Bitter Sweet Symphony: the impact of sugars on autoimmunity

Kissel, T.

General introduction

Autoantibodies in Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is an autoimmune disease causing chronic inflammation of the joints and other associated tissues. Characteristic is a symmetrical and persistent inflammation of the synovial tissue of small joints, particularly of the hands and feet. RA is classified based upon the American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) criteria combining clinical manifestations (joint involvement, duration of symptoms) and laboratory tests (serology, acute phase reactants)[1]. RA has a slowly evolving disease course including arthralgia, a phase in which synovial inflammation is still absent, as an important pre-disease manifestation. Autoantibodies are an important hallmark of RA and about 60-70% of patients display rheumatoid factors (RF), antibodies directed against the fragment crystallizable (Fc)-tail of IgG, when these are complexed with antigen. The effective treatment with B-cell targeted therapies emphasizes that B cells and/or their secreted antibodies are central players in the disease progression[2,3].

The most disease-specific antibodies were first identified in 1964 as "anti-perinuclear factors"[4]. In 1998 it was found that these "anti-perinuclear factors" were directed against citrulline, a "non-encoded" amino acid generated by a post-translational modification (PTM) of arginine via protein-arginine deiminase (PAD) enzymes[5,6]. The therefore called anti-citrullinated protein antibodies (ACPAs) are present in about 50-70% of RA patients. ACPAs can already occur several years before the onset of RA, in the phase of clinically "silent" autoimmunity, although to a rather limited extent[7]. Intriguingly, ACPA-positive patients have a more severe disease progression and a lower chance to achieve long-term drug-free remission as compared to ACPA-negative patients[8]. ACPA responses are dynamic and an increase in levels combined with a broader citrullinated epitope recognition profile associates with the transition towards RA[9]. Remarkably, ACPAs can react towards a broad spectrum of citrullinated antigens, such as α-enolase, fibrinogen, filaggrin, vimentin and type II collagen[6,10-14]. Next to ACPAs, also other autoantibodies targeting different PTMs, such as carbamylated-(homocitrulline-presenting) and acetylated-antigens, have been identified. Anti-carbamylated protein antibodies (ACarPAs) are present in ~45% of patients with RA, while anti-acetylated protein antibodies (AAPAs) can be found in ~40% of RA patients[15-17]. The concurrent presence of different autoantibody systems in patients with RA is surprising and suggests cross-reactivity of one autoantibody to various PTMs[18-21]. Nevertheless, ACPAs, ACarPAs and AAPAs may possibly also represent three distinct autoantibody families[15,17]. This assumption is reinforced by the fact that the "modified" epitopes, citrullinated-arginine residues and carbamylated-/acetylated-lysine residues, occur at different positions in the protein backbone surrounded by distinct flanking regions. In addition, the three amino acid modifications are structurally dissimilar, especially when comparing carbamylation to acetylation.

Currently it is unclear if and how the autoantibodies in RA are involved in the inflammation of the synovium, but it has been postulated that ACPAs can contribute to disease pathogenesis via the complement system. The complement system can be activated via three distinct cascades – classical, lectin, and alternative – each leading to a common terminal pathway[22]. It has been shown that ACPAs can activate both the classical and the alternative complement pathway in an antigen-dependent in vitro assay[23].

B-cell response in Rheumatoid Arthritis

The identification and isolation of citrullinated protein (CP)-directed, ACPA secreting B cells from RA patients allowed for the first time the detailed characterization of these cells[24]. These B cells were identified using labelled citrullinated antigen-(cyclic citrullinated peptide 2) tetramers. Flow cytometry experiments revealed that they mainly circulate in the peripheral blood as class-switched CD20 and CD27 positive memory B cells at a mean frequency of 1:10,000 B cells. In addition, long-lived ACPA secreting plasmablasts and plasma cells have been detected, producing immunoglobulins (Ig) of several classes (IgM, IgG and IgA)[25]. Interestingly, a recent study showed that CP-directed memory B cells in patients with recent-onset RA display an activated and proliferative phenotype as opposed to tetanus toxoid (TT)-directed B cells[26]. This activated phenotype, represented by an increased expression of CD80, CD86 and Ki67, did not correlate with disease activity, indicating that autoreactive B cells in RA are continuously active and fail to reach a state of quiescence irrespective of treatment. This is also consistent with findings that ACPA-positive patients rarely seroconvert and that ACPA levels remain elevated compared with RF levels[27], suggesting that ACPAs are produced by long-lived plasma cells residing in niches of the bone or (chronically) inflamed tissues. Furthermore, a high positivity for HLA-DR was observed pointing towards their ability to interact with T cells in RA. Next to the increased activation status, CP-directed B cells also down-regulate the inhibitory receptor CD32 and thus escape CD32-mediated negative regulation mechanisms. Furthermore, CP-directed B cells are enriched in synovial fluid and have been detected in synovial tissue mainly as plasmablasts or plasma cells[26,28]. CP-directed B cells isolated from the inflamed synovial compartment secreted functionally active proinflammatory cytokines, particularly IL-8, potentially able to attract neutrophils. This phenotype was less prominent in individuals with arthralgia, indicating that an activated phenotype of CP-directed B cells might be predictive for disease development[26]. Remarkably, CP-directed B cells have introduced a high amount of somatic mutations into their B-cell receptors (BCRs), implying once again continuous T-cell help. However, despite the high amount of mutations, the ACPA response undergoes limited avidity maturation[29] pointing towards a different selection mechanism of the autoreactive B cells as compared to "conventional" B cells and B-cell responses against e.g. recall antigens.

Genetic factors contributing to Rheumatoid Arthritis

A consistent and reproducible genetic contribution to RA was reported for variants within the human leukocyte antigen (HLA) region on chromosome 6 (6p21.3). More specifically, it has been shown that RA associates with alleles of the class II HLA-DRB1-gene, that encode a conserved amino acid sequence in the third hypervariable region (HVR3). This region, shared by most HLA-DRB1-genes associated with RA, is referred to as the shared epitope (SE)[30], and hence these alleles are called HLA-SE alleles. Differences in the predominantly RA-associated alleles have been found for different ethnicities: the *0401 and *0404 alleles are associated with RA in Caucasians, *0405 in Asian populations and *0101 in Israeli Jews. The SE hypothesis suggests that these class II molecules are directly involved in the pathogenesis of RA, although the exact mechanism is still unknown. As the SE alleles are located in the antigen-binding groove of the HLA molecule, it is compelling to assume that they influence the binding and presentation of specific peptide epitopes, including arthritogenic epitopes[31]. Interestingly, the HLA-SE alleles do not predispose to RA as such, but to ACPA-positive RA, but not to RF-positive RA[32]. This association is mainly present for individuals with ACPA-positive disease[32,33] and mostly lost in ACPA-positive healthy individuals[34]. These findings are of importance and indicate that T helper cells are involved in the transition from an ACPA-positive healthy to disease state (Figure 1).

N-linked protein glycosylation

A key characteristic, found on more than 90% of the RA-specific autoantibodies, ACPA IgG, is the abundant presence of N-linked glycans in their antigen-binding domains[35,36]. Proteins can be co- or post-translationally modified by the attachment of monosaccharides (also known as sugars, glycans or carbohydrates). The most common type of glycosylation, is the N-linked glycosylation where glycans are attached to a nitrogen atom of an asparagine residue (N). N-glycans are attached to an asparagine in the presence of N-glycosylation consensus sequences, asparagine-X-serine/threonine (N-X-S/T), where "X" can be any amino acid except for proline[37]. To a lower degree, N-glycans are also attached to asparagine-X-cysteine (N-X-C) motives[38,39]. More recently, N-linked glycans were also observed on reversed N-linked consensus sequences (S/T-X-N)[40] or non-consensus sequences (A-N-S-G)[41]. The most common N-glycan building blocks are monosaccharides, cyclic structures consisting of five carbons and one oxygen atom within the ring structure. Two monosaccharides can be enzymatically linked by condensation between the hemiacetal group of one monosaccharide and one of the hydroxyl groups of the other monosaccharide. As one monosaccharide contains several hydroxyl groups, multiple glycosidic bonds can be formed which results in branched glycan structures.

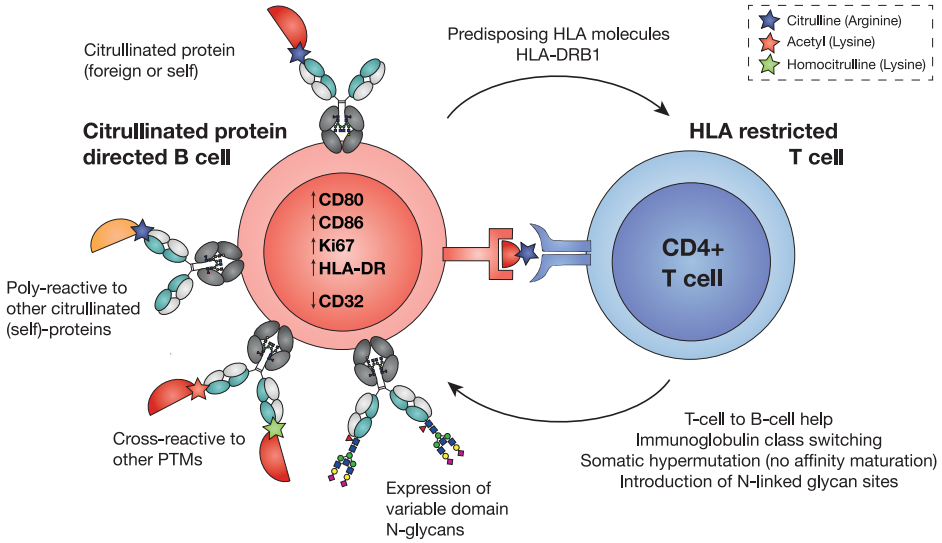

Figure 1. Schematic depiction of citrullinated protein (CP)-directed B cells. CP-directed IgG B-cell receptors (BCRs) interact with citrullinated proteins (foreign or self). CD4+ T cells recognize the citrullinated proteins presented by predisposing HLA molecules. T cells provide help to B cells followed by somatic hypermutation, which results in broadly poly- and cross-reactive BCRs and the introduction of N-linked variable domain glycans. The continuously activated CP-directed B cells depict an activated phenotype (increased expression of CD80, CD86 and Ki67, decreased expression of CD32).

Glycans are attached to proteins co- or post-translationally in the endoplasmatic reticulum (ER) (Figure 2). In a first step, a precursor N-glycan consisting of two N-acetylglucosamines (GlcNAc), nine mannoses (Man) and three glucoses (Glc) is transferred from a dolichol anchor to the asparagine in the consensus sequence. The terminal Glc are then enzymatically removed. This functions as a folding-quality control mechanism and only after correct folding, the protein enters the Golgi. In the Golgi the Man residues are trimmed down to a structure containing two GlcNAcs and five Man. Later on, glycans can be extended with a GlcNAc resulting in a hybrid-type glycan. An ongoing interplay between glycosidases and glycosyltransferases shapes the formation of complex-type N-glycans and contributes to a plethora of N-glycan forms. They can be extended by GlcNAc branches (antennae), a bisecting GlcNAc (β1,4), core and/or antennary fucoses (Fuc), antennary galactoses (Gal), N-aceylgalactosamines (GalNAc) and N-acetylneuraminic acids (Neu5Ac, α2,6 in humans)[42]. Neu5Ac are large monosaccharides belonging to the group of sialic acids (Sia) and contain a carboxylic acid functional group[43]. The final N-glycan composition is most likely influenced by the expression levels and localization of glycosyltransferases, glycan transporters, and glycosidases, the accessibility of the specific N-linked glycosylation sites and the immunological and metabolic state of the cell, which might influence the duration of the glycoproteins in the ER and the Golgi apparatus. Each individual glycan modification can have an substantial impact on the structure and function of the protein or its interactions with surrounding molecules[44].

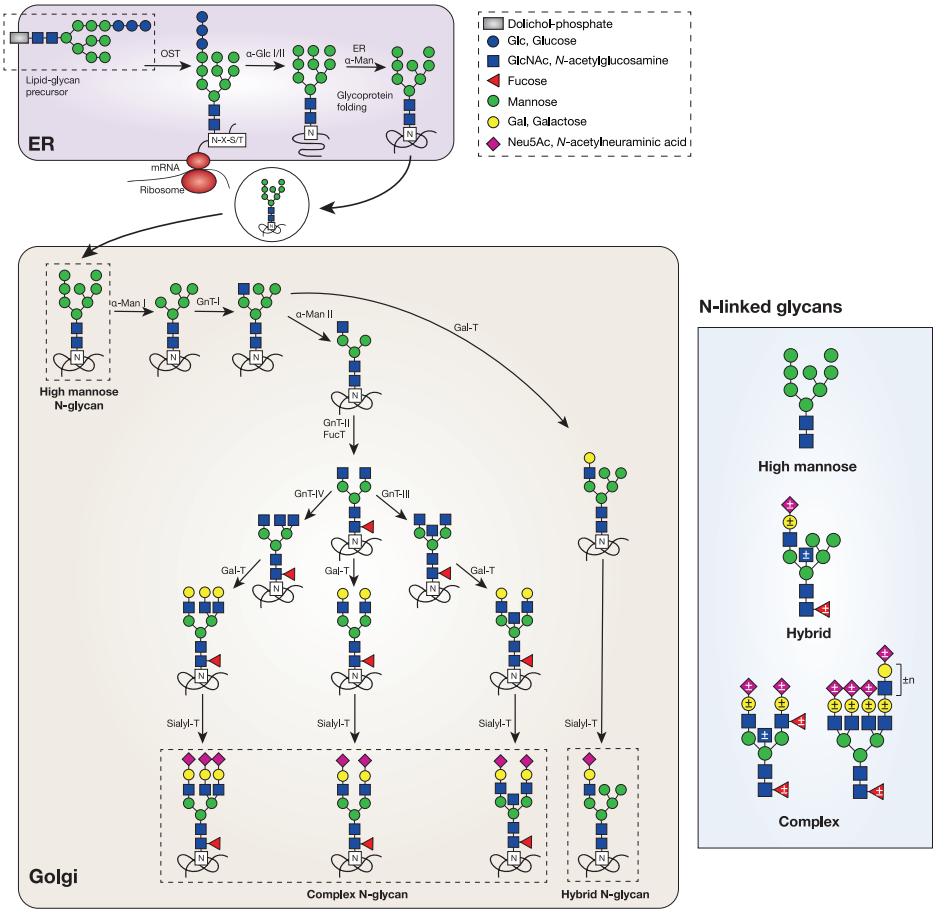

Figure 2. Biosynthesis of N-linked glycans. The biosynthesis of N-linked glycans is initiated in the endoplasmic reticulum (ER) by the transfer of a lipid/glycan precursor (dolichol phosphate bound glycan with Gcl_3 Man_9 $GlcNAc_2$) to the nitrogen atom of an asparagine residue by the oligosaccharyltransferase (OST) enzyme. The glucose residues are trimmed down by two α-glucosidases (α-Glc I-II) and an initial mannose residue is removed by the α-mannosidase (ER α-Man). The correctly folded glycoprotein is transported via vesicles to the Golgi apparatus where the N-glycans are further trimmed by α-mannosidase I and II (α-Man I-II) and modified by glycosidases and transferases [GlcNAc-transferase I-IV (GnT-I-IV), Fucosyltransferase (FucT), β1,4 galactosyltransferase (Gal-T), α2,6 sialyltransferase (Sialyl-T)]. This results in the three most common types of N-glycan structures attached to human proteins: high mannose-, hybrid- and complex-type glycans (carrying optional core and antennary monosaccharide residues).

Glycosylation of immunoglobulin G

IgG is a highly abundant glycoprotein in human serum consisting of two heavy (H) and two light (L) chains both including constant (C_H1, C_H2, C_H3 and C_L) and variable regions (V_H and V_L) (Figure 3). The fragment antigen-binding (Fab) moiety is formed by the C_L, C_H1, V_L and V_H and the fragment crystallizable (Fc) region consists of the C_H2 and C_H3 domains, which are linked by a flexible hinge region. Almost 100% of human IgG in the circulation contain a N-glycosylation consensus sequence (N297) in the C_H2 domain of the Fc region (Figure 3)[45]. Mainly complex-type and diantennary N-glycans are attached to the IgG Fc domain carrying a high amount of core fucosylation (~94%). IgG Fc N-glycans can carry a bisecting GlcNAc (~10%), present antennary galactoses (~67%) and terminal *N*-acetylneuraminic acids (~10%)[46,47]. The conserved Fc glycans have been studied extensively and the glycosylation profile can vary with disease (e.g. Rheumatoid Arthritis), gender and age, particularly with respect to the level of galactosylation[48-50]. Fc glycans are essential for the structure and function of the Fc domain and already minor changes can alter the Fc conformation, change the interaction with Fc-gamma receptors (FcγRs) or the complement component C1q and thus modulate IgG effector functions[51].

Additionally to the aforementioned conserved C_H2 N-glycans, ~15-25% of IgG variable regions can carry N-glycosylation consensus sequences (Figure 3)[47,52]. In comparison to Fc glycans, variable domain glycans (VDGs) depict higher levels of galactosylation (~94%), sialylation (~72%) and bisection (~45%), while fucosylation is reduced (~69%)[47,53]. In addition low levels of high-mannose type structures have been described[47,53], depending on the consensus amino-acid motif or the location of the N-glycosylation sites[54]. N-glycosylation sequences are mainly introduced into the variable domains following somatic hypermutation (SHM) during antigen-specific immune responses[55] and sequencing analyses of (auto)antibody repertoires revealed that this is likely a selective process. The naïve human B-cell antibody repertoire is almost devoid of such sites, with an exception of the IGHV1-8, IGHV4-34, IGHV5-10-1, IGLV3-12 and IGLV5-37 alleles[52]. The introduced VDG sites primarily emerge near antigen-binding regions in both H and L chains[56]. IgG subclass analyses disclosed distinct VDG levels for different isotypes. Thus, lower levels were observed for IgG1 (12%), IgG2 (11%) and IgG3 (15%) as compared to IgG4 (44%)[56], which might be partially explained by their ability to Fab-arm exchange or by their slightly higher mutation rate as compared to other subclasses.

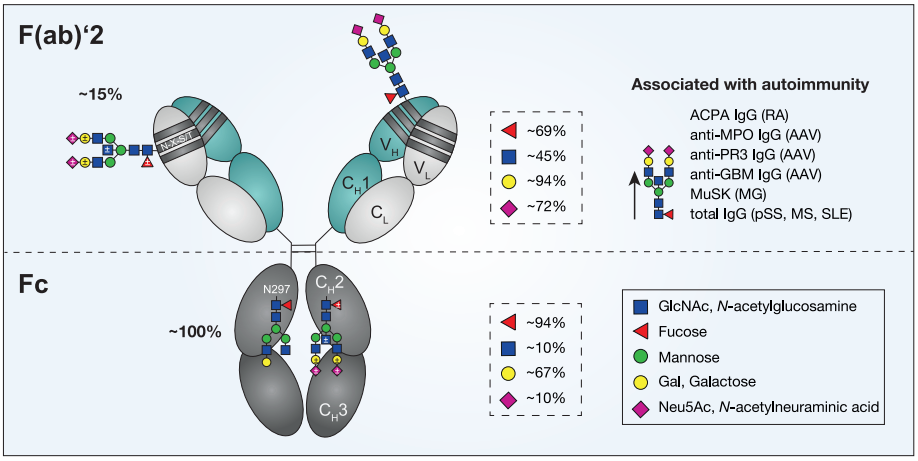

Figure 3. N-linked glycosylation of IgG. Schematic representation of an IgG molecule consisting of two heavy (H) and two light (L) chains both including constant (C_H1, C_H2, C_H3 and C_L) and variable domains (V_H and V_L). The fragment antigen-binding (Fab)'2 moiety is formed by the C_L, C_H1, V_L and V_H of both antibody chains linked by a flexible hinge region. The fragment crystallizable (Fc) region consists of the C_H2 and C_H3 domains. The Fc domain is ~100% N-glycosylated at N297 and the Fab domain is ~15% N-glycosylated at N-X-S/T consensus motifs in the hypervariable regions (variable domain glycans, VDGs). The percentage of fucosylation, bisection, galactosylation and sialylation of the Fc and Fab N-glycan is depicted. Variable domain glycosylation is increased on ACPA IgG from RA patients, anti-MPO/PR3 and GBM IgG from AAV patients and on total IgG from pSS, MS and SLE patients.

Variable domain glycosylation in disease

Notably, enhanced IgG variable domain glycosylation seems to be a common feature among autoimmune diseases. A hyperglycosylation of the variable domain has been shown for the RA-specific ACPA IgG, as evidenced by an increased molecular weight of the variable fragments and mass spectrometry[35]. Sambucus nigra agglutinin (SNA)-binding studies revealed an increased binding, and thus most probably the presence of α2,6-linked sialic acid containing VDGs, for anti-neutrophil cytoplasmic antibodies (ANCA) directed against myeloperoxidase (MPO) or proteinase 3 (PR3) and for anti-glomerular basement membrane (GBM) autoantibodies from ANCA-associated vasculitis (AAV)-patients as opposed to total IgG[57]. These results have been confirmed by mass spectrometry studies of anti-MPO enriched IgG depicting disialylated and bisected glycan traits specific for the Fab domain of antibodies[58]. Additionally, primary sjorgen's syndrome (pSS), multiple sclerosis (MS) and systemic lupus erythematosus (SLE) patients show a higher degree of IgG sequences with N-linked glycan sites compared to healthy donors[59,60]. Recently, N-linked glycosylation motifs were also found to be present in the variable domains of monoclonal muscle-specific kinase (MuSK) autoantibodies suggesting a potential role for the autoimmune disease myasthenia gravis (MG)[61,62]. Interestingly, also inflammation-associated

antibodies, such as anti-hinge antibodies (AHAs), revealed an increased variable domain glycosylation of 53% as determined by SNA-chromatography[56]. Also anti-drug antibodies that emerge in patients treated with adalimumab or infliximab depict increased VDG levels suggesting that the introduction of N-glycans into the immunoglobulin variable domain is triggered by a chronic and systemic antigen exposure[56]. Intriguingly, an abundant amount of N-linked glycosylation sites in the variable domain of immunoglobulins has not only been observed for several autoimmune diseases, but also for patients with follicular lymphoma, especially with diffuse large B-cell lymphoma and Burkitt's lymphoma, suggesting a key role in lymphomagenesis[63].

Despite all these observations, little is known about the functional role of glycans attached to the hypervariable region of (surface) immunoglobulins, most likely due to their high heterogeneity in terms of distribution, frequency and glycan composition. Also, it remains unclear why and how chronic antigen exposure facilitates the accumulation of N-glycosylation sites in BCR V-regions.

Variable domain glycosylation of ACPA IgG

ACPA IgG depict a characteristic high expression of VDGs. N-glycans were found in the variable domain on more than 90% of ACPA IgG[35,36] and autoantibodies isolated from the site of inflammation, the synovial fluid, presented even over 100% of VDGs, implying that multiple glycans are attached to the variable region of one ACPA molecule[36]. The underlying N-linked glycosylation sites are selectively and abundantly introduced into the ACPA IgG hypervariable regions following somatic hypermutation (SHM) and no sites were found to be present in the hypervariable regions of ACPA IgM or germline encoded sequences[64,65]. Structural analysis revealed that ACPA IgG VDGs carry a high amount of bisection, galactosylation and terminal sialic acids[36]. Intriguingly, recent studies show that the abundant presence of VDGs on ACPA IgG in healthy first nation Canadian individuals is a strong predictor for progression towards RA[66]. As VDGs are selectively introduced, present in the vast majority of ACPA IgG molecules and predictive for disease development, it is tempting to speculate that their expression provides a selective advantage for ACPA-expressing B cells allowing them to persist for several years. The glycosylation of the hypervariable regions might provide an additional pathway for the autoreactive B cells to regulate tolerance. However, further investigations are needed to unveil the first occurrence of these glycans and to understand the biological impact of VDGs for ACPA-expressing B cells.

Influence of variable domain glycosylation on IgG structure and function

Although glycosylation is a well-known post-translational modification able to modulate the structure and function of proteins and thus to affect humoral responses, little is known about the functional impact of sugar moieties attached to the variable domains of (surface) immunoglobulins. Interestingly, more and more studies suggest that VDGs might be involved in the pathophysiology of certain autoimmune diseases and can have immunomodulatory effects. As mentioned above, N-glycosylation consensus sequences in the variable domain occur mainly near antigen-binding regions and VDGs thus potentially influence the affinity/ avidity for antigens. Indeed, previous studies have shown differential impacts of VDGs on antigen binding. Dependent on the amount and the location of the N-linked glycosylation sites[56], VDGs can increase[67-69] or decrease[69,70] binding to their cognate antigens and thus modulate the specificity of an antibody. For example, it has been hypothesized that VDGs allow BCRs to move away from auto-reactivity, while cross-reactivity to foreign-antigens can be retained[71]. Furthermore, VDGs, especially their composition, can have a modulatory impact on the half-life of immunoglobulins[69,72]. In the absence of terminal sialic acid residues, asialoglycoprotein receptors presented on liver cells can interact with terminal galactoses or *N*-acetylgalactosamine residues, expressed on the variable domain of the immunoglobulins, leading to a shortened half-life[73]. In addition high-mannose structures, that can be attached to N-glycan sites in the variable domain, are more easily cleared by mannose receptors[74]. Further VDGs, without the need of terminal sialic acids, can improve antibody thermostability, possibly by shielding hydrophobic residues[75]. Depending on their location and charge, VDGs could also potentially alter binding to Fc gamma receptors (FcγR), including the neonatal FcR (FcRn), thereby affecting the serum half-life of IgG. In addition to an effect on FcγRs, VDGs may also affect the propensity of immunoglobulins to form immune complexes/ aggregates[76], potentially affecting complement activation, another IgG effector mechanism. A further important functionality reported for VDGs, and glycans in general, is the interaction with glycan-binding proteins termed lectins. It has been suggested that a variable domain glycosylation of BCRs allows interactions with lectins in vicinity to the BCR (in *cis*) or on neighboring cells (in *trans*), which will provide the B cell with survival signals[56,71,77-79]. This has for example been proposed for follicular lymphoma cells, where the interaction between mannosylated VDGs and lectins free these cells from the dependence on antigen and enhance their survival through selection based on glycan interactions[63,80,81].

Nonetheless, despite these possible roles assigned to VDGs on BCRs, the function or consequences of VDGs on ACPA and/or CP-directed BCRs is unknown. Given the abundant presence of these glycans on ACPA, additional insight into the role of VDGs on the biology of the ACPA response will likely yield relevant information on the emergence of this highly specific autoantibody-response hallmarking RA.

Scope of this thesis

The aim of the studies described in this thesis is to better understand the concurrent presence of the different anti-modified protein antibody responses in RA and to obtain functional insights into the role of VDGs on the biology of autoreactive B cells and the autoantibodies they produce. More specifically, the studies not only aim to illustrate the cross-reactive nature of autoreactive B cells in RA or the occurrence of VDGs across different disease stages, they also intend to gain understanding how both characteristics are potentially involved in the breach of B-cell tolerance.

In **Chapter 2** several mechanism on how human autoreactive B cells might overcome peripheral B-cell "tolerance" checkpoints are reviewed. We highlight the autoimmune disease RA and elaborate on how RA-specific autoreactive B cells, expressing ACPAs, might bypass these B-cell checkpoints. We specifically focus on the cross-reactivity of these B cells and on the abundant expression of VDGs on their (surface) immunoglobulins.

The high cross-reactive nature of RA-specific antibodies is illustrated in **Chapter 3**. This chapter describes the cross-reactivity of ACPAs on the monoclonal level and on the level of CP-directed B-cell responses. The reactivity of monoclonal ACPAs to antigens carrying distinct PTMs, such as citrullination, homcitrullination (carbamylation) or acetylation, is assessed and the joined term of anti-modified protein antibodies (AMPAs) introduced. Furthermore, the chapter illustrates the activation potential of CP-directed B cells after the exposure to different PTMs, which is highly relevant to further the understanding of the "evolution" of the autoimmune responses in RA.

The possible role of VDGs in the development of RA is reiterated in **Chapter 4**. This chapter describes the emergence of VDGs on ACPA IgG in the phase before the onset of clinical symptoms. Associations between ACPA carrying VDGs and the most prominent genetic risk factor predisposing to RA, the HLA-SE alleles, are assessed. **Chapter 5** presents additional in depth studies on the association between the HLA-SE risk alleles and ACPA IgG harboring VDGs in the phase prior to the onset of RA in three distinct cohorts. It further highlights that the HLA-SE alleles primarily associate with the highly sialylated VDGs on ACPA IgG and not with ACPA as such.

The emergence and abundance of ACPA IgG VDGs across several clinical stages of RA is illustrated in **Chapter 6**. Dynamic changes of VDGs across different disease stages are analyzed in a large data set of 1500 samples. ACPA IgG glycan profiles of 7 different cohorts including ACPA-positive healthy individuals, pre-symptomatic individuals, individuals with arthralgia, patients at the onset of RA and RA patients 4,8 and 12 months after disease-onset and treatment are assessed. Additionally, ACPA IgG VDG profiles of individuals in whom long-term drug-free remission is achieved are illustrated.

After analyzing the emergence of VDGs on ACPA IgG in the studies presented in the aforementioned chapters, **Chapter 7** focuses on the biological consequences of VDGs for autoreactive B cells and their secreted antibodies. In particular, this chapter deals with the impact of ACPA IgG VDGs on citrullinated (auto)antigen binding assessed amongst others by crystallography and antigen-binding studies. Furthermore, we studied the impact of highly sialylated VDGs on B-cell activation and BCR downmodulation to dissect the key characteristics of VDGs on human autoreactive B cells.

Chapter 8 focuses on the biological impact of VDGs on an important effector function of IgG, the activation of the complement system. Monoclonal antibodies expressing identical Fc-glycan profiles, but various amounts of N-linked glycans in their variable domains, were used to identify VDG-specific effects on complement activation.

Finally, the work described in this thesis comes together in **Chapter 9**, the general discussion, with a specific focus on the involvement of VDGs in the breach of tolerance of autoreactive B cells in RA.

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