Complement in neuroinflammation
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Discussion & Summary

Neuroinflammation caused by complement effects inherited and acquired

disease progression
Discussion & Summary

Neurodegeneration is a key aspect of a large number of diseases including; Parkinson’s disease, Alzheimer, Amyotrophic lateral sclerosis and many more. Although these are different diseases, they have a common feature; immune activation and inflammation. Immune activation has physiological roles and maintains homeostasis in the body. But unbalanced or uncontrolled activation of the immune system may result in inflammation and pathology in different neurodegenerative diseases.

Mechanisms underlying neuroinflammation have been a focus of research in the past decades. Emerging evidence indicates that neuroinflammation is caused by a local immune response and contributes to neurodegeneration. Neuroinflammation is of interest because it might contribute to neuronal dysfunction, loss of neurons and axons in neurodegenerative diseases due to the production of neurotoxic mediators. A better understanding of the interaction of the inflammatory components of the immune system with damaged or stressed tissue is crucial to determine the beneficial effects for therapeutic strategies. A major unanswered question is whether pharmacological inhibition of inflammatory components or inflammation pathways is able to slow down the course of disease in man.

Both the innate and adaptive immune response are involved in neurodegeneration. Pattern Recognition Receptors (PRRs) are an important part of the immune system. Cells of the innate immune system express these receptors, which recognize pathogen-associated molecular patterns (PAMPs) from pathogens or danger-associated molecular patterns (DAMPs) from damaged or stressed tissue.

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The recognition of PAMPs and DAMPs include the detection of altered self cells and discrimination from non-self that is not dangerous and pathogens [1]. When this discrimination between dangerous signals and non-harmful signals is not made this leads to autoimmunity.

The complement system has been regarded as a key player in adaptive immunity [2]. It is a bridge between the innate and adaptive immune response. It consists of soluble and membrane associated proteins, that get activated via three pathways whereby one protein promotes the sequential binding of the following protein [3]. Regardless of the trigger, activation results in the cleavage of C3, followed by cleavage of C5 and formation of the membrane attack complex (MAC), which forms pores in the cell membrane resulting in lysis of the target cell. The complement system plays an important role in the defense against pathogens by identifying, opsonizing and lysis of the infected cells. Activation of the complement system by PAMPs induces C3 and C5 cleavage and generates anaphylatoxins C3a and C5a as well as the opsonin C3b [4]. Different phagocytic cells take up opsonized pathogens and provoke their lysis by MAC [5] and anaphylatoxins mediate the migration and recruitment of immune cells to the site of infection, were acute inflammatory reaction is initiated [4, 6].
Humans deficiencies of complement components can result in a wide range of diseases [7, 8]. Deficiencies in the classical pathway components C1 and C4 are associated with an increased incidence of immune complex disease and recurrent bacterial infection. A common immune complex disease is systemic lupus erythematosus (SLE) [9]. Complement deficiency in this case leads to the failure to clear circulating immune complexes. Consecutively this leads to the deposition of the complexes in tissues and an associated inflammatory response. Deficiencies for C2 are also associated with frequent bacterial infection and an increased risk of cardiovascular disease. In addition, deficiencies in complement component C3 results in problems with the coating of pathogenic cells with opsonin to promote phagocytosis. This deficiency can lead to severe recurrent infections, such as sepsis early in life. On the other hand deficiencies of the components of the terminal complement pathway (C5-C9) result in less serious infections and have a better prognosis.

Complement also plays an important role in synapse remodeling during development, showing a direct link between nerve elimination and complement. Activated complement components are soluble and can drift from their site of activation to adjacent areas, MAC can damage adjacent healthy tissue and enhance inflammation [5, 10], thereby resulting in more tissue damage. We have shown that formation of the MAC contributes to early clearance of myelin proteins and to axonal damage after traumatic injury of the peripheral nerve, while inhibition of MAC formation reduces nerve damage and improves regeneration and functional recovery [11-13]. Activation of the complement system occurs early in the disease process and persists while disease progresses. Regulators of the complement system permit elimination of pathogens or dead cells without injuring the host. When this balance is disrupted,
complement activation causes injury to the host and contributes to pathology in various diseases [14-16]. In this way complement can be a continuous source of neuroinflammation.

Complement proteins are abundantly present in most neurodegenerative diseases and can have pathological roles in neurological conditions offers broad scope for therapeutic intervention. Our hypothesis is that MAC may play an important role in nerve damage and that inhibition of MAC, using a C6 antisense/ RNA antagonist, is protective. In this thesis I describe research in which we tested whether this is the case in an animal model of *M.leprae* induced nerve damage and in the SOD1<sup>G93A</sup> mouse model of Amyotrophic lateral sclerosis.

**MAC contributes to pathology in a model of *M. leprae* induced nerve damage**

Nerve damage in leprosy is widely regarded as an important problem in patients, persisting long after the patients have completed treatment [17]. However, the nerve damage should be regarded as an early sign of leprosy, because the loss of sensation in patients with suspected leprosy is considered the hallmark of early disease [18]. Despite advances in our knowledge of the pathogenesis of leprosy spectrum, the understanding of the mechanisms of nerve damage in leprosy-associated neuropathy remains poor. Progress has been limited by the lack of established experimental models for studying leprosy-induced neuropathy. Here we used a mouse model of *M. leprae*-induced nerve damage to test whether inhibition of MAC using an C6 antisense/ RNA antagonist is protective.
Previous studies showed that loss of myelin proteins can be induced by *M. leprae* in the absence of lymphocytes in Rag knockout mice [19]. These data suggest the existence of host innate factors that interact with a pathogen-associated molecule (PAM) causing the initial damage. Mannose Binding lectin (MBL) contains carbohydrate recognition domains. During bacterial infections MBL mediates defence phagocytosis and extracellular complement activation via the lectin pathway.

In **Chapter 2** of this thesis we demonstrated that *M. leprae* lipoarabinomannan (LAM) is the most dominant activator of the complement system mainly via MBL/ the lectin pathway. We suggest that LAM interacts with the nerve and initiates complement activation resulting in the *in situ* formation of the MAC, causing nerve damage. In a model of *M. leprae* induced nerve damage we show that preventing MAC formation by antisense oligonucleotide-based therapy protects the nerve from *M. leprae*-induced damage, implying a role for the complement pathway in *M. leprae* associated nerve damage. Antibodies to mycobacterial antigens, such as lipoarabinomannan (LAM) are found in leprosy patients, suggesting an immune response to the *M. leprae* antigens [20]. Deposits of MAC are detected in thickened cutaneous sensory nerves of leprosy patients, suggesting a role for MAC in leprosy pathology [21].

We explored the extent of complement deposition, including MAC, in series of nerve biopsies from patients with full blown leprosy at either of the two poles of the disease spectrum, showing an association between the amount of MAC deposition and LAM immunoreactivity in nerves of leprosy patients. LAM persists in lesions of leprosy patients long after treatment. Antigens from dead bacilli can provoke immunological reactions, such as reversal reaction, causing serious nerve damage and subsequent disabilities. *M. leprae* antigens can trigger complement activation. A previous studies showed the localization of persisting *M. leprae* antigens in leprosy patients with
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Terminal Complement Complex elevated In Reactional Leprosy

Leprosy patients can change in clinical and immunohistopathological status during the course of the disease. This is common for patients during or after treatment, that could develop a reaction. Previous studies have also indicated an important role for complement in the disease, showing a normal or increased level of complement components by serological and pathological studies [26]. Therefore we examined whether increased systemic levels of complement activation could be detected in leprosy patients and whether complement products and regulators might be useful markers of leprosy disease state. In Chapter 3 we show that the activation products terminal complement complex (TCC), C4d and iC3b were specifically elevated in Bangladeshi patients with reaction at intake compared to endemic controls. In addition, levels of the regulator Clusterin were also elevated in MB patients irrespective of a reaction. Similar analysis of the Ethiopian cohort confirmed that irrespective of a reaction, serum TCC levels were significantly increased in patients with reactions compared to patients without reactions. Our data also showed that TCC levels stayed elevated after treatment, this suggests that treatment with either MDT or steroids does not lower complement activation in reaction patients, reinforcing the possibility that complement contributes to the nerve damage in these
patients. These findings imply that the analysis of the complement system might be of value for the diagnosis and prognosis of leprosy disease status. Unfortunately, the limitations of this study such as the samples being collected in the field situations and the lack of EDTA in the serum samples, any elaborate interpretation regarding the mechanism of high level of TCC in leprosy patients with reaction are not possible at this stage of the study. The lack of especially EDTA in collection tubes might have resulted in \textit{in vitro} generation of TCC which continues in serum. On the other hand, all the samples studied were processed similarly; therefore, any comparative values relating the disease state is considered as true reflection. The best way forward would be a prospective study on complement activation in leprosy, to confirm conclusively whether circulating complement activation products such as TCC can be applied as biomarkers for diagnosis of patients at a risk of developing a reaction.

\textbf{A role for complement C3d on T cell function in leprosy}

We demonstrated that \textit{M. leprae} specific component LAM activates the complement system and is associated with complement activation in nerves of leprosy patients. Therefore, we were interested whether LAM deposition is also seen in conjunction with the complement components C3d and MAC in the skin lesions of leprosy patients and which cells are positive for complement activation products. This is important to understand the role for complement in skin lesion pathology of leprosy patients. In Chapter 4 I describe an analysis of complement and inflammatory cells in skin lesions of leprosy patients. I demonstrate that C3d, MAC and LAM deposition was significantly higher in the skin biopsies of multibacillary compared to paucibacillary, with a significant association between the bacterial index/ LAM and
C3d or MAC in the skin biopsies of leprosy patients. In addition, MAC positivity was increased in both ENL and RR skin lesions compared to non-reactional leprosy patients.

Co-stimulation is essential for the development of an effective immune response. It is well known that paucibacillary leprosy patients have a stronger cell mediated immune response. For the activation of lymphocytes both antigen specific signal from their antigen and co-stimulation are required. Co-stimulation for B cells can also be provided by complement receptors. During an infection complement may be activated resulting in C3b binding to the pathogens. C3b is degraded into a fragment iC3b or C3b then cleaved to C3dg, and eventually to C3d. B cells express complement receptor CR2 or CD21 to bind to C3d. CR2 on mature B cells forms a complex with CD19 and CD81. This additional co-stimulation by C3d results in B-cell being more sensitive to the antigens of this pathogen. Interestingly, different studies have shown that a population of T cells also has a CR2 receptor for C3d binding [27-29]. In skin lesions of paucibacillary patients we found C3d positive T-cells in and surrounding granulomas, but hardly any MAC deposition compared to multibacillary patients. C3d on the T-cells might be involved in co-stimulation by binding CR2 on T-cells resulting in an enhanced cell mediated immune response in paucibacillary patients. We considered that C3d might bind to CR2 expressed on the surface of T cells and, by ligand–receptor interaction, result in T cell stimulation and enhancement of the adaptive immune response. In this way, C3d might play an important role in the inflammation in skin lesions of paucibacillary patients. Axons were hardly detected in skin lesions of paucibacillary patients, probably the nerves are destroyed.
MAC that was found deposited on the axons and co-localizing with LAM in lesions of multibacillary patients. This suggests that MAC is attacking the axons in skin lesions with LAM as a trigger for complement activation.

We conclude that signatures of complement activation are abundantly present in skin lesions of leprosy patients, even after treatment, suggesting that inflammation triggered by complement activation might contribute to nerve damage in the lesions of these patients and that this should be regarded as an important factor in *M. leprae* pathology.
In the second part of my thesis I describe work on ALS, a progressive neurodegenerative disease. ALS was chosen because of the previous work describing complement activation products in both patients and animal models of this disease.

Complement is deposited at the presymptomatic stage on motor end-plates of SOD1\textsuperscript{G93A} mice

Mechanisms leading to the neurodegenerative disease ALS are still unclear. Both cell autonomous and non-cell autonomous mechanisms are involved in the disease [30-32]. Different studies suggest an important role for neuroinflammation in the disease [33-35]. The involvement of complement as an inflammatory component in the pathogenesis of ALS in man is suggested by different researchers. Elevated levels of complement activation products in serum and cerebrospinal fluid were detected in ALS patients. Levels of mRNA for C1q and C4 and protein levels of complement proteins C1q, C3 and MAC were elevated in spinal cord and motor cortex of patients with sporadic ALS [36]. In the murine mouse model of ALS C1q and C4 were upregulated in motor neurons [37, 38]. Other studies have also shown upregulation of the major proinflammatory C5a receptor, during disease progression in mouse motor neurons [39]. SOD1\textsuperscript{G93A} rat treated with C5aR antagonist had an extension of survival time and a reduction in end-stage motor scores compared to the untreated rats. This data suggests an important role for complement in the progression of ALS [35]. Also, increased expression of complement components C1qB, C4, factors B, C3, C5 and a decrease in the expression of the regulators CD55 (regulator of C3) and CD59a (regulator of MAC) was detected in the lumbar spinal cord of SOD1\textsuperscript{G93A}
mice [40]. This data points towards a disrupted regulation of the complement system in this model.

In the SOD1\textsuperscript{G93A} rodent model, an early involvement of the motor end-plate has been shown [41, 42]. Retrograde degeneration is detected in ALS patients [43, 44]. In addition, several physiological and morphological alterations have been reported on the muscle end-plates from \textit{in vivo} and \textit{ex vivo} mouse and rat preparations [45-51]. These data suggest that some parts of ALS pathology start at the muscle end-plates and might proceed to the spinal cord and subsequently the brain [52, 53], “a dying back mechanism”.

In chapter 5 we determined complement expression and activation in the SOD1\textsuperscript{G93A} mouse model of familial ALS (fALS). We show that complement components C1q and C3 are deposited on the motor end-plates at the presymptomatic, symptomatic and endstage of the disease. At the end stage of the disease, C3 mRNA was upregulated in spinal cord and C3 protein accumulated in astrocytes and motor neurons. While, complement activation products C3/C3b and C1q were detected at the motor end-plates of SOD1\textsuperscript{G93A} mice before the appearance of clinical symptoms and remained detectable at the symptomatic stage, suggesting that complement activation on the motor end-plates precedes neurodegeneration and plays an early role in this model [54].
MAC deposition on motor endplates of ALS patients

In Chapter 5 we did not show deposition of MAC on motor end-plates of SOD1\textsuperscript{G93A} mice. Since our hypothesis is that MAC contributes to tissue damage in disease, we show in Chapter 6 of this thesis that MAC is also deposited on the motor end-plates of SOD1\textsuperscript{G93A} mice at the presymptomatic stage.

We determined that complement components C1q and C3 are deposited on the motor end-plates of SOD1\textsuperscript{G93A} mice and suggest that the complement system might play an important role in disease. An important question is whether what we observe in a mouse model of the disease is also what happens in the patient. In Chapter 6 we show that complement activation products and regulators are deposited on the motor end-plates of ALS patients. In intercostal muscle biopsies of ALS patients we see two patterns of MAC immunoreactivity. On the end-plates with a weak α-BTX immunoreactivity, strong signal for MAC immunoreactivity was seen. By contrast, a weak MAC immunoreactivity was detected on end-plates with strong α-BTX immunoreactivity. This is in line with a model in which MAC deposition occurs before loss of the end-plates. Moreover, MAC was found deposited on motor end-plates that were innervated by nerves, indicating that complement activation may precede motor-endplate denervation. C1q, C3 and MAC were also detected on the motor nerve-terminal and terminal Schwann cells. In general, regulators protect cells from complement-mediated damage. We show that the regulators CD55 and CD59 are both expressed on the motor end-plates, indicating an attempt to control the activation. This process is probably not efficient enough because MAC can still be detected on the α-BTX positive motor end-plates. Since a role for MAC in the pathology of neurological disorders is suggested [55], detecting complement deposited at the end-plates of ALS donors, before the end-plates are lost,
complement activation could be an early event in ALS and might play an important role in the motor end-plate pathology in ALS. This observation is in line with earlier studies suggesting a "dying-back" mechanism in ALS, meaning the disease probably starts at the motor end-plates [52]. However, this study is performed using post-mortem tissue and thus analysing the end stage of disease. So we cannot detect the early changes. Although there may be some limitations to our conclusions about complement being involved in motor end-plate degeneration, this study adds to the understanding of ALS pathology in man.

C6 inhibition in female SOD1\(^{G93A}\) mice decreases neurological disability

Complement products are abundantly present in tissue of ALS patients, including MAC which is found in serum, cerebrospinal fluid, spinal cord, motor cortex and at the neuromuscular junction of SOD1\(^{G93A}\) mice and ALS patients. We propose that MAC might be involved in causing secondary damage driving the progressive loss of motor neuron function in ALS. Despite the evidence of complement activation in ALS, the role of the complement system and its contribution to disease progression in animal models for ALS is controversial [56, 57]. Therefore, we tested whether inhibiting MAC formation with a C6 antisense/ RNA antagonist (C6 ODN) results in a delay of disease progression in a murine model of ALS.

In Chapter 7 describes the effect of complement inhibition using 1 mg/kg/day C6 ODN in SOD1\(^{G93A}\) mice on the survival, body weight and neurological score. No significant effects on median onset or survival in either female or male C6 ODN treated SOD\(^{G93A}\) mice compared to vehicle controls. The onset and survival curve of the C6 ODN treated female SOD\(^{G93A}\) mice showed a different disease progression...
compared to the controls, but there were no statistical difference in onset and survival. At the end-stage of the disease (day 120) the vehicle control mice dropped in weight while the treated female SOD1\textsuperscript{G93A} mice that were still alive maintained their body weight. Interestingly, C6 ODN treated female SOD\textsuperscript{G93A} mice, progressed in a manner that was slower than vehicle controls ($p=0.002$). Together with the maintained body weight this suggests that these mice were performing better than the vehicle controls.

The treated male SOD1\textsuperscript{G93A} mice progressed in the same manner as the vehicle controls. No significant effect on the onset and survival was observed compared to the vehicle controls. In addition, the male treated SOD1\textsuperscript{G93A} mice showed a decrease in body weight similar to control and the neurological score of was not different from the vehicle controls, suggesting no effect of treatment on the male SOD1\textsuperscript{G93A} mice.

The difference in progression of the disease in treated male and female SOD1\textsuperscript{G93A} mice is probably due to the C6 mRNA levels in these animals. We treated both with 1mg/kg/day, while C6 mRNA is much lower in female than male SOD1\textsuperscript{G93A} mice (ten-fold difference). This study suggests that complement inhibition in female SOD\textsuperscript{G93A} mice might reduce disease severity, based on the results of the female SOD1\textsuperscript{G93A} mice. The lack of effects in male SOD1\textsuperscript{G93A} mice prevents us to convincingly show that the hypothesis is correct. Therefore, we suggest further investigations with a higher concentration of C6 ODN which might have an effect on the outcome of the disease in male SOD1\textsuperscript{G93A} mice.

In summary, the data presented in this thesis shows that complement activation occurs in various neuroregenerative conditions, both inherited and acquired. I see associations between complement activation products and disease severity in
leprosy and ALS. Preventing terminal pathway activation has an effect on demyelination in a model for M. leprae induced nerve damage, and on the disease severity in SOD\textsuperscript{G93A} mice. Overall, these experiments show that complement components are putative targets for future therapies modulating disease severity.
References


