Bitter Sweet Symphony: the impact of sugars on autoimmunity

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General discussion and perspectives

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Loss of humoral tolerance is a hallmark of many autoimmune diseases and leads to the appearance of self-reactive B cells and the autoantibodies they secrete. This also applies to the prototypical human autoimmune disease Rheumatoid Arthritis (RA), which is characterized by inflammation of the synovial tissue of the joints. The pathways of autoreactive B cells to overrun tolerance checkpoints and thereby drive the development of RA are largely unknown. Nevertheless detailed analyses of the RA-specific autoantibody responses in the past decades, have provided important insights into how autoreactive responses could arise in RA and how checkpoints could be violated. RA is a heterogeneous autoimmune disease that emerges most likely through the accumulation of genetic, epigenetic and environmental factors, such as the microbiome. Immunologically, the autoimmune disease can be divided into at least two different subgroups, characterized by the presence or the absence of the disease-specific autoantibodies, anti-citrullinated protein antibodies (ACPAs). The fact that ACPA-positivity associates with more severe clinical outcomes[1] and that these autoantibodies appear already many years before the onset of the disease[2], suggests an early break in B-cell tolerance and highlights the importance of autoantibodies and the underlying B-cell response for the development and maintenance of RA. Additionally, the most important genetic risk factor for RA, the HLA-shared epitope (SE) alleles predispose only to the development of ACPA-positive RA[3]. Thus, it is likely that autoreactive citrullinated protein (CP)-directed B cells in RA can recruit HLA-SE restricted T-cell help necessary for the induction and the maturation of the ACPA-specific B-cell response. Several studies indicate that ACPA responses differ from conventional antibody responses as they are characterized, despite an extensive amount of somatic mutations, by a relatively low avidity for antigen[4] combined with a high promiscuity, a reactivity to several antigenic-backbones and post-translational modifications (PTMs)[5-14]. Moreover, unlike most antigen-specific antibody responses, most ACPA in RA harbor N-linked glycans in their hypervariable regions[15].

The presented work in this thesis deals with these specific features of autoreactive B cells and their secreted autoantibodies in RA. The aspect of cross-reactivity is described on the monoclonal antibody level and on the level of autoreactive B-cell responses (**Chapter 3**). Furthermore, the emergence of variable domain glycans (VDGs) (**Chapter 4**, **Chapter 5** and **Chapter 6**) and their functional consequences for CP-directed B cells and their secreted autoantibodies (**Chapter 7** and **Chapter 8**) are evaluated. These aspects will be discussed in the following sections along with the latest findings and a focus on how these characteristics might help ACPA-expressing B cells to overrun peripheral B-cell checkpoints, which are highlighted in **Chapter 2**.

The promiscuity of anti-modified protein antibodies in RA

The molecular nature of the antigens recognized by most of the disease-specific antibodies, anti-citrullinated protein antibodies (ACPAs), in RA, was identified in 1998 and has since been used as a biomarker to aid in RA diagnosis. ACPAs are already detectable years before the onset of inflammation and persist in patients with established RA and in individuals with drug-free sustained remission. Hence it has been hypothesized that the "quality" of the ACPA response rather than their first emergence determines its involvement in the inflammatory responses observed in RA. This includes their fine-specificity (epitope recognition)[16], the degree and type of glycosylation in the Fc and the variable domain[15,17], their isotype usage[18-20], their avidity[4] and the potential of ACPA to activate the complement cascade[21]. An important characteristic of ACPAs in "full-blown" RA is for example their extensive poly-reactivity to several citrullinated epitopes. Next to that, ACPAs can cross-react to multiple post-translationally modified epitopes, including carbamylated-lysine and acetylated-lysine residues. Initial studies suggested that ACPAs, anti-carbamylated protein antibodies (ACarPAs) and anti-acetylated protein antibodies (AAPAs) represent three independent autoantibody systems[22], due to the positional and structural differences of the targeted modified epitopes. However, there is more and more evidence indicating that a large proportion of these antibodies are cross-reactive and relate to a single antibody family, defined in **Chapter 3** as anti-modified protein antibodies (AMPAs)[13]. Particularly, characterizations of fourteen RA-derived monoclonal ACPAs confirmed their cross-reactivity and highlighted their promiscuity to modified amino acid residues that share similar physicochemical and structural properties with citrulline (**Chapter 3**). However, it should also be noted that not all ACPAs exhibit the same degree of cross-reactivity towards all modifications (**Chapter 3**). This has not only been observed at the monoclonal antibody level, but autoantibody-positive patients have also been described to exhibit reactivity to either one, two or all three modifications[23,24].

The first crystal structures of monoclonal ACPA Fab domains provided structural background that could explain the extensive cross-reactivity of ACPAs[25]. The superposition of crystal structures from three ACPA Fabs co-crystallized with diverse citrullinated antigens, revealed that next to their individual structural features and differential electrostatic potentials, all Fab domains share two deep polar and hydrophobic binding pockets able to accommodate a citrullinated residue (**Chapter 7**). Importantly, the primary interactions were observed between the terminal nitrogen and oxygen atoms of the citrulline side chain, and secondary interactions only with the peptide backbone of the flanking amino acids. Hence, except for the citrulline modification, no "specific" interactions to determined flanking amino acids are needed for ACPAs to interact with their cognate antigens. The antibodies can promiscuously accommodate different citrullinated peptide variants as long as the modified residue is in a favorable and accessible position (Figure 1A). The accessibility of the modification also depends on the nature

of the secondary contacts formed with the surrounding peptide scaffold. Thus, antigens could be excluded from the binding repertoire due to steric repulsion with certain spatially demanding amino acids proximal to the citrulline (Figure 1A). This might explain why glycine residues, with only minor spatial requirements are potentially preferred amino acids in the surrounding of the modification[11,25,26]. The importance of secondary contacts is likely to be different for different ACPAs accommodating different antigens. It has been postulated that certain ACPAs that require only interactions with the modification are more "promiscuous", whereas others that bind depending on the specific flanking regions are more "private specific"[27]. The rather "shallow" binding mode of RA-specific autoantibodies might also explain the low avidity/affinity of ACPAs towards many of their antigens, despite the fact that ACPA-expressing B cells undergo extensive somatic hypermutation (SHM), a process that is usually accompanied by affinity maturation[11,28,29]. It further explains the broad cross-reactivity of ACPAs to other post-translational modifications (PTMs) harboring terminal nitrogen and oxygen atoms and comparable structural requirements to citrulline, such as acetylated- and carbamylated-lysine residues (Figure 1, A and B). All three modified residues exhibit similar physicochemical properties, such as size, shape, charge and hydrophobicity.

Thus, existing theories regarding the main role of citrullination as the driver of the autoimmune response connected to RA might be incorrect as possibly other post-translational modifications (PTMs), such as carbamylation or acetylation, presented by for example microbe derived antigens, might be involved in the initial breach of tolerance. This hypothesis is reinforced by our findings showing that autoreactive B cells expressing B-cell receptors (BCRs) directed against one particular PTM can also be activated by antigens expressing different modifications (**Chapter 3**). Additionally, recent studies revealed already a high cross-reactive nature and thus promiscuity of ACPA IgM, despite limited mutational load. Intriguingly, cross-reactivity even remained in two antibody variants which were mutated back to germline further emphasizing that multiple antigens could be involved in the initial B-cell activation and that the breach of tolerance towards several PTMs occurs already before extensive SHM and class switching[30]. It is tempting to speculate that a particular PTM initiates the AMPA response and that further exposure to other PTMs determines its direction towards a more ACPA-, ACarPA- or AAPA-like response. Furthermore, it can be hypothesized that this response is highly dynamic and can be "re-routed" up upon a new encounter with a different modification (Figure 1C). This dynamic behavior of the AMPA response associated with the plasticity of the paratope-epitope interactions is highlighted in a recent study showing that the AMPA recognition profile is skewed towards the most recently encountered PTM, suggesting that the PTM responsible for the initial induction of the response and the PTM predominant in established RA are not necessarily identical[31]. Indeed, an ongoing antigen triggering, with potentially different modifications, is also in line with the activated proliferative phenotype of ACPA-expressing B cells in RA[32].

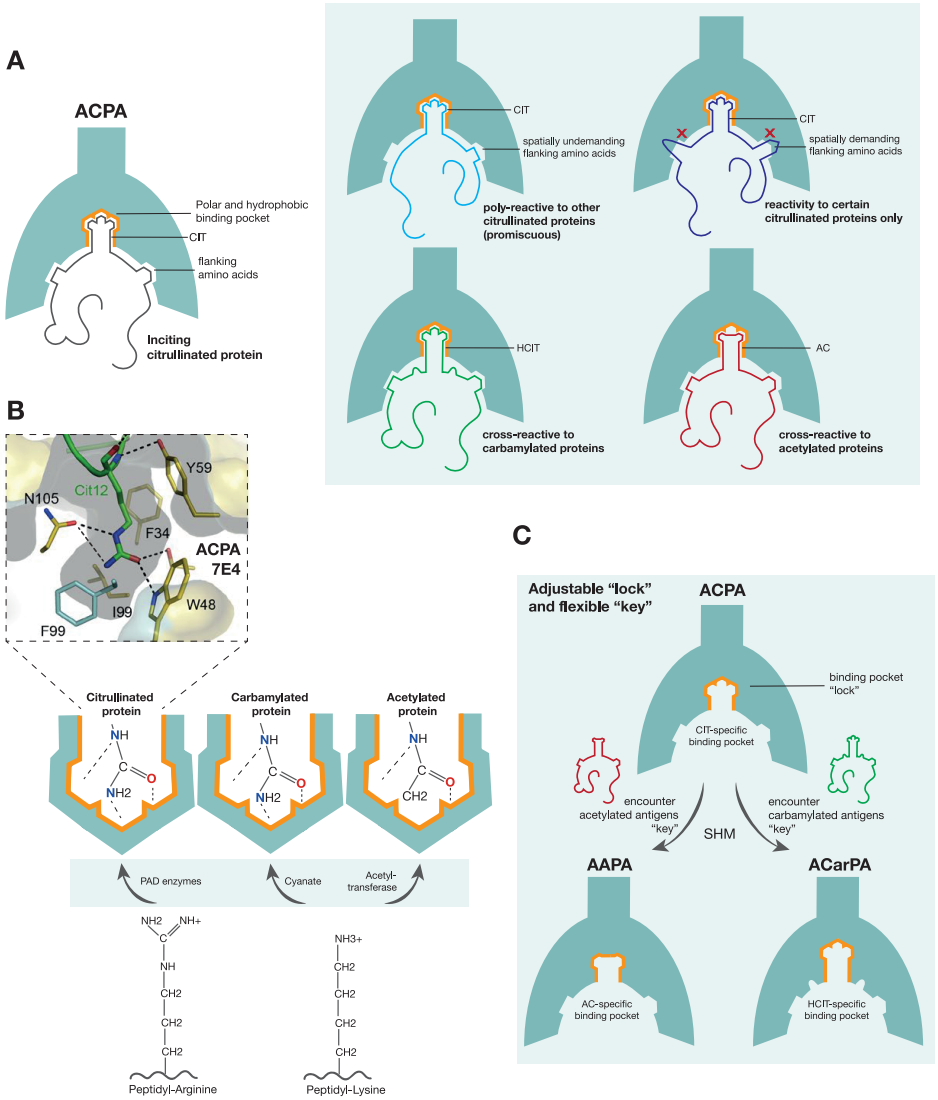

Figure 1. The binding mode of anti-citrullinated protein antibodies (ACPAs). (**A**) Schematic representation of the polar and hydrophobic ACPA binding pocket accommodating a citrulline (CIT) side chain. Illustration of the promiscuous binding mode to CIT proteins with flanking amino acids of minor spatial requirements and specificity for only certain CIT proteins due to steric repulsion with demanding flanking amino acids. Cross-reactivity to carbamylated (homocitrullinated, HCIT) and acetylated (AC) proteins with similar structural requirements. (**B**) Depiction of the formation of citrullination, carbamylation and acetylation. Illustration of the main hydrogen-bond interactions formed between the antibody binding pocket and the terminal nitrogen and oxygen atoms of the respective modification. Crystal structure of the CIT binding pocket of the monoclonal ACPA 7E4 is depicted. (**C**) Plasticity of the ACPA binding pocket (adjustable "lock") upon encounter of a differently modified antigen (flexible "key") and somatic hypermutation (SHM).

Nevertheless, it remains unknown which modification, or which modified antigen incites the autoantibody specific B-cell response in RA. Although previous studies have identified ACPAs as the dominant autoantibody response in patients with established RA, it still needs to be further investigated whether ACPAs are already prevalent years before the onset of clinical symptoms or if the pre-RA response is characterized by the presence of AAPAs and/or ACarPAs. It can also be speculated that the AMPA response is initiated by acetylated or carbamylated foreign-antigens presented at mucosal surfaces[33] or by acetylated antigens generated upon cell death and NETosis[12]. That carbamylation might play an important role for the initiation of the AMPA response, is also in line with the findings that the extracellular matrix protein collagen, which has a long half-life and represents one of the potential autoantigens in RA, can be easily carbamylated[34]. This response may then be the bridge to the production of ACPAs in RA by a skewing of the immune response to citrullinated proteins presented at the side of inflammation[35,36]. However it is also conceivable that carbamylation and acetylation represent epitopes that have no relevance for disease pathogenesis.

Overall, it can be concluded that the generation of cross-reactive antibodies directed against a broad range of potentially unrelated self or non-self-proteins carrying structural epitopes that mimic the primary immunogen, is adding another layer of complexity to the already heterogeneous autoimmune disease[37]. Further studies, focusing on the cross-reactive nature of AMPAs in the phase prior to the onset of arthritis combined with more comprehensive analyses of AMPA epitope structures, are evidently important to understand the initiation, the evolution and variety of the autoreactive responses observed in RA. Furthermore, more structural insights will allow us to improve the diagnostic assays used in the clinic to detect autoantibodies and therefore to improve disease diagnosis.

The emergence of variable domain glycans during the ACPA B-cell development

N-linked glycosylation sites, a pre-requisite for the introduction of N-glycans, were found to be present in almost 90% of ACPA IgG variable regions, which by far exceeds the frequency of such sites in the healthy repertoire[29]. However, it has been hardly described when, during the autoreactive B-cell development, N-glycans are introduced into the ACPA IgG variable domains. Based on previous studies it can be assumed that N-linked glycan sites are introduced into the ACPA variable domains following class-switch recombination (CSR) and SHM, as no sites were found to be preset on ACPA IgM[38] or in the germline-encoded ACPA IgG V-gene repertoire[29,39]. SHM occurs in germinal centers (GC) following T-cell help provided to B cells. It can therefore be assumed that N-glycans are accumulated in the ACPA IgG variable domains during the repeated passage of ACPA-expressing B cells through GCs and are thus part of the maturation process of

the ACPA B-cell response. However, GC responses, CSR and SHM are common processes that occur in all humoral immune responses and do not normally lead to a frequent accumulation of N-glycans in the variable domain of the BCR. It can therefore be assumed that "specific" T cell contacts are required and that the introduction of glycans in the variable domain is not just the result of the accumulation of multiple somatic mutations. Indeed, the frequency of N-glycosylation sites does not correlate with the mutational load of ACPA IgG BCRs, suggesting that the sequons are introduced during a selective process[29].

Furthermore this hypothesis was confirmed by our findings, presented in **Chapter 4** and **Chapter 5**, showing that the most prominent genetic risk factor predisposing to ACPA-positive RA, the HLA-SE alleles, associates with ACPA IgG presenting N-glycans in their variable domains. More specifically, the data depict an association between ACPA IgG carrying VDGs and the HLA-SE alleles in the pre-disease phase as shown for healthy individuals from Japan, pre-symptomatic individuals from Sweden and individuals with arthralgia from the Amsterdam area of The Netherlands. This finding is intriguing and indicates that SE-restricted T-cell help might drive the introduction of N-linked VDGs. The fact that two different T helper cell responses are at play during the ACPA B-cell development is also in line with the finding that no association between ACPA and HLA-SE was observed in healthy individuals, despite class switching to IgG, but in individuals with ACPA-positive disease that also abundantly incorporated VDGs into their ACPA IgG. Thus, N-glycosylation sites are potentially accumulated during SHM following help of T cells restricted to the predisposing HLA molecules. In line with this assumption we observed an increased association between the presence of VDGs and the HLA-SE alleles after correcting for ACPA-positivity. Thus, HLA-SE alleles predominantly associate with variable domain glycosylated ACPA IgG and not with ACPA IgG as such.

We observed not only an association between the HLA-SE alleles and the presence of VDGs, but also identified increased VDG levels in HLA-SE-positive individuals, highlighted in **Chapter 5,** and no association between HLA-SE and ACPA levels after correcting for the presence of VDGs. Additionally, our data depicted primarily an association between HLA-SE and the most prominent bisected and disialylated glycan trait (G2FBS2) found on the ACPA IgG variable domains and again this association remained after correcting for ACPA levels. Thus, these data have solved another piece of the jig-saw puzzle unraveling the development of RA as it can now be hypothesized that, in the phase preceding the onset of RA, T cells restricted to the HLA molecules predisposing to RA development provide help to ACPA-expressing B cells, which results in extensive SHM leading to the introduction of N-linked glycosylation sites and hence the abundant expression of VDGs. The HLA-SE risk effect together with the subsequent expression of VDGs on ACPA IgG can thus be seen as an accelerating factor and important "hit" for the development of the autoimmune disease RA.

This notion is in line with the findings presented in **Chapter 5** showing that individuals with an "incomplete" VDG profile (< 75%) were more prone to develop RA, if they were HLA-SE-positive. The presence of HLA-SE alleles will likely lead to an accelerated introduction of VDGs which will conceivably promote the transition to RA. That increased VDG levels in the asymptomatic phase are a strong predictor for the development of inflammatory arthritis has also been shown in a previous study within a healthy Canadian population[40]. Together with the observation that N-glycans are selectively introduced into the variable regions of ACPA BCRs and are not the results of a random accumulation of somatic mutations[29], and the finding that ACPA B cells do not undergo avidity maturation[4], these observations point towards a different selection mechanism of ACPA-expressing B cells, in which post-translational glycan modifications most likely play a crucial role.

However, our results also indicate that, in certain individuals, VDGs can already be abundantly present several years before the actual onset of RA (**Chapter 4** and **Chapter 5**) indicating that several "hits", over a period of multiple years, are likely involved in the transition to RA. To investigate the emergence and the momentum of VDGs on ACPA IgG in greater detail and to understand their contribution to the autoreactive B-cell response in RA, we cross-sectionally investigated their presence and abundance in 1498 samples from individuals in various disease stages (**Chapter 6**). The percentages of ACPA IgG VDGs were analyzed in the "pre"-RA phase in samples of healthy individuals from Japan and Canada, of pre-symptomatic individuals from Sweden and of individuals with arthralgia from the Amsterdam and the Leiden area of The Netherlands. Additionally, the abundance of ACPA IgG VDGs in the "post"-RA phase was determined including the influence of treatment and changes upon drug-free remission (DFR) or late disease flares. We therefore made use of a well-controlled treatment strategy trial and furthermore analyzed individuals whose RA achieved long-term drug free remission (DFR) or late flares and had been followed up to 16 years. The data show that VDGs were already abundantly (58%) expressed on ACPA IgG isolated from healthy individuals. Levels increased further towards the arthralgia phase (75%) and towards RA-onset (93%). Thus, in agreement with the results presented above, VDGs are already present on ACPA IgG of asymptomatic healthy individuals and their abundance rises further during the progression to arthralgia and ultimately clinically detectable arthritis/ early RA. In established RA, we observed a stable expression of the glycan modification with a slight decrease upon start of immunosuppressive therapy. Furthermore, especially in the "pre"-RA phase, we identified a correlation between the abundance of VDGs and the evolution of the ACPA immune response, as expressed by an increase in ACPA levels and epitope spreading, implying again the involvement of VDGs in the progression of disease. Notably, our data illustrated that patients whose RA achieved long-term DFR later in time had introduced significantly less glycans into their variable domains compared to patients with persistent or relapsing disease.

Thus the data, presented in **Chapter 4**, **Chapter 5** and **Chapter 6** highlight the association of VDGs with the transition from autoimmunity to autoimmune disease as well as with the persistence of the disease. Therefore, it is relevant to uncover and understand the biological impact of VDGs on autoreactive B cells and their secreted autoantibodies in general and on the RA-specific ACPA response in particular. Further it remains to be elucidated, whether VDGs are presented by all AMPA classes and to what extend the selective introduction of VDGs is influenced by the nature of the antigen.

Functional impact of VDGs on secreted autoantibodies

Current findings showing that ACPAs do not undergo strong avidity maturation, but selectively and abundantly introduce N-glycans into their variable domains, strongly indicate that VDGs are involved in the selection and regulation mechanisms involved in the expansion of ACPA-expressing B cells. This assumption is further reinforced by the predictive value of VDGs for disease development and their association with the most prominent genetic risk factor, the HLA-SE alleles. Intriguingly, not only ACPAs, but also other human (auto)antibody responses, including anti-MPO or anti-PR antibodies from ANCA-associated vasculitis patients[41], are characterized by a hyperglycosylation of the variable domain.

Despite the probably important involvement of VDGs in the mechanisms leading to the maturation of the autoimmune responses underlying human autoimmune diseases, we currently only have a glimpse on the functional role of these glycan modifications for autoreactive B cells and the antibodies they secrete. In the studies described in **Chapter 7**, we identified the impact of complex-type, bisected and disialylated ACPA IgG VDGs on (auto)antigen binding by analyzing the binding mode of six monoclonal antibodies with and without their naturally occurring VDGs. N-linked glycosylation sites presented in the variable domains are, contrarily to Fc N-glycans, not necessarily occupied with a glycan and constrained by the accessibility of the N-glycosylation sites[42]. Thus, it might be exceptional that almost all glycan sites expressed in the variable domain of the ACPA IgG mAbs were found to be completely occupied by a complex-type N-glycan. With an exception of the N-linked glycosylation site in the LC of two mAbs, which appeared to be only partially occupied. It is also exceptional that some monoclonal ACPA IgG presented up to a total of eight glycans in their variable domains.

Based on the crystallographic data of three ACPA Fab domains and dynamic modeling, we were able to show that VDGs attached to the FR3 or the CDR1 regions can interact with amino acids in or in close proximity to the antigen-binding pocket and thus potentially compete with the antigens for binding. The suspected negative impact on binding was confirmed for all six mAbs by ELISA-based antigen-binding studies. Particularly low-affine antigens were outcompeted by

the (potentially higher affine) VDGs, while the binding towards high-affine antigens was less affected. The impact of VDGs on antigen binding depends on the specific glycan structure and the location of the N-glycan in the variable domain. In this respect it is noteworthy that a differential impact of VDGs on antigen binding has been reported in literature[43,44]. Further site-specific analyses are necessary to draw more comprehensive conclusions on the impact of VDGs on antigen binding. Indeed, it is also conceivable that N-glycans interact with the antigens upon binding, holding them in the binding-pocket and thereby enhancing binding. This would be in line with the decreased apparent dissociation rates observed in our SPR experiments presented in **Chapter 7**. Next to the effect on antigen binding, VDGs can increase the thermostability of antibodies, possibly by shielding hydrophobic residues (Figure 2D). This positive impact on antibody stability is not mediated by the terminal sialic acids[45].

Next to the impact on stability, VDGs may affect the propensity of immunoglobulins to form immune complexes/ aggregates[46] and thus potentially impact on IgG effector functions. In the studies described in **Chapter 8** we investigated the influence of VDGs on hexamer formation of IgG molecules and the subsequent effect on complement activation. Monoclonal antibodies with identical Fc glycan patterns, but different amounts of VDGs depicted a clearly reduced classical complement pathway activation in the presence of N-linked VDGs. This difference is likely caused by a decreased ability of variable domain glycosylated IgG to form hexamers, due to steric repulsion, leading to a lowered binding to the classical pathway initiator C1q (Figure 2E). These results indicate that not only Fc glycans, but also glycans presented on the IgG variable domain can have immunomodulatory effects and impact on the effector functions of antibodies. Therefore, VDGs might be thought to affect not only complement but also binding to Fc gamma receptors (FcγR), including the neonatal FcR (FcRn), which is critical for the half-life of serum IgG.

Functional impact of VDGs on autoreactive B cells

In addition to the functional impact of VDGs on secreted antibodies, it is of importance to investigate the effects of VDGs for surface-bound immunoglobulins, BCRs. In this way, we can determine whether glycans can interfere with B-cell functions and thus could be a potential trigger for B-cell selection and disease development. Interestingly, an inhibitory effect on binding to low-affine antigens was also observed for the CP-directed BCRs harboring N-glycan modifications in their variable regions. Thus, it can be hypothesized that VDGs reduce the self-antigen recognition profile in the GC preventing negative selection and promoting B-cell survival only through a retained cross-reactivity to potentially higher affine foreign-epitopes (Figure 2A). This "redemption" hypothesis is in line with a study in mouse models showing that self-reactive antibodies mutate away from autoreactivity through the introduction of N-linked

variable domain glycans and thereby prevent elimination through clonal deletion or receptor editing[47]. Another important functionality reported for glycans on BCRs is the interaction with glycan-binding proteins, lectins. For follicular lymphoma B cells it has been proposed that high mannosylated VDGs presented on the BCR variable domain interact with mannose-binding lectins causing B-cell activation without the need for antigen[48-50]. These B cells are thus selected for the presence of VDGs, which is in line with the abundant expression of N-linked glycosylation sites in the variable regions of follicular lymphoma BCRs[51]. Concordantly, it has been suggested that the abundant expression of glycans in the hypervariable domains of CP-directed BCRs provide a selection/ survival advantage to ACPA-expressing B cells through the interaction with lectins in vicinity to the BCR (in *cis*) or on neighboring cells (in *trans*). In line with this hypothesis, we identified an increased activation potential of B cells carrying variable domain glycosylated BCRs (**Chapter 7**, Figure 2B). This observation was made by transducing human Ramos B cells with two CP-directed BCRs from RA patients. We consistently observed an increased and prolonged BCR signaling after antigenic-stimulation or BCR cross-linking. Consequently, VDGs seem to change the activation threshold of RA-specific B cells which could lead to the loss of control of the self-reactive response and thus may play an important role for the pathogenesis of the disease. This activation/ survival advantage will in turn lead to the selection of these B cells in the germinal center reaction and thereby the secretion of abundantly variable domain glycosylated autoantibodies.

However, against the hypothesis that lectins provide this survival signal[52], we could not identify an impact of the negative regulator, the sialic acid-binding immunoglobulin-type lectin (Siglec) CD22, on the VDG-mediated effects on B-cell signaling. CD22 knock-outs showed no impact on the increased activation status of N-glycosylated BCRs, despite the fact that we mainly identified complex-type disialylated glycans expressed by the BCR variable regions. Nevertheless, VDGs might interact with other (soluble) lectins leading to an increase in activation. It has for example been suggested that galectin-9 facilitates interactions between N-glycosylated IgM BCRs and either of the inhibitory proteins CD45 or CD22 and thus attenuating B-cell signaling upon antigen-stimulation[53,54]. Therefore galectin-9 interactions could also play a role for signaling of CP-directed B cells carrying a large amount of N-glycans in the variable regions of their BCRs although binding has been reported primarily for *N*-acetyllactosamine (LacNAc) containing multi-antennary N-glycans[55]. Additionally, we observed decreased antigen uptake and BCR downmodulation rates in the presence of VDGs (**Chapter 7**, Figure 2C). The prolonged surface expression of antigen-bound BCRs might be an explanation for the observed differences in signaling strength and duration. This would be in line with previous studies showing an increased BCR signaling after inhibition of BCR uptake[56]. The impact of VDGs on BCR downmodulation may also affect the turnover rate of the surface immunoglobulins. It is conceivable that non variable domain glycosylated BCRs are less stably expressed on the B-cell surface and have thus a lower turnover rate. This may cause BCR surface expression to decrease over time,

which in turn may affect the overall signaling strength of the B cell and its activation. The stable and abundant expression of N-glycosylated CP-directed B-cell receptors on the surface of autoreactive B cells in RA might explain why a large population of actively proliferating autoreactive B cells are found that do not "die out" by negative selection or "exhaustion" even after more than 30 years of disease. Nevertheless further studies, using for example endocytosis inhibitors, are needed to delineate whether the observed effects of VDGs on BCR signaling and downmodulation are directly linked to these cellular phenotypes.

In addition, the altered B-cell activation installed by VDGs might also be explained by altered nanoscale organization of the BCR structures. It could be envisioned that the incorporation of VDGs by a BCR will lead to the formation of distinct clusters in a resting or an activated state which could in turn influence signaling. The negatively charged sialic acids terminating the N-linked glycan structures on the variable domain of the BCR could repel similarly sialylated glycoproteins in close proximity as e.g. neighboring BCRs. This could lead to an "open" BCR conformation, which according to the "dissociation activation model" could determine the activated state of variable domain glycosylated B cells, particularly after monovalent antigen exposure[57]. According to this model, BCRs form a closed autoinhibited oligomeric structure on the surface of resting B cells. Antigen binding disturbs this structure leading to an opening and thus activation of the BCRs as evidenced by proximity ligation assays[57]. Further insights could be obtained by tracking the BCRs with and without VDGs after antigenic-stimulation using live cell imaging. This would enable us to detect potential spatial differences of BCRs due to the presence of VDGs. A differential formation of BCR signaling-active caps in the presence of spatially demanding N-glycans could lead to an altered organization of the molecular adaptor Cbl with the tyrosine kinase Syk, causing altered ubiquitination of phosphoryltated Syk and thus differences in BCR signaling[58]. In addition, it is conceivable that VDGs impact on the metabolic fitness of B cells. The two major pathways that provide energy for cells are glycolysis and oxidative phosphorylation or in short OXPHOS. Glycolysis is a relatively inefficient, but rapid way of generating energy for cells as compared to OXPHOS. Recently it has been shown that germinal center B cells acquiring higher-affinity mutations and thereby undergoing clonal expansion and positive selection, showed elevated levels of OXPHOS genes[59]. Therefore, it is also conceivable that ACPA-expressing B cells, that potentially have a selective advantage through the introduction of VDGs, show an increased oxidative phosphorylation. BCRs have been shown to affect the metabolic fitness of B cells[60], and it can be hypothesized that N-glycans bound to these receptors and affecting B-cell activation may thus also be able to control the metabolic state of the cells.

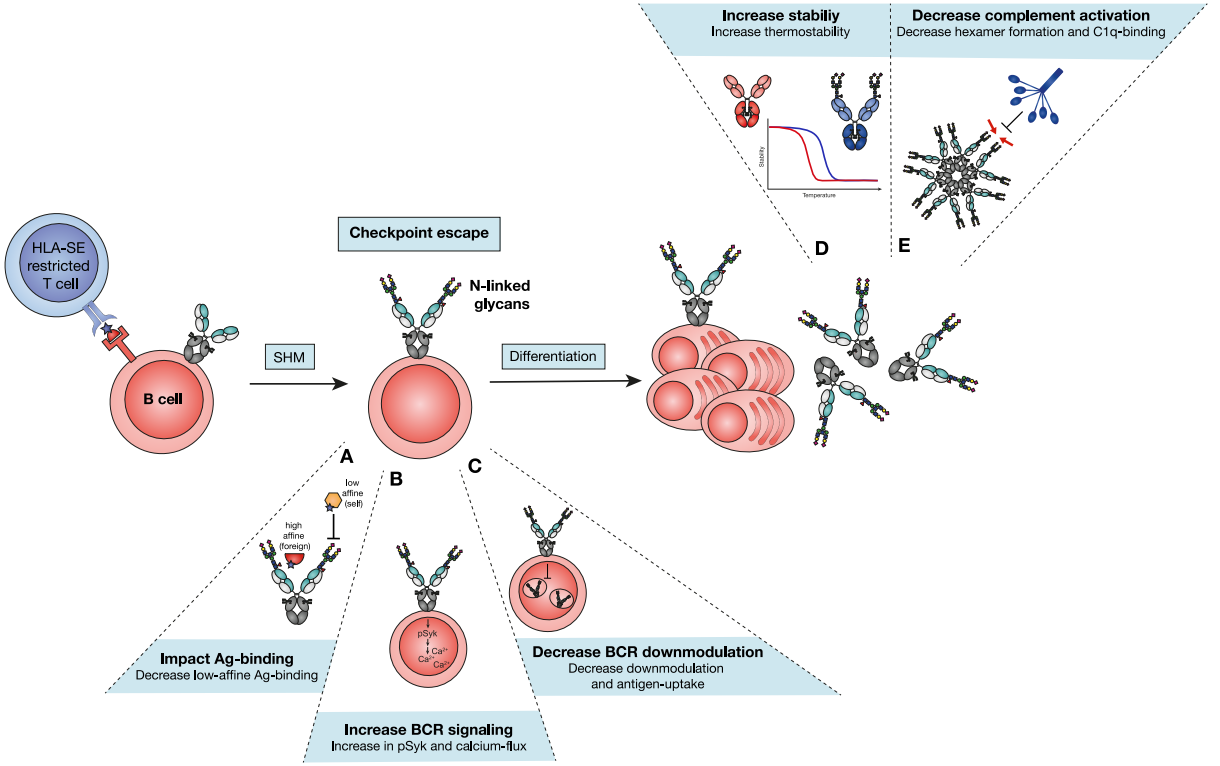

Figure 2. The functional impact of variable domain glycans (VDGs) for autoreactive citrullinated-protein (CP) directed B cells and their secreted ACPAs. N-linked glycans are introduced into the variable domains following HLA-SE restricted T-cell help and somatic hypermutation (SHM). VDGs might allow the B cells to overcome checkpoint control mechanisms by (**A**) lowering binding to low-affine (self)-antigens, while retaining binding to high-affine (potentially foreign)-antigens. (**B**) Increasing B-cell receptor (BCR) signaling and thus decreasing the activation threshold. (**C**) Decreasing BCR downmodulation and antigen-uptake. VDGs potentially also impact on (**D**) the thermostability of secreted ACPAs, (**E**) the propensity of ACPA IgG to form hexamers and thus on C1q-binding and complement activation.

Thus, our data, together with the current literature presented above, indicate that VDGs seem to have important immunomodulatory effects on autoreactive B cells and their secreted autoantibodies. Variable domain glycans can affect antigen recognition, positively influence the stability of immunoglobulins, impact on the effector functions of immunoglobulins (complement activation) and importantly influence antigen uptake, BCR downmodulation and signaling. Further explorations of the functional consequences of VDGs could therefore provide more insights into the regulation of humoral immune responses and possibly the pathogenesis of B cell-mediated autoimmune diseases.

The evolution from autoimmunity to autoimmune disease – "multiple hit" model

The development of ACPA-positive RA is thought to be a multistep process which is here described as a "multiple-hit" model (Figure 3). The initial immunological trigger for the induction of an ACPA immune response might be a combination of environmental risk factors, such as smoking, and/or post-translationally modified microbe derived antigens. Recently, we showed that the first responding naïve IgM B cells can already express cross-reactive BCRs[30] that promiscuously accommodate citrullinated/ carbamylated and/or acetylated foreign- and/or self-proteins in their binding pockets. The high cross-reactive nature complicates the identification of the "inciting" antigen underlying the first activation of the ACPA immune response that is responsible for the recruitment of T-cell help. A "wealth" of antigens could activate the CP-directed B cells, and different antigens may play a role in different patients and at different stages of disease. However, it is conceivable that the inciting B cells are activated by a normal response against (opsonized) microbes presenting foreign-epitopes that mimic self-epitopes as presented in the "mucosal origin hypothesis"[61]. Such antigens are then recognized by citrullinated protein specific T cells, conceivably non-self-directed, that provide help to the B cells, followed by SHM and class switch recombination. This can be defined as a "1st hit" responsible for B cell-mediated immunity to PTM proteins, including self-proteins through cross-reactivity. Although autoantibodies occur, individuals are still healthy, and the induction of the "PTM protein-directed immunity" will only lead to the transition to an autoimmune disease in conjunction with other factors (hits), possibly including certain genetic risk factors. This probably also explains why some individuals are more prone to transition to disease than others.

The most prominent genetic risk factor for ACPA-positive RA are the HLA-DRB1 shared epitope (SE) alleles. That HLA-SE alleles are not involved in the initial breach of tolerance, but rather facilitate a further maturation of the ACPA response occurring before RA-onset, is supported by the findings showings that HLA-SE alleles only associate with ACPA-positive disease and not with ACPA-positivity in healthy individuals[62,63]. Thus, it is likely that in a 2nd immunological

hit, modified (citrullinated) antigens facilitate the help of additional T cells directed against citrullinated antigens presented by HLA-SE molecules. The findings described in this thesis, now indicate that this "2nd" HLE-SE restricted T-cell response expedites the introduction of N-linked glycosylation sites into the variable regions of the CP-directed BCRs through SHM (**Chapter 4** and **Chapter 5**). Thus, upon repetitive passages of CP-directed B cells through germinal centers and the accompanied T-cell help, N-linked glycans will be presented in the variable domains of the autoreactive BCRs and their secreted autoantibodies. The introduced VDGs alter antigen binding and the activation threshold of the CP-directed B cells (**Chapter 7**) and may thus be considered as "the spark that lights the fire". VDGs potentially help the B cells to proliferate and to overcome important B-cell checkpoints ultimately leading to the induction of the autoimmune disease RA. This concept of VDGs as a "master switch" for the expansion of the ACPA immune response, is reinforced by the fact that ACPAs display a relatively low avidity to their antigens, even after extensive SHM[4,64]. Apparently, CP-directed B cells are not following "conventional" affinity maturation concepts that underly the outgrowth of the "fittest" B cells in germinal centers, but rather acquire crucial survival and proliferation signals through their ability to incorporate N-glycans into their variable regions. Thus, the second expansion of ACPA B cells is most likely regulated through the selective introduction of N-glycans into the variable regions rather than by avidity maturation. This is also in line with previous studies showing that the introduction of VDGs is associated with the transition towards disease[40] and with the findings presented in **Chapter 6** indicating that their abundance rises significantly towards disease progression. In addition, the high abundance of VDGs on ACPA IgG in all patients indicates that they are likely an important pre-requisite for disease development. As evidenced by the results presented in **Chapter 7**, the introduction of VDGs potentially explains the activated phenotype of the CP-directed B cells. The abundant expression of VDGs might hinder the autoreactive B cells to reach a state of quiescence through a continuous activation, potentially even in the absence of antigens, via e.g. the interaction with other glycan-binding molecules. However, further studies are needed to bring these two findings into context. It is also conceivable that the hyperresponsiveness of CP-directed B cells can be explained by the fact that these B cells almost exclusively interact with post-translational modifications that are repeatedly present on the respective antigens. Thus, BCRs are efficiently cross-linked through multiple interactions with the repeated epitopes, resulting in extensive and rapid B-cell activation. In comparison, tetanus toxoid directed B cells, which can only interact with one specific epitope, receive only monovalent antigenic stimulation and therefore show a reduced activation status.

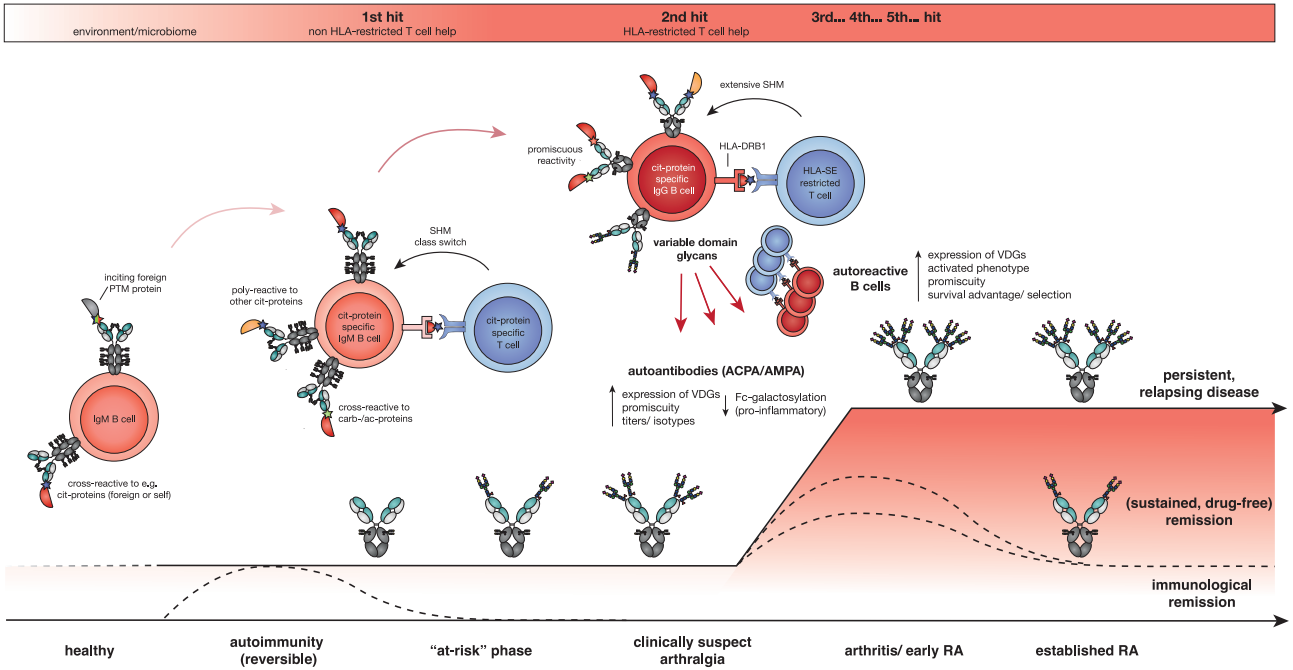

Figure 3. "Multiple hit" model potentially explaining the development of ACPA-positive RA inspired by[65]. Environmental factors or the microbiome generate post-translational modified proteins likely involved in the earliest breach of self-tolerance. Naïve IgM B cells able to accommodate these modified-proteins [citrullinated (blue), carbamylated (green), acetylated (red)] via their cross-reactive (promiscuous) BCRs, receive help from PTM protein specific T cells followed by somatic hypermutation (SHM) and class switching (1st hit). These early events lead to autoimmunity as evidenced by the presence of ACPAs and already occurs several years before the onset of arthritis. Further hits are likely at play involving the ongoing help of HLA-SE restricted T cells (2nd hit) followed by the ongoing and ultimately abundant introduction of N-linked variable domain glycans (VDGs) via SHM (3rd, 4th, 5th hit). VDGs likely give a selective advantage to ACPA-expressing B cells which is eventually instrumental in the transition to an autoimmune disease. The "quality" of the ACPA response, characterized by its cross-reactivity, antibody titres, isotype-usage, Fc glycosylation and expression of VDGs, potentially predetermines the transition to a persistent, relapsing disease or the chance that the RA of patients achieves remission at a later stage.

The "multiple hit" model also explains why ACPAs can already be present several years before the onset of clinical symptoms. The "constitution" of the ACPA response changes however during disease development, as evidenced by an increase in ACPA titres, isotype-usage, an expansion of the recognition profile (increased cross-reactivity), an increase in the introduction of VDGs as well as a change in the Fc-glycan profile (reduced Fc-galactosylation). These changes are likely obtained via "multiple hits" requiring multiple rounds of repetitive HLA-SE restricted T-cell help over a period of several years and are thus responsible for the transition from a (potentially reversible) autoimmunity to autoimmune disease. The "type" of the ACPA response also correlates with outcomes, more specifically, if individuals will develop an irreversible (chronic), relapsing disease or if their disease achieves remission at a later time point. This was also demonstrated in **Chapter 6,** where individuals who had introduced a lower amount of VDGs into the ACPA variable domains had a higher chance of achieving drug-free remission at a later stage. The results presented in this chapter indicate that once a specific ACPA response is present in disease, it seems to be relatively stable over time. The ACPA response in individuals with persistent disease is characterized by low avidity, but this is balanced by a high cross-reactive nature and an abundant expression of N-linked glycans in the variable domains. The high expression of VDGs can possibly shift the B cells into a more active phenotype by e.g. affecting the metabolic state. This might explain why these B cells can persist for several years and eventually cause the onset of RA.

Concluding remarks

The last few years, we have made great progress in our understanding of the immunological processes underlying the human autoimmune disease Rheumatoid Arthritis (RA) and identified mechanisms potentially involved in the breach of tolerance. The data described in this thesis reinforce the hypothesis that the disease-specific autoantibodies (ACPAs, ACarPAs and AAPAs) are highly cross-reactive to several post-translational modifications (PTMs) and thus potentially belong to one family of anti-modified protein antibodies (AMPAs). The studies show that ACPA-expressing B cells can be activated by multiple PTM antigens indicating that various modifications can be involved in the initial breach of tolerance. In addition, the studies unravel the important role of variable domain glycans (VDGs) for the disease progression to RA. Our data show that VDGs associate with the most prominent genetic risk factor for ACPA-positive RA, the HLA-SE alleles, and that the abundance of VDGs can be predictive for the development of a chronic, persistent disease or the chance that RA patients achieve remission at a later time. Functionally, we identified that VDGs can impact complement activation and reduce binding to low-affine (potential self) antigens, while binding to high-affine (potential foreign) antigens is retained. Importantly, VDGs were able to decrease the activation threshold of CP-directed B cells and thus display important immunomodulatory functions. The results presented in this

thesis show that autoreactive B-cell responses in RA most likely evolve via "multiple hits", and that both, the highly cross-reactive nature of the response and the abundant expression of VDGs might help the B cells to overcome important immune checkpoints and to breach tolerance. Stratifying patients with RA on the basis of their cross-reactivity and the abundance of VDGs together with the specific Fc glycosylation profile might therefore allow us to identify more homogeneous patient groups, with respect to both disease course and the response to treatment. We are looking forward to the coming years and the many more important discoveries that are waiting to be made to unravel the role of variable domain N-glycans (sweet) on autoreactive B cells and their secreted autoantibodies (bitter) and thus to understand this Bitter Sweet Symphony.

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