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C1q and anti-C1q autoantibodies in (auto)immunity

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CHAPTER 1

General introduction

Douwe J. Dijkstra

The human immune system

The immune system is a major contributor to human health by protecting against various threats from both inside and outside the body. Humans are constantly exposed to pathogens like bacteria and viruses. However, the immune system is usually able to quickly eliminate these pathogens before we get very sick. The importance of our immune system is underscored by the fact that large parts of it are evolutionarily conserved among mammals and to a degree among other jawed vertebrate species [1]. The human immune system consists of both cellular and humoral (proteins and other molecules secreted by both immune and non-immune cells) components.

The human immune system can be divided into two parts: the innate part and the adaptive part. While these two parts heavily supplement and even depend on each other, they fundamentally differ in their mode of operation. The innate immune system comprises multiple cell types and sets of proteins (such as the complement system), which can react very fast to any threat, are often short-lived, and do not have a 'memory' of past encounters with pathogens [2]. Although there are exceptions to all of these characteristics, the cells and molecules of innate immunity can be seen as first responders, they start acting within seconds to minutes of the appearance of a potential threat. They are important in clearing the bulk of pathogens, but also dead cells and debris, and are mainly generalists which rely on a limited set of pattern recognition receptors to identify their target [3]. Upon recognition and elimination of pathogens, innate immune cells can also communicate with each other, through the release of cytokines to either enhance or decrease the local state of inflammation and immune activation. This process is especially important for the activation and instruction of the adaptive immune system.

The adaptive or acquired immune system takes a longer time (in the order of days up to one or two weeks) to develop a full response upon the first encounter, which will then be very specific for the current threat. The main cell types in adaptive immunity are B and T lymphocytes, of which there is a continuous generation of a pool of naïve cells. Each of these cells have a different receptor with which they recognize a specific target, their antigen. When these B and T cells encounter their antigen, they require an extra stimulatory signal instructing them to proliferate and specialize, from a combination of other activated immune cells, the activated complement system or other danger signals [4]. This instruction of adaptive immune cells by the innate immune system is an important example of the communication between the two different parts of the immune system. However, the interaction can also be in the other direction, from

adaptive to innate, for instance when immunoglobulins produced by B cells bind their antigen and instruct complement and innate cells to react against this antigen.

When a particular threat (like an infection) has passed, it is crucial that the cellular immune response contracts and the inflammation stops. At the same time, some cells of the adaptive immune system, so-called memory cells, continue to exist for years and have the ability to quickly proliferate upon re-infection. The retaining of memory cells helps the adaptive immune elicit a faster and stronger response upon the next encounter of the same pathogen and thereby provides better protection. This process is exploited in the case of vaccination. A vaccine typically contains a dead bacterium or virus, or one of its components, to mimic an infection, and to which the body raises an initial immune response including memory cells. Subsequently, when an actual infection with this particular pathogen occurs, there are already specific B and/or T cells in place to quickly reactivate and respond against the infection.

The complement system

A collection of interacting proteins called the complement system forms an integral part of the innate immune system. While the complement system was initially named because it 'complemented' immunoglobulins in the killing of bacteria, many additional functions for complement have since been described [5]. The complement system consists of the classical, lectin and alternative pathways, which all converge into the terminal pathway. The chain of activating molecules starting from the classical pathway are termed C1 to C9, while cleaved products are denoted with the addition of an 'a' or 'b' [6]. Various inhibitors, which can be found both in fluid phase and on the surface of cells, act on most of the intermediate molecules to regulate these activation pathways. In this thesis, there is much focus on C1q, which is the recognition protein for the classical pathway. It consists of six arms with an N-terminal collagen-like region (CLR) and a C-terminal globular head (GH), which are linked at the CLR. Each arm is a heterotrimer of polypeptides (A, B and C chains) connected through disulfide bonds. The elaborate architecture of 18 polypeptide chains gives C1q its large size of approximately 450 kDa and it is abundantly present in the circulation, with a serum concentration around 0.2 mg/ml. While the majority of complement proteins are synthesized in the liver, C1q is, in contrast, produced by several types of immune cells, mainly monocytes, macrophages and dendritic cells [7].

The GHs of C1q can bind to various isotypes of immunoglobulins, C-reactive protein (CRP) and dead or dying cells, and thereby allow its associated enzymes C1r and C1s

to cleave C4 and C2. The activated fragments C4b and C2a together deposit on the cell surface of the target and form the convertase that activates C3. Similarly, the lectin pathway is initiated by mannose-binding lectin (MBL), collectins or ficolins, which also use C4 and C2 to activate C3 and the further complement cascade. Addition of a cleaved C3b to C4b and C2a completes the convertase which activates C5. The alternative pathway is constitutively activated by the spontaneous hydrolysis of C3, which binds factor B and allows factor D to cleave and activate another molecule of C3 into C3b and C3a and thereby continuously challenges defense by complement regulators on the cells in its immediate environment. Foreign cells and particles are not protected by human complement regulatory proteins and will therefore be subject to alternative pathway complement activation. Because the deposition of C3b and subsequent recruitment of factor B can cause the activation of yet more C3, the alternative pathway also serves as an amplification loop for the classical and lectin pathways.

After the activation pathways converge at C3 activation, the complement cascade reaches its end when the complement proteins C5b, C6, C7, C8 and 18 copies of C9 form the membrane attack complex (MAC), thereby puncturing the cell membrane, aimed at causing cell lysis [8]. Apart from direct lysis by the MAC, the deposition of activated complement components, especially C3b, activates immune cells with complement receptors to act against the opsonized target. Both the complement system as a whole, and individual proteins outside the context of complement activation, also have functions other than defense against pathogens [9]. For example, when antibodies bind their target and form immune complexes in the blood, complement deposition allows their clearance from the circulation through interaction with complement receptor 1. Similarly, binding of C1q and a controlled amount of complement activation on dying or dead cells can be beneficial. It helps phagocytes in their waste disposal task via complement receptors, while moderation is needed to prevent formation of the MAC and spilling of cellular content into the environment [10]. Deficiency of C1q or other classical complement pathway proteins is associated with inadequate clearance of cellular debris, and often leads to autoimmune disease, which will be discussed later. This adds to the status of the complement system as an indispensable component of healthy human physiology.

B cells and antibodies

The human adaptive immune system has three major classes of molecules to recognize antigens: antibodies (also called immunoglobulins; Ig), T-cell receptors and the major histocompatibility complex (MHC, also called human leukocyte antigen; HLA) proteins

[4]. Of these, antibodies are able to bind the broadest range of antigens, with proteins as well as lipids, polysaccharides and small chemicals as possible targets, and have the highest potential binding strength. Antibody classes are split into five isotypes based on the structure of their HC constant domains: IgA, IgD, IgE, IgG and IgM. Of these, IgA can be further divided into subclasses IgA1 and IgA2, while IgG similarly consists of subclasses 1 to 4. IgG is the most abundant isotype in human blood, and is the focus of antibody research in this thesis. IgG antibodies are produced as a symmetric molecule of four peptide chains which are linked by disulphide bonds: two identical heavy chains (HC) and two identical light chains (LC) (Figure 1). Other isotypes generally follow a similar architecture, although modification and/or multimerization occur for certain isotypes. Each LC consists of one variable domain and one constant domain, which can be either of the kappa or lambda type. An HC also contains one variable domain, but three or four constant domains, depending on the isotype. The LC together with the variable domain and first (counting from the N terminus) constant domain of the HC form the 'Fragment, antigen binding' (Fab), while the further constant domains of the two HCs form the 'Fragment, crystallizable' (Fc) [11]. Both Fabs are linked to the Fc at the hinge region, which is also the site where the two HCs are covalently linked through disulphide bonds. The specificity of an antibody is determined by the composition of the variable domains, particularly by the complementarity determining regions (CDRs) within. These CDRs contain the amino acids that come into contact with the antigen, the part of the antigen which interact with the antibody is called epitope.

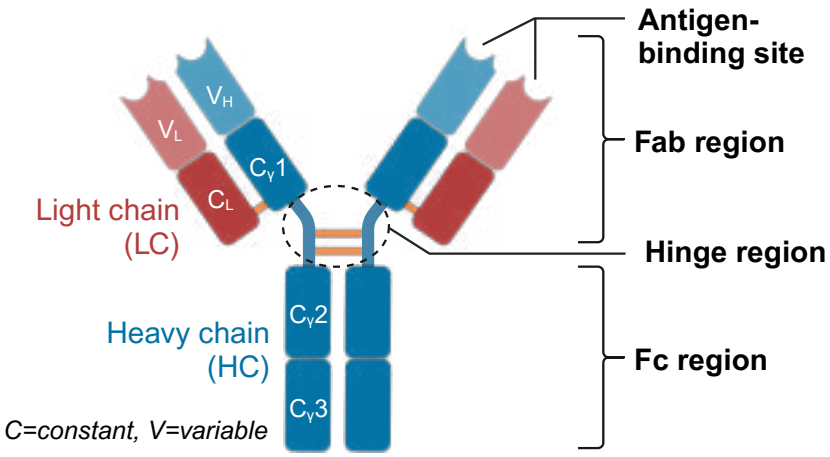

Figure 1. Overview of the major structural components of antibodies, represented by IgG, which is the most abundant isotype in human blood. Created with BioRender.com

The production of antibodies is performed by plasma B cells, whose progenitors originate from stem cells in the bone marrow. When these cells differentiate into pre-B cells, their specificity is determined for the first time. In stem cells, the germline DNA sequence from which the variable domains of antibodies will later be made contain many V, D and J genes, but pre-B cells randomly select one of each in a process called V(D)J recombination [12]. This process occurs separately for the HC and the LC, and may be repeated if an unproductive rearrangement has been made. The HC locus contains about 45 functional V genes, 23 D genes and 6 J genes, giving rise to a large number of unique combinations and large diversity between HCs. The LC locus does not have D genes, but contains 35 V genes and 5 J genes for kappa LC, or 30 V genes and 4 J genes voor lambda LC, multiplying the number of unique HC-LC pairings even further [4]. Another large contributing factor to diversity among antibodies is the addition or deletion of nucleotides at the junctions between V, D and J genes (or between V and J for the LC) during the recombination process, leading to billions of possible clones. When these steps have occurred, the cell has become an immature B cell, with a unique specificity which it expresses as a membrane-bound immunoglobulin molecule, the B-cell receptor. These cells then leave the bone marrow to mature in peripheral lymphoid tissue, such as lymph nodes and the spleen.

Most naïve B cells are follicular B cells, which travel though the blood stream from one lymphoid organ to another until it encounters an antigen. When a B cell recognizes an antigen with its B-cell receptor, other receptors on the B cell can provide extra stimulation (Figure 2A). This can be facilitated for instance by complement receptor 2 binding to deposited C3 fragments on the antigen, or by Toll-like receptors binding their targets of bacterial or viral origin [13]. Activation of these receptors functions like an additional verification that an antibody response against this particular antigen is indeed desirable. In some cases, especially with non-protein antigens which are multivalent, B cells can activate and proliferate without the help of other cells [14]. However, responses to most proteins require the stimulation of helper T cells. A protein antigen bound by the B-cell receptor is internalized, processed by the internal machinery and then presented in the form of peptide fragments in the MHC class II molecules on the cell membrane [15]. An activated helper T cell recognizes specific peptides embedded in MHC class II and provides a stimulating signal to the B cell via membrane proteins such as CD40 (on the B cell) and CD40 ligand (on the T cell), as well as cytokines produced by the helper T cell [16]. Development of an antibody response therefore requires not only a B cell recognizing the antigen, but often also a specific T cell.

Activated B cells migrate into the lymphoid follicles of lymphoid organs to form a germinal center, where they rapidly proliferate. Under stimulation from specialized follicular T

helper cells and follicular dendritic cells, B cells undergo the so-called germinal center reaction, including affinity maturation and isotype switching. Isotype switching consists of a DNA recombination event where the B cell switches to produce another constant domain for the HC [17]. Affinity maturation is caused by somatic hypermutation, a process where variable domain-encoding DNA in proliferating B cells is subject to an exceptionally high rate of mutation [18]. When this process leads to a higher affinity B-cell receptor, this clone has favorable competition for antigen capture and T cell stimulation, and therefore a higher chance of survival and further proliferation. After undergoing the germinal center reaction, B cells differentiate into either long-lived plasma cells, which produce antibody while residing in the bone marrow, or into circulating memory cells [19]. B cells can also be stimulated extrafollicularly, where limited isotype switching occurs and short-lived plasma cells are generated.

The antibodies produced by B cells after a germinal center reaction have high avidity for their antigen and are generally of an isotype other than IgM. For their role in immune defense, antibodies rely on several effector functions (Figure 2B), which may be altered by structural modification of the antibody [20]. Firstly, binding to a microbial antigen may allow neutralization of this microbe by blocking critical surface components or neutralization of toxins [21]. Secondly, the Fc domain of antibodies is the ligand for Fc receptors on various immune cells. Antibodies have varying avidities for the different types of Fc receptor depending on isotype and subclass [22]. Through Fc receptors, antibodies may allow phagocytosis by phagocytes, or antibody-dependent cellular cytotoxicity by natural killer cells, granulocytes or macrophages. Lastly, clustered Fc domains of most IgG subclasses and IgM also form a ligand for C1q after a conformational change upon antigen binding [23]. Activation of the complement system may result in direct lysis of the targeted microbe, opsonization with deposited complement fragments and stimulation of inflammation. These effector functions benefit greatly from enhanced avidity by binding antigens with multiple Fab arms. Furthermore, interactions between the Fc regions of antibodies allow clustering upon antigen binding, thereby further increasing total avidity for the antigen and for several receptors and C1q [24]. Antibodies of the most common isotype in blood, IgG, can remain in the circulation for a long time, with a half-life up to 3 weeks for most IgG subclasses. To accomplish this, IgG binds to the neonatal Fc receptor, which rescues IgG from degradation in lysosomes and recycles it to the cell surface. The combination of these effector functions, the broad range of possible targets and the capacity for high avidity and specificity make antibodies a potent part of the immune system, and a highly-explored option for therapeutics, of which already over 100 have been approved.

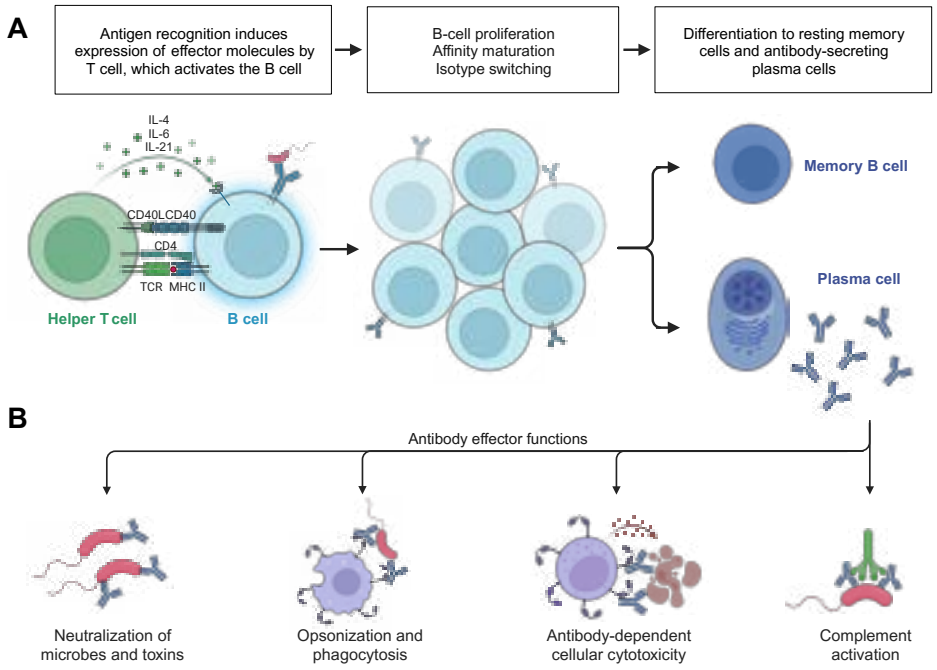

Figure 2. B cell development and antibody effector functions. (A) In addition to recognizing their antigen, B cells must receive stimulatory signals, often from helper T cells, in order to be activated. They can then enter a germinal center, in which they mature their specificity and switch antibody production to a new isotype. These B cells can differentiate into long-lived memory cells, or antibody-producing plasma cells. (B) Antibodies have various effector functions, each with varying effectiveness per antibody isotype. Created with BioRender.com

Tolerance, autoimmunity and autoantibodies

As the immune system is capable of mounting a strong and aggressive response to a wide range of antigens, it is of great importance that all molecules produced by the organism itself are tolerated. In the case of both B cells and T cells, antigen receptors with a large repertoire of specificities are generated through random events like recombination and mutation. Sometimes, these antigen receptors may turn out to recognize a self-antigen, causing a risk of attacking the body's own tissues. Therefore, when a naïve lymphocyte in its generative lymphoid organ recognizes a self-antigen, it either undergoes apoptosis, edits its receptor to no longer recognize the self-antigen (in the case of B cells), or develops into a regulatory cells (in the case of some T cells) [25]. As opposed to this central tolerance mechanism for naïve lymphocytes during their maturation, peripheral tolerance can be formed in mature lymphocytes. When mature lymphocytes recognize their antigen in the absence of stimulating or inflammatory signals, they die by apoptosis or enter a state of anergy, where they are no longer

responsive to their antigen [26]. Additionally, peripheral tolerance is maintained by regulatory T cells, which suppress lymphocytes specific to self-antigens.

Under certain conditions, a break in the tolerance towards self-antigens may occur and the immune system may react against autologous antigens. The recognition of self-antigens by the immune system is called autoimmunity, and when this has pathogenic effects and causes symptoms, it is termed autoimmune disease. Autoimmune diseases can be either systemic or local depending on the distribution of the self-antigen, and are often chronic due to the continued availability of antigen and the amplification mechanisms in the activated immune system. While the exact cause of autoimmune diseases is often difficult to pinpoint, they are generally ascribed to certain genetic risk factors, environmental factors and triggers of immunity, such as infections or tissue injury [27, 28]. Inadequate disposal of waste and cellular debris can cause exposure to DNA, histones and other intracellular antigens which are spilled from these cells. These self-antigens may provide a target for autoreactive lymphocytes which would not be available under physiologic conditions.

In this thesis, the most prominent aspect of autoimmunity is the occurrence of antibodies against autologous proteins, so-called autoantibodies. These autoantibodies result from B cells which have escaped the regulatory pathways of the immune system that control self-antigen recognition. This may be because central tolerance failed to eliminate an autoreactive naïve B cell, or because a B cell originally recognizing a (slightly) different antigen matured and developed cross-reactivity to a self-antigen. One type of autoantibody, which is discussed in several chapters of this thesis, recognizes C1q. Anti-C1q autoantibodies occur in a few percent of the general population, meaning that a breach in the tolerance to this self-antigen is relatively common. However, anti-C1q is also strongly associated with the occurrence and severity of kidney injury (lupus nephritis) in patients with systemic lupus erythematosus [29]. One reason that anti-C1q autoantibodies are relatively common may be that they specifically recognize solid-phase C1q (Figure 3). When C1q binds one of its ligands, like a cluster of IgG Fc domains, it docks on to this ligand with its six globular heads. In this process, a conformational change occurs in the C1q molecule, C1q in this state is called solid-phase C1q [30]. The conformational change makes the epitope of anti-C1q autoantibodies available [31]. Due to this property, C1q and its autoantibody do not interact in the solution, but only where ligands of C1q are present. For this reason, it is possible for individuals to have anti-C1q autoantibodies and C1q abundantly present in the blood, while remaining healthy.

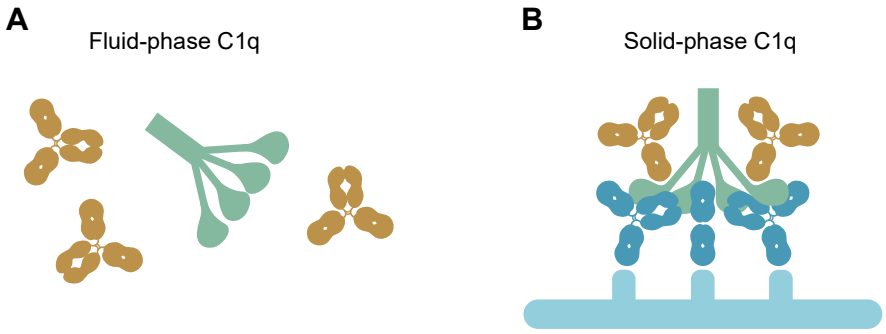

Figure 3. C1q can exist in multiple conformational states, which determines whether anti-C1q autoantibodies will bind. (A) When C1q is in solution, not bound to any ligand, the epitope of anti-C1q autoantibodies is not available. (B) Upon binding to a ligand such as a hexameric cluster of IgG antibodies, C1q changes conformation. The epitope for anti-C1q autoantibodies now becomes available and the autoantibodies bind to this 'solid-phase' C1q.

Scope of this thesis

This thesis investigates multiple facets of the complement system, specifically the classical pathway initiator C1q, and autoantibodies to complement proteins in various diseases. **Chapter 2** reviews the different roles complement activation and regulation can play in the rheumatic diseases systemic lupus erythematosus, rheumatoid arthritis and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis. Previous research has identified C1q as a possible biomarker for active lung infection by tuberculosis bacteria, **chapter 3** confirms and builds on this. This work investigates the value of C1q as a diagnostic tool for active tuberculosis infection and specifically uveitis complications.

In **chapter 4**, we continue to examine C1q, but also alternative complement pathway regulator Factor H and autoantibodies against these complement proteins. The setting of this chapter is pregnancy, and the common pregnancy complication preeclampsia. Anti-C1q autoantibodies are also the subject of **chapter 5**, where we find that the presence of these autoantibodies is not fit as a biomarker for involvement of the lung in systemic sclerosis. Subsequently, **chapter 6** reports on the isolation of human anti-C1q autoantibodies and their characterization on a monoclonal level, which may help to better understand their involvement in disease. Finally, **chapter 7** summarizes and discusses the results and impact of the research presented in this thesis, and relates it to the available literature.

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