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SPECIAL ARTICLE

Sweet but dangerous – the role of immunoglobulin G glycosylation in autoimmunity and inflammation

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Glycosylation is well-known to modulate the functional capabilities of immunoglobulin G (IgG)-mediated cellular and humoral responses. Indeed, highly sialylated and desialylated IgG is endowed with anti- and pro-inflammatory activities, respectively, whereas fully deglycosylated IgG is a rather lame duck, with no effector function besides toxin neutralization. Recently, several studies revealed the impact of different glycosylation patterns on the Fc part and Fab fragment of IgG in several autoimmune diseases, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Here, we provide a synoptic update summarizing the most important aspects of antibody glycosylation, and the current progress in this field. We also discuss the therapeutic options generated by the modification of the glycosylation of IgG in a potential treatment for chronic inflammatory diseases. *Lupus* (2016) **25,** 934–942.

Key words: Glycosylation; sialylation; fucosylation; galactosylation; Fc fragment; autoantibody; autoimmunity; inflammation

Introduction

One of the mechanisms fostering the development of immunoglobulin G (IgG)-mediated inflammatory autoimmune diseases is the impaired clearance of cell-derived remnants, which results in a breakdown of tolerance to self and the initiation of an autoimmune response.[1] B cells exert their pro-inflammatory effects by producing pathogenic IgG autoantibodies (AAbs). In systemic lupus erythematosus (SLE) IgG AAbs interact with dsDNA, and DNA- and RNA-associated nuclear proteins.[2] In rheumatoid arthritis (RA), pathogenic IgG AAbs recognize citrullinated epitopes.[3] In diseases affecting the skin, such as epidermolysis bullosa acquisita or bullous pemphigoid, pathogenic IgG AAbs specifically bind collagen type VII and XVII, respectively.[4] Pathogenic IgG AAbs tend to form immune complexes (IC) with their cognate autoantigens (AAgs). These activate complement and recruit inflammatory effector cells expressing Fcγ receptors (FcγRs), which ultimately contribute to tissue damage.[5] The glycosylation pattern of an antibody crucially influences its conformation, if it binds to FcγRs and complement, as well as its aggregation behaviour. The pro-inflammatory effector functions of IgG AAbs are mainly modulated by their Fc N-linked glycan patterns. IgG profiling of glycan structures employing denaturating analytical methods have shown that hypoglycosylation of IgG AAbs correlates with pro-inflammatory immune responses and disease severity in patients with SLE or RA.[6,7] In the following paragraphs we provide an overview of findings concerning the role of IgG glycosylation in the course of inflammation and autoimmune disease. Furthermore, we aim to encourage researchers to develop new therapeutic strategies shaping the immune response based on the IgG glycans.

Immunoglobulin Fc fragment glycosylation

It is well known that IgG recognizes pathogens and toxic products in order to neutralize and tag them

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for elimination from the host (opsonization). IgG is a hetero-tetrameric protein consisting of two heavy (HC) and two light chains (LC) linked by disulfide bonds.[8] Papain cleaves IgG into two functional units: two fragment antigen binding (Fab) fragments specifically recognizing the antigen, and the fragment crystallizable (Fc) part mediating the interactions of IgG with FcγRs on cellular surfaces and with complement. Human IgG is mainly glycosylated with biantennary oligosaccharide chains covalently attached to asparagine 297 (Asn 297) of each HC (CH2 domain). The N-linked glycan is composed of a core heptasaccharide (GlcNAc2-Man3-GlcNAc2) (Figure 1).[9] Glycosylation starts in the endoplasmatic reticulum, but the complex processing of the glycan tree occurs mainly in the Golgi apparatus.[10] Variable additions to, and modifications of, fucose, galactose and sialic acid involving this core heptasaccharide can generate a total of 32 unique oligosaccharide chains.[11] In addition, asymmetric addition of oligosaccharides to each HC can generate more than 400 glycoforms. In average human serum, 16%, 35% and 35% of the IgG carry terminal galactosyl residues on 2, 1 or 0 of the glycan chains, respectively, and are, therefore, referred to as IgG-G2, G1 and G0, respectively. The remaining 14% are terminally sialylated at one (S1) or two glycan arms (S2) (Figure 1). The IgG-associated glycan depends on the age and gender of the individual and the state of a disease. As an example, Parekh and colleagues reported a minimum of IgG-G0 glycan in individuals of 25 years of age.[12] The IgG glycans are thought to act as spacers between the two HCs. The interactions of protein and sugar residues are critical for the structural stability and functional activity of the IgG molecule influencing the outcome of the immune response.[13–15]

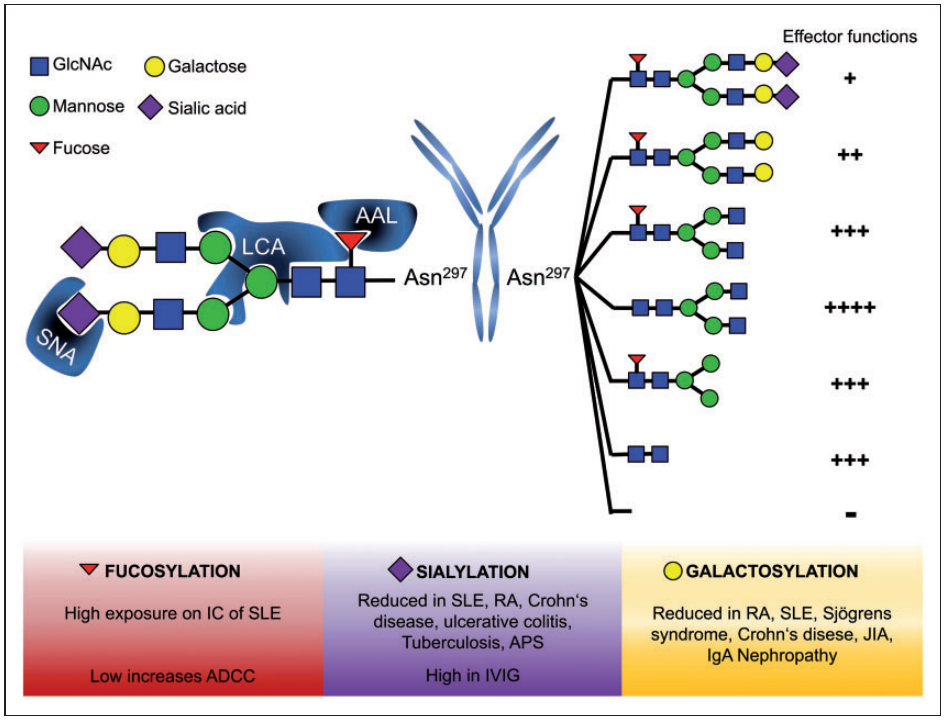

Figure 1 N-Glycosylation of immunoglobulins and its implications in autoimmune diseases. The human IgG is a hetero-tetrameric protein consisting of two heavy (HC) and two light (LC) chains linked by disulfide bonds. It is mainly glycosylated with biantennary oligosaccharide chains covalently attached to Asparagine 297 (Asn 297). The core heptasaccharide of N-acetylglucosamine (GlcNAc, blue square) and mannose (green circle) can be altered by addition of variable sugar moieties, namely fucose (red triangle), galactose (yellow ellipse) and sialic acid (purple diamond). In addition to galectins or MBL, several plant lectins recognize specific glycans and are used for in vitro analyses of protein glycosylation. *Aleura aurantia* lectin (AAL), *Lens culinaris* agglutinin (LCA) and *Sambucus nigra* lectin (SNA) reportedly bind to fucose, fucosylated tri-mannose N-glycan core sites and sialic acid, respectively. The glycosylation pattern of IgG crucially impacts subsequent cellular effector functions mediated by binding of the antibody to Fc receptors (see Figure 2). Lower glycosylation results in a more pronounced immune response (including ADCC). However, complete removal of the Fc sugar residues abrogates IgG activity. Increased IgG autoantibody (AAb) activity is often associated with a higher pathogenicity of AAb in autoimmune diseases, including SLE, RA, APS and inflammatory bowel diseases (Crohn's disease, ulcerative colitis). In contrast, enrichment of sialylation by therapeutic intravenous IgG (IVIG) can be utilized to ameliorate pro-inflammatory effects.

Thirty percent of serum IgG carries N-linked carbohydrates on the Fab region, attached to the variable regions of LC or HC. The composition of these oligosaccharides is shifted towards higher galactosylation and sialylation when compared to the Fc fragment.[15] The function of these oligosaccharides has not been fully elucidated, although studies on monoclonal antibodies suggest that glycosylation of the variable regions can have positive, neutral or negative influences on antigen binding.[15] Several plant lectins recognize specific glycans and are used for in vitro analyses of protein glycosylation. *Aleura aurantia* lectin (AAL), *Lens culinaris* agglutinin (LCA), *Sambucus nigra* lectin (SNA) and Jacalin (from Jackfruit) reportedly bind to fucose, fucosylated tri-mannose N-glycan core sites, sialic acid and N-acetylgalactosamine, respectively (Figure 1).

Furthermore, a recent study used *Narcissus poeticus* lectin binding oligomannose N-glycans, and *Viscum album* agglutinin binding desialylated glycans, specifically targeting subpopulations of human polymorphonuclear leukocytes undergoing apoptosis.[16]

Receptors sensing IgG glycosylation

Effector functions of IgG are mediated by the interaction of its Fc fragment and the IgG-specific FcγRs (FcγRs or type I FcRs). Type II FcRs include C-type lectins, SIGLECs and the low-affinity IgE receptor CD23 (Fcε R), which mediate inhibitory or immunomodulatory signals. The IgG-specific FcγR is a type I transmembrane glycoprotein, which is primarily expressed on leucocytes. The FcγR consist of an extracellular part of two or three immunoglobulin-like domains, one transmembrane and one cytosolic domain. The signal is transduced via phosphorylation of immunoreceptor tyrosine-based activating motifs (ITAM) by SRC family kinases. The ITAM motif is located on the cytoplasmic domains of the activating receptors FcγRIIA and FcγRIIC and on an associated γ-chain of the activatory FcγRI and FcγRIIIA.[17]

In humans there are three FcγRs: the high affinity FcγRI and two low affinity receptors, FcγRII and FcγRIII.[18] Most isoforms are activatory, including high-affinity FcγRI and low-affinity FcγRIIA, FcγRIIC, FcγRIIIA and FcγRIIIB. When aggregated IgG-Fc fragments bind to FcγRs, they induce cross-linking of two or more receptors and, subsequently, trigger receptor signalling. Subsequent immune responses include antibody-dependent cellular cytotoxicity (ADCC), secretion of inflammatory mediators, regulation of antibody production, oxidative burst and/or phagocytosis (Figure 2). FcγRIIB is the main inhibitory receptor, present on B cells, dendritic cells (DC), macrophages, activated neutrophils, mast cells and basophils. Cross-linking of FcγRIIB and the B cell receptor decreases the B cell activation threshold and ameliorates the humoral response. Also, in the case of other immune cells, engagement of FcγRIIB suppresses the immune response (Figure 2).[19] Each subclass of IgG binds to a specific FcγR. In humans, IgG1 and IgG3 mainly interact with FcγRI.[17] The binding affinity for functional FcγR by specific IgG subclasses is described as a result of an activatory/inhibitory (A/I) ratio. Glycosylation is vitally involved in affinity binding and, therefore, in shaping the immune response. The fact that each cell subset has an overlapping repertoire of FcγRI makes it tough to identify the precise effector cells that mediate a certain antibody function.[20]

Consistent evidence for the modulation of effector functions by specific IgG glycosylations was reported by Kaneko et al. in 2006.[21] Antibodies that recognize a platelet integrin efficiently depleted platelets in vivo only if they are enriched for low sialylated IgG isoforms. This reduction correlated with a lower binding affinity of the IgG for the respective FcγRs. In murine models of experimental autoimmune encephalomyelitis (EAE), inflammatory arthritis and immune thrombocytopenia (ITP), removal of the glycan tree abrogated its anti-inflammatory function confirming the importance of the oligosaccharide chain.[20]

Low fucosylation of IgG1 increases binding to FcγRIIIa, leading to enhanced cytotoxic activity in vivo. It is yet unknown if the absence of fucose alters the conformation of the HC or interferes with the interaction with the FcγRIIA.[22] However, it is known that the presence or absence of fucose does not affect the binding of IgG1 to the neonatal FcR or C1q. Importantly, differential glycosylation was found to alter the A/I ratio of specific murine IgG subclasses for FcγR.[5] Defucosylation of IgG2a and IgG2b, increasing the A/I ratio, resulted in higher affinity to FcγRIIb and IV, and subsequently increased ADCC. In contrast, defucosylation of IgG1 did not alter affinity to FcγRIIB or FcγRIII. It was suggested that non-fucosylated IgG displays a larger surface on the Fc fragment, making it available for additional interactions with glycan residues on the FcγR.[23] Several studies aiming to generate therapeutic monoclonal Ab revealed that IVIG carrying bisecting N-acetylglucosamine residues shows enhanced ADCC activity,

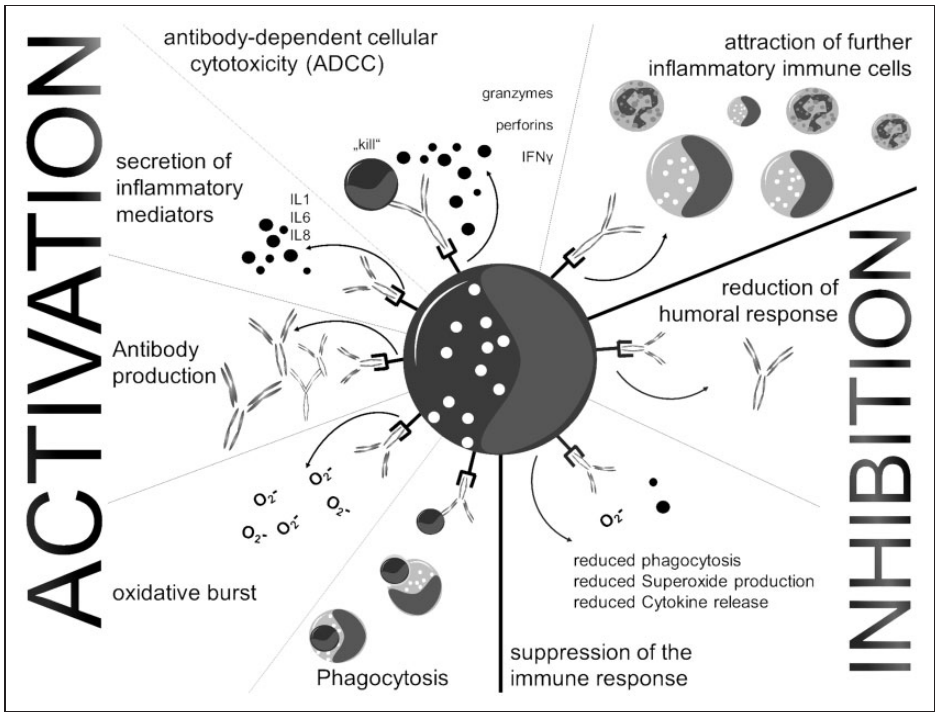

Figure 2 Fc receptor-mediated effector functions. Effector functions of IgG are mediated by the interaction of its Fc fragment and the IgG-specific FcγR. *Activation*. Most isoforms are activatory, including high-affinity FcγRI and low-affinity FcγRIIA, FcγRIIC, FcγRIIIA and FcγRIIIB. When aggregated IgG-Fc fragments bind to FcγR, they induce cross-linking of two or more receptors and trigger receptor signalling. Subsequent immune responses include antibody-dependent cellular cytotoxicity (ADCC), secretion of inflammatory mediators, regulation of antibody production, ROS production or phagocytosis. *Inhibition*. FcγRIIB is the main inhibitory receptor, present on B cells, dendritic cells (DC), macrophages, activated neutrophils, mast cells and basophils. Its engagement suppresses immune responses and inhibits phagocytosis. Cross-linking of FcγRIIB and the B cell receptor decreases the B cell activation threshold and reduces the humoral response.

since the early addition of Glc-NAc inhibited subsequent addition of fucose.[24]

In 2011, Biburger and colleagues showed that, despite expression of relevant activating FcγR on various phagocyte populations, the majority of these cell types were dispensable for IgG activity in vivo.[25] The $CX3CR1^{hi}Ly6C^{lo}CD11c^{int}$ monocyte subset was crucial to mediating IgG-dependent effector functions.

Sialylation

Recently, several studies revealed that sialylation of the Fc part of IgGs has a critical influence on their pro- or anti-inflammatory activities (Figure 2). An anti-inflammatory and immunomodulatory activity has been observed when IgG glycovariants are rich in terminal sialic acid and galactose residues, resulting from a loss of affinity for type I FcR. This is often accompanied by a simultaneous gain of the capacity to bind type II FcR.[26–28] Consistently, patients suffering from RA, Crohn's disease, ulcerative colitis and tuberculosis show increased levels of IgGs lacking terminal sialic acid and galactose residues (Figure 1).[29–31] Vice versa, the recovery of IgG sialylation of therapeutic intravenous IgG (IVIG) is associated with an anti-inflammatory effect that counteracts AAb pathogenicity.[21,32,33] Tetra-Fc sialylation of IVIG (S4-IVIg) enhances its anti-inflammatory activity up to 10-fold in comparison to random IVIG across different animal models.[34]

Consistent with these findings, we recently detected a significantly lower sialylation of IgG recognizing β2GP-1 isolated from sera of patients with clinically apparent APS when compared to IgG of asymptomatic carriers.[35] Interestingly, healthy children also carry high levels of non-pathogenic, highly sialylated, specific anti-β2GP-1 IgG. This suggests that an increased sialylation is an important factor limiting the pathogenicity of specific AAbs.

Terminal sialic acid residues reportedly control the arrangement of the carbohydrate chains,

prevent conformational modifications and provide antibody binding to receptors on the surfaces of immune cells (macrophages, neutrophils, mast cells and many others) and complement component C1q, thereby avoiding inflammation and induction of autoimmune reactions.[36] We have demonstrated that random IgG and specific anti-dsDNA/anti-histone antibodies isolated from the sera of patients with SLE reveal completely distinct profiles of the glycan residues they expose.[37] Histone IgG AAb displayed significantly lower sialylation than random IgG of the same individual, suggesting that these prototypic SLE AAbs possess pro-inflammatory properties. Functional analyses of ex vivo phagocytosis revealed that the purified desialylated anti-SNEC AAbs mediate the uptake of SNEC preferentially into polymorphonuclear cells without inducing anti-inflammatory cytokine responses. In contrast, the sialylated IgG fraction reduced phagocytosis by monocytes of SNEC and induced a switch of the cytokine profile from IL-6/IL-8 to TNF-α/IL-1β.

Recently, Harre et al. reported an association between desialylation of IgG and bone loss in patients with RA.[38] Desialylated IC enhanced osteoclastogenesis in vitro and in vivo. The state of Fc sialylation of random IgGs and specific IgG AAbs is associated with bone pathology in patients with RA. Furthermore, mice treated with the sialic acid precursor N-acetylmannosamine, causing increased IgG sialylation, were less susceptible to inflammatory bone loss. This suggests a protective role of sialylated IgG in autoimmune-mediated bone destruction.

Taken together, terminal sialylation of IgG controls its action regarding cytotoxicity and anti- or pro-inflammatory effects.[39,40] Carrying low sialylated glycans, IgG binds to activating FcγR and hence displays cytotoxic activity. However, sialylation of the Fc-linked glycan reduces FcγR binding and induces the loss of the cytotoxic capability and, consequently, converts IgG into an unreactive, non-inflammatory molecule. In addition, IgG can actively suppress inflammatory responses mediated through 2,6-linked sialic acid residues binding to a cognate receptor. Importantly, a recent study revealed that, in distinct combinations of FcγR alleles and human IgG subclasses, the interaction of certain non-FcγR-binding IgG variants was not abrogated if present in large IC.[41] This study emphasizes the importance of investigating the interaction of IgG with FcγR in a cellular and physiological context (i.e. as part of IC).

Fucosylation

The state of IgG-fucosylation has been shown to be essentially implicated in antibody-dependent cellular cytotoxicity (ADCC). In general, addition of fucose residues decreases ADCC.[5,42] IgG1 carrying Fc glycans without fucose residues showed dramatically enhanced binding to human FcγRIIA and increased ADCC in vitro.[22]

In 2015, we reported on the impact of fucosyl exposure on native circulating IgG complexes in patients with SLE.[43] Our results showed significantly higher levels of AAL and LCA binding sites exposed on IgG complexes of patients with SLE than on those of normal healthy donors (Figure 1). Moreover, disease activity in patients positively correlated with exposure of AAL-reactive fucosyl residues on immobilized IgG complexes. The increased exposure of these glycans may have resulted from an increased exposure on either the canonical N-glycan of the Fc fragment or on an IgG binding non-IgG molecule, such as complement or CRP. In both cases, the complexed IgG may be alternatively targeted to lectin receptors of effector cells, for example, dendritic cells.

Galactosylation

Agalactosyl glycoforms of IgG (IgG-G0) are markedly increased in patients with RA and positively correlate with disease activity.[44,45] IgG-G0 is also directly associated with pathogenicity of murine collagen-induced arthritis.[44–46] Moreover, high serum levels of these IgG glycoforms are associated with further autoimmune diseases, including Crohn's disease, juvenile onset chronic arthritis, SLE complicated by Sjögren's syndrome and tuberculosis.[47–49] The presence of agalactosyl glycoforms in RA has been shown to be associated with lowered galactosyltransferase (GTase) activity.[50] Inflammation in the joints was once thought to be mediated by MBL binding to clustered IgG-G0 and IgG-G0 ICs, which had been deposited in the synovial tissue, subsequently initiating the MBL-dependent pathway of complement activation.[51] However, later it was reported that the activity of IgG-G0 in mice with a genetic deletion of MBL (MBL-null mice) is unimpaired, but fully dependent on the presence of activating FcγR.[32,52,53] However, RA patients carrying an MBL mutant allele show an earlier onset of disease, confirming the current paradigm that MBL, as well as other complement components, protects from the development of autoimmune diseases.[54] Clarification of

the role of MBL in the pathogenesis of RA is still needed.

Moreover, a role for differential glycosylation of IgG rheumatoid factor (IgG-RF) has been described. The levels of sialylation and galactosylation were significantly lower in IgG-RF than in RF-depleted IgG.[55] In addition, decreased levels of galactosylation and sialylation of RF-IgG positively correlated with increased RF avidity, suggesting an increased pathogenicity of galactose-free and less-sialylated IgG-RF.

IC composed of galactosylated IgG1 has been shown to inhibit complement component 5a (C5a) receptor-mediated phosphorylation of extracellular-signal-regulated kinase 1/2 (ERK1/2).[56] This blocked C5a effector functions in vitro and C5a-dependent inflammatory responses in vivo (peritonitis and experimental epidermolysis bullosa acquisita).

Furthermore, terminal galactose has been shown to be crucial for antibody-mediated phagocytosis. In IgA nephropathy, reduced levels of this residue lead to hampered clearance of both IgA and serum IC-containing IgA1 by hepatic asialoglycoprotein receptor, thus resulting in their deposition in the kidneys.[57,58] The activation of complement by enhanced binding of MBL to agalactosyl IgA in the IC deposits essentially contributes to the induction of inflammation in renal mesangium.[59]

Polyclonal IgG isolated from sera of patients with ANCA-associated vasculitides was shown to display a marked deficit in galactosylation when patients were experiencing an acute inflammatory episode.[60,61] Under these conditions, the Fc part of ANCA IgG was hypogalactosylated, whereas the Fab fragment displayed normal galactosylation and sialylation. Thus, the IgG Fc hypogalactosylation most likely results from a conformational change of the IgG Fc portion. The glycan processing machinery seemed unaffected.

Immunoglobulin Fab fragment glycosylation

Thirty percent of serum-derived Fab fragments contain N-glycosylation motifs which differ significantly from those found in the Fc domain.[62] Fab portions contain about 14% of the total carbohydrate chains of IVIG preparations. The relative distribution of glycans in the Fab fragment has been reported to be 21% S0; 43% S1; 36% S2. The distribution within S0 is 14% G0; 34% G1; 52% G2. Compared to Fc fractions, Fab portions contain much higher proportions of sialic acid and biantennary fucosilated residues.[63]

The role of the sialylation of the $F(ab')_2$ part in the function of IVIG therapy is controversial.[64] In one study, high $F(ab')_2$ sialylation resulted in a reduction of the anti-inflammatory activity in a murine model of idiopathic thrombocytopenic purpura.[65] In another study, it promoted the production of prostaglandin E2 by monocytes, which in turn suppressed TLR-mediated IFN-α secretion by pDC.[66] Since the enrichment of sialylated fractions of IVIG are mostly made by *Sambucus nigra* lectin fractionation, the evaluation of the role of $F(ab')_2$ sialylation needs further investigation and more specific approaches to draw definitive conclusions. Similarly, contradictory reports are found regarding the biological activity of therapeutic antibodies in terms of affinity and aggregation properties.[67,68]

The importance of Fab glycosylation has been recently illustrated by an elegant study on anti-citrullinated peptide antibodies (ACPA) in RA. This study reported that the vast majority of ACPA-IgG harbour N-glycans in their variable domains. The glycosylation consensus sites of the mutated variable regions were absent in their 'germline-counterparts'. This suggests that the N-glycosylation sites in ACPA variable domains have been introduced by somatic hypermutation. This specific modification of ACPA modulates the binding activity to citrullinated AAg, thereby conferring a selective advantage to ACPA-producing B cells in RA.[69] A similar mechanism has been proposed in the case of follicular lymphoma B cells. Mannose-rich Fab glycans on cell surface BCR create a functional bridge with micro-environmental lectins, thereby providing survival signals.[70] It is not yet clear whether this feature is unique for ACPA. Fab glycosylation also influences ACPA fine specificity. Patients who are seropositive for low avidity ACPA display the highest rate of erosive joint destruction.[71] Thus, it is possible that ACPA Fab glycosylation is responsible for the overall low avidity of the citrulline-specific immune response, which opens a novel perspective for the study of AAb function in autoimmunity.

Concluding remarks and outlook

The evidence published so far clearly shows that glycosylation of antibodies plays a crucial role in modulating antibody-mediated responses. Rheumatic diseases offer the opportunity to analyse the impact on disease development and the progress of glycosylation patterns of IgG. The availability of antibody-based therapies has provided further

insights into this complex field. Recently, it was demonstrated that the therapeutically most widely-used IgG subclasses, including human IgG1 and IgG3, surprisingly remained fully functional in a minimally glycosylated form solely carrying a mono- or di-saccharide sugar moiety.[72] Thus, enzymatic processing of heterogeneously glycosylated IgG preparations may represent a strategy to generate well-defined and highly-active therapeutic antibody preparations. As an example, obinutuzumab (Gazyva™), a humanized and glycoengineered therapeutic antibody, has been recently approved, showing increased ADCC against B-cell malignancies.[73]

Fully deglycosylated autoreactive IgG recognizing cellular debris might also be a promising tool for the treatment of SLE. Deglycosylated IgG targeting circulating SNEC might be able to neutralize this material without inflammatory FcγR signalling.[74] Treatment with endoglycosidase S (EndoS), an IgG glycan-hydrolyzing bacterial enzyme from *Streptococcus pyogenes*, has already been shown to have beneficial effects in several experimental animal models for chronic inflammatory diseases.[75,76] Moreover, a study investigating the pro-inflammatory properties of IC in vitro suggests that EndoS treatment has the potential to serve as therapy for SLE.[7]

Analysis and quantification of the glycosylation state of IgG are mostly based on denaturating methods such as mass spectrometry. These methods cannot resolve the spatial configuration of the hetero-tetrameric IgG molecule in vivo. Glycans of IgG and IC are usually recognized by lectin receptors on immune cells. Several plant lectins possess highly specific carbohydrate recognition domains, and they have been used to fractionate differently glycosylated IgG. Those lectins can also be exploited for the development of a new generation of diagnostic tools that detect functionally relevant alterations of the glycosylation pattern in patients with rheumatic diseases.

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