Nervous immunity: A study on the role of complement system in neuronal degeneration and regeneration

Sta, M.

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General discussion: Nervous immunity, is it always beneficial?

M. Sta
Nervous immunity, is it always beneficial? The answer to this question is: “No it is not”. Activation of the inflammatory system in the peripheral- and central nervous system is not always associated with a reduction of disease severity or better prognosis for regeneration. In the nervous system, the adaptive immune system is often activated via primary innate immune responses, some of which are induced by the complement system that becomes activated after trauma or in disease. However, activation of the complement system has a dual role in neurodegenerative disorders. The research described in this thesis deals with the role of the complement system in neurodegeneration and regeneration after trauma or in disease. Chapter 1 of this thesis gives an overview of previous research on the role of complement in various animal models of neurological disease and of complement activation in neuronal development. In this thesis we used a model of peripheral nerve trauma in complement sufficient and deficient animals to shed light on the role of complement activation in neurodegeneration, its effect on regeneration and the downstream adaptive immune responses induced by the complement system. We show that the time course of degeneration and regeneration of the peripheral nerve is in part dependent on the amount of complement activation, which in turn is determined by the severity of the initial damage.

Peripheral nerves

In degenerative diseases of the peripheral nervous system complement activation is observed in the affected nerves. For example, in Charcot Marie Tooth (CMT) patients, complement is activated and high levels of membrane attack complex (MAC) deposition are observed in diseased nerves.

Our group previously showed that inhibition of MAC formation resulted in delayed Wallerian degeneration (WD) (Ramaglia et al., 2007; Ramaglia et al., 2008) and improved regeneration in a rat sciatic nerve crush model (Ramaglia et al., 2009b). Inhibition of MAC formation was achieved both pharmacologically by addition of soluble Cr1 and by using rats deficient for complement C6 (C6\(^{-/-}\)), which are unable to form MAC, but in which all upstream complement components are normal (Ricklin et al., 2010). When C6 was reconstituted in the C6\(^{-/-}\) animals, WD and regeneration displayed the normal WT phenotype. In a nerve crush mouse model, mice with a loss of function mutation in CD59, an inhibitor of MAC, WD was exacerbated (Ramaglia et al., 2009a).

Macrophages or MAC?

Complement deficiency not only leads to a delay in WD, but also results in a reduced influx and activation of hematogenous macrophages, in chapter 2 we studied whether the observed delay in WD in the C6\(^{-/-}\) animals could be caused by the lack of influx and activation of hematogenous macrophages. In order to do so, we depleted wild type (WT) animals’ hematogenous macrophages and studied alterations in the morphology as well as the amount of C9 deposition.

This study shows two things; First, macrophages are responsible for rapid myelin degeneration during WD, but axonal damage is independent of macrophages and is possibly induced by MAC. Second, we found that the amount of C9 deposition correlated with the number of activated macrophages in the injured nerve. We propose that activated macrophages can induce complement activation. A possible explanation is that the presence of infiltrated, activated macrophages induces additional damage to the nerve, by disruption of the environment and thus by exposing new complement activation epitopes.
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So we propose that at least part of the complement activation observed in normal WD is a result of an amplification of complement activation by the influx and activation of macrophages in the injured nerves. The inability to form MAC of C6-/- animals results in reduced influx and activation of (hematogenous) macrophages. This in turn reduces complement activation, which is partially dependent on the presence of activated macrophages. In this way, the different amounts of activated macrophages might contribute to a difference in upstream complement activation, contributing to the difference in clearance of debris from the injured nerve between WT and C6-/- animals.

Gene expression profiling

In chapter 3 we describe a study on the alteration in gene expression profiles after injury in WT and C6-/- nerves and dorsal root ganglions.

We found that two master regulators of DNA transcription, c-jun and c-fos, were differentially expressed in WT and C6-/- animals. Accordingly we found alterations in gene expression in many different biological pathways. We focused this study on pathways associated with the inflammatory response, alterations in extracellular matrix and neurite outgrowth. These pathways are likely directly involved in WD and regeneration and might provide an answer to the question whether C6-/- animals have a different strategy to clear debris from the injured nerves. Additionally, we wanted to determine whether there were genetic clues that explain why the C6-/- animals show improved regeneration.

We did find differences in the inflammatory response to damage. Analysis of chemokine pathways showed that WT and C6-/- animals upregulate expression of genes encoding for chemokines that attract macrophages. Expression of the Ccl2 gene encoding a chemokine that attracts macrophages is upregulated in WT and C6-/- animals, but the protein could not be detected in C6-/- nerves. This could be an indication that Ccl2 is subject of post transcriptional regulation (Berkman et al., 1995; Yiakouvaki et al., 2012). It has been described that IL1b stabilizes the mRNA of Ccl2, whereas IL10 destabilizes the mRNA, which explains why there is only very little Ccl2 protein detected in the C6-/- injured nerves. One of the activators of macrophages, Ccl5, is downregulated in C6-/- animals. The difference in Ccl2 protein and down regulation of Ccl5 explains why the C6-/- animals show a lack of influx and activation of hematogenous macrophages after injury.

An analysis of interleukin expression disclosed indications that both WT and C6-/- animals have a secondary response to nerve damage. After initial attraction and activation of macrophages, signs of a T-helper (Th) cell response were detected. The nature of this response was different in WT and C6-/- animals. In WT animals we primarily observed a pro-inflammatory Th1 response and only few anti-inflammatory Th2 markers. Th1 cells can induce an M1 type (pro-inflammatory) macrophage response. In the C6-/- animals we also observed Th1 cell markers, but less than in WT animals. We did, however, find an increase in markers for pro-inflammatory Th17 cells. The presence of markers for T regulatory cells (Treg) and anti-inflammatory markers associated with Th2 cells indicates that the inflammatory response is regulated. In contrast to Th1 cells, Th17 cells do not affect macrophage response, but Th2 cells can induced an M2 type (anti-inflammatory) macrophage response (Prajeeth et al., 2014). This indicates that the macrophage response observed in WT is predominantly M1 macrophage and in C6-/- deficient animals an M2 macrophage response. These findings were confirmed by expression data.
Previous studies suggested that the balance of T cells can influence the outcome of disease, as in Guillain Barré syndrome, where the presence of Th2 cells and M2 macrophages is neuroprotective (Zhang et al., 2013).

Gene expression analysis also showed that genes associated with neurite outgrowth are differentially regulated in WT and C6\(^{-/-}\) animals. We observed more factors associated with neurite outgrowth in C6\(^{-/-}\) animals, than in WT animals. The changes were already visible at 2 days post injury (d PI).

Taken together gene expression analysis showed that C6\(^{-/-}\) animals have a different response to nerve damage. The C6\(^{-/-}\) animals fail to mount a rapid clearance by macrophages, instead a pro inflammatory Th17 response is induced. Th2 and Treg cells tightly regulate this response. This regulation via Th2 and Treg results in a neuroprotective environment. Additionally, factors that promote neurite outgrowth attain higher levels in C6\(^{-/-}\) animals. Both differences might explain why C6\(^{-/-}\) animals show faster recovery than WTs.

Ex vivo electrophysiology, a tool to study early regeneration

Our analysis showed that the faster recovery of C6\(^{-/-}\) nerves is found in combination with an altered response to nerve damage. The expression analysis showed that early events associated with the degeneration process are different. Already at 2 d PI factors that promote neurite outgrowth are upregulated in the C6\(^{-/-}\) animals. Therefore, both early and late events might affect the regenerative process. Unfortunately, the tests for functional recovery, measure it only at the late stages of regeneration, because these methods can measure recovery of function only when regeneration has reached the end of the limb.

In order to study the restoration of function early in the regeneration process, we developed a new method to measure functional regeneration over the entire length of the sciatic nerve. This method uses the capacity of the nerve to generate a compound action potential (CAP) after electrical stimulation. The CAP is recorded and amplitude and shape can be studied to provide information on functional state of the nerve. This ex vivo electrophysiological method, was compared with traditional measurements and morphological analysis and corresponded to the findings in all methods (Sta et al., 2014a).

This new ex vivo method is a good tool to study early recovery of nerve function, in and around the crush area. However, to study sensory and motor function recovery at later stages, the traditional methods are more sensitive. Ex vivo electrophysiology measures the CAP generated by the whole nerve bundle, whereas the in vivo functional tests are so sensitive that a few regenerated axons are enough to evoke a response. Morphological analysis is required to distinguish between degenerative and regenerative phenotypes.

Intrinsic regrowth capacities

In Chapter 5 we investigated if C6\(^{-/-}\) animals have an underlying trait that induces faster neuronal regeneration. We used a crush injury model with low initial complement activation. The new ex vivo electrophysiology method was employed to study early functional recovery, and monitor its progression along the whole length of the nerve. This study showed that the C6\(^{-/-}\) show a similar regeneration progress to WT animals, when the role of complement is minimized by reducing initial activation. Functional recovery could be observed as early as 2 d PI in WT and C6\(^{-/-}\) animals. Over time the observed maximum amplitude of the CAP and the distance it travels...
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over the nerve increased. At 3 weeks post injury (wk PI) sensory recovery could be observed in the rat’s paw.

Morphological analysis showed that at 5 mm distal of the crush and 3 d PI the degeneration process was similar in WT and C6−/− animals: in both types of injured nerves, myelin and axonal debris could be found. Also changes in nerve morphology followed similar patterns in WT and C6−/− animals over time. It was shown that unmyelinated fibres were present in the distal tibial nerve at 1 wk PI. However it is unclear whether these are degenerating or regenerating fibres. At 2 wk PI the unmyelinated fibres found in the nerve are denser and are most likely regenerated axons. The axons show myelination at 3 wk PI, the amount of myelinated fibres is increased at 4 wk PI. No differences were observed between WT and C6−/− animals (Sta et al., 2014b).

So, if initial complement activation and MAC deposition are low, no difference in WD and subsequent regeneration is observed between WT and C6−/− nerves. This indicates that both animal types have the same intrinsic capacities to regenerate nerve and that the differences previously observed are in fact caused by a large difference in MAC deposition.

This also implies that inhibition of MAC formation to improve nerve regeneration is only beneficial in the presence of substantial complement activation.

**Transection model**

WD and subsequent regeneration are well studied in crush injury models of the sciatic nerve. To determine if complement activation has similar effects in other types of injury to the sciatic nerve we studied a transection-resuture model. Many cases of peripheral nerve trauma are cause by transection of the nerve. In chapter 6 we describe the findings in the transection-resuture model. We found there are major differences between the crush and transection models. The integrity of the epineurium is disrupted in the transection resuture model, allowing easier influx of hematogenous factors into the damaged nerves. We observed a massive influx of hematogenous cells into the transection site, in WT and C6−/− animals. The majority of these cells were mononuclear and CD68 negative. We did observe influx of CD68+ cells too. The infiltration of these CD68+ cells was observed mainly in and directly adjacent to the transection area, the CD68+ and CD68− cells showing little spread distal from it. No differences were found in myelin and axonal damage and complement deposition between the WT and C6−/− transected nerves at 3 d PI. Although, the spread of damage to myelin and axons as well as complement activation were delayed in distal spread at 3 d PI relative to the findings in crush injured nerves.

Regeneration of the nerves in the transection model differs from regeneration after crush injury. After transection, beginning of motor function recovery is observed after 4 wk PI regardless of animal type. Sensory recovery is observed in WT at 5 wk PI and at 6 wk PI in C6−/− animals, this one week difference remains significant (P < 0.05) until week 8, when it diminishes. More importantly, the transected nerves never regain full function: at 11/12 wk PI the regeneration process reaches its maximum at approximately 30-70% of normal function. This underscores the differences between the injury types. In the transection injury the initial complement activation is very low. We attribute this to the lack of exposure of complement activation epitopes by the injury. In contrast to a crush injury where the initial damage is in the range of millimeters, the damage inflicted in the transection-resuture model is only micrometers wide. Additionally, the remaining epitopes are rapidly removed by the infiltrated macrophages. This results in reduced spreading of complement activation distally over the damaged nerve.
These findings are a possible explanation for the observed delay in WD at 5 mm distal of the injury.

Although the degeneration follows similar patterns in WT and C6⁻/⁻ animals and the slightly earlier start of sensory recovery observed in WT animals, no difference was observed in motor function recovery between the two animal types. At 12 wk PI no differences in either motor or sensory recovery could be observed. These findings were confirmed by morphological analysis of the regenerated tibial nerve, which did not show a difference between WT and C6⁻/⁻ animals either. Both showed recovery of the nerve morphology with similar timing, small regenerated fibres at 8 wk PI. At 12 wk PI most regenerated fibres are remyelinated and inter-axonal space is small.

These data confirms that MAC is necessary for rapid WD following peripheral nerve degeneration, but it also suggests that low levels of complement activation do not delay the regeneration process. Maybe, in case of the transection injury low levels of initial complement activation during the early degeneration phase are needed to trigger alternative responses to clear debris from the injured nerve. Schematic representation of the responses following peripheral nerve injury is shown in figure 1.

Our data on the response of a peripheral nerve to crush injury point in the direction that it is in fact MAC that induces rapid WD, clearance of myelin and axonal debris. The previously observed effect of MAC inhibition inducing improved nerve regeneration, is most likely the result of a difference in this clearing strategy and not due to an intrinsic non-complement related trait in the C6⁻/⁻ animals. WT and C6⁻/⁻ animals displayed similar regrowth capacities in a model with low initial complement activation. Genetic analysis showed that the clearance strategy with high initial complement activation after injury is different in the two animal types. The WT animals rapidly recruit and activate macrophages and display a Th1 response pushing the macrophage phenotype into the M1, pro-inflammatory, type. In contrast, C6⁻/⁻ animals fail to recruit and activate macrophages. Instead they induce a pro-inflammatory Th17 response regulated by anti-inflammatory Th2 and Treg cells. These latter two result in a neurite outgrowth friendly environment, reflected by the higher expression levels of neurite outgrowth promoting factors. The finding that MAC triggers a pro-inflammatory, Th1 and M1 response is underscored by the study of Ydens et al. (Ydens et al., 2012). This study reports that after nerve injury by transection Th2 and M2 responses are triggered. In chapter 6 we showed that in a transection-resuture model there is almost no MAC deposition in the injured WT nerves, in contrast to our crush injury model where a large amount of MAC was found in injured WT nerves. An overview of the injury type and the presence of MAC are given in table 1.

We propose that the delay in degeneration is a consequence of the difference in clearance strategy in presence or absence of MAC as well.

<table>
<thead>
<tr>
<th>Initial damage</th>
<th>2.0 mm crush</th>
<th>1.5 mm crush</th>
<th>Transection-resuture</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAC deposition in WT animals*</td>
<td>high</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>Time to maximal sensory recovery between WT and C6⁻/⁻</td>
<td>WT &gt; C6⁻/⁻</td>
<td>WT = C6⁻/⁻</td>
<td>WT = C6⁻/⁻</td>
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Table 1: Overview of injuries: initial damage, MAC deposition and regeneration time. *No MAC deposition is observed in C6⁻/⁻ animals
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We attribute the lack of an effect of complement C6 status in the smaller crush and the transection-resuture injury to the low levels of complement activation, MAC deposition, in these models. Low levels of complement activation, MAC deposition, result in a different clearance response involving M2 type macrophages and Th2, Th17 and T regulatory cells.

However, if the injury type does not trigger substantial complement activation, there is not much room for inhibition and the effect will be small. We therefore propose that inhibition of MAC formation to improve peripheral nerve regeneration after injury or disease is only useful when the levels of initial complement activation are high.

Central nervous system

Activation of the complement system is also associated with neuroinflammation and neurodegeneration in the central nervous system. However, in the central nervous system the complement system not only plays a role in degeneration and inflammation, it is also necessary for normal development. Complement activation via C1q is necessary for synapse elimination during the development of the brain. If the complement system is not activated during neurogenesis, serious dysfunctions as a consequence of hyper connectivity occur in the brain (Stevens et al., 2007; Yuzaki, 2010). Various studies reported that complement activation during...
early stages of neurodegenerative disease has a neuroprotective effect. In the same diseases, activation of the complement system in their later stages is believed to negatively affect disease progression (Alexander et al., 2008; Fonseca et al., 2004a; Fonseca et al., 2004b; Sjöberg et al., 2009).

Complement activation and adaptive immune cells in human sporadic amyotrophic lateral sclerosis (ALS)

In chapter 7 of this thesis we investigated whether activation of the innate and adaptive immune system was associated with disease progression in human sporadic ALS (sALS) patients.

In this study we analyzed spinal cord and motor cortex of sALS patients with a rapid disease progression (survival less than 6 months), patients with a slower progression of the disease (survival longer than 24 months) and human spinal cord and motor cortex from individuals that had died of other than neurodegenerative causes. This study shows that complement activation was observed in spinal cord and cortex of all sALS but not in tissue from controls. Levels of upstream complement components, C1q and C3 were not associated with disease progress. Levels of C5b-9 showed a trend that levels were higher in the spinal cord of rapidly than of slowly progressing sALS patients. This trend was more visible in motor cortex.

We also observed differences in the amount of inflammatory cells between control, rapidly and slowly progressing sALS spinal cord and motor cortex. Only few inflammatory cells could be detected in control tissue. We found that the presence of activated microglia was associated with slow disease progression, whilst the presence of higher levels of dendritic cells and CD8 positive T cells in the spinal cord and motor cortex was associated with rapid disease progression. No CD4 (Th) positive cells were observed in the spinal cord and motor cortex of the sALS patients in this study (Sta et al., 2011).

We propose that in ALS, the stressed motor neuron and its surrounding glial cells, triggers complement activation by the production and secretion of C1q and other complement factors (e.g. C4). Upon binding of C1q to damaged cells, the complement system is activated via the classical activation pathway. Complement activation results in a positive feedback loop, since complement activates microglia which serves as a positive feedback loop, because activated microglia produces more complement proteins and pro-inflammatory cytokines. Pro-inflammatory cytokines result in more complement activation, leakage of the blood-brain-barrier and attraction of specific adaptive immune cells. Neuronal stress itself, complement activation and activated adaptive immune cells all can ultimately cause neuronal loss. In sALS dendritic, antigen presenting cells and CD8 positive cytotoxic T cells are both associated with rapid disease progression. The presence of microglia in the spinal cord and motor cortex is associated with slowly progressing sALS.

Little is known about early stages of complement activation in human ALS, because study is limited to post-mortem material. In a study of a transgenic mouse model for ALS, these mice carry a mutated superoxide dismutase gene of human origin (SODG93A). In the SODG93A mouse complement activation was seen already before the development of physical signs of disease, suggesting that complement activation occurs very early in the disease process (Heurich et al., 2011). GWAS studies have identified regulators of the complement system as risk factors for AD (for details view articles on Cr1 and clusterin b (Brouwers et al., 2012; Crehan et al., 2012; Hazrati et al., 2012; Lambert et al., 2009). We propose that modifying the activation state of the complement system and more specifically MAC formation during the (pre-)symptomatic stage of
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the disease might slow down disease progression, because it stops the positive feedback loop of complement and the induction of neuronal damage.

**Triggers of inflammation in sALS**

ALS is a disease in which the inflammatory process is not only induced by complement activation, but is also triggered by Toll-like receptors (TLRs). TLRs are part of innate immunity and each receptor recognizes a specific structure, pattern or protein to trigger the innate and adaptive inflammatory response as a first line of defense against a wide range of pathogenic or potentially harmful substances. Chapter 8 describes which TLR response was observed in spinal cord and motor cortex of our sALS group. It showed that the TLRs associated with ALS are TLR2 and TLR4, both of which showed upregulated expression levels in spinal cord and motor cortex of sALS patients. TLR2 is found mainly expressed by microglial cells and TLR4 is present on astrocytes and neurons. Other factors associated with neurodegeneration and TLR signaling like the receptor for advanced glycation end products (RAGE) and the transcription factor HMBG1 are also observed in these sALS patients (Casula et al., 2011).

These findings suggest activation of the inflammatory response in glial cells via TLR2 and in astrocytes via the TLR4/RAGE signaling pathway. Additionally, TLR4 is found on neuronal tissue as well, where it regulates excitability and neurotoxicity via the NMDA receptor. This selective role of TLR4 in motor neurons could play a role in the progressive motor neuron loss in ALS. Targeting TLR signaling and the interaction of TLRs with RAGE and the transcription factor HMBG1 might be a tool to decrease motor neuron loss during ALS.

The fact that the inflammatory process in ALS is not only induced by activation of complement, but also by other pattern recognition molecules, indicates that targeting the inflammatory process to decrease motor neuron loss in ALS will remain complex. Inhibiting complement is just one of the strategies to influence the inflammatory response, but will never fully stop the recruitment and activation of inflammatory cells in spinal cord and motor cortex of sALS patients.

**Conclusion**

This thesis describes research that shows that activation of the immune system is it not always beneficial.

An activated immune system in the brain or in the peripheral nerves acts as a dual edged sword. A little activation is required for normal function, clearance and inhibiting disease progression. Chronic and massive activation of the immune system often results in excess damage and a more rapid disease progression and/or poorer outcome of the disease. Inhibition of the complement system can result in improved regeneration and delay of the disease progression, but the activation state of the complement system in each given stage of the disease should be carefully considered prior to inhibiting the terminal complement activation, MAC formation.

**Future scopes**

The difference in regrowth properties of nerves in presence and absence of high levels of MAC deposition should be studied in more detail, for example by using the *ex vivo* method on the wide crush model and compare between WT and C6-/- animals.
Another step could be to determine the type of macrophage response in each crush model and determine how exactly the macrophage response can induce higher levels of proteins that enhance neurite outgrowth. Additionally, the timing and amount of the various cytokines could be determined. It has already been found that difference in cytokine response are associated with neuropathic pain, but maybe the influence of cytokines on the neuronal response also affect recovery.

It would be interesting to determine if differences in macrophage and T-helper cell responses are associated with the clinical outcome of CMT and other peripheral nerve diseases. Additionally one can study whether the T-helper cell response is also activated in degenerative diseases of the central nervous system.

Although we did not observe a CD4 response in our sALS cohort, it could be involved in other neurodegenerative diseases of the CNS, such as Alzheimer’s disease, traumatic brain injury or multiple sclerosis.

Identifying systemic markers that are associated with the different cellular responses during neurodegeneration might prove a powerful tool to identify- and predict the clinical outcome of neurological trauma and neurodegenerative diseases.

Reference List


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