two galactose residues being 40% and 20%, respectively. It was found that 11.6% of the monogalactosylated structures carried a sialic acid and over 50% of the digalactosylated molecules were sialylated. Importantly, there was a lot of variability within these values. For example, the agalactosylated structures ranged from 14% to 70% of the total neutral glycans, while the number of neutral glycans without core fucosylation ranged from 1.3% to 19%.

In terms of trends, age was found to be the best predictor of the variance within the proportion of agalactosylated structures, in that agalactosylation increased parallel to the increases in bisecting GlcNAc. Interestingly, sex was not associated with glycan changes in this study (Pucic et al. 2011). However, these trends do not hold up in all populations or in all studies. For example, in a study involving 735 controls and 138 thyroid cancer patients, the authors report differences in IgG glycosylation among healthy control females, particularly at puberty and menopause (Chen et al. 2012). Consistent with differences between males and females, a 2017 study demonstrated that menopause was associated with increased agalactosylated glycoforms, but that hormone replacement therapy reversed this effect (Ercan et al. 2017). Likewise, males deprived of gonadal hormones with goserelin had decreased galactosylated IgG glycoforms, which was reversible with testosterone treatment. Finally, in a recent study of Mtb-infected patients from two populations—South Africa and Texas/Mexico—there were large differences in IgG glycan composition reported (Lu et al. 2016). In the South Africa cohort, patients were found to have 90% or greater of their IgG glycans fucosylated and 15–20% sialylated. Yet, in the Texas/Mexico cohort, fucosylation was closer to 75–80% and sialylation was

between 30% and 45%. Even the amount of digalactosylated glycans was distinct, with higher levels in South Africa.

The overall theme of these results is that IgG glycosylation is quite variable within and across populations, though it follows general trends. The sialylation and bisecting GlcNAc rates tend to be relatively low, fucosylation relatively high and galactosylation somewhere in the middle, making the interpretation of small changes as a function of any variable within a population difficult. In addition, the HILIC method, which has made a major positive impact on the field, has limitations in the amount of compositional detail. For example, the linkage of sialic acid, which has an impact on function (Jassal et al. 2001), is not typically reported. Also, the number of peaks analyzed is lower than the total number of glycoforms identified on IgG using more stringent, albeit much slower and laborious, methods such as mass spectrometry.

Nonetheless, it is reassuring that many of the trends in IgG glycosylation can be associated with genome-wide association study (GWAS) results, which identified four genes closely linked to the expected changes (Lauc et al. 2013). These were *ST6GAL1*, *B4GALT1*, *FUT8* and *MGAT3*, which encode the enzymes responsible for α 2,6-linked sialylation, galactosylation, core fucosylation and bisecting GlcNAc addition within typical N-glycans, respectively. Another report from 2018 suggested an association between IgG glycosylation and DNA methylation among smokers (Wahl et al. 2018), although a causal relationship was not established. Many other recent articles fall into a similar category, aligning GWAS results and/or some characteristic of the subjects with the changes in IgG glycoforms: Smoking and longevity are two such correlations (Wahl et al. 2018); depression is another (Park et al. 2018). IBD (Klasic et al. 2018); hypertension (Liu et al. 2018b); dyslipidemia (Liu et al. 2018a); COPD (Pavic et al. 2018); metabolic syndrome, hypertriglyceridemic waist phenotype and abdominal obesity (Wang et al. 2019); and so on.

Whether these associations are functional or causally related to disease requires further mechanistic studies. Moreover, the source of nucleic acid for the GWAS studies is, in most cases, not the B-cell compartment. As a specific example, promoter methylation of the *MGAT3* gene, which produces the enzyme responsible for bisecting GlcNAc, was reported as being associated with IgG glycans and IBD (Klasic et al. 2018). Yet, the epigenetics was performed with whole peripheral blood, making it difficult to know the extent of epigenetic changes in the B-cell compartment. Collectively, these studies are exciting because they form the foundation for new hypotheses and fuel much needed mechanistic research to fully understand the biology.

IgG glycans and regulation

Considering the impact of IgG glycosylation on the function of an IgG antibody, renewed interest in vaccination is palpable from the perspective that a protective antibody response to a vaccine may hinge upon the glycoforms of the antibodies generated. However, there is substantial debate about how IgG glycosylation is regulated, and whether it is possible that IgG glycoforms can be selectively and permanently elicited at all.

A recent study showed that immunization of patients with the seasonal influenza vaccine resulted in shifting glycoforms over time (Wang et al. 2015), similar to what was reported previously (Lastra et al. 1998). Interestingly, this was extended using mouse models in which immunization with immune complexes comprised of human IgG and hemagglutinin, where the IgG was either partially sialylated

or enzymatically desialylated, resulted in higher affinity IgG when α 2,6-sialylated IgG was used (Wang et al. 2015). It was proposed that this effect was through upregulation of Fc γ RIIB, but the precise mechanism remains unclear given that increased Fc γ RIIB signaling on the B cell was previously reported to induce apoptosis (Pearse et al. 1999).

In healthy human samples collected from vaccinated individuals at varied time points following exposure, it was found that the amount of galactosylation and total sialylation on vaccine-specific IgG varied over time. Both were low at first, increased later, and then established a baseline at the latest time point. Interestingly, the nonvaccine-specific IgG did not change over the same period (Selman et al. 2012). Similarly, in 2016, it was reported that despite the large variability in IgG glycosylation across multiple geographical locations, that immunization “overcomes” these differences and yields antigen-specific antibodies with similar antibody glycosylation patterns, and that these patterns were influenced by the vaccination regimen (Mahan et al. 2016). In support of this interpretation, previous work using primary human B cells harvested and stimulated to produce IgG with and without other stimuli demonstrated that CpG (TLR9 agonist) and IL-21 increased galactosylation and reduced bisecting GlcNAc, whereas all-trans retinoic acid decreased galactosylation and total sialylation levels (Wang et al. 2011).

These data have been interpreted as suggesting that specific IgG glycoforms are permanently encoded within a B cell and can be specifically elicited, and that the associated glycoforms are optimized for the required protective function of that particular IgG target (Mahan et al. 2016). While the data are clear, the interpretation is debatable. In this study, all of the glycan analyses were performed at 2 weeks postfinal immunization, but it is clear that the glycan profiles change over time after immunization, with 2 weeks being a peak in that difference (Lastra et al. 1998, Wang et al. 2015). Moreover, since all IgG is antigen specific and produced by a clonal population of B-cell-derived cells, the changes in IgG glycoforms with age, sex, inflammation and disease reviewed herein runs counter to the notion of hardwired programming of IgG glycosylation.

Consistent with a dynamic glycoform model of IgG glycosylation whereby the glycan composition is driven by the immediate in vivo environment, patients with multiple sclerosis (MS) showed reduced fucose and galactose and increased bisecting GlcNAc in IgG localized within the central nervous system (CNS), yet IgG from serum did not reflect those same changes (Wuhrer et al. 2015). This could mean that the antibody-producing plasma cells localized within the inflamed CNS produce antibodies with divergent glycoforms compared to the plasma cells outside of the localized inflammation characteristic of MS. It would be interesting to know if this difference holds for clonal IgG with identical specificity produced in both anatomical/inflammatory environments.

Another line of evidence comes from studies in mice where the sialyltransferases responsible for IgG α 2,6-sialylation (Hennet et al. 1998) was manipulated. First, in mice lacking the P1 region of the ST6Gal1 promoter, IgG α 2,6-sialylation is reduced substantially (Jones et al. 2012). Since B-cell expression of ST6Gal1 is reportedly independent of the P1 region, these data suggested that B cells were not responsible for IgG α 2,6-sialylation. Supporting those findings, mice in which ST6Gal1 was selectively deleted from B cells showed no change in IgG α 2,6-sialylation (Jones et al. 2016). These data demonstrate that IgG α 2,6-sialylation is not controlled by IgG-secreting plasma cells but is sialylated after release by circulatory ST6Gal1 in the plasma (Jones et al. 2012, 2016). Perhaps more importantly, the ability of IgG to be sialylated after release from an

antibody-producing plasma cell directly supports a model in which IgG glycans can be dynamically remodeled long after secretion into the circulatory environment. Indeed, it is now known that Neu1 and Neu3, sialidases which remove sialic acids from glycans, are released into the circulation as well (Yang et al. 2015), though it is not yet clear whether this release directly alters IgG sialylation. Finally, while these studies were performed in mice, it is important to note that ST6Gal1 is also known to exist in human plasma.

Translating the glycomics, revisited

With these profound differences in function, the notion of manipulating the IgG glycome as a novel approach to disease therapy has matured rapidly. Using collagen antibody-induced arthritis as a model, it was demonstrated that disease-causing collagen-specific antibodies could be rendered nonpathogenic by removing the N-glycans with EndoS treatment (Nandakumar et al. 2007). Perhaps more important from a translational perspective, this was extended to direct in vivo injections of EndoS to remove most of the glycan. Treated mice were protected in the CIA model (Albert et al. 2008). Supporting these findings, EndoS deglycosylation of antiaquaporin-4 autoreactive IgG, which is causative for neuromyelitis optica, was also shown to not only eliminate its pathogenic efficacy but also converted the IgG into a robust therapeutic antibody that blocked disease (Tradtrantip et al. 2013).

In 2016, it was reported that sialylation of RA-associated antibodies converted them into potent inhibitors of arthritogenic activity and suppresses CIA development in treated mice (Ohmi et al. 2016). A recent and more directly translational extension of this work was published in *Cell*, wherein investigators administered engineered glycosyltransferases (B4GalT1 and ST6Gal1, which catalyze N-glycan galactosylation and α 2,6-linked sialylation respectively) to mice. These increased galactosylation and sialylation of IgG and reduced severity in a Goodpasture Disease model of autoimmunity (Pagan et al. 2018).

Cancer is also a primary target for glycomic engineering of drugs. There is a recent and comprehensive review on the relationship between fucose and anticancer antibody drugs, which provides details that go far beyond the scope of this review (Pereira et al. 2018). However, it is important to recognize that there are a number of strategies being employed to translate the core fucosylation effect into pharmaceuticals. For example, recombinant systems producing anticancer IgG have been manipulated to delete Fut8, the fucosyltransferase responsible for core fucosylation. In this case, afucosylated anti-CD20 (the target of rituximab) showed a two-fold increase in ADCC (Yamane-Ohnuki et al. 2004). Others are working to delete the nucleotide-sugar transporter Slc35c1, which is responsible for providing GDP-fucose into the Golgi for all fucosylation reactions (Chan et al. 2016). And still others are attempting to make mutants that will mimic the fucose structural effect leading to enhanced Fc γ RIIA binding and activation (Jung et al. 2010; Shields et al. 2001).

Finally, there are efforts to harness the sialylation effect to improve IVIg efficacy. As an example, researchers have produced an IVIg in which all IgG molecules carry fully α 2,6-sialylated glycans at N297. This drug candidate was reported to have a 10-fold higher anti-inflammatory activity compared to conventional IVIg using a variety of animal models, including collagen antibody-induced arthritis, K/BxN arthritis, ITP and epidermolysis bullosa acquisita (Washburn et al. 2015).

Conclusions

In this article, a concerted effort has been made to provide a thorough historical perspective interlaced with an up to date review of what is known about IgG glycosylation. The field is broad, and the impact cannot be overstated. IgG is central as a drug platform, defense against microbial pathogens and prevention of inappropriate inflammation characteristic of many diseases. Yet, many questions remain. Can IgG glycoforms be programmed, or are they dynamic? Or, is the truth somewhere in between? Will glycomic engineering of antibody-based drugs increase efficacy? Can endogenous IgG glycans be manipulated directly in vivo as a therapeutic strategy, and would that have unintended consequences? The more learned about the effects of glycans on IgG, how their composition and structure are regulated and methods to manipulate them, the better armed the research community will be at finally opening the gates to the use of the glycome as the next pharmaceutical frontier.

Conflict of interest statement

None declared.

Acknowledgements

The author would like to thank Lori SC Kreisman for critical evaluation of this manuscript, and Douglas Oswald for assistance in basic editing. Funds supporting the creation of this manuscript were provided by a NIH/NIGMS grant to BAC (R01GM115234).

Conflict of interest statement

None declared.

Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-mediated cytotoxicity; CIA, collagen-induced arthritis; CNS, central nervous system; Fc, fragment crystallizable; GPA, granulomatosis with polyangiitis; GWAS, genome-wide association study; HILIC, hydrophilic interaction liquid chromatography; ITP, murine immune thrombocytopenia; mAb, monoclonal antibody; MBP, mannose-binding lectin; MS, multiple sclerosis; OA, osteoarthritis arthritis; OST, oligosaccharyltransferase; PIT, passive immune thrombocytopenia; PMNs, polymorphonuclear cells; PSA, prostate-specific antigen; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SPF, specific pathogen-free; SPR, surface plasmon resonance; TNF α , tumor necrosis factor α .

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