Complement biology in health and disease

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CHAPTER 1

GENERAL INTRODUCTION

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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**).

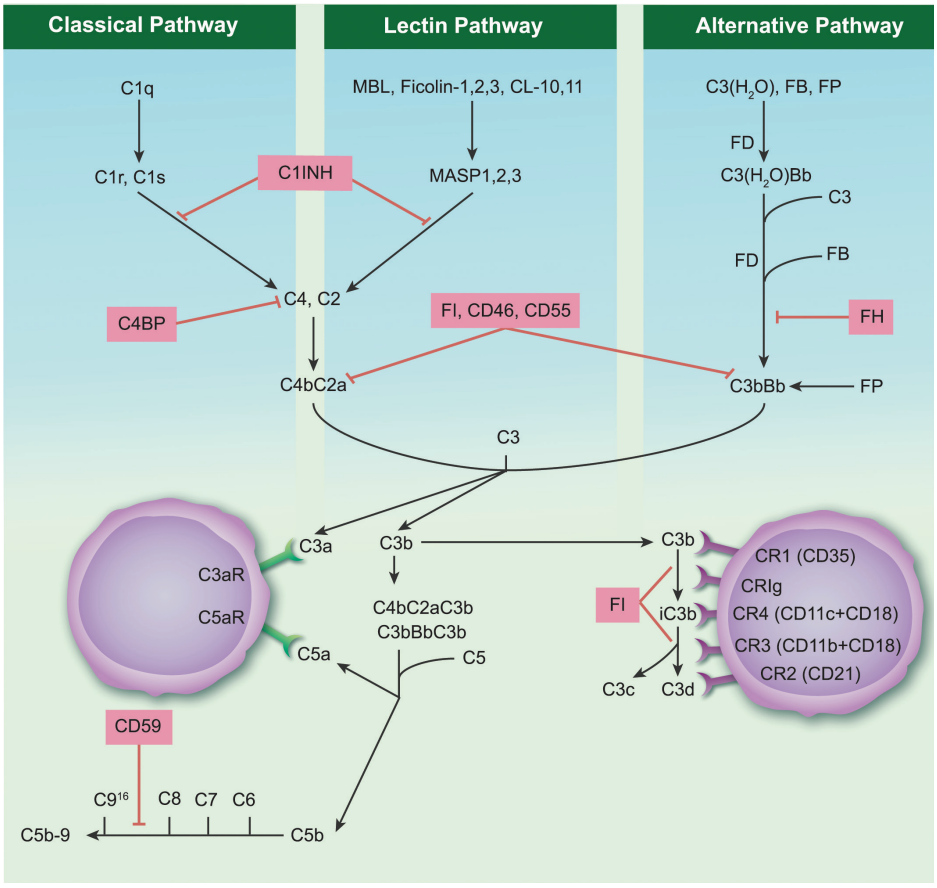

Figure 1. Schematic representation of the complement system.
The complement system can be activated via three different pathways: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). These pathways have their own sequential manner in forming a C3 convertase: C4b2a or 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].

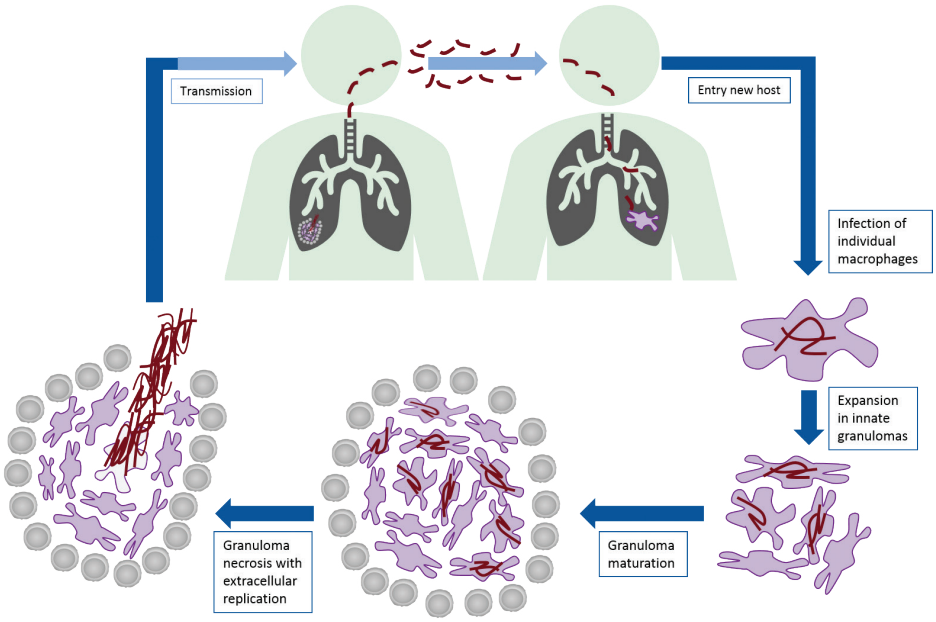

Figure 2. Schematic representation of the pathogenic life cycle of *Mycobacterium tuberculosis* (adapted from Cambier et al Cell 2014 [45]).
Infection with *Mycobacterium tuberculosis* (*Mtb*) occurs when fine aerosol particles containing *Mtb* are coughed up by an individual with active tuberculosis (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].

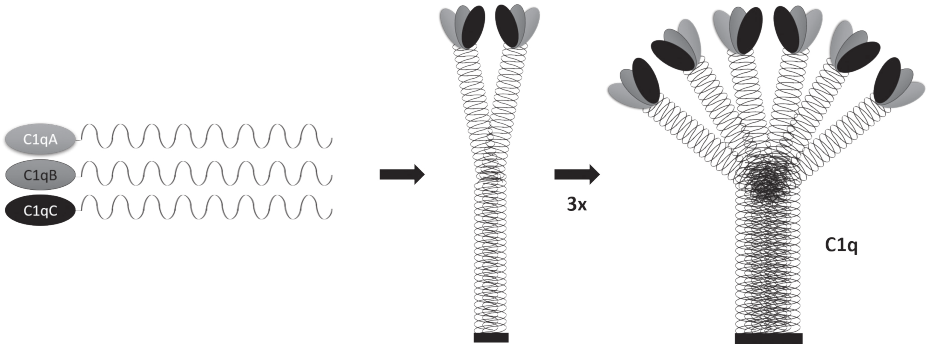

Figure 3. Schematic representation of the assembly of the C1q molecule [63]. C1q is assembled from three different chains, A, B and C. Each chains has a globular domain at the C-terminus (gC1q).

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.

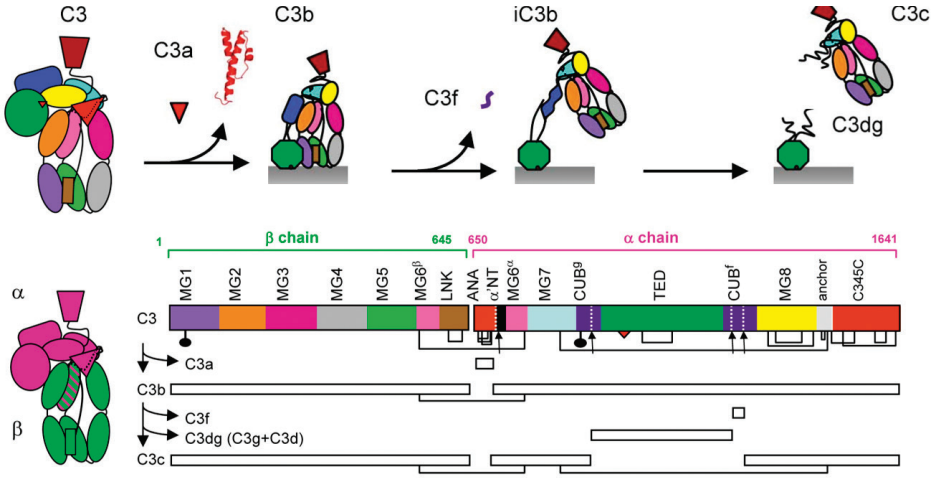

Figure 4. Schematic representation of the C3 molecule. Schematically the conformational changes to the C3 molecule upon activation of the C3 molecule and subsequent inactivation of C3b (top). Representation of the arrangement of the domains of C3 (bottom) [65, 66].

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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CHAPTER 2

COMPLEMENT COMPONENT C1Q IS PRODUCED BY ISOLATED ARTICULAR CHONDROCYTES

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ABSTRACT

Objective: Inflammation and innate immune responses may contribute to development and progression of Osteoarthritis (OA). Chondrocytes are the sole cell type of the articular cartilage and produce extracellular-matrix molecules. How inflammatory mediators reach chondrocytes is incompletely understood. Previous studies have shown that chondrocytes express mRNA encoding complement proteins such as C1q, suggesting local protein production, which has not been demonstrated conclusively. The aim of this study is to explore C1q production at the protein level by chondrocytes.

Design: We analysed protein expression of C1q in freshly isolated and cultured human articular chondrocytes using western blot, ELISA and flow cytometry. We examined changes in mRNA expression of collagen, MMP-1 and various complement genes upon stimulation with pro-inflammatory cytokines or C1q. mRNA expression of C1 genes was determined in articular mouse chondrocytes.

Results: Primary human articular chondrocytes express genes encoding C1q, *C1QA*, *C1QB*, *C1QC,* and secrete C1q to the extracellular medium. Stimulation of chondrocytes with pro-inflammatory cytokines upregulated *C1QA*, *C1QB*, *C1QC* mRNA expression, although this was not confirmed at the protein level. Extracellular C1q bound to the chondrocyte surface dose dependently. In a pilot study, binding of C1q to chondrocytes resulted in changes in the expression of collagens with a decrease in collagen type 2 and an increase in type 10. Mouse articular chondrocytes also expressed *C1QA*, *C1QB*, *C1QC*, *C1R* and *C1S* at the mRNA level.

Conclusions: C1q protein can be expressed and secreted by human articular chondrocytes and is able to bind to chondrocytes influencing the relative collagen expression.

INTRODUCTION

Chondrocytes are the only cells present in the healthy cartilage matrix. They are responsible for the synthesis and turnover of the articular extracellular cartilage matrix (ECM). Originating from mesenchymal stem cells, chondrocytes are located in matrix cavities called lacunae. The two major ECM macromolecules produced by chondrocytes are collagen type 2 and aggrecan [1]. Healthy cartilage mainly consist of these two components, but in the development of osteoarthritis (OA) the composition of the cartilage changes. In OA the chondrocytes can dedifferentiate and thereby reverse to a fibroblast phenotype, and the chondrocytes can terminally differentiate to a hypertrophic phenotype. Dedifferentiation is characterized by expression of Collagen type 1 and decreased expression of ACAN and Collagen type 2. Whereas hypertrophy is characterized by the expression of Collagen type 10, runt-related transcription factor 2 (RUNX2) and matrix metalloproteinase 13 (MMP13) [2]. In the early stages of OA it has been shown that the cartilage content changes from mainly collagen type 2 to collagen type 1 [3]. Furthermore, chondrocytes that have been isolated from patients with OA also produced collagen type 10[4].

The complement system is an important part of the innate immune defence, which is able to remove dying cells, immune complexes and kill pathogens. The complement system can be activated via three pathways; the classical pathway, the lectin pathway and the alternative pathway. C1q is the recognition molecule of the classical pathway and together with the proteases C1r and C1s it forms the C1 complex [5]. C1q can activate the classical pathway (CP) by binding different ligands such as IgG and IgM antibodies, but also DNA, C-reactive protein (CRP) and lipopolysaccharides [5-7]. Following activation of the CP, a C3 convertase is generated which cleaves C3 into two functional fragments, C3b which functions as an opsonin and C3a which is a chemoattractant promoting further involvement of the innate and/or adaptive immune system. The final step in the CP is the formation of the membrane attack complex (MAC) which creates a pore on the target membrane inducing lysis (**Figure 1A**). While most of the complement proteins are made by the liver, an essential set of complement proteins is made by cells of the immune system [8, 9]. C1q producing cells originate from haematopoietic stem cells, such as macrophages, immature dendritic cells and mast cells [7, 10-12]. However, other reports suggest that decidual endothelial cells, microglia and osteoclasts can also produce C1q [13-15].

Previous studies described that matrix molecules like aggrecan, fibromodulin and osteoadherin can bind C1q and thereby activate the classical pathway of the complement system [16-18]. The cartilage oligomeric matrix protein (COMP) is a strong ligand for C1q. It is hypothesised that COMP interferes with the binding site of C1r and C1s resulting in inhibition of the classical pathway [19]. This interference is also reported for the cartilage fragments decorin and biglycan [20]. Taken into account the ability of

C1q to interact with matrix molecules, it is relevant to determine whether C1q is locally produced in the articular cartilage and whether they can cause detrimental changes in chondrocytes.

Proteomic data from synovial fluid from osteoarthritic patients shows the presence of complement components [21, 22]. Wang et al. described that complement is important in the pathogenesis of OA [23], as the presence of the MAC, which is the terminal product of the complement cascade, could be detected in the cartilage. Although the authors speculated that complement activation takes place outside the cartilage and then attacks damaged chondrocytes, it cannot be excluded that chondrocytes initiate complement activation leading to MAC deposition. Similarly, in a bovine cartilage model it was shown that terminal pathway complement components were detected upon degradation [24]. Additionally, in the early 90's it was already described that articular chondrocytes express mRNA for C1q and that deposits of the C1q molecule were present in the cartilage. However at that time, the source and function of the C1q was not clear [25]. Articular cartilage is avascular and relies on diffusion of molecules, the structure is heterogenous and the possibility to proteins to diffuse into the cartilage decreases with increasing molecular size [26, 27]. C1q exists out three different polypeptide chains: C1qA (27,5 kilo Dalton (kDa)), C1qB (25,2 kDa) and C1qC (23,8 kDa). From each chain six copies are required, resulting in a total of 18 polypeptide chainswhich assemble together (460 kDa) before the full C1q molecule is secreted by the cell (**Figure 1B**). OA causes disruption in the cartilage structure and therefore may become more permeable. The size limit of proteins that can move freely in cartilage is estimated to be around 65 kDa [25]. Diffusion of IgG (150 kDa) has been demonstrated but exhibited unexpectedly slow diffusion through the superficial region [27], C1q is an even larger protein with a total molecular mass of 460 kDa, which makes it unlikely that it can readily diffuse into cartilage.

In this study, we have investigated whether primary human articular chondrocytes are able to express and secrete C1q proteins. We have also determined if the expression of C1q is modulated under inflammatory conditions, frequently present in OA.

MATERIALS AND METHODS

Patient

Cartilage was obtained as left-over tissue from OA patients undergoing total knee-replacement surgery in the Alrijne hospital, Leiden. The study was approved by the local medical ethical committee.

Isolation of chondrocytes

Chondrocytes were isolated from cartilage obtained from total knee-replacement surgery. Cartilage pieces were incubated in 20 ml PBS with pronase (2 mg/ml, Roche)

for 90 minutes, followed by overnight incubation with collagenase type 2 (225 U/ml, Worthington) in 20 ml F12 DMEM culture medium (Gibco) supplemented with 1% penicillin and streptomycin. After digestion of the tissue, the chondrocytes were passed over a cell strainer (mesh width 70 µM) and were pelleted. The chondrocytes were cultured, without extra passages, for further stimulation purposes or directly lysed for Western blot analysis and RNA isolation. The purity of the chondrocytes upon isolation was determined by Flow Cytometry using CD14 (BD Biosciences cat#555399) and CD45 (BD Biosciences cat#555482) expression to check for contamination of hematopoietic cells.

Stimulation of chondrocytes

Primary chondrocytes were plated in a cell density of $0.5*10^5/cm^2$ in F12 DMEM culture medium (Gibco) supplemented with 10% BSA (Bovine Serum Albumin), L-glutamine, penicillin and streptomycin. Chondrocytes were stimulated for 24 hours with IL-1β (30 ng/ml, R&D systems), TNF-α (100 ng/ml, R&D systems), TGF-β (10 ng/ml, peprotech), LPS (100 ng/ml, Sigma) or C1q (1 / 10 / 100 µg/ml. Quidel) and RNA was isolated as described below. For western blot analysis primary chondrocytes were lysed and C1qA, C1qB, C1qC was determined as described previously [28]. For ELISA, lysates and culture supernatants of chondrocytes (unstimulated or stimulated with 30 ng/ml IL-1β) were harvested after 72h hours and C1q levels were determined as described before [28]. The supernatants were concentrated according to manufacturer's protocol (Amicon Ultra-0.5 Centrifugal Filter, Merck) before analysis by ELISA was performed.

ELISA

C1q levels were measured using an in-house developed ELISA. Maxisorp plates (nunc) were coated overnight with mouse anti-human C1q (2204), (Nephrology department, LUMC) in coating buffer (0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH9.6). Plates were washed and blocked with PBS/1%BSA for one hour at 37°C. After washing, a serial dilution of a pool of normal human serum (NHS) was applied as a standard and samples were added in dilution buffer and incubated for one hour at 37°C. After washing, plates were incubated with rabbit anti-human C1q (Dako cat#A0136) for one hour at 37°C and for detection a goat anti-rabbit HRP (Dako cat#P0448) was used which was also incubated for one hour at 37°C. All washing steps were performed with PBS/1%BSA/0.05%Tween. The substrate was added to the plates using 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS) and the signal was measured at an absorbance level of 415 nm using Biorad iMark Microplate Absorbance Reader.

Western Blot

Using western blot the composition of C1q was examined by detection of the three chains of the C1q protein. *Ex vivo* chondrocytes were lysed using NP-40 lysis buffer (Invitrogen, cat#FNN0021) in combination with a protease inhibitor cocktail (Sigma cat#P2714) according to manufacturer's protocol, next these lysates were applied in

reduced conditions. Proteins were separated by SDS/PAGE using Tris-glycine gels (Biorad cat#456-1033) under reducing conditions which were loaded with equal amounts lysed chondrocytes. Next proteins were transferred on a Trans-Blot Turbo Transfer pack: mini, 0.2 µM PVDF (Bio-Rad). The membrane was blocked one hour in PBS containing 0.05% Tween and 3% skimmed milk on room temperature. The rabbit anti-human C1q (Dako) was preincubated in C1q depleted serum (Quidel) for one hour. Next, the blot was incubated with the preincubated rabbit anti-human C1q overnight on 4°C. After washing with PBS/0.1% Tween the membrane was incubated with goat anti-rabbit HRP (Dako) for one hour at room temperature. Finally, the blot was washed and C1q was visualized using ECL Western Blotting Analysis system (GE Healthcare).

Intracellular flowcytometry staining C1q

Isolated chondrocytes were fixed and permeabilized by using the Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD). Next, the chondrocytes were incubated with rabbit anti-human C1q-FITC (Dako) or with an isotype control rabbit Ig-FITC (Dako) for 20 minutes on ice in the dark. After incubation the chondrocytes were washed and measured on LSRII. Analyses were performed using FlowJo version 10.

Surface binding C1q chondrocytes

Chondrocytes were incubated for 45 minutes at RT with or without purified C1q in different concentrations (1 / 10 / 100 µg/ml). C1q incubation of chondrocytes was performed on a plate shaker at 600 rpm to prevent pelleting of the cells. After incubation the cells were washed and binding of C1q to the surface of chondrocytes was detected using rabbit anti-human C1q-FITC (Dako). Rabbit Ig-FITC (Dako) was used as isotype control. Samples were measured on LSRII and analyses was performed using FlowJo version 10.

qPCR Human chondrocytes

RNA was isolated from cultured chondrocytes using either RNeasy mini kit (Qiagen) or mirVana™ miRNA Isolation Kit (Invitrogen) and concentration was determined with the NanoDrop (NanoDrop Technologies). Subsequently, the RNA was treated with DNase I, Amplification Grade (Invitrogen) and cDNA was synthesized using superscript III (200U/µl, Invitrogen). The cDNA was diluted and qPCR was performed using SensiFast Sybr no-ROX (Bioline). Primers specific for *MMP1*, collagen type 1, type 2, type 10, *C1R*, *C1S*, *C3*, *C5* and the C1q genes *C1QA*/*B*/*C*, as well as the reference *RPL5* were used (see **Table 1** for primer sequences). The qPCR was performed on the real time PCR system (BioRad CFX-384) with an activation step of 3 min on 95°C (hot start polymerase activation), a melting temperature of 95°C for 5 seconds and at an optimized annealing temperature per primer for 5 seconds followed by an elongation step for 20 seconds on 72°C for 40 cycles. At the end of the protocol melting curves were performed from 65°C to 95°C to test specific binding of SensiFast Sybr.

Table 1. Human primers

Gene	5' Forward	3' Reverse
MMP1	CTGGCCACAACTGCCAAATG	ATTCTGTCCCTGAACAGCCC
Collagen type 1	CATGTTCAGCTTTGTGGACCTC	ATGGTACCTGAGGCCGTTCT
Collagen type 2	TCCTAAAGGAGCCCGAGGTGCC	CAGGAGCTTGGAGGCCGGGTT
Collagen type 10	CCCAGCACGCAGAATCCATCT	TTCCAGCCGGTCCAGGGATT
C1QA	CCAGGAAGAACCGTACCAGA	GACGATGGACAGGCAGATTT
C1QB	TCCAGATATGGAGGCCTGAC	TTCACTCAGCAGCATTCACC
C1QC	AAGGARGGGTACGACGGACT	GTAAGCCGGGTTCTCCCTTC
C1R	TCACAGTCCCCACGGGATAC	CCAGTGGAGAACCCAGTTGC
C1S	CTGCAGAGGGAGCGTCAA	TGGTAGGCTCAGCATAAACCC
C3	TGGCCAATGGTGTTGACAGA	GCGTAGACCTTGACTGCTCC
C5	TACCTTGCCTGTTGAAGCCC	CCAGGGAAAGAGCATACGCA
RPL5	TGGAGGTGACTGGTGATG	GCTTCCGATGTACTTCTGC

Immunofluorescence staining of C1q in chondrocytes

Freshly isolated chondrocytes were cultured on poly-d-lysine (Sigma 50 µg/ml) chamber slides (Thermo Scientific) and fixated and permeabilised using 1% paraformaldehyde and acetone. Cells were subsequently incubated with rabbit anti-C1q (Dako) or isotype control rabbit Ig (Dako) for one hour at RT. After washing, slides were incubated with goat anti-rabbit ALEXA 488 (Invitrogen) and ActinRed 594 (Applied bioprobes) for one hour at RT. Finally, the slides were covered with Vectashield containing DAPI (Vector laboratories) to stain nuclei and analysed on a confocal microscope (Leica SP8 confocal).

qPCR Mouse chondrocytes

Articular cartilage from tibia plateaus, femoral condyles were harvested from 5-6 days new born mice. The isolation protocol was adapted from literature [29, 30], in short, chondrocytes were seeded in 12-wells plates (200.000 cells/well) after matrix digestion with "liberase, Roche" (two digestions of 45 minutes with 0.1 mg/ml liberase and a third digestion with 0.025 mg/ml overnight). DMEM (Gibco Life Technomogies, France) supplemented with 10 % fetal bovine serum (FBS), 2% L-glutamine and 1% of antibiotic was used for this culture [30]. Isolated articular chondrocytes from several pups from the same litter were pooled, sample #1 is one pooled litter and sample #2 is a pool from another different litter. RNA was isolated using and cDNA synthesis was performed as previously described [31]. Next, real-time qPCR was performed with primers specific for mice *C1QA*/*B*/*C*, *C1R* and *C1S* (see **Table 2** for primer sequences). qPCR was performed on the real time PCR system (BioRad CFX-384) with an activation step of 3 min on 95°C (hot start polymerase activation), a melting temperature of 95°C for 5 seconds and at an optimized annealing temperature per primer for 10 seconds followed by an elongation

step for 15 seconds on 72°C for 40 cycles. At the end of the protocol melting curves were performed from 65°C to 95°C to test specific binding of SensiFast Sybr. After qPCR the products were analysed on agarose gel with Nancy-520 fluorescent DNA binding dye.

Table 2. Primers mouse

Gene	5' Forward	3' Reverse
C1QA	CCATCAGCAAAGGGCTGGAG	TACTAGGGTCATGGTCAGCA
C1QB	GACTTCCGCTTTCTGAGGAC	TGTGTCTTCATCAGCTCAGC
C1QC	GAC GTC TCT GTG ATT AGGCC	AGGGCCAGAAGAAACAGCAG
C1R	GAGGAGAATGGGACATCAT	GACACAGATGTTGGCATCGG
C1S	GTTTGGTCCTTACTGTGGTA	CCAAGGGTTCTTTTGCCCC

Statistics

Statistical analyses were performed using Graphpad Prism version 7. To compare relative expression levels or ratio the Wilcoxon test or Kruskall-Wallis test with Dunn's multiple comparisons test were used. To analyse the effect of increasing concentrations of C1q, compared to unstimulated, on relative expression the Friedman test with Dunn's multiple comparisons test was used, p-value's <0.05 were considered significant. In all graphs the mean with SEM is shown, or a representative figure.

RESULTS

Chondrocytes produce and secrete C1q protein

We isolated primary human chondrocytes from articular cartilage obtained during surgical procedures. First, we validated the purity of the isolated chondrocyte population, using flow cytometry. As shown in Figure 1A, chondrocytes do not express the hematopoietic marker CD45 and the myeloid marker CD14 (**Figure 1C**). Additionally, we used flow cytometry to analyse the presence of intracellular C1q after isolation. Indeed, we observed with intracellular staining that C1q is present in chondrocytes directly *ex vivo* (**Figure 1D**). To confirm these results, we subsequently let the chondrocytes adhere for at least one hour at 37°C on chamber slides followed by the analysis of the presence of C1q using confocal imaging. Also by microscopy we detected intracellular expression of C1q in the chondrocytes (**Figure 1E**). Finally, freshly isolated chondrocytes were lysed and protein extracts were prepared for western blot analysis to determine C1q expression. Also the lysates of the chondrocytes stained positive for C1q (**Figure 1F**). Collectively, these data indicate that chondrocytes express C1q protein.

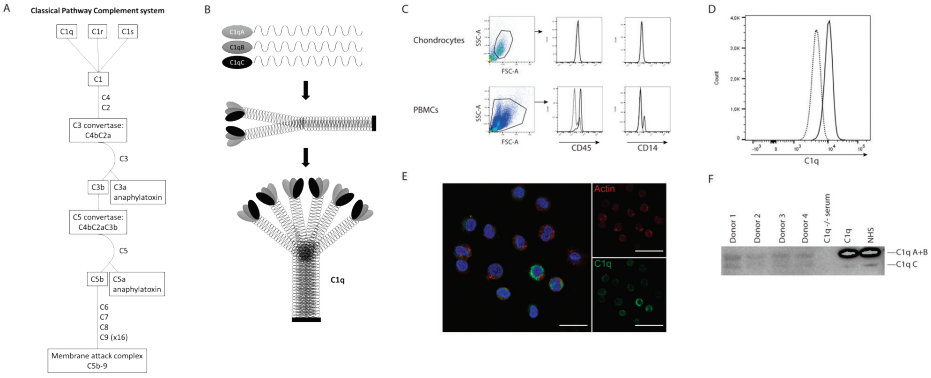

Figure 1. Chondrocytes express C1q protein

(A) Schematic presentation of the classical pathway of the complement system. (**B**) Schematic representation of the assembly of the C1q molecule. C1q is assembled from three different chains, A, B and C. (**C**) Flow cytometry of chondrocytes isolated from human cartilage compared to Peripheral blood mononuclear cells (PBMCs), dashed lines correspond with the isotype staining and solid lines to the antibody. (**E**) Flow cytometry of intracellular C1q in chondrocytes after isolation, the dashed line correspond with the isotype staining and the solid line to the antibody. (**E**) Cultured chondrocytes on chamber slides. C1q is visualized with ALEXA488. Actin is used as counterstaining at 594 nm. The nucleus is stained with DAPI, 630x magnification, the scale bars indicates 6.3 µm. (**F**) Western blot analysis of lysates of isolated chondrocytes from 4 different donors, purified C1q and normal human serum (NHS) serve as positive control whereas C1q-/- serum served as a negative control.

C1q mRNA expression is increased in the presence of OA relevant stimuli

Next, we aimed to determine whether C1q expression is modulated in an inflammatory milieu. To this end, mRNA expression of the C1q chains, *C1QA*, *C1QB* and *C1QC*, was determined in cells that were cultured with or without inflammatory stimuli that are thought to be relevant for OA, namely IL-1β, TNFα, and LPS and growth factor TGF-β [32]. Stimulation with these mediators resulted in differences in expression of *C1QA*, *C1QB* and *C1QC*, as analysed with Kruskall-Wallis (respectively: p=0.0282, p=0.0283 and p<0.0001). After adjusting for multiple comparisons the only remaining significant difference is IL-1β versus LPS in *C1QC* expression, probably due to low number of donors (n=3) (**Figure 2A-C**). Subsequently, we also wished to determine whether C1q-protein expression is also elevated by inflammatory stimuli. To this end, we stimulated chondrocytes with IL-1β, since this cytokine displayed the strongest effect on mRNA expression, albeit not statistically significant. After 72 hours, both the cell lysates and the culture supernatants were analysed. Although we detected C1q protein expression in both lysates and concentrated supernatant, we did not observe a difference between control and IL-1β stimulated chondrocytes (**Figure 2D**, **E**). Moreover, we noticed high variability in C1q production among donors.

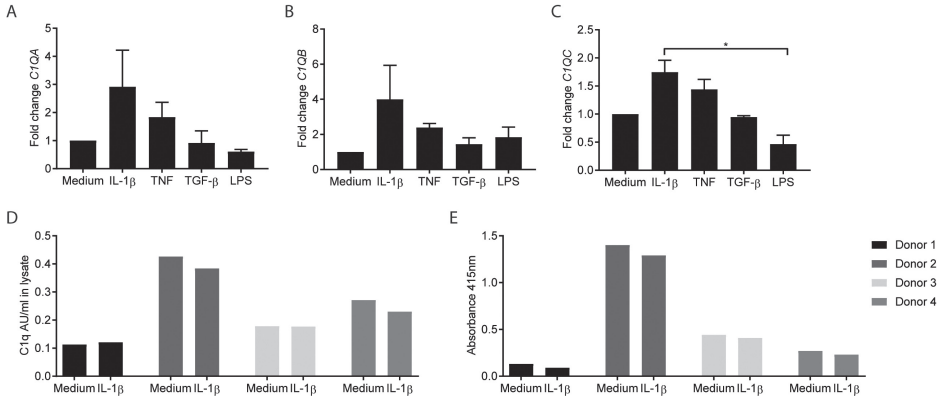

Figure 2. C1q after stimulation with pro-inflammatory cytokines

(A-C) Fold change expression after correction with *RPL5* of *C1QA*, *C1QB* and *C1QC*. Data represents mean and SEM of 3 different OA donors measured in duplicate after 24 hours stimulation (N=3). Differences in expression were analysed with Kruskall-Wallis, after adjusting for multiple comparison the individual significant adjusted p-values were plotted when p<0.05 (*). (**D**) Chondrocytes were stimulated with IL-1β for 72 hours, after stimulation the supernatant was collected and concentrated, additionally, the chondrocytes were lysed. Next, C1q was measured by ELISA. C1q measured in chondrocyte lysates by ELISA expressed as AU/mL in 4 donors. (**E**) Supernatant was collected and concentrated and C1q was measured and is expressed as the absorbance measured at 415nm. To compare relative expression levels Kruskall-Wallis test with Dunn's multiple comparisons test was used for statistical analysis and p-value's <0.05 (*) were considered significant.

Complement and matrix component expression in pro-inflammatory environment

Next, we sought to study the expression pattern of different collagens produced by chondrocytes upon stimulation with pro-inflammatory cytokines. TGF-β is known to increase the expression of Collagen type 1 in chondrocytes [33], although in our experiment the increase was only significant compared to IL-1β stimulation condition (**Figure 3A**). TGF-β also caused a slight non-significant upregulation in the expression of collagen type 2 and type 10 (**Figure 3B, C**). No effect was observed upon TNFα or LPS stimulation. IL-1β caused a trend towards decreased expression in all collagen types, type 1 (95% CI 0.33 – 0.52) type 2 (95%CI -0.03 – 0.20) and type 10 (95%CI -0.07 – 0.53). Also *MMP1* expression was determined for the different stimulations, we see differences between the stimulations, but these are not significant (**Figure 3D**). Furthermore, we investigated whether the expression of various complement components could be modulated by the inflammatory cytokines. *C1R* and *C1S* expression was analysed since these molecules in combination with C1q make one complete C1 complex, which activates the classical pathway of the complement system. C3 and C5 are further downstream in the complement system and important in the cascade for their ability to be cleaved into pro-inflammatory molecules. Stimulation with IL-1β, TNF and LPS seems to be accompanied by changes in the expression of *C1R*, *C1S* and *C3* (**Figure 3E**, **F** and **G** respectively), however, probably due to inter-donor variability, these changes

are not significant. *C5* expression seems to be unaffected by all different stimulations after 24 hours of stimulation (**Figure 3H**). Overall, the pro-inflammatory stimulation resulted not only in an upregulated expression of the C1q genes also in slightly enhanced expression of C1r and C1s, allowing the formation of the C1-complex.

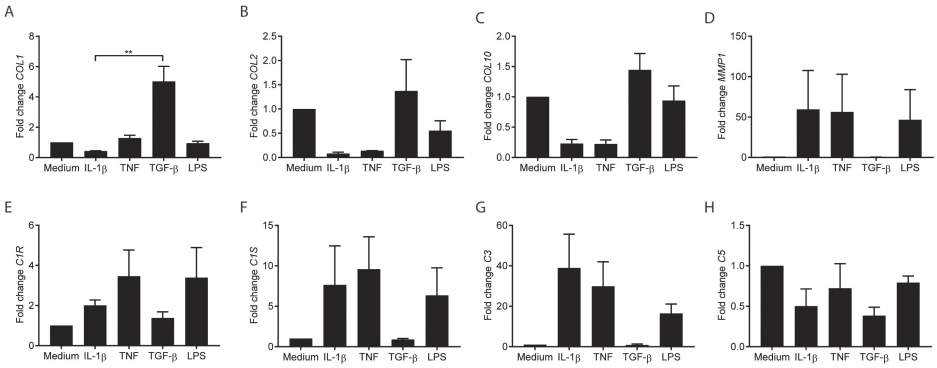

Figure 3. mRNA expression of complement and matrix genes after 24 hours stimulation (N=3). Fold change expression after correction with *RPL5* of (**A**) *COLA1*, (**B**) *COL2A1*, (**C**) *COL10*, (**D**) *MMP1*, (**E**) *C1R*, (**F**) *C1S*, (**G**) *C3* and (**H**) *C5*. Data represents mean and SEM of 3 different OA donors measured after 24 hours stimulation in duplicate. To compare relative expression levels Kruskall-Wallis test with Dunn's multiple comparisons test was used and and p-value's <0.05 were considered significant (p <0.01 **).

C1q can bind to chondrocytes

After showing that chondrocytes can produce C1q, we wished to assess the functional relevance of such local C1q production. First, we tested whether C1q protein was able to bind to chondrocytes. Chondrocytes were incubated with different concentrations of purified human C1q protein and surface binding was analysed by flow cytometry. We observed a dose-dependent binding of C1q to chondrocytes (**Figure 4A**). Next, we investigated whether C1q has a regulatory effect in chondrocytes on the mRNA expression of a set of complement genes (*C1QA/B/C*, *C1R*, *C1S*, *C3* and *C5*), different types of collagen (type 1, 2 and 10), *MMP-1*, *MMP13*, and on chondrocyte specific genes *SOX9* and *ACAN*. Chondrocytes were incubated for 24 hours with the same concentrations of C1q as used for flow cytometry, after which RNA was isolated and qPCR performed. Treatment with 100 µg/ml C1q induced significant decrease in *MMP13* expression (95% CI: 0.11 – 0.60), and a non-significant decrease in *COLL2* (95% CI 0.45 – 0.92) and *MMP1* (95% CI 0.30 – 0.60). In addition, we observed a non-significant increase in *C1QB* (95% CI: 1.012 – 2.12). *SOX9* and *ACAN*, two chondrocytes specific genes, were unaffected by the stimulation with C1q (**Figure 4B**). With lower concentrations of C1q no differences were observed (data not shown). It has been previously demonstrated that OA cartilage has a more dedifferentiate phenotype [34]. Therefore we analysed the ratio between expression of the genes encoding Collagen type 2 and 10, as an increased Collagen type 10 over Collagen type 2 expression which could be indicative

of hypertrophic chondrocyte differentiation. Stimulation of chondrocytes with 100 µg/ml C1q led to a significant increase in Collagen type 10 over Collagen type 2 expression. Overall, the data provide preliminary evidence that stimulation of chondrocytes by C1q affects expression of various genes (decreased *MMP13* and ratio change *COLL2*:*COLL10*) by the chondrocytes and could therefore affect the composition of the cartilage.

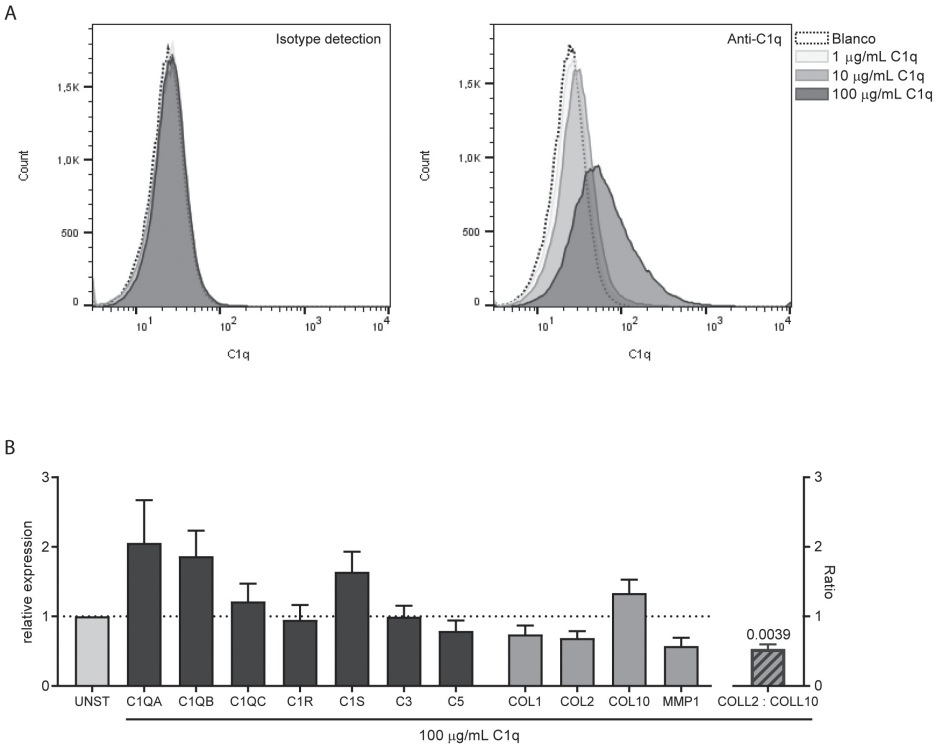

Figure 4. C1q effects on chondrocytes

(A) Representative figure for detection of C1q by flow cytometry on the surface of chondrocytes after incubation with an increasing concentration of C1q (n=3). (**B**) Chondrocytes were incubated in the presence of an increasing concentration of C1q (1 / 10 /100 µg/ml C1q) for 24 hours, after stimulation RNA was isolated and analysed for expression of both complement, matrix and chondrocyte specific genes. Stimulation and isolation was performed on biological triplicate and qPCR was performed with technical duplicate, mean with SEM are shown (n=9). Only the data from 100 µg/ml C1q stimulation is shown, on the right hand side the ratio of COL2:COL10 is presented. The dashed line at y=1 represents the unstimulated (UNST) level expression. The relative expression was analysed with Kruskall-Wallis test with Dunn's test for correction of multiple testing, the ratio was analysed with Wilcoxon-rank test. P-value's <0.05 were considered significant.

Mouse chondrocytes express all genes required to form the C1 complex
Finally, we aimed to demonstrate the presence of C1q transcripts in mouse articular chondrocytes. To this end, mRNA was isolated from articular mouse chondrocytes, from two different litters, and qPCR was performed for mouse *C1QA*, *C1QB*, *C1QC*, *C1S* and *C1R*. For both samples we observed expression and PCR product for all genes analysed. The PCR products were further analysed on agarose confirming the correct size of the bands and single bands. (**Figure 5**). These data indicate that, like human articular chondrocytes, mouse articular chondrocytes similarly express transcripts for the genes encoding the C1 complex, allowing further studies into the functional impact of C1 expression in experimental murine models.

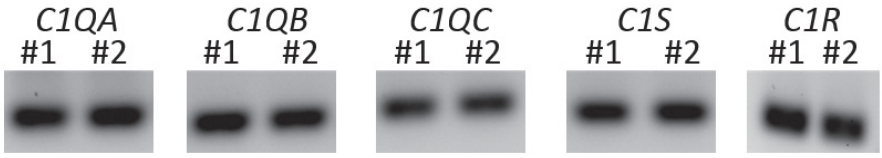

Figure 5. C1 expression in mouse articular chondrocytes
Articular chondrocytes from mouse pups that were 6 days old were collected from two different litters. Both samples are a pool of isolated articular chondrocytes from several pups from the same litter, sample #1 is one pooled litter and sample #2 is a pool from another litter. Agarose gel of PCR products from isolated mRNA analysed for *C1QA*, *C1QB*, *C1QC*, *C1S* and *C1R* expression.

DISCUSSION

The main cell types described to produce C1q are derived from haematopoietic stem cells [8]. Chondrocytes are derived from mesenchymal stem cells and are important in the production of extracellular matrix molecules to build up the cartilage. Wang et al. described that the dysregulation of complement system in the synovial joints has a central role in the pathogenesis of OA [23]. Also, in a transcriptomic approach complement genes were differentially expressed in OA knee cartilage [35]. Although in the early 90's, various complement components have been detected in cartilage including C1q mRNA, the presence of C1q at the protein level could at that time not be confirmed *in vitro*. Likewise, the possible function of C1q in cartilage was not addressed [25]. Here, we demonstrate that isolated chondrocytes indeed produce C1q protein.

To evaluate which factors can modulate the production of C1q by chondrocytes, we stimulated the chondrocytes with cytokines known to be present in the joint during inflammation [32]. The expression patterns of C1q genes were different compared to the expression of the collagen genes after TGF-β stimulation. As expected, the expression of the different types of collagen showed an increase of expression after stimulation of TGF-β [36]. In contrast, the expression of the C1q genes was mostly unaffected. Stimulation with IL-1β and TNF-α led to an increase in mRNA expression for the C1q

genes compared to unstimulated chondrocytes. However, an increase in C1q secretion at protein level was not observed in our experimental conditions, which could be due to a later translation. The production of C1q was very variable among donors, which may be related to the inflammatory state of the cartilage sample, or other unknown factors. Unfortunately, due to limitations in the samples; low numbers and the samples being obtained from left-over tissue, there is not enough information regarding patient demographics, to draw conclusions regarding C1q production and disease state.

C1q released by the chondrocytes could deposit on the cartilage or reach the synovium which is damaged by inflammation. However, C1q is a large 460 kDa protein and the size limit of proteins that can move freely in cartilage is estimated to be around 65 kDa [25]. Therefore, it is likely that C1q produced by the chondrocytes plays a local rather than systemic function. Conceivably, C1q would function in an autocrine setting in stimulating the chondrocytes, or alternatively C1q would be involved in local complement activation and / or cartilage biology, involving C1r and C1s directly outside the chondrocyte. We speculate that C1q and complement activation may be involved in the maintenance of a lacuna for the chondrocyte. In such a scenario the released C1q, possibly by the action of the enzymes C1r and C1s, may trigger the degradation of matrix molecules that are (too) close to the cell body of the chondrocytes.

Previous studies have demonstrated that the serine protease C1s can degrade collagen type 1 and type 2 when it is activated [37] and that it can play a role in the degenerative cartilage matrix in rheumatoid arthritis [38]. We also observed an increase in RNA expression of C1s and C1r after stimulation. In *ex vivo* chondrocytes we were also able to detect C1s in the lysates by Western blot (data not shown). This could indicate that under pro-inflammatory conditions, complement activation can occur both in the cartilage, as well as on the synovial surface of the cartilage. Together with the production of C1q and C3, it is conceivable that the production of different complement components by chondrocytes has a local function, contributing to cartilage degradation.

Next to a direct effect of C1q on complement activity via C1s on cartilage remodelling, C1q could also impact chondrocyte biology by binding C1q receptors. Several molecules have been coined to be C1q receptors [39], but which of these receptors would be relevant in the context of chondrocytes is unknown. The isolated chondrocytes represented a pure cell population as evidenced by FACS and as evidenced by cell culture. On the coverslips the cell population appears homogeneous. The cells may appear somewhat small as compared to their cultured and passaged counterparts, but this is likely the consequence of the fact that these are freshly isolated and non-passaged cells. We have shown that C1q can bind to the surface of chondrocytes but we currently have no insight into which receptors or ligands C1q is binding to. In a first study with a limited number of patients, we analysed the effect of C1q on the gene expression profile of chondrocytes. After incubation with 100 µg/ml C1q, a significant

change in the ratio between collagen type 2 and 10 expression levels was shown, in favour of more collagen type 10, suggesting that exposure to C1q (in high concentration) can contribute to a switch in the relative proportion of collagen expression, and tissue degrading enzyme(s). The concentration of C1q used to stimulate the chondrocytes (100 µg/ml) is roughly the same as the serum concentration of C1q, however if such a concentration is achievable locally in the cartilage is unclear. These data provide evidence that C1q can have functional effects on articular chondrocytes and provide the basis for future, more in-depth studies with larger cohorts of patients.

In our study we only used cartilage and chondrocytes derived from patients who underwent a total knee replacement due to OA, indicating that the cartilage could already be in inflammatory conditions. Therefore, for further investigations towards the role of complement in the cartilage it would be interesting to compare these results with healthy donors. The observation that also mouse primary chondrocytes express all genes required to make the C1 complex (*C1QA*, *C1QB*, *C1QC*, *C1R* and *C1S*) indicates that also experimental murine models can be employed to study the *in vivo* role of C1q in cartilage biology and pathology.

In summary, we show that isolated human articular chondrocytes express and secrete C1q protein, C1q is able to bind to chondrocytes and provide first evidence that C1q could induce changes in extracellular matrix molecule expression. Collectively, these data indicate a potential role in locally produced C1q in OA pathogenesis.

CONTRIBUTIONS

RL, RAvS, AIF and LAT designed the study and interpreted the data. RL, RAvS, JCK, NEWL, AMB, SM and CC collected and assembled the data. RJL, RM and AIF provision of study material or patients. RET, AIF and LAT obtained funding. All authors critically revised and approved the manuscript.

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CONFLICT OF INTEREST:

The authors report no conflict of interest.

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CHAPTER 3

COMPLEMENT C3 CLEAVAGE BY CATHEPSIN-L DOES NOT GENERATE C3A BUT C3A-DESARG AND IS NOT ESSENTIAL FOR SURVIVAL IN A HAP-1 MODEL CELL LINE

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Manuscript in preparation

ABSTRACT

Introduction: A novel intracellular role has been described for complement protein 3 expressed in CD4+ T-cells, by indicating that intracellular C3 can be cleaved by Cathepsin-L (CTSL), resulting in signalling by C3a via the intracellular C3aR. This intracellular C3 pathway was hypothesized to be pivotal for the survival of cells. Here, we further explored the CTSL cleavage site in C3 and survival of C3 deficient cells.

Material and methods: Western blots were performed to detect C3 in lysates. C3 protein was incubated together with activated CTSL and the fragments generated were analysed on SDS-page gel and by mass spectrometry (MS). As a model, a C3 knock out cell line was generated from homozygous HAP-1 cells using CRISPR/Cas9. The wild-type cells, mock cells, and C3-deficient cells were analysed regarding morphology, proliferation, and metabolism.

Results: Lysates of cells cultured in the absence of human serum did not reveal the presence of intracellular C3, while lysates of cells incubated in the extracellular presence of normal human serum did reveal C3 bands. *In vitro* exposure of purified C3 to CTSL resulted in the cleavage of C3. Interestingly, this did not result in C3a formation, but in the generation of C3a-desArg, since the arginine was still attached to the N-terminus of the C3b molecule. To study if C3 is essential for cell survival, we generated multiple cells lines from HAP-1 with CRISPR/Cas9. No differences were observed regarding proliferation, morphology and metabolism between the wildtype and C3 deficient HAP-1 cell lines.

Discussion: We demonstrate that processing of C3 by CTSL predominantly generates C3a-desArg and not C3a, and further that C3 or its derived products are not essential for cell survival in a HAP-1 cell line model.

INTRODUCTION

The complement system is an important humoral part of the innate immune system and comprises multiple soluble and membrane bound proteins. Activation of the complement system can occur via three different pathways, each consisting of sequential enzymatic reactions. The main functions described for the complement system are: opsonization, chemotaxis and lysis [1]. Currently, the complement system is no longer viewed as an isolated segment of the innate immune system, as several connections to the coagulation and the adaptive immune system have been identified [2, 3]. This includes the recent description that some complement proteins not only function extracellulary, but also intracellulary independent of the complement system [4, 5].

Studies on intracellular complement have focussed on C3 and C5, referring the new attributed functions as the ''complosome'' [4, 6, 7]. The first description of intracellular complement is provided by Liszewski et al. [5]. Intracellular C3 was reported to be intracellularly cleaved by Cathepsin-L (CTSL) in CD4+ T-cells, resulting in the formation of C3a and C3b. Subsequently, the intracellular C3a bound to the intracellular C3aR where it was involved in the phosphorylation of mTOR. Instead, C3b was reported to shuttle to the cell membrane. Interestingly, CD4+ T-cells from a C3-deficient patient, who is deficient for C3 in the circulation, were reported to be positive for intracellular C3a as analysed by confocal microscopy [5]. In addition, it was found that complement was involved in the metabolism of both CD4+ and CD8+ T-cells via CD46 [8, 9]. The main conclusion of these studies indicate that C3 serves an important role in cell homeostasis research. Subsequently, it was reported that intracellular C3 was only detected after incubation of cells with normal human serum (NHS). Allowing take-up of C3 as hydrolysed C3 ($C3H_2O$) which could then interact with CTSL [10]. Additionally, it was demonstrated that apoptotic cells internalized Factor H (FH), where it could regulate endogenous C3. Interestingly, it was proposed that FH has the potential to bind CTSL and thereby, functions as a cofactor, bringing these two proteins in close proximity to each other [11]. Most recently, intracellular C3 has been connected to the prevention of autophagy in pancreatic islets [12].

The central component of the complement system, C3, is conventionally cleaved by a C3 convertase in to two functional fragments, the larger fragment (170kDa) C3b acts as an opsonin whereas the smaller fragment (10kDa) C3a is a chemoattractant. C3a is, after its generation, quickly modified by carboxypeptidases, removing the C-terminal arginine and thereby generating C3a-desArg [13, 14]. C3a-desArg, also known as Acylation Stimulating Protein (ASP) is no longer able to bind to the C3aR [15]. Conflicting data are reported on the ability of ASP/C3a-to bind the receptor C5L2 [16]. ASP has been linked to metabolism, in which ASP is able to enhance triglyceride synthesis and glucose uptake in adipocytes and cultured skin fibroblasts [17, 18].

Thus, together, a connection between complement and basal mechanisms of the cell homeostasis have been reported. However, different interpretations of reported data have been proposed and expression of intracellular complement components have not been confirmed unambiguously at the protein level. For example, in the study studying the role of C5 in the activation of the inflammasome in CD4+ T-cells, no presence of intracellular C5 has been demonstrated at the protein level [19]. Besides, regarding the presence of intracellular C3, so far this has not been replicated on protein level, also because the antibody originally used for the studies described above is no longer available [5]. Here, we aimed to develop a method to measure C3 in various lysates at the protein level. In addition, we analysed the cleavage product of C3 and CTSL and developed a C3-deficient cell line with the CRISPR/Cas9 system to establish the proposed fundamental requirement of C3 for cell survival / physiology.

MATERIALS AND METHODS

Western blot

Cultured and freshly isolated cells were lysed using NP-40 lysis buffer (Invitrogen, cat#FNN0021) in combination with a protease inhibitor cocktail (Sigma cat#P2714) according to manufacturer's protocol, next these lysates were applied in reduced or non-reduced conditions. For the NHS loaded cells, cells were incubated prior to lysis with NHS at 37°C, after incubation they were washed six times in PBS and the last supernatant was also collected to control for carry over, as previously described [10]. Proteins were separated by SDS/PAGE using Tris-glycine gels (Biorad cat#456-1033). Next proteins were transferred on a Trans-Blot Turbo Transfer pack: mini, 0.2 µM PVDF (Bio-Rad). The membrane was blocked one hour in PBS containing 0.05% Tween and 3% skimmed milk on room temperature. Next, the blot was washed and incubated with the primary antibody overnight on 4°C. After washing with PBS/0.1% Tween the membrane was incubated with the proper secondary antibody labelled with HRP (Dako) for one hour at room temperature. Finally, the blot was washed visualized using ECL Western Blotting Analysis system (GE Healthcare).

Cleavage assay

Protein C3 was cleaved by recombinant CTSL as previously described [5]. Recombinant CTSL and CTSG were activated according to manufacturer's protocol, in short, CTSL (40 ng/µl) was incubated 15 min on ice in the activation buffer (1M MES/5mM DTT/0.0035% Brij35 or 1M MES/5mM /0.2% Rapigest, pH 6.15) without shaking to activate the enzyme. The activated cathepsin was then incubated with commercial purified C3 for different time points at 37°C. If an inhibitor was used, than the inhibitor was incubated with C3 one hour before the activated cathepsin was added. Reactions were stopped by adding 4x Laemmli sample buffer or 10% formic acid, and frozen till further analysis. Reaction mixtures were analysed for C3 fragments using Western blot or Mass spectrometry analysis.

Mass spectrometry

Gel slices containing C3 derived proteins were subjected to reduction with dithiothreitol and alkylation with iodoacetamide using Proteineer DP digestion robot (Bruker). Next, N-terminal amines were acetylated using acetic acid anhydride in order to discriminate between N-termini generated by CTSL (they are acetylated) and peptides generated by trypsin (they are not acetylated) during mass spectrometry work-up. Slices were incubated for 1 hour at room temperature in 20 %(v/v) acetic anhydride, 60 %(v/v) methanol and 10 mM NH4HCO3 pH 8.4. Next, they were washed three times sequentially with acetonitrile and 10 mM NH4HCO3 pH 8.4 prior to protein digestion with trypsin. Peptides were lyophilized, dissolved in 95/3/0.1 v/v/v water/acetonitrile/formic acid and subsequently analyzed by on-line C18 nanoHPLC MS/MS with a system consisting of an Easy nLC 1200 gradient HPLC system (Thermo, Bremen, Germany), and a LUMOS mass spectrometer (Thermo). Fractions were injected onto a homemade precolumn (100 µm × 15 mm; Reprosil-Pur C18-AQ 3 µm, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (30 cm × 50 µm; Reprosil-Pur C18-AQ 3 um). The gradient was run from 10% to 40% solvent B (20/80/0.1 water/acetonitrile/formic acid (FA) v/v) in 30 min. The nano-HPLC column was drawn to a tip of ~5 µm and acted as the electrospray needle of the MS source. The LUMOS mass spectrometer was operated in data-dependent MS/MS mode for a cycle time of 3 seconds, with a HCD collision energy at 32V and recording of the MS2 spectrum in the orbitrap. In the master scan (MS1) the resolution was 120,000, the scan range 400-1500, at an AGC target of 400,000 @maximum fill time of 50 ms. Dynamic exclusion after n=1 with exclusion duration of 10 s. Charge states 2-5 were included. For MS2 precursors were isolated with the quadrupole with an isolation width of 1.2 Da. First mass was set to 110 Da. The MS2 scan resolution was 30,000 with an AGC target of 50,000 @ maximum fill time of 60 ms.

In a post-analysis process, raw data were first converted to peak lists using Proteome Discoverer version 2.2.0.388 (Thermo Electron), and then submitted to the Uniprot database (452772 entries) using Mascot v.2.2.04 (Matrix Science) for protein and peptide identification. Mascot searches were with 10 ppm and 0.02 Da deviation for precursor and fragment mass, respectively, and trypsin as enzyme with up to two missed cleavages allowed. Methionine oxidation and acetylation were set as variable modification. Carbamidomethyl on cysteïne was set as a fixed modification. False discovery rate was set to 1% and the Mascot ion threshold score to 35. Acetylated spectra were also inspected manually. Data were also processed and visualized using Scaffold 4.10.0 software.

CRISPR/Cas9

Lentiviral vectors for expression of the guide RNAs (**Table 1**) were constructed by cloning synthetic oligonucleotides in the vector pLKO1-Puro-U6-BfuA-stuffer. The pLKO.1-puro U6 sgRNA BfuAI stuffer plasmid was a gift from Rene Maehr & Scot Wolfe

(Addgene plasmid # 50920). This vector allows stable selection for transduced clones using puromycin selection. Lentivirus vectors were produced on 293T cells, titrated, stored, and used as described previously [20]. After several passages with puromycin selection, the Cas9 protein was expressed either via lentiviral (pLV-Cas9-Blast; Sigma-Aldrich Chemie NV, Zwijndrecht, The Netherlands) or adenoviral vectors [21]. Cells were passaged 3 times before, gDNA was collected and clonal cell lines were cultured via limiting dilution.

Table 1. Sequence guides CRISPR

Guide CRISPR	Sequence
C3a Guide 1	TTGGGGTACTTGCCGACTGC GGG
C3a Guide 5	GCAGCTCACGGAGAAGCGAA TGG

T7 Endonuclease I assay

T7 Endonuclease I (T7EI) assay was performed to analyse the CRISPR/Cas9 procedure [22]. Briefly, genomic DNA was extracted from cells using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's handbook. primers were developed to ensure that the guides were not in the middle of the PCR product. PCR was performed and the PCR product was split in two, one part remained untreated. The other part was denatured and reannealed in NEBuffer 2 (New England Biolabs) using a thermocycler with the following protocol (to re-anneal the PCR products at suboptimal temperature to create mismatched base pairs): 95^{o}C 10min; ↓Ramp to 85^{o}C at 2.0^{o}/s + 85^{o}C 1min; ↓Ramp to 75^{o}C at 0.3^{o}/s + 75^{o}C 1min; ↓Ramp to 65^{o}C at 0.3^{o}/s + 65^{o}C 1min; ↓Ramp to 55^{o}C at 0.3^{o}/s + 55^{o}C 1min; ↓Ramp to 45^{o}C at 0.3^{o}/s + 45^{o}C 1min; ↓Ramp to 35^{o}C at 0.3^{o}/s + 35^{o}C 1min; ↓Ramp to 25^{o}C at 0.3^{o}/s + 25^{o}C 1min; 16^{o}C ∞. The mismatched strands were treated with T7EI for 17 minutes at 37°C (induces double strand breaks at mismatch sites). The initial PCR product and the T7EI product were run on 2% agarose gel to analyse the fragmentation pattern.

Table 2. Sequence primers gDNA C3

Target	5' Forward	3' Reverse
gDNA C3	CCG CCT CCA CCA CCA CCT AGT AAA	TGGCACGAGAACCTCATGGG

Sanger sequencing

C3 from the HAP-1 clonal cell lines was amplified by PCR with primers (**Table 2**). PCR products were visualized on a 1% agarose gel and subsequently sequenced with Sanger sequencing [23]. Sequences were compared to the reference C3 sequence obtained from the UCSS genome browser (NM_000064).

Tritium
The clones were cultured in a flat-bottom 96-wells plate in a concentration of 10.000 cells/well for six hours before incubation with tritium. To a volume of 100uL cell culture, 50uL of 10uCi ^{3}H was added to establish a concentration of 0.5uCi ^{3}H per well. After incubation for 18, 42 or 66hrs, cells were resuspended and transferred to a round-bottom 96-wells plate for harvesting. Cells were harvested with the TOMTEC Harvester 96 according to manufacturer's protocol. 3H-positive cells were counted with the Microbeta Trilux 1450 scintillation counter.

Phase microscopy
Cells were seeded in T75 culture flasks in DMEM, pictures were taken after 24 and 72 hours of culture with Olympus SC30 microscope 20x enlargement.

Flow cytometry
Cells were stained for surface and/or intracellular markers. For intracellular staining the cells were fixed and permeabilized by using the Cytofix/Cytoperm™ Fixation/ Permeabilization Solution Kit (BD). For staining isotype controls were used. After incubation with the fluorescent antibodies, the cells were washed and measured on LSRII. Analyses were performed using FlowJo version 10.

Direct Cellular Metabolism Measurement
Seahorse XF Cell Mito Stress Test (Kit 103015-100, Agilent Technologies, US) was performed according to manufacturer's protocol. In short, cells were seeded in the Seahorse XF Cell Culture Microplate overnight in DMEM. The sensor cartridge was hydrated with Seahorse XF Calibrant at 37°C in a non-CO_2 chamber overnight. Next, the assay medium was prepared (Seahorse XF base medium supplemented 1mM pyruvate/2 mM glutamine/10 mM glucose) and the compounds were loaded in the sensor cartridge (final concentrations: Oligomycin 1µM; FCCP 3 µM; Rotenone & antimycin A both 0,5µM). Standard assay was performed and measured by using the Wave software. Finally, the data was normalized on BCA content, the BCA content was measured using Pierce BCA protein assay kit (cat#23227 Thermo Scientific).

RESULTS

Detection of intracellular C3 in cell lysates
To study the role of intracellular C3, we first set out to replicate the original findings that C3 is present in cell lysates and cleaved by CTSL. Unfortunately, the antibody originally used by Liszweski et al. was not available anymore by the supplier. Therefore, several antibodies were tested for their capacity to detect (intracellular) C3 in lysates. First, we analysed the alternative antibody suggested by Abcam (**Figure 1A**). Unfortunately, this mouse anti-C3d only detectsC3 in the positive control NHS sample, but not in the lysates, despite the use of different lysis procedures. We continued testing various

other antibodies, including the DAKO rabbit anti-C3d seemed promising as it was able to detect C3 fragments on WB and showed positive detection in multiple lysates, both from cell culture and isolated cells (**Figure 1B**). Under reducing conditions, a clear band at the height of 110kDa was visible, corresponding, to the α-chain of C3. Next, we analysed this antibody under non-reducing conditions. As a positive control, lysates from human liver were used, showing a band at 180kDa, corresponding with intact C3. As an additional control for the procedure, C3 was added to Jurkat cells prior to lysis, also giving rise to a clear band at 180 kDa. Unexpectedly, in the lysates from the T-cell lines and $CD4^+$ cells from three different donors, there was a band observed at 110kDa. This band is migrating at the same height as the band observed under reducing conditions (F**igure 1C**). Therefore, as C3 is 180KDa, the protein detected with an apparent molecular weight does not correspond to intact C3. Nonetheless, intact C3 can be detected using this DAKO rabbit anti-C3d in the human liver lysate, the NHS, purified C3 samples as such and spiked in lysates. Thus, together, these data indicate that this rabbit serum recognizes besides intact C3, also another protein with an apparent molecular weight of 110 kDa and hence was not suitable for further use in our studies.

During the course of our experiments it was reported [10] that cells only test positive for C3 upon exposure to NHS, because of ingestion of C3 from the NHS, prior to lysis. Therefore, we continued reproducing these findings using the goat anti-C3 serum (CompTech cat#A213). The cells incubated with NHS were washed repeatedly with PBS and the last supernatant prior to lysis was also run an WB to control for possible carry over of the protein, these ''wash supernatants'' were negative. The lysates of cells, incubated with NHS prior to lysis, show an increase in C3 signal (**Figure 1D**). Additionally, we verified that the mouse anti-C3(a) antibody only recognizes C3, and not the processed form C3b (**Figure 1E**). This mouse anti-C3(a) antibody was used for follow-up experiments, where C3 was incubated in the presence of CTSL *in vitro*.

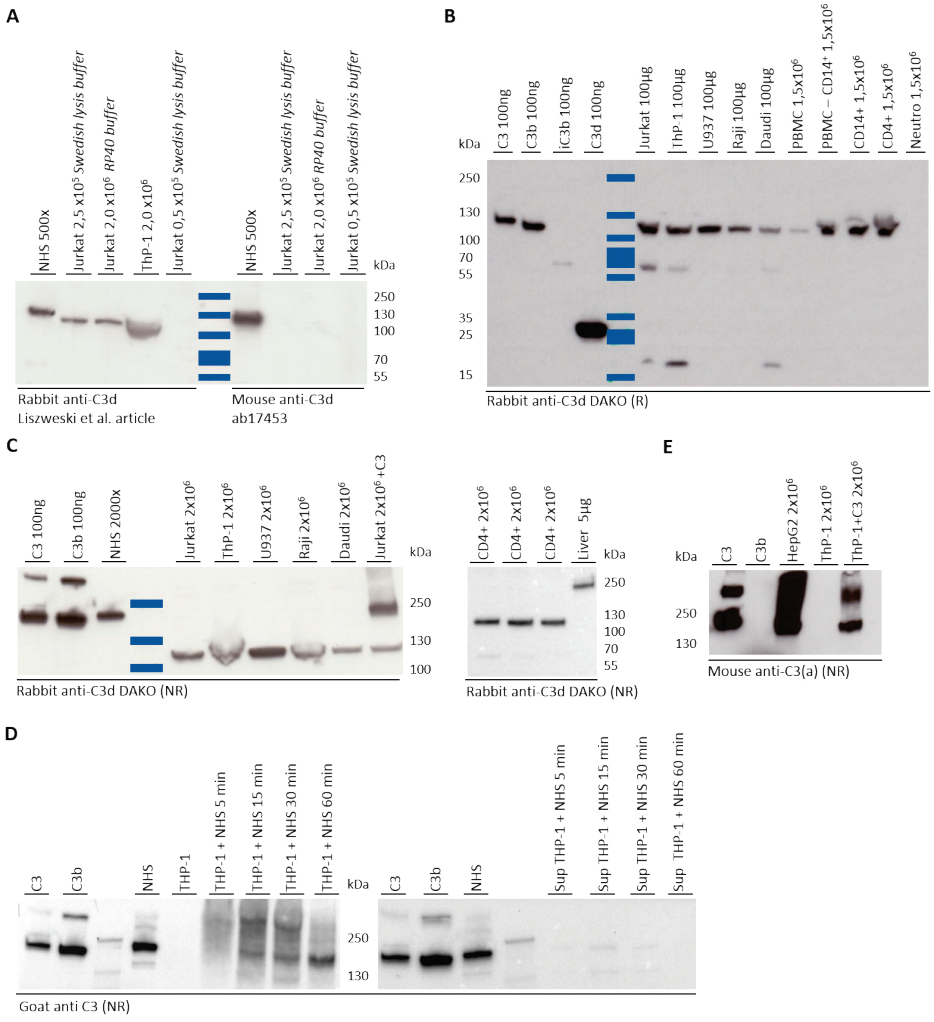

Figure 1. C3 detection in lysates on Western Blot

Western Blot (WB) results from lysates and C3 detection by different antibodies. (**A**) WB of lysates detected by two different antibodies, left is the rabbit anti-C3d from the Liszweski article which is no longer produced and the right part is the alternative (ab17453) offered by the company. (**B**) WB of lysates from various cell lines and freshly isolated cells, detected with rabbit anti-C3d antibody from DAKO under reducing (R) conditions, Peripheral blood mononuclear cells (PBMCs). (**C**) WB of lysates from various cell lines (one Jurkat sample had C3 added during the lysis process to control for the procedure) detected with rabbit anti-C3d antibody from DAKO under non-reducing conditions (NR). (**D**) WB of lysates with or without prior incubation in normal human serum (NHS), the wash supernatants (sup) are on the right. (**E**) WB of proteins and lysates detected with mouse anti C3(a) under NR conditions.

Cathepsin-L cleavage of C3 does not generate C3a

Whether C3 is intracellularly retained after synthesis or taken up from the circulation may both indicate a role for C3-processing in intracellular processes, which is believed to occur after the cleavage of C3 in a convertase-like manner by CTSL. Therefore, we next incubated commercial, purified C3 with activated CTSL and analysed the fragments generated by CTSL-mediated cleavage. Fragments were analysed both by WB and MS. By using the C3(a) antibody we confirm that C3 is cleaved by CTSL and that the reaction can be inhibited with a specific CTSL inhibitor (CTSLi), but not with a non-specific Cathepsin inhibitor (CTSi) (**Figure 2A**). In addition, the disappearance of intact C3 is specific for CTSL and does not occur when C3 is incubated with Cathepsin-G (CTSG) (**Figure 2B**). The products formed by CTSL-cleavage were run on a high percentage SDS-page gel (20%) to visualize the C3a(desArg) using the C3(a) antibody. A relatively faint signal at the height of (commercial) C3a(desArg) was observed after C3-processing by CTSL (**Figure 2C**). To further confirm the conversion of C3 into C3a(desArg), protein N-termini were acetylated using acetic anhydride in order to discriminate between N-termini generated by CTSL and generated during protein digestion. The latter peptides will not contain an acetylated N-terminus. In doing so, we found RSNLDEDIIAEENIVSR which was formed CTSL-dose dependently (**Figure 2D**). Interestingly, CTSL cleaves C3 one amino acid upstream to that of convertase, leaving an N-terminal R on the "C3b α "-chain (**Figure 2E**). These data suggest that C3 is specifically cleaved by CTSL resulting in the generation of C3a-desArg . Although MS analysis could demonstrate that CTSL leaves the arginine (essential for C3a function) on C3b, we have been unsuccessful to detect the C3a-desArg cleavage product despite several attempts on orbitrap and MALDI instruments. In contrast, convertase-generated control C3a could be detected with high yield and full coverage .

CRISPR/Cas9 mediated knockdown of C3 in HAP-1 cells

To analyse the potential role of intracellular C3 we set out to develop C3-/- model cell line(s), by using CRISPR/Cas9. For Cas9 delivery two different viral transvectors were used, either adenovirus or lentivirus. To control for successful use of CRISPR/Cas9, the T7EI assay was performed in the bulk of the cells after Cas9 transfection. Since it has been published that C3 can be taken up from circulation and it is unknown, given the speculated role of C3 in cell survival [5], whether this C3 is essential for survival, both adenoviral and lentiviral generated Cas9 cells were cultured, in limited dilution, in the presence of either FCS or complement active NHS. This resulted in four different culture conditions: adenovirus with FCS (AF); adenovirus with NHS (AN); lentivirus with FCS (LF); lentivirus with NHS (LN). Since all conditions show additional bands after T7EI treatment, CRISPR/Cas9 was successful in all culture conditions (**Supplementary Figure 1**). After limiting dilution, 90 viable clones were collected and gDNA was isolated. Of these 90 clones, 46 were further analysed by Sanger sequencing (**Supplementary Figure 2**), excluding those with double bands or absent bands after PCR. The sequences obtained are summarized in **Table 1**. After CRISPR/Cas9 in 18 out of 46 analysed sequences a

perfect match was found with the reference sequence. In 15 out of 46 sequences we found various mutations in the gDNA: three remained a perfect match on cDNA level; one had a deletion but did not lead to a frameshift mutation; 11 sequences had deletions which resulted in a frameshift mutation (including two sequences with early stopcodons). Interestingly, some mutations were found in more than one clone. For example, clones LNE8.1, LFE5, LFG11, AFH8, ANG11 and AFG8 displayed the same 203bp deletion in the gDNA, resulting in a 11bp deletion on cDNA level and a frameshift which prohibits intact C3 translation. Since this particular mutation is found in all four culture conditions, there seems to be no selection in Cas9 viral delivery and the use of either FCS or NHS supplemented medium. Additionally, clones AND8 and LNA4 had a matching mutation, where there was an even larger deletion of 214bp in gDNA and 22bp in cDNA, prohibiting C3 translation.

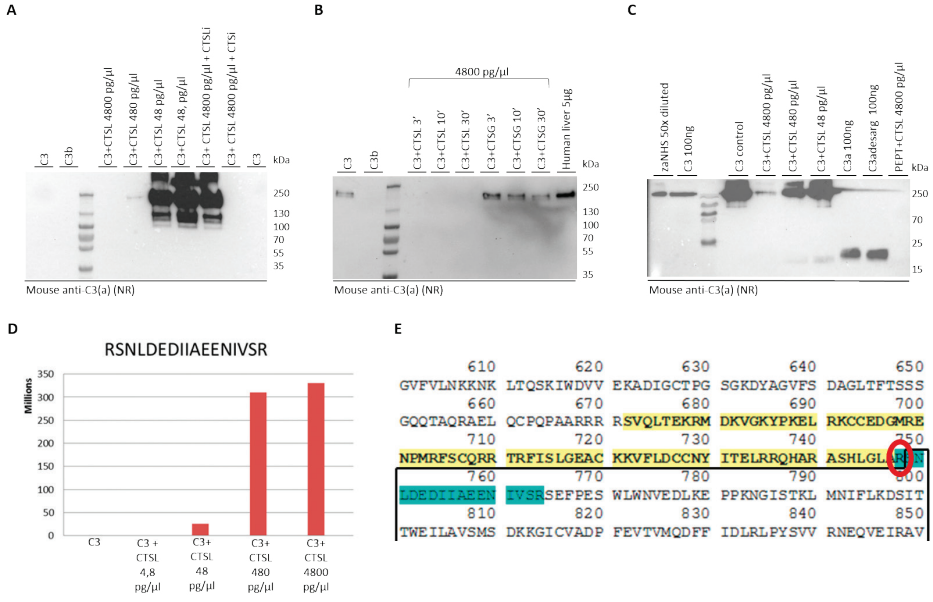

Figure 2. C3 cleavage by Cathepsin-L

(A) Western blot (WB) intact C3 after incubation with Cathepsin-L in various conditions. With decreasing concentration of CTSL, in the presences of a CTSL inhibitor (CTSLi) or non-specific CTS inhibitor (CTSi). (**B**) WB intact C3 after incubation with CTSL or Cathepsin-G (CTSG) for different incubation times. (**C**) WB of C3 with decreasing concentration of CTSL incubated samples analysed on 20% gel, detection of C3 and C3a/C3adesArg. (**D**) Peptide abundance measured by mass spectrometry as a function of increasing concentrations of CTSL. RSNLDEDIIAEENIVSR was the only peptide that showed a dose dependent response to the concentration of CTSL. These results are representative for two independent experiments (**E**) Schematic presentation of the peptide found with MS analysis, the C3a and C3b sequences are separated with the black solid line, the C3a sequence is highlighted in yellow and the N-terminal peptide found for C3b in blue. The arginine (R) that should be on the C3a, but is found attached to the C3b is circled in red.

Table 1. Sequence results of CRISPR-Cas9 of C3a in HAP-1 cell line

Cas9	Culture	Name	Deletion DNA	Deletion cDNA	Insertion nt	Mutation	Frameshift	Comment sequence analysis
Adeno	*FCS*	**AF_A10**	*x*	*x*	*x*	-	*x*	low quality sequence
Adeno	FCS	**AF_A3**	-	-	1 "C"	-	YES	early stopcodon
Adeno	FCS	**AF_A7**	-	-	-	-	NO	perfect match
Adeno	*FCS*	**AF_B5**	-	-	1? "T"	-	YES?	sequence pattern inconclusive
Adeno	FCS	**AF_B6**	-	-	-	-	NO	perfect match
Adeno	FCS	**AF_D10**	-	-	-	-	NO	perfect match
Adeno	FCS	**AF_E10**	-	-	-	-	NO	perfect match
Adeno	FCS	**AF_F12**	-	-	-	-	NO	perfect match
Adeno	FCS	**AF_G10**	-	-	-	-	NO	perfect match
Adeno	FCS	**AF_G11**	-	-	-	-	NO	perfect match
Adeno	*FCS*	**AF_G4**	-	-	-	-	*NO*	sequence pattern inconclusive
Adeno	*FCS*	**AF_G6**	-	-	*3?*	-	*YES?*	sequence pattern inconclusive
Adeno	FCS	**AF_G8**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Adeno	FCS	**AF_H8**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Adeno	NHS	**AN_C1**	-	-	-	-	NO	perfect match
Adeno	*NHS*	**AN_C11**	-	-	-	*G->C?*	*NO*	sequence pattern inconclusive
Adeno	NHS	**AN_C4**	-	-	-	-	NO	perfect match
Adeno	*NHS*	**AN_D1**	-	-	*1 "T"*	-	*YES?*	sequence pattern inconclusive
Adeno	NHS	**AN_D5**	13	0	-	-	NO	perfect match on cDNA level

Table 1. Continued.

Cas9	Culture	Name	Deletion DNA	Deletion cDNA	Insertion nt	Mutation	Frameshift	Comment sequence analysis
Adeno	NHS	**AN_D8**	214	22	-	-	YES	AN_D8, LN_A4
Adeno	NHS	**AN_F4**	-	-	-	-	NO	perfect match
Adeno	*NHS*	**AN_F9**	-	-	*1? "C"*	-	*YES?*	sequence pattern inconclusive
Adeno	NHS	**AN_G11**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Adeno	NHS	**AN_G2**	-	-	1? "G" in intron	-	NO	perfect match on cDNA level (extra G just before start exon)
Adeno	NHS	**AN_H10**	-	-	1? "G" in intron	-	NO	perfect match on cDNA level (extra G just before start exon)
Lenti	FCS	**LF_A10**	-	-	1 "C"	-	YES	early stopcodon
Lenti	FCS	**LF_A12**	-	-	-	-	NO	perfect match
Lenti	FCS	**LF_A3**	-	-	-	-	NO	perfect match
Lenti	FCS	**LF_B4**	-	-	-	-	NO	perfect match
Lenti	*FCS*	**LF_B6**	*222*	*30*	-	-	*NO*	sequence pattern inconclusive
Lenti	*FCS*	**LF_C5**	-	-	-	-	*NO*	sequence pattern inconclusive
Lenti	FCS	**LF_E2**	-	-	-	-	NO	perfect match
Lenti	FCS	**LF_E5**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Lenti	FCS	**LF_F3**	73	63	-	-	NO	AA deletion, not leading to frameshift
Lenti	FCS	**LF_G1**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8

Table 1. Continued.

Cas9	Culture	Name	Deletion DNA	Deletion cDNA	Insertion nt	Mutation	Frameshift	Comment sequence analysis
Lenti	NHS	**LN_A4**	214	22	-	-	YES	AN_D8, LN_A4
Lenti	*NHS*	**LN_B4**	-	-	-	-	NO	sequence pattern inconclusive
Lenti	NHS	**LN_C1**	-	-	-	-	NO	perfect match
Lenti	*NHS*	**LN_C7**	-	-	-	-	*NO*	sequence pattern inconclusive
Lenti	*NHS*	**LN_E3**	-	-	-	-	*NO*	sequence pattern inconclusive
Lenti	NHS	**LN_E8-1**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Lenti	NHS	**LN_G4**	-	-	-	-	NO	perfect match
Lenti	NHS	**LN_G9**	-	-	-	-	NO	perfect match
Lenti	NHS	**LN_H1**	203	11	-	-	YES	LN_E8_1, LF_E5, LF_G11, AF_H8, AN_G11, AF_G8
Lenti	NHS	**LN_H10**	-	-	-	-	NO	perfect match
Lenti	*NHS*	**LN_H8**	-	-	*1? "T/C"*	-	*YES?*	sequence pattern inconclusive
n.a.	n.a.	**WT**	-	-	-	-	NO	perfect match

Sequence results from CRISPR/Cas9 clones summarized. Clones were generated by using either adenovirus or lentivirus for Cas9 delivery, subsequently, the clones were culture in either normal human serum (NHS) or fetal calf serum (FCS). The gDNA sequence results were translated to cDNA for further translation of the mutations and analysed whether the mutation resulted in a frameshift. Further comments per sequence are annotated in the last column.

CRISPR/Cas9 mediated knockdown of C3 does not affect survival, morphology and metabolism of model cell line HAP-1

For further analysis of the effects of C3 deficiency on basal cellular processes, two clones (AFE10 (E10) and AND8 (D8)) were selected alongside the WT cell line HAP-1 for further analyses. E10 was selected as a control and D8 as the C3- clone (22bp deletion on cDNA (**Supplementary Figure 2**)). We first explored whether receptors for C3 and C5 anaphylatoxins are expressed intracellularly to determine whether HAP-1 is able to respond to intra-cellular C3a/C5a .In all cell lines tested we observed a clear signal for intracellular C3aR whereas surface staining is negative (**Figure 3**). Likewise, C5aR1/CD88 can be detected intracellularly as an enhanced MFI signal is detected for the intracellular detection. In contrast, surface expression was absent as no specific signal was observed after staining with an anti- C5aR1/CD88-antibody. Similar data were obtained for C5L2. Overall, no differences in the expression patterns of WT, E10 and D8 cell line for C3aR and C5aR-receptors was observed, indicating that all cell lines could, potentially, react in response to C3a/C5a.

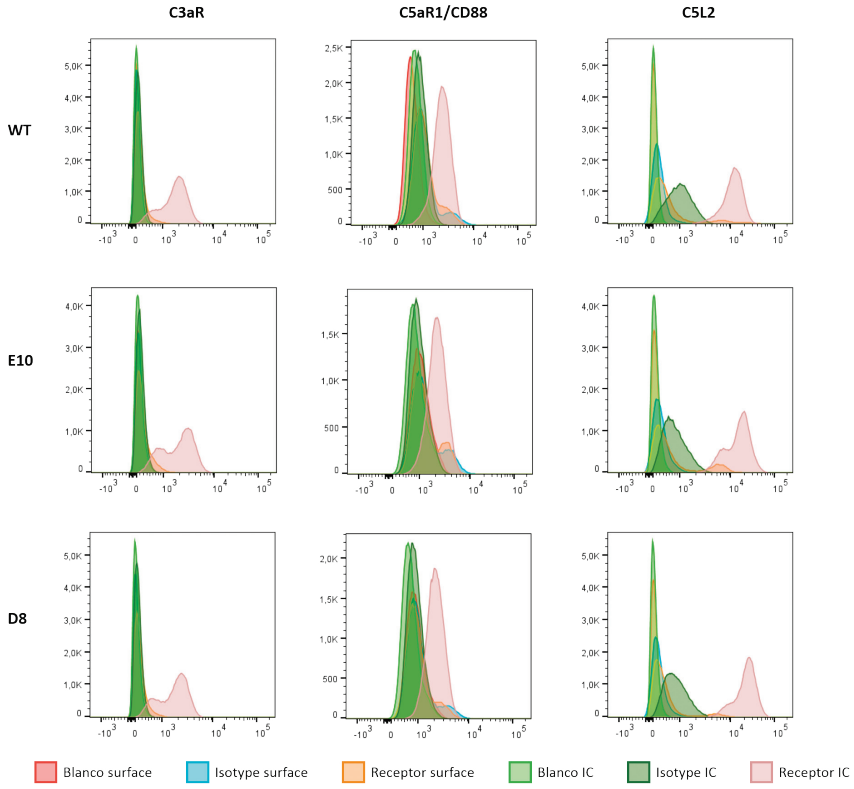

Figure 3. HAP-1 clones intracellular expression C3aR, C5aR1 and C5L2

Histograms of various receptor expression are presented both surface detection and intracellular (IC) after gating for single cells as measured by LSR II an analysed by FlowJo. Wildtype (WT) is the HAP-1 wildtype cell line, E10 and D8 are the clonal cell lines analysed. On the y-axes the counts are depicted and the x-axes show the mean fluorescence intensity.

Next, proliferation of the different cell lines was analysed over a course of 72 hour culture in T75 culture flasks. No differences in proliferative capacity were found between the cell lines in three independent experiments (**Figure 4A**). Next, the basal metabolism of the cells was analysed using the Mito Stress test on a Seahorse XFe96 Analyzer. First, three measurements were performed to obtain a basal measurement per cell line. Next, oligomycin was injected, which inhibits ATP synthase resulting in a reduction in mitochondrial respiration or OCR. The decrease observed in the WT, E10 and D8 cell lines was quite minor. Then FCCP is injected, resulting in the disruption of mitochondrial membrane potential, and uninhibited electron transport chain (ETC) allowing maximum oxygen consumption of cells. The final drugs injected is a combination of Rotenone and Antimycin A, which shuts down the ETC resulting in the non-mitochondrial respiration [24]. Afterwards the BCA content was measured to normalize the results, no differences in mitochondrial respiration or glycolysis was observed between the three cell lines, indicating that C3 does not play a prominent in these basal cellular processes. (**Figure 4B**). Also in this setting no differences were found between the cell lines, it does seem that there is a tendency for D8 to have a higher OCR, but upon the injection of the different drugs, the relative changes in OCR are the same for all three cell lines tested. Likewise, morphology of the cells did not change analysed by phase microscopy after 24 hours of culture and on the forward and side ward scatter with flow cytometry (**Figure 4C, D**). Together, these results indicate that knock out of C3 has no effect on cell survival, morphology, and metabolism in a HAP-1 cell line.

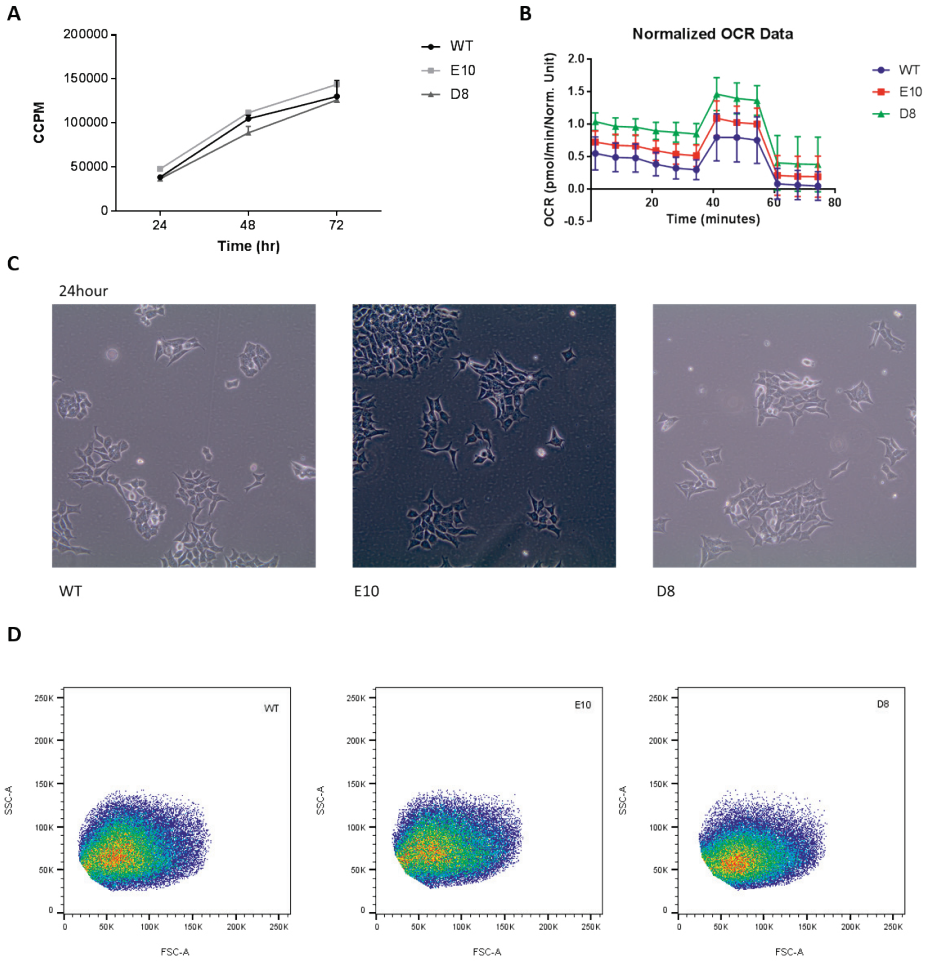

Figure 4. HAP-1 clones gDNA, proliferation, mitochondrial respiration and morphology
(A) Cell proliferative capacity as measured of incorporation of labelled tritium by the different HAP-1 cell lines over the course of 72 hour, taken at 24hour intervals. Shown is a representative of three experiments and all measurements were done in triplicate. (**B**) Oxygen Consumption Rate (OCR) data as measured by the Mitostress test, normalized on BCA content. Olig = Oligomycin, FCCP = Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone, R / A = Rotenone + Antimycin A. (**C**) Images from the different clones taken with the Olympus SC30 phase microscopy after a 24 hour culture period with a seeding concentration of $1x10^6$ cells in 75 cm^2. (**D**) Forward and sideward scatter (FSC and SSC respectively) from the different clones after gating for single cells as measured by LSR II an analysed by FlowJo.

DISCUSSION

Intracellular complement, or the ''complosome'' emerged recently as an interesting new perspective in the field of complement immunology. However, some precaution may be warranted, since replication of published articles remains challenging and necessary. Here, we set out to further explore intracellular C3 with the emphasis on studying the intriguing role that has been attributed to intracellular C3 processing and signalling.

The replication of the findings that C3 is present intracellularly on the protein level using western blot was challenging given the discontinuation of the detection antibody originally used in the studies showing, the presence of intracellular stores containing C3 by confocal microscopy [5]. Therefore, various antibodies were tested for specific reactivity to C3, but, unfortunately, no convincing results by western blot could be obtained. Interestingly, one polyclonal antibody (Dako C3d), which detected the alfa chain at the correct expected molecular weight in almost all lysates, also detected commercially available C3 fragments at the predicted molecular weight. Unfortunately, the polyclonal antibody does not appear to be specific for C3 as it detected a protein with a molecular weight of 110 kDa, (same as the alfa-chain of C3) in lysates of various cellular origins in reducing but also in non-reducing conditions, which is not compatible with C3 [25]. Therefore, we consider it most likely that this antiserum did not visualize C3 in these circumstances. These data are relevant as they point to the absence of C3-expression in these cell lines. Likewise also other antibodies failed to detect a signal in the lysates analysed. Thus, together, we were not able to convincingly show the presence of intracellular C3 on the protein level. In contrast, when the cells were incubated with NHS prior to lysis, we were able to detect C3 in the lysate, indicating that C3 can be detected intracellularly after incubation with NHS as published before [10], which also indicates that the intracellular C3 is either absent or too low to detect prior to this incubation. Thus, together, these data indicate that, under the experimental conditions used, C3-expression cannot be detected in freshly isolated CD4+ T-cells and PBMCs using the various antibodies tested.

Next, we wished to analyse the cleavage of C3 by CTSL and subsequent generation of C3a. We show that CTSL specifically and reproducibly cleaves C3 *in vitro*. However, as shown by MS, the terminal arginine remained attached to the C3b part of the protein, indicating that the fragment generated by CTSL is likely not exactly the same sequence as C3a since. The loss of the terminal arginine on C3a indicates the formation of “ASP”/ C3a-desArg. In contrast to the complete C3a, this variant is not able to bind to the C3aR. Interestingly, ASP has been linked to metabolism [17, 18] and so has (intracellular) complement [8, 9] which could show that metabolism and complement are more intrinsically linked. The observations that CTSL cleavage of C3 produces C3a-desArg instead of C3a is in disagreement with the results and interpretations presented by Liszweski et al. [5], concluding that intracellular C3 processing induced the activation

of intracellular C3aR. The authors argued that intracellular C3 was essential for survival of cells and demonstrated that C3 deficient patients would also express intracellular compartments containing C3 [5]. However, the notion that C3 was essential for cell survival was mainly derived from experiments involving CTSL inhibition and inhibition of C3aR with siRNA. In these experiments, reduced phosphorylation of mTOR was observed, which is essential for cell viability [26]. To study C3 deficiency, we knocked-out of C3 in a model cell line by CRISPR/Cas9. For this purpose, the HAP-1 cell line was used, which is a haploid cell line. By analysing growth, morphology and the metabolic capacity of the cells, no differences between the C3 sufficient and deficient cells was observed, despite the expression of the required receptors intracellular. Nonetheless, although widely used for several purposes, HAP-1 cells are different from other cells due to its haploid nature. Ideally, the use of freshly isolated CD4+ T-cells should be pursued for future CRISPR/Cas9 C3 knock out research. Nonetheless, the ability of cells to survive and grow is also supported by recent studies in which C3 was knock-out, like our study by CRISPR/Cas9, in the lung epithelial cell line A549 cells and an insulin secreting cell line (INS-1). They demonstrated that C3 expression was required for normal autophagy regulation in INS-1 cells [12]. They also state that autophagy can be regulated via mTOR activation, though they found no differences in the phosphorylation of mTOR comparing C3 sufficient and deficient INS-1 cells.

In conclusion, our results indicate the expression of intracellular C3 only after cells are incubated with NHS prior to lysis. We demonstrate that processing of C3 by CTSL is likely generating C3a-desArg. Finally, we showed in a HAP-1 cell line model that C3a is not essential for survival.

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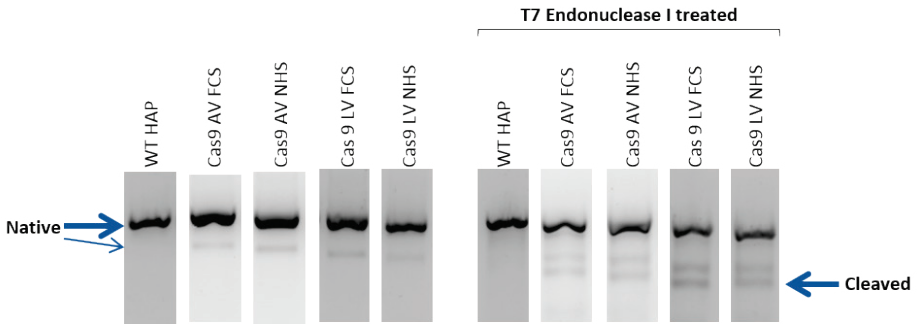
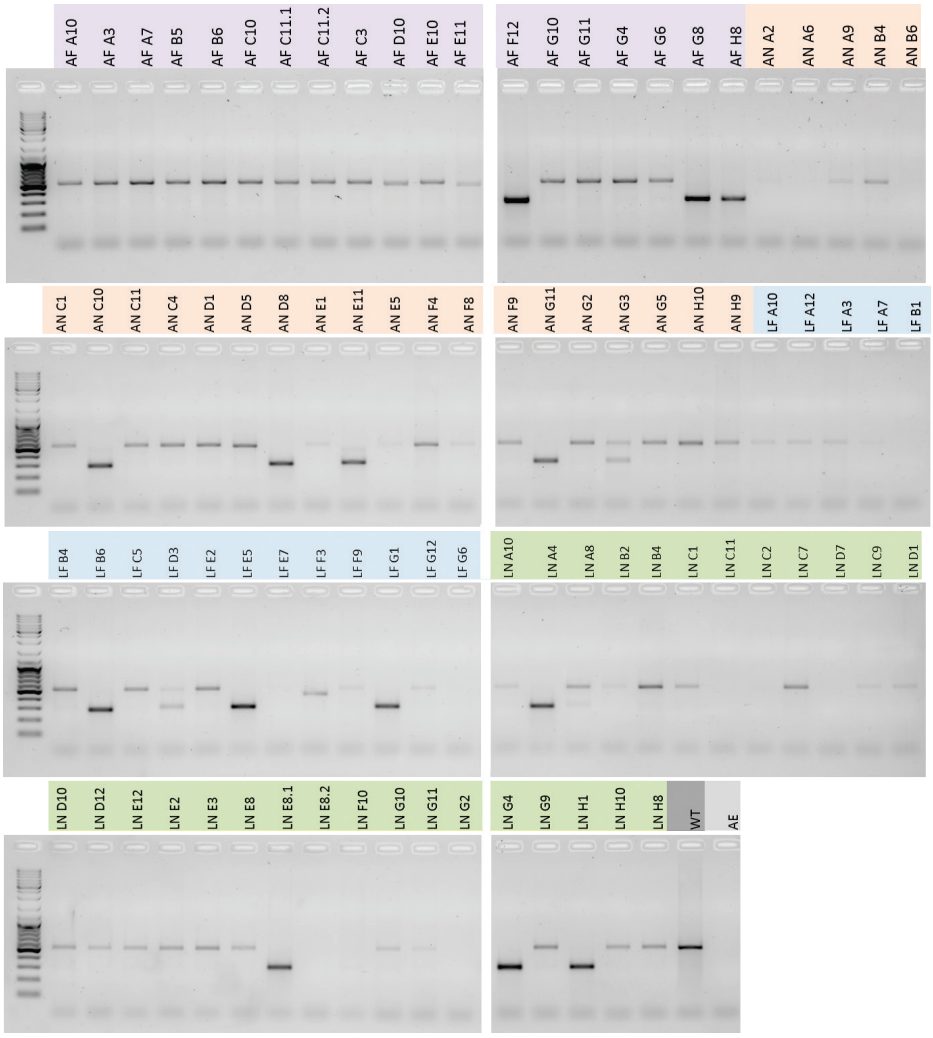
SUPPLEMENTARY FIGURES



Supplementary Figure 1. T7 Endonuclease I assay bulk CRISPR/Cas9 gDNA
The PCR products of the C3a containing the CRISPR sites is visualized on agarose gel for the different culture conditions after CRISPR/Cas9. Bulk genomic DNA was isolated from HAP-1 wildtype (WT); adenovirus with FCS (AF); adenovirus with NHS (AN); lentivirus with FCS (LF); lentivirus with NHS (LN). The PCR product without and with T7 Endonuclease I treatment were run on agarose gel to analyse fragmentation pattern, indicative for successful CRISPR/Cas9 procedure.



Supplementary Figure 2. Agarose PCR product CRISPR/Cas9 clones C3a

The PCR products of the C3a containing the CRISPR sites is visualized on agarose gel for the different clones generated after CRISPR/Cas9. This method resulted in four different culture conditions: adenovirus with FCS (AF); adenovirus with NHS (AN); lentivirus with FCS (LF); lentivirus with NHS (LN). WT is genomic DNA from HAP-1 wildtype and AE is the elution buffer used for genomic DNA isolation.

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CHAPTER 4

COMPLEMENT COMPONENT C1Q AS SERUM BIOMARKER TO DETECT ACTIVE TUBERCULOSIS

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ABSTRACT

Background: Tuberculosis (TB) remains a major threat to global health. Currently, diagnosis of active TB is hampered by the lack of specific biomarkers that discriminate active TB disease from other (lung) diseases or latent TB infection (LTBI). Integrated human gene expression results have shown that genes encoding complement components, in particular different C1q chains, were expressed at higher levels in active TB compared to LTBI.

Methods: C1q protein levels were determined using ELISA in sera from patients, from geographically distinct populations, with active TB, LTBI as well as disease controls.

Results: Serum levels of C1q were increased in active TB compared to LTBI in four independent cohorts with an AUC of 0.77 [0.70 ; 0.83]. After six months of TB treatment, levels of C1q were similar to those of endemic controls, indicating an association with disease rather than individual genetic predisposition. Importantly, C1q levels in sera of TB patients were significantly higher as compared to patients with sarcoidosis or pneumonia, clinically important differential diagnoses. Moreover, exposure to other mycobacteria such as *M. leprae* (leprosy patients) or BCG (vaccinees) did not result in elevated levels of serum C1q. In agreement with the human data, in non-human primates challenged with *Mycobacterium tuberculosis*, increased serum C1q levels were detected in animals that developed progressive disease, not in those that controlled the infection.

Conclusions: In summary, C1q levels are elevated in patients with active TB compared to LTBI in four independent cohorts. Furthermore, C1q levels from patients with TB were also elevated compared to patients with sarcoidosis, leprosy and pneumonia. Additionally, also in NHP we observed increased C1q levels in animals with active progressive TB, both in serum and in broncho-alveolar lavage. Therefore, we propose that the addition of C1q to current biomarker panels may provide added value in the diagnosis of active TB.

INTRODUCTION

Tuberculosis (TB) is a major global health threat, which is caused by infection by *Mycobacterium tuberculosis* (*M.tb*) [1]. Current estimations indicate that a quarter of the global population is infected with *M.tb*, with a life-long risk to develop active TB disease. Particular regions, such as South-East Asia, Western-Pacific, and Africa regions account for more than 80% of infected individuals [2].Annually over 6 million people are diagnosed with TB disease, a serious and highly contagious condition, resulting in 1.3 million deaths in 2016 only [1]. While most infected individuals remain asymptomatic latently infected (LTBI), a minority (5-10%) of these individuals progress to active TB. Given the high rate of infections with *M.tb* in some regions it is important to discriminate infection from disease, which is difficult with the currently available tests. At present, only *M.tb* detection in sputum using smear, PCR or culture is definitive proof of TB disease. Early diagnosis and treatment of TB disease is important to reduce transmission of infection and prevent disease associated mortality [1].

Diagnosis of active TB is made by microbiological or genetic detection of *M.tb* in sputum (or other specimens in case of extrapulmonary TB), but this can be expensive and time-consuming depending on bacterial burdens or requires complex methodology and infrastructure. Current immunological tests can detect infection with *M.tb* but often fail to discriminate active disease from latent infection [3]. Therefore, there is an urgent need to identify biomarkers that can discriminate active and latent TB infection in order to promptly initiate treatment to prevent mortality and further spread of the pathogen, in particular in areas where *M.tb* is highly endemic. Ideally, such biomarkers should also be able to discriminate between TB and other respiratory infections that present with similar symptoms and abnormalities on chest X-rays.

Many studies have identified potential biomarkers that discriminate active TB from LTBI, or that are predictive of which individuals will progress to active TB [4; 5; 6; 7; 8; 9; 10]. Differential gene expression profiles between patients with TB and LTBI or other (lung) diseases resulted in identification of an array of potential biomarkers, such as *FCGR1A* [11; 12; 13] and *GBP5* [5; 6; 14]. Recently, complement has been highlighted as candidate biomarker for active TB disease [15; 16; 17; 18; 19] also in the presence of HIV co-infection [20]. Most currently identified biomarkers have been identified at the transcriptomic level; however, easy, robust markers that can be measured at the protein level would be more ideal candidates for application in the field. Therefore, validation of markers previously identified at the mRNA level at the protein level would provide important insights into the applicability of such markers in clinical practice.

Next to biomarker studies in humans, there are various experimental models to study the host-pathogen interaction. The best available model is the non-human primate model (NHP), infection of rhesus macaques with *M.tb*, resulted in TB disease which

closely resembles human TB as they experience similar lesions and clinical courses as humans, suggesting a common pathophysiology [21]. The NHP model adds important information on kinetics of disease development following *M.tb* infection and can be manipulated with e.g. different dosages of infection, different infecting strains, but also with vaccines.

The complement system is an important part of the innate immune system and functions as a proteolytic cascade. The classical complement pathway is initiated by binding of C1q to ligands such as immune complexes. Following the binding of C1q to ligands, enzymatic processes lead to the release of inflammation stimulating peptides, C3a and C5a, formation of the opsonin C3b and formation of the membrane attack complex, resulting in target cell lysis [22]. Furthermore, C1q can bind several receptors that contribute to other important functions such as phagocytosis or myeloid cell modulation, outside traditional complement system activation [23; 24]. For instance, C1q is involved in neovascularization during pregnancy, coagulation processes and neurological synapse function [25]. C1q is a 480kDa protein composed of six arms, each comprising one A, B and C peptide chain [26]. These three chains are encoded by individual genes, *C1QA, C1QB* and *C1QC,* located on chromosome 1p. In contrast to most complement proteins, C1q is not produced by hepatocytes but mainly by monocyte derived cells such as macrophages and immature dendritic cells [27; 28; 29] and by mast cells [30]. Increased expression of mRNA for C1q has been associated with TB disease [16; 19].

Here, we analyzed differential expression of complement genes in patients with TB. Since in publicly available datasets, C1q expression was most pronouncedly upregulated, we validated C1q at the protein level in samples from various patient groups as a biomarker for active TB. Patients with active TB disease were compared to latently infected individuals, vaccinnees and to patients with clinical conditions that are important differential diagnoses in clinical practice. Finally, to obtain more insight in the pathophysiology, kinetic analyses were performed samples obtained from NHP animal models of TB.

MATERIALS AND METHODS

Patients and controls

Demographic data and classification of the cohorts are presented in Table 1. Below we have specified the specific inclusion criteria per cohort.

Tuberculosis

Smear, GeneXpert or sputum culture positive pulmonary TB patients, LTBI patients and treated TB patients as well as endemic controls (in different combinations) were included from various demographic locations: Italy [31], the Gambia, Korea and South

Africa (Table 1). Patients with active pulmonary TB disease, referred to as 'TB' in the manuscript, were diagnosed based on local, routine methodology. Active pulmonary TB was sputum-culture confirmed (BACTEC™, Becton-Dickinson, USA), or based on positive Xpert Mtb/RIF assay (Cephaid Inc., Sunnyvale, CA, USA), patients were included within 7 days of TB treatment initiation. Latent TB infection, LTBI, was determined by Quantiferon TB Gold-in tube positivity (Qiagen, The Netherlands).

In the cohort from South Africa people suspected for TB were used as controls, these people were presenting with symptoms compatible with active TB but had negative X-ray and negative sputum cultures for TB. These suspected TB patients were seen again after two months and had recovered spontaneously or after appropriate (non-TB related) treatment. All TB patients were HIV-negative, as were the endemic controls. Additionally, from Italy both LTBI (QuantiFERON TB Gold-In-tube-positive individuals) and successfully treated TB patients (2-72 months after end of therapy) were included. TB patients from the Gambia were followed over time (one, two and six months after diagnosis), until completion of successful treatment.

Other mycobacterial diseases and vaccination

Leprosy patients (mostly immigrants with mixed ethnic backgrounds) at diagnosis of primary leprosy were included in the Netherlands. In addition, patients with type-1 reactions were enrolled in Brazil, Nepal and Ethiopia. Furthermore, we measured C1q in healthy Dutch individuals who were vaccinated with BCG Danish strain 1331 (Statens Serum Institut, Denmark) and followed over time [32].

Other pulmonary diseases

Patients with community acquired pneumonia were included in the Netherlands, one cohort comprised patients admitted to the intensive care unit in a tertiary care hospital in Leiden (one patient was HIV-infected with a normal CD4 count and one suffered from sarcoidosis) and the other cohort comprised patients admitted to a hospital ward of a non-academic teaching hospital in Nieuwegein. From both groups of pneumonia patients paired samples from the time of diagnosis and after recovery (10-124 days later) were available. Finally, samples were included prior to initiation of treatment from sarcoidosis patients in the Netherlands that had pulmonary involvement.

Additional control group

As a reference group we included a panel of Dutch healthy controls, not suffering from major infections or autoimmune disease.

Ethics statement

Blood was obtained from individuals upon signing an informed consent. All studies comply with the Helsinki declaration. The use of the samples in this study was approved by local ethical committees. For Italy, Ethical Committee of the L.Spallanzani National

Institute of Infectious diseases (02/2007 and 72/2015); The Gambia (SCC1333); Korea, Institutional Review Board for the Protection of Human Subjects at YUHS; South Africa, Health Research Ethics Committee of the Faculty of Medicine and Health Sciences at Stellenbosch University (N13/05/064); Brazil, National Council of Ethics in Research and UFU Research Ethics Committee (#499/2008); Nepal, Health Research Council (NHR#751); Ethiopia, Health Research Ethical Review committee Ethiopia (NERC#RDHE/127-83/08); The Netherlands leprosy patients, (MEC-2012-589); sarcoidosis patients, Medical research Ethics Committees United of the St Antonius (#R05.08A); BCG vaccinated individuals, Leiden University Medical Center Ethics Committee (P12.087); control group, (P237/94); pneumonia Leiden (P12.147); pneumonia Nieuwegein (C-04.03 and R07.12).

Table 1. Description of the cohorts.

Country	Classification	N	Age mean (range)	Sex (%male)
Italy	Control	15	38 (25-57)	40
	Latent TB	18	37 (21-77)	33
	Active TB	18	38 (23-67)	89
	Treated TB	17	39 (18-70)	35
The Gambia	Control	50	31 (15-60)	30
	Active TB	50	34 (17-62)	62
Korea	Control	10	23 (21-25)	90
	Active TB	10	51 (24-77)	40
South Africa	Suspect TB	31	32 (18-56)	26
	Active TB	20	32 (19-57)	65
Multiple*	Leprosy reactions	53	(18-69)	68
the Netherlands	Leprosy†	33	34 (18-57)	62
	Sarcoidosis	50	43 (26-57)	60
	Control	80	38 (21-67)	36
	BCG vaccinated	12	27 (23-57)	33
	Pneumonia (Leiden)	40	66 (23-93)	60
	Pneumonia (Nieuwegein)	28	73 (34-91)	57

In the table the different cohorts are described regarding the country of origin of the samples, the disease classification, and the total number of samples per group as well as the demographic info on age and sex.

* Nepal, Brasil, Ethiopia

† Diagnosis made in the Netherlands

Gene expression analysis

Global transcriptomic analyses have been performed to compare patients with active TB disease with latently infected individuals. In addition, transcriptomes from patients with TB disease were compared with patients with other diseases such as sarcoidosis, pneumonia or lung cancer [5; 6; 7; 33; 34; 35; 36; 37]. Microarray data from these studies, publically available in Gene Expression Omnibus (GEO) (GSE37250, GSE19491, GSE39941, GSE28623, GSE73408, GSE34608, GSE42834, GSE83456), were retrieved from

GEO and re-analyzed. Several of the studies described multiple independent cohorts, which we analyzed separately for each population (from Malawi (2), South Africa (3), United Kingdom (2), Kenya (only TB vs LTBI), The Gambia (only TB vs LTBI), USA and Germany (only TB vs other diseases))., All data were extracted from GEO and compared in the same way using GEO2R, thus not relying on the analysis performed in the original manuscript. *GEO2R* compared two or more groups of samples in order to identify genes that are differentially expressed across experimental or clinical conditions. Here, lists of differentially expressed genes between TB and LTBI or TB and other diseases (with a significance of $p>0.05$ and a factorial change of >2 or <0.5) were generated. A list of complement genes and two reference genes was used to assess possible differential expression for each individual gene for each study population. All studies/ populations with significant differential expression for a particular gene between patients with TB compared to LTBI/ other diseases were enumerated and expressed as the percentage of the total number of comparisons investigated. E.g. differential expression of C1QA between TB and LTBI was observed in only 2/9 populations investigated (22%) whereas C1QB differed in 8/9 populations (88%). For all populations with significant differential expression, the factorial change (the difference between gene expression in TB patients compared to LTBI/ other diseases) was calculated and plotted.

Animals

Non-human primate (NHP) serum was available from a biobank of samples collected from earlier TB studies in healthy, purpose-bred rhesus macaques (*Macaca mulatta*) for which ethical clearance was obtained from the independent ethical authority according to Dutch law. All housing and animal care procedures were in compliance with European directive 2010/63/EU, as well as the "Standard for Humane Care and Use of Laboratory Animals by Foreign Institutions" provided by the Department of Health and Human Services of the US National Institutes of Health (NIH, identification number A5539-01). Longitudinal banked serum samples were available for C1q analysis. Animals were non-vaccinated or vaccinated with BCG (BCG Danish 1331 (Statens Serum Institute, Denmark)) (n=23) and experimentally infected via bronchoscopic instillation of 500 CFU of *M.* Erdman. Prior to infection and 3, 6, 12, 24, 36 and 52 weeks post infection samples were collected and stored. Animals were sacrificed when reaching early humane endpoints (acute progressors) or after reaching the pre-defined end-point (52 weeks) (non-progressors). Broncho-alveolar lavage (BAL) samples were available from six animals infected with 1 to 7 CFU of M. Erdman prior to infection and 6 or 12 weeks post infection.

Detection of C1q by ELISA

C1q levels in sera were measured using an in-house developed ELISA. Maxisorp plates (nunc) were coated overnight with mouse anti-human C1q (2204) [38], Nephrology department, LUMC) in coating buffer (0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH9.6). Plates were washed and blocked with PBS/1%BSA for one hour at 37°C. After washing, a serial dilution of a pool of normal human serum (NHS) was applied as a standard and samples

were added in dilution to the plate, all in duplicate, and incubated for one hour at 37°C. Human sera were diluted 1:8000 and NHP sera 1:4000, the BAL fluids were diluted 1:1. After washing, plates were incubated with rabbit anti-human C1q (Dako cat#A0136) for one hour at 37°C and for detection a goat anti-rabbit HRP (Dako cat#P0448) was used which was also incubated for one hour at 37°C. All washing steps were performed with PBS/1%BSA/0.05%Tween. Plates were stained using ABTS and measured at absorbance of 415nm, the measured C1q is expressed in µg/mL as compared to a C1q standard.

Immunohistochemical staining of lung tissue for C1q

Samples were collected at autopsy from patients with fatal TB disease (n=3), fatal pneumonia patients (n=4) and a control that died of vascular disease (n=1) at the National Institute for Infectious Diseases, Rome, Italy under local ethical approval (72/2015). Paraffin sections of 4µm thickness were subjected to heat-induced antigen retrieval using tris/EDTA (pH 9.0) at 96°C for 30 minutes, and then stained with rabbit anti-human C1q (1:1000; Dako cat#A0136) in PBS/1%BSA for one hour at room temperature, followed by an anti-rabbit Envision (Dako) HRP conjugated antibody also for one hour at room temperature, with DAB+ as the chromogen. Negative control rabbit immunoglobulin fraction (Dako) was used as a negative control in the same concentration as the primary antibody. Sections were counterstained with Haematoxylin (Klinipath; 4085.9001).

Statistics

Statistical analyses were carried out using SPSS statistics version 23 (IBM) or Graphpad Prism version 7. To compare C1q levels the Mann-Whitney U test, Kruskall-Wallis and Dunn's multiple comparisons test were used. In all graphs the median is shown unless indicated otherwise. Receiver operating characteristic (ROC) analysis was performed to assess the sensitivity and specificity of C1q as biomarker and was expressed as Area Under the Curve (AUC).

RESULTS

***C1Q* expression is upregulated in patients with active TB disease**

Publically available microarray data from TB patients were retrieved from Gene Expression Omnibus, all data were ranked as differentially expressed between TB patients and either LTBI or other diseases [5; 7; 33; 34; 35; 36; 37]. These studies contained information from diverse populations (Malawi, South Africa, United Kingdom, Kenya, The Gambia, USA, and Germany). A list of complement gene expression patterns was generated and the number of microarray studies that reported differential complement gene expression between patients with TB and LTBI (Figure 1A) or other diseases (Figure 1B) was enumerated. Complement genes *C1QB, SERPING1* were expressed at higher level (more than two-fold) in 8/9 (88%) of studies comparing patients with active TB to LTBI (Figure 1A). C1QC was expressed at a higher level in

TB patients in 7/9 studies (78%), whereas C1QA expression was only increased in TB patients in 2/9 studies (22%) (Figure 1A). The observed factorial changes in the expression of these complement genes between TB patients and LTBI individuals were comparable with the changes as seen for *FCGR1A* and *GBP5*, which were previously described as promising and highly consistent biomarkers of active TB (Figure 1C). A similar pattern, although less pronounced, was seen when comparing TB patients with patients having another lung-disease (Figure 1 B, D). As C1q is abundantly present, easy to measure and stable, therefore we continued to analyse C1q protein levels.

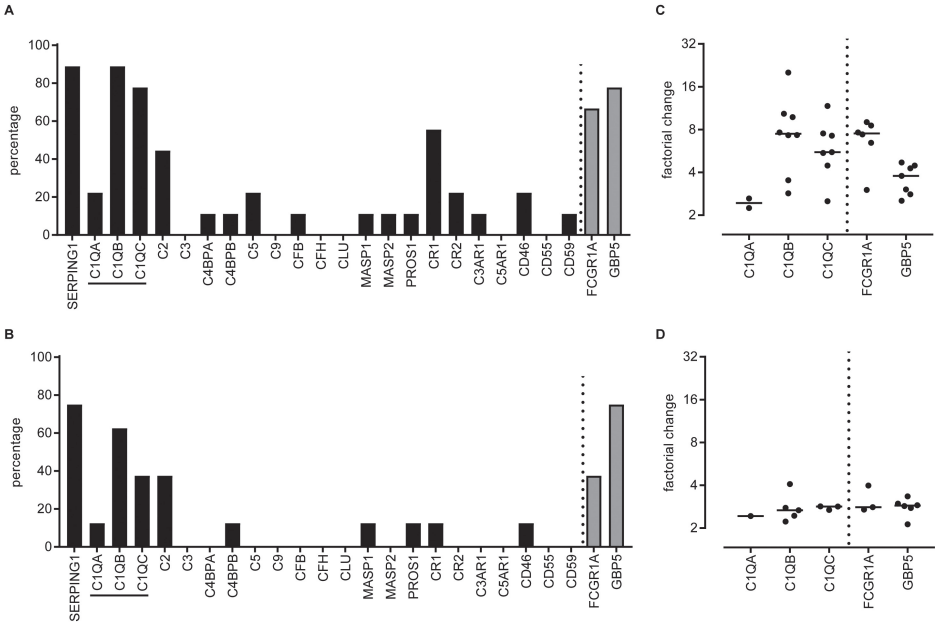


Figure 1. Differentially expressed complement genes in whole blood tuberculosis transcript signatures.

Tuberculosis specific transcript signatures, from various populations, were investigated for the presence of differentially expressed complement genes using a tuberculosis RNA biomarker database. Publically available transcriptome data was retrieved from Gene Expression Omnibus [5; 6; 7; 33; 34; 35; 36; 37] and analyzed using GEO2R. Data were available for 9 populations comparing active TB with LTBI and for 8 populations comparing active TB with other diseases.. For each population we determined if the complement family genes were differentially expressed between TB and LTBI or other diseases. Differential expression was defined as an adjusted p-value < 0.05and more than 2-fold change. Differential expression of a gene between TB and LTBI or other diseases was expressed as percentage of the total number of populations investigated. The differential expression of complement genes was scored (**A**,**B**) as well as the mean factorial change for the C1q genes, *C1QA, C1QB* and *C1QC* (**C**,**D**), for the comparisons TB versus LTBI (A,C) and TB versus other diseases (B,D). As a reference two other highly upregulated potential diagnostic TB markers, *FCGR1A* and *GBP5*, were included in the analyses.

C1q is significantly increased in serum of TB patients

C1q protein levels were measured in sera from TB patients and controls from independent and geographically distinct cohorts (Fig 2A-D). The levels of C1q were significantly higher in sera from patients with active TB as compared to their respective controls in all cohorts: Italy (Fig 2A), The Gambia (Fig 2B), Korea (Fig 2C) and South Africa (Fig 2D). LTBI individuals and successfully treated TB patients had serum C1q levels similar to controls (Fig 2A). Combined analysis of all TB patients, control groups, LTBI and the successfully treated TB patients from the different cohorts revealed that serum C1q protein levels are significantly ($p<0.001$) increased in active TB (Fig 2E).

The Gambian TB patients were followed over time which allowed us to investigate C1q levels during successful treatment. At one month of treatment the median serum C1q level was still increased, however, the level of C1q began to decrease after two months ($p=0.0650$), resulting in complete normalization compared to the TB contacts after six months of successful treatment ($p<0.001$) (Fig 2F). Thus, serum C1q protein levels were significantly elevated in patients with active TB, and levels decreased to the level of the control population during successful treatment. This further indicates that increased C1q levels are associated with active TB disease and do not reflect genetic variation in C1q expression.

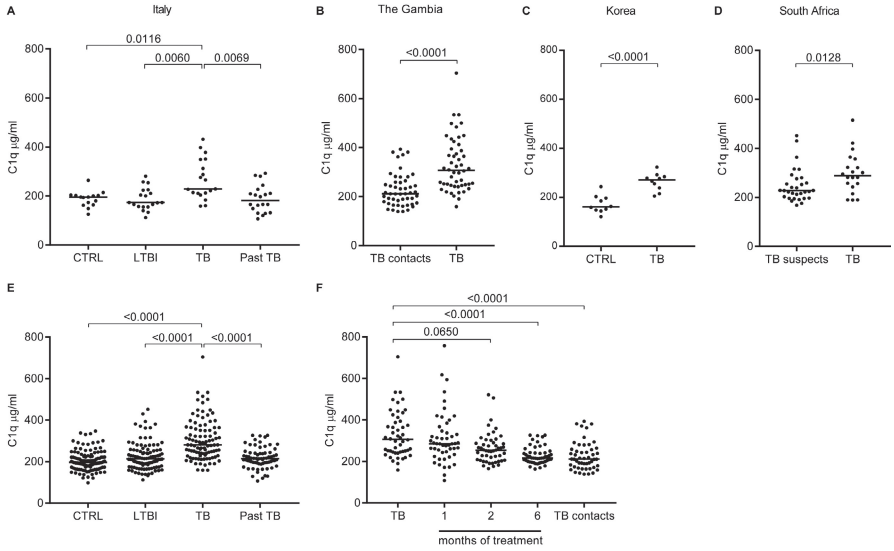


Figure 2. C1q serum levels are increased in patients with pulmonary Tuberculosis.

C1q levels (µg/ml) were measured with ELISA in sera from TB patients (active disease) and controls from different cohorts. First the results from the independent and geographically different cohorts are depicted. TB patients from Italy were compared to Latent TB infected (LTBI), to Past TB (patients that were successfully treated for TB) and to endemic controls (**A**). TB patients from The Gambia were compared to TB contacts (**B**). TB patients from Korea were compared to endemic controls (**C**). From South Africa the TB patients were compared to patients suspected for TB but confirmed non-TB (**D**).

Subsequently, data from the cohorts were pooled: Control (CTRL) comprises Dutch healthy controls, combined with the CTRL from Italy and Korea; moreover, healthy individuals prior to vaccination with BCG were included (n=117). Latent TB infected (LTBI) comprises LTBI from Italy, TB suspects (confirmed non-TB) from South Africa and TB contacts from the Gambia (n=100). Active tuberculosis (TB) from Italy, the Gambia, Korea and South Africa (n=99); Past TB are patients that were successfully treated for TB and combined the past TB from Italy and the Gambia samples after six months of treatment (n=71) (**E**). From the Gambia the TB patients were followed over time during treatment, TB contacts are shown on the right (n=50) (**F**). Results were analyzed using the Mann-Whitney U test, >2 groups Kruskall-Wallis and Dunn's multiple comparisons test. The treatment months were compared to TB diagnosis with Friedman Test and Dunn's multiple comparisons test.

Vaccination with BCG does not increase C1q levels in serum

To investigate if vaccination with *M. bovis* BCG, a live replicating mycobacterium, induced a similar increase in serum C1q levels, samples were taken before and after BCG vaccination of healthy Dutch volunteers and C1q levels were measured (Fig 3). Samples taken at screening and directly before vaccination showed minimal variation in C1q levels, reflecting normal variation within individuals. BCG vaccination did not induce fluctuations in C1q levels larger than this naturally observed variation. Thus, BCG vaccination did not increase C1q levels in contrast to what was observed in TB disease, despite the presence of live, replicating mycobacteria.

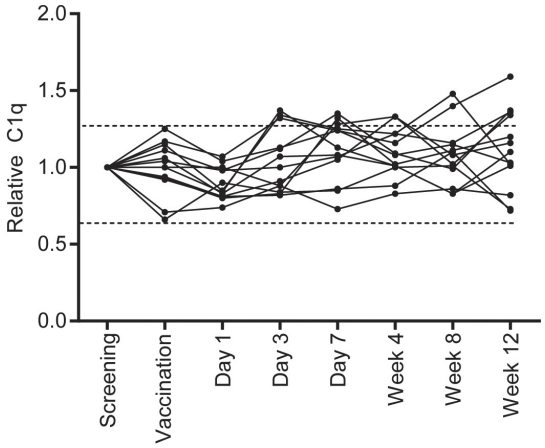


Figure 3. BCG vaccination does not induce a similar C1q upregulation.
Healthy individuals who were vaccinated with BCG (n=13), were followed over time and C1q serum levels were measured. C1q levels were first calculated to µg/ml and for each individual set to 1 using the measurement of the C1q level before vaccination. The dotted lines indicate the variation that is present in C1q levels at the time of screening and prior to vaccination.

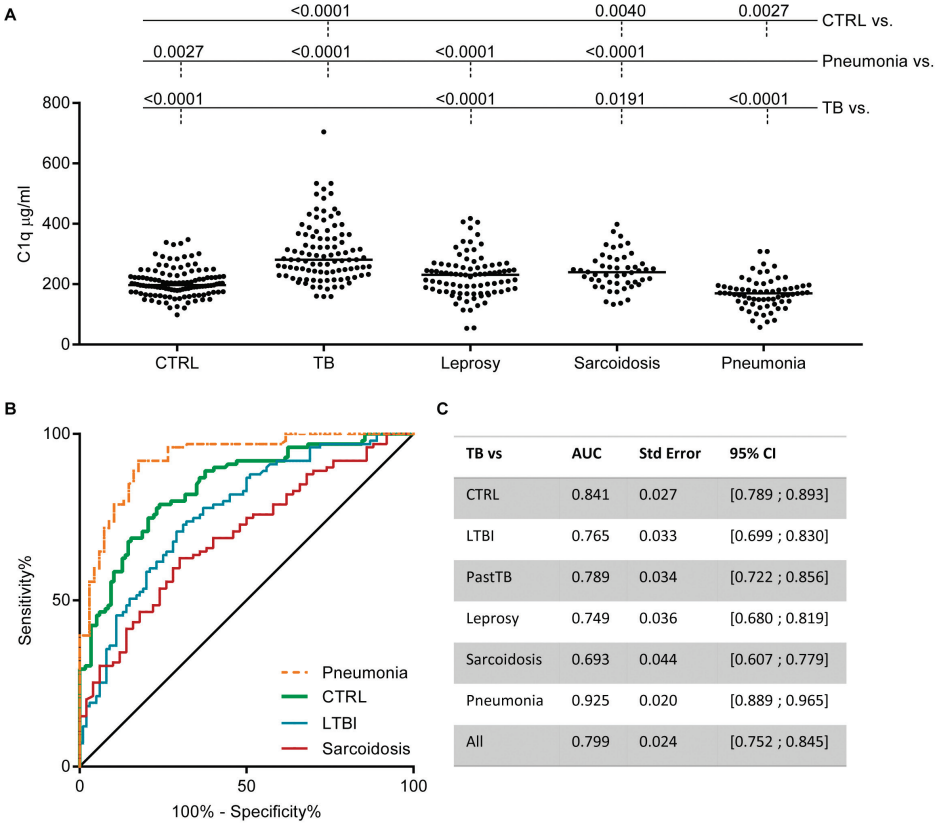
C1q levels are increased in active TB compared to other diseases

To investigate the specificity of increased C1q levels for active TB, sera from patients with other diseases with similar symptoms and radiological abnormalities (pneumonia, sarcoidosis) and sera from patients with other mycobacterial disease (leprosy, primary disease or type 1 (acute pro-inflammatory) reactions) were analyzed. C1q levels in sera from patients with TB were significantly higher compared to sera from patients with leprosy, pneumonia or sarcoidosis (Fig 4A, data from TB patients and controls same as used in Fig 2E). Some individual leprosy patients had C1q protein levels above the median of TB patients but this was not related to either primary disease or having type I reactions (Supplementary Figure E1A). Patients with sarcoidosis showed a slight increase in C1q levels compared to the controls. In contrast, patients with community acquired pneumonia showed a significant decrease in C1q levels compared to the control population. The reduced levels observed in patients with pneumonia at diagnosis was associated with the disease state since samples included from the same individuals at later time points had normal C1q levels (Supplementary Figure E1 B & C).

To assess the value of C1q as possible TB biomarker, the sensitivity, specificity and the positive likelihood ratio (LR+) were calculated from C1q concentration cut-offs [10]. With a cut-off at the 95th percentile of the control population (300.2 µg/ml C1q) the sensitivity is 42% with a specificity of 91% resulting in a LR+ of 4.96. Application of a cut-off at the maximum of the control population (347.6 µg/ml) resulted in a sensitivity of 29% and a specificity of 97% resulting in a LR+ of 8.99. The capacity of serum C1q to discriminate active TB from LTBI, pneumonia and sarcoidosis was also analyzed using ROC analyses and expressed as AUC (Fig 4B and 4C). The AUC of C1q levels for TB versus LTBI was 0.77, for TB versus sarcoidosis 0.69, and for TB versus pneumonia even an AUC of 0.93 was achieved.

C1q is locally present in the lungs of TB patients

So far circulating RNA and protein levels of C1q have been analyzed, which reflect the systemic response to TB. Additionally, we analyzed the local C1q production or deposition in response to *M.tb* infection by staining lung tissue. Staining lung tissue from a control revealed scarce C1q staining with only few C1q positive macrophage-like cells in the lung parenchyma and in the intra-alveolar space (Fig 5). In contrast, lung tissue of fatal TB patients revealed, next to the intra-alveolar C1q positive cells also a pronounced C1q staining both in the necrotic centers of the granulomas and in the surrounding lung tissue with predominantly macrophage-like cells staining positive. Lung-tissue from patients that succumbed from pneumonia showed C1q staining predominantly in the intra-alveolar space. Staining of consecutive sections with an isotype control did not reveal any staining, also not in the necrotic centers, confirming specific staining for C1q. Thus, C1q protein is locally detected at an increased level in the lungs of TB patients (n=3) compared to tissue samples from a non-pulmonary disease control (n=1) or pneumonia patients (n=4).



C

TB vs	AUC	Std Error	95% CI
CTRL	0.841	0.027	[0.789 ; 0.893]
LTBI	0.765	0.033	[0.699 ; 0.830]
PastTB	0.789	0.034	[0.722 ; 0.856]
Leprosy	0.749	0.036	[0.680 ; 0.819]
Sarcoidosis	0.693	0.044	[0.607 ; 0.779]
Pneumonia	0.925	0.020	[0.889 ; 0.965]
All	0.799	0.024	[0.752 ; 0.845]

Figure 4. Increased C1q serum levels are associated with TB.

C1q was measured in various cohorts by ELISA. The pooled data from figure 2E control population (CTRL) and TB (active disease) is now compared to other diseases: leprosy (n=86), sarcoidosis (n=50) and community acquired pneumonia (n=68) (**A**). Differences between groups were analyzed using Kruskall-Wallis and Dunn's multiple comparisons test. Both the leprosy and the pneumonia cohort comprise two patient groups. The data for these individual groups is visualized in Supplementary Fig E1. ROC analysis of the ability of C1q to distinguish TB from CTRL, LTBI, sarcoidosis and pneumonia are plotted together (**B**), and for all comparisons the Area Under the Curve (AUC) was calculated and summarized in the table (**C**).

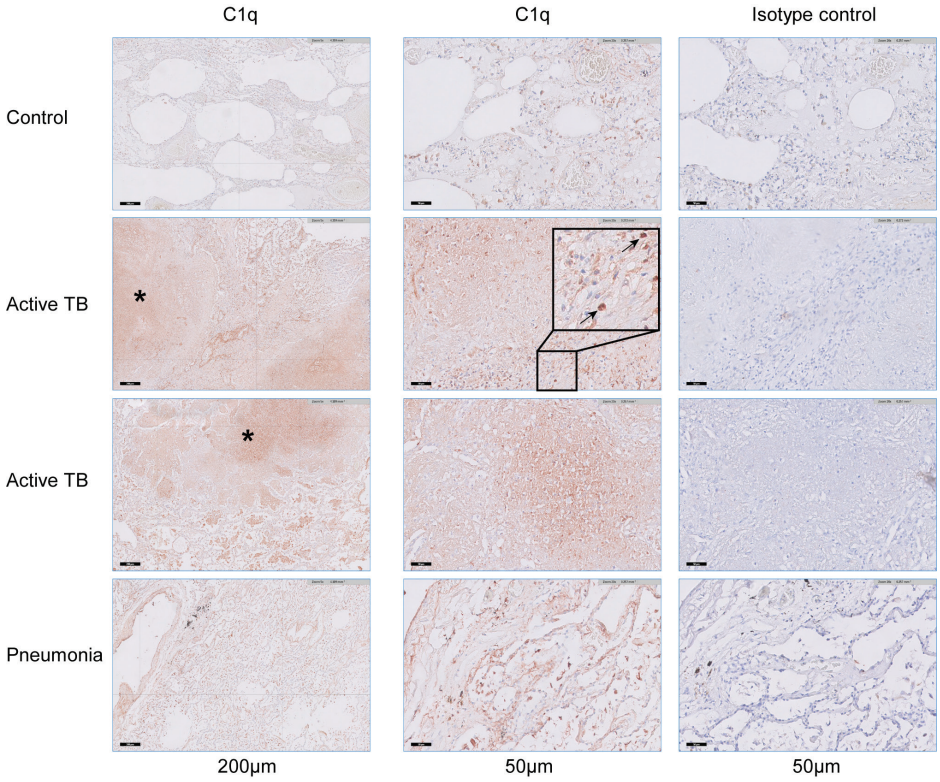


Figure 5. C1q accumulates in lung tissue of patients with fatal pulmonary TB.
Lung tissue, obtained at autopsy from a non-pulmonary disease control (n=1), fatal active TB patients (n=3), or patients with lethal pneumonia (n=3) were stained for the presence of C1q. The left column shows the presence of C1q in a section of the control, two active TB patients and a pneumonia patient, scale bar at 200µm. The middle column shows the same samples stained for C1q, while the right column shows consecutive tissue slides stained with a matched control antibody, scale bars at 50µm. Necrotic areas that stain positive for C1q are highlighted with an asterisk (*) and individual cells that stain positive for C1q are highlighted with arrows.

Non-human primates with active TB disease also display increased serum C1q levels

Non-human primate (NHP) *M.tb* infection models are widely used to study pathogen-host interactions and for pre-clinical evaluation of vaccine candidates [39]. After infection, rhesus macaques develop TB disease which closely resembles human TB in most aspects. Sera banked over the course of a long-term follow-up study in rhesus macaques were used to determine C1q levels after experimental *M.tb* infection.14 Out of 16 animals with active progressive disease, that had reached an early humane endpoint due to exacerbation of TB disease, had increased C1q levels compared to their baseline C1q levels before infection (Fig 6A). Such a rise in C1q levels was absent in six out of seven animals that did not develop overt disease, but controlled the infection over an extended period of time up to 1 year post-infection (Fig 6A). Additionally, in an

separate cohort of *M.tb* infected NHPs we detected elevated C1q levels in five out of six broncho-alveolar lavage (BAL) fluid samples taken before necropsy, while no C1q could be detected in paired BAL fluids taken prior to infection (Fig 6B). The observed differences could not be explained by any differences in BAL volume recovery and thus these data reflect a true local increase in C1q after *M.tb* infection.

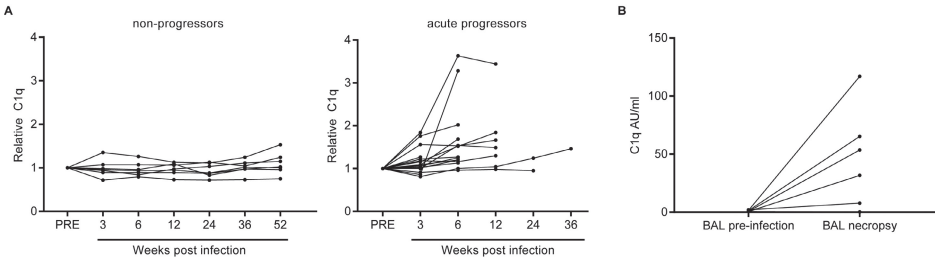


Figure 6. Rhesus macaques infected with Mycobacterium tuberculosis display increased levels of C1q in serum and broncho-alveolar lavage fluid.
C1q levels were measured in serum samples from rhesus macaques (n=23) which were infected with *Mycobacterium tuberculosis* and followed over time. C1q levels were first calculated as Arbitrary Units (AU) per ml after which the C1q level of each animal at baseline was set as 1. Animals which controlled the infection are termed non-progressors and animals reaching a premature humane endpoint due to active disease progression are termed acute progressors (**A**). Separately, C1q was measured in broncho-alveolar lavage (BAL) fluid obtained from animals before and six or 12 weeks after infection with *M.tb* (n=6) and calculated as AU/ml (**B**).

DISCUSSION

The accurate and fast identification of patients with active TB remains challenging, largely because of limitations in the current diagnostic tools to differentiate active TB from other diseases, as well as LTBI. Extensive searches for biomarkers that can discriminate active TB from other diseases with similar clinical presentation as well as from LTBI have been recently reported [3; 40]. Several studies reported genes encoding for complement components to be upregulated in TB [15; 16; 17; 18; 19]. Here, we compiled available genome wide gene expression data for TB compared to LTBI and TB compared to other lung diseases [5; 6; 7; 33; 34; 35; 36; 37] and observed that in particular C1q encoding genes were highly upregulated. Interestingly, these observations were made in studies using RNA from whole blood, indicating increased transcription of C1q genes in circulating blood cells, most likely monocytes/ macrophages. Since C1q is not a typical acute-phase protein we were interested to confirm and validate these findings at the protein level. We have therefore measured C1q protein levels in serum and confirmed increased levels in patients with active TB, but not in other diseases with a similar clinical presentation such as pneumonia or sarcoidosis, or mycobacterial exposure. C1q protein is also present in the lung tissue of deceased TB patients. The

increased levels of local and circulating C1q in TB were replicated independently in a NHP TB infection model.

Literature suggests that expression of the three C1q genes *C1QA, C1QB* and *C1QC* is regulated in a similar manner [41]. However, upon IFNγ-stimulation upregulation of *C1QB* is higher than *C1QC*, which is higher than *C1QA* [41]. Similarly, our data also showed upregulated expression of *C1QB* and *C1QC* genes and *C1QA* was less frequently observed in active TB, also in the analysis from Cai et al. the extend of increase in the expression of *C1QA* was less pronounced as compared to the increase in expression of *C1QB* and *C1QC*. Since C1q protein production requires equal ratios of all three chains, the detected increase in C1q protein levels in TB patients indicates that all chains are expressed. The low detection of *C1QA* may thus be technical and reflect a poor capture of *C1QA* expression on the microarrays in general.

We measured C1q protein levels in four different geographical cohorts from Italy, The Gambia, Korea and South Africa. C1q levels were increased in patients with active TB compared to all relevant control populations. Importantly, treatment normalized serum C1q levels to those of endemic controls, indicating that the upregulation of C1q was associated with the disease and not intrinsic to the individuals. BCG vaccination, although being a live replicating mycobacterial vaccine, did not induce increased C1q levels. Although leucocytes of patients with leprosy reactions were reported to express increased levels of *C1QA, B* and *C* [42], the cohorts analyzed here did not universal show increased serum C1q levels. Individual patients might have somewhat increased levels, both in this study (type 1 reaction) and a previous report (type 2 reaction) [43], which warrants more detailed analyses. We speculate that the different pathophysiologies of TB and leprosy, in particular the different levels of systemic inflammation and immune activation which are generally higher in active TB than in leprosy are responsible for the difference in C1q levels between the two diseases, even though both are caused by pathogenic mycobacteria.

To further evaluate the potential of C1q as a biomarker for active TB, C1q levels were compared to those of disease relevant controls as patients with untreated pulmonary sarcoidosis or pneumonia. Patients with TB had significantly higher circulating C1q levels as compared to patients with sarcoidosis or patients with pneumonia. Thus, the upregulation of C1q likely does not reflect a general response to inflammation. Patients with pneumonia rather had decreased C1q levels compared to controls. Treatment of pneumonia normalized the levels of circulating C1q, suggesting that the observed decrease in C1q is non-genetic and likely associated with the disease process. ROC analysis indicated that C1q, even as a single marker, readily discriminated active TB from all other diseases investigated here. Furthermore, also in a setting of an experimental NHP model of TB disease we observed in both sera and BAL fluid increased C1q levels in animals with symptomatic TB disease compared to the level prior to infection with

M.tb. This was not seen in animals that did not progress to TB disease, suggestion again an association with TB disease rather than infection only. These data fully agree with and support the observations described above in the human cohorts.

Longitudinal follow up of TB patients during treatment revealed that circulating C1q levels normalized to the level of endemic controls. However, here protein levels did not completely normalize until the six month time point. Previously published data showed a rapid decrease in C1q mRNA expression levels following treatment [16; 19]. Cliff et al. show that already after one week of treatment the blood C1q gene expression decreased [16]. Cai et al. reported a significant decrease in the expression of the C1q genes at 3 months of anti-TB chemotherapy whereas they also described a reduction in C1qc protein levels after six months [19]. Our data presented here substantially expand the number of populations, including populations from different regions of the world. Transcriptomic analysis of genes encoding complement proteins were now assessed in 9 independent populations from different TB endemic as well as non-endemic regions, strongly supporting an increase in expression of C1Q as well as SERPING1 during active TB disease in cells present in peripheral blood. Moreover, 4 independent cohorts as well as the data obtained in the NHP model show increased circulating C1q plasma levels during active TB disease, but not infection. In addition to conforming the data previously published for a Chinese population (Cai et al.) in 4 different TB cohorts, we also showed the specificity for TB disease in comparison to clinically important differential diagnoses such as sarcoidosis and pneumonia. As C1q is produced by cells of monocytic origin, it reflects another component of the immune space compared to most currently applied TB biomarkers such as C-reactive protein and IP-10 [44; 45; 46; 47]. In addition, C1q levels are technically easy to measure and the C1q protein is not sensitive to degradation. Therefore, we hypothesize that addition of C1q to current biomarker panels or platforms, will have additive value in discrimination of TB patients.

Low levels of C1q have been reported in several inflammatory and autoimmune diseases, such as Systemic Lupus Erythematosus. This is largely the result of C1q consumption because of immune complex mediated disease and in some rare cases caused by genetic C1q deficiency [26]. However, the increased levels of C1q, as occur in TB, are observed very rarely. So far the only other clinical condition in which increased levels of C1q have been reported is Kala Azar [48]. The mechanism behind the increased C1q levels observed in TB patients, or the possible functional consequences for the host are unknown, and will need further investigation. The availability of the NHP model of tuberculosis for C1q research, as demonstrated for the first time in this study, should greatly accelerate and facilitate such work.

In conclusion, we show here that circulating C1q expression is increased in 9 different populations with TB disease, moreover, elevated C1q plasma levels were observed in 4 cohorts of TB patients compared to LTBI or endemic controls. Specifically, C1q levels

in TB patients were significantly increased compared clinically relevant diseases such as sarcoidosis, leprosy and pneumonia. Moreover, we show that increased C1q levels decreased to the level of the control population during successful treatment. In analogy with human TB, C1q also validated as a biomarker of TB disease in rhesus macaques, in both serum and BAL. Increased C1q levels were only observed in animals that progressed to active disease and not in those that controlled the infection, suggesting a direct association with disease rather than with infection. Therefore, we propose that the addition of C1q measurements to current biomarker panels may provide added value in the diagnosis of active TB.

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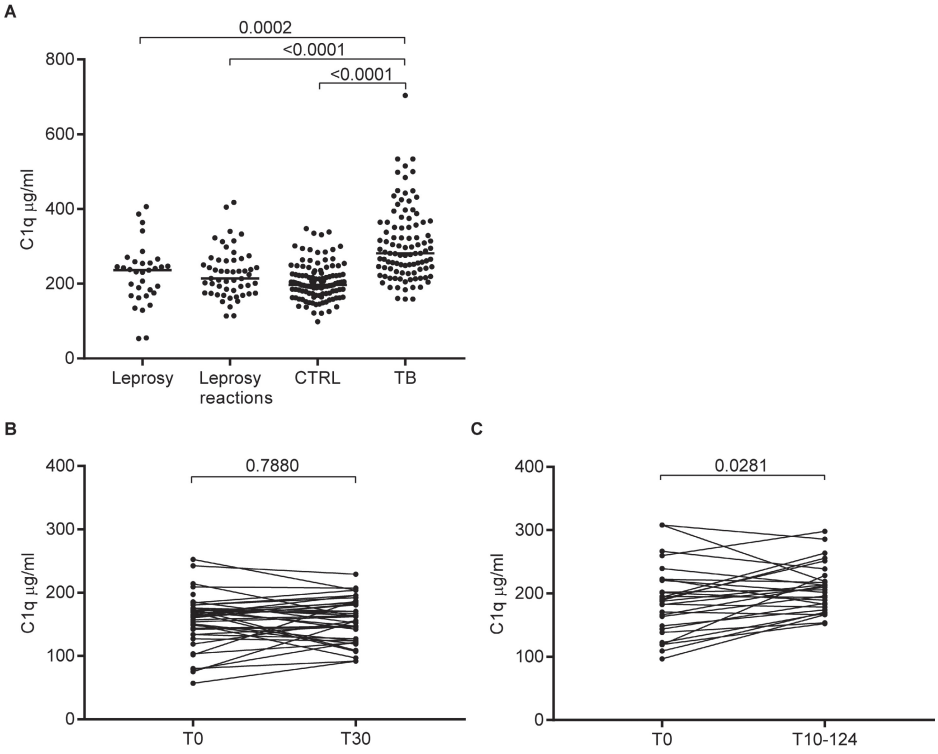
Author Contributions Statement
RL, FV, AG, TO, SJ and LT designed the study. RL, RP, KD, IB and KG performed analyses. JS, DG, CM, MV, SV, WB, LP, FN, GW, and GG oversaw recruitment and collection of specimens. RL, SJ and LT interpreted the data. All authors critically revised and approved the manuscript.

Conflict of Interest Statement
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY DATA



Supplementary Figure E1. C1q levels in leprosy and community acquired pneumonia.
From leprosy two different cohorts were measured, one consists out of patients that were included in the Netherlands at the moment of diagnosis, the other out of sera samples from patients included at the moment they presented with a leprosy reaction. Reference C1q levels both the control groups as the pooled data from the active TB patients are depicted from Figure 2E (**A**). For the community acquired pneumonia cohorts, samples were available from the moment the patients were included and a follow up sample after recovery from both in Leiden (**B**) and in Nieuwegein (**C**).

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CHAPTER 5

EXPRESSION AND PRODUCTION OF THE *SERPING1*-ENCODED ENDOGENOUS COMPLEMENT REGULATOR C1-INHIBITOR IN MULTIPLE COHORTS OF TUBERCULOSIS PATIENTS

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ABSTRACT

Background: To facilitate better discrimination between patients with active tuberculosis (TB) and latent TB infection (LTBI), whole blood transcriptomic studies have been performed to identify novel candidate host biomarkers. *SERPING1*, which encodes C1-inhibitor (C1-INH), the natural inhibitor of the C1-complex has emerged as candidate biomarker. Here we collated and analysed *SERPING1* expression data and subsequently determined C1-INH protein levels in four cohorts of patients with TB.

Methods: *SERPING1* expression data were extracted from online deposited datasets. C1-INH protein levels were determined by ELISA in sera from individuals with active TB, LTBI as well as other disease controls in geographically diverse cohorts.

Findings: *SERPING1* expression was increased in patients with active TB compared to healthy controls (8/11 cohorts), LTBI (13/14 cohorts) and patients with other (non-TB) lung-diseases (7/7 cohorts). Serum levels of C1-INH were significantly increased in The Gambia and Italy in patients with active TB relative to the endemic controls but not in South Africa or Korea. In the largest cohort (n=50), with samples collected longitudinally, normalization of C1-INH levels following successful TB treatment was observed. This cohort, also showed the most abundant increase in C1-INH, and a positive correlation between C1q and C1-INH levels. Combined presence of increased levels of both C1q and C1-INH had high specificity for active TB (96%) but only very modest sensitivity 38% compared to the endemic controls.

Interpretation: *SERPING1* transcript expression is increased in TB patients, while serum protein levels of C1-INH were increased in half of the cohorts analysed.

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). Most people infected with *Mtb* remain latently infected (LTBI) and only a minority (5-10%) of those infected progress to active TB disease [1]. Most recent estimates from the World Health Organization (WHO), showed that, 10 million people became infected with *Mtb* in 2017 and 1.6 million died from TB that year (0.3million co-infected with HIV). Although diagnostic testing and successful treatment has increased survival globally, there is a clear need for better, user friendly diagnostic tools, particularly to provide easy access in remote areas [1].

Current clinical diagnostic tests, other than mycobacterial culture, are based on detection of immunological memory (i.e. IFNγ production by T-cells) towards *Mtb* antigens and therefore fail to discriminate between individuals that are latently infected and those that have active disease [2] or cured TB [3]. In addition, treatment efficacy monitoring of TB, could benefit from more accurate biomarkers of TB disease activity [3]. Many recent studies have assessed general transcriptional biomarkers, such as *GBP5* and *FCGR1A* [4-11]. Upregulation of the expression of genes encoding proteins of the complement system have been identified as components of TB biomarker signatures [12-17]. Recently, we reported that increased levels of C1q can be used a host serum biomarker that can discriminate active TB disease from LTBI and non-TB pulmonary infections or sarcoidosis [18].

Next to activators, the complement system also includes several inhibitors, such as C4b-binding protein (C4BP), Factor H (FH) and the degrading enzyme Factor I [19]. In addition, membrane-bound inhibitors exist such as CD46, CD55 and CD59. In serum, C1q forms a complex with C1r and C1s to generate the full C1 molecule which can activate the classical pathway of the complement system. Importantly, the C1 complex is regulated by a natural inhibitor, namely C1-inhibitor (C1-INH). Transcriptional biomarker signatures have indicated that expression of *SERPING1,* which encodes the inhibitor C1-INH, discriminates between active and latent TB disease. The expression of *SERPING1* was increased in active TB patients compared to LTBI subjects [5, 6, 13, 20-23] and also in LTBI individuals that subsequently progressed to active TB [23]. Furthermore, *SERPING1* expression levels declined upon treatment initiation [24]. Most recently, *SERPING1* expression was also shown to associated with TB disease progression in mice and macaques [25]. However, information on the circulating levels of C1-INH are lacking.

C1-INH is a member of the serine protease inhibitor family and regulates both the classical and the lectin pathway of the complement system [26]. Additionally, C1-INH is a regulator of the contact system, which is a system of proteins related to coagulation, as an inhibitor for Factor XIa, Factor XIIa and kallikrein [27]. C1-INH protein functions as a suicide inhibitor, it binds to its target, and upon enzymatic cleavage C1-INH undergoes

a permanent conformational change and therefore C1-INH can only work once. C1-INH is synthesized as a single-chain protein which is heavily glycosylated. C1-INH, C4BP and FH are mainly produced by hepatocytes but can also be produced by monocytes and macrophages [28-30]. C1q is also produced by cells from the myeloid lineage: monocytes, macrophages and dendritic cells [31], but not by hepatocytes [29]. C1-INH is an acute phase protein which can be increased upon inflammation, deficiency of C1-INH is clinically associated with recurrent episodes of angio-oedema [32, 33].

Here we have determined serum levels of *SERPING1* encoded C1-INH in four geographically diverse cohorts of patients with active TB, LTBI, disease controls and patients that have been successfully treated for TB, to investigate possible application as host biomarker for active TB.

MATERIALS AND METHODS

Patients and controls

The demographic data and classification of the cohorts are presented in **Table 1** [18]. Serum samples were collected from all individuals locally following written informed consent and shipped to the laboratory at LUMC for assessment. Maximal transport time was 48 hours, the shipments were dry ice monitored and samples frozen upon arrival. Below, the inclusion criteria per cohort are specified.

Patients with pulmonary TB, latent TB and controls

Smear, PCR or culture positive pulmonary TB patients, LTBI patients and treated TB patients as well as endemic controls were included from various geographic locations: Italy [18, 34], the Gambia, South Korea and South Africa (**Table 1**) [18]. Patients with active pulmonary TB disease, referred to as 'TB' below, were diagnosed based on local guidelines and routine methodology. Active pulmonary TB was sputum-culture confirmed (BACTEC™ MGIT™, Becton-Dickinson, USA), or based on positive Xpert Mtb/RIF assay (Cephaid Inc., Sunnyvale, CA, USA), patients were included within 7 days of TB treatment initiation. LTBI was defined as Quantiferon TB Gold-in tube positivity (Qiagen, Germany) in absence of signs of active disease.

Stored samples from participants recruited in South Africa from six public health care clinics around Tygerberg Academic Hospital in Northern Cape Town. These included 20 newly diagnosed pulmonary TB cases (4 HIV+) and 31 healthy controls (no HIV+) from the same area. TB cases were confirmed by a positive sputum MGIT culture for *Mtb* or GeneXpert in addition to a chest X-ray suggestive of active pulmonary TB [35]. The non-TB controls were asymptomatic individuals from the same communities, with a negative sputum culture for *Mtb* and a chest X-ray not suggestive of TB.

Additionally, from Italy both LTBI (QuantiFERON TB Gold-In-tube-positive) individuals and successfully treated TB patients (2-72 months after end of therapy) were included. TB patients from the Gambia were followed over time (one, two and six months after diagnosis), until completion of successful treatment.

Other mycobacterial diseases

Patients infected with *Mycobacterium leprae* (mostly immigrants with mixed ethnic backgrounds) were included in the Netherlands after having been diagnosed with leprosy without reactions. In addition, patients with type-1 leprosy reactions were enrolled in Brazil, Nepal and Ethiopia [36, 37].

Other pulmonary diseases

Hospital admitted patients with community-acquired pneumonia in the Netherlands were included. One cohort comprised patients admitted to a tertiary care hospital in Leiden (one patient was HIV-infected with a normal CD4 count and one suffered from sarcoidosis) and the other cohort comprised patients admitted to a hospital ward of a non-academic teaching hospital in Nieuwegein. From both groups of pneumonia patients paired serum samples from the time of diagnosis and after recovery (10-124 days later) were available. Lastly, serum samples of patients with sarcoidosis from the Netherlands that had pulmonary involvement were included, obtained prior to initiation of treatment.

Additional control group

As a reference group we included a panel of Dutch healthy controls (n=92), not suffering from major infections or autoimmune diseases.

Table 1. Description of the cohorts

Country	Classification	N	Age mean (range)	Sex (%male)
Italy	Control	15	38 (25-57)	40
	Latent TB	19	37 (21-77)	33
	Active TB	19	38 (23-67)	89
	Treated TB	20	39 (18-70)	35
The Gambia	Control	50	31 (15-60)	30
	Active TB	50	34 (17-62)	62
South Korea	Control	10	23 (21-25)	90
	Active TB	10	51 (24-77)	40
South Africa	Control	31	32 (18-56)	26
	Active TB	20	32 (19-57)	65
Multiple*	Leprosy reactions	53	(18-69)	68
	Leprosy†	33	34 (18-57)	62
	Sarcoidosis	50	43 (26-57)	60
the Netherlands	Control	92	37 (21-67)	36
	Pneumonia (Leiden)	40	66 (23-93)	60
	Pneumonia (Nieuwegein)	28	73 (34-91)	57

* Nepal, Brazil, Ethiopia

† Diagnosis made in the Netherlands

Ethics statement

Blood was obtained from individuals upon signing informed consent. All studies comply with the Helsinki declaration. The use of the samples in this study was approved by local ethical committees. For Italy, Ethical Committee of the Lazzaro Spallanzani National Institute of Infectious diseases (02/2007 and 72/2015); The MRC/Gambian government joint ethics committee (SCC1333); South Korea, Institutional Review Board for the Protection of Human Subjects at YUHS; South Africa, Health Research Ethics Committee of the Faculty of Medicine and Health Sciences at Stellenbosch University (N13/05/064); Brazil, National Council of Ethics in Research and UFU Research Ethics Committee (#499/2008); Nepal, Health Research Council (NHR#751); Ethiopia, Health Research Ethical Review committee Ethiopia (NERC#RDHE/127-83/08); The Netherlands leprosy patients, (MEC-2012-589); sarcoidosis patients, Medical research Ethics Committees United of the St Antonius (#R05.08A); control group, (P237/94); pneumonia Leiden (P12.147); pneumonia Nieuwegein (C-04.03 and R07.12).

Gene expression analysis

Microarray data from comparative studies [4, 5, 7, 8, 20, 22, 38-44], publicly available in Gene Expression Omnibus (GEO) (GSE37250, GSE19491, GSE34608, GSE39941, GSE42834, GSE70478, GSE25534, GSE28623, GSE56153, GSE74092, GSE73408, GSE62525, GSE83456), were retrieved from GEO and re-analysed. Several of the studies described multiple independent populations, which we analysed separately. All data were extracted from GEO and compared in the same way using GEO2R, thus not relying on the analysis performed in the original manuscript. GEO2R compared two or more groups of samples in order to identify genes that are differentially expressed across experimental or clinical conditions. Here, lists of differentially expressed genes between TB and LTBI or TB and other diseases (with a significance of $p<0.05$ and a factorial change of >2 or <0.5) were generated. A list of complement protein inhibitors (*SERPING1*, *CFH*, *C4BPA*, *CD46*, *CD55* and *CD59*) and two reference genes (*FCGR1A* and *GBP5*) was used to assess possible differential expression for each individual gene for each study population. In addition, information on the differential expression of the three different chains for C1q (*C1QA*, *C1QB* and *C1QC*) was retrieved from the same datasets for comparative purposes. All studies/ populations with significantly differential expression for a particular gene between patients with TB compared to healthy controls/ LTBI/ other diseases were enumerated and expressed as the percentage of the total number of comparisons investigated.

Detection of C1-INH by ELISA

C1-INH levels in sera were measured using an in-house developed ELISA. Maxisorp plates (nunc) were coated for two hours at room temperature on a shaker with mouse anti-human C1-INH (RII A280 2.8 RUCHIRA) in coating buffer (0.1M NA_2CO_3, 0.1M $NaHCO_3$, pH 9.6). Plates were washed with PBS/0.05%Tween and the samples were diluted in PBS/0.2%Gelatin/0.1%Tween (PTG). Human sera were diluted 1:1000. A serial dilution of a pool of normal human serum (NHS) was applied as a standard with a known concentration and samples were added to the plate, all in duplicate, and incubated for one hour at room temperature on a shaker. After washing, plates were incubated for one hour on the shaker at room temperature with rabbit-anti-human C1-INH labelled with biotin (Sanquin) and diluted in PTG/1% normal mouse serum. Plates were washed and incubated with streptavidin-HRP (GE Healthcare cat#GERPN1231) for a maximum of 30 minutes on the shaker. Next, the plates were washed and TMB (BD cat# 555214) was added as a substrate, the signal was stopped using 2M H_2SO_4. Signal was measured as the absorbance at 450nm minus the absorbance at 540nm with the SpectraMax i3x. The measured C1-INH is expressed as µg/ml as compared to the standard. the levels of C1-INH were compared to the levels of C1q that were previously determined in the same samples by ELISA as described [18].

Statistics

Statistical analyses were carried out using SPSS statistics version 23 (IBM) or Graphpad Prism version 8. To compare C1-INH levels the Mann-Whitney U test, Kruskall-Wallis and Dunn's multiple comparisons test were used. In all graphs median values are shown unless indicated otherwise. Receiver operating characteristic (ROC) analyses were performed to assess the sensitivity and specificity of C1-INH serum levels and was expressed as Area Under the Curve (AUC). Increased serum levels were based upon the 95th percentile of a reference group, Dutch healthy controls, of either C1q [18] or C1-INH.

RESULTS

Patients with active TB have upregulated expression of *SERPING1*

Publicly available microarray data were retrieved from GEO from thirteen studies [4, 5, 7, 8, 20, 22, 38-44], from different populations across the world, and all data were ranked according to differential expression between TB patient and Healthy Controls (HC), LTBI or other lung-diseases. The number of studies that reported on differential gene expression between patients with TB and HC (**Figure 1A**), LTBI (**Figure 1B**) or other lung-diseases (**Figure 1C**) was plotted for complement system inhibitors: *SERPING1*, *CFH*, *C4BPA*, *CD46*, *CD55* and *CD59*. In addition, two genes, *FCGR1A* and *GBP5*, that are consistently top-differentially expressed genes in many of the TB cohorts, were included for comparative analysis. *SERPING1* expression was increased in TB patients compared to HC (8/11 studies), LTBI (13/14 studies) and patients with other lung-diseases (7/7 studies). Other genes encoding complement inhibitory proteins were not consistently increased in active TB disease. Subsequently, in the studies that found differential gene expression the factorial changes were analysed for *SERPING1*, and control *FCGR1A* and *GBP5* (right panel **Figure 1**). The factorial changes for these three genes were in the same order of magnitude. Since *SERPING1* expression results distinguished TB patients from both LTBI subjects and patients with other lung-diseases, we next measured the *SERPING1* protein product, C1-INH.

C1-INH protein levels in serum of patients with active TB and other diseases

Sera from cohorts of patients with pulmonary TB from distinct geographical locations were analysed for C1-INH protein levels by ELISA. Sera collected from TB patients in South Korea or South Africa showed no differences in C1-INH levels compared to controls from the same area (**Figure 2A, B**). In samples from the Italian cohort, however, C1-INH levels were significantly increased ($p=0.026$) in TB patients compared to controls (**Figure 2C**). Similarly in samples from Gambian TB patients there was a significant increase in serum C1-INH levels compared to endemic controls (LTBI; $p<0.0001$) (**Figure 2D**).

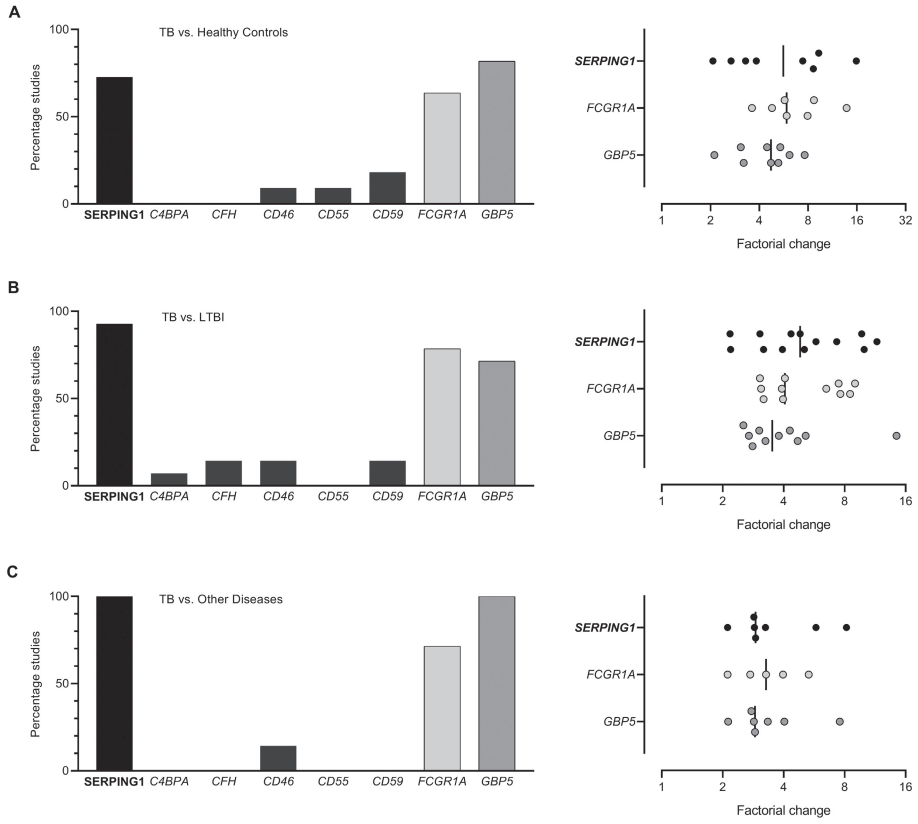


Figure 1. Differentially expressed complement inhibitor genes in whole blood tuberculosis transcript signatures

Tuberculosis (TB) specific blood transcript signatures, from various populations, were investigated for the presence of differentially expressed genes encoding inhibitors of the complement system and two well-known biomarkers, *FCGRIA* and *GBP5*. Publicly available transcriptome data was retrieved from Gene Expression Omnibus [4, 5, 7, 8, 20, 22, 38-44] and analysed using GEO2R. Data were available for comparing patients with active TB to healthy controls for 11 cohorts (**A**); compared to Latent TB for 14 cohorts (**B**) and to other diseases for 7 cohorts (**C**). The left side of the figure shows the number of times the gene was significantly differentially expressed as percentage of the total number of studies analysed. The right side of the figure shows the factorial change measured in the positively reporting studies for *SERPING1* and *FCGR1A* and *GBP5*.

Next, C1-INH levels were measured in samples from patients with other lung diseases (sarcoidosis and community-acquired pneumonia) that present clinical symptoms comparable to those found in active TB, as well as patients with a non-TB related mycobacterial disease (leprosy). These were compared to a control group which comprised Dutch healthy controls (n=92) (**Figure 2E**). In none of these patient groups C1-INH was increased compared to healthy controls, however, the C1-INH levels in

pneumonia patients were significantly decreased compared to the controls (p=0.021) and sarcoidosis (p=0.004). The elevation of serum levels of C1-INH in TB patients (found in two out of four cohorts) thus seems related to *Mtb* infection and not an indirect consequence of other infections or inflammation. However, it is unclear why a significant increase in serum C1-INH protein levels was only present in the Italian and Gambian the cohort analysed, whereas on transcriptomic level the *SERPING1* is more consistently increased.

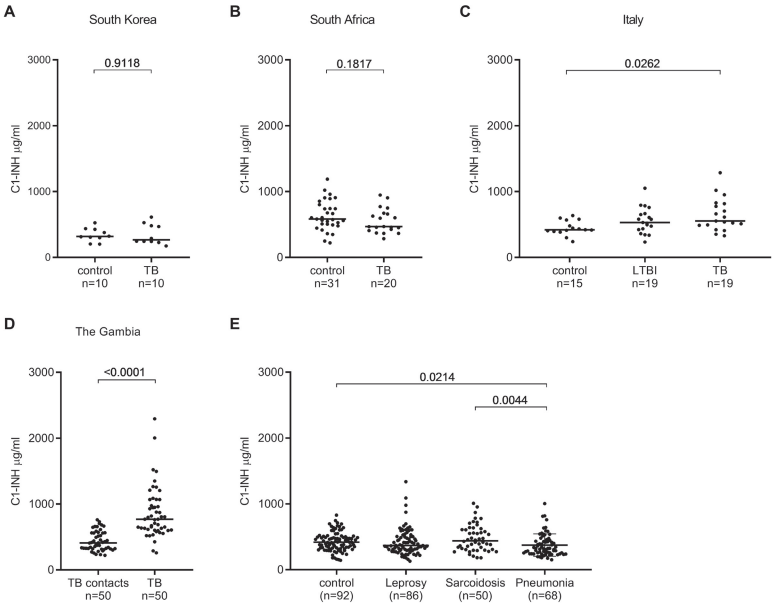


Figure 2. C1-inhibitor serum levels in various patient cohorts worldwide
C1-inhibitor (C1-INH) levels (µg/ml) were measured by ELISA in sera from patients with active tuberculosis (TB) and controls from different cohorts. The results from the independent and geographically different TB cohorts are depicted: South Korea (**A**), South Africa (**B**), Italy (**C**) and The Gambia (**D**). Dutch healthy controls (n=92) were compared to other diseases: leprosy (n=86), sarcoidosis (n=50) and community acquired pneumonia (n=68) (**E**). Differences between groups were analysed using using the Mann-Whitney U test, >2 groups Kruskall-Wallis and Dunn's multiple comparisons test and a p-value of <0.05 was considered statistically significant.

C1-INH serum levels normalize over course of successful TB treatment

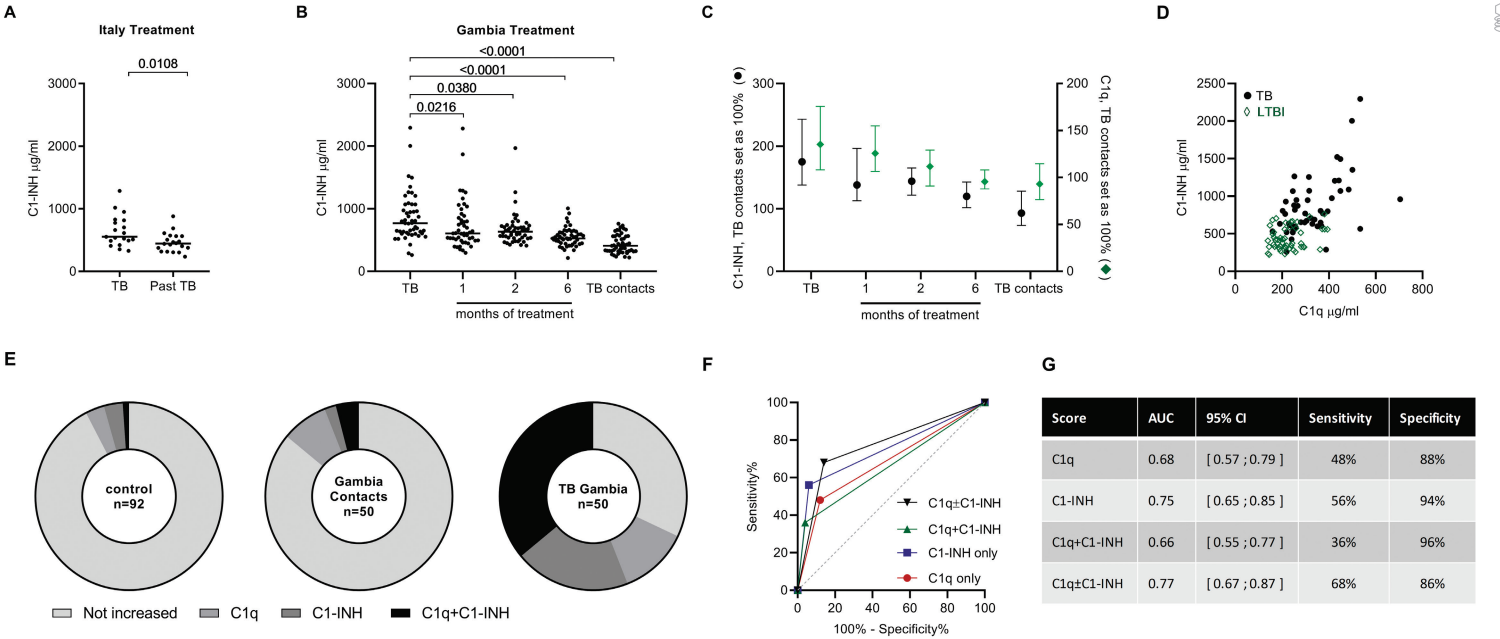
The Italian and The Gambian cohort showed significantly increased serum levels of C1-INH in TB. From these two cohorts additional samples were available from TB patients that were successfully treated for pulmonary TB or longitudinal samples of patients on treatment. From the Italian cohort, samples from patients with active TB and patients successfully treated for TB were collected cross-sectionally. C1-INH serum levels were significantly lower in the treated patients as compared to the untreated active TB samples (**Figure 3A**). In The Gambia, samples were collected longitudinally

from TB patients over the course of their treatment, after one, two and six months (completion of therapy). Already after one month of treatment the C1-INH levels significantly dropped and even further decreased after two and six months of therapy, albeit not completely to the level of the control population (**Figure 3B**).

Next to C1-INH levels, C1q serum levels of these Gambian patients were also available [18]. First, the average serum levels of C1-INH or C1q were both set at 100% for the control group (TB contacts from the Gambia), such that serum values could be expressed as relative levels compared to controls. The medians with the 25th-75th percentiles were plotted for the patients during treatment compared to the TB-contacts (**Figure 3C**). The C1q levels were 135% higher during active TB whereas C1-INH was nearly doubled compared to TB contacts (175%). C1-INH levels had already decreased after one month of treatment, whereas C1q levels did not decrease up to two months of treatment. These data indicate that the patients do not have a genetic predisposition to high C1q or C1-INH levels as treatment resulted in complete normalisation of C1q serum level and a significant decrease of C1-INH serum level.

Combining C1q and C1-INH measurement has added value in identifying patients with TB in The Gambia

In the samples from TB patients and controls, collected in The Gambia, we combined C1q levels (previously published in [18]) with C1-INH levels determined in this study, in order to investigate if improved discriminatory value could be achieved or whether these molecules are possibly co-regulated. On the transcriptomic level the factorial changes were plotted (from the publicly available transcriptome data as also analysed in **Figure 1**) for TB compared to LTBI for the different C1q chains and *SERPING1* (**Supplementary Figure 1**). Spearman analyses showed significant correlations between *SERPING1* vs *C1QB* (p=0.0098; r=0.75) and *SERPING1* vs *C1QC* (p= 0.028; r=0.79). C1-INH and C1q protein levels showed a significant (p<0.0001) moderate correlation (r=0.56) (**Figure 3D**). Subsequently, we scored each sample for containing increased concentrations of either C1q or C1-INH, or both. A sample was scored as 'increased' if the value exceeded the 95th percentile of the Dutch healthy control group. These scoring results are visualized as a fraction of the total per group in **Figure 3E**. Increased protein concentrations were categorized as: not increased, increased for C1q only, increased for C1-INH only, or increased for both C1q and C1-INH.



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Score	AUC	95% CI	Sensitivity	Specificity
C1q	0.68	[0.57 ; 0.79]	48%	88%
C1-INH	0.75	[0.65 ; 0.85]	56%	94%
C1q+C1-INH	0.66	[0.55 ; 0.77]	36%	96%
C1q±C1-INH	0.77	[0.67 ; 0.87]	68%	86%

Figure 3. C1-inhibitor and C1q serum levels following treatment and combined scoring in The Gambian population
C1-inhibitor (C1-INH) was measured by ELISA in TB patients and Past TB (patients that were successfully treated for TB, cross-sectionally enrolled) from Italy. Results were analysed using the Mann-Whitney U test (**A**). In patients from The Gambia, C1-INH was measured by ELISA and combined with C1q data that was available for these patients. These TB patients were followed over time during treatment, TB contacts are shown on the right (n=50). The treatment months were compared to TB diagnosis with Friedman Test and Dunn's multiple comparisons test (**B**). The level of C1-INH or C1q was set at 100% for the TB contacts from the Gambia and the median with the 25th -75th percentile was plotted for the Gambia active TB samples with treatment follow up (**C**). The C1-INH and C1q levels were analysed with Spearman (**D**). Subsequently, we further analysed this cohort and scored each sample for positivity for either C1q or C1-INH, or both. Positivity was based on the respective level of the Dutch healthy controls group (n=92) at 95th percentile, when higher, the sample scored positive. Positivity was categorised in: not increased, single positive for C1q, single positive for C1-INH and finally double positive. These scoring results are visualized as a fraction of the total per group in doughnut charts (**E**). Additionally, these scores were analysed and the Area Under the Curve (AUC) was calculated comparing TB with the TB contacts. A separate ROC was generated based on a sample being positive for either one of the proteins (or both) and was annotated as C1q±C1-INH (**F**). All AUC were calculated and summarized in the table, accompanied by the respective sensitivity and specificity (**G**).

Additionally, these results were analysed with ROC, comparing TB at diagnosis with TB contacts. Next to the categories in **Figure 3D**, a separate ROC was generated based on a sample being increased for either one of the proteins (or both) and was annotated as C1q ± C1-INH (**Figure 3F**, **G**). C1q alone resulted in an AUC of 0.68, whereas when C1q, C1-INH or both were increased an AUC of 0.77 was obtained. By using this scoring system, the presence of increased levels of both proteins is highly specific (96%) but is accompanied by a relatively low sensitivity (36%) in this cohort from the Gambia. Discrimination of TB patients from the control group based on C1q levels [18] resulted in a sensitivity of 48% and a specificity of 88%. In this comparison, incorporating the measurement of the C1-INH serum levels led to a remarkable increased sensitivity up to 68% with a minor reduction of the specificity to 86% in this specific cohort. Therefore, combination of C1q and C1-INH protein levels in serum, resulted in both acceptable specificity and sensitivity for active TB detection in the cohort collected in The Gambia.

Overall, in contrast to the apparent abundant expression changes in *SERPING1*, measurement of C1-INH protein levels did not permit discrimination of TB patients from latently infected individuals in all cohorts. However, there may be added value in combined measurement of C1-INH in particular cohorts that warrant further investigation in the potential value of C1-INH as biomarker of TB disease.

DISCUSSION

The major health threat posed by tuberculosis can likely only be tackled efficiently when a proper and fast identification of patients with active TB is available. Recently, complement gene expression and/or complement protein levels have been put forward as possible markers for such fast identification. In this study we collected available gene expression data and determined serum levels of C1-INH in four separate cohorts. We observed that *SERPING1* gene expression was higher in patients with active TB compared to healthy controls, LTBI and patients with other lung-diseases. This increase in *SERPING1* appears to be unique, as C4BP and FH, which are also soluble complement inhibitors with similar production sites as C1-INH, were not differently expressed in whole blood. For application in the field, it could be more feasible to measure protein levels using field friendly tests instead of gene expression, the latter being more expensive, labour intensive, time consuming and requiring more infrastructure. Therefore, we measured C1-INH protein serum levels, the product of *SERPING1*, in various worldwide cohorts of patients with active TB and other lung-diseases or mycobacterial exposure.

C1-INH serum levels were significantly increased in TB patients compared to endemic controls in two out of four cohorts analysed. In addition, in the Gambian cohort with TB patients followed over time the increased C1-INH decreased rapidly upon initiation of TB treatment. It is interesting that *SERPING1* expression is consistently increased in patients with TB whereas its protein product C1-INH is found to be increased in only half of the cohorts analysed in this study. It is unclear why C1-INH upregulation was observed in only two out of four cohorts. Possible explanations may be sought in the production sites of C1-INH. C1-INH has multiple sources of production, like C1q, C1-INH is produced by circulating immune cells. But unlike C1q, C1-INH is also produced by hepatocytes and therefore may display a less clear relationship between mRNA levels in circulating cells versus protein levels in the circulation. The differences in expression in the microarray studies were measured in blood, which indicates that peripheral regulation may be affected by TB disease but this is not necessarily influencing circulating protein levels.

C1-INH levels were also measured in sera from patients that were diagnosed with diseases that resemble the symptoms of active TB, such as sarcoidosis and pneumonia, or caused by *Mycobacterium leprae*, leprosy. In contrast to active TB, in none of these patient cohorts, C1-INH was increased compared to the control group. Therefore, the increased C1-INH serum levels, observed in the two TB cohorts, seem to be specific for TB disease and not for inflammation in general.

TB patients from the Gambia were followed during treatment and samples were available over the full course of therapy. At recruitment, 28 of the 50 samples had C1-INH levels above the 95th percentile of the Dutch healthy control group. Of those 28 samples, 20 had decreased C1-INH levels after 1 month, 24 after 2 months, and 25 after

6 months of treatment. A significantly decreased C1-INH level was already observed after one month of therapy, whereas C1q levels at one month were indistinguishable from C1q levels at diagnosis [18]. An explanation for this difference could be that different cellular sources have different response properties. Perhaps, hepatocytes decrease their production of C1-INH faster, resulting in a faster decrease of C1-INH in circulation, compared to the production of C1q. In the Gambia, where we found C1-INH to be significantly increased in TB compared to endemic controls, there was a positive correlation for C1q and C1-INH serum levels and the combination of these results had added value in discriminating active TB patients in this particular cohort. Moreover, it has been shown that the magnitude of the systemic inflammatory perturbation in pulmonary TB may be associated with different qualitative changes in inflammatory profiles [45] in samples from individuals coming from countries at different TB prevalence (Italy, Gambia, Korea, South Africa) and likely with different *Mtb* lineages, as previously shown [46].

Overall, upon comparing C1q and C1-INH protein level in the different populations analysed, C1q was uniformly increased in TB cohorts whereas C1-INH in two out of four TB cohorts. We exclude that these differences between C1q and C1-INH in the measured TB cohorts were caused by freeze-thaw cycles or other technical issues with the samples, since the exact same samples were used for both measurements. For the differential diagnosis, leprosy, sarcoidosis and pneumonia, neither C1q nor C1-INH was increased. Both C1q and C1-INH decrease after successful TB treatment. How and why *Mtb* infection leads to increased levels of C1q and C1-INH in a part of the population is currently unknown, but it is interesting to speculate about the possible implications. C1q has been shown to be of importance in $CD8^+$ T-cell biology where C1q dampens the $CD8^+$ T-cell responses [47]. Given that *Mtb* is an intracellular pathogen it could be of importance for *Mtb* to stimulate higher C1q production which will subsequently contribute to decreased $CD8^+$ T-cell anti-microbial activity. Simultaneous increases in C1q and C1-INH could limit activation of the classical pathway of the complement system. Recently, it was demonstrated in a *SERPING1*$^{-/-}$ mice that upon *Mtb* infection no differences were detected compared with the wildtype. However, more inflammation was observed in the *SERPING1*$^{-/-}$ mice, possibly due to the lack of complement inhibition [25].

In this study, the microarray data analysis demonstrated consistent increased expression of *SERPING1* in patients with TB compared to controls. However, the protein product of *SERPING1*, C1-INH, was increased in two out of four cohorts of patients with active TB analysed in this study. Thus, as yet, we conclude that C1-INH is not generally applicable as single protein host biomarker for TB. Cohorts with and without significant increases in C1-INH levels were from different geographical regions, however, at present it is unknown whether that explains the different outcomes. Additional, yet unidentified, factors may contribute as well and more (replication) studies are needed to provide insight into this finding.

The observation that increased C1-INH serum levels were only detected in cohorts of patients with active TB, but not in patients with similar clinical conditions and the notion that C1-INH decreased rapidly during TB treatment, suggests that *Mtb* is actively regulating these complement proteins. An increase in C1q and C1-INH could therefore represent an immune-escape mechanism of *Mtb* enabling immunosuppressive actions of C1q, without enhancing the classical pathway activity.

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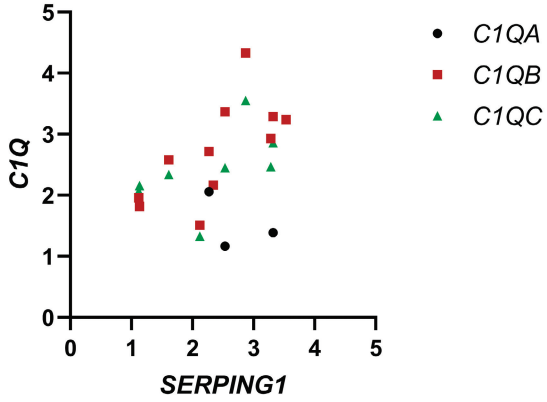
AUTHOR CONTRIBUTIONS STATEMENT

RL, AG, TO, SJ and LT designed the study. RL, RP and KG performed analyses. JS, DG, CM, MV, SV, WB, LP, STM, FN, GW, and GG oversaw recruitment and collection of specimens. RL, SJ and LT interpreted the data. All authors critically revised and approved the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY DATA



Supplementary Figure 1. Fold increase in TB compared to LTBI correlation for C1Q and SERPING1 in whole blood tuberculosis transcript signatures

Tuberculosis (TB) specific transcript signatures, from various populations, were investigated for the presence of differentially expressed, complement genes encoding C1q chains (A, B and C) and C1-inhibitor encoded by *SERPING1*. Publicly available transcriptome data was retrieved from Gene Expression Omnibus [4, 5, 7, 8, 20, 22, 38-44] and analysed using GEO2R. Data were available for comparing patients with active TB and Latent TB infected for 14 comparisons. The factorial changes were plotted if both genes (*C1QA/B/C* and *SERPING1*) were significantly differently expressed.

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CHAPTER 6

SYSTEMIC AND PULMONARY C1Q AS BIOMARKER OF PROGRESSIVE DISEASE IN EXPERIMENTAL NON-HUMAN PRIMATE TUBERCULOSIS

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ABSTRACT

Tuberculosis (TB) causes 1.6 million deaths annually. Early differential diagnosis of active TB infection is essential in optimizing treatment and reducing TB mortality, but is hampered by a lack of accurate and accessible diagnostics. Previously, we reported on complement component C1q, measured in serum by ELISA, as a candidate biomarker for active tuberculosis. In this work we further examine the dynamics of C1q as a marker of progressive TB disease in non-human primates (NHP). We assessed systemic and pulmonary C1q levels after experimental infection using high or low single dose as well as repeated limiting dose *Mycobacterium tuberculosis* (*Mtb*) challenge of macaques. We show that increasing C1q levels, either peripherally or locally, correlate with progressive TB disease, assessed by PET-CT imaging or post-mortem evaluation. Upregulation of C1q did not precede detection of *Mtb* infection by a conventional interferon-gamma release assay, confirming its association with disease progression. Finally, pulmonary vaccination with Bacillus Calmette Guérin also increased local production of C1q, which might contribute to the generation of pulmonary protective immunity. Our data demonstrate that NHP modelling of TB can be utilized to study the role of C1q as a liquid biomarker in TB protection and disease, complementing findings in TB patients.

INTRODUCTION

Tuberculosis (TB) remains a highly significant burden to global health. In 2018, 6.4 million new cases of TB were officially notified to the World Health Organization (WHO)[1]. However, the WHO estimates the actual number of TB cases to be 10 million[1], implying an underestimation of the true number of TB cases. Closing this gap in TB detection has the potential to prevent millions of deaths and to curb further dissemination of TB. Accordingly, the WHO has made early diagnosis of tuberculosis an integral part of their EndTB strategy.

After infection with *Mycobacterium tuberculosis* (*Mtb*), the causative agent of TB, individuals may develop latent TB infection (LTBI) or active progressive disease (although some may also clear the infection). Definitive diagnosis of active TB can only be made by detection of *Mycobacterium tuberculosis* (*Mtb*) in sputum, either by smear microscopy, culture or GeneXpert technology. These assays, however, are time-consuming, require specific (expensive) infrastructure and suffer from sampling error and low sensitivity. Immunological tests, such as Tuberculin Skin Testing (TST) or Interferon Gamma Release Assays (IGRAs) can detect exposure to mycobacteria, but are unable to differentiate between active and latent TB infection[2,3]. These host response measures can remain positive even after treatment and clearance of infection. Accurate tests for risk of relapse after drug-treatment or risk of progression to active disease are currently unavailable. A wide range of new TB diagnostics are currently under development, investigating the diagnostic potential of pathogen-derived components as well as host-derived biomarkers in various body fluids[2,3].

Over the last few years many host-derived candidate biomarkers of progressive TB disease have been identified, and amongst them several components of the complement system[4-9]. The complement system consists of a number of serum proteins with the capacity to recognize and neutralize invading pathogens by opsonization and lysis. One of these components, C1q, can bind to pathogen-bound C-reactive protein, IgG or IgM, thereby activating the complement cascade through its associated serine proteases C1s and C1r. This binding results in subsequent deposition of the opsonin C3b on the surface of the pathogen and, ultimately, the formation of the membrane attack complex (MAC). Additionally, C1q enhances phagocytosis and has been implied, amongst others, in tissue repair, synaptic pruning and macrophage polarization (reviewed in [10,11]). More recently, a role for complement components in shaping innate and adaptive immune responses has been established. For instance, in a model for systemic lupus erythematosus, C1q was found to control auto-reactive CD8+ T-cell responses, by modulating the mitochondrial metabolism of these T-cells[12,13]. C1q is mainly produced by cells of the myeloid lineage[14], and especially by immature dendritic cells[15], macrophages[16] and mast cells[17].

We and others have previously described serum C1q as a biomarker of active TB disease[18,19]. In patients with active TB we observed significantly higher levels of serum C1q compared to individuals with latent *Mtb* infection or individuals with non-mycobacterial pneumonia. Furthermore, we showed increase of C1q after high dose TB challenge of Non-Human Primates (NHPs).

NHP and macaque species (*Macaca spp*) in particular, are considered highly relevant models for TB, due to their close phylogenetic relationship to man, outbred nature and large similarity in TB pathogenesis. Macaques are applied across the whole spectrum of TB research, both in preclinical evaluation of TB vaccines and therapeutics as well as basic research on TB disease development[20-22]. Modelling in these species presents the advantage of having controlled and accurate time-response-conditions relative to infection. Depending on macaque (sub)species, *M.tuberculosis* strain and challenge dose, TB disease manifestation in macaques mimics the diversity seen in humans[23-25].

In this work, we exploited diversity in TB disease manifestation in NHPs to examine the dynamics of C1q as a biomarker of TB disease in more detail. We assessed C1q levels in plasma and bronchoalveolar lavages (BALs) in multiple independent cohorts of *Mtb* infected macaques under varying experimental challenge conditions. By profiling circulating and pulmonary C1q levels at various timepoints after infection we show that increasing C1q levels correlate with increased TB pathology and with decreased survival following challenge with high or low dose *Mtb*. However, neither peripheral nor local upregulation of C1q preceded IGRA conversion, suggesting its association with progressive disease but not TB infection per se. Lastly, we show that pulmonary vaccination with Bacillus Calmette Guérin (BCG), which is superior in inducing protection against TB compared to standard intradermal vaccination[23,26,27], also results in an increase in pulmonary C1q and discuss its potential role in the generation of a protective immune responses. Our observations confirm and further support C1q as a marker of progressive TB disease.

RESULTS

C1q is a predictive marker of disease progression in NHP TB

In addition to having described serum C1q as a biomarker of active TB in human patients, we also reported on elevated C1q levels in a preliminary analysis of serum and BAL samples from *Mtb*-infected non-human primates[18]. Here we set out to further investigate C1q as a marker of TB disease, under various model conditions in NHPs.

We started with retrospective measurement of C1q in serum from rhesus macaques (*Macaca mulatta*) that were either vaccinated or not with a standard human dose of intradermal BCG and subsequently challenged with a high dose (500 Colony Forming Units (CFU)) of *Mtb* strain Erdman, previously described in part in Lubbers et al (2018)[18].

These animals were monitored for 1 year after infectious challenge, or until reaching a humane endpoint due to progressive TB disease. In this cohort, prior BCG vaccination was efficacious and resulted in significant improvement of survival after *Mtb* challenge ($p = 0.0031$, **Figure 1a**) and reduction of TB associated pathology ($p = 0.0196$, **Figure 1b**).

Serum C1q levels did not differ between the two groups prior to infection (**Supplemental Figure 1a**). Assessing changes over time, we observed most prominent upregulation in non-vaccinated controls (nv.ctrls) already from 3 weeks after infection with *Mtb* (**Figure 1c**). In some individuals, an increase in C1q levels preceded a humane endpoint event by several weeks. By week 6 post-*Mtb* infection, group median serum C1q in non-vaccinated controls was significantly higher compared to BCG vaccinees (**Figure 1d**, left panel). Despite reducing TB pathology by prior BCG vaccination (**Figure 1b**), when comparing C1q levels at individuals' endpoints, non-vaccinated controls and BCG vaccinated animals showed no significant difference in serum C1q (**Figure 1d**, right panel).

To determine the potential of serum C1q as a prognostic marker regardless of prophylactic treatment and prior to the incidence of humane endpoints, we divided all animals in two groups irrespective of treatment: those exhibiting week 6 C1q levels below the median value (C1q.lo, 2 nv.ctrl and 9 BCG) and animals with week 6 levels above the median (C1q.hi, 9 nv.ctrl and 2 BCG). When comparing Kaplan-Meier curves for time-to-endpoint, animals with the highest C1q levels displayed significantly reduced survival ($p = 0.0081$, **Figure 1e**). Furthermore, we found a strong statistical correlation between the total TB pathology score at necropsy and serum C1q levels at 6 weeks post-infection (Spearman's rho = 0.772, $p < 0.0001$, **Figure 1f**, left panel). Individual C1q levels at endpoint correlated significantly with the total amount of pathology (Spearman's rho = 0.591, $p = 0.003$, **Figure 1f**, right panel). Taken together, these observations suggest that C1q is an early marker of progressive disease after experimental infection of rhesus macaques.

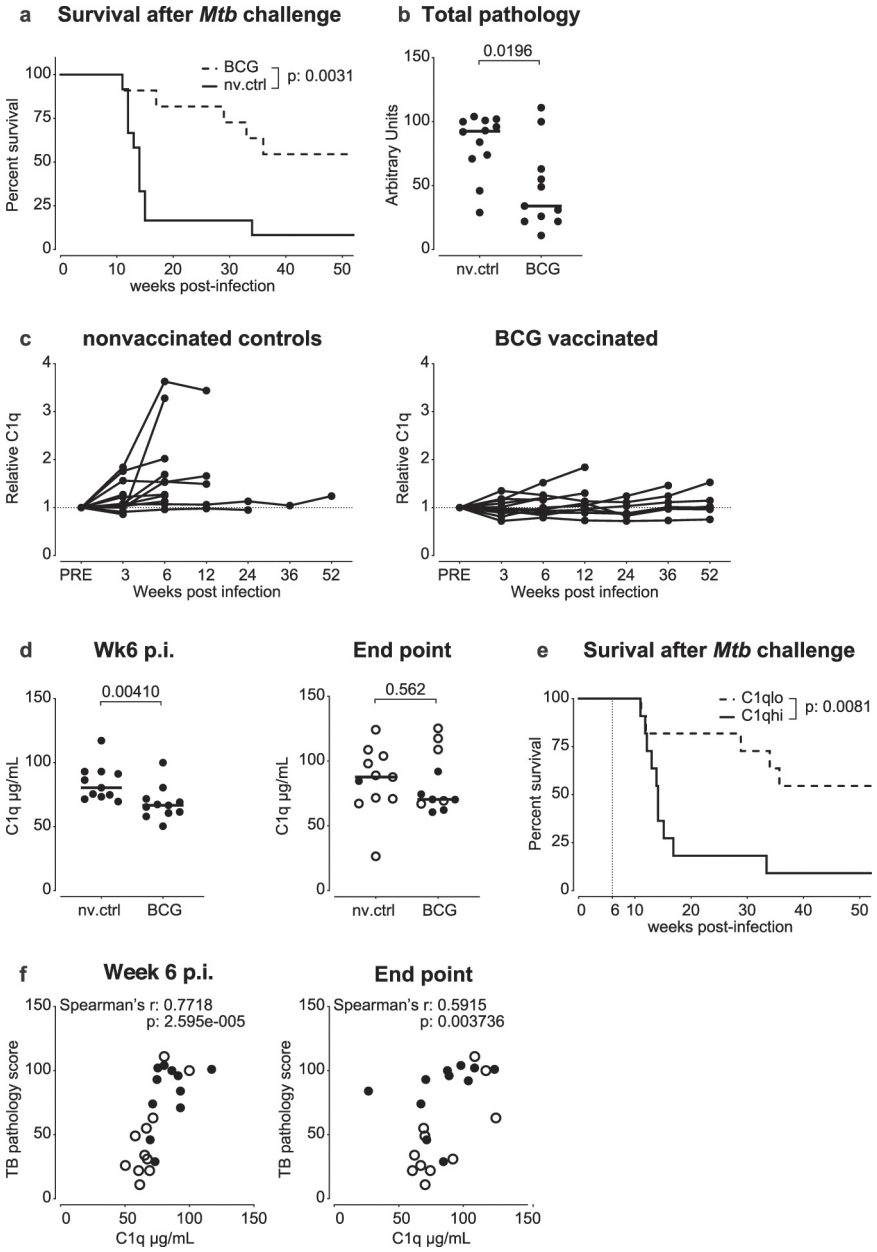


Figure 1. C1q is a predictive marker of disease progression in NHP TB

Increase in serum C1q correlates with disease manifestation after high dose *Mtb* challenge. **a**) Kaplan-Meier curves of time-to-endpoint (survival) for non-vaccinated (solid line, n=12) and BCG vaccinated (dashed line, n=11) rhesus macaques after infection with 500 CFU of *Mtb*. **b**) Total post-mortem tuberculosis pathology scores, showing reduction of TB disease by prior BCG vaccination. **c**) Serum C1q levels relative to pre-infection values of nonvaccinated controls and BCG vaccinees. **d**) Group-comparison of serum C1q concentration 6 weeks post-infection (p.i.) and at

endpoint. Open symbols in right panel represent animals that reached a humane endpoint. **e**) Kaplan-Meier curves of time-to-endpoint (survival) for animals with high/above median (solid line) or low/below median C1q levels (dashed line) at 6 weeks post-infection. **f**) Correlation of serum C1q levels at 6 weeks post-infection and at endpoint, with total tuberculosis pathology scores from BCG vaccinated animals represented by open symbols.
Horizontal line**s** in **b**) & **d**) indicate group medians. Statistical significance of group differences determined by two sided Mann-Whitney. Statistical curve comparison in **a**) and **e**) by Mantel-Cox Log-rank test. Correlations in **f**) calculated with Spearman's rank-order test.

C1q is a marker of differential disease severity in distinctive rhesus cohorts

We next sought to confirm the correlation of C1q upregulation with disease severity in an independent cohort of *Mtb* infected NHP. As a species, rhesus macaques can be divided into distinct subpopulations based on genetic variation that is associated with their geographical distribution[28]. When comparing Chinese versus Indian type rhesus macaques head-to-head, we found the latter to exhibit a more severe TB disease phenotype, 12 weeks after single high dose (500 CFU) *Mtb* challenge ([23] & **Figure 2a**, non-vaccinated controls). Prior intradermal BCG vaccination reduced TB associated pathology in Indian but not Chinese type rhesus macaques. We measured C1q in serum collected at 3 weekly intervals and assessed the association of C1q levels with TB pathology.

As observed previously, serum C1q levels increased during experimental infection with *Mtb*, from 3 weeks post-infection onward (**Figure 2b**). The strongest increase in serum C1q was again observed in the animals with the highest disease severity, the non-vaccinated Indian rhesus macaques (**Figure 2b**). Reduced TB pathology, as seen in Chinese type rhesus macaques or after prior BCG vaccination of Indian type rhesus macaques, is reflected in the limited upregulation of C1q in these groups (**Figure 2b**). Since we observed a trend of higher C1q levels prior to infection in BCG vaccinated Chinese rhesus macaques in particular (**Supplemental Figure 1b**), we used individual fold-increase of C1q (a relative measure of C1q) in subsequent analyses. When comparing values 6 weeks post-infection, relative C1q values were significantly higher in unvaccinated Indian rhesus macaques compared to BCG vaccinated Indian rhesus macaques or unvaccinated Chinese type macaques (**Figure 2c).** Non-parametric Spearman's analysis of the relative C1q levels at week 6 versus total TB pathology scores revealed a strong correlation (rho = 0.7014, p = <0.0001, **Figure 2d**), corroborating the association between C1q upregulation and tuberculosis disease severity.

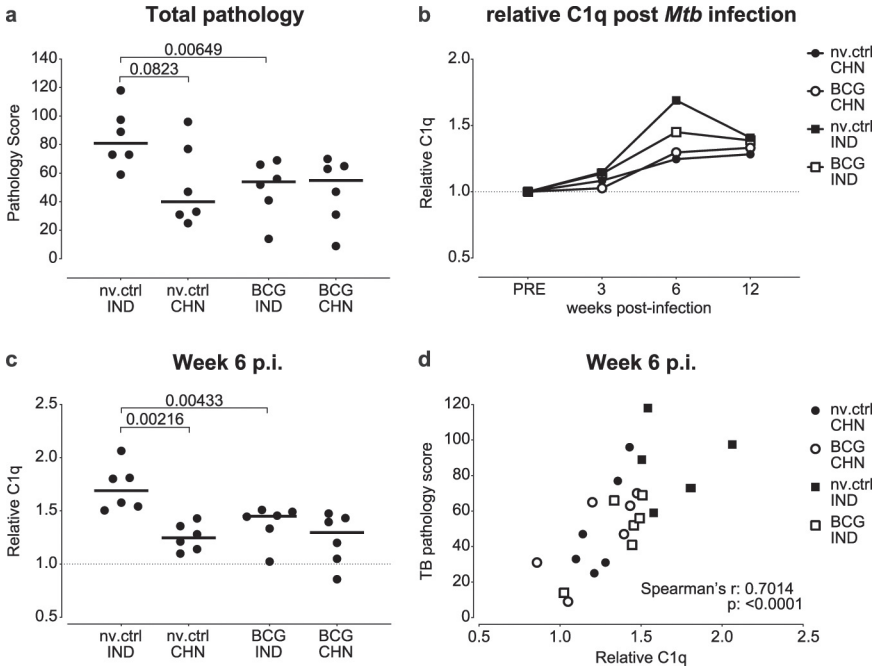


Figure 2. C1q is a marker of differential disease severity in distinctive rhesus cohorts
Serum C1q levels after *Mtb* infection of two genotypic cohorts of rhesus macaques. **a**) Total tuberculosis pathology scores after infection of unvaccinated (nv.ctrl) or BCG vaccinated rhesus macaques with 500 CFU of *Mtb*. IND: Indian genotype, CHN: Chinese genotype. N=6 per group. **b**) Group medians of relative C1q levels over the course of *Mtb* infection. **c**) Group-comparison of relative C1q levels at 6 weeks post-infection (p.i). **d**) Correlation of relative C1q levels with total tuberculosis pathology scores. Horizontal lines in **a**) and **c**) indicate group medians. Statistical significance of group differences determined by two sided Mann-Whitney. Correlation in **d**) calculated with Spearman's rank-order test.

Increased C1q is observed in the pulmonary space after *Mtb* infection

Over time we have moved away from high dose infection studies, since macaques appeared highly susceptible to infection and natural exposure to *Mtb* likely occurs at much lower doses than the 500 CFU applied in the studies described above[29]. To investigate whether increasing serum C1q still associates with disease severity after low dose *Mtb* infection, we assessed serum C1q dynamics in a cohort consisting of rhesus and cynomolgus macaques (*Macaca mulatta* and *Macaca fascicularis,* respectively) that were infected with a low dose (<10 CFU) of *Mtb*. The two species differ in the extent of TB pathology development after experimental infection; rhesus macaques typically tend to develop more severe TB pathology (**Figure 3a**, unpublished data & [30]). These animals were sacrificed either 6 or 12 weeks after *Mtb* infection, and displayed significantly less TB pathology compared to the animals from the studies described in **Figure 1** and **Figure 2** (**Supplemental Figure 2**).

Baseline pre-infection levels of serum C1q did not differ between the two species (**Supplemental Figure 1c**). After infection with low dose *Mtb*, overall we observed a modest increase in serum C1q only, most apparent between 3 and 6 weeks post-infection (with the exception of one rhesus showing a progressive increase) **(Figure 3b**). However, despite increased TB disease levels in rhesus over cynomolgus macaques, there was no significant difference in C1q levels between the two species, neither when comparing C1q levels at week 6 post-infection nor at endpoint (that is pooling endpoint values of animals sacrificed at week 6 or 12) (**Figure 3c**, left and right panel, respectively).

Considering the relatively low serum C1q levels in these animals after low dose *Mtb* challenge, we interrogated C1q at the site of infection by measuring C1q in BAL fluids collected at various timepoints post-infection. Prior to *Mtb* infection, C1q was virtually undetectable in BAL fluids from either species (**Supplemental Figure 1c**, right panel). However, from 3 to 6 weeks post-infection we observed a marked increase in C1q in the BALs of rhesus macaques (**Figure 3d**), though C1q levels dropped between week 6 and week 12. In contrast, this marked increase in local C1q was absent in cynomolgus macaques (except for one animal with an (unexplained) outlier measurement at week 3). When comparing C1q values at 6 weeks post-infection and at study endpoint, increased C1q levels were observed in the rhesus but not the cynomolgus cohort (**Figure 3e**). As expected from the above findings, serum C1q levels did not correlate with TB disease scores (**Figure 3f**). But, when analyzing C1q expression in BAL 6 weeks post-challenge, we did find a statistically significant correlation with TB pathology (Spearman's rho = 0.599, p = 0.005, **Figure 3g**). Thus, also after low dose *Mtb* challenge and subsequent mild(er) TB disease, increasing C1q levels remain associated with disease progression and pathological involvement, albeit locally rather than peripherally.

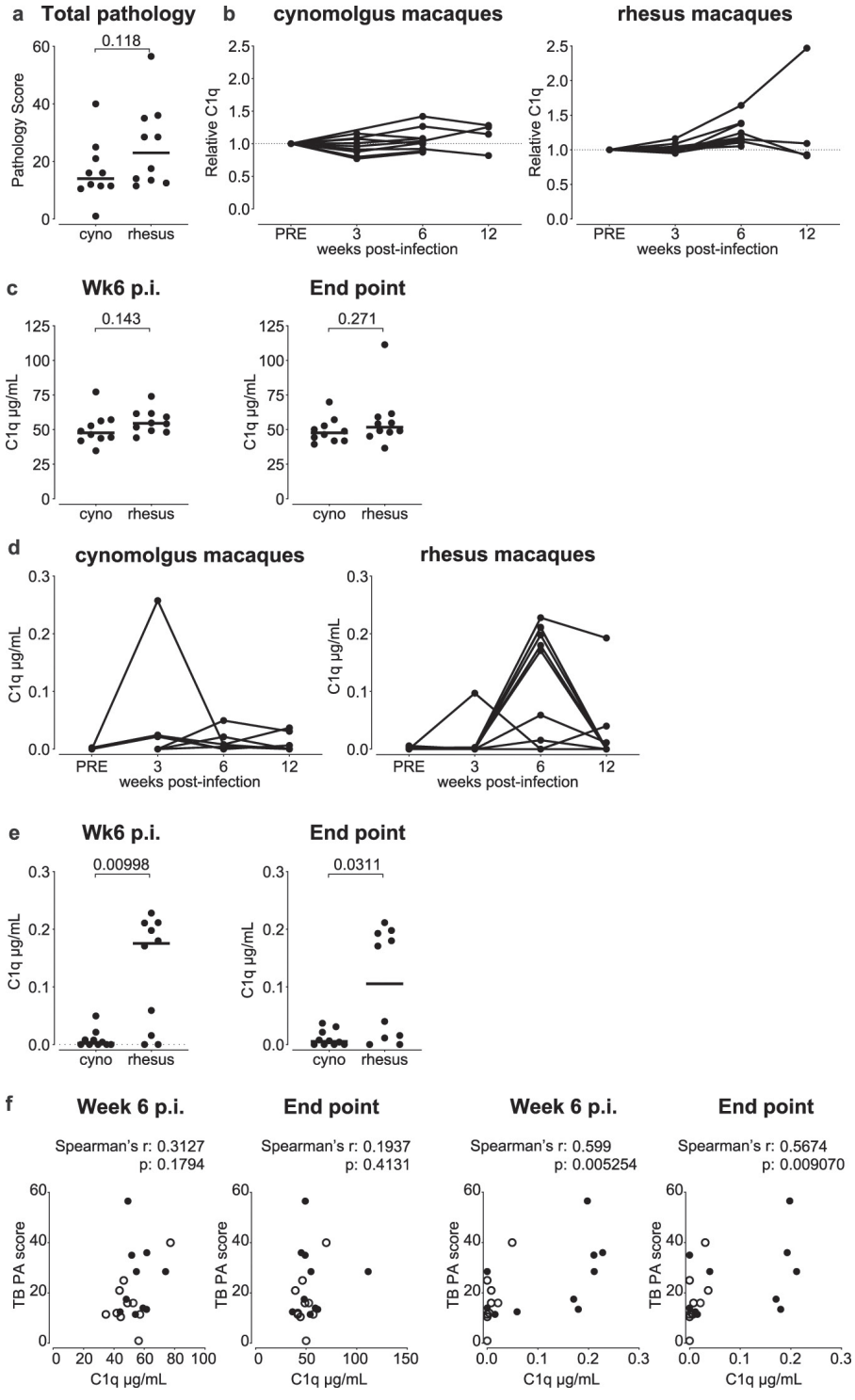

a
Total pathology
0.118
Pathology Score
cyno
rhesus
b
cynomolgus macaques
rhesus macaques
Relative C1q
PRE
3
6
12
weeks post-infection
c
Wk6 p.i.
End point
0.143
0.271
C1q µg/mL
d
cynomolgus macaques
rhesus macaques
e
Wk6 p.i.
End point
0.00998
0.0311
f
Week 6 p.i.
End point
Week 6 p.i.
End point
Spearman's r: 0.3127
p: 0.1794
Spearman's r: 0.1937
p: 0.4131
Spearman's r: 0.599
p: 0.005254
Spearman's r: 0.5674
p: 0.009070
TB PA score
C1q µg/mL

Figure 3. Increase in local C1q in TB disease susceptible rhesus but not cynomolgus macaques. Analysis of C1q levels in serum and broncho-alveolar lavage (BAL) fluid after low dose (1-7 CFU) *Mtb* challenge of rhesus and cynomolgus macaques (n=10 per species). **a**) Total tuberculosis pathology scores after *Mtb* infection of rhesus versus cynomolgus macaques. **b**) Relative levels of serum C1q over the course of *Mtb* infection for the two species. **c**) Species comparison of serum C1q concentration 6 weeks post-infection (p.i, left panel) and at endpoint, being either week 6 or week 12 post-infection by predefined study plan (right panel). **d**) BAL C1q levels over the course of *Mtb* infection for both species. **e**) Species comparison of BAL C1q levels 6 weeks post-infection (p.i) and at endpoint. Correlation of **f**) serum C1q and **g**) BAL C1q levels, both at 6 weeks post-infection and at endpoint, with total amount of tuberculosis pathology.
Horizontal lines in **a**), **c**) and **e**) indicate group medians. Open symbols in **f**) and **g**) represent cynomolgus macaques; closed symbols rhesus macaques. Statistical significance of group differences determined by two sided Mann-Whitney. Correlations in **f**) and **g**) calculated with Spearman's rank-order test.

C1q upregulation does not precede IGRA conversion

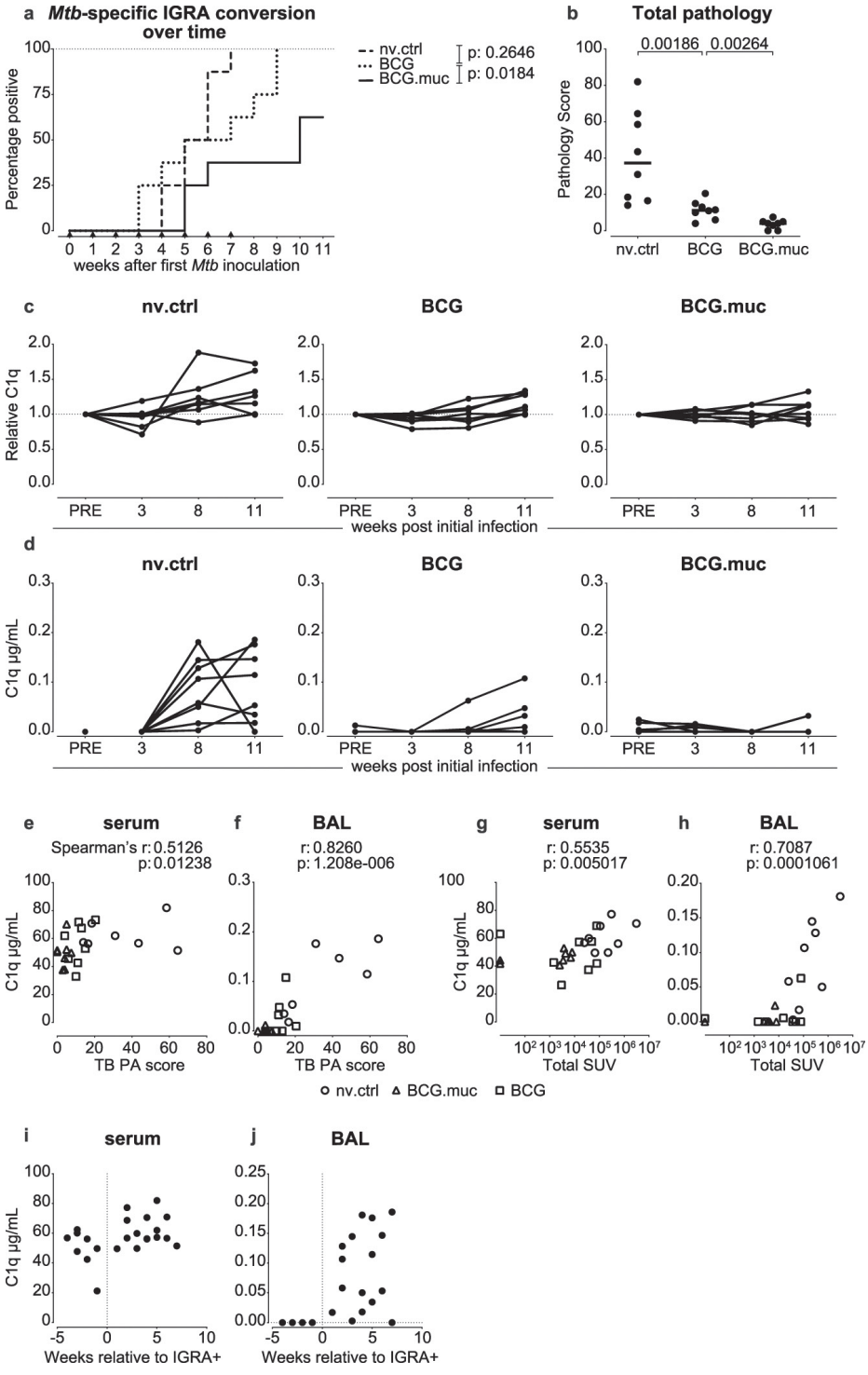
Having established that C1q is upregulated either peripherally and/or locally in association with various TB (challenge and) disease levels, we subsequently wanted to investigate if this increase in C1q could serve as a diagnostic marker of *Mtb* infection, with superior sensitivity to a conventional IFNg Release Assay (IGRA). To address the diagnostic potential of C1q measurement, we profiled peripheral and local C1q levels in a challenge study in rhesus macaques in which we deployed a novel repeated limiting dose (RLD) *Mtb* challenge modality. In this RLD challenge model, we have demonstrated prevention of infection and disease after mucosal BCG vaccination (**Figure 4a&b**, previously published and further detailed in Dijkman et al (2019)[26], and depicted here only to provide relevant background).

As observed after single low dose infection, RLD *Mtb* challenge resulted in a modest increase in serum C1q over the course of the infection phase, in particular in non-vaccinated control animals, which, expectedly displayed the highest tuberculosis pathology levels at study end-point (**Figure 4c**). Animals protected from severe TB disease by prior BCG vaccination (by either standard intradermal or pulmonary mucosal route of administration) did not show any increase in relative serum C1q. Similarly, C1q is markedly increased in BAL fluids of the non-vaccinated control group, but not after prior BCG vaccination (with the exception of a few intradermally BCG vaccinated animals) (**Figure 4d**). Also in this study, we found BAL as well as serum C1q levels to statistically correlate with the level of TB pathology at endpoint, once more confirming C1q upregulation to be associated with disease development and severity rather than infection (**Figure 4e&f**). Along *Mtb* infection (rather than post mortem), disease severity can be assessed by means of PET-CT imaging, in which inflammation and metabolically active granulomas can be visualized using ^{18}F-fluorodeoxyglucose (FDG) as a PET tracer[31]. To investigate whether C1q levels correlate with TB pathology as measured by PET-CT, we plotted summed lung and bronchoalveolar Standard Uptake Values (SUVs) obtained

8 weeks after initial challenge against C1q levels measured in serum and BAL collected at that same timepoint. Again, we found both serum and BAL fluid C1q concentrations to correlate significantly ($p<0.05$) with PET-CT signals of TB pathology (Spearman's rho 0.554 and 0.709 respectively, **Figure 4g&h**).

We next set out to investigate if C1q upregulation preceded detection of *Mtb* infection by IGRA conversion and therefore aligned the C1q responses in the non-vaccinated control animals to their time point of IGRA conversion. As can be expected for a marker associated with progressive disease, we did not observe an increase in C1q prior to IGRA conversion, neither in serum nor in BAL (**Figure 4i&j**). Not earlier than two weeks post-IGRA conversion local C1q levels were elevated over baseline. This observation further asserts the association of C1q with progressive, rather than latent or incipient, tuberculosis disease. Finally, to further assert the relation between serum C1q and disease progression, we pooled the data of all studies and correlated serum C1q levels with the amount of "local" pathology in the lung and with the amount of disseminated disease, i.e. lung draining lymph node pathology and extra-thoracic pathology. As expected, serum C1q levels determined at end-point correlated most strongly with disseminated disease (for lung draining lymph node: Spearman's rho = 0.697, P = <0.0001, for extra thoracic pathology: Spearman's rho = 0.635, P = <0.0001). Correlation of serum C1q with the amount of lung pathology was significant, but weak (Spearman's rho = 0.311, P = <0.0001).

Figure 4. Infection-associated IGRA conversion precedes peripheral and local C1q increase. Analysis of C1q levels in serum and broncho-alveolar lavage (BAL) fluid after repeated limiting dose (RLD) *Mtb* challenge of rhesus macaques that were either vaccinated by standard intradermal (BCG) or pulmonary mucosal (BCG.muc) BCG administration or left untreated (nv.ctrl). **a**) Rate of IGRA conversion after RLD *Mtb* challenge in vaccinated and non-vaccinated animals. **b**) Total tuberculosis pathology scores per treatment group after RLD *Mtb* challenge. C1q levels over the course of *Mtb* infection in **c**) serum and **d**) BAL fluid. Correlation between **e**) serum C1q and **f**) BAL C1q levels 11 weeks after the first exposure to *Mtb* and the total amount of tuberculosis pathology. Correlation between **g**) serum C1q and **h**) BAL C1q levels and total lung standard uptake values (SUV) as measured by PET-CT 8 weeks after initial Mtb challenge. **i**) Serum and **j**) BAL C1q levels of non-vaccinated control animals over time, aligned relative to IGRA conversion. Horizontal lines in **b**) indicate group medians. Statistical significance of group differences determined by two sided Mann-Whitney. Correlations in **e-h**) calculated with Spearman's rank-order test.


a Mtb-specific IGRA conversion over time
Percentage positive
weeks after first Mtb inoculation
nv.ctrl
BCG
BCG.muc
p: 0.2646
p: 0.0184
b Total pathology
0.00186
0.00264
Pathology Score
nv.ctrl
BCG
BCG.muc
c
nv.ctrl
BCG
BCG.muc
Relative C1q
PRE
weeks post initial infection
d
C1q µg/mL
e serum
Spearman's r: 0.5126
p: 0.01238
TB PA score
f BAL
r: 0.8260
p: 1.208e-006
g serum
r: 0.5535
p: 0.005017
Total SUV
h BAL
r: 0.7087
p: 0.0001061
nv.ctrl
BCG.muc
BCG
i serum
j BAL
Weeks relative to IGRA+

Pulmonary BCG vaccination increases local C1q production

C1q is considered to contribute to control of infection by enhancing phagocytosis of pathogens, either by opsonization and specific uptake or by activating phagocytes through engagement of C1q-binding receptors[32]. As we have previously found mucosal BCG to be superior over intradermal BCG in preventing TB infection and disease, we here investigated BCG's capacity to induce upregulation of C1q. We determined C1q in serum and BAL fluid from intradermal and mucosal BCG vaccinated animals from the repeated low dose challenge study described above and in **Figure 4**. Similar to what has been described for humans, BCG vaccination in rhesus macaques by either route of administration, does not lead to upregulation of serum C1q (**Figure 5a&b**). Interestingly, pulmonary, but not intradermal, BCG vaccination resulted in a marked increase in local C1q either 3 or 8 weeks after vaccination, to return to baseline levels by week 12 after vaccination (**Figure 5c&d**).

As we observed a local, but not peripheral, elevation of C1q levels, we hypothesized that local production of C1q could underlie the increase observed in the mucosally vaccinated animals. We sought to verify the possibility of local C1q production by *in vitro* stimulation of BAL cells versus Peripheral Blood Mononuclear Cells (PBMCs) from BCG vaccinated rhesus macaques (intradermal as well as mucosal, at 8 weeks after vaccination), and from low dose *Mtb*-infected animals (at 11 weeks after infection) and untreated controls. Cells were incubated either with culture medium to assess *ex vivo* C1q production or with dexamethasone plus IFNg (Dex/IFNg), a positive control stimulus known to induce C1q release[17], to assess the potency of these cells to produce C1q.

In PBMCs, a trend towards higher production by unstimulated PBMCs from vaccinated or *Mtb* infected animals (**Figure 5e,** left panel) was observed when compared to unvaccinated/ uninfected control animals. Prior BCG vaccination also seemed to potentiate C1q secretion of PBMCs, as reflected by the increase in C1q production in response to Dex/IFNg stimulation in the vaccinees (**Figure 5e,** right panel). Locally, *ex vivo* C1q production was highest in unstimulated BAL cells of animals that were exposed to either mucosal BCG or *Mtb* (**Figure 5f,** left panel). After Dex/IFNg stimulation, lower C1q production was observed in BCG vaccinated animals compared to non-vaccinated controls, potentially reflecting alterations in the cellular composition of the BAL (**Figure 5f,** right panel)[26]. Collectively, our data show that pulmonary mycobacterial exposure can induce the production of local C1q, likely by resident alveolar macrophages.

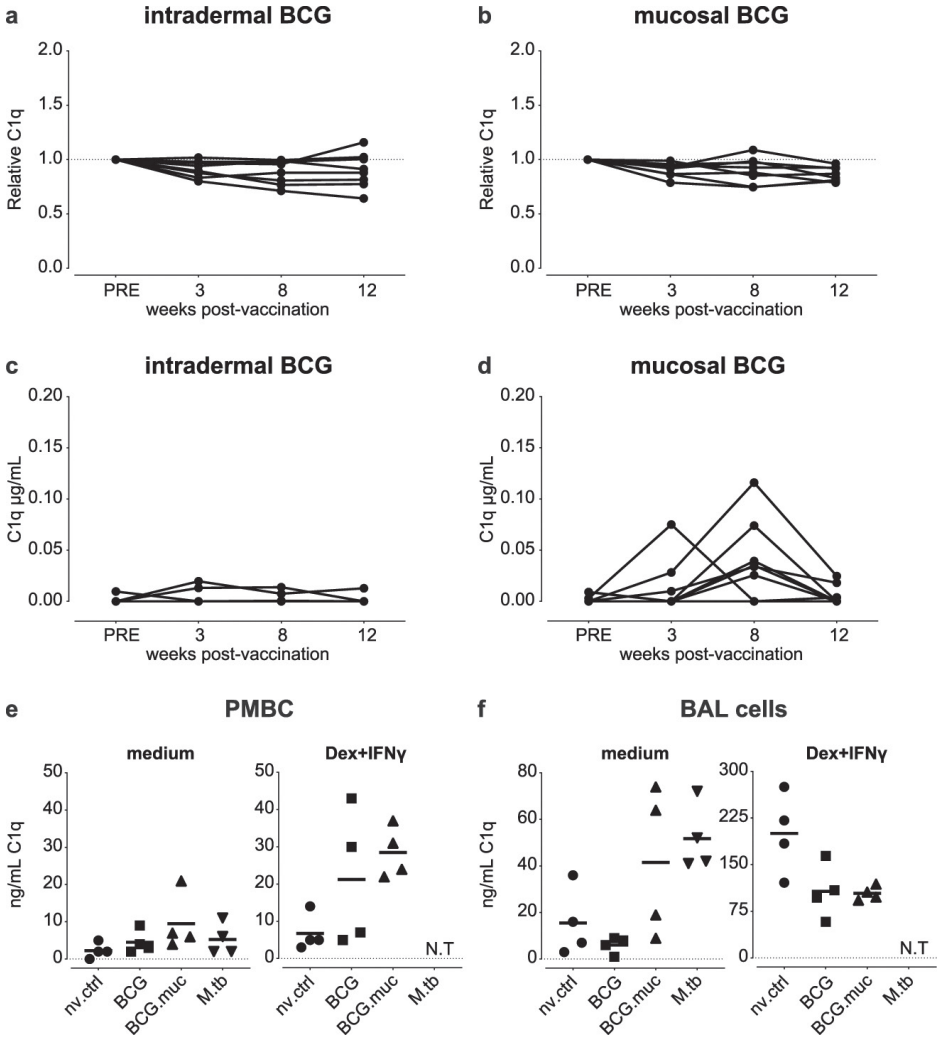


Figure 5. Pulmonary but not intradermal BCG vaccination results in upregulation of local production of C1q in rhesus macaques.

(a-d) C1q levels in serum (**a**-**b**) and broncho-alveolar lavage (BAL) fluid (**c**-**d**) after BCG vaccination of rhesus macaques either through intradermal injection (BCG, **a** & **c**) or endobronchial instillation (BCG.muc, **b** & **d**). C1q production after stimulation with culture medium or dexamethasone plus interferon-g (Dex+IFNg) of **e**) PBMC and **f**) BAL cells, comparing from left to right non-vaccinated (nv.ctrl), standard BCG vaccinated, BCG.muc vaccinated, and low dose (15 CFU) *Mtb* infected rhesus macaques.

N.T = not tested. Horizontal lines in **e** & **f** indicate group medians.

DISCUSSION

In this work, we show that increasing C1q levels in serum and BAL are associated with TB disease severity in various non-human primate TB studies. High C1q levels did not precede detection of *Mtb* infection by IGRA conversion, but did correlate with TB pathology after both high (500 CFU) and low (<10 CFU) dose *Mtb* challenge in different macaque species. Likewise, in a long-term follow up (up to 1 year) setting, we found increasing C1q levels to be associated with reduced survival. Contrarily to what has been described for serum C1q, increasing pulmonary C1q was not exclusive to infection with *Mtb*; pulmonary vaccination with BCG also resulted in a temporal increase of C1q in BAL fluid. Whether this local C1q production plays a role in protection against *Mtb* infection and disease remains to be investigated.

Our data demonstrates that, in addition to being a marker of active TB in humans, C1q can also serve as a marker of progressive disease in experimental *Mtb* infection studies in NHP. C1q levels measured after *Mtb* infection correlated with disease severity measured post-mortem, but also with disease severity measured during infection by PET-CT. As C1q can be readily measured over the course of *Mtb* infection, it could therefore be applied to monitor TB disease progression in a resource-limited setting. Especially in cynomolgus macaques, which are known to develop latent TB infection[24], measuring C1q at regular intervals after *Mtb* infection would be informative when assessing the occurrence and reactivation of LTBI. While the cohort of cynomolgus macaques measured in this study was not followed long enough to confirm the establishment of such a latent infection, the lack of C1q upregulation in the majority of this group might be indicative of TB latency development in these animals. As no post-infection increase in serum C1q could be observed after protective BCG vaccination, C1q can be used to assess efficacy along the infection phase when evaluating new drug or vaccine regimens in the NHP model.

Next to being a marker of progressive disease, it is tempting to speculate that C1q might also play a role in shaping the protective immune response observed after mucosal vaccination with BCG. Macrophages, including alveolar macrophages, express receptors, such as gC1qR and cC1qR (calreticulin), that can interact with either the globular head or collagen-like tail of C1q[11]. Not only does binding of (pathogen-bound) C1q to these receptors improve pathogen uptake[32], C1q-receptor interaction can also modulate macrophage inflammatory status and cytokine responses. Monocytes incubated with C1q have been described to produce higher amounts of anti-inflammatory IL-10[33], and it has been shown that C1q can polarize macrophages to an anti-inflammatory M2 phenotype, associated with the resolving of inflammation and tissue repair[34]. After mucosal BCG vaccination we observed an increase in pulmonary C1q (**Figure 5b**), which coincides with increased PPD-specific IL-10 production by BAL cells[26]. This increased IL-10 production in response to PPD is sustained up to several weeks after *Mtb* challenge,

and might be required to counterbalance the inflammatory response induced by BCG/*Mtb*, to prevent inflammation-induced damage[35]. However, both IL10 production and M2 polarization have also been identified as detrimental in the context of *Mtb* infection[36,37], so the exact contribution of C1q to TB disease development remains to be elucidated. Lastly, C1q has the capacity to reduce production of IFNa[38,39], which has been implied as detrimental in the (late phase) host immune response to *Mtb*[40,41]. In addition to modulating innate immune responses, C1q has also been described in the regulation of adaptive immune responses, either indirectly through regulating APC function or through direct interaction with C1q-receptors expressed on T-cells. However, reports on the capacity of C1q to directly activate T-cells are contradictory and need further investigation[42,43]. To fully appreciate the mechanistic role of C1q in shaping the protective immune response after mucosal BCG vaccination further research is required.

In summary, this work corroborates and extends on findings in human TB patients and links increased C1q levels with TB disease severity. It demonstrates that macaques can serve as informative model animals to study the role of (pulmonary) C1q in TB pathogenesis and/or protective immunity.

MATERIALS & METHODS

Animals & Ethics

All housing and animal care procedures took place at the Biomedical Primate Research Centre (BPRC) in Rijswijk, the Netherlands. The BPRC is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and is compliant with European directive 2010/63/EU as well as the "Standard for Humane Care and Use of Laboratory Animals by Foreign Institutions" provided by the Department of Health and Human Services of the US National Institutes of Health (NIH, identification number A5539-01). Before the start of each study ethical approval was obtained from the independent animal ethics committee (in Dutch: Dierexperimentencommissie, DEC), as well as BPRC's institutional animal welfare body (in Dutch: Instantie voor Dierwelzijn, IvD).

Animals included in each study were screened negative for prior exposure to mycobacteria by means of tuberculin skin testing with Old Tuberculin (Synbiotics Corporation, San Diego, CA) and an IFNg ELISPOT against Purified Protein Derivative (PPD) from *Mycobacterium bovis*, *Mycobacterium avium* (both Fisher Scientific, USA) or *Mycobacterium tuberculosis* (Statens Serum Institute, Copenhagen, Denmark).

For the duration of the study animals were socially housed (pair-wise) at animal biosafety level 3. Animal welfare was monitored daily. Macaques were provided with enrichment in the form of food and non-food items on a daily basis. Animal weight was

recorded prior to each blood collection event. To limit possible discomfort due to severe TB disease humane endpoints were predefined. All animal handling and biosampling was performed under ketamine sedation (10 mg/kg, by intra-muscular injection). When performing endobronchial challenge with *Mtb* or BAL, ketamine sedation was supplemented with intramuscular medetomidine (0.04 mg/kg) and an analgesic sprayed into the larynx. At the end of the study or when reaching a humane endpoint, animals were euthanized by intravenous injection of pentobarbital (200 mg/kg) under ketamine sedation. Veterinary staff and animal care-takers were blinded to animal treatment.

***Mtb* challenge**

In all studies, animals were challenged with *Mycobacterium tuberculosis* Erdman K01 strain (BEI Resource, VA, USA). Every *Mtb* challenge occurred by endobronchial instillation, targeting the lower left lung lobe. All challenge events were executed in a single session within 2-3 hours from preparing the inoculum from frozen *Mtb* stock, challenging animals in random order. *Mtb* challenge dose, verified in each study by quality control plating, is indicated in the relevant results sections and figure legends.

Serum and BAL-fluid collection

Serum and BAL fluid were collected at various timepoints after *Mtb* challenge or BCG vaccination, as indicated in the figure axes. For serum, peripheral blood was collected in serum separator tubes by means of venipuncture. Tubes were spun for 10 minutes at 1000g to harvest cell-free serum, which was stored at -80°C pending further analysis. BAL was performed by targeting the lower left lung lobe by bronchoscope, followed by instillation and recovery of three times 20 mL prewarmed 0.9% saline solution. BALs were first passed over a 100 mm filter to remove mucus and debris, followed by centrifugation for 10 minutes at 400g. Supernatant was decanted and stored at -80°C pending further analysis. Serum and BAL fluid were filter-sterilized by centrifugation through 0.2 mm PVDF membrane plates (Fisher Scientific) before analysis.

Post-mortem pathology scoring

Post-mortem tuberculosis pathology was scored by a semi-quantitative grading system (adapted from[44]) based on lesion size, manifestation and frequency, and lymph node involvement. Over time, from one to the other study, the scoring system has been adjusted slightly on minor details to more accurately describe disease manifestation, but the overall algorithm, as outlined in the paragraph below, has remained the same. When comparing disease severity between studies, pathology scores have been expressed as a percentage of the maximal possible score.

After euthanasia, the thoracic cavity, including the heart, ribcage, vertebrae and diaphragm were all macroscopically scored for the presence of granulomas and pleural adhesions. Lungs were isolated and lobes were separated from the trachea. Subsequently, lung lobes were cut in 5mm thick slices and scored for the amount of

pathology. Lung draining lymph nodes were removed from the trachea and scored for size and extent of involvement. Extra-thoracic organs such as kidneys, spleen, pancreas and liver were macroscopically assessed for the presence of lesions. The "Total Pathology score" that is depicted throughout represents the summed score of all these organs.

C1q production by PBMC and BAL cells

To assess production of C1q by PBMC and BAL cells, freshly isolated cells were taken up in Roswell Park Memorial Institution 1640 medium (RPMI), supplemented with 10% Fetal Calf Serum (FCS), glutamine and penicillin/streptomycin. Cells were seeded at 200.000 cells per well in triplicate in 96-well round bottom plates and stimulated with 4 g/mL dexamethasone (Merck) + 200 U/mL IFNg (Peprotech, UK), or left untreated. Supernatants were harvested after 96 hours, pooled and filter-sterilized by centrifugation through 0.2 mm PVDF membrane plates (Fisher Scientific) before storage at -80°C and subsequent analysis by ELISA.

C1q ELISA

C1q levels in sera, BAL fluid and culture supernatants were measured using an in-house developed ELISA, as described previously[18]. In brief, 96-well Maxisorp plates (Nunc) were coated overnight with mouse anti-human C1q (Nephrology department, LUMC) in coating buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH9.6). The next day, plates were washed with PBS/0.05% Tween and blocked with PBS/1%BSA. After subsequent washing, samples (serum diluted 1:4000, BAL fluid 1:1, culture supernatant 1:1) were added to the plates. A serially diluted pool of normal human serum (NHS) was taken along as a standard. Bound C1q was detected by incubation with rabbit anti-human C1q, followed by goat anti-rabbit HRP (both Dako) and ABTS substrate. C1q concentration is calculated in µg/mL from a human C1q standard.

Statistics

Statistical analyses were performed using Graphpad Prism, software version 7. Significance of differences between groups was calculated by two-sided Mann-Whitney testing. All correlations were calculated by Spearman's log rank testing.

Data availability

The datasets generated and analyzed are available from the corresponding authors upon reasonable request. Likewise, biomaterials remaining from the studies described could be shared for further research.

ADDITIONAL INFORMATION

Competing Interest statement
The authors declare no competing interests

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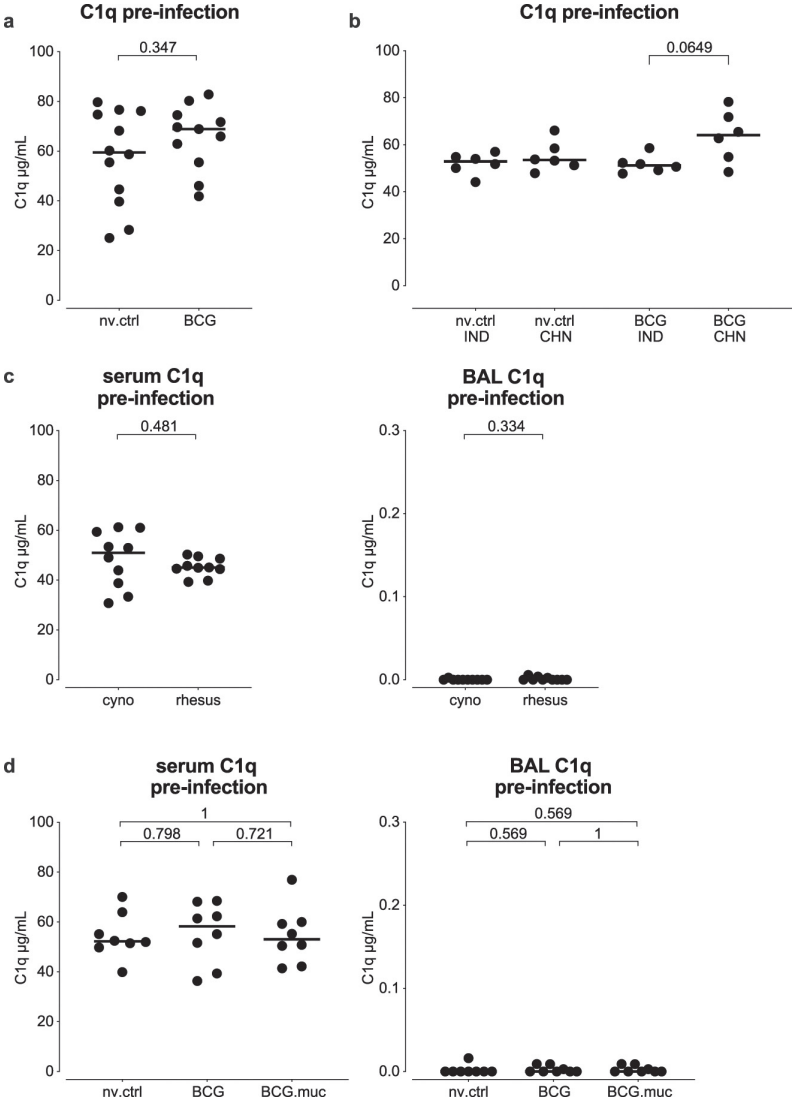
Mycobacterium tuberculosis strain Erdman K01 (TMC107, NR-15404) was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH.

Non-human primate samples used in this study were banked from previous studies performed with financial support from Aeras or from the European Commission under the Framework Programme 7 project TRANSVAC, contract number 228403, governed by the European Vaccine Initiative (EVI, Heidelberg), and the Horizon2020 project TBVAC2020, contract number 643381, governed by the Tuberculosis Vaccine Initiative (TBVI, Lelystad).

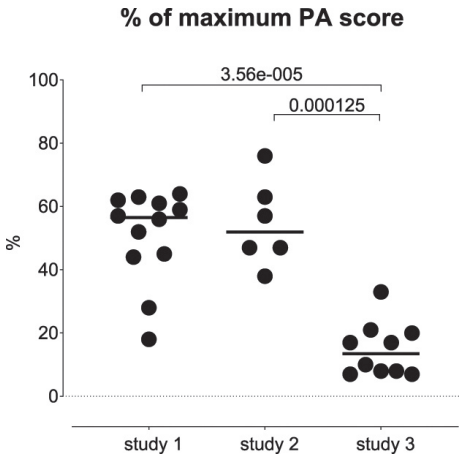
Author contributions
KD, RL, THMO, SAJ, LAT, and FAWV were involved in conceptualization and experimental design. KD, RL and NVB acquired and analyzed the data. KD & FAWV wrote the manuscript. RL, NVB, SAJ, LAT and THMO reviewed and edited the manuscript.

SUPPLEMENTARY FIGURES



Supplemental Figure 1. Pre-infection C1q levels for each study

C1q levels before infection with *Mtb* for each of the cohorts described in the main body text. **a)** Pre-infection serum C1q levels per treatment group from the high-dose *Mtb* challenge with long (1 year) follow-up described in **Figure 1**. **b)** Idem from the high-dose *Mtb* challenge study with short (3 months) follow-up described in **Figure 2**. **c**) Pre-infection serum (left panel) and BAL C1q levels (right panel) from the low dose *Mtb* challenge study described in **Figure 3**. **d**) Idem from the repeated limiting dose *Mtb* challenge study described in **Figure 4**. Horizontal lines in **a-d** indicate group medians. Statistical significance of group differences determined by two sided Mann-Whitney.



Supplemental Figure 2. Comparison of TB associated pathology after high and low dose Mtb challenge.

Pathology (expressed as percentage of the maximal possible score) measured at end-point after high dose *Mtb* challenge (500 CFU), long-term follow up (study 1), high dose, short-term follow up (study 2) and low dose (<10 CFU), short-term follow up (study 3). Horizontal lines indicate group medians. Statistical significance of group differences determined by two sided Mann-Whitney.

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CHAPTER 7

COMPLEX MEDICAL HISTORY OF A PATIENT WITH A COMPOUND HETEROZYGOUS MUTATION IN *C1QC*

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ABSTRACT

Introduction: C1q is an essential part of the classical pathway of complement activation. Genetic deficiencies, caused by homozygous mutations in one of the C1q genes are rare and are strongly associated with development of Systemic Lupus Erythematosus (SLE). Here we describe a C1q deficient patient with a compound heterozygous mutation.

Material and methods: Serum was analysed with ELISA and western blot for the presence of C1q and DNA and RNA sequencing was performed to identify the mutations and confirm that these were located on different chromosomes.

Results: The medical history of the patient includes SLE diagnosis at the age of eleven with cerebral involvement at the age of thirteen, various infections, osteonecrosis and hemophagocytic syndrome. Using ELISA and western blot we confirmed the absence of C1q in the serum of the patient. Using DNA sequencing two mutations in the *C1QC* gene were identified: c.100G>A p.(Gly34Arg) ; c.205C>T p.(Arg69X). With RNA sequencing we confirmed that the mutations are located on different chromosomes.

Discussion: The patient described in this case report has a compound heterozygous mutation in *C1QC* resulting in C1q deficiency.

INTRODUCTION

The complement system consists of both soluble and membrane bound proteins. Activation can occur via three different pathways: the classical pathway (CP), the lectin pathway and the alternative pathway. The CP is activated when C1q is bound to IgM, immunocomplexes or among others pentraxins[1]. Many complement proteins are produced by hepatocytes, but also cells of the immune system produce several complement proteins[2]. Importantly, C1q is not produced by hepatocytes but largely by cells from the myeloid lineage. C1q has been described to have other functions outside of the complement system cascade: in the remodelling of the maternal decidua during pregnancy, during embryonic development, in neurological synapse function and in the coagulation process[3]. The C1q protein is a molecule of 480kDa which has six identical arms. Each arm consists out of three combined peptide chains: A, B and C. These peptide chains are generated from three different genes: *C1QA*, *C1QB* and *C1QC* which have a synchronized transcription[4].

C1q deficiency is a rare condition with just over 70 documented cases from at least 45 different families[5]. These deficiencies all are the result of homozygous mutations in one of the three C1q genes, except in one case with a compound heterozygous mutation in *C1QA*[6]. Patients with C1q deficiency have various clinical presentations and outcome[7]. Most common is the diagnosis of Systemic Lupus Erythematosus (SLE) in early childhood and recurrent infections[6-8]. Other common clinical manifestations are alopecia, Raynaud's phenomenon, involvement of the central nervous system, and also Sjögren's syndrome and Hyper IgM syndrome have been reported[5-7]. Treatment of C1q deficient patients mainly focuses on the treatment of the symptoms. For combating the C1q deficiency itself, intravenous administration of fresh frozen plasma (FFP) is used. In rare cases allogenic hematopoietic stem cell transplantation has been performed[9, 10].

Here we describe a patient with C1q deficiency based on a compound heterozygous mutation in the *C1QC* gene. This patient was treated with FFP for over a decade, over time it has resulted in various adverse reactions ranging from mild to anaphylactic which led to discontinuation of FFP therapy.

MATERIAL AND METHODS

Patient

The patient is a 29 years old Dutch woman, diagnosed with C1q deficiency since early childhood. Blood was obtained from the patient upon signing an informed consent in compliance with the Helsinki declaration.

Samples
Blood was collected from the patient in order to obtain serum as well as Peripheral Blood Mononuclear Cells (PBMCs) using Ficoll-Paque density gradient centrifugation.

Western blot
With western blot the availability of C1q was examined by detection of the three chains of the C1q protein. Serum of the patient and normal human serum (NHS), which was used as a positive control, were applied in reduced and non-reduced SDS conditions. The western blot was performed using previously described methods[11].

ELISA
C1q measurement by an in-house developed ELISA was performed as previously described[12]. In short, plates were coated with mouse anti-human C1q (2204), Nephrology department, LUMC) in coating buffer (0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH9.6), samples were incubated at 37°C and detection was performed with rabbit anti-human C1q (Dako cat#A0136) for one hour at 37°C and subsequently a goat anti-rabbit HRP (Dako cat#P0448) which was also incubated for one hour at 37°C. The substrate was added to the plates using 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS) and the signal was measured at an absorbance level of 415 nm using Biorad iMark Microplate Absorbance Reader.

RNA isolation and cDNA preparation
PBMCs (1 x 10^6 cells/ml) of the patient were cultured for 72 hours in RPMI (Gibco) culture medium supplemented with +1% Penicillin/streptomycin, +1%Glutamax and +8%FCS. PBMCs were either cultured in medium alone or in the presence of Dexamethason (10 µM, Pharmacy LUMC) and IFN-γ (200 U/ml, Peprotech), to increase *C1QA/B/C* expression[13]. After 72 hours RNA was isolated from the PBMCs using the mirVana miRNA isolation kit (Life Technologies cat# AM1561) according to manufacturer's protocol. The isolated RNA was subsequently treated with DNase I, Amplification Grade (Invitrogen) and cDNA was synthesized using superscript III (200U/µl, Invitrogen).

Ion Torrent sequencing
An Ampliseq™ custom panel (Thermo Fisher Scientific, Waltham, MA USA) was used to sequence the coding regions of the following genes: *C4A*, *C2*, *C1S*, *C1R*, *DNASE1L3*, *TREX1*, *MASP2*, *C4B*, *C1QA*, *C1QB*, *C1QC*, *PLG* and *SERPING1*. Library preparation and sequencing was performed according to manufacturer protocols on a S5 system (Thermo Fisher Scientific).

PCR
To confirm mutations found by Ion Torrent sequencing and to test whether the mutations found were on different alleles, mutation (allele) specific PCR was used on cDNA of the patient. We used the Rapid Cycler technology (BioFire Diagnostics, Salt Lake

City, UT USA) with 50 cycles of 5s at 95°C, 30s at 65°C and 60s at 72°C, in 15 µl of PCR buffer containing 2 U of Taq polymerase (Promega Benelux, Leiden, The Netherlands), 2 U of TaqStart antibody (Takara, Mountain View, CA, USA), 50 ng of each primer 200 µM for each of the dNTPs, 50 mM KCl, 1.5 mM MgCl2, 0.1% (v/v) Triton X-100, 10 mM Tris, pH 9.0 at 25°C, in 10-µl glass capillaries (BioFire Diagnostics). The *C1QC* primer sequences for cDNA are in **Table 1**.

Table 1. *C1QC* primer sequences

Name primer	sequence
C1QC-exon2-fw	GCCCCTCAGGGGCCAAGCCAACAC
C1QC -exon2-mut-fw	GGGCCAAGCCAACACAGGCTGCTTCA
C1QC -exon2-wt-fw	GGGCCAAGCCAACACAGGCTGCTTCG
C1QC -exon3-rev	GGTAAGCCGGGTTCTCCCTTCTGC
C1QC -exon3-mut-rev	TCCCTTCTGCCCTTTGGGTCCACA
C1QC -exon3-wt-rev	TCCCTTCTGCCCTTTGGGTCCACG

Sanger sequencing

PCR products from genomic DNA and cDNA were sequenced on an automated fluorescent sequencer (ABI 3730; Thermo Fisher Scientific) with the use of Big Dye Terminator (v.1.1) chemistry (Thermo Fisher Scientific). Primers used for sequencing were the same as those used for the PCR. For cDNA numbering, the A of the ATG translation initiation codon was taken as 1. This codon is codon 1. NM_172369.4 was used as reference sequence.

RESULTS

Patient medical history

Here we describe a Dutch woman born from two Caucasian non-consanguineous parents. She has two half-brothers who are reported to be healthy. C1q deficiency was diagnosed at the age of five. The patient has encountered many different clinical problems including infections, neurological-, vascular- and bone complications. The case will be presented per disease manifestation and not in chronological order. The use of FFP throughout her medical history will be discussed separately.

Infections

During the first year of her life she suffered from recurring otitis and gingivitis. She was reported to be a non-responder for Hepatitis B vaccination. Additionally, at the age of six she developed sepsis caused by *Streptococcus pneumoniae*. She experienced a herpes zoster infection when she was 12 years old. During adulthood, she was hospitalized with hemophagocytic lymphohistiocytosis (confirmed with a bone marrow biopsy) and pancytopenia which was potentially induced by co-trimoxazole. During this

hospitalization various infections were also diagnosed and treated accordingly; *Escheria coli* and candidiasis.

Systemic Lupus Erythematosus From the age of four, clinical symptoms were compatible with SLE-like disease, with butterfly rash. At the age of eleven, the diagnosis of SLE was established based on butterfly rash, oral ulcers, thrombocytopenia and positive antibodies (positive ANA, positive anti-Sm and positive anti SSA). Anti-dsDNA and antiphospholipid antibodies were not present. Furthermore, she experienced recurrent fevers with lymphadenopathy and vasculitis lesions of hand and feet. Initially, the SLE was treated with hydroxychloroquine and prednisolone, with serious side effects, including weight gain (Cushingoid), osteoporosis and bone infarctions. Furthermore, symptoms of fatigue, headache and arthralgia occurred during attempts to taper prednisolone treatment. On the basis of the diagnosis SLE in combination with the earlier established C1q deficiency, and both the side effects and the inability to taper prednisolone, treatment with FFP was initiated.

Cerebral involvement
When the patient was 14 years old she was hospitalized with a fever, paraesthesia and difficulties with speech. Infectious causes were excluded. MRI scans of the brain was normal. EEG showed left parieto-occipital irritative abnormalities. Liquor analysis revealed enhanced protein content without elevated cell count. With the working diagnosis of TIA / partial epileptic seizure due to cerebral vasculitis she was treated with prednisolone and carbasalate calcium. Subsequent visits at the age of 24, 25 and 28 at the Leiden University Medical Center multidisciplinary neuropsychiatric SLE (NPSLE) clinic [14] because of anxiety and difficulty in speech, did not reveal signs of active inflammatory NPSLE.

Vascular problems
At the age of 26 she developed a deep venous thrombosis, although no anti phospholipid antibodies were detected (ACA IgM/IgG, anti-B2GPI IgM/IgG and LAC). When the patient was 27 years old, she experienced a spontaneous abortion at a gestation of 8 weeks.

Bone lesions
At the age of nine she developed an avascular necrosis of the humerus which led to a destructed right shoulder. During the recent years she developed extensive bone infarctions around the knee. Because of the osteonecrosis she underwent total hip replacement surgery at the age of 29. Osteoporosis was identified already during childhood.

Therapy
Initially, prednisolone and hydroxychloroquine were used to treat the SLE. Because she was unable to taper the prednisolone and she was already diagnosed with

C1q deficiency, FFP treatment was started at the age of 11 with 15 mL/kg. The FFP infusions were administered 1-4 times a month and were preceded by clemastine and prednisolone intravenously. CP activity was measured preceding each FFP infusion. When FFP took place each week the CP activity was 80-90%, when the FFP was every two weeks the CP activity dropped below 50%, which was in line with previous reports[15]. Anti-C1q antibodies were detectable, though not increased. There were several adverse reactions to the FFP therapy, ranging from mild urticarial to anaphylactic reaction. Despite these adverse reactions, the patient preferred the FFP therapy, because of reduction of fatigue, arthralgia and number of infections. However, because of a serious anaphylactic reaction at the age of 25, FFP treatment was discontinued and her current treatment regimen consists of: hydroxychloroquine, azathioprine, low dose prednisolone, clopidogrel, bisphosphonates and cholecalciferol.

Complete absence of C1q in the serum

With western blot analysis sera of both the patient and normal human serum (NHS; pool of four healthy adults) were analysed for the presence of C1q. The same amount of serum was applied in native, denaturing or reducing conditions. Only in the NHS lane C1q was detected (**Figure 1 A-C**). Therefore, we confirmed the absence of circulating C1q in the serum of the patient. Additionally, patient serum was tested for C1q in ELISA format, next to 21 healthy female controls (age (26-32). The C1q levels in the healthy control had an average of 171 µg/mL C1q, while in the sera of the patient the C1q level was below the detection limit of 0,065 µg/mL (**Figure 1D**).

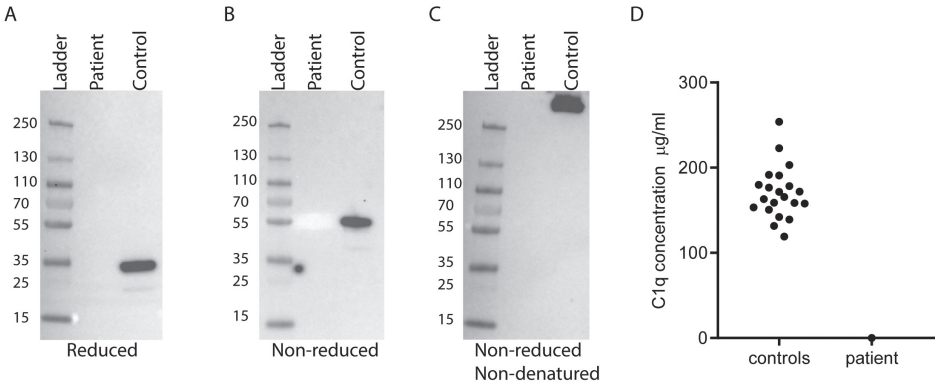

Figure 1. Western blot analysis shows no C1q in serum patient

Western blot analysis of serum from the patient and a control (normal human serum). The serum samples were either prepared under reducing condition (A), denaturing and non-reduced condition (B) or non-reducing and non-denaturing conditions (C). Measurement of C1q with ELISA in the sera of healthy female age matched controls (n=21) and the patient (D).

Sequencing

To determine what the mutation(s) are in this patient and where they are located, DNA and RNA sequencing was performed. Two previously described mutations were identified in the *C1QC* gene of the patient: c.100G>A p.(Gly34Arg) ; c.205C>T p.(Arg69X). With RNA sequencing we confirmed that both mutations were heterozygous, meaning these mutations are compound heterozygous (**Figure 2**).

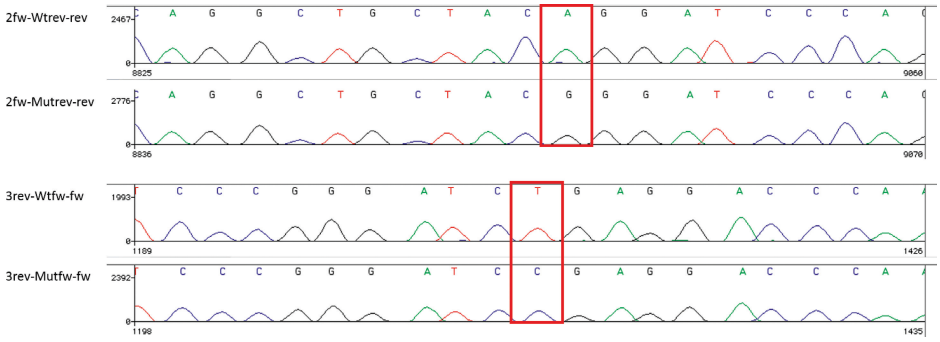

Figure 2. Compound heterozygous mutation located in *C1QC* gene

RNA sequence analysis revealed heterozygous mutations of C1QC highlighted by the red box: c.100G>A p.(Gly34Arg) ; c.205C>T p.(Arg69X).

DISCUSSION

C1q deficiency is a rare genetic disorder which is often accompanied with development of SLE. The patient described here was already diagnosed with both C1q deficiency and SLE in early childhood. She has suffered from recurrent infections, which is also a hallmark for C1q deficiency[12]. Another part of her medical history involves bone lesions. Interestingly, osteoclasts are able to produce and secrete C1q, which could be suggestive for a direct relation between C1q deficiency and development of bone lesions, although the function of C1q is unknown in this environment[16]. However, it is unclear and difficult to determine whether these bone lesions and recurrent infections are a consequence of the C1q deficiency, the SLE, the steroid treatment, or a combination of these factors. C1q is important in the clearance of apoptotic material and immune complexes. Next to activation of the CP of the complement system, C1q has various functions independent of CP activation[3].

Previously, 15 C1q deficient patients with NPSLE have been described in literature, with the most frequent presenting symptom being seizures (67%). which is much higher than observed in conventional SLE patients[12]. Cerebral vasculitis has also been reported in 27% of the C1q deficient NPSLE patients. The patient described here has experienced neurological symptoms in adolescence and was diagnosed as a TIA / partial epileptic seizure due to cerebral vasculitis. It is known that C1q inhibits interferon production.

In patients with C1q deficiency high interferon levels are observed in serum and cerebrospinal fluid. Recently, it was found that type I interferon stimulates microglia to engulf synaptic material, resulting in synaptic loss in the central nervous system [17, 18]. This could contribute to neuropsychiatric involvement in C1q deficiency.

This patient has been treated with FFP for almost 14 years. Shortly after infusion C1q levels reach their maximum and rapidly decline, the CP activity was sustained for a longer period of time. Even though the C1q levels and CP activity effects were relatively short lived, the symptomatic relief and substantial improvement in quality of life of the FFP treatment was sustained for various weeks. Empirically it had been established, for this patient, that two units of FFP every two weeks was most optimal. The FFP therapy has been accompanied by adverse events upon infusion, to even anaphylactoid reactions, although anti-C1q antibodies were not increased in this patient.

From the C1q deficient patients that have been described so far, all except one have been reported to have a homozygous mutation in on the C1q genes. Here, we report the second case of C1q deficiency with a compound heterozygous mutation, in this case located in *C1QC*: c.100G>A p.(Gly34Arg) ; c.205C>T p.(Arg69X).

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CHAPTER 8

CARBAMYLATION REDUCES THE CAPACITY OF IGG FOR HEXAMERISATION AND COMPLEMENT ACTIVATION

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ABSTRACT

Introduction: Carbamylation is a post-translational modification that can be detected on a range of proteins, including immunoglobulin G (IgG), in several clinical conditions. Carbamylated IgG (ca-IgG was reported to lose its capacity to trigger complement activation, but the mechanism remains unclear. Since C1q binds with high affinity to hexameric IgG, we analysed whether carbamylation of IgG affects binding of C1q, hexamerisation and Complement Dependent Cytotoxicity (CDC). Synovial tissues of rheumatoid arthritis (RA) patients were analysed for presence of ca-IgG *in vivo*.

Materials and methods: Synovial tissues from RA patients were analysed for presence of ca-IgG using Mass Spectrometry (MS). Monomeric or hexameric antibodies were carbamylated *in vitro* and quality in solution was controlled. The capacity of ca-IgG to activate complement was analysed in ELISAs and cellular CDC assays.

Results and conclusions: Using MS we identified ca-IgG to be present in the joints of RA patients. Using *in vitro* carbamylated antibodies, we observed that ca-IgG lost its capacity to activate complement in both solid-phase- and CDC assays. Mixing ca-IgG with non-modified IgG did not result in effective inhibition of complement activation by ca-IgG. Carbamylation of both monomeric IgG and pre-formed hexameric IgG greatly impaired the capacity to trigger complement activation. Furthermore, upon carbamylation the pre-formed hexameric IgG, dissociated into monomeric IgG in solution, indicating that carbamylation influences both hexamerisation and C1q binding. In conclusion, ca-IgG can be detected *in vivo* and has a strongly reduced capacity to activate complement, which is in part, mediated through a reduced ability to form hexamers.

INTRODUCTION

Post-translational modifications (PTM) of proteins following biosynthesis are common in the human body, and are important in the regulation of activity, stability and folding of proteins [1, 2]. Dysregulation of PTMs has been linked to inflammatory and autoimmune conditions [1]. Besides dysregulation, PTMs can also cause the formation of neoepitopes on extracellular proteins during environmental exposure and aging which subsequently give rise to autoantibodies [3, 4]. The PTM carbamylation is the chemical conversion of a positively charged lysine into an uncharged homocitrulline. This conversion is mediated by cyanate, which is in equilibrium with urea and the availability of cyanate can be increased by inflammation through the release of myeloperoxidase (MPO) from neutrophils [5]. Carbamylation is therefore especially interesting in the context of inflammatory and autoimmune diseases. Carbamylated proteins are frequently targeted by autoantibodies in patients suffering from Rheumatoid Arthritis (RA) [6, 7]. Several proteins have reported to be carbamylated *in vivo* e.g. Albumin [8], Alpha 1 anti-trypsin [9], but interestingly also IgG [10, 11]. It has been reported that carbamylation of IgG impacts on its capacity to activate complement [10].

The complement system is a well described sequential autolytic cleavage system which can be activated via three different pathways: the classical pathway (CP), the lectin pathway and the alternative pathway. The CP is commonly activated upon binding of C1q to IgM or multiple copies of antigen-bound IgG [12]. One report described that upon carbamylation of IgG, there was a dose-dependent decrease in complement activation due to the loss of capacity to bind C1q, and hence loss of ability to initiate the CP, as measured in ELISA-based and tumour opsonisation assays [10]. Recently, it has been demonstrated that binding of C1q requires a hexameric arrangement of monomeric IgG complexes, which assemble via non-covalent Fc:Fc interactions [13, 14]. Moreover, cryo-electron microscopy analyses have shown that also the C1 complex binds to hexameric IgG1 complexes [15] and IgM [16]. This could indicate that the reduced capacity of carbamylated IgG (ca-IgG) to activate complement may directly or indirectly be caused by reduced hexamerisation. If this is the case, then carbamylation of IgG may be utilised to dampen inflammatory responses triggered by non-modified IgG. By the introduction of three mutations (E345R, E430G and S440Y) in the Fc domain, the ability of IgG1 to form hexamers was enhanced, both in solution and on the cell surface [13, 14]. This hexameric IgG is able to bind C1 in solution and activate the complement system. Here we studied the effects of IgG carbamylation on complement binding, activation and CDC in both normal monomeric IgG1 and hexameric conformations. In addition, we investigated the possibility of using ca-IgG to inhibit IgG mediated complement activation.

In this study we extend the observation that ca-IgG is present in vivo and unable to activate complement, by showing that the carbamylation impacts both on the C1q

binding to the IgG Fc, but also impairs hexamerisation of IgG which is essential for effective C1q binding and classical pathway activity.

MATERIALS & METHODS

Carbamylation

For carbamylation and subsequent experiments various (therapeutic) antibodies were used: intravenous immunoglobulin (IvIg) (Nanogram, Sanquin), alemtuzumab (Genzyme), rituximab (MabThera) and IgG1-DNP (RGY) (described below). These different antibodies were carbamylated by incubation in 0.1M potassium cyanate (KOCN) (cat#215074, Sigma-Aldrich) or incubated in phosphate buffered saline (PBS) as a control at 37°C for different periods of time. After incubation, the preparations were dialysed against PBS for 48 hours, or a buffer exchange was performed with PBS (pH 7.4).

Expression and purification of anti-DNP antibodies

The IgG1-DNP antibody consists of the variable domains of mouse mAb G2a2 against the hapten dinitrophenyl (DNP) combined with the constant domains of human IgG1 and the kappa light chain [17]. A triple mutant variant of the IgG1-DNP was produced containing three mutations (E345R, E430G and S440Y) in the Fc domain, which enhance the ability of the antibody to form hexamers both in solution and on the cell surface (designated IgG1-DNP-RGY), [13, 14]. Gene constructs for heavy and light chain were separately ordered (Thermo Fisher Scientific GeneArt, Regensburg, Germany) and cloned into a pcDNA3.3 vector (Thermo Fisher Scientific). Antibodies were expressed by transient transfection of Expi293F™ cells with equimolar amounts of heavy and light chain plasmid, using the ExpiFectamine™ 293 Transfection Kit (Thermo Fisher Scientific) according to the manufacturers guidelines. Secreted antibodies were harvested from the supernatant five days post-transfection, 0.2 µm filtered and purified on a column of protein A Sepharose (Centocor, Leiden, the Netherlands). Solution phase hexamerisation of IgG1-DNP-RGY was verified using High Pressure Size Exclusion Chromatography (HP-SEC) analysis as previously described [14].

Western Blot

10% Tris-glycine gels (Biorad Cat# 456-1033) were loaded with equal amounts of untreated IgG and ca-IgG under reducing conditions. Carbamylation was analysed using anti-carbamyl-lysine antibody (cat#STA-078, Cell Biolabs). Next, loading was visualized by stripping the western blot and re-probing for human IgG (DAKO cat# P0214).

Mass spectrometry carbamylation

Mass spectrometry (MS) was carried out as described previously [9, 18, 19]. Synovial fluid (SF) was centrifuged at 2000 rpm for five minutes, the supernatant collected and stored in aliquots at -80°C. Next, SF samples (500 µg protein) were depleted, according to the instructions of the supplier, for the top-12 most abundant serum proteins (Pierce/

Thermo). Subsequently, the depleted sample (50 µg) was subjected to filter-aided sample preparation (FASP II) [20] using ^{13}C-urea instead of regular ^{12}C-urea, in order to distinguish artificial *in vitro* ^{13}C carbamylation during the FASP procedure from genuine *in vivo* ^{12}C carbamylation events. After FASP II procedure no *in vitro* carbamylation events were observed.

The cartilage and synovium samples (after their extraction with hot sodium dodecyl sulfate (SDS) to remove adherent and easily soluble protein) were digested with trypsin using the following procedure: Samples were incubated in 100 µL Dithiothreitol (DTT) (100 mM) and NH_4HCO_3 (25 mM) for 20 min at 54°C. After five minutes centrifugation at max speed, the supernatant was saved and the pellet was incubated in 150 µL iodoacetamide (15 mM) and NH_4HCO_3 (25 mM) for 30 min at room temperature. After five minutes centrifugation at max speed, the supernatant was saved and the pellet was incubated in 200 µL 25 mM NH_4HCO_3 containing 10 µg trypsin for four hours at 37°C. The supernatants from the DTT and iodoacetamide incubation were combined and concentrated on a 30 kDa filter (Microcon, Millipore), washed three times with 100 µL 25 mM NH_4HCO_3 and incubated with 1 µg trypsin for four hours at 37°C. Finally, the supernatant containing digested protein from the pellet was added to the digest on the filter. The filter was washed once with 100 µL 0.5 M NaCl. Peptides were recovered from the filtrate and subjected to solid phase extraction on C18 cartridges (Oasis HLB Waters).

ELISA to detect carbamylation

Maxisorp plates (Nunc) were coated with 10 µg/mL IgG in coating buffer (0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH 9.6). Plates were blocked with PBS/1% BSA and carbamylated signal was detected using rabbit anti-carbamyl-lysine antibody (cat#STA-078, Cell Biolabs) and a goat anti-rabbit Horseradish Peroxidase (HRP) secondary antibody (DAKO cat# P0448). The substrate was added using 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS) and the carbamylation signal was measured at an absorbance level of 415 nm using Biorad iMark Microplate Absorbance Reader.

Complement activation ELISA

Maxisorp plates (Nunc) were randomly coated with 10 µg/mL (ca-)IgG in coating buffer (0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH 9.6) In mixing experiments the final concentration was 10 µg/mL with different ratios between the ca-IgG and non-modified IgG, unless indicated otherwise. Plates were blocked with PBS/1% BSA and incubated with 1% normal human serum (NHS, pooled from 4 healthy donors) or heat-inactivated NHS as a control (diluted in GVB++; 0.1% gelatin, 5mM Veronal, 145mM NaCl, 0.025% NaN_3, 0.15mM $CaCl_2$, 0.5mM $MgCl_2$, pH 7.3). Complement binding or deposition was analysed with rabbit anti-human C1q (DAKO cat# A0136), goat anti-human C4 (Quidel cat# A305), rabbit anti-human C3c (DAKO cat# A0062) and mouse anti-human C5b9 (DAKO cat# M0777) with corresponding HRP-labelled secondary antibodies in PBS/1% BSA/0.05%

Tween. Finally the substrate was added using ABTS and absorbance measured at 415 nm using Biorad iMark Microplate Absorbance Reader.

Complement dependent cytotoxicity

CDC assays with B-lymphoma cell lines (Daudi and Wien-133) were performed using 100,000 target cells opsonized with antibody concentration series in the presence of pooled NHS (20% final concentration) as a complement source (Sanquin, The Netherlands) as previously described [21]. Cells were incubated for 45 minutes at 37℃ and killing was calculated as the percentage of propidium idodide (PI, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) positive cells determined by flow cytometry. Curves were generated using non-linear regression (sigmoidal dose-response with variable slope) analyses within GraphPad Prism software (GraphPad Software, Sand Diego, CA, USA).

Antibody binding

Antibody binding assays with B-lymphoma cell lines (Daudi and Wien-133) were performed using 100,000 target cells opsonized with antibody concentration series and incubated for 30 minutes at 4℃. Next, cells were washed and incubated for 30 minutes at 4°C with R-Phycoerythrin (PE)-conjugated goat-anti-human IgG $F(ab')_2$ (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; cat #: 109-116-098). Cells were washed and analysed by determining the mean fluorescent intensity (MFI) using flow cytometry. Binding curves were generated using non-linear regression (sigmoidal dose-response with variable slope) analyses within GraphPad Prism software (GraphPad Software, Sand Diego, CA, USA).

Complement-mediated liposomal lysis assay

Liposomes were prepared using Dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), cholesterol and DNP-cap-PE, purchased from Avanti Polar Lipids (Alabama, USA). Lipid films were composed of DMPC:DMPG:cholesterol:DNP-cap-PE (45:5:49:1 mol%). Components were dissolved in chloroform:methanol (9:1 v/v) before drying under nitrogen gas and desiccation overnight. Films were rehydrated at 37°C for 30 minutes with a self-quenching concentration of sulforhodamine B (20 mM; S1402 from Sigma Aldrich, Missouri, USA) in PBS to a final lipid concentration of 0.8 mg/mL. The sulforhododamine B-liposome mixture was sonicated for five minutes at 37°C in a water bath. Purification of liposomes was performed through size-exclusion chromatography by using a prepacked NAP-25 column (17-0852-01 GE, Healthcare, Little Chalfont, UK).

To analyse complement activity via membrane attack pore membrane attack complex (MAC) mediated dye leakage, purified liposomes were diluted 10× in PBS and mixed with NHS (10% v/v final concentration) from Complement Technologies (Tyler, TX, USA). Sulforhodamine B fluorescence was measured with an excitation wavelength

of 565 nm and emission wavelength of 585 nm using a CLARIOstar microplate reader (BMG Labtech, Offenburg, Germany). Fluorescence was measured at 21°C for 100 s before different antibodies (IgG1-DNP and IgG1-DNP-RGY, both non-modified and carbamylated) were added to final concentrations of 4.35 µg/mL.ml before assaying for a further 10 minutes. Total lysis was performed by adding 70% ethanol after assay. Experiments were performed in triplicate.

Statistics

Statistical analysis was performed using GraphPad Prism version 7.02. Statistical differences were determined using T-tests, a p-value of <0.05 was considered statistically significant. Data is either representative for multiple experiments or the mean with standard deviation (SD) is shown.

RESULTS

Identification of *in vivo* occurring ca-Ig(G) using mass spectrometry

Previously, ca-IgG was detected in synovial fluid from two RA patients [10]. Therefore, we investigated various tissue samples from RA patients. In both synovium and synovial fluid from either the knee or hip, carbamylated immunoglobulin peptides were found using MS (**Table 1**). Interestingly, the same carbamylated immunoglobulin peptides were found in independent donors, for example VGVETTkpSkQSNNkYAASSYLSLTPEQWK (k = carbamylation on Lysine p = oxidation on Proline) found in two out of 14 donors (14%). Analysis of the relative abundance of the carbamylated immunoglobulin peptides revealed that, at a reasonable coverage of the immunoglobulin proteins (on average 63%,ranging from 25% to 100%), we detected the carbamylated peptides roughly at a 1000 fold lower abundance as compared to the abundance of the protein from which the peptide is derived. Moreover, in various OA tissue samples (2x synovial fluid, 2x synovium) no carbamylated Ig peptides were found. These data indicate that carbamylation of immunoglobulins, including IgG, is occurring *in vivo* as exemplified using samples from RA patients.

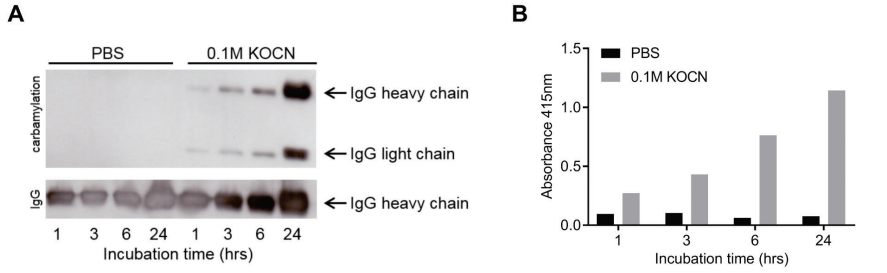
Table 1. Identification of *in vivo* occurring ca-IgG using Mass spectrometry

Diagnosis	*Sex*	*Age*	*Location*	*Tissue*	*Protein Group Accessions*	*Sequence*	*Protein Descriptions*
RA	Female	76	Knee	Synovium	A0A087X130;A0A075B6H6	H**k**VYAcEVTHQGLSSPVTK	Ig kappa chain C region
					P01857;A0A087WYE1;A0A087WYC5	KVEP**k**ScD**k**THTcppcp APELLGGPSVFLFPPKPK	Ig gamma-1 chain C region
RA	Male	56	Hip	Synovium	A0A087X130;A0A087WZW8; A0A075B6H6	H**k**VYAcEVTHQGLSSPVTK	Ig kappa chain C region
					P01876	TFTcTAAYPES**k**TPLTATLSK	Ig alpha-1 chain C region
					A0A087X130;A0A087WZW8; A0A075B6H6	VYAcEVTHQGLSSPVT**k**SFNRGEc	Ig kappa chain C region
					A0A087X130;A0A087WZW8; A0A075B6H6	ADYE**k**HKVYAcEVTHQGLSSPVTK	Ig kappa chain C region
					A0A087WYE1;A0A087WYC5	**k**VEP**k**ScDKTHTcPpcp ApELLGGPSVFLFPPKPK	Ig gamma-1 chain C region
					P01861	VD**k**RVES**k**YGppcpScp ApEFLGGPSVFLFPPKPK	Ig gamma-4 chain C region
RA	Male	49	Knee	Synovial Fluid	A0M8Q6	VGVETT**k**pS**k**QSNN**k**YAASSYLSLTPEQWK	Ig lambda-7 chain C region
RA	Male	67	Knee	Synovial Fluid	A0M8Q6	VGVETT**k**pS**k**QSNN**k**YAASSYLSLTPEQWK	Ig lambda-7 chain C region

Carbamylated immunoglobulins detected *in vivo* in samples from Rheumatoid Arthritis (RA) patients, as analysed by mass spectrometry, the carbamylated lysines are annotated (**k**) and the carbamylated lysines that are identical to those observed in the *in vitro* preparation are underlined (**k**) . k = Carbamyl, c = Carbamidomethyl, p = Oxidation.

Successful carbamylation of IgG preparations

To study the biology of carbamylation of antibodies, different IgG antibodies (alemtuzumab, rituximab, IgG1-DNP, IgG1-DNP-RGY) and IVIg were carbamylated by 0.1 M KOCN (cyanate) at 37°C for different time points (1, 3, 6 and 24 hours). Carbamylation of all antibody preparations were successful as detected by western blot and an ELISA based assay. Both the western blot and the ELISA show a time-dependent increase in carbamylation for all antibodies, as exemplified by rituximab (**Figure 1 A, B**). Moreover, the 24 hour carbamylated rituximab sample was analysed with MS to identify the carbamylated lysines (**Figure 1C)**. MS analysis showed extensive presence of carbamylation, several of the carbamylated lysines were identical to the carbamylated lysines found in the *in vivo* samples.



C

Rituximab heavy chain coverage 79%

QVQLQQPGAE LVKPGASVKM SC**k**ASGYTFT SYNMHWV**k**QT PGRGLEWIGA IYPGNGDTSY NQ**k**FK**G**K**k**ATL TAD**k**SSSTAY MQLSSLTSED SAVYYCARST
YYGGDWYFNV WGAGTTVTVS AAST**k**GPSVF PLAPSS**k**STS GGTAALGCLV K**k**DYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTQ
TYICNVNHKP SNTKVDKKAE P**k**SCD**k**THTC PPCPAPELLG GPSVFLFPP**k** PKDTLMISRT PEVTCVVVDV SHEDPEV**k**FN WYVDGVEVHN AKT**k**PREEQY
NSTYRVVSVL TVLHQDWLNG **k**EY**k**C**k**VSNK ALPAPIE**k**TI S**k**A**k**GQPREP QVYTLPPSRD ELT**k**NQVSLT CLVKGFYPSD IAVEWESNGQ PENNY**k**TTPP
VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K

Rituximab light chain coverage 100%

QIVLSQSPAI LSASPGE**k**VT MTCRASSSVS YIHWFQQ**k**PG SSP**k**PWIYAT SNLASGVPVR FSGSGSGTSY SLTISRVEAE DAATYYCQQW TSNPPTFGGG
T**k**LEIKRTVA APSVFIFPPS DEQL**k**SGTAS VVCLLNNFYP REA**k**VQW**k**VD NALQSGNSQE SVTEQDS**k**DS TYSLSSTLTL S**k**ADYE**k**H**k**V YACEVTHQGL
SSPVT**k**SFNR GEC

k indicates carbamylated lysines observed in *in vitro* modified Rituximab
k indicates the carbamylated lysine positions as observed in gecarbamyleerd IgGs *in vivo*
The yellow colour highlights the coverage

Figure 1. Successful carbamylation of rituximab

The results from rituximab are shown and are representative for all carbamylated antibodies (IVIg, alemtuzumab, DNP variants). Antibodies were either incubated in PBS as a control (buffer only) or in 0.1M potassium cyanate (KOCN). (**A**) Western blot analysis of 2 µg (ca)rituximab, carbamylation was visualised using a carbamyl-lysine specific antibody. Equal loading of the preparations was shown by detection of human IgG. Incubation time is indicated in hours. (**B**) Carbamylation of rituximab as analysed by ELISA, where an equal amount of antibody (10 µg/mL) was used to coat the well. Detection was performed using a carbamyl-lysine specific antibody. (**C**) Mass spectrometry analysis of rituximab that was carbamylated for 24 hours. The carbamylated lysines are annotated (**k**), coverage is indicated with yellow highlight.

Carbamylation of IgG completely blocks the complement activating potential of IgG
Complement activation assays were performed using 10 µg/mL (ca-)IgG coated ELISA plates. Binding of C1q and deposition of C4, C3c and C5b9 was significantly decreased upon carbamylation of IVIg, rituximab and alemtuzumab, when compared to the non-modified counterpart (**Figure 2**). We did analyse whether carbamylation affected the antibody's immobilization in the ELISA plate and although we did observed some differences, these are too small to explain the observed biological effects (**Supplementary Figure 1**). Previously, it has been demonstrated that optimal complement activation via IgG is achieved when the IgGs are in a hexameric arrangement. Therefore, we wondered whether adding ca-IgG to IgG would inhibit this process by limiting the possibilities to form productive hexamers. Mixing experiments were performed to analyse whether the presence of ca-IgG indeed affects the ability of non-modified IgG to activate the complement system. Mixing ca-IgG antibodies with non-modified antibodies, while maintaining a similar end concentration, resulted in a decrease in complement activation (at the levels of C1q, C4, C3c and C5b9) (**Figure 3**), which was proportional to the decreasing concentration of coated non-modified IgG. Although ca-IgG was unable to activate complement by itself, we did not observe an inhibitory effect of ca-IgG on the complement activation induced by non-modified IgG other than the simple dose response effect of diluting the non-modified IgG. As the coated antibodies in ELISA plates are known to form a high density surface of IgGs that bind C1q without a need to form hexamers [14], the data indicate that the carbamylation on Lysine residues in the Fc impact directly on the binding of C1q.

CDC is not affected by the presence of ca-IgG
Because of the observed differences in complement binding and activation by plate-immobilized IgGs, we next explored the effect of carbamylation on antibody binding and complement dependent cytotoxicity (CDC) using tumour cell lines. IgG hexamerisation is required for optimal C1q binding and complement activation in this setting. Binding of non-modified or carbamylated rituximab and alemtuzumab was analysed using Daudi and Wien-133 cell lines, respectively. The carbamylated variants of rituximab and alemtuzumab displayed a slightly lower binding compared to their non-modified counterparts, which actually might reflect lower binding efficiency of the anti-IgG detection conjugate antibody to the ca-IgG as compared to the non-modified IgG (**Supplementary Figure 2**). Next, we investigated whether carbamylated IgG affects the ability of non-modified IgG to induce CDC. CDC assays were performed with different ratios of non-modified or carbamylated rituximab and alemtuzumab (**Figure 4**). No CDC was observed in the presence of carbamylated rituximab or alemtuzumab only, which cannot be solely attributed to the reduced binding of ca-IgG to the surface. Non-modified rituximab and alemtuzumab induced substantial CDC, while titrating in their carbamylated counterparts resulted in a decrease in killing capacity. For rituximab the decrease in killing capacity was gradual with the titrating of the carbamylated variant. Whereas for alemtuzumab there is a sharp decrease in CDC after the carbamylated

variant exceeded the 50% ratio. These results indicate that at the chosen antibody concentrations and ratios there is no dominant negative effect of ca-IgG presence on the capacity of non-modified IgG to induce CDC.

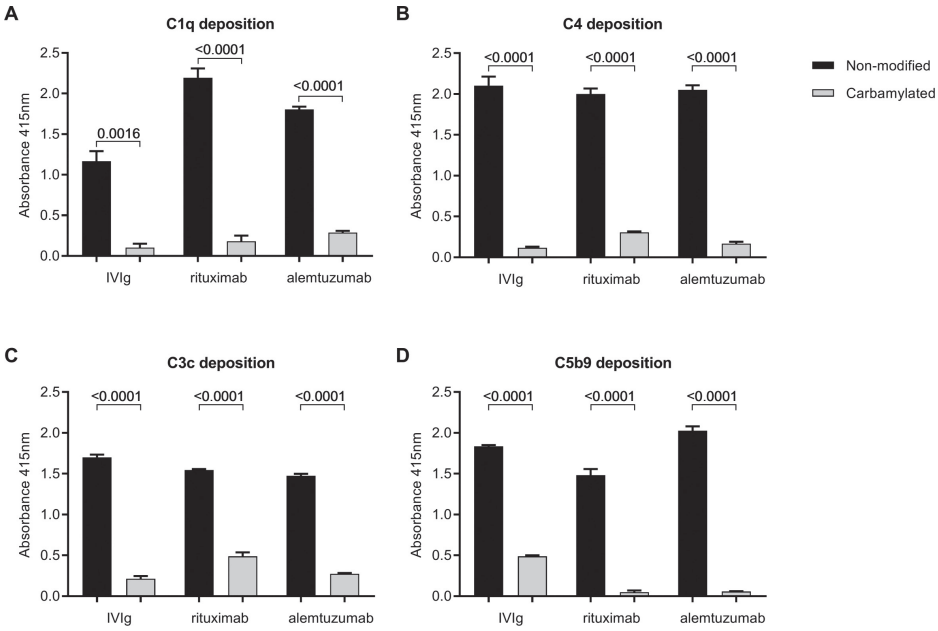


Figure 2. Complement deposition of C1q, C4, C3c and C5b9 on non-modified and carbamylated IgG

Complement deposition ELISAs were performed using 10 µg/mL plate bound non-modified or carbamylated IVIg, rituximab and alemtuzumab. C1q (**A**), C4 (**B**), C3c (**C**) and C5b9 (**D**) deposition after incubation for one hour with 1% normal human serum (NHS) was measured at an absorbance wavelength of 415 nm. Data are shown as mean with standard deviation from technical triplicate. All differences in complement deposition between non-modified antibodies and their carbamylated counterparts were significant, as analysed with T-test (all $p < 0.05$). Shown is a representative experiment performed at least twice.

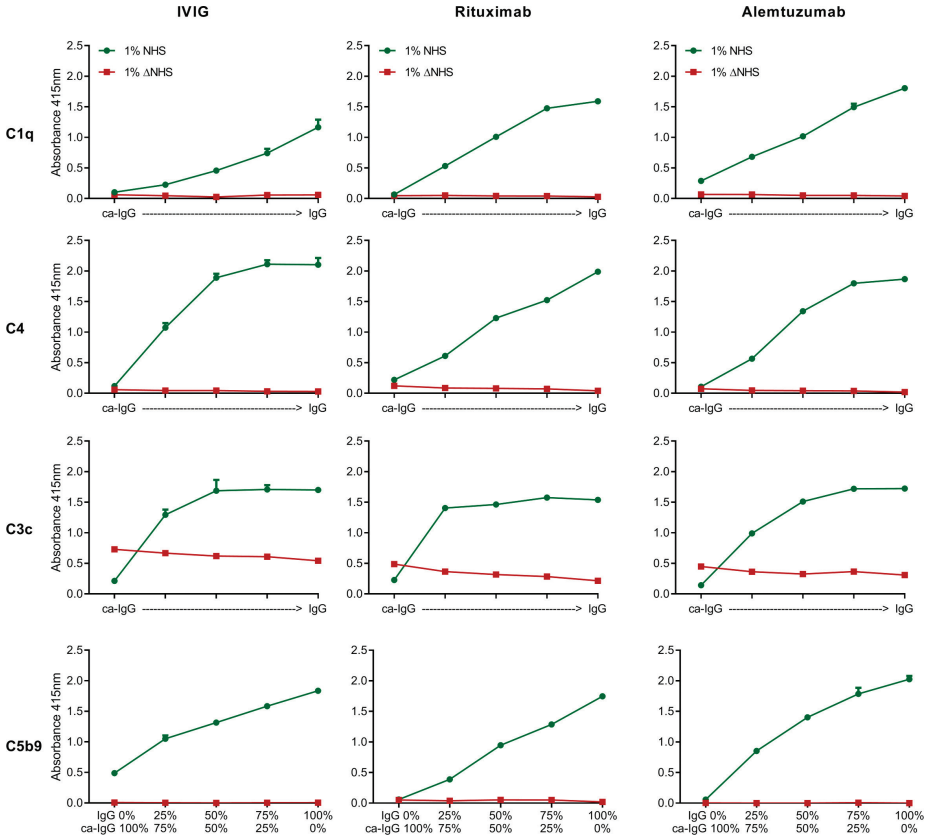


Figure 3. Deposition of different complement components is not affected by the presence of ca-IgG

Complement deposition ELISAs were performed using plate bound non-modified or carbamylated IVIg, rituximab and alemtuzumab, with a sum of 10 µg/mL coating mixed in different ratios, and for rituximab a sum of 2.5 µg/ml (C4) or 5 µg/ml (C1q, C3c, C5b9). From left to right ca-IgG vs non-modified IgG: 100%:0%, 75%:25%, 50%:50%, 25%:75%, 0%:100%. Deposition of complement components C1q, C4, C3c and C5b9 were measured at an absorbance wavelength of 415 nm. Green circles depict the complement deposition after incubation with 1% normal human serum (NHS), red squares depict complement deposition after incubation with 1% heat-inactivated NHS (ΔNHS) for one hour. Shown is a representative experiment which has been performed independently at least twice.

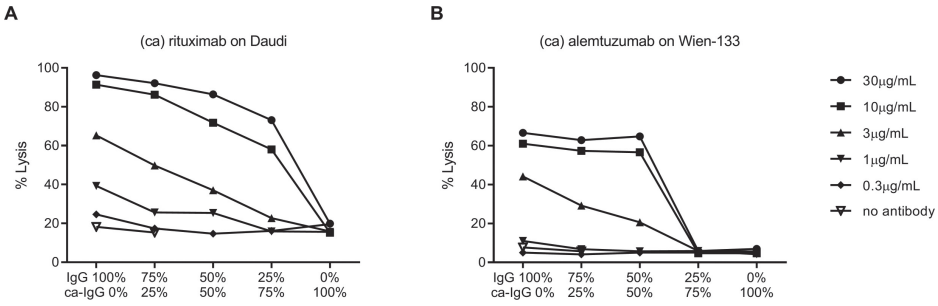


Figure 4. Complement-dependent cytotoxicity is not affected by the presence of ca-IgG
Complement dependent cytotoxicity (CDC) assays were performed with Daudi (**A**) and Wien-133 (**B**) cells opsonized with various concentrations of non-modified and/or carbamylated rituximab or alemtuzumab respectively in the presence of 20% pooled NHS. CDC assays were performed using different ratios of mixed non-modified (IgG) and carbamylated (ca-IgG) antibody variants. Ratios of ca-IgG versus non-modified IgG from left to right are: 100%:0%, 75%:25%, 50%:50%, 25%:75%, 0%:100%, each depicted for in total five different final antibody concentrations. Shown is a representative experiment which has been performed independently twice.

Monomeric and hexameric IgG

CDC activity is highly dependent of IgG1 hexamer formation, and in the CDC assay it was unclear whether ca-IgGs form hexamers in the presence of non-modified IgGs. Therefore, we explored whether the effect of reduced complement activation by ca-IgG is also present when using pre-formed ca-IgG-hexamers. DNP antibodies were produced containing three point mutations in the Fc domain (DNP-RGY), which causes the IgGs to form stable hexamers in solution [14] (**Figure 5A**). Next, these preparations were also carbamylated and the ability of IgG-RGY mutants to hexamerise in solution was analysed using size exclusion chromatography (HP-SEC). Control IgG1-DNP-RGY that primarily existed as a hexamer: an early eluting peak was observed for hexameric IgG (58.6%) and a second peak for monomeric IgG (39.4%). In solution both species are in equilibrium with a relatively high fraction forming hexameric complexes. Carbamylated IgG clearly only showed a monomeric IgG peak (98%) (**Figure 5B**) indicating that hexamerisation was abrogated. First we tested the effect of carbamylation on monomeric IgG or on hexameric IgG regarding complement activation in a liposome lysis assay, a setting in which the bound antibodies can move freely and form hexamers on the surface. In a liposome lysis assay, complement activation is measured by sulforhodamine B release from liposomes as a consequence of MAC formation [22]. In non-modified conditions, both anti-DNP antibody preparations are able to lyse liposomes (the lysis caused by DNP-RGY is higher since pre-formed hexamers enhance complement activation) [14]. Carbamylation of both these antibodies leads to a complete loss of liposome lysis (**Figure 5C**, **D**).

Next, the RGY variant of alemtuzumab was generated and carbamylated (or treated with PBS as control), and CDC assays performed. Again, carbamylation of RGY resulted in loss of CDC capacity (**Figure 5E**). Collectively, the data on the size exclusion chromatography, the liposome lysis assay and the CDC assays indicate that carbamylation removes the capacity of IgG-RGY mutants to spontaneously from hexamers in solution.

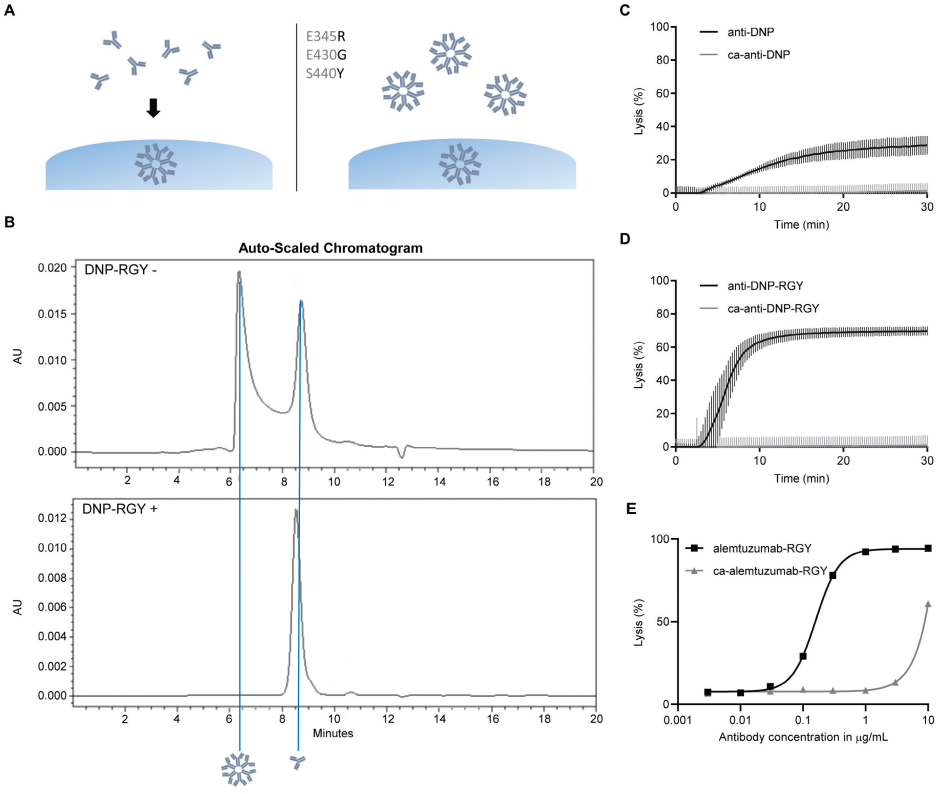


Figure 5. Monomeric and hexameric IgG both lose liposomal lysis capacity upon carbamylation
(A) Schematic representation of monomeric IgG1-DNP which forms hexamers on the cell surface (left) and IgG1-DNP-RGY which forms hexamers in both solution and on the cell surface (right). (**B**) The effect of carbamylation of IgG1-DNP-RGY on hexamerisation in solution was analysed with HP-SEC (High Pressure Size Exclusion Chromatography). (**C**, **D**) Liposomes expressing DNP were lysed in the presence of NHS and 4.35 µg/ml (carbamylated (ca))anti-DNP(RGY) antibodies. Fluorescence of sulforhodamine B is normalised to total-lysis by addition of ethanol, which is set at 100%. Data shown are the mean with SD from technical triplicate and results are representative of three independent experiments. (**E**) Alemtuzumab-RGY was carbamylated and complement dependent cytotoxicity (CDC) assays on Wien-133 were performed. Cells were opsonized with various concentrations of non-modified and/or (ca) alemtuzumab-RGY in the presence of 20% pooled NHS.

DISCUSSION

Several carbamylated proteins are known to be present *in vivo;* carbamylated albumin was identified in patients suffering from renal failure [23, 24], and carbamylated fibrinogen and A1AT were discovered in samples from RA patients. In addition, ca-IgG was present in synovial tissue of RA patients [10]. It has also been shown that carbamylation of IgG1 occurs *in vivo,* resulting in loss of C1q binding [10, 11] and in a subsequently reduced complement activation. These observations may have interesting implications. In RA anti-CarP antibodies are present that target carbamylated proteins [3, 6], possibly including ca-IgG, which in such a scenario would have rheumatoid factor-like properties. Furthermore, ca-IgG will behave differently in the inflamed joint of the patient. For several therapeutic antibodies, such as the CD20-specific antibody rituximab, the mechanism of action involves CDC. When therapeutic antibodies become compromised by carbamylation *in vivo,* their functionality may be affected, as well as their half-life. Therefore, we were interested to reproduce the previously known inhibitory effects of ca-IgG on the complement system and CDC, and specifically analyse ca-IgG in the presence of non-modified IgG and variants of IgG that form hexamers in solution.

We confirmed that carbamylation of IgG results in a decrease in complement activation, both in ELISA and in CDC assays. For plate bound assays we observed that the effect on the level of C3c deposition of ca-IgG was less pronounced, which could be related to some alternative pathway activation of the plastic wells at high serum concentrations. The binding of rituximab and alemtuzumab to cell surfaces appeared reduced upon carbamylation, however this could also be the result of reduced detection efficiency by the anti-IgG detection antibodies used, as the detection of plate bound IgG, a condition not depending on the antigen binding by the IgG, was similarly impaired. Nevertheless, when the carbamylated variants were mixed with the non-modified IgG there was no dominant inhibitory effect on complement activation. However, for alemtuzumab there is a sharp decrease in CDC once the ca-IgG exceeds the 50% ratio. This is in agreement with other published data where a less potent CDC antibody had negative effect when exceeding the 1:1 ratio [25]. These data on alemtuzumab suggest that the ability of non-modified IgG to form hexamers and consequently bind C1q was not affected by the presence of ca-IgG at ratios below 50%. To investigate whether carbamylation interferes with antibody hexamer formation (Fc-Fc interactions), we used DNP antibodies and the DNP-RGY mutant, the latter of which forms spontaneous hexamers in solution, and analysed the ability of these antibodies to lyse DNP-coated liposomes. Upon carbamylation, the IgG1-DNP-RGY was no longer able to hexamerise in solution, indicating that carbamylation negatively affects Fc:Fc interaction resulting in the loss of C1q binding by IgG.

Using MS we identified several peptides of human immunoglobulins to be carbamylated *in vivo* in the synovial fluid of RA patients. These peptides were not found in all samples analysed, which may suggest an accumulation in a specific disease condition, but may also be the result of limited sensitivity to observed these modified peptides. Although the number of peptides containing homocitrulline was limited, we did observe similar peptides in several patients. Whether or not also therapeutic antibodies may become carbamylated *in vivo* in e.g. the inflamed joint or the tumour micro-environment is currently unknown. We still expect the majority of IgG to be unmodified even in an inflammatory environment. Based on our titration experiments, used to mimic the biological scenario, it is unlikely that the *in vivo* carbamylation of IgG would have a major impact on the complement activating potential of these therapeutic antibodies. It is interesting to note that by carbamylating a therapeutic IgG preparation it is possible to completely avoid any risk of complement activation by these antibodies. This is especially relevant for antibodies that should bind to cell surfaces where they should modify receptor ligand interactions without killing or activating the cell, such as e.g. with checkpoint blockade in anti-tumour therapy.

In conclusion, the inability of ca-IgG to activate the complement system is the combined effect of both decreased binding of C1q to the modified Fc of IgG and the reduced capacity of ca-IgG to form hexamers.

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FUNDING

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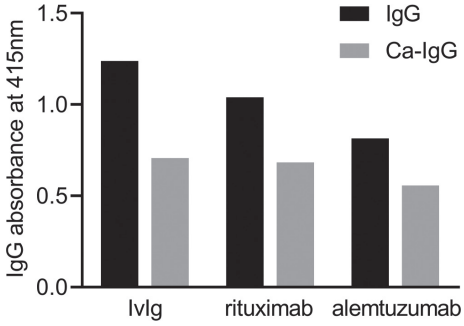
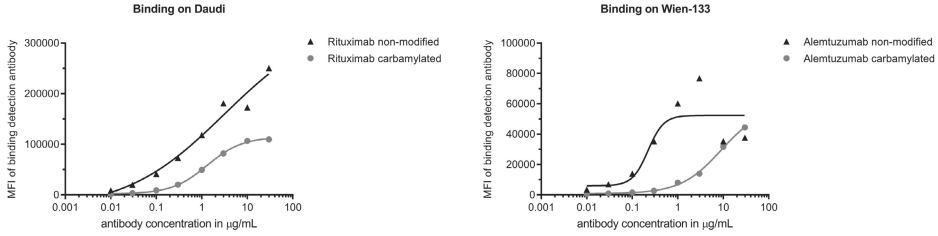
CONFLICT OF INTEREST STATEMENT

P.A.v.V and L.A.T. are listed as inventors on a patent application on the detection of anti-CarP antibodies in RA.

SUPPLEMENTARY DATA



Supplementary Figure 1. Coating of (ca-)IgG to the plate
Antibody binding to the plate of carbamylated Igs (ca-IgG) and their non-modified counterpart (IgG) was performed by directly detecting the coated antibodies with anti-human IgG-HRP labelled antibody.



Supplementary Figure 2. Cellular binding (ca-)IgG
Antibody binding was measured using flow cytometry for non-modified or carbamylated rituximab and alemtuzumab, on Daudi and Wien-133 cells respectively. Data shown are representative for two experiments.

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CHAPTER 9

SUMMARY AND GENERAL DISCUSSION

SUMMARY

The complement system is an integral part of the immune system and tissue homeostasis. The complement system has long been viewed as a straightforward proteolytic cascade to combat invading pathogens, however, the complement system has been shown to be far more complex and our knowledge and understanding of the complement system is still expanding. In this thesis we report on local complement production and possible significance of complement proteins outside of the traditional complement system activity. We focus in this thesis on C1q in autoimmunity and tuberculosis (TB), in addition, we studied the relatively new concept of intracellular C3.

Complement component C1q is locally produced by isolated articular chondrocytes
Inflammation and innate immune responses may contribute to development and progression of Osteoarthritis (OA). Chondrocytes are the sole cell type in articular cartilage and produce extracellular-matrix molecules. How inflammatory mediators reach chondrocytes is incompletely understood. Previous studies have shown that chondrocytes express mRNA encoding complement proteins such as C1q, suggesting local protein production, which has not been demonstrated conclusively. **Chapter 2** describes the analyses of the chondrocytes from osteoarthritic cartilage, which were isolated from patients that underwent total knee replacement. The chondrocytes isolated were able to produce and secrete C1q *ex vivo*, as well as able to bind C1q *ex vivo*. Since chondrocytes were able to secrete and bind C1q we explored whether incubation *in vitro* with C1q could affect RNA expression of various complement and collagen genes. In a pilot experiment we demonstrated that incubation with 100 µg/ml C1q for 24 hours, negatively affected the expression of *MMP13* and the *COLL2*:*COLL10* ratio. We concluded therefore, that C1q protein can be expressed and secreted by human articular chondrocytes and that C1q is able to bind to chondrocytes influencing the relative collagen expression.

Complement C3 cleavage by Cathepsin-L leads to formation of C3a-desArg and is not essential for survival in a HAP-1 model cell line
A novel intracellular role has been described for complement protein C3 expressed in CD4+ T-cells, by indicating that intracellular C3 can be cleaved by Cathepsin-L (CTSL), resulting in signalling by C3a via the intracellular C3aR. This intracellular C3 pathway was hypothesized to be pivotal for the survival of CD4+ T-cells. The studies described in **Chapter 3** explored the presence of protein C3 in cell lysates, the CTSL cleavage site in C3 and survival of C3 deficient cells. We were unable to detect C3 in cell lysates without incubation with NHS prior to lysis. The analysis of C3 cleavage by CTSL *in vitro* by mass spectrometry indicated that the C3 cleavage by CTSL results in the formation of C3ades-Arg, since the arginine was still attached to the N-terminus of the C3b molecule. This is an interesting finding, since prior research indicated that intracellular

C3 was downstream modulated by the C3aR. However, C3ades-Arg is unable to bind the C3aR, indicating that the hypothesized intracellular pathway could not occur. In addition, we generated a C3 deficient HAP-1 cell line by the use of CRISPR/Cas9, and no differences were observed regarding proliferation, morphology and metabolism between the wildtype and C3 deficient HAP-1 cell lines. In conclusion, we demonstrate that processing of C3 by CTSL likely generates C3a-desArg and not C3a, and further that C3 or its derived products are not essential for cell survival in a HAP-1 cell line model.

Complement component C1q as serum biomarker to detect active tuberculosis

Tuberculosis (TB) is a major global health threat, which is caused by infection by Mycobacterium tuberculosis (*Mtb*). Diagnosis of active TB is hampered by the lack of specific biomarkers that discriminate active TB disease from other (lung) diseases or latent TB infection (LTBI). Early diagnosis and treatment of TB disease is important to reduce transmission of infection and prevent disease associated mortality. Recently, complement has been highlighted as candidate biomarker for active TB disease. Based on integrated human gene expression results from literature, genes encoding for the different C1q chains and *SERPING1* (encoding for the C1-inhibitor (C1-INH)) were increased in TB disease. Therefore, we analysed the protein levels for C1q (**Chapter 4**) and C1-INH (**Chapter 5**). In the studies described in **Chapter 4** we measured C1q protein levels in four different geographical TB cohorts from Italy, The Gambia, Korea and South Africa. C1q levels were increased in patients with active TB compared to all relevant control populations. Importantly, longitudinal follow up of TB patients during treatment revealed that serum C1q levels normalized to those of endemic controls, indicating that the upregulation of C1q was associated with disease and not intrinsic to the individuals. To further evaluate the potential of C1q as a biomarker for active TB, C1q levels of TB disease patients were compared to those of patients with conditions for which differential diagnoses are difficult to make in clinical practice. Patients with TB had significantly higher circulating C1q levels as compared to patients with sarcoidosis or patients with pneumonia. Likewise, higher C1q levels were found in patients with TB compared to patients with leprosy, that were analysed as additional control. Thus, the upregulation of C1q likely is does not reflect a general response to inflammation. Moreover, C1q protein was locally detected at an increased level in the lungs of TB patients (n=3) compared to controls. In analogy with human TB, C1q also validated as a biomarker of TB disease in rhesus macaques, in both serum and bronchoalveolar lavage (BAL). Increased C1q levels were only observed in animals that progressed to active disease and not in those that controlled the infection. In summary, C1q levels were elevated in patients with active TB compared to LTBI in four independent cohorts. Therefore, we propose that the addition of C1q measurements to current biomarker panels may provide added value in the diagnosis of active TB.

Analysis of the endogenous complement regulator C1-inhibitor, the product of *SERPING1*, in active tuberculosis

We already reported in **Chapter 4** that increased levels of C1q can be used a host serum biomarker that can discriminate active TB disease from LTBI and non-TB pulmonary infections or sarcoidosis. In serum, C1q forms a complex with C1r and C1s to generate the full C1 molecule which can activate the classical pathway of the complement system. Importantly, the C1 complex is regulated by a natural inhibitor, namely C1-inhibitor (C1-INH). Transcriptional biomarker signatures have indicated that expression of *SERPING1*, which encodes the inhibitor C1-INH, discriminates between active and latent TB disease. The studies described in **Chapter 5** aimed to determine serum levels of *SERPING1* encoded C1-INH in four geographically diverse cohorts of patients with active TB, LTBI, disease controls and patients that have been successfully treated for TB. The aim of these studies was to investigate a possible application as host biomarker for active TB. C1-INH serum levels were significantly increased in TB patients compared to endemic controls in two out of four cohorts analysed. In addition, in the Gambian cohort with TB patients followed over time the increased C1-INH decreased rapidly upon initiation of TB treatment. It is interesting that SERPING1 expression is consistently increased in patients with TB whereas its protein product C1-INH is found to be increased in only half of the cohorts analysed in this study. It is unclear why C1-INH upregulation was observed in only two out of four cohorts. Overall, upon comparing C1q and C1-INH protein level in the different populations analysed, C1q was uniformly increased in TB cohorts whereas C1-INH in two out of four TB cohorts. The observation that increased C1-INH serum levels were only detected in cohorts of patients with active TB, but not in patients with similar clinical conditions and the notion that C1-INH decreased rapidly during TB treatment, suggests that *Mtb* is actively regulating these complement proteins. An increase in C1q and C1-INH could therefore represent an immune-escape mechanism of *Mtb* enabling immunosuppressive actions of C1q, without enhancing the classical pathway activity.

Systemic and pulmonary C1q as biomarker of progressive disease in experimental non-human primate tuberculosis

Non-human primate (NHP) *Mtb* infection models are widely used to study pathogen-host interactions and pre-clinical evaluation of vaccine candidates. NHP and macaque species (*Macaca spp*) in particular, are considered highly relevant models for TB, due to their close phylogenetic relationship to man, outbred nature and large similarity in TB pathogenesis. Macaques are applied across the whole spectrum of TB research, both in preclinical evaluation of TB vaccines and therapeutics as well as basic research on TB disease development. Modelling in these species has the advantage of having controlled and accurate time-response-conditions relative to infection. Depending on macaque (sub)species, *Mtb* strain and challenge dose, TB disease manifestation in macaques mimics the diversity seen in humans. After the demonstration presented in **Chapter 4** that C1q was increased after high dose TB challenge in NHP, we exploited the diversity

in TB disease manifestation in NHPs to examine the dynamics of C1q as a biomarker of TB disease in more detail. The results of these studies are presented **Chapter 6**. We assessed systemic and pulmonary C1q levels after experimental infection using a high or low single dose as well as repeated limiting doses *Mtb* challenge of macaques. We show that increasing C1q levels, either peripherally or locally, correlate with increased TB pathology and with decreased survival following challenge with high or low dose *Mtb* in different macaque species. Upregulation of C1q did not precede detection of *Mtb* infection by a conventional interferon-gamma release assay, confirming its association with disease progression but not TB infection per se. Lastly, we show that pulmonary vaccination with Bacillus Calmette Guérin (BCG), also results in a temporal increase in pulmonary C1q. However, whether this local C1q production plays a role in protection against *Mtb* infection and disease remains to be investigated. Our observations confirm and further support C1q as a marker of progressive TB disease. As C1q can be readily measured over the course of *Mtb* infection, it could therefore be applied to monitor TB disease progression in a resource-limited setting.

Complex medical history of a lupus patient with a compound heterozygous mutation in *C1QC*

Genetic deficiencies, caused by homozygous mutations in one of the C1q genes are rare and are strongly associated with development of Systemic Lupus Erythematosus (SLE). C1q deficiency is a rare condition with just over 70 documented cases from at least 45 different families. These deficiencies are all the result of homozygous mutations in one of the three C1q genes, except in one case with a compound heterozygous mutation in *C1QA*. Patients with C1q deficiency have various clinical presentations and outcome. Most common is the diagnosis of Systemic Lupus Erythematosus (SLE) in early childhood and recurrent infections. In **Chapter 7** we describe a C1q deficient patient with a compound heterozygous mutation in the *C1QC* gene: c.100G>A p.(Gly34Arg); c.205C>T p.(Arg69X). The medical history of this patient was complex and involved various infections, SLE, cerebral involvement, vascular problems and bone lesions. This patient was treated with fresh frozen plasma (FFP) for over a decade. Even though the C1q levels and CP activity effects were relatively short lived, the symptomatic relief and substantial improvement in quality of life following FFP treatment was sustained for various weeks. Over time there were several adverse reactions to the FFP therapy as well. However, despite these adverse reactions, the patient preferred the FFP therapy, because of reduction of fatigue, arthralgia and number of infections. Nonetheless, because of a serious anaphylactic reaction, FFP treatment was eventually discontinued.

Carbamylation reduces capacity of IgG for hexamerisation and complement activation

Post-translational modifications (PTM) of proteins following biosynthesis are common in the human body, and are important in the regulation of activity, stability and folding of proteins. Dysregulation of PTMs has been linked to inflammatory and autoimmune

conditions. Carbamylation is the chemical conversion of a positively charged lysine into an uncharged homocitrulline. Carbamylated proteins are frequently targeted by autoantibodies in patients suffering from Rheumatoid Arthritis (RA). Several proteins have reported to be carbamylated *in vivo*, interestingly, it was reported that carbamylation of IgG impacts on its capacity to activate complement. Moreover, cryo-electron microscopy analyses have demonstrated that binding of C1q requires a hexameric arrangement of monomeric IgG complexes, which assemble via non-covalent Fc:Fc interactions. The studies described in **Chapter 8** were performed to better comprehend a possible role of a prominent post-translational modification associated rheumatic disease that is formed after carbamylation. More specifically, we studied the interaction between carbamylated IgG in relation to the ability to activate the complement system. We identified several peptides of human immunoglobulins (including IgG) to be carbamylated *in vivo* in the synovial fluid of RA patients. We confirmed that carbamylation of IgG results in a decrease in its ability to mediate complement activation, both in ELISA and in complement dependent cytotoxicity (CDC) assays. Nevertheless, when the carbamylated variants were mixed with the non-modified IgG, there was no dominant inhibitory effect on complement activation. CDC activity is highly dependent of IgG1 hexamer formation, and in the CDC assay it was unclear whether ca-IgGs form hexamers in the presence of non-modified IgGs. Therefore, we explored whether the effect of reduced complement activation by ca-IgG is also present when using pre-formed ca-IgG-hexamers. Next, we analysed the effect of carbmaylation on hexamerisation and complement activation by making use of a triple mutant variant of the IgG1-DNP which enhance the ability of the antibody to form hexamers both in solution and on the cell surface (designated IgG1-DNP-RGY). Upon carbamylation, the IgG1-DNP-RGY was no longer able to hexamerise in solution, indicating that carbamylation negatively affects Fc:Fc interaction resulting in the loss of C1q binding by IgG. In conclusion, the inability of ca-IgG to activate the complement system is the combined effect of both decreased binding of C1q to the modified Fc of IgG and the reduced capacity of ca-IgG to form hexamers.

GENERAL DISCUSSION

Circulating Complement

In this thesis we have explored both low and high C1q serum concentrations in relation to disease and disease manifestations. On one hand we described a patient with C1q deficiency in **Chapter 7** [1], on the other hand in **Chapter 4**, we described the presence of elevated C1q levels in patients with active (pulmonary) TB [2]. C1q deficiency is associated with the development of Systemic Lupus Erythematosus (SLE) [3-5], and we reported on a new identified case with a rare mutation, a compound heterozygous mutation in *C1QC*, as described in **Chapter 7**. This patient has a complex medical history with a wide variety of clinical manifestations, indicative that C1q is, either directly or indirectly, involved in various biological processes. The notion that C1q has functional properties independent of the complement system is also indicated by the hierarchical association of complement deficiencies and the risk to develop SLE. C1q deficiency has the largest risk for SLE development, while in C1r/s deficiency this risk is some-what lower, for C4/C2 deficiency the risk is even less, and C3 deficiency is not a very strong risk factor [6, 7]. If all effects of C1q where via the CP then all deficiencies in that pathway should have the same risk. It is remarkable that genetic deficiencies of the CP predispose to SLE rather than that they protect against SLE development since in most SLE patients complement activation is suggested to contribute to inflammation and organ damage [8]. For C1q it is known that it plays an important role in the clearance of apoptotic cells [9]. Proper clearance of apoptotic cells is important to prevent the release of immunogenic material which could trigger development of an autoimmune diseases such as SLE and glomerulonephritis. Moreover, recently, C1q was reported to act as an inhibitor for T-cell responses, suggesting that a C1q deficiency could give rise to T cells aiding autoreactive B-cells [10]. The complement system is both friend and foe in the pathogenesis of SLE. Because of the complexity of SLE, both in clinical presentation and multifactorial aetiology, it is unlikely that one process is sufficient on its own is causative for disease onset.

On the opposite side of C1q deficiency, is an increased concentration of circulating C1q. In **Chapter 4**, we described the presence of increased C1q levels in patients with active TB. We assume that the increased levels of C1q we observed are rare since the only other clinical condition in which increased levels of C1q have been reported is visceral leishmaniasis, also known as kala azar [11]. Whether the increased C1q levels are protective for the host or the pathogen, more specifically *Mtb* as described in **Chapter 4**, is unknown. Interestingly, the infection itself occurs rather local, in the macrophages of the lungs, whereas the increased C1q expression is found systemically, in circulating cells collected from peripheral blood. Also *SERPING1* expression is elevated, though this increase did not translate to uniformly increased C1-INH protein levels in the measured cohorts as demonstrate in **Chapter 5**. Other components from the complement system do not appear to be increased in various studies in the circulation. These results are

interesting as they suggest an independent role of C1q outside of complement system. TB is caused by an intracellular pathogen and C1q has been shown to be of importance in $CD8^+$ T-cell biology where C1q dampens the $CD8^+$ T-cell responses [10]. Given that *Mtb* is an intracellular pathogen it could be of importance for *Mtb* to stimulate higher C1q production which will subsequently contribute to decreased $CD8^+$ T-cell anti-microbial activity. It is interesting to speculate that the rise in C1q levels serves a functions outside of the complement system cascade since the other component of the CP do not seem to be increased based on the expression profiles. Whether the *Mtb* directly upregulates the C1q expression or indirect is unclear. In the NHP model, increased C1q levels were associated with pathology and was found to be a marker of progressive TB disease and not the infection itself as demonstrated by the studies presented in **Chapter 6**. These observations correspond to the observation made in the human situation. We hypothesize that the increase in C1q is not a direct consequence of infected macrophages, but an indirect consequence of the milieu created by infected macrophages. For example, it is conceivable that infected macrophages produce mediators which stimulate surrounding cells to increase the production of C1q. This concept can be further studied *in vitro* by adding the supernatant of cultured infected macrophages to naïve cultured PBMCs / monocytes. The increased levels of C1q would lead to a potentially more active CP, which would conceivably contribute to enhanced clearance of *Mtb*. For increased levels of C1q to impact on T-cell biology without the concomitant effect of enhanced CP activity, C1-INH also needs to be present in increased levels to prevent further cascade activation.

Tuberculosis and biomarkers

Common diagnostic tests as the tuberculin skin test and interferon (IFN)-γ release assay are unable to discriminate between active and latent TB infection. It is important to discriminate active TB from LTBI in order to promptly initiate treatment to prevent mortality and further spread of the pathogen, here, it appears that host biomarkers play a significant role. In the search for biomarkers it is important to take along LTBI, since only comparing active TB with healthy controls is not representative or informative for an environment where TB is endemic. Ideally, biomarkers should be able to discriminate between TB and other respiratory infections that present with similar symptoms and abnormalities on chest X-rays. In this thesis, **Chapter 4** and **Chapter 5**, we have focussed on complement proteins as biomarker for TB. From a feasibility perspective, we preferred protein measurements instead of RNA expression profiles for biomarker signatures, since protein measurements are often easier, more stable, more accessible, cheaper and less time consuming compared to RNA procedures. In **Chapter 4** we demonstrated that increased C1q levels were specifically increased in active TB, not in LTBI or other respiratory infections. The ROC analysis of C1q resulted in an AUC of 0.77 for active TB versus LTBI, which may seem unimpressive, in the TB field, for a single protein biomarker, this is considered quite good. C1q is abundantly present, easy to measure and stable, therefore, it would be interesting to further evaluate its

potential in biomarker panels for TB diagnostics. The availability of the NHP model of tuberculosis for C1q research, as we demonstrated in **Chapter 4** and **Chapter 6**, could greatly accelerate and facilitate further research into biomarkers for TB. Further research can also be performed more into the host-pathogen interactions in the NHP for TB, but for a biomarker it is most important that it has a high specificity and sensitivity, this can be explored independent of its role in the pathogenesis.

Local Complement

The bulk of the complement proteins that are present in serum are produced and secreted by the liver, in particular by hepatocytes. However, serum does not reach all sites in the body where complement activation is needed. There are also cells that produce complement proteins locally at serum restricted sites. Local complement production and activation play a role in the initiation phase of the immune response. This activation impacts on the permeability of the local vasculature that subsequently will allow more systemic plasma proteins to leave the vessel and contribute to, or even take-over, the initial local response. We have reviewed the literature and found that there was also a profound ability for immune cells to produce local complement components [12]. Some cells are capable of producing all components needed for a functional pathway (monocytes), whereas others are only able to produce one or two complement components. This could point to other local functions of these complement proteins outside the complement system. We have further explored local production of complement and demonstrated that C1q can be produced by chondrocytes, as described in **Chapter 2.** In diseased joints, as found in rheumatoid arthritis (RA) and osteoarthritis (OA), higher levels of complement components are detected in the synovial fluid. This could be due to leakage from serum in pathogenic conditions. However, we found that C1q was produced by chondrocytes in unstimulated conditions *ex vivo*. This is interesting, especially considering the nutrient poor environment of the articular cartilage. From the cells perspective it could be disadvantageous to use a large amount of energy for the production of a protein without functionality. Therefore, it is tempting to speculate that C1q must have some functionality for the cartilage environment, as otherwise the cell would not produce this molecule. Articular cartilage is avascular and relies on diffusion of molecules. The structure is heterogenous and the ability of proteins to diffuse into the cartilage decreases with increasing molecular size [13, 14]. C1q is a large, 460 kDa, protein and the size limit of proteins that can move freely in cartilage is estimated to be around 65 kDa [15]. Therefore, it is likely that C1q produced by the chondrocytes has a local rather than systemic function. Conceivably, C1q would function in an autocrine setting in stimulating the chondrocytes, or alternatively C1q could be involved in local complement activation and / or cartilage biology. We speculate that C1q and complement activation may be involved in the maintenance of a lacuna for the chondrocyte. In such a scenario the released C1q may trigger the degradation of matrix molecules that are (too) close to the cell body of the chondrocytes. There are local interactions between complement proteins and matrix

molecules [16-20] and given the relation between complement and arthritic symptoms [21], it is interesting to explore the contribution of local production of C1q to cartilage morphology and biology with for example dedicated experiments with C1q deficient mice as a model.

We also explored the effect of the post translation modification (PTM) homocitrulline, formed by carbamylation, on complement activity. Carbamylation is the chemical conversion of a positively charged lysine into an uncharged homocitrulline and can occur on all proteins. Carbamylated proteins are frequently targeted by autoantibodies in patients suffering from RA [22, 23]. Upon carbamylation of IgG (ca-IgG), the IgGs were incapable of activating the complement system via the CP, which is initiated by C1q binding to a hexameric formation of IgGs. Some therapeutics, such as cell-depleting (monoclonal) antibodies, rely on complement activity for their functionality, so in theory, these could become ineffective by Carbamylation *in vivo*. In **Chapter 8** we demonstrate that IgGs can indeed become carbamylated *in vivo* in the synovial fluid of RA patients. Therapeutic antibodies (-mabs) could therefore also become carbamylated and lose their complement dependent effects. However, with our titrating experiments we demonstrated that for a mixture of antibodies to lose their complement effect, the majority has to become carbamylated. We still expect the majority of IgG to be unmodified, even in an inflammatory environment, and therefore the effect of *in vivo* carbamylation on therapeutics is considered negligible. Next to therapeutics that rely on complement activity for their mode of action, there are also antibodies for which complement activation is an unwanted side effect. For therapeutic antibodies for which complement activation would be considered to be a risk factor [24], the simple carbamylation of the antibodies would render the antibodies completely inert regarding complement activity. However, if therapeutics are administered in a carbamylated version, this could also give rise to anti-carbamylated antibodies, which can consequently form immune complexes, depositing in the glomeruli which then still results in the activation of the complement system. Another possibility is to explore the effectivity and safety of those therapeutics by replacing the lysines in the C1q binding region of the Fc portion of the IgG, thereby preventing the possibility to carbamylate lysines. However, this could influence the effectivity and safety of the therapeutics or enhance their immunogenicity and should therefore be thoroughly evaluated.

Intracellular Complement component C3

Complement component C3 is, together with Factor B, one of the ''oldest'' proteins in the complement system. Intracellular complement, or the ''complosome'' emerged recently as an interesting new perspective in the field of complement immunology [25-28]. However, some precaution put forward in this recent literature may be warranted, since replication of published articles remains necessary and has been challenging, as demonstrated by the studies described in **Chapter 3**. The new notion of intracellular complement has been met with mixed reactions in the field. Challenges lie in, but

are not limited to, the definition of ''intracellular complement'' since all proteins are made intracellular and are at some point fully formed inside the cell before they are secreted. Another terminology that is being challenged is ''C3 deficiency'', since confocal microscopy data from a C3 deficient patient demonstrated presence of intracellular C3, though this data cannot be replicated independently because of the antibodies used in these experiments are not available anymore. In this thesis we have aimed to find a new alternative antibody but were unsuccessful in finding one that gave similar results as published by Liszweski et al.. This also begs for the question that if only one antibody is able to show these results, is it justified to rely on this one polyclonal antibody? More data has been published pointing to an intricate and complex relation between: complement, metabolism and autophagy [29-34]. There are still information gaps in these networks which could be relevant for understanding the possible role for intracellular complement in biology. I do believe we are collecting the pieces to finish this puzzle but it remains challenging to allocate them. For example, Liszweski et al. proposed that intracellular C3a, resulting from intracellular C3 cleavage by CTSL, would signal via the C3aR and have further downstream effect in the mTOR pathway [28]. In **Chapter 3** we have analysed the cleavage fragments of C3 by CTSL and found the terminal arginine attached to the C3b instead of the C3a, indicating production of C3a-desArg. Interestingly, this C3a-desArg is unable to bine C3aR [35, 36] and consequently the pathway proposed earlier involving the intracellular C3aR would not be relevant. Moreover, C3a-desArg, which is also annotated as Acylation Stimulating Protein (ASP) in literature, has been linked to lipid metabolism [37]. In **Chapter 3** we also analysed the necessity of C3 for cell survival in the HAP-1 cell line as a model. We found no differences between C3 sufficient and deficient cells so far regarding survival and growth. This is supported by a recent study where C3 was also knocked out in different cell lines [32], however, they also demonstrated that C3 expression was required for normal autophagy regulation. Ideally, the use of freshly isolated CD4+ T-cells should be pursued for future intracellular C3 research. These are exiting new times which could possibly open up to a new area for complement therapeutics, though we should remain critical how to interpret these novel data and continue to replicate and validate those findings.

Conclusion

In this thesis we have explored the role and presence of complement proteins in both auto-immune and infectious diseases as well as cell biology of C1q production and intracellular C3. The diversity of the findings presented in this thesis further demonstrate the complexity and intrinsic relations that exist in the human body and urges the need to have a broad focus and challenges to look beyond the often conventional compartmentalized research perspectives. New evidence in this thesis has been presented that supports the consensus that complement proteins have conceivably other, local, functions outside of the scope of the complement system.

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ADDENDUM

NEDERLANDSE SAMENVATTING

Het complementsysteem is een integraal onderdeel van het immuunsysteem en draagt bij aan weefselhomeostase. Het complementsysteem werd lange tijd gezien als een eenvoudige proteolytische cascade van eiwitten om binnendringende pathogenen te bestrijden, maar het complementsysteem is veel complexer gebleken en onze kennis en begrip van het complementsysteem breidt zich nog steeds uit. Dit proefschrift rapporteert over de lokale complementproductie en de mogelijke betekenis van complementeiwitten buiten de traditionele activiteiten van het complement systeem. Hierbij richten de studies in dit proefschrift zich op C1q bij auto-immuniteit en tuberculose (TB), alsmede op de biologie van intracellulair C3.

Complementcomponent C1q wordt lokaal geproduceerd door geïsoleerde articulaire chondrocyten

Ontstekingen en immuunreacties van het aangeboren immuunsysteem kunnen bijdragen aan de ontwikkeling en progressie van artrose (OA). Chondrocyten zijn het enige celtype in gewrichtskraakbeen en produceren extracellulaire matrixmoleculen. Hoe inflammatoire mediatoren chondrocyten bereiken, is nog niet geheel bekend. Eerdere studies hebben aangetoond dat chondrocyten mRNA tot expressie brengen dat voor complement-eiwitten zoals C1q codeert, wat duidt op lokale eiwitproductie. De C1q eiwitproductie was nog niet overtuigend aangetoond. **Hoofdstuk 2** beschrijft de analyses van de chondrocyten uit kraakbeen, die werden geïsoleerd van patiënten die een totale knievervanging ondergingen vanwege artrose. De geïsoleerde chondrocyten waren in staat om C1q *ex vivo* te produceren en uit te scheiden, en waren ook in staat om C1q *ex vivo* te binden. Omdat chondrocyten C1q konden uitscheiden en binden, is onderzocht of incubatie *in vitro* met C1q de RNA-expressie van verschillende complement- en collageengenen zou kunnen beïnvloeden. In een kleinschalig proefopzet hebben we aangetoond dat incubatie met 100 µg / ml C1q gedurende 24 uur de expressie van *MMP13* en de *COLL2*:*COLL10* verhouding negatief beïnvloedde. Daarom concluderen we dat C1q eiwit tot expressie kan worden gebracht en uitgescheiden door menselijke articulaire chondrocyten en dat C1q kan binden aan chondrocyten die de relatieve collageenexpressie beïnvloeden.

Complement C3-splitsing door Cathepsin-L leidt tot vorming van C3a-desArg en is niet essentieel voor overleving van een HAP-1-cellijn

Er is recent een nieuwe intracellulaire rol beschreven voor complementeiwit C3 aanwezig in CD4+ T-cellen. Intracellulair C3 kan worden gesplitst door Cathepsin-L (CTSL), resulterend in signalering door C3a via de intracellulaire C3aR. Er werd verondersteld dat deze intracellulaire C3-route cruciaal is voor de overleving van CD4+ T-cellen. De studies beschreven in **Hoofdstuk 3** verkenden de aanwezigheid van het C3 eiwit in cellysaten, de CTSL-splitsingsplaats in C3 en de overleving van C3-deficiënte cellen. We konden C3 niet detecteren in cellysaten zonder dat de cellen eerst C3

hadden opgenomen na incubatie met NHS voorafgaand aan lysis. De analyse van C3-splitsing door CTSL *in vitro* door massaspectrometrie gaf aan dat de C3-splitsing door CTSL resulteert in de vorming van C3ades-Arg, aangezien het arginine nog steeds was gehecht aan het N-uiteinde van het C3b-molecuul. Dit is een interessante bevinding, aangezien uit eerder onderzoek was gebleken dat intracellulair C3 na CTSL knip via C3a de C3aR zou activeren. C3ades-Arg kan de C3aR echter niet binden, wat aangeeft dat de veronderstelde intracellulaire route niet zou kunnen voorkomen. Daarnaast genereerden we een C3-deficiënte HAP-1-cellijn met behulp van CRISPR/Cas9. Er werden geen verschillen waargenomen met betrekking tot proliferatie, morfologie en metabolisme tussen de wildtype en C3-deficiënte HAP-1-cellijnen. Samengevat, onze proeven laten zien dat de verwerking van C3 door CTSL waarschijnlijk C3a-desArg genereert en niet C3a, en verder dat C3 of de afgeleide producten niet essentieel zijn voor de overleving van een cel in een HAP-1 cellijnmodel.

Complement component C1q als serum biomarker om actieve tuberculose te detecteren

Tuberculose (TB) is een grote wereldwijde bedreiging voor de gezondheid, welke wordt veroorzaakt door infectie met *Mycobacterium tuberculosis* (*Mtb*). De diagnose van actieve TB wordt bemoeilijkt door het ontbreken van specifieke biomarkers die actieve TB ziekte onderscheiden van andere (long) ziekten of latente TB-infectie. Vroege diagnose en behandeling van TB is belangrijk om de overdracht van de infectie te verminderen en sterfte door ziekte te voorkomen. Onlangs zijn complement componenten naar voren gekomen als kandidaat-biomarker voor actieve TB. Op basis van geïntegreerde resultaten van humane genexpressie uit de literatuur, werden verhoogde niveaus gevonden van RNA-moleculen die coderen voor de verschillende C1q-ketens en *SERPING1* (coderend voor de C1-esterase-remmer (C1-INH)) bij actieve TB. Daarom hebben we de eiwitniveaus voor C1q (**hoofdstuk 4**) en C1-INH (**hoofdstuk 5**) geanalyseerd. In de studies beschreven in **Hoofdstuk 4** hebben we C1q eiwitniveaus gemeten in vier geografisch verschillende TB-cohorten uit Italië, Gambia, Korea en Zuid-Afrika. De C1q serumconcentraties waren verhoogd bij patiënten met actieve TB in vergelijking met alle relevante controlepopulaties. Belangrijk is dat longitudinale follow-up van TB patiënten tijdens de behandeling aan het licht bracht dat de C1q serumconcentraties normaliseerden naar die van endemische controles. Dit geeft aan dat de verhoging van C1q geassocieerd was met ziekte en niet intrinsiek was voor de individuen. Om het potentieel van C1q als biomarker voor actieve TB verder te evalueren, werden de C1q serumconcentraties van patiënten met actieve TB ziekte vergeleken met die van patiënten met aandoeningen waarvoor differentiële diagnoses in de klinische praktijk moeilijk te stellen zijn. Patiënten met actieve TB vertoonden significant hogere circulerende C1q serumconcentraties in vergelijking met patiënten met sarcoïdose of patiënten met longontsteking. Evenzo werden hogere C1q serumconcentraties gevonden bij patiënten met actieve TB in vergelijking met patiënten met lepra, die werden geanalyseerd als aanvullende controle. De verhoging van C1q

is dus waarschijnlijk geen weerspiegeling van een algemene respons op ontsteking. Bovendien werd C1q eiwit lokaal aangetroffen op een verhoogd niveau in de longen van TB patiënten (n=3) in vergelijking met controles. In analogie met humane TB werd C1q ook gevalideerd als biomarker voor TB ziekte bij resusapen, zowel in serum als in bronchoalveolaire lavage (BAL). Verhoogde C1q concentraties werden alleen waargenomen bij dieren waar er sprake was van progressie naar actieve ziekte en niet bij de dieren die de infectie onder controle hadden. Samengevat waren de C1q serumconcentraties verhoogd bij patiënten met actieve TB in vergelijking met latente TB in vier onafhankelijke cohorten. Daarom stellen we voor dat de toevoeging van C1q metingen aan huidige biomarkerpanels een meerwaarde kan bieden bij de diagnose van actieve TB.

Analyse van de endogene complementregelaar C1-remmer, het product van SERPING1, bij actieve tuberculose

We rapporteerden al in **Hoofdstuk 4** dat verhoogde C1q concentraties gebruikt kunnen worden als serum biomarker die actieve TB kan onderscheiden van latente TB en niet-TB-longinfecties of sarcoïdose. In serum vormt C1q een complex met C1r en C1s om het volledige C1-molecuul te genereren dat de klassieke route van het complementsysteem kan activeren. Belangrijk is dat het C1-complex wordt gereguleerd door een natuurlijke remmer, namelijk C1-INH. Transcriptionele biomarkersignaturen hebben aangegeven dat expressie van *SERPING1*, dat codeert voor de remmer C1-INH, onderscheid maakt tussen actieve en latente TB. De studies beschreven in **Hoofdstuk 5** waren gericht op het bepalen van serumniveaus van *SERPING1* gecodeerde C1-INH in vier geografisch diverse cohorten van patiënten met actieve TB, latente TB, ziektecontroles en patiënten die succesvol zijn behandeld voor TB. Het doel van deze studies was om een mogelijke toepassing als biomarker voor actieve TB te onderzoeken. De C1-INH-serumconcentraties waren significant verhoogd bij actieve TB patiënten in vergelijking met endemische controles in twee van de vier geanalyseerde cohorten. Bovendien nam in het Gambiaanse cohort met actieve TB-patiënten die in de loop van de tijd werden gevolgd, de verhoogde C1-INH serumconcentraties snel af bij het starten van de TB-behandeling. Het is interessant dat *SERPING1*-expressie consistent verhoogd is bij patiënten met actieve TB, terwijl het eiwitproduct C1-INH in slechts de helft van de cohorten blijkt te zijn verhoogd die in deze studie zijn geanalyseerd. Het is onduidelijk waarom C1-INH-verhoging werd waargenomen in slechts twee van de vier cohorten. Over het algemeen was C1q bij vergelijking van het C1q- en C1-INH-eiwitniveau in de verschillende geanalyseerde populaties uniform verhoogd in TB-cohorten, terwijl C1-INH in twee van de vier TB-cohorten de serumconcentratie was verhoogd. De observatie dat verhoogde C1-INH-serumconcentraties alleen werden gedetecteerd in cohorten van patiënten met actieve TB (maar niet bij patiënten met vergelijkbare klinische aandoeningen), en de observatie dat C1-INH snel afnam tijdens TB-behandeling, suggereert dat *Mtb* deze complementeiwitten actief reguleert. Een toename in C1q en C1-INH zou daarom een immuun-ontsnappingsmechanisme van *Mtb*

kunnen vertegenwoordigen dat immunosuppressieve acties van C1q mogelijk maakt, zonder de klassieke route van het complement systeem te versterken.

C1q concentraties in de circulatie en in de long als biomarker van progressieve ziekte bij experimentele niet-menselijke tuberculose bij primaten

Voor het bestuderen van pathogeen-gastheerinteracties en preklinische evaluatie van vaccinkandidaten, word veel gebruik gemaakt van *Mtb*-infectiemodellen in niet-menselijke primaten (NHP). NHP, en met name makaken (*Macaca spp*), worden beschouwd als relevante modellen voor TB, vanwege hun nauwe fylogenetische verwantschap met de mens, genetische diversiteit en de grote gelijkenis in de pathogenese van TB. Makaken worden ingezet over het hele spectrum van TB-onderzoek, zowel bij de preklinische evaluatie van TB-vaccins en -therapieën als bij fundamenteel onderzoek naar de ontwikkeling van TB-ziekten. Proefdieronderzoek bij deze soorten heeft het voordeel dat ze gecontroleerde en nauwkeurige tijd-responscondities hebben met betrekking tot infectie. Afhankelijk van de makaak (onder)soort, de *Mtb*-stam en de blootstellingsdosis, bootst de manifestatie van tuberculose bij makaken de diversiteit na die bij mensen wordt waargenomen. Na het aantonen, gepresenteerd in **Hoofdstuk 4,** dat C1q verhoogd was na een hoge dosis TB-blootstelling in NHP, hebben we de diversiteit in TB-ziekteverschijnselen in NHP's benut om de dynamiek van C1q als een biomarker van TB-ziekte in meer detail te onderzoeken. De resultaten van deze studies worden gepresenteerd in **Hoofdstuk 6**. We hebben de systemische en pulmonale C1q-niveaus na experimentele infectie beoordeeld met een hoge of lage enkele dosis en met herhaalde limietdoses *Mtb*-blootstellingen van makaken. We laten zien dat toenemende C1q-niveaus, hetzij perifeer of lokaal, correleren met verhoogde tuberculose-pathologie en met verminderde overleving na blootstellingen met hoge of lage dosis *Mtb* bij verschillende makaken. Verhoging van C1q ging niet vooraf aan detectie van *Mtb*-infectie door een conventionele interferon-gamma-afgiftetest, wat de associatie met ziekteprogressie bevestigt, maar niet met TB-infectie als zodanig. Ten slotte, laten we zien dat pulmonale vaccinatie met Bacillus Calmette Guérin (BCG) ook resulteert in een tijdelijke toename van pulmonale C1q. Of deze lokale C1q-productie een rol speelt bij de bescherming tegen *Mtb*-infectie en ziekte, moet echter nog worden onderzocht. Onze waarnemingen bevestigen en ondersteunen C1q als een marker van progressieve TB-ziekte. Aangezien C1q gemakkelijk kan worden gemeten tijdens het verloop van *Mtb*-infectie, kan het daarom worden toegepast om de voortgang van de TB-ziekte te volgen in een omgeving met beperkte middelen.

Complexe medische geschiedenis van een lupus patiënt met een samengestelde heterozygote mutatie in *C1QC*

Genetische deficiëntie, veroorzaakt door homozygote mutaties in een van de C1q-genen, zijn zeldzaam en hangen sterk samen met de ontwikkeling van Systemische Lupus Erythematosus (SLE). C1q-deficiëntie is een zeldzame aandoening met iets meer dan 70 gedocumenteerde gevallen uit ten minste 45 verschillende families. Deze deficiënties

zijn allemaal het gevolg van homozygote mutaties in een van de drie C1q-genen, behalve in één geval met een samengestelde heterozygote mutatie in *C1QA*. Patiënten met C1q-deficiëntie hebben verschillende klinische presentaties en uitkomsten. De meest voorkomende diagnose is de diagnose van SLE bij vroege kinderjaren en terugkerende infecties. In **Hoofdstuk 7** beschrijven we een C1q-deficiënte patiënt met een samengestelde heterozygote mutatie in het *C1QC*-gen: c.100G> A p. (Gly34Arg); c.205C> T p. (Arg69X). De medische geschiedenis van deze patiënt was complex en omvatte verschillende infecties, SLE, cerebrale betrokkenheid, vaatproblemen en botlaesies. Deze patiënt werd gedurende meer dan tien jaar behandeld met vers ingevroren plasma (FFP). Hoewel de C1q-waarden en CP-activiteit relatief kortstondig waren, hielden de symptomatische verlichting en substantiële verbetering van de kwaliteit van leven na FFP-behandeling gedurende verschillende weken aan. In de loop van de tijd waren er ook verschillende bijwerkingen op de FFP-therapie. Ondanks deze bijwerkingen gaf de patiënte de voorkeur aan de FFP-therapie vanwege de vermindering van vermoeidheid, artralgie en het aantal infecties. Echter werd de FFP-behandeling uiteindelijk stopgezet vanwege een ernstige anafylactische reactie.

Carbamylatie vermindert de capaciteit van IgG voor hexamerisatie en complement activatie

Post-translationele modificaties (PTM) van eiwitten na biosynthese komen veel voor in het menselijk lichaam en zijn belangrijk bij de regulering van activiteit, stabiliteit en vouwing van eiwitten. Ontregeling van PTM's is in verband gebracht met inflammatoire en auto-immuunziekten. Carbamylatie is de chemische omzetting van een positief geladen lysine in een ongeladen homocitrulline. Gecarbamyleerde eiwitten zijn vaak het doelwit van auto-antilichamen bij patiënten die lijden aan reumatoïde artritis (RA). Van verschillende eiwitten is gemeld dat ze *in vivo* worden gecarbamyleerd. Interessant genoeg werd gerapporteerd dat carbamylering van IgG invloed heeft op het vermogen om complement te activeren. Bovendien hebben cryo-elektronenmicroscopie-analyses aangetoond dat binding van C1q een hexamere rangschikking vereist van monomere IgG-complexen, die samenkomen via niet-covalente Fc:Fc-interacties. De studies beschreven in **Hoofdstuk 8** zijn uitgevoerd om de mogelijke rol van een prominente PTM, geassocieerd met reumatische ziekte, die wordt gevormd na carbamylering beter te begrijpen. Specifieker hebben we de interactie tussen gecarbamyleerd IgG bestudeerd in relatie tot het vermogen om het complementsysteem te activeren. We identificeerden verschillende peptiden van menselijke immunoglobulinen (inclusief IgG) die *in vivo* gecarbamyleerd waren in de synoviale vloeistof van RA-patiënten. We hebben bevestigd dat carbamylatie van IgG resulteert in een afname van het vermogen tot complement activatie, zowel in ELISA als in complement-afhankelijke cytotoxiciteitstesten (CDC). Desalniettemin, wanneer de gecarbamyleerde varianten werden gemengd met het niet-gemodificeerde IgG, was er geen dominant remmend effect op complementactivatie. CDC-activiteit is sterk afhankelijk van IgG1-hexameervorming en in de CDC-assay was het onduidelijk of ca-IgG's hexameren vormen

in aanwezigheid van niet-gemodificeerde IgG's. Daarom hebben we onderzocht of het effect van verminderde complementactivering door ca-IgG ook aanwezig is bij gebruik van voorgevormde ca-IgG-hexameren. Vervolgens analyseerden we het effect van carbamaylatie op hexamerisatie en complement activatie door gebruik te maken van een drievoudige mutantvariant van de IgG1-DNP die het vermogen van het antilichaam om hexameren te vormen zowel in oplossing als op het celoppervlak (aangeduid als IgG1-DNP- RGY). Na carbamylatie was het IgG1-DNP-RGY niet langer in staat om in oplossing te hexameriseren. Dit geeft aan dat carbamylatie de Fc: Fc-interactie negatief beïnvloedt, en resulteert in het verlies van C1q-binding door IgG. Concluderend, het onvermogen van ca-IgG om het complementsysteem te activeren, is het gecombineerde effect van zowel verminderde binding van C1q aan de gemodificeerde Fc van IgG, als het verminderde vermogen van ca-IgG om hexameren te vormen.

CURRICULUM VITAE

Rosalie Lubbers is geboren op 18 januari 1990 in Hardenberg. Na het behalen van haar Gymnasium diploma in 2008 aan het Vechtdal college in Hardenberg, begon ze met de bachelor Biomedische wetenshappen aan de Radboud universiteit te Nijmegen. Tijdens haar bachelor heeft ze stage gelopen bij het Radboudumc op de afdeling Craniofaciale Biologie. Ze vervolgde haar studie met de master Biomedische Wetenschappen aan de Radboud universiteit te Nijmegen, waarbij zij koos voor de specialisatie pathobiologie en in haar vrije keuze ruimte voor immunologie. Tijdens haar master heeft ze haar internationale stage uitgevoerd aan de Baskische universiteit in Leioa, Spanje. Haar afstudeerstage volbracht ze op de afdeling Interne Geneeskunde aan het Radboudumc. In 2014 slaagde ze bene meritum voor haar master Biomedische Wetenschappen.

In 2014 begon Rosalie aan haar promotie onderzoek op de afdeling Reumatologie aan het Leiden Universitair Medisch Centrum, onder de begeleiding van prof. Huizinga, prof. Toes en dr. Trouw. Tijdens haar promotie heeft ze onderzoek gedaan naar verschillende complement eiwitten, zowel in de context van immuniteit als auto-immuniteit. De resultaten van haar promotie onderzoek staan beschreven in dit proefschrift.

Momenteel is Rosalie werkzaam als trialcoördinator aan het Radboudumc Technology Center Clinical Studies op de afdeling Hematologie.

LIST OF PUBLICATIONS

Systemic and pulmonary C1q as biomarker of progressive disease in experimental non-human primate tuberculosis
Dijkman K, Lubbers R, Borggreven NV, Ottenhoff THM, Joosten SA, Trouw LA, Verreck FAW.
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Expression and production of the SERPING1-encoded endogenous complement regulator C1-inhibitor in multiple cohorts of tuberculosis patients
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Carbamylation reduces the capacity of IgG for hexamerization and complement activation
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