C1q and anti-C1q autoantibodies in (auto)immunity

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CHAPTER 7

General discussion

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Complement and complement therapeutics in rheumatic disease

The human complement system plays a major role in immune defense and maintenance of homeostasis, but through overactivation, inadequate regulation or improper function, complement is also involved in disease processes. This detrimental role of the complement system is apparent for example in several rheumatic diseases, as we have reviewed in **chapter 2**. In rheumatoid arthritis (RA), the observation that activated complement protein fragments are present in the synovium and in the circulation was an initial clue for a role for complement activation in the disease processes of RA [1-7]. This was experimentally elaborated in mouse models of collagen antibody-induced arthritis (CAIA), where especially the alternative complement pathway was essential for disease development, with only a minor role for the classical pathway [8-10]. Meanwhile, the influence of complement on systemic lupus erythematosus (SLE) can take on of two forms. Complement deposition in affected organs and decreased concentrations of circulating complement proteins due to massive consumption of complement is often observed in SLE patients and correlates with disease severity, implicating that complement activation contributes to the disease processes of SLE [11-13]. On the other hand, individuals who are genetically deficient for C1q (and to a lesser extent also C1r, C1s, C4 and C2) are highly likely to develop SLE disease [14, 15]. According to the waste disposal hypothesis, the inability of the classical complement pathway to help clear apoptotic cells and debris leads to the exposure of intracellular antigens and formation of autoantibodies against these antigens. Additionally, C1q is reported to have a regulatory effect on T cells, interacting with $CD8^+$ T cells with their globular head to modulate T-cell metabolism [16]. The absence of this pathway may increase the occurrence of autoimmunity.

Even though a large body of evidence points towards the involvement of the complement system in both RA and SLE, therapeutics targeting complement have so far not yielded convincing results. Eculizumab, an antibody that prevents the cleaving and activation of C5, was clinically tested in both RA and SLE patients, but did not progress to pivotal clinical trials and regulatory approval [17]. Off-label use of eculizumab anecdotally demonstrated positive outcomes in lupus nephritis, as reported by in several single cases [18, 19], but no large-scale study has been performed. These results show that complement activation, at least when inhibited at the level of C5, is not likely to play an essential role in the immunopathology of established RA and SLE, and C5 is therefore not the best target for therapy of these diseases.

Successful intervention by complement-targeting therapeutics is possible in other diseases, exemplified by the approval of anti-C5 antibodies eculizumab and ravulizumab in paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS) [20]. In **chapter 2**, the C5a receptor-blocking small molecule avacopan was described to be in clinical trials, and it has since been approved for the treatment of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) [21-23]. In AAV, neutrophils are attracted and activated through their C5a receptor and subsequently cause more complement activation by secreted factors and neutrophil extracellular traps (NET). This feedback loop is broken by blockade of the C5a receptor, as has now been shown in human studies with avacopan [24, 25].

With these and other complement-targeting therapeutics on the market, and several more under clinical investigation, every trial allows the scientific community to learn more about the relative contribution of complement in the respective disease [20]. In this sense, it will be interesting to follow a recently developed antibody against C1q, which aims to deplete free C1q from the circulation [26]. As C1q deficiency is associated with development of SLE disease, long-term treatment with this anti-C1q antibody may have as a side-effect the development of autoimmune disease. The currently approved complement-targeting therapeutics work systemically, which may have a great impact on side effects. Even though safety concerns for eculizumab are less prominent than once feared, inhibiting (parts of) complement systemically to treat a localized disease remains sub-optimal [27]. This leaves a great opportunity for future therapeutics in targeting specific locations within the body. Localizing the therapeutic to the affected organ or tissue will allow the next generation of complement drugs to maintain normal complement functionality throughout the rest of the body [van de Bovenkamp *et al.*, submitted].

C1q as biomarker in tuberculosis disease

While complement is a therapeutic target in some diseases, complement components may serve as a biomarker for other diseases. Tuberculosis (TB) is a clear example of such a disease where current diagnostic approaches are either very time-consuming in the case of bacterial culture from sputum, or incapable of distinguishing between active disease, latent infection or past infection in the case of immunological tests. This raises the need for better TB diagnostics, and complement protein C1q has been identified as a possible biomarker. We and others have found that the C1q concentration in serum is increased in patients with active pulmonary TB disease compared to latently infected patients and relevant disease controls [28-30]. Whole blood transcriptomics analyses also

revealed other possible biomarkers, including differential expression of certain interferon-stimulated genes. One particular study selected 10 of these genes to form a whole blood transcriptional type-1 interferon signature for use as a diagnostic marker [31]. In **chapter 3**, we observed an inverse correlation between C1q and the interferon signature in active pulmonary TB patients. As a result, all patients in the cohort were positive for high C1q concentration or for high type-1 interferon signature, with some double positive patients. Importantly, none of the active TB patients were double negative, while a large majority of healthy controls fell within the double negative quadrant. We conclude that the use of both C1q and the interferon signature yields improved diagnostic value based on the results in our cohort, compared to either of the single biomarkers.

TB can spread to many different organs, and we also investigated involvement of the eye, in the form of TB uveitis, which is especially difficult to diagnose as it may present itself clinically similarly to other ocular conditions. Additionally, association between uveitis and positive immunological tests for TB may be coincidental in regions with high rates of latent TB infection. In uveitis of unknown cause, we could stratify patients into groups based on low or high risk of TB uveitis by using both C1q and the interferon signature as combined biomarkers. Although further development and validation are still needed, the inverse correlation between C1q concentration and the type-1 interferon signature we observed for active pulmonary TB in **chapter 3** means they could work well together. The main advantage over the current immunological tests is their ability to distinguish active disease from past exposure. Bacterial culture from a biological sample can also achieve this distinction, but may take weeks to deliver results. The use of C1q concentration and interferon signature therefore offers the opportunity to start anti-tuberculosis treatment rapidly, without the risk of overzealous treatment in patients with past exposure to TB. We investigated TB in the context of the lungs and eyes, however it is likely that a similar strategy would work for TB involvement with other organs.

To persist in the body, *Mycobacterium tuberculosis*, the causative agent of TB infection, needs to evade the immune system. At a later time however, the bacterium also causes inflammation to aid transmission [32]. It is likely that the increased C1q concentration during active TB disease is a result of immunomodulation by the bacterium. According to our current hypothesis, it could be beneficial for *M. tuberculosis* to stimulate C1q production, which is known to have a regulatory influence on T cells [16]. Actual complement activation may not be increased, since an increase in systemic levels of the regulator C1-INH was also observed [33]. Higher C1-INH levels can counteract any increased complement cascade initiation that could otherwise be caused by higher C1q

levels. It is currently unknown exactly how *M. tuberculosis* would instruct the human body to produce more C1q.However, we do know that the additional C1q production occurs systemically, as both increased C1q protein and increased C1q gene expression are detected in whole blood. We recently identified that among the circulating cells a specific subsets of monocytes are the main producers of C1q during active TB disease [34]. Further research will have to elucidate through which signals and pathways *M. tuberculosis* or TB-infected macrophages in the lung granuloma instruct upregulation of C1q in circulating cells.

Increased C1q concentration can serve as a biomarker for active TB disease, and may be used in clinical diagnostics to identify active TB disease, possibly in combination with other biomarkers. In light of spreading (multi)drug-resistant TB strains, therapy to stop immune evasion by *M. tuberculosis* and allow the immune system itself to clear the bacterium, should be investigated [35]. As increased C1q may play an immunomodulatory role during active TB disease, it could be a target in those cases where traditional drugs fail to eradicate the infection. Systemically lowering the C1q concentration over a prolonged period of time may be difficult and may have side-effects. However, blocking the downstream effects of high C1q concentrations may provide an opportunity. The initial literature describing interaction between C1q and T cells in autoimmunity hypothesized that C1q can alter $CD8^+$ T-cell function through metabolism [16]. According to their theory, this would involve the receptor for the globular heads of C1q (gC1qR), which is expressed primarily on mitochondria, but also on the cell surface [36]. When the interactions between C1q and T cells are further elucidated, an inhibitor of this interaction or its downstream signaling could be interesting in unleashing T cells to fight TB infection, analogous to the immune checkpoint inhibition which has revolutionized cancer treatment in the last decade [37].

The role of complement in pregnancy and preeclampsia

We investigated C1q and also Factor H in a very different situation in **chapter 4**: pregnancy and the pregnancy complication preeclampsia. During pregnancy, shaping and maintaining the placenta requires a large amount of blood vessel formation, tissue remodeling and subsequently the resulting cellular debris has to be cleared. These are processes in which C1q is known to play a role throughout the body. It is therefore interesting that we observed higher serum C1q concentrations in pregnant women than in nonpregnant controls. Similarly, we also observed higher serum factor H concentration in pregnant women compared to nonpregnant controls, as has been reported before [38, 39]. We postulate that C1q is upregulated during pregnancy to

perform its role in neovascularization and removal of debris from tissue remodeling and invasion of fetal trophoblasts, while increased factor H helps limit alternative pathway complement activation which is known to occur on apoptotic and necrotic bodies [40].

Preeclampsia is an important complication of pregnancy, and a large contributor to maternal and fetal morbidity. It presents during the second half of pregnancy with increased blood pressure and typically also proteinuria. While the etiology is not clearly understood, this condition is thought to originate in improper placentation, a partly dysfunctional placenta, and the stress signals this causes when the fetus grows larger and needs more nutrition [41]. In our research in **chapter 4** we showed that serum factor H concentration was lower in preeclampsia patients than in pregnant women in a control group without preeclampsia. This difference was particularly found in early-onset preeclampsia patients (defined as gestational age below 34 weeks at delivery), which are generally the more severe cases. These findings are in agreement with earlier reports of increased concentrations of the alternative pathway activation fragment Bb in preeclampsia cases [39, 42, 43].

The only known cure for preeclampsia is delivery of the fetus and placenta, although preventative administration of aspirin during high-risk pregnancies has been used with positive results [44]. A better understanding of preeclampsia is therefore needed to improve treatment [45]. The study presented in **chapter 4** indicates a role for (the lower concentration of) factor H in preeclampsia, however it has not been established whether it would be involved in the cause of preeclampsia, or rather be a step in the later pathology. Alternatively, the lower factor H levels may also be a result of increased binding to tissues in the prevention of further pathology. On account of the relatively small differences in factor H concentration between preeclampsia and control pregnancies, and the fact that our study was performed on samples from the end of pregnancy, factor H would not be an obvious biomarker candidate based on this study. A follow-up of this study should therefore focus on a further contribution to the understanding of preeclampsia. To make more definitive statements on a role for factor H in the cause of preeclampsia, factor H would have to be monitored prospectively, before the onset of preeclampsia, in a large cohort of pregnancies. Such a study should aim to learn at which point during the pathogenesis of preeclampsia the decreased concentration of factor H develops, and subsequently whether a therapeutic strategy involving factor H would be feasible.

Autoantibodies to solid-phase C1q

Autoantibodies against C1q are investigated in **chapters 4, 5 and 6** of this thesis. These autoantibodies have been known for decades and are associated with autoimmune disease, mostly with lupus nephritis [46-48]. Interestingly however, anti-C1q autoantibodies are also present in a few percent of the general population, without current signs, or evidence for future development, of autoimmune disease [49]. Interestingly, these healthy individuals with anti-C1q autoantibodies often have normal circulating levels of C1q. This indicates that the anti-C1q antiantibodies apparently do not make complexes with C1q that would lead to their clearance. This is likely explained by the peculiar characteristic of these autoantibodies that they specifically bind to solid-phase C1q, which means C1q that is bound to a ligand or surface. In this situation, C1q undergoes a conformational change, opening up a cryptic epitope which anti-C1q autoantibodies bind to. As these autoantibodies bind to C1q specifically when C1q is engaged with a ligand, they may cause unwanted amplification of immune responses in this location.

Due to the association with autoimmune disease and the hypothesis that anti-C1q binding could contribute further immune activation in a local environment, anti-C1q autoantibodies have been investigated as a biomarker in many diseases. An association between anti-C1q and a certain disease means testing for anti-C1q could aid diagnosis. One such report identified anti-C1q as a possible biomarker for lung involvement in systemic sclerosis [50]. Systemic sclerosis is characterized by fibrosis of the skin and internal organs, with most related deaths attributed to involvement of the lungs and heart. In **chapter 5**, we examined the relation between anti-C1q autoantibodies, systemic sclerosis and its associated lung conditions, most commonly pulmonary fibrosis and pulmonary arterial hypertension. Due to the severity of these conditions, it would be of considerable importance to have a prognostic marker for them as several treatment options are available [51]. However, our study did not support a prognostic value of anti-C1q autoantibodies in systemic sclerosis or its related lung conditions. The systemic sclerosis patients positive for anti-C1q did show a much higher incidence of anti-topoisomerase antibodies, which are themselves reported to associate with lung complications and more severe disease [52]. The overlap between anti-C1q and anti-topoisomerase antibodies would detract from any prognostic value of anti-C1q, therefore anti-C1q autoantibodies are unlikely to be an adequate biomarker for systemic sclerosis or its related lung conditions.

Anti-C1q autoantibodies have been studied in the context of pregnancy as well, multiple studies previously linked anti-C1q to different negative pregnancy outcomes,

but not to preeclampsia [53-56]. In **chapter 4**, we also studied anti-C1q in pregnancy and in preeclampsia patients, and found no significant association between anti-C1q and preeclampsia in the three pooled cohorts from the Netherlands, Finland and Norway. A recent study from Italy concluded that anti-C1q autoantibody levels were actually lower in preeclampsia than in control pregnancy, which we also observed when looking only at the cohort from the Netherlands [57]. However, our other two cohorts did contain equal or higher percentages of anti-C1q positive individuals among preeclampsia patients compared to controls. Careful investigation of demographic and clinical data available did not explain these differences in the population from Italy and the Netherlands versus Finland and Norway. Overall, the two studies combined do not indicate a role for anti-C1q in the pathogenesis of preeclampsia.

Characterization of anti-C1q autoantibodies

Anti-C1q autoantibodies have been studied for decades, largely using serum as a source. This approach has multiple drawbacks, such as the forced focus on polyclonal mix of antibodies and the high salt concentration required to distinguish anti-C1q from immune complexes. To overcome these drawbacks, we set out to isolate C1q-reactive B cells and recombinantly reproduce human anti-C1q autoantibodies in **chapter 6**. We were indeed able to successfully obtain 9 unique clones from 4 different donors, allowing their characterization. We showed that at least two unique epitopes on the C1q protein are targeted by anti-C1q autoantibodies, showcasing their diversity, and that these antibodies indeed specifically bind solid-phase C1q as was described in literature for polyclonal autoantibodies [47, 58, 59]. Since we studied a relatively limited selection of antibodies, it is entirely possible that even more unique epitopes for anti-C1q exist. Furthermore, electron microscopy revealed that multiple molecules of the same anti-C1q monoclonal antibody could bind to the same C1q protein. Although this is not unexpected due to the radial symmetry of C1q, no prior study had found evidence that multiple antibodies would bind to one C1q molecule. All 9 anti-C1q antibodies characterized in this study originated from healthy donors. In ELISA competition assays, we observed that the anti-C1q monoclonals and antibodies purified from SLE patients compete with each other for binding to C1q, showing they target the same or similar (overlapping) epitopes on C1q. Additionally, we showed that the anti-C1q monoclonal antibodies are specific for solid-phase C1q and they were of the IgG isotype, which is also the main isotype of anti-C1q autoantibodies in SLE patients [60, 61]. Based on these factors, we argue that the results in this study are very likely to translate to the anti-C1q autoantibodies found in SLE patients.

The notion that anti-C1q autoantibodies in healthy subjects are identical to those in SLE patients, where they especially associate with lupus nephritis, raises the question why these autoantibodies are harmful in one person but not in the other. It is conceivable that for local pathology to occur, the target of these autoantibodies must be abundantly present and this organ or location must be vulnerable to the effector mechanisms mediated by the autoantibodies. In the case of lupus nephritis, this location is the kidney, more specifically the glomerulus. Indeed, in a mouse model of lupus nephritis, renal pathology by anti-C1q is only observed in the presence of C1q-containing immune complexes [62]. This same study also found that renal damage depended on C1q, complement activation and also on Fcγ receptors. In **chapter 6**, we found that addition of anti-C1q to C1q-containing immune complexes did not increase complement activation. We also investigated Fcγ receptors and observed that their binding to immune complexes markedly decreased by addition of C1q, probably because C1q occupies or masks the binding site for Fcγ receptors on IgG as reported recently [63]. Reversely, binding of Fcγ receptors increased again after subsequent addition of anti-C1q antibodies. We unite these results in **chapter 6** and earlier results by Trouw *et al.*, to hypothesize that complement activation by immune complexes deposited in the glomeruli attracts immune cells, while anti-C1q antibodies on the C1q-containing immune complexes engages Fcγ receptors on these cells to cause the actual renal pathology of lupus nephritis (Figure 1). This model is further supported by a recent finding that administration of C5a receptor-blocking small molecule avacopan in a mouse model of lupus nephritis severely reduced influx of granulocytes and prevented kidney damage [64].

In order for anti-C1q antibodies to be present in the circulation, whether in a healthy individual or in an SLE patient, C1q-reactive B cells must arise. Immature B cells that recognize self-antigens with high affinity in the bone marrow or spleen should undergo apoptosis [65]. In the case of anti-C1q it is less likely that immature B cells will encounter solid-phase C1q however, which may allow them to escape and mature, whereas any B cells recognizing fluid-phase C1q would fall victim of negative selection. Mature B cells may be stimulated upon encountering their antigen in a T cell-dependent or independent manner. T cell-independent stimulation usually occurs for non-protein antigens, based on multivalency to crosslink the B-cell receptor and C3d deposition to stimulate complement receptor 2 [66]. Interestingly, C1q is also multivalent, as we showed that multiple antibodies can bind at once, and solid-phase C1q is in an activated state and will therefore be in close proximity of deposited C3. Still, based on the characteristics of anti-C1q autoantibodies, it is likely they are the product of

T-cell stimulated B cells. The autoantibodies we isolated in **chapter 6** were all of the IgG isotype and many contained a reasonable amount of V-gene mutations. These features are typical of germinal center reactions, which require T-cell help based on peptides presented in class II major histocompatibility complex (MHC) on the B cell. It is unlikely that these helper T cells specifically recognize a peptide of C1q, as they would have been selected out, but they likely recognize a foreign protein to which C1q has bound. A B cell producing anti-C1q would thereby receive help from a T cell recognizing an unrelated foreign protein, analogous to the hapten-carrier effect, but now with two proteins in complex. This is a possible way how anti-C1q-producing B cells can arise and continue to exist and produce these potentially harmful autoantibodies.

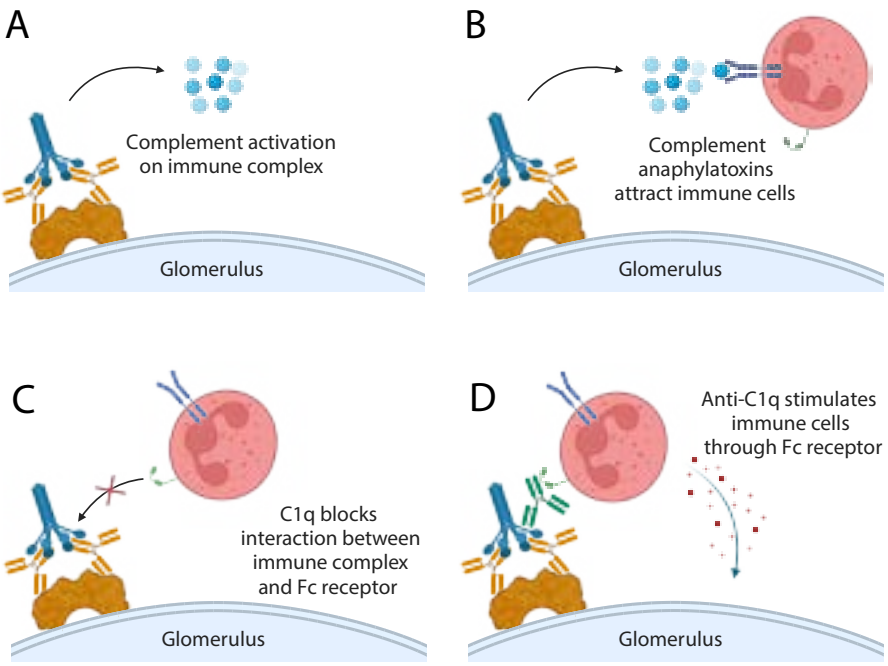

Figure 1. The proposed influence of complement activation, Fc receptors and anti-C1q autoantibodies in the pathology of lupus nephritis. (A) Deposited immune complexes in the glomerulus activate the complement system. (B) Several products of complement activation serve as anaphylatoxins, which are able to attract immune cells such as granulocytes to the glomerulus. (C) The Fc receptors on the newly attracted immune cells are blocked from interacting with immune complexes by the presence of C1q, which masks the binding site for Fc receptors on antibodies. (D) The binding of anti-C1q autoantibodies to C1q-containing immune complexes offers a target for Fc receptors, which causes the immune cells to activate. This process then leads to glomerular inflammation and renal damage in lupus nephritis patients. Created with BioRender.

While anti-C1q autoantibodies can contribute to autoimmune disease, their isolation and reproduction, as described in **chapter 6**, opens the door to new opportunities as well. By specifically recognizing solid-phase C1q, these antibodies are essentially directed towards location where classical pathway complement activation occurs. When coupled to a tracer, these antibodies or their derived Fab fragments may be used to trace C1q binding in tissues, for instance in patients with suspected autoimmune disease or transplant rejection, without the need to take a biopsy. Engineered anti-C1q may also be employed in a therapeutic setting. An aspect of the anti-C1q antibody research we did not describe in this thesis, involves bispecific antibodies targeting both solid-phase C1q and a complement inhibitor such as factor H or C4b binding protein. These engineered antibodies are able to (partially) inhibit complement-mediated cell death in vitro. While further research is required to show efficacy and specificity in more complex models, the engineered anti-C1q antibodies do have potential to concentrate autologous complement inhibitors at the site of C1q activation. Thereby, the antibodies isolated in **chapter 6** may be developed into useful therapeutics for the treatment of disease where classical pathway complement activation plays a deleterious role, such as lupus nephritis.

The research described in this thesis underlines the many roles the complement system, and particularly C1q, play in human physiology and disease. Our research underlines the potential diagnostic value of C1q concentration in active TB disease and ocular involvement in TB. Next to C1q's role as a biomarker, it may also be a target for therapy in TB patients with high C1q levels. If C1q is indeed vital in the regulation of T-cell activity in TB, as proposed here, blocking C1q or its downstream signaling offers great possibilities for therapy to unlock the immune system. C1q also is the target of relatively common human autoantibodies. By studying these autoantibodies at a monoclonal instead of polyclonal level, we gained new insights into both their molecular characteristics and opened the door to new therapeutic opportunities. Most of all, this research urges continuation and validation to increase our understanding of canonical and non-canonical complement biology and hopefully contribute to future therapies.

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