Complement biology in health and disease

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CHAPTER 1

GENERAL INTRODUCTION

Adapted from Clin Exp Immunol. 2017 May;188(2):183-194.

THE HUMAN IMMUNE SYSTEM

Humans have evolved alongside various microorganisms and many of these microorganisms can be pathogenic. Therefore, a defence system has to be in place to achieve resistance against pathogenic organisms. The immune system is versatile and consist of many components. Traditionally, the immune system has been split in to two different subsystems; the innate and adaptive immune system. The innate immune system is, next to physical barriers, the first line of defence. It is able to distinguish self from non-self and attack invaders in an orchestrated manner very rapidly. The adaptive immune system adds to an ongoing innate immune response. The adaptive immune receives information from the innate immune system, and tailors the response towards the specific pathogen encountered. Therefore, the adaptive immune response is a delayed reaction and is also able to develop memory. Upon encountering the same pathogen again, the adaptive immune system will recognise it and respond much faster and stronger. This principle forms the basis for vaccine development. Both the innate and adaptive immune systems consists not only of cells, but also of a humoral components. The complement system is a major humoral part of the innate immune defence [1].

THE COMPLEMENT SYSTEM

The complement system is an "ancient" cascade of proteins which has been first described in the 19th century [2]. The complement system has been shown to have many different functions, and three main functions have especially been well documented: opsonisation, chemotaxis and lysis.

The complement system is part of the innate immune defence and it functions as a cascade of proteases that in an enzymatic fashion activate each other. Complement comprises, next to a set of soluble proteins, also of several membrane bound complement regulators and receptors. The complement cascade can be activated via three different pathways, the classical pathway (CP) the lectin pathway (LP) and the alternative pathway (AP). These pathways are activated via different recognition molecules and activation results in the formation of C3 convertases, C4b2a by the CP and the LP and/or C3bBb via the AP. The C3 convertase cleaves C3 into C3a and C3b, where C3a serves as a chemo-attractant and C3b serves as an opsonin. C3b becomes covalently bound to its target via its thioester that binds to amine and carbohydrate groups on the activating surface. When a threshold of activation is reached and another C3b binds to the C3-convertase, the C5-convertase is formed. The C5-convertase will cleave C5 and this initiates the terminal pathway resulting in the generation of C5a and a multimeric complex called the membrane attack complex (MAC) C5b-C9 which eventually can cause lysis of cells [3] (**Figure 1**).

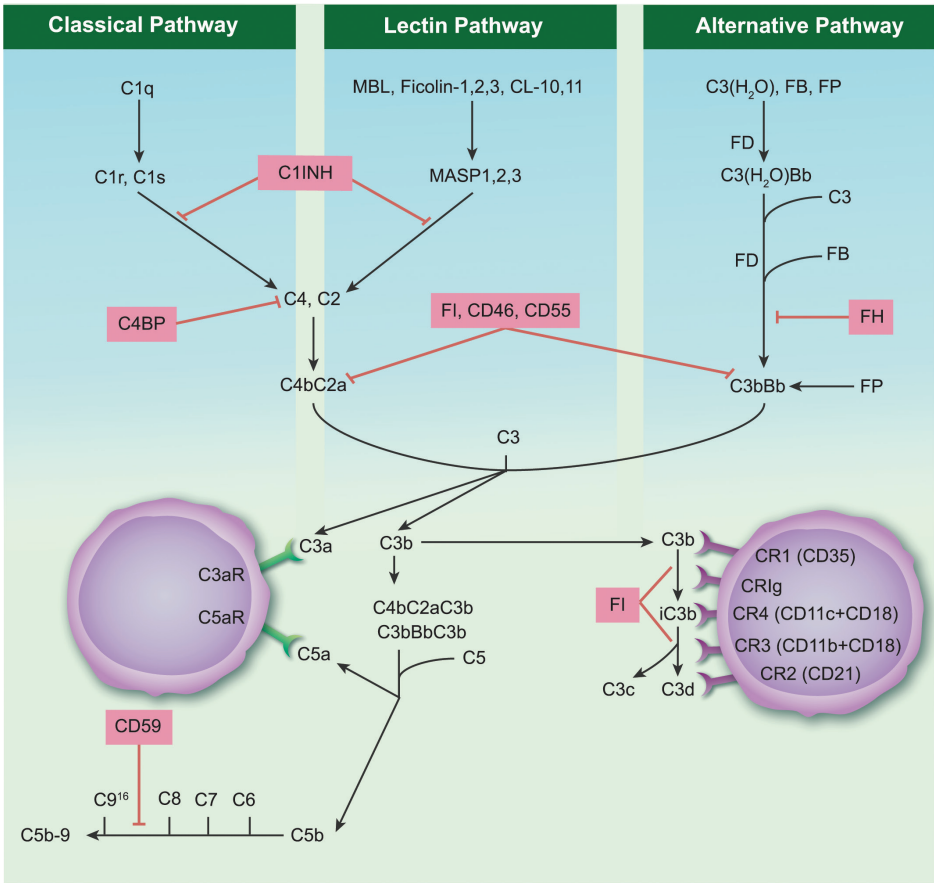

Figure 1. Schematic representation of the complement system.
The complement system can be activated via three different pathways: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). These pathways have their own sequential manner in forming a C3 convertase: C4b2a or C3bBb. These C3 convertases cleave the central component C3 generating two activation fragments, C3a and C3b. The C3a is able to bind its anaphylatoxin receptor the C3aR, whereas C3b can opsonize a target membrane. C3b and its further degradation products, iC3b, C3c and C3d/C3dg are able to bind various complement receptors (CRs). Additionally, C3b can bind to the former C3 convertase which then results in formation of the C5 convertase: C4bC2aC3b or C3bBbC3b. The C5 convertase cleaves C5 in two activation fragments C5a and C5b. C5a can bind to its anaphylatoxin receptors C5aR1 and C5aR2, whereas C5b marks the start of the formation of the membrane attack complex (MAC). In a sequential manner C5b, C6, C7, C8 and up to 16 molecules of C9 bind together to form a MAC. Various inhibitors of this system are marked in pink boxes. MBL = mannose binding lectin; MASP = MBL-associated serine protease; FB = factor B; FP = factor P; FD = factor D; FH = factor H; C1INH = C1 inhibitor; FI = factor I (FI), C4BP = C4b-binding protein; CR = complement receptor.

Apart from these "traditional" activities of the complement system in attacking invading pathogens, it has become clear that its effector functions extend to instruction of the adaptive immune system and to several physiological processes. A large part of these effects are translated into cellular effector functions via a set of complement receptors (CR), specific for proteolytically-cleaved complement fragments. CR1 (CD35) is both a complement receptor for C3b, iC3b and C4b and a complement inhibitor, by competing with FB for C3b binding and by functioning as a cofactor for FI [4]. CR2 (CD21) binds C3b, iC3b and C3d while CR3 (MAC-1, CD11b/CD18) and CR4 (gp150/95, CD11c/CD18) only bind iC3b [5]. Also, for C1q several receptors have been described, although the relative contributions of these receptors and their functions is not resolved yet [6-9]. Next to receptors for the complement opsonins, also a set of receptors can be triggered by the anaphylatoxins, C3a and C5a. For C3a one receptor is known, the C3aRwhereas for C5a two receptors have been identified; C5aR1 (CD88) and C5aR2 (Figure 1). The different cellular expression profiles of these receptors will be outlined below.

Next to activators, the complement system comprises of both fluid phase and membrane bound regulators to keep complement activation in check. C1-inhibitor (C1INH) is a circulating complement regulatory protein which can inactivate C1r, C1s, MASP1 and MASP2, thereby preventing/limiting complement activation via both the CP and the LP. C4b-binding protein (C4BP) acts as an inhibitor by accelerating the decay of the C3 convertase and is a cofactor for FI mediated cleavage of C4b and C3b. In a similar way, FH serves as a cofactor for FI mediated cleavage of C3b/iC3b. Likewise, the membrane bound regulators of complement, CD46 or Membrane Cofactor Protein (MCP), serves as a cofactor for FI. Additionally, CD46 can bind the C3 activation fragments. CD55, Decay Accelerating Factor (DAF) accelerates the decay of C3 convertases. Finally, CD59 or protectin inhibits the binding of C9 to the C5b-8 complex thereby preventing the last step needed for MAC formation [10]. The membrane bound regulators, CD46, CD55 and CD59, are expressed on all circulating cells, including all the cell types addressed in this review [11]. It is conceivable that the expression of the whole arsenal of membrane bound complement inhibitors is necessary to protect these cells for the high levels of complement in circulation or in the local environment where they reside.

COMPLEMENT PROTEIN PRODUCTION

Complement has been mostly considered in the systemic compartment and serum levels of most components of the complement system, including C3, C4 and MBL are derived from the liver and produced by hepatocytes [12, 13]. Nonetheless, other tissues also contain cells capable of complement production. For example endothelial and epithelial cells are also able to secrete various complement components [14] (Figure 2). However, for C1q, Properdin/Factor P (FP) and Factor D (FD) it has been shown that the major/only site of production is outside the liver [14-20]. Even for complement proteins which have their major source within the liver, there is increasing evidence

that many cells can produce these complement factors locally, thereby contributing to local processes (**Figure 2**).

We have reviewed the production of complement proteins by cells of the immune system [21]. From several cell types such as the monocytes, macrophages and the dendritic cells (DC) their complement secretion is well studied and these cells seem to possess the capacity to produce locally all proteins needed to form fully functioning complement pathways. On the other hand, for other cell types the repertoire of complement proteins that is produced is less well documented. Secretion of the recognition molecules of the LP by immune cells is hardly addressed. The same holds true for the natural killer (NK) cells where the focus seems to have been on expression of complement receptors. Also other innate immune cells such as eosinophils and basophils have not been elaborately studied in relation to complement secretion.

Local complement production not only adds to the total pool of complement proteins that circulates, but influences other local processes via paracrine or autocrine interactions. An important example is the production, targeted secretion and local activation of complement in the T cell – DC synapse [22]. Another exciting example is the production and intracellular activation of C3 and C5 as recently reported to be operational in human T cells [23, 24].

Taken together, it seems that various immune cells have the capacity to form fully functioning complement pathways in their direct environment. This is especially of importance for sites where the access to serum complement is initially restricted. Because of the presence of additional C3/C5 cleaving enzymes, local secretion of C3 and C5, and the expression of the anaphylatoxin receptors, various cells are capable to create an environment that is required for autocrine stimulation with complement proteins which acts independent of the “traditional” complement cascade.

COMPLEMENT PROTEINS HAVE FUNCTIONS OUTSIDE THE COMPLEMENT SYSTEM

There is a growing body of evidence indicating that local secretion of complement proteins plays an important role in regulating physiological processes even in the absence of further complement activation. For example, C1q has effector functions that are outside the scope of “traditional” complement activation. C1q exerts effects during pregnancy (where it is involved in remodelling of the maternal decidua), embryonic development, coagulation process and neurological synapse function (reviewed by Nayak et al. [25]). C1q can also serve, in the tumour microenvironment, as a tumour promoting factor by favouring cell adhesion migration and proliferation, independent of complement activation [26]. More recently, C1q has been implicated to be of importance in the metabolic reprogramming and regulation of activated CD8+

T cells [27]. Altogether, it is clear that complement proteins also function outside its traditional functions, an area that is still relatively ill defined. Thus, complement can have an important role in immune regulation and immune cells have been identified as an additional source for local complement activation. Likewise, a new dimension has been provided by the observation that there might even be an intracellular role for complement and complement activation.

INTRACELLULAR COMPLEMENT ACTIVATION

The paradigm that complement solely affects the extracellular space has quite recently been challenged. For example, it was described that $CD4^+$ T cell have intracellular stores of C3 and C3a, and that C3a can be generated intracellular by Cathepsin-L (CTSL). Subsequently, the newly generated C3a is able to bind to the intracellular C3aR where it is linked towards a survival mechanism mediated by mTOR [24]. It is suggested, that the phenomena associated with intracellular C3 are not exclusive to the $CD4^+$ T cells, but that C3/C3a stores are also found in both other immune cells and non-immune cells. Furthermore, it was recently reported that FH can be internalized by apoptotic cells (Jurkat T cells) where it did not become degraded but instead could directly bind to CTSL. The FH was then able to function intracellular as a cofactor for CTSL mediated cleavage of C3. Therefore, it was hypothesized that this could be a consequence of FH binding to both CTSL and C3, thereby bring them in proximity of each other [28]. Besides a role for intracellular C3 and C3aR, it is reported that there is also a role for intracellular C5 and C5aR1 in human T cells. In these studies, the hypothesis was put forward that the NLRP3 inflammasome in T cells receives signals via intracellular engagement of C5aR1, which increases the expression of *IL1B* and induces the production of ROS, thereby activating the inflammasome [23]. It therefore appears that several complement components are involved in intracellular processes. This new concept for intracellular complement has been referred to by Kolev et al. as the ''Complosome'' [29]. These newly described functions for complement components opens up exiting new opportunities to endeavour.

COMPLEMENT IN DISEASE

Most systems in biology need to be balanced properly, too little or too much activation or inhibition can cause a variety of problems. This also holds true for the complement system. Too much activation can cause tissue damage which can give rise to further immune responses and improper healing of the damaged tissue [30]. Too little complement activity can make the body vulnerable to various infections and even predispose to autoimmunity. In this thesis, several aspects of the regulation and activation of complement proteins have been investigated in relation to three difference diseases/conditions. These will be introduced shortly below.

Osteoarthritis

Osteoarthritis (OA) is a heterogenous joint disease which has long been viewed as the result of wear and tear of the joint. In recent years, however, it has become clear that the disease is much more complicated. Clinical symptoms of OA are pain, stiffness and disability of the affected joints. On the histological level, there is cartilage loss and structural abnormalities of both the joint and the soft tissue [31]. The pathophysiology of OA has been thought to be cartilage driven, however, additional roles for both bone and synovial tissue have been identified. Inflammation and innate immune responses may contribute to development and progression of OA by negatively affecting the balance of cartilage matrix repair and degradation [32]. There is evidence that complement plays a role in the pathogenesis of OA and the MAC has been detected in cartilage [33]. Additionally, C5 deficient mice were resistant to disease development in a collagen-induced arthritis model [34]. Released cartilage fragments may trigger complement activation and thereby promote further joint pathology [35-37].

Tuberculosis

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). Most people infected by *Mtb* remain latently infected (LTBI) and only a minor portion (5-10%) of these infected subjects progress to active TB disease [38]. Most recent estimates from the World Health Organization (WHO) suggest that 10 million people developed TB disease and that TB caused 1.3 million deaths in 2018. Clinical symptoms of active TB are coughing (possibly including blood), chest pain, fatigue, fever, night sweat and loss of appetite. The prognosis of TB is good if the treatment protocols are completed. Prognosis also depends on other factors such as co-infection with HIV and development of complications (such as pulmonary fibrosis, disseminated TB) [39]. Although diagnosis and successful treatment has averted mortality in many cases, there is a clear need to improve the gap that still exists between detection and treatment [38]. The pathogenic life cycle of *Mtb* starts when an individual with active TB coughs and the *Mtb* spreads via droplets in the air. The *Mtb* can be inhaled by an individual and deposit in the lower lungs of a new host. The *Mtb* attracts macrophages to the surface which subsequently become infected. The infected macrophages can then migrate to deeper tissues, further transporting the bacteria. Next, a granuloma structure can be formed and the *Mtb* can remain hidden and dormant for decades in this structure. These individuals, though infected, are asymptomatic and therefore classified as LTBI (**Figure 2**). Currently, most tests fail to discriminate between individuals that are latently infected and those that have active disease since they rely on the identification of immune recognition of *Mtb* [40]. Both the diagnosis and the monitoring of the treatment efficacy, could be aided by biomarkers of active TB disease. In various (biomarker) studies, complement genes have been indicated to be of interest for this purpose [41-44].

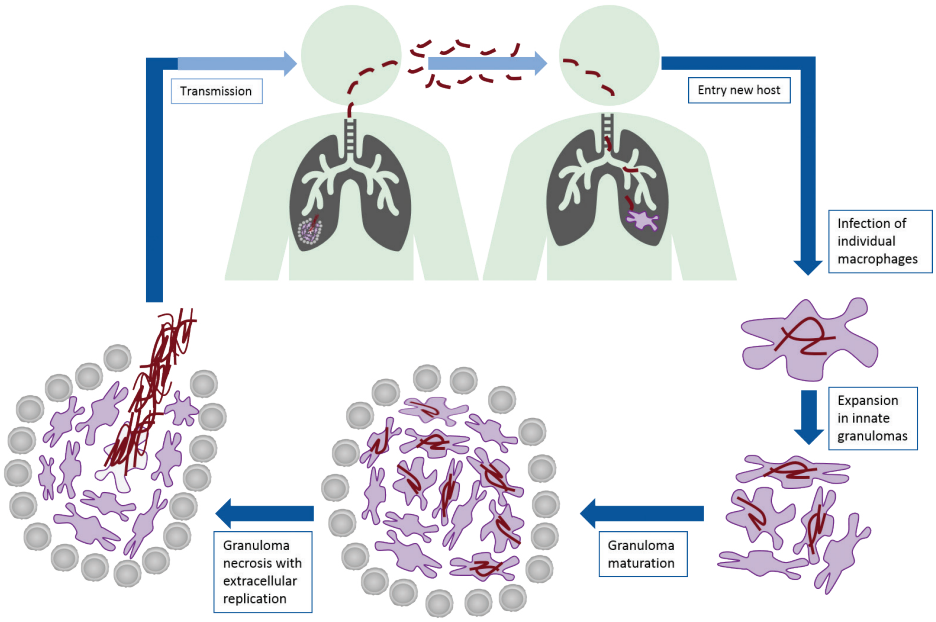

Figure 2. Schematic representation of the pathogenic life cycle of *Mycobacterium tuberculosis* (adapted from Cambier et al Cell 2014 [45]).
Infection with *Mycobacterium tuberculosis* (*Mtb*) occurs when fine aerosol particles containing *Mtb* are coughed up by an individual with active tuberculosis (TB) disease. These particles are deposited in the lower lungs of a new host where the bacteria recruits macrophages to the surface of the lung. These macrophages become infected and transport the bacteria across the lung epithelium to deeper tissue. New macrophages are recruited to the original infected macrophage, initiating the formation of a granuloma. The granuloma is an organized aggregate of macrophages and other immune cells, as lymphocytes. In the early stage of the granuloma the infection can still expand to newly arriving macrophages, later on, as adaptive immunity develops, the granuloma can restrict the bacterial growth. In this stage a patients is latent infected with TB (LTBI). In a minority of the LTBI patients (5-10%) the disease can progress to active TB, where the infected granuloma undergoes necrosis resulting in the formation of a necrotic core that supports bacterial growth and allows transmission to the next host.

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disorder involving multiple organ systems. SLE is presumably caused by a combination of genetic susceptibility and environmental triggers and the incidence is higher in women [46]. Autoantibodies are present in SLE and many of these antibodies target nuclear antigens, which are commonly found already before the onset of SLE [47]. SLE has a heterogenous clinical presentation which often involves skin rashes (butterfly rash), fatigue, joint pain, joint swelling, anaemia, fever and weight loss. There is currently no cure for SLE and because of the heterogenous clinical symptoms combined with the complexity of the factors associated with disease onset and pathogenesis, the treatment is often directed at

immunosuppression focussing on symptomatic relief and prevention of organ damage. Although in most SLE patients complement activation is suggested to contribute to inflammation and organ damage, it is remarkable that genetic deficiencies of the classical pathway predispose to SLE rather than protect against SLE development. C1q deficiency provides the largest risk for development of SLE, while C1r/s deficiency is somewhat less, C4 and C2 deficiency even less and C3 deficiency is not a very strong risk factor for SLE [48, 49].

COMPLEMENT COMPONENTS STUDIES DESCRIBED IN THIS THESIS

The complement system comprises of many proteins, as outlined above. However, in this thesis the focus will mainly be on two components of the complement system: C1q and C3. Both these proteins will be further introduced below with respect to their biology and structure.

Complement component C1q

Complement component C1q is a large protein (460kDa) which consists of six arms. Each arm comprises three polypeptide chains: A (34kDa), B (32kDa) and C (27kDa) (**Figure 3**). These polypeptide chains are derived from *C1QA*, *C1QB* and *C1QC*, which are clustered on chromosome 1p and have a synchronized expression [50]. The genes are arranged in the order A-C-B on the chromosome. The full C1q molecule is produced by various cells of myeloid origin such as monocytes [16, 18, 51], macrophages [52-56], immature dendritic cells [15] and mast cells [57]. C1q is a pattern recognition molecule and can bind to various ligands to activate the CP. Although, it was already known that multiple copies of IgG were required to bind C1q, recently it was elucidated that those IgGs need to be organised in a hexameric structure for optimal binding of C1q [58, 59]. C1q forms a complex with two esterases, C1r and C1s, this process is Ca^{2+} dependent and the full molecule ($C1qC1r_2C1s_s$) is named C1. C1q deficiency has been reported in literature with under 80 cases identified to date [60, 61].

Deficiency of C1q is associated with recurrent skin lesions, chronic infections, SLE or SLE-like diseases. Also, kidney involvement as mesangial proliferative glomerulonephritis and pronounced neuro-psychiatric problems have been reported in C1q deficient patients [60-62].

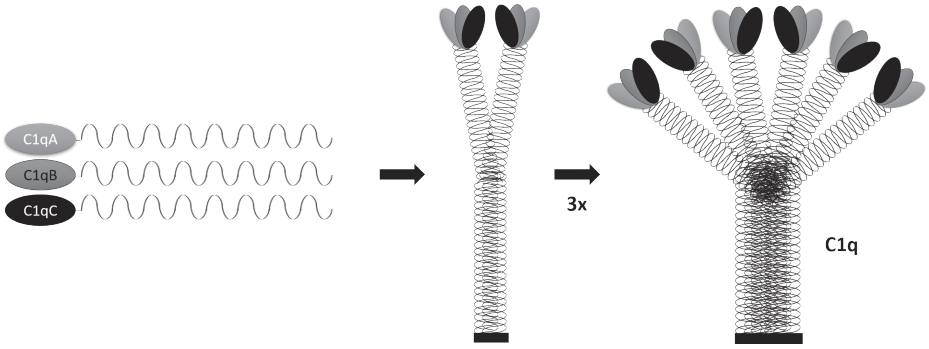

Figure 3. Schematic representation of the assembly of the C1q molecule [63]. C1q is assembled from three different chains, A, B and C. Each chains has a globular domain at the C-terminus (gC1q).

Complement component C3

C3 is the central component of the complement system and is also one of the most "ancient" proteins of the complement system. The origin of the C3 gene could be traced back before the divergence of Cnidaria and Bilateria and is therefore estimated to have originated 1300 million years ago [64]. C3 is encoded on chromosome 19 and is translated as a single peptide. The preprotein is further processed to generate the mature protein of 180kDa which consists out of a total of 13 domains, divided over the alpha and beta chain [65]. C3 undergoes various conformational changes upon activation and subsequent inactivation [66] (**Figure 4**). C3 is activated via a C3 convertase leading to the production of C3b and the anaphylatoxin C3a (~10kDa). C3a is a very potent anaphylatoxin and in circulation is rapidly modified by carboxypeptidases, which remove the terminal arginine generating C3a-desarg. C3a-desarg is also referred to in literature as Acylation Stimulating Protein (ASP). Upon generation of C3b the highly reactive thiol ester domain (TED) is exposed which allows covalent binding of C3b to the surface. Next, C3b can be further proteolyzed by FI and appropriate cofactors to inactive C3b (iC3b), which also results in the release of C3f. Finally, iC3b is further cleaved by FI generating the C3dg fragment and the fluid-phase C3c. The conformational changes that occur during these processes give rise to neo-epitopes which can be exploited to generate antibodies able to discriminate between the various C3 fragments. The bulk of circulating C3 is produced by hepatocytes, though numerous other cells, both immune and non-immune cells, can also produce C3 [12-14, 21]. C3 deficiency, which has been described in 27 patients from 19 different families worldwide to date [67], mainly associates with recurrent infections with gram negative bacteria.

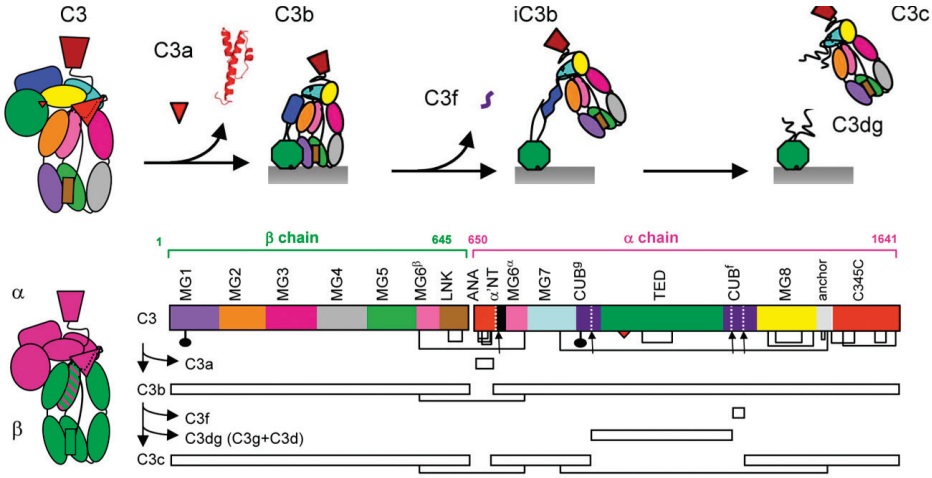

Figure 4. Schematic representation of the C3 molecule. Schematically the conformational changes to the C3 molecule upon activation of the C3 molecule and subsequent inactivation of C3b (top). Representation of the arrangement of the domains of C3 (bottom) [65, 66].

SCOPE AND OUTLINE OF THIS THESIS

In this thesis several aspects of complement proteins are described, from circulating levels in blood to their intracellular presence and from autoimmunity to the infectious disease tuberculosis. We explored the local production of complement and we describe in **Chapter 2** the production of C1q by chondrocytes. Additionally, because of the interest in the new role that has been attributed to C3 intracellularly, studies addressing the potential intracellular C3 role are described in **Chapter 3**. The potential role of the complement system as biomarker was investigated by addressing the presence and concentrations of C1q in serum of patients with active tuberculosis and controls. The results of these studies are described in **Chapter 4**. Like C1q, we also investigated the expression and concentration of the natural inhibitor C1-INH. The results of these studies, which were also performed in the context of tuberculosis, are described in **Chapter 5**. C1q protein was further analysed as biomarker for tuberculosis in experimental non-human primate models. The results of these studies are described in **Chapter 6.** While these studies are the first to describe aberrant an C1q levels in relation to tuberculosis, C1q is most notably known in the clinical context in relation to SLE as C1q-defiency is often associated with the development of SLE. In this thesis, a newly identified case of a lupus patient is described with a complex medical history and a compound heterozygous deficiency of C1q in **Chapter 7**. To better comprehend a possible role of a prominent post-translational modification associated rheumatic disease, carbamylation, the interaction between carbamylated IgG was investigated in relation to the ability to activate the complement system. These studies are described in **Chapter 8**. Finally, the results presented in this thesis are summarized and discussed in **Chapter 9**.

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