The Anti-Citrullinated Protein Antibody immune response and its effector functions in Rheumatoid Arthritis

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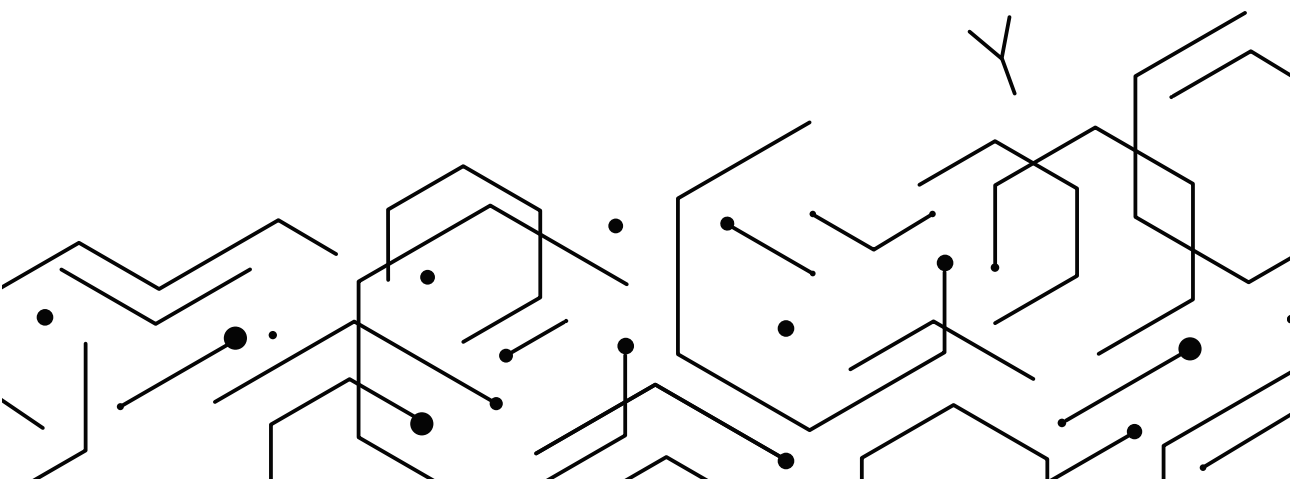
CHAPTER 7

Summary and discussion



In the studies described in this thesis, we aimed to obtain insight into specific aspects of the Anti-Citrullinated Protein Antibody (ACPA) immune response and its biological effector functions in rheumatoid arthritis (RA). ACPA, produced by citrullinated antigen-directed B cells, are highly specific for RA and ACPA-positive RA is associated with a severe and destructive disease course [1-3]. Several studies so far have shown that the polyclonal ACPA response differs from conventional antibody responses (such as, for example, anti-vaccine responses) by the remarkably low avidity of secreted ACPA molecules, by its limited avidity maturation, altered Fc glycosylation and extensive glycosylation in the Fab region [4-7]. Moreover, ACPA promote several biological effector functions *in vitro* associated with the induction of inflammatory processes observed *in vivo* [8-11]. Although these studies hint towards a pathogenic role for ACPA or ACPA-expressing B cells in RA, the exact underlying mechanisms of the involvement of ACPA in RA development and severity, and the role of the aberrant glycosylation in disease pathogenesis has not yet been elucidated. Therefore, we here set out to study the origin and function of ACPA, of ACPA glycosylation and of B cells in RA pathogenesis.

The presence of a citrullinated antigen-directed immune response demonstrates, if identified in healthy individuals, that immunological tolerance of citrullinated 'self'-proteins has been broken. Frequently, this occurs prior to the occurrence of clinical symptoms. To date, it is unclear which triggers or events cause this loss of tolerance although it is suggested that both environmental or stochastic events might be involved. Following the initial break of tolerance, the ACPA response is thought to mature under the influence of helper T cells that provide help to ACPA-expressing B cells. Eventually, this cascade of several immunological 'hits' may lead to the onset and perpetuation of RA. In the asymptomatic initiation phase as well as during established disease, it is largely unknown how and where ACPA-expressing B cells are generated and maintained. Some evidence has suggested constant regeneration of citrullinated antigen-directed B cells from naïve precursors because of the presence of IgM-ACPA during undifferentiated arthritis, early RA and established RA [12]. However, it is likely that ACPA-expressing B cells also differentiate from memory B cells into plasma cells that eventually reside in supportive survival niches and promote a constant level of ACPA production, as has been demonstrated for other pathogenic antibody-secreting cells [13-15]. With these considerations in mind, we studied, as described in **chapter 2**, ACPA-expressing plasmablasts and plasma cells at the site of inflammation, the synovial compartment, to investigate if the synovial compartment can provide a tissue environment supportive of the long-term survival of ACPA-expressing B cells and the production of ACPA. Next, we considered that ACPA produced by citrullinated antigen-directed B cells can be present long before the onset of RA. However, it is upon the expansion of ACPA in

serum titres, isotypes and antigen recognition that clinical arthritis can develop. This expansion, which is presumably based on T-cell dependent processes, is remarkable as the overall avidity of ACPA remains low. This hints towards another survival signal that selects these low-avidity ACPA for further maturation. In this context, we focussed on a recently discovered, unique feature of ACPA: the abundant presence of glycans in the variable region. We set out, as described **in chapter 3,** to study which ACPA subclasses harbour these Fab-glycans in order to determine if Fab-glycans could result from and/or contribute to the T-cell dependent process during the development of the ACPA-specific B cell response. Next, we focussed on the IgG-Fc domain of ACPA during the mature phase of the ACPA response to determine how ACPA can contribute to RA pathogenesis. As most antibody effector mechanisms are based upon interaction with Fc-gamma receptors (FcγR), we studied ACPA immune complex binding characteristics to individual FcγR as described in **chapter 4**, assuming that this could lead to more insight in the downstream pathogenic effects of ACPA. Of note, the aberrant expression of glycans in the Fc region of ACPA, that is associated with a more pro-inflammatory profile, could additionally influence the interaction with other proteins. In this context, we investigated the interaction of ACPA and rheumatoid factors (RF) which are often implicated together when it comes to RA disease pathogenesis. The studies presented in **chapter 5** aimed to determine if RF preferentially bind to ACPA-IgG over non-ACPA-IgG because of the lower Fc-galactosylation of ACPA. In such a scenario, the aberrant Fc glycosylation of ACPA could trigger a set of downstream events that could lead to enhanced inflammation and contribute to disease progression. This further led us to investigate whether it would be possible to modulate the Fc glycosylation of IgG secreted by plasma cells by micro-environmental factors in order to prevent disease aggravation. We were interested whether the Fc glycosylation of IgG antibodies secreted by terminally differentiated plasma cells could be altered as these plasma cells are the main producers of antibodies. To this end, the studies described in **chapter 6** addressed the Fc glycan modulation of secreted IgG by a micro-environmental factor relevant for the development and/or maturation of B cells. Together, the studies presented in this thesis demonstrate various aspects of the ACPA immune response that can contribute to the maturation of ACPA and subsequently to the inflammatory processes in RA.

ACPA RESPONSE AT THE SITE OF INFLAMMATION

Autoantibodies produced by autoreactive plasma cells can constitute an important component in the initiation and perpetuation of the inflammatory cascade observed in autoimmune diseases. This is relevant because long-lived plasma cells can survive for extended periods of time in the presence of a supportive stromal microenvironment

[16]. Besides the bone marrow, non-dividing, long-lived plasma cells have been shown to reside in other niches such as tonsils and the intestine where they can survive and secrete (autoreactive) antibodies for weeks [17, 18]. In ACPA-positive RA, citrullinated antigen-directed B cells are thought to play a crucial role in the initiation, aggravation and/or perpetuation of the disease. Previous findings demonstrated the presence of circulating plasmablasts and plasma cells in peripheral blood of patients with ACPA-positive RA which spontaneously secreted ACPA in culture [19]. These observations, together with the notion that sustained and abundant levels of ACPA-IgM could be observed in the synovial fluid, the site of inflammation, suggest an ongoing, active immune response against citrullinated antigens in RA [20]. However, it was so far unknown whether these ACPA-secreting plasmablasts/cells compete for survival niches in, for example, secondary lymphoid structures or whether the synovial compartment of the inflamed joint in RA could provide such a niche.

To characterise ACPA-expressing plasmablasts/cells from the synovial compartment and to investigate whether this compartment can provide a micro-environment maintaining the ACPA-expressing B cell response, we studied citrullinated antigen-directed B cells from the synovial fluid of ACPA-positive RA patients as described in **chapter 2**. We hypothesized that ACPA-secreting plasma blasts/cells would be more frequent in the synovial compartment compared to peripheral blood and that the synovial microenvironment could provide a niche supporting the long-term survival of these antibody secreting cells. Therefore, we isolated mononuclear cells from peripheral blood and synovial fluid of ACPA-positive RA patients and cultured these cells without any additional stimuli. From these cultures, we could identify a high frequency of differentiated, spontaneously ACPA-IgG secreting plasmablasts/cells in synovial fluid, which was much lower in peripheral blood. Furthermore, we showed that mononuclear cells from the synovial fluid maintained a stable and spontaneous production of ACPA-IgG secreted from pre-existing plasma cells and differentiated plasma blasts/-cells for several months. These data indicate that cells within the synovial fluid can indeed provide a niche for the prolonged survival of antibody-secreting cells and, hence, promote the sustained production of ACPA-IgG. This substantially contributes to the concept of an ongoing ACPA B cell immune response that could sustain the chronicity of synovial inflammation. Future research on the molecular and cellular components of the synovial fluid is needed to identify and target possible contributors to the prolonged survival of antibody secreting cells in order to shorten the lifespan of ACPA-secreting plasmablasts/cells, which could be evaluated as a therapeutic intervention in RA.

ACPA Fab GLYCOSYLATION

ACPA variable domain glycosylation is a recently reported novel molecular feature of ACPA-IgG. Almost all ACPA-IgG molecules were found to carry these highly sialylated Fab glycans whereas non-ACPA IgG and a selected group of other autoantibodies analysed thus far did not show this feature [7, 21, 22]. This indicates that extensive Fab glycosylation is a specific feature of ACPA-IgG in RA and can therefore be used as a biomarker in ACPA-positive disease. The extensive presence of Fab glycans on ACPA-IgG was recently found to result from *N*-glycosylation sites in ACPA-IgG variable domains that have been generated by somatic hypermutation [22, 23]. So far, it is not known why *N*-glycosylation sites accumulate in ACPA-IgG and at what stage during B cell development this event occurs. In **chapter 3**, we describe studies on ACPA-IgM and ACPA-IgG subclasses and the presence of Fab glycosylation to gain more insight in the development of Fab glycosylated ACPA and the potential role of these glycans in RA pathogenesis. We hypothesized that variable domain glycosylation would be absent from ACPA-IgM but present in all ACPA-IgG subclasses, as the ACPA response presumably matures under the influence of T-cell help due to its association with the HLA-region [24, 25]. Indeed, we could demonstrate the absence of Fab glycosylation of ACPA-IgM as there was no increase in molecular weight of ACPA-IgM compared to its non-citrullinated counterpart. Surprisingly, we did not obtain evidence indicating the presence of variable domain glycosylation of ACPA-IgG3. This could, potentially, be explained by its location within the IGH gene locus. Because of its “front” position on the IGH gene locus, IgG3 is the first IgG subclass to be produced upon class-switch recombination and is associated with less mutations compared to the other IgG subclasses [26]. It is therefore possible that ACPA-IgG3 is expressed before the accumulation of *N*-glycosylation sites occurs. Furthermore, all ACPA-IgG subclasses, with the exception of ACPA-IgG3, demonstrated a higher molecular weight indicating the presence of ACPA Fab glycosylation. These results, together with the findings that variable domain glycosylation results from somatic hypermutation and the strong HLA association with ACPA-positive RA, implies that the introduction of *N*-glycans in the variable domain is dependent on T cell help in or outside the germinal center. It also provides an argument for the hypothesis that the maturation of the ACPA response is dependent on T-cell help which may contribute to the breach of tolerance checkpoints and to the maturation of citrullinated antigen-directed B cells in RA.

Functionality of ACPA Fab glycosylation

So far, it is unknown what role Fab glycans play in the functionality of ACPA-IgG. Generally, glycans in the antibody variable domain have been implicated to modulate several functions in different ways. For example, Fab glycans located close to the antigen

binding site may affect antigen binding [27]. Indeed, it was demonstrated that Fab glycans on monoclonal ACPA may influence antigen binding, as depletion of the variable domain glycans enhanced or decreased binding to the CCP2 antigen, albeit to a limited extent [22]. Others have suggested that ACPA Fab glycosylation does not influence antigen binding based on a CCP3 binding assay with PNGaseF treated cloned ACPA-IgG [21]. Another important functionality reported for glycans is the interaction with lectins. One hypothesis is that introduction of Fab glycosylation in the B cell receptor (BCR) via somatic hypermutation allows interaction of the BCR with lectins in the germinal center and thereby provide survival signals to self-reactive B cells [28]. This phenomenon has been reported before in follicular lymphoma where the interaction of lymphoma cells with lectins "free" these cells from dependence on antigen and enhance the survival through selection based on glycan interaction [28-30].

Because of the abundant presence of terminally sialylated Fab glycans on ACPA-IgG and the overall low affinity of ACPA-IgG compared to conventional recall antigens [4, 7], it is possible that B cells producing Fab glycosylated ACPA could have a survival advantage through selection based on the presence of *N*-linked Fab glycans rather than on affinity for their cognate antigen. Moreover, this process could contribute to the breach of tolerance checkpoints during the "second hit" of the two-hit model for the development of ACPA-positive RA. However, this does not exclude the possibility that ACPA-IgG Fab glycans accumulate over time by multiple "hits" or that some ACPA may carry Fab glycans from the start. Although the function of ACPA Fab glycosylation in the pathogenesis of RA remains to be determined, there are hints towards the involvement of Fab glycans in the selection and survival of low avidity citrullinated antigen-directed B cells. Further research is needed to determine whether Fab glycosylation is also present in other Anti-Modified Protein Antibodies in RA, and to gain insight in the biological processes underlying the hyperglycosylation of ACPA-IgG.

BIOLOGICAL EFFECTOR FUNCTIONS OF ACPA

Fc gamma receptor binding

Antibody effector functions are mostly regulated via the antibody constant region (Fc tail). Various Fc-mediated effects have been described for ACPA under which the activation of the complement system, of neutrophils and of immune cells via Fc gamma receptor (FcγR) binding [8, 10, 11, 31]. So far, ACPA-mediated effector functions have been associated mostly with FcγRIIA by demonstrating that ACPA-containing immune complexes can induce TNF-α secretion by macrophages and PBMCs via this receptor [5, 32-34]. In addition, in *in vivo* studies, mice expressing FcγRIIA had a more rapid onset of collagen-

induced arthritis than mice that did not express this receptor [35]. As it is likely that ACPA immune complexes can be formed *in vivo* because of the abundant presence of ACPA immunoglobulins and its citrullinated antigens in the synovial fluid of RA patients, these ACPA-IC induced FcγR-mediated effects are of importance in the context of synovial inflammation. However, the Fc receptor system is complex and within the RA synovium distinct immune cells are present which express all kinds of combinations and levels of FcγR that can balance ACPA-IC mediated effects.

As presented in **chapter 4**, we therefore set out to study binding characteristics of ACPA-IgG IC to various FcγR individually to further dissect potential effector mechanisms by which ACPA could contribute to RA pathogenesis. To this end, we used isolated ACPA and non-citrulline specific IgG from the plasma and synovial fluid of ACPA-positive RA patients which we combined with fluorescently labelled Fab_2 antibodies directed against the Fab_2 of IgG or citrullinated fibrinogen to generate ACPA-IgG and IgG IC. These IC were incubated with single FcγR-transfected CHO cells which allows to control for the expression level of each individual FcγR using an unique experimental setting. To translate our findings in a more representative setting, we also incubated ACPA-IC with human neutrophils in the presence or absence of FcγR blocking antibodies. Using the (ACPA-)IgG Fab_2 IC we demonstrated that ACPA-IgG IC predominantly bind to FcγRI and FcγRIIIA. We verified these results with ACPA-IgG IC made with citrullinated fibrinogen, which represent a more natural occurring IC in RA. In comparison with IgG-IC, the binding of ACPA-IgG IC to FcγRIIIA was notably lower. As the presence of core-fucosylation is known to prevent or weaken binding of antibodies to FcγRIIIA, this reduced affinity of ACPA-IgG IC to FcγRIIIA could be explained by the relatively high level of core-fucosylation within the Fc glycan of ACPA [36, 37]. This observation might suggest that FcγRIIIA-mediated effector mechanisms such as antibody-dependent cellular cytotoxicity are less likely to contribute to ACPA-mediated effector functions [38]. We further extended our research to human neutrophils as neutrophils represent the main cell type present in synovial fluid of RA patients. Moreover, neutrophils also provide a good model to study the binding with FcγRI as this receptor can be induced on neutrophils, while the expression of FcγRII and FcγRIII remain stable. Our results demonstrated that ACPA-IC can bind FcγR on neutrophils and indeed, we could confirm binding of ACPA-IC to FcγRII. However, blocking FcγR on activated neutrophils also revealed that FcγRI was responsible for almost 30% of the ACPA-IC binding to neutrophils. These findings suggest a role for FcγRI expressed by activated neutrophils as relevant receptor for ACPA-IC in the context of synovial inflammation, in addition to the known effector functions of ACPA that are mediated via FcγRIIA. Given that the majority of immune cells in the synovial fluid of RA patients are activated neutrophils expressing FcγRI and the abundant presence of

ACPA-IgG IC within the synovial fluid, it is conceivable that FcγRI triggering of neutrophils by ACPA-IC is of importance in RA [20, 39-42]. Together, these observations provide an additional argument for a potentially pathogenic role of ACPA-IgG in the synovial inflammation in RA. Further downstream analysis of the ACPA-IC triggered FcγRI signalling is needed to determine the exact Fc-mediated effector mechanisms.

Rheumatoid factor binding to ACPA-IgG

Rheumatoid factor (RF) and ACPA are often found together in RA and suggested to contribute to disease pathogenesis. Moreover, the presence of both ACPA and RF showed an increased association with RA compared to the presence of ACPA only [43]. RF is commonly expressed as the IgM isotype and has a binding specificity towards the Fc region of IgG antibodies. This binding region of RF is situated near IgG Fc-glycans, making it likely that glycosylation can influence the binding of RF to IgG. More specifically, it was suggested in older studies that IgM-RF might prefer binding to IgG with low Fc galactosylation levels [44, 45]. In RA, ACPA-IgG has an altered Fc-glycosylation profile, which is characterized by low galactosylation and sialylation levels compared to IgG antibodies without citrulline specificity [6, 36]. In addition, ACPA-IgG in RF-positive RA patients have a lower galactose content compared to the ACPA-IgG from RF-negative RA patients [6]. Together, these observations led us to the hypothesis that RF could preferentially bind to ACPA-IgG, rather than to non-citrulline specific IgG. To assess the binding between IgM-RF and (ACPA-)IgG, we performed surface plasmon resonance imaging arrays with RA patient derived ACPA-IgG and non-citrulline-specific IgG in combination with serum from RF-positive and RF-negative RA patients as described in **chapter 5**. We observed comparable binding profiles for serum RFs binding ACPA-IgG or non-citrulline specific IgG, indicating that RF do not preferentially bind to ACPA-IgG compared to non-ACPA IgG. Using glyco-engineered monoclonal IgG1 antibodies with different degrees of Fc galactosylation, we could confirm that there are no differences in binding of RFs to IgG with normal, low or high galactosylation content. Therefore, we concluded that ACPA-IgG are not necessarily better targets for RF than non-ACPA IgG. However, this observation does not exclude the more frequent presence of ACPA-RF IC than IgG-IC in RA patients. High ACPA titres within the synovial environment and the multiplicity of ACPAs binding citrullinated antigens may provide enhanced opportunities for RFs to bind and form ACPA-IC [20, 46, 47]. In this way, ACPA-RF IC could contribute to RA-specific inflammation, such as the RF enhanced macrophage cytokine production by ACPA-IC stimulation *in vitro* [48, 49]. Together with our observation that binding of RF to ACPA is not dependent on Fc-galactosylation levels, these data suggest that potential RF enhancing effects on ACPA-mediated inflammation may contribute to RA pathogenesis even before changes in ACPA Fc-glycosylation develop.

MODULATION OF IgG Fc GLYCOSYLATION

Our studies presented indicated that ACPA immune complexes could be important in RA mediated inflammation and bind to FcγRI through which they may exert their effector mechanism. In addition, we have also studied whether the binding of RF to ACPA-IC was dependent on the decreased ACPA-IgG galactosylation levels as antibody effector functions (including FcγR binding, activation of immune cells and activation of the complement system) and interactions with other proteins are greatly depend on glycosylation in the Fc region [50]. Changes in Fc glycosylation, as observed in pathogenic conditions such as RA, could therefore contribute to inflammatory processes in disease pathogenesis. Although the altered Fc glycosylation of ACPA-IgG did not seem to affect RF binding towards ACPA-IgG compared to non-ACPA IgG, it is remarkable that the ACPA Fc-glycosylation profile shows a more pro-inflammatory phenotype around 3 months prior to diagnosis of RA [36]. The altered Fc glycosylation of ACPA-IgG seems to trigger a set of downstream events that leads to the onset of arthritis. This hypothesis is supported by evidence from an experimental arthritis model in which IgG glycosylation levels were affected by IL-23-activated Th17 cells which induced a pro-inflammatory autoantibody profile and triggered the onset of arthritis. If Fc-glycosylation is such a critical factor in the onset of RA, one of the potential solutions to prevent disease aggravation would be to modulate the IgG Fc glycosylation.

Here, we studied whether it is possible to structurally modulate the Fc glycans of IgG antibodies secreted by terminally differentiated plasma cells in order to determine if antibody effector functions can be modulated while the antibody secreting cell is in its most terminal phase of differentiation. Recent observations have already indicated that micro-environmental factors such as cytokines and hormones can influence IgG Fc glycosylation secreted by $CD19^+$ B cells [51-53]. As such, we were interested whether an environmental factor relevant for the development and/or maturation of B and plasma cells could influence the Fc glycosylation of IgG antibodies. Therefore, the studies presented in **chapter 6** set out to investigate the influence of IL-10 on the IgG1 Fc glycosylation of antibody secreting B cells (ASC), containing late plasma blasts, and $CD19^+$ cells depleted of ASC, containing mostly naïve and memory B cells. We isolated the ASC and $CD19^+$ cells depleted of ASC from human tonsils and cultured them in the absence or presence of IL-10, after which we analysed the IgG1 Fc glycosylation. IL-10 is an important regulatory cytokine involved in the suppression of inflammation during pregnancy [54-56] and in B cell survival, proliferation and differentiation [57, 58]. Upon stimulation of ASC with IL-10 we observed lower galactosylation and sialylation levels of IgG1. Interestingly, upon IL-10 stimulation of the $CD19^+$ population depleted of ASC, we observed increasing

galactosylation and sialylation levels suggesting that IL-10 has contrasting effects on the different B cell populations. Together, these data indicate that the modulatory effect of IL-10 on the IgG1 Fc glycosylation is depended on the differentiation status of the B cells. Further research is needed to determine if these results also apply to B cells derived from other tissues (e.g. blood and bone marrow) which are in another maturation state. Moreover, our data suggest that the Fc glycosylation of IgG1 secreted by plasma blasts and plasma cells can still be modified under the influence of micro-environmental factors. These micro-environmental factors may well be a part of the micro-environment maintaining the ACPA-expressing B cell response in the synovial compartment as described in **chapter 2**. In this way, ACPA-IgG Fc glycosylation could be influenced by the synovial environment supporting the sustained production of ACPA-IgG which may result in its pro-inflammatory phenotype of the Fc glycans. As pathogenic plasma cells secreting autoantibodies are highly resistant to therapy and are difficult to target, modulation of their IgG Fc glycosylation by micro-environmental factors would allow intervening with the pathogenicity of these autoantibodies.

CONCLUSION

The ACPA immune response has been implied to contribute to inflammatory processes in RA for many years. By gaining insight in the relation between ACPA, ACPA glycosylation and B cells in RA, we provide multiple arguments for the contribution of ACPA in the chronic inflammation of RA. This thesis highlights the importance of the synovial environment during active disease for the survival of citrullinated antigen-directed B cells in the continuously ongoing ACPA immune response sustaining RA chronicity. Moreover, for maturation of the ACPA immune response, introduction of variable domain glycosylation in ACPA-IgG is likely to play an important role in the breach of tolerance checkpoints and may provide a survival signal for low-avidity ACPA. In this way, Fab glycosylation may contribute to the ‘second hit’ that promotes RA development into clinical disease. Synovial inflammation by the binding of ACPA immune complexes to FcγR and RF binding to ACPA immune complexes further enhance and persevere clinical disease. These effector mechanisms are largely dependent on the IgG-Fc glycosylation profile which was shown to be influenced by micro-environmental factors. As micro-environmental factors are able to modulate inflammation, it also provides a potential solution to intervene with pathogenic antibodies as a therapeutic strategy in RA pathogenesis. ACPA glycosylation and the environment in which ACPA develop are both important for the breach in B cell tolerance, the survival of citrullinated antigen-directed B cells and subsequently in the maturation of ACPAs and its immune response, thereby actively contributing to the inflammatory processes in RA.

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