From the Neuroimmunology Unit
Department of Clinical Neuroscience
Karolinska Institutet, Stockholm, Sweden

Impact of Genetic Variability on Early Immune Reactions following Nerve Injury

Rickard P.F. Lindblom

Karolinska Institutet

Stockholm 2013

All previously published papers and images were reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by Larserics Digital Print AB, Sweden.

© Rickard Lindblom, 2013
ISBN 978-91-7549-286-5

The great tragedy of science-
the slaying of a beautiful hypothesis by an ugly fact.

Thomas Henry Huxley, 1870, (1825-1895).

TO MY BELOVED ONES

ABSTRACT

Injury to the central nervous system (CNS) is frequently associated with significant morbidity, in worst case mortality. This in turn leads to considerable disease burden for the individual and also for society. The spectrum of conditions that cause damage to the CNS is wide and highly diverse, ranging from acute trauma to degenerative diseases that progress over decades. But in spite of the heterogeneity, there exist common aspects. For instance, there is often interplay between the immune and nervous systems in one stage or another of disease. Also, the genetic background of the individual influences both susceptibility to and severity of the disease. This thesis focuses on the early immune reactions that occur after CNS injury in rodents, based on the hypothesis that early immune reactions affect important downstream events, not least nerve cell death.

In **study I** a large rat experimental cross F2(DAxPVG) was created from the inbred strains DA and PVG. The global transcriptome in the spinal cord was screened using microarrays five days after ventral root avulsion (VRA), a reproducible nerve injury model. In parallel, whole genome mapping was performed. The expression and genetic data was co-analyzed to identify gene regions regulating components of the complement system, in turn associated with loss of synapses. Lastly, we identified a link between the cholinergic and complement systems, which was confirmed *in vitro*.

In **study II**, data from the F2(DAxPVG) cross was used to analyze regulation of complement receptors following injury. We found a strong cis-regulatory influence acting on the expression of complement receptor 2 (CR2), a receptor mostly associated with B cell functions, but with unknown role in the adult CNS. CR2 was up regulated on astrocytes and protected from injury induced synapse loss in rodents. Levels of CR2 were increased in the cerebrospinal fluid (CSF) of rats following injury, but also in the CSF of patients with multiple sclerosis as compared to controls, implicating a possible role for CR2 also in context of human disease.

Traumatic brain injury (TBI) is one of the leading causes of death in the young population. Secondary events following initial injury contribute to the damage, but are not fully understood. In **study III** TBI was performed on DA and PVG rats and the ensuing injury processes analyzed at both the molecular and cellular levels using microarrays and flow cytometry. Large strain differences in complement activation and the size and composition of multiple immune cell populations were found. Complement was also found to label axons of injured neurons and the degree of complement expression correlated with the levels of neurofilament-light in CSF, a marker of axonal injury.

C-type lectins (CLECs) are a group of immune molecules structurally similar to members of the complement family. Study I demonstrated several loci co-regulating the expression of complement and CLEC transcripts, thus providing a link between the two families. In **study IV** we examined the properties of a congenic rat strain, Aplec, which onto DA background has a very small genomic insert from PVG consisting of 7 CLEC genes. The Aplec rat displayed improved survival of motor neurons following VRA compared to DA, which in turn was associated with increased infiltration of T cells. This demonstrates that CLECs convey neuroprotection after nerve root injury, potentially through T cell pathways.

These results provide insights into the molecular pathways regulating inflammation after mechanical nerve injuries, in turn of relevance for nerve injury-induced synaptic remodeling and neurodegeneration.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Skador på centrala nervsystemet (CNS), d.v.s. hjärna och ryggmärg, leder ofta till allvarliga och bestående handikapp, med långtgående konsekvenser både för individen och dennes omgivning. Traumatiska hjärnskador är den ledande orsaken till död hos unga i många länder. Samtidigt har medicinska landvinningar inom andra områden lett till att folk lever allt längre och en växande andel av befolkningen utgörs av äldre. I och med en ökande andel äldre i befolkningen ökar också förekomsten av neurodegenerativa sjukdomar, såsom Alzheimers sjukdom, med stor inverkan på individ, anhöriga och samhälle.

Även om orsakerna till skador på och sjukdom i CNS skiljer sig mycket åt finns det ett antal gemensamma nämnare, t.ex. att graden av nervcellsdöd korrelerar till funktionell nedsättning och handikapp oavsett sjukdom, liksom att det finns tecken till aktivering av immunsystemet, antingen som en direkt orsak till sjukdom, eller till följd av skadan. Likaså har individer olika känslighet för att utveckla neurologiska sjukdomar. Dessutom skiljer sig förloppet hos de som drabbas och hur väl man svarar på behandling. Allt detta påverkas i hög grad av individens genetiska uppsättning.

I denna avhandling tillämpas olika skademodeller på genetiskt modifierade råttor och möss i syfte att identifiera faktorer som påverkar den aktivering av immunsystemet som följer av en traumatisk nervskada, liksom hur denna aktivering påverkar utfallet av skadan. Vissa av de identifierade faktorerna, d.v.s. generna, har föranlett vidare studier av motsvarande genprodukt, d.v.s. protein, hos patienter som drabbats av multipel skleros, MS, en sjukdom där immunförsvaret angriper och skadar det egna nervsystemet. Eftersom en gen kodar för ett protein som sedermera är inblandat i skadeförloppet utgör identifierade gener potentiella måltavlor för riktade terapier.

LIST OF PUBLICATIONS

I. Rickard PF Lindblom, Mikael Ström, Matthias Heinig, Faiez Al Nimer, Shahin Aeinehband, Alexander Berg, Cecilia A Dominguez, Swetha Vijayaraghavan, Xing-Mei Zhang, Karin Harnesk, Johan Zelano, Taher Darreh-Shori, Staffan Cullheim, Margarita Diez, Norbert Hübner, Fredrik Piehl.
Unbiased expression mapping identifies a link between the complement and cholinergic systems in the rat central nervous system.
Submitted Manuscript

II. Rickard PF Lindblom, Alexander Berg*, Mikael Ström*, Shahin Aeinehband, Faiez Al Nimer, Cecilia A Dominguez, Nada Abdelmagid, Matthias Heinig, Johan Zelano, Karin Harnesk, Kerstin Sandholm, Norbert Hübner, Bo Nilsson, Kristina Nilsson Ekdahl, Mohsen Khademi, Margarita Diez, Staffan Cullheim, Fredrik Piehl.
Complement receptor 2 is up regulated in the spinal cord following injury and protects from synapse elimination.
Manuscript

III. Faiez Al Nimer, Rickard Lindblom, Mikael Ström, André Ortlieb Guerreiro-Cacais, Roham Parsa, Shahin Aeinehband, Tiit Mathiesen, Olle Lidman, Fredrik Piehl.
Strain influences on inflammatory pathway activation, cell infiltration and complement cascade after traumatic brain injury in the rat.
Brain, Behaviour & Immunity, 2013 Jan;27(1):109-22.

IV. Rickard PF Lindblom, Shahin Aeinehband, Roham Parsa, Faiez Al Nimer, Mikael Ström, Xing-Mei Zhang, Cecilia A Dominguez, Margarita Diez, Fredrik Piehl.
Genetic variability in the rat Aplec C-type lectin gene cluster regulates lymphocyte trafficking and motor neuron survival after traumatic nerve injury.
Journal of Neuroinflammation, 2013 May 8;10:60.

* Equal contribution.

List of publications not included in the thesis

I. Diez M, Abdelmagid N, Harnesk K, Ström M, Lidman O, Swanberg M, Lindblom R, Al-Nimer F, Jagodic M, Olsson T, Piehl F.
Identification of gene regions regulating inflammatory microglial response in the rat CNS after nerve injury.
Journal of Neuroimmunology, 2009 Jul 25;212(1-2):82-92.

II. Al Nimer F, Beyeen AD, Lindblom R, Ström M, Aeinehband S, Lidman O, Piehl F.
Both MHC and non-MHC genes regulate inflammation and T-cell response after traumatic brain injury.
Brain, Behavior and Immunity, 2011 Jul;25(5):981-90.

III. Mikael Ström, Faiez Al Nimer*, Rickard Lindblom*, Jens Rendel, Fredrik Piehl.
Naturally occurring genetic variability in expression of Gsta4 is associated with differential survival of axotomized rat motoneurons.
Neuromolecular Medicine, 2012 Mar;14(1):15-29.

IV. Faiez Al Nimer,* Mikael Ström,* Rickard Lindblom, Jens Nyengaard, Shahin Aeinehband, Olle Lidman, and Fredrik Piehl.
Naturally occurring variation in the glutathione-S-transferase 4 gene determines neurodegeneration after traumatic brain injury.
Antioxidants & Redox Signaling, 2013 Mar 1;18(7):784-94.

V. Taher Darreh-Shori, Swetha Vijayaraghavan, Shahin Aeinehband, Fredrik Piehl, Rickard PF Lindblom**,** Bo Nilsson, Kristina Nilsson Ekdahl, Bengt Långström, Ove Almkvist, Agneta Nordberg.
Functional variability in butyrylcholinesterase activity regulates intrathecal cytokine and astroglial biomarker profile in patients with Alzheimer's disease.
Neurobiology of Aging, 2013 Nov;34(11):2465-81.

VI. Swetha Vijayaraghavan, Azadeh Karami, Shahin Aeinehband, Homira Behbahani, Alf Grandien, Bo Nilsson, Kristina Nilsson Ekdahl, Rickard PF Lindblom, Fredrik Piehl, Taher Darreh-Shori.
Regulated extracellular choline acetyltransferase activity - the plausible missing link of the distant action of acetylcholine in the cholinergic anti-inflammatory pathway.
PLoS One, 2013 Jun 19;8(6):e65936.

VII. Shahin Aeinehband*, Rickard PF Lindblom*, Mikael Ström, Faiez Al Nimer, Swetha Vijayaraghavan , Kerstin Sandholm, Mohsen Khademi, Tomas Olsson, Bo Nilsson , Kristina Nilsson Ekdahl , Taher Darreh-Shori, Fredrik Piehl.
Co-regulation of cholinergic and complement pathways associates with clinical disability in Multiple Sclerosis.
Submitted Manuscript

* Equal contribution.

CONTENTS

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's Disease
AIL	Advanced Intercross Line
ALS	Amyotrophic Lateral Sclerosis
APC	Antigen Presenting Cell
Aplec	Antigen Presenting Lectin-like receptor gene Complex
BBB	Blood-brain Barrier
BChE	Butyrylcholinesterase
BCR	B Cell Receptor
ChAT	Choline Acetyltransferase
CLECs	C-type Lectins
CR2	Complement Receptor 2
CSF	Cerebrospinal Fluid
DA	Dark Agouti
DC	Dendritic Cell
Dcir	Dendritic Cell Immunoreceptor
EAE	Experimental Autoimmune Encephalomyelitis
eQTL	Expression Quantitative Trait Loci
GWAS	Genome-wide Association Study
HKG	House-keeping Gene
HLA	Human Leukocyte Antigen
IHC	Immunohistochemistry
KO	Knock-out
MAC	Membrane Attack Complex
MHC	Major Histocompatibility Complex
MS	Multiple Sclerosis
NFL	Neurofilament-light
PD	Parkinson's Disease
PPMS	Primary Progressive Multiple Sclerosis
PVG	Piebald Virol Glaxo
QTL	Quantitative Trait Loci
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RRMS	Relapsing-remitting Multiple Sclerosis
SCI	Spinal Cord Injury
sCR2	Soluble Complement Receptor 2
SNT	Sciatic Nerve Transection
SPMS	Secondary Progressive Multiple Sclerosis
TBI	Traumatic Brain Injury
TCR	T Cell Receptor
VRA	Ventral Root Avulsion
WT	Wild-type

1 AIMS OF THIS THESIS

General aims

The global aim of this thesis was to identify new immunological pathways and understand the relevance of the immune activation that occurs early after injury to the CNS. A genetic approach was chosen, and the most important tool was genetically modified rodents. The rationale for this is as follows: since, hypothetically, in the optimally controlled experimental setting only the genes vary between the genetically modified animals, any observed difference in outcome after injury can be attributed the genetic variation.

Specific aims of the individual studies

Study I: To identify pathways that underlie the activation of the complement system that occurs in the spinal cord following traumatic nerve root injury, ventral root avulsion (VRA). A further aim was to assess the functional outcome of the complement activation.

Study II: In this study we identified a strong and unexpected up regulation of complement receptor 2, CR2, in the rat spinal cord following VRA. As CR2 is not well characterized in the CNS the aim of this study was to establish the relevance of CR2 in neuroinflammation in rats, mice and humans.

Study III: The events occurring after traumatic brain injury (TBI) are complex and severe. The aim of this study was to thoroughly assess and characterize the local TBI response at both expressional and cellular level using global transcriptional profiling and flow cytometry.

Study IV: In study I we found that several components of the complement system were co-regulated with C-type lectins (CLECs). In this study we aimed to explore the role of CLECs following traumatically induced spinal cord inflammation.

2 THE CENTRAL NERVOUS SYSTEM IN HEALTH AND DISEASE

2.1 THE HEALTHY CENTRAL NERVOUS SYSTEM

The central nervous system (CNS) is perhaps the most complex organ system in the human body. Macroscopically the CNS consists of the brain, cerebellum and spinal cord, all of which in turn can be divided into grey and white matter. Simplified, the grey matter is nerve cell dense and the white matter is made up of myelin, a fatty-protein mix that surrounds the axons and optimizes neural transmission. The nerve cells, or rather the cells populating the CNS, are a mixed population which can be grossly divided into neurons and glia. The glial cells consist mainly of astrocytes, microglia and oligodendrocytes, which will be briefly described below, but also a number of other, smaller populations such as vascular endothelial smooth muscle cells, perivascular macrophages and ependymal cells. The glial-neuron ratio is a topic filled with myths and beliefs, and it is often stated that there are 10 times more glia than neurons (1, 2). However, this statement is not fully supported by later scientific evidence, as a number of studies in higher mammals, including humans, have suggested that the ratio is closer to 1:1 (3, 4). A compromise is to say that half the cellular volume of the CNS consists of neurons and the other of glia (4). An ambitious study found that in the neocortex of the human brain i.e. the frontal, parietal, temporal and occipital lobes, there were in average 21.4 and 26.3 billion neurons in females and males respectively and 27.9 and 38.9 billion glial cells in females and males respectively giving a neuron:glia ratio of 1.2 (5). Oligodendrocytes were the most numerous glia cell type, 28.8 billion (75.6%), astrocytes the second largest population, 7.8 billion (17.3%), and microglia the smallest, 2.0 billion (5.2%). This was in middle aged men without neurological disease but the numbers and proportions for matched females were similar (5). The size of the glia populations is also something that changes with age, as in the young human brain oligodendrocytes constitute the largest glia population, but while they decrease in number with time the astrocyte population remains stable (5). The neuron:glia proportion in the spinal cord is also a subject of much debate; another extensive study performed in a 8 different species of primates found that about 10% of the cells in the spinal cord were neurons (6). This study also pointed out that the larger the animal (and the longer the spinal cord), the fewer the neurons, as the non-neuronal cells expanded faster than the neurons as the cord grew. In summary- it is still not settled the exact ratio between neurons and glia, but there are likely more than 1 glia but less than 10 glia cells per neuron in the human CNS.

However, it is important to state that there are large differences in the composition of the CNS between species, and interestingly; the more complex the species, the larger number of glia, especially astrocytes (7), relative to neurons exist, which may sound counterintuitive at first. Whereas humans have more than 1 astrocyte/neuron the rats have only 0.4 and the mice even less (3, 7). This may reflect the many and indispensable functions of the astrocytes which, in summary, aim to provide support (metabolic, structural, signaling etc.) and homeostasis for the neurons (7). However, increasing interest is also directed towards the immunologic role of astrocytes (8).

Microglia constitute 5-20% glia population (5) and are the main CNS resident immune-, phagocytic- and antigen-presenting cells (9, 10). There has since long been a discussion regarding the embryologic origin of microglia, whether they are of neural or myeloid origin, but the current opinion is that they are of myeloid lineage, i.e. stem from primitive macrophages in the yolk sac during embryogenesis and infiltrate and populate the CNS during development (11, 12). However in the inflamed adult brain there is an influx of monocyte-derived macrophages, and there is evidence, from rodents, that these can differentiate to microglia (12). The last major glia population are the oligodendrocytes, whose most acknowledged role is to produce the myelin sheaths that surround the axons and improve neuronal signaling (13). But also the oligodendrocytes can likely participate to some degree in immune responses (14).

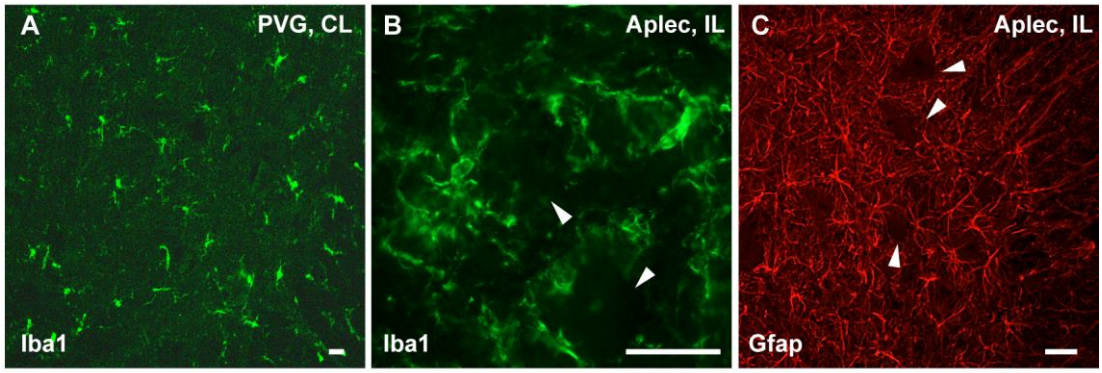

Figure 1. Astrocytes and microglia.
The contralateral (CL) side of the spinal cord of a PVG rat stained with Iba1 demonstrates the ramified, resting microglia (A). The ipsilateral (IL) side, at higher magnification, of an Aplec rat 5 days after VRA, where Iba1 staining demonstrates the activated microglia, which have become more round (B). The IL side of an Aplec rat 5 days after VRA stained with Gfap shows astrocytes (C), recognizable by their stellate processes. The white arrow heads point at selected motor neurons, seen only as “dark holes” in these micrographs. The scale bar corresponds to approximately 40um.

2.2 THE DAMAGED CENTRAL NERVOUS SYSTEM

Severe injury to the CNS often causes lifelong morbidity or even mortality. The functional deficit depends both on the extent of neuronal death and the loss of axonal and synaptic connections. The region of the brain/spinal cord that is affected is also of importance, as for instance a cervical spinal cord transection leads to significantly increased morbidity compared to a distal lumbar transection. Unlike glial cells, adult neurons in the CNS largely lack regenerative capability. This is one of the major differences between the peripheral nervous system (PNS), i.e. the nervous tissue outside of the brain, cerebellum and spinal cord, which, contrary to the CNS has a significant regenerative capability (15). This fundamental difference depends on multiple factors, where perhaps the most obvious one is that in the PNS there are Schwann cells rather than oligodendrocytes that support the axons, and these provide better trophic support than the glia cells in the non-growth permissive CNS milieu (15). However, there are also intrinsic differences between the neurons in the CNS and PNS (16). Another factor that hinders regeneration in the CNS is the formation of a glial and fibrotic scar at the site of injury, which prevents the regrowth of axons, which for anatomic reasons is of special importance in the spinal cord (17). In spite of the lack of, or at least very limited, regeneration of nerve cells in the CNS some recovery of function may still be possibly after injury. This is possible due to the process of synaptic plasticity, which means that synapses can be removed and reformed to create new neural circuits, that by-pass the non-functional parts of the CNS. Synaptic plasticity can occur both in the brain and spinal cord (18, 19), but is not always of good, as it may also lead to harm and malfunction exemplified by neuropathic pain (20) and epilepsy (21). It has been demonstrated that the spinal cord is more vulnerable and sensitive to trauma than the brain with a more pronounced inflammatory response (22, 23) which may in part explain why the capacity to recover after injury is less in the spinal cord than the brain. Another reason for this is likely the relatively small size and tube shape of the spinal cord. A comparatively small injury can consequently damage a large proportion of the cord and thereby transect a large number of axons and neural tracts that for many of the above described reasons will not be able to connect again.

2.2.1 A brief overview of archetypal CNS injuries

CNS diseases can like most pathological conditions be classified as acute or chronic. In practice though, this is not always helpful, as many of the acute CNS diseases, like trauma and ischemia often lead to chronic disability and the disease processes frequently continue and cause secondary damage over long time after initial onset (24, 25). Also, some of the chronic diseases can manifest in acute or sub-acute ways, like for instance the first bout of Multiple Sclerosis (MS). In common for most CNS diseases is that they often have severe consequences for the individual, and cause a significant burden of disease to society (26). The sections below aim to provide an overview of the different clinical scenarios which cause CNS disease. However detailed disease pathomechanisms will not be explained here, neither will infectious CNS diseases be discussed, as the focus of this thesis is on the inherent responses of the CNS rather than on those triggered by exogenous pathogens.

2.2.2 Acute CNS disease

Trauma and vascular pathology are both common causes of acute CNS injuries. Whereas traumatic brain and spinal cord injuries often affect the younger population, vascular injuries like ischemic or hemorrhagic stroke more commonly affect older people.

2.2.2.1 Traumatic Brain Injury

TBI is one of the leading causes of neurological disability and death among younger individuals worldwide. Trauma to the brain results in a primary mechanical damage characterized by disruption of the brain parenchyma and blood vessels, which initiates a complex cascade of molecular events and physiological processes. Some of these processes may lead to further injury on nerve cells and axons, i.e. secondary brain damage, that takes place in hours and days after the primary insult. The TBI panorama displays a high degree of variability, and ranges from mild injuries, such as commotio, without any obvious macroscopic signs of injury, to widespread tissue damage, that can lead to death or severe neurologic impairment. Also, the location of the lesion can have very different impact on the individual's neurologic performance. This heterogeneity could in part explain the somewhat disparate, and disappointing results from different pharmacological treatment studies (27).
Initial neurosurgical and intensive care is lifesaving and the basis of treating TBI (28, 29), but there remain major challenges in reducing subsequent injury. Antioxidants are being evaluated and have shown promising neuroprotective results after traumatic brain injuries in preclinical experiments, but these have not as convincingly been reproduced in the clinical setting (30). An interesting observation is that people exposed to TBI suffer an increased risk of developing both Parkinson's (PD) and Alzheimer's (AD) diseases later in life illustrating that the processes triggered by TBI (31, 32) can affect disease susceptibility even many decades after the initial injury.

2.2.2.2 Spinal cord injury

The term spinal cord injury (SCI) most often refers to traumatic injury, but the spinal cord can also be injured secondary to ischemia (33), further described below. As for TBI, initial fast neurosurgical and intensive care is lifesaving and the basis of limiting post-injury complications (28, 29).
For complete spinal cord lesions, i.e. with no motor and no sensory function distal of the lesion (34), there is most often little or no recovery, and if there is recovery this usually occurs within the first months (35), and seldom after 18 months. Incomplete SCI have a more unpredictable prognosis, and 20-50% of those who present with a complete motor injury, but have a retained sensory function can display recovery of motor functions (35).
The treatment options, and results, for severe SCI have since long been very limited and restricted to corticosteroids, a treatment with moderate evidence based support. However, a recent meta-analysis demonstrated that high-dose steroids is the only clinically used pharmacological treatment that has shown positive effect and improve neurologic outcome after acute SCI (36), which demonstrates the urgent need for more randomized trials for the development of new medical treatments after SCI. As for TBI,

antioxidants have been tested, mostly experimentally, but may turn out useful (30). Also stem cells have shown promising results experimentally (37) and trials in humans have recently started (38, 39), which gives hope to this group with otherwise poor prognosis. Lastly, intense rehabilitation and training can improve spinal circuits and reflexes in spite of lacking supraspinal input and thus constitutes an extremely important part of post-injury treatment (40).

2.2.2.3 Vascular brain and spinal cord injuries

The most common type of brain injury caused by vasculopathy are the ischemic lesions, in turn caused by arterial embolization due to atrial fibrillation or plaque rupture as a consequence of atherosclerosis (41). Brain damage can also be caused by rupture of intracerebral arteries, which causes secondary ischemic injury (42). There is a wide range of other conditions that also can cause ischemic damage to the brain, for instance dissection of the aorta (43), the carotid or the vertebral arteries respectively (44). Ischemic brain damage can also be iatrogenic, as a consequence of complex aortic arch (45) or carotid artery surgery (46).
The most common cause of ischemic SCI is aortic disease, that can be either dissecting or aneurysmal (33), but also surgery, open as well as endovascular, on the thoracoabdominal aorta is associated with considerable risk of developing spinal cord ischemia (47, 48). What can be said about ischemic SCI in general, is that compared to ischemic brain injuries, the capacity for return of function is even less (49).
Treatment in vascular pathology is directed towards addressing the underlying cause and preventing new insults. This includes treating atrial fibrillation, often with anti-coagulants, surgical treatment of significant carotid stenosis, anti-platelet therapy after ischemic events, and also managing other cardiovascular risk factors, like cessation of smoking, dietary control and treatment of hypertension and hypercholesterolemia as well as to strive for optimal blood glucose control for patients with diabetes etc. (50). As for the traumatic lesions fast and extensive rehabilitation following the insult is of outmost importance.
Another area where several global brain injury occurs is after cardiac arrests; almost 70% of the deaths in patients that are resuscitated after out-of hospital cardiac arrest are a consequence of severe brain injury (51). However, new frontline research in pigs has demonstrated remarkable results, where salvage of a brain with restoration of almost normal neurological function, in spite of normothermic, global cerebral ischemia for 30 minutes is possible (52). This was accomplished through surgically assisted controlled reperfusion of the ischemic brain, using extra-corporeal circulation, leukocyte filtration and anti-oxidative treatment (53, 54). Although a lot of work likely remains before this method can be taken into clinical practice the results can be described as a paradigm shift in the view of ischemic brain lesions (55).

2.2.3 Chronic CNS disease

Examples of chronic CNS diseases are MS, AD, PD and Amyotrophic Lateral Sclerosis (ALS). Chronic CNS diseases constitute a heterogeneous group and there has classically been a distinction between neuroinflammatory (e.g. MS) and neurodegenerative diseases (e.g. AD). However, this concept is becoming revisited as the neurodegenerative aspects in MS are pronounced, and ultimately, the degree of

neuronal loss best correlates with clinical disability. Also, the inflammatory events in for instance AD have received more and more attention, with inflammatory genes being linked to disease (56, 57).

2.2.3.1 Multiple Sclerosis

MS illustrates the classic neuroinflammatory disease where a yet unidentified trigger causes an autoimmune reaction directed against the myelin surrounding the axons in the CNS. There are typically focal lesions in the white matter of subcortical or periventricular areas, the optic nerve or in brainstem and spinal cord. In the lesions infiltration of both CD8 and CD4 T cells as well as macrophages, monocytes and microglia together with soluble immune system components like complement and immunoglobulins can be seen (58). But apart from the white matter inflammation there with time also occurs a significant degeneration of grey matter and neuronal loss (59, 60). As for most autoimmune diseases there is a clear gender bias with increased susceptibility among women (61). Disease is driven by immune activation and is in its most typical form characterized by bouts and disease-free periods i.e. relapsing-remitting MS (RRMS). As disease progresses it takes on a more chronic degenerative form (58, 61, 62), secondary progressive MS (SPMS). There also exists a form of MS with a constant progressive development from start; primary progressive MS (PPMS). In most MS patients disease starts out as RRMS, whereas 10-15% will suffer from PPMS from the beginning (63), and the higher the age at onset, the more likely it is that the disease will have a primary progressive course. Unlike for the traumatic injuries, there has been a rapid advance in the MS treatments (64, 65), but these treatments in general target lymphocytes and affect the relapsing-remitting phase, whereas effective treatments are still is lacking for the progressive forms, perhaps illustrating the poorer understanding of the mechanisms active in this disease phase.

2.2.3.2 Alzheimer's disease and other dementias; global brain degeneration

AD is the most known and common form of dementia, estimated to further increase in prevalence (66). The disease is characterized by progressive and general loss of grey substance, neurons, and thus illustrates an example of global brain degeneration. Histopathologically in the AD brain there are intracellular neurofibrillary tangles composed of abnormally phosphorylated Tau protein and extracellular formation of senile plaques consisting of misfolded amyloid-beta (67). The pathophysiology in AD consists of a complex interplay between multiple processes. One hypothesis is that an unknown trigger induces a low-degree local and systemic inflammation and stress (62) to the by age more susceptible neurons that gradually lose function (67) which leads to neuronal disconnection (68) and metabolic disturbances with subsequent accumulation plaques and tangles. There is a strong genetic component contributing to the risk of developing sporadic AD, with the strongest effect conveyed by the apolipoprotein E4 (ApoE4) allele (69), but also a number of other genes have been uncovered by genome-wide association studies (GWAS) (70), several of them implicating involvement of the immune system (56). Also genetic variants in butyrylcholinesterase (BChE), an enzyme that hydrolyzes acetylcholine (ACh), have been linked to more severe AD development (71). There exist multiple other types of dementia, all of which are more common with increasing age, however, the normal ageing process should not lead to dementia, but should rather be seen as the strongest risk factor (67).

Treatments for AD are directed at inhibiting the activity of the cholinesterase's (acetylcholinesterase; AChE and BChE) and aim to increase the levels of ACh (72), a neurotransmitter important for most cognitive processes but also increasingly recognized for its anti-inflammatory properties (73). The treatments are to some degree able to slow down cognitive deterioration (71).

2.2.3.3 Amyotrophic lateral sclerosis and Parkinson's disease; loss of specific neuronal populations

ALS is characterized by a gradual loss of motor neurons in the spinal cord, brain stem and motor cortex, which leads to progressive muscle weakness and ultimately to respiratory failure and death. Disease pathology in ALS is unknown, but analysis of familial cases identified mutations in the SOD1 (Superoxide dismutase) gene, involved in detoxifying oxygen radicals, which suggests the involvement of oxidative pathways in the disease (74). However 90-95% of ALS cases are sporadic, i.e. without known familial/genetic predisposition, which implies that there are multiple disease pathways active in ALS, where also inflammatory activation of microglia and astrocytes is known occur. Contrary to most other diseases, extensive physical activity and slenderness are independent risk factors for developing ALS (75). To date there is no causative treatment for ALS.

PD is the second most common neurodegenerative disease after AD (76) and is like ALS also caused by loss of specific neuronal population, the dopaminergic neurons in the substantia nigra, which leads to the characteristic motoric disturbances with tremor, ataxia and difficulty to initiate movements. But later in the disease course the PD patients also often tend to suffer from dementia and psychiatric diseases. Histopathologically neuronal loss is seen, together with formation of intraneuronal Lewy bodies that are aggregates of an abnormal form of α-synuclein protein. The causes behind PD development are still not well known, but as for AD the major risk factor is age, although exposure to certain pesticides has been suggested to increase the risk, whereas smoking has been linked to decreased risk of developing PD (76). As for ALS, the genetic influence is not so strong as 90-95% of the cases are sporadic (77). Recent advances in genetic technology has led to the identification of multiple new disease-associated genes that are involved in oxidative stress, mitochondrial and lysosomal dysfunctions (77) which tell us more about disease pathophysiology. Although there is no curative treatment, symptomatic treatment of motoric symptoms can be achieved quite well pharmacologically or neurosurgically (78). Since more than 30 years there has been a vision to replace the lost dopaminergic neurons with new neurons or stem-cells, however, results have been somewhat disappointing (79).

2.2.4 Neurological injury and disease- summary

Medical improvements have increased the survival from all kinds of acute injuries as well as the overall life expectancy in general in most countries. But with more TBI survivors and a growing number of older aged the incidence of neurodegenerative diseases, like PD, ALS and AD as well as of stroke will also increases, meaning that disease burden for affected individuals, families and society will escalate. Neurologic

diseases are therefore something that concern us all, both as medical professionals and private persons- and we still have a lot to learn.

Clearly, there are large differences in pathophysiology between acute insults and slower progressing diseases, but there are still some basic principles that hold true for most CNS pathology:

- The degree of neuronal loss is what correlates best with extent of disability.
- Genetic variability in the population can partly explain both susceptibility and the heterogeneity of disease course in affected individuals.
- Even though direct genetic influence is not always strongly linked to disease development the identification of genes has taught us a lot about disease pathogenesis and involved pathways.
- Immune reactions occur at some point in the disease process- either as a direct cause of disease, or as a consequence of the insult, but are in both settings of relevance to disease progression.

As a last interesting link between the nervous and immune systems, a well-studied phenomena is the fact that CNS injury, either severe brain (80, 81) or spinal cord (82) injury also influences the immune system, instead of only in the opposite direction, leading to a state of immune suppression. The role of this is not clear, as infections are one of the leading causes of death following severe neurological injury; but it is speculated that the immunodepression could be beneficial for the harnessed CNS, as an excessive inflammation could exaggerate the injury (80).

3 THE IMMUNE SYSTEM- WITH FOCUS ON ITS ROLE IN THE CNS

Immune reactions are, as mentioned, implicated in most CNS disease states. However, the immune system is too complex and diverse to describe in detail, and the following section will therefore give a brief and simplified overview, focused on the role of the immune system in the CNS.

It was long believed that CNS was an immunoprivileged organ, separated from the rest of the body by the blood-brain barrier (BBB). This is not the case, although the immune system in the CNS is somewhat different, as large molecules, like acute-phase proteins, complement and antibodies do not readily cross the BBB, at least not in the healthy state. There are in general less immune cells in the CNS as compared to the periphery, as for instance T cells are only rarely encountered in the intact CNS (83, 84). However, in different disease states, the BBB becomes leaky, or even ruptured, which exposes the CNS to factors from the general circulation, but it also exposes the general circulation to substances from the CNS (85). The role of the immune system in the CNS is to protect from infection but also to maintain homeostasis and prevent development of autoimmunity by clearing debris (86, 87).

The immune system is classically divided into the innate and the adaptive arm, where the innate is constitutive and acts rapidly but stereotypically after insults, whereas the adaptive system requires a series of triggers to function effectively. Both systems can in turn be further subdivided into the cellular compartment, i.e. the different immune cell types, and the humoral compartment, i.e. soluble proteins and substances, which participate in immune responses. In most immune responses there is however a constant interplay between the innate and adaptive arms, where the bottleneck and bridge between the two is the process of antigen presentation.

3.1.1 Antigen presentation, MHC molecules and HLA haplotypes

The process of antigen presentation constitutes both the interface and the dividing line between innate and adaptive immunity, and also explains the mechanistic background to development of autoimmune disease. Adaptive immune responses are initiated by antigen presenting cells (APCs). There are a few types of cells that can act as APCs, the most acknowledged are: dendritic cells (DCs), macrophages, microglia and B cells, all which will be further described below. An antigen is any substance recognized by a T cell receptor (TCR) or an antibody, for instance part of a bacterial cell wall or a fragmented viral surface receptor. Antigens can also be derived from the organism itself, but the immune system is supposed not to react to these as this would cause a pathological and potentially harmful autoimmune response (88). There are several variants of the antigen presentation process, the most classic form starts when a foreign agent, for instance a bacterium is phagocytized by an APC. The bacterium is enzymatically digested in the phago-lysosomes into, amongst other things, small peptide fragments. In the lysosome the peptide fragments come into contact with a special group of immune molecules, the major histocompatibility complex class II molecules (MHCII), localized on the inner membrane of the lysosome. The MHCII

molecules have a unique three-dimensional structure where a cleft is formed in a part of the molecule into which a specific peptide fragment, i.e. the antigen, can bind like a key in a keyhole. The antigen binding part of the MHC molecule is assembled from randomized blocks of DNA sequences from within the MHC locus, meaning that all APC clones carry a unique set of MHCII molecules (88). The technical assembly process is highly complex and out of the scope of this thesis to describe in detail. But in practice, there are nearly an unlimited number of possible combinations into which the DNA segments can be arranged, which means that among the APCs in the body there should be those with an MHCII molecule that can bind the antigens present in the lysosomes. Only antigens that specifically fit the cleft of the MHCII molecule will attach to the MHCII molecule in the lysosome, with one peptide per MHCII (Figure 2).

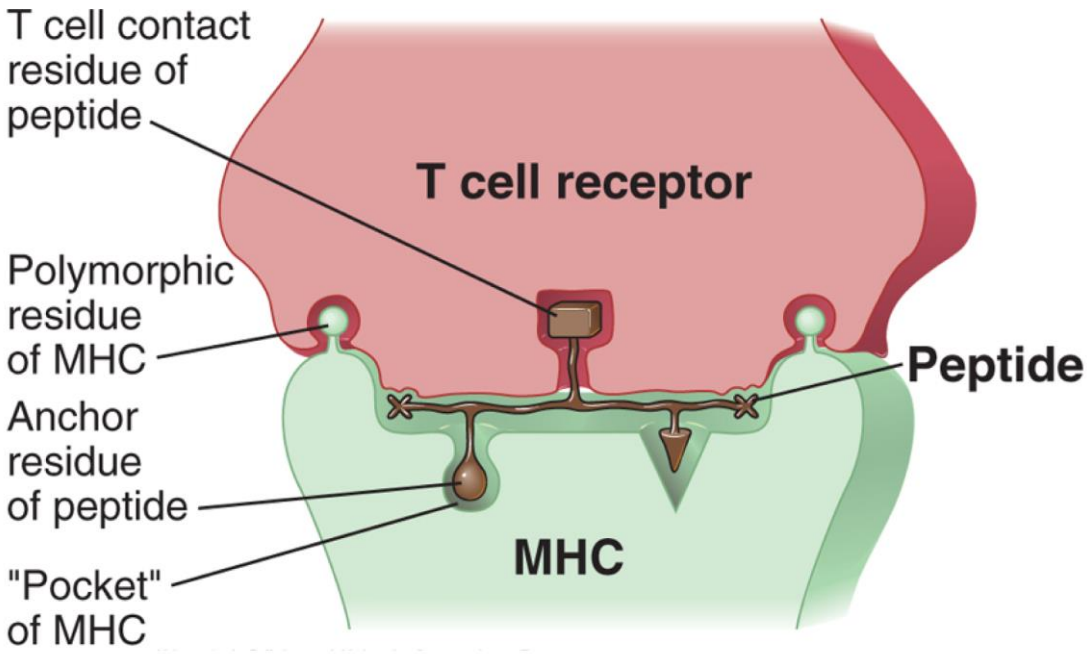

Figure 2. MHC and T cell interaction.
Illustration of the physical interaction between the T cell receptor on the T cell which recognizes the MHC-antigen complex on the antigen presenting cell, where the antigen fits in the cleft of the MHC molecule. Image adopted from:
Abbas, AK., Lichtman, AH., Pillai, S. Cellular and Molecular Immunology, 2010 (6th edition). Reproduced with permission from the publisher.

The antigen-MHCII complex is next recirculated to the external cell membrane of the APC and exposed to the environment outside the cell, where the complex (antigen-MHCII molecule) can come into contact with T helper cells (Th), also called CD4 cells. The Th cells have a TCR with a physical configuration that is supposed to match the three-dimensional shape of the antigen-MHCII complex (89) (Figure 2). If this happens, subsequent activation of the Th cell occurs with propagation of the adaptive immune response. The above process describes presentation of exogenous antigens (Figure 3) which is a task exclusively performed by the professional APCs.

Another form of antigen presentation occurs when endogenous antigens, i.e. antigens that derive from inside the cell, are presented to T cells. This process can, in contrast to

the presentation of exogenous antigens, occur in most cell types. In all cells there is a constant turn-over of proteins, which are degraded into peptides that are put back together into new proteins. This degradation occurs in the proteasome (90). In analogy to what happens with exogenous antigens in the lysosome, endogenous antigens come in contact with MHC molecules, but in this case MHCI molecules, in the proteasome (90). A complex is formed between a matching peptide and MHCI molecule, and the complex is transported to the cell surface and then exposed to the outside world, where the MHCI-antigen complex can interact with the TCR on a T cell (Figure 3). However, MHCI molecules do not interact with Th cells but with cytotoxic T cells (Tc), also called CD8 cells, further described below.

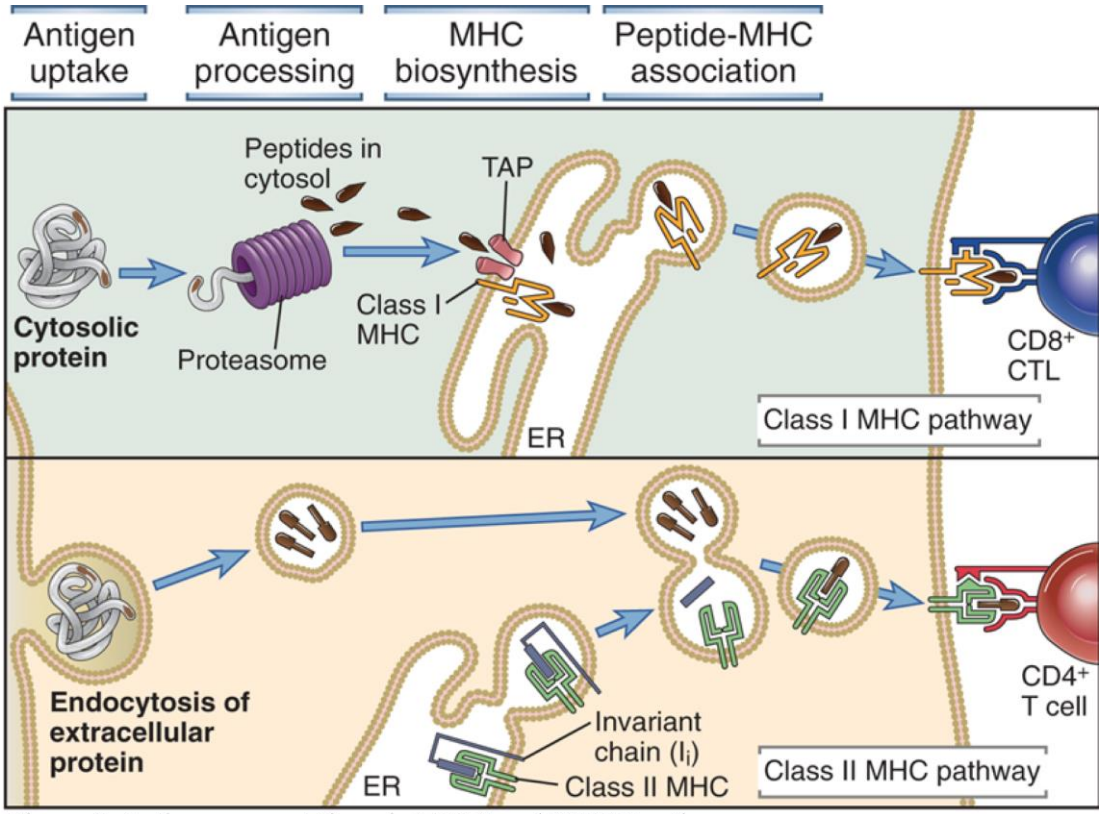

Figure 3. Antigen presentation via MHCI and MHCII pathways.
The MHCI pathway occurs in all cells, whereas the MHCII pathway is specific for professional antigen presenting cells. Image adopted from:
Abbas, AK., Lichtman, AH., Pillai, S. Cellular and Molecular Immunology, 2010 (6th edition). Reproduced with permission from the publisher.

In humans the MHC molecules are instead called human leukocyte antigens (HLA), class I and II respectively. The terminology and genetics in classifying HLA genotype would render a long explanatory section and is therefore left out. Within the HLA II region there are several genes that code for different HLA proteins, for instance HLA-DR, HLA-DP and HLA-DQ. These genes are inherited *en bloc*, and the combination of these genes is called haplotype. Importantly, the strongest genetic linkage to multiple autoimmune diseases, like Type 1 Diabetes, Coeliac Disease, Psoriasis, Inflammatory Bowel Disease, Multiple Sclerosis and Rheumatoid Arthritis, has been demonstrated towards certain HLA haplotypes (91). This is logical as CD4 cells play an instrumental role in these diseases and the HLA II molecules interact with the T cells. There is often large variation in HLA haplotypes between different population groups and ethnicities,

whereas within specific ethnic groups the variation can be quite small. This partially explains why some ethnic groups are more disposed to certain autoimmune diseases than others. It is believed that this is a result of evolutionary selection, as some HLA haplotypes can mount more efficient antimicrobial responses than others (92), but with the price of being more prone to autoimmunity. Knowledge of the HLA haplotype is also of outmost importance in transplantation immunology, as severe mismatch in HLA haplotype between donor and recipient will lead to development of antibodies and an immune response that in worst case causes rejection of solid organs (93), or a severe immune reaction towards the host, in case of bone-marrow transplantation.

3.1.2 Innate immune cells in the CNS

In the healthy CNS there are, apart from the microglia, few immune cells in the tissue. However, after acute injuries innate immune cells infiltrate the CNS. Neutrophils, the largest granulocyte population, strongly implicated in the defense against bacterial infections, are among the first cells to infiltrate the CNS (94), as early as within hours after injury (95). Neutrophils can contribute to tissue injury through multiple mechanisms and have mostly been linked to unfavorable consequences following CNS injury (95, 96).
The other main type of innate immune cells that infiltrate the CNS after injury are the monocyte derived macrophages (94, 97), often simplified into one population. Monocytes/macrophages constitute a heterogeneous cell population with multiple functions, and can act as both APCs and phagocytes that clear the environment of both tissue debris and pathogens. Macrophages are therefore important in both initiating, shaping and perhaps also dampening inflammatory responses, which they achieve through production of different cytokines. The panel of secreted cytokines are in turn dependent on the bi-directional macrophage/T cell interactions (98). Given the diversity and plasticity of macrophages it is not surprising that they have been assigned dual roles also after CNS injury. There is compelling evidence for the negative role that blood- and bone marrow derived macrophages have following CNS injury, for instance by inflicting additional axonal injury (99), contributing to inflammation and inhibiting axonal sprouting (100) that worsens motor function. However the opposite effect, where blood-derived macrophages improve motor outcome after spinal cord injury has also been demonstrated (101). This diversity suggests that there could exist subtypes of macrophages, which is the case, as at least two different subsets of macrophages, M1 and M2, have been identified, which convey protection from (M2) and exaggeration of (M1) injury respectively (102).

In the healthy state, microglia are considered to be in a resting condition and morphologically display a highly ramified shape in order to scan the CNS microenvironment and detect changes that may require action. In the case of a trigger the microglia become activated and undergo morphological changes, from the dendritic to a more round shape (103). The microglia are, like the macrophages, highly versatile, and can perform a number of tasks, like phagocytosis, antigen presentation and further immune propagation (103). And in analogy with the macrophages, there likely exists heterogeneous subpopulations of microglia.
Microglia constitute the main APC of the CNS (9), and are therefore important for eliciting subsequent immune responses, which includes recruitment of immune cells

both from the innate and adaptive compartment. As microglia are constantly present in the CNS it is not surprising that they are involved in all aspects of neurodegenerative disease, from beginning to end. And given their multi-faceted function it is easiest to conclude that microglia as a group probably contribute both with factors that are beneficial for the injured CNS as well with those that further propagate disease (104).

Astrocytes, are generally not considered immune cells, however they are increasingly recognized as an important part of CNS immunity (8, 105) as they can produce a number of cytokines that influence inflammatory responses. There is also data suggesting that astrocytes can act as APCs (106), although they are generally considered poor at this. Instead, the most acknowledged role of astrocytes in neuroinflammatory states is perhaps regulatory, even though their tendency to form glial scars often is seen as something negative following injury.

Natural killer cells (NK cells) are of lymphocyte origin, and alike the T and B cells they have to be educated during development in order not to be self-reactive and provoke autoimmunity (107). However, in one way NK cells can be said to have a function opposite to that of T cells; whereas T cells interact with MHC molecules in order to become activated, NK cells do the opposite- they kill cells that *do not* present MHCI on the surface. This is quite common in for instance virus infected or tumor cells (108), which often down regulate MHCI to avoid having their antigens presented on the surface and risk being killed by Tc's. However, there are also other signals which can override and activate the NK cell in spite of MHCI presence on the cell surface (108). NK cells have received an increasing amount of attention and been attributed with both protective and detrimental properties in the setting of neuroinflammation. For instance, a recent publication demonstrated an interesting role of NK cells in experimental autoimmune encephalomyelitis (EAE), the animal model for MS, where NK cells depleted auto-reactive and harmful microglia and thus contributed to neuroprotection (109). Also in MS there are signs that NK cells could be protective, as reduced numbers of NK cells correlated with increased number of relapses (110). However, as for many of the above described cell types, the roles and function of NK cells in CNS disorders are highly conflicting, as also multiple detrimental effects of NK cells has been demonstrated, recently reviewed by Poli et al. (111).

DCs are the most efficient APC of all cells, and as such key players in shaping immune responses. In the healthy CNS, DCs constitute a very small population, even though they can increase in number in pathological conditions (112). They are most commonly found in the perivascular spaces at the BBB, where they most efficiently can monitor the microenvironment. DCs are probably mostly of interest in triggering adaptive immune responses and of lesser interest in acute injuries. However there may be a role for DCs even in acute injury, as injection of DCs immunized with whole spinal cord homogenate improved outcome after SCI in both mice (113) and higher mammals (114).

3.1.3 Adaptive immune cells in the CNS

The cells from the adaptive immune system, i.e. the T and B lymphocytes, have been shown to confer both protective and deleterious effects following CNS injuries (87,

115, 116). This is perhaps not surprising as there, especially among the T cells, exist multiple different subtypes, and likely, all are not yet fully characterized. A simplified presentation of the lymphocytes follows below.

T lymphocytes are a highly diverse population, primarily divided into Th/CD4 and Tc/CD8 cells. All T cells carry the surface antigen CD3, which is a co-receptor to the TCR. The TCR is the molecular signature of the individual T cell and all T cells in the body thus carry different TCRs. This is not really true in practice, as activation of a specific T cell causes clonal expansion of this cell that leads to a large number T cells with this specific TCR. The TCR consists of several chains, which in turn are translated from genes that are assembled randomly from DNA segments from within a specific locus during T cell development (88) (Figure 4). The highly complex process of assembling the different genes of the TCR chains is somewhat similar to the assembly of the MHC genes. This is also one of the keys to why adaptive immune responses are exactly that- adaptive- and able to respond to almost all kinds of pathogens.

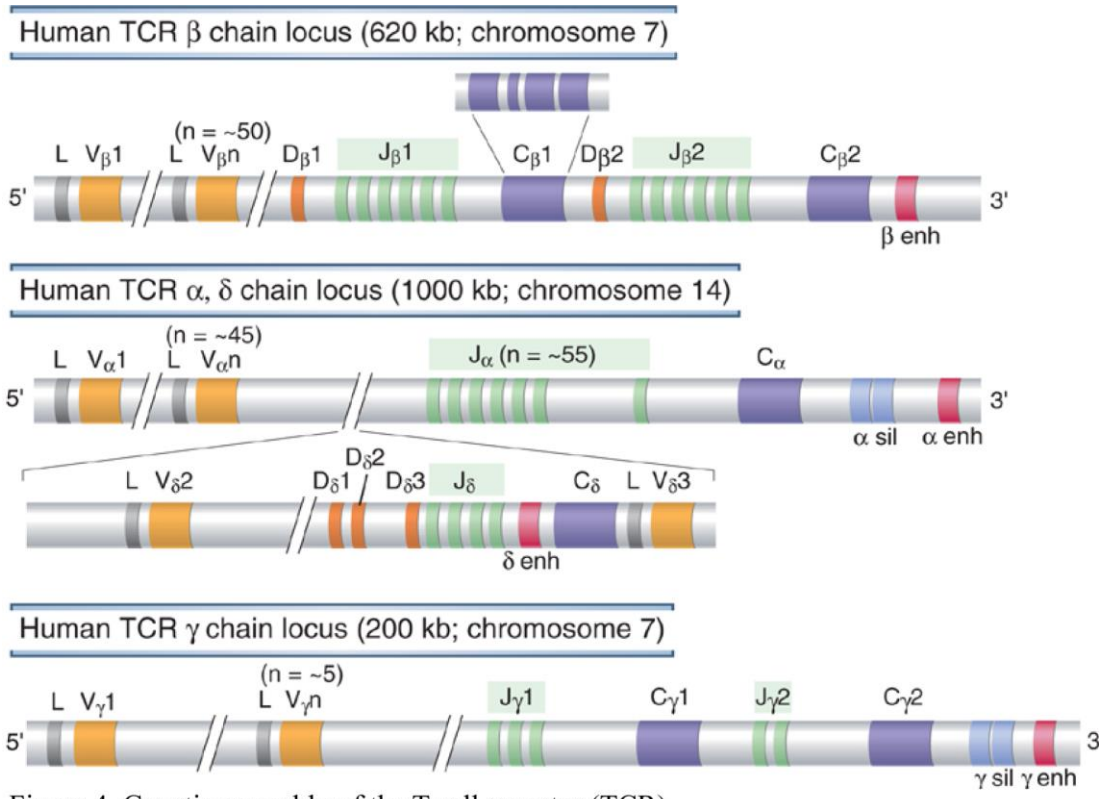

Figure 4. Genetic assembly of the T cell receptor (TCR).
In brief, the antigen/MHC binding part of the TCR is stochastically assembled from the rearrangement of specific DNA segments within the TCR gene. This can give rise to a near infinite number of combinations, which constitutes the basis for the plasticity that is necessary for the T cells to be able to interact with any presented antigen. Image adopted from: Abbas, AK., Lichtman, AH., Pillai, S. Cellular and Molecular Immunology, 2010 (6th edition). Reproduced with permission from the publisher.

CD4 cells interact with professional APCs that present exogenous antigens on MHCII complexes, as above. It is only when the CD4-APC interaction occurs that the CD4 cell becomes activated, and develops to a functional Th that next can continue to activate both Tc's and B cells, as well as macrophages and other cells from the innate immune compartment. Depending on the additional stimulations the CD4 cell receives, i.e.

cytokines and other molecular signals from the environment, the Th can be primed to different profiles, lineages. Examples of Th subclasses are the pro-inflammatory Th1 and Th17 cells, and the Th2 cells which instead promote B cell stimulation and antibody responses (117). The CD4 cells can also differentiate into regulatory T cells (Treg's) that are important for regulating and terminating adaptive immune responses. The plasticity of the Th cells enables adaptation of the immune response to optimally target the pathogen in question, but is also one of the reasons why immune responses can become misdirected and in worst case lead to autoimmunity (117).

The CD8 cells mediate action more directly than the CD4 cells, namely by killing of cells through cellular lysis. The CD8 cells monitor the body and scan for infected, and perhaps also tumor cells, by examining the antigens presented on MHCI molecules. They thus interact with most cell types, as MHCI receptors are present on almost all enucleated cells. If the CD8 cell recognizes an antigen that is not supposed to be found within the cell, for instance a virus antigen, this cell will be killed. For this to occur the CD8 cell needs to be equipped with a TCR that matches the MHCI-antigen complex. When this occurs the CD8 cell will become activated, kill the potentially infected cell and go through clonal expansion so that more Tc's with this specificity emerge and can help the body clear the infection. However, as the Tc's are potentially very harmful and can cause tissue damage an extra step needs to occur before the Tc is fully activated. This is mediated through Th cell signaling (88), even though exceptions from this Th mediated activation exists.

The B cells have dual roles- they partly produce antibodies, but they are also MHCII positive and can act as APCs and activate CD4 cells. However, in order to itself become a fully mature, antibody producing B cell, the B cell itself needs to be activated by a Th cell. The B cells carry a B cell receptor (BCR), which is specific for each B cell clone, in analogy with the TCR for T cells. The BCR is in practice an antibody anchored to the surface of the B cell and binds an antigen specific for that clone. BCRs are assembled similarly to the TCRs. The immature B cell circulates in the body to the secondary lymphoid organs, i.e. lymph nodes and the spleen, and when the BCR binds an antigen the antibody-antigen complex is internalized, processed and presented on the MHCII to a CD4 cell. The CD4 cell then becomes activated and amplifies the immune response, including activation of the B cell (88). The activated B cell undergoes clonal expansion and develops into a plasma cell, its final maturation stage. The plasma cell produces large quantities of antibodies that have the same specificity as the initial BCR, i.e. towards the antigen that started the immune reaction (88) (Figure 5).

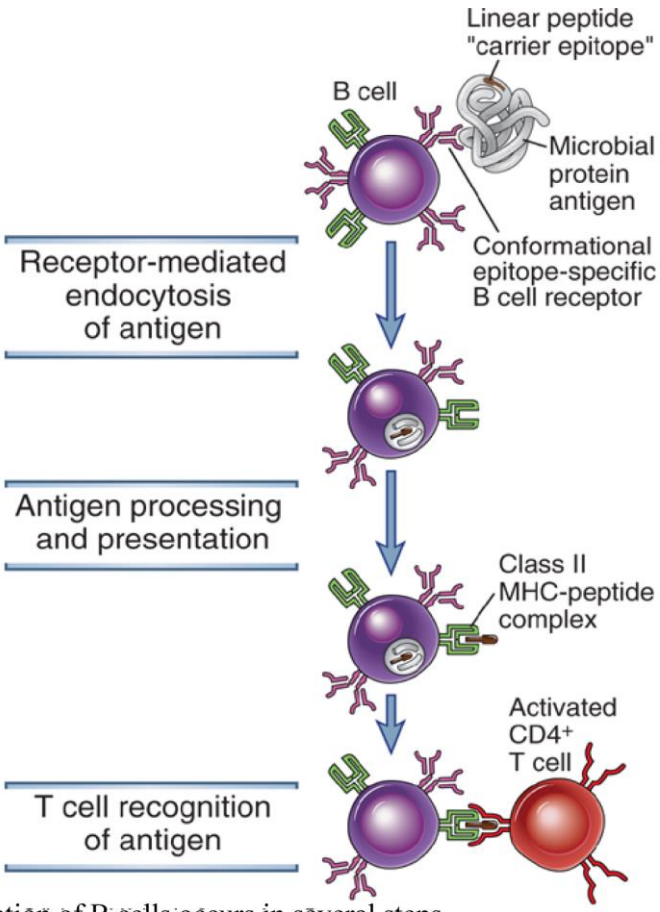

Figure 5. Activation of B cells occurs in several steps.
First the antigen is bound by the BCR which in the next step is internalized. The antigen is processed in the lysosome, bound to MHCII and recirculated to the surface where the MHCII-antigen complex is presented to a CD4 cell, which then activates the B cell. Image adopted from: Abbas, AK., Lichtman, AH., Pillai, S. Cellular and Molecular Immunology, 2010 (6th edition). Reproduced with permission from the publisher.

3.1.4 Cytokines and immune signaling

Cytokines are a diverse group of proteins essential for the intercellular signaling that occurs in all immune reactions; as such they cannot be classified as being a part of the innate or the adaptive immune system. In general, there exists pro- and anti-inflammatory cytokines that either amplify or attenuate immune responses. Examples of pro- inflammatory cytokines, like interleukin 1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) are secreted by activated macrophages at sites of inflammation and trigger and exaggerate immune responses (98). IL-4 and IL-10 are examples of anti-inflammatory cytokines, which can be secreted by the anti-inflammatory type 2 macrophages. But many cytokines can in different settings or combinations with other cytokines act either pro- or anti-inflammatory. A good example of this is transforming growth factor beta (TGF-β), which drives both pro-inflammatory Th17 and anti-inflammatory Treg development (118). Also IL-6 has dual roles, it is not only is one of the main pro-inflammatory cytokines, but also has well-established neurotrophic effects giving it a complex role following CNS injury (119).

A concrete example demonstrating the importance of the cytokines is seen in modern medical therapies, as some of the most potent drugs available for treating autoimmune diseases target cytokines. For instance biological compounds/monoclonal antibodies

that target TNF-α are highly efficient in treating multiple forms of arthritis, psoriasis as well as inflammatory bowel diseases (120). One of the major drawbacks of these powerful treatments, apart from that they are very expensive, is that they can lead to increased susceptibility to infections, and also reactivate dormant tuberculosis (121), which again demonstrates the significance of the cytokines. And, paradoxically anti-TNF treatments have been shown to lead to demyelination, both aggravating disease in patients with MS, as well as triggering demyelination in patients treated for other disease (122).

3.1.5 The humoral immune system

The term humoral immune system mostly refers to antibodies, although also the complement system often is seen as a part of the humoral immune system.

3.1.5.1 Antibodies

Antibodies, also called immunoglobulins (Ig), form an integral part of immunity and are one of the most refined end products of a successfully activated immune response. The antibodies are in practice slightly modified BCRs. The process of antibody production is complex, but will be briefly described below. All B cell clones carry a BCR, a membrane anchored antibody from the beginning of its development from the hematopoetic stem cell in the bone marrow. The part of the antibody anchored to the cell is the invariant part and does not vary largely in configuration between different clones, so that when the antibody is secreted, this side can be bound by Fc-gamma receptors (Fcgr) that are expressed on a variety of cells like macrophages and NK cells. The other end of the antibody is the antigen binding part; this is what varies between clones enabling the binding of the plethora of different antigens available. In simple, the antibodies can be considered Y-shaped, with the forked part the antigen binding end and the straight end the invariant end that binds Fcgr.
The antibodies are powerful effectors of the immune response and convey an array of actions, including direct binding and neutralization of microbes and toxins, enhancing phagozytosis of microbes/toxins, contributing to antibody mediated cellular lysis via NK cells and also to phagozytosis and cellular lysis via activation of complement (88). An additional strength of antibodies is that they are able to bind carbohydrates, lipids and more compound substances, as opposed to TCRs that only recognize peptides. The initial secreted antibodies belong to the IgM class, but there will also be a class switch towards IgG and IgA. The different classes of Ig's have different properties regarding solubility and function, but will not be discussed further here. As immunoglobulins are found in the serum of a patient that has been exposed to, or vaccinated towards, a certain pathogen they can be measured and detected as an immunological "scar", this is part of the immunological memory, and as there is a class switch during the response, from IgM to IgG, it is also possible to conclude the actuality of the exposure.

The role of antibodies in CNS disease is interesting, as normally, there should be very few B cells in the CNS, and also, the healthy BBB is not permeable for large proteins, like antibodies. The presence of antibodies in the CSF, seen as oligoclonal bands in electrophoresis assays of CSF, is indicative of B cell/plasma cell activity within the CNS and is present in 95% of MS patients (123). Even though systemic depletion of B cells has a good effect in reducing disease burden (123) in MS the specificity of the

antibodies in the CSF and not least their function in relation to disease has yet to be proven. A related observation is that following experimental brain injury in rats, antibodies directed against both neuronal and glial epitopes in the brain can be detected in serum (124). But, again, the significance of these antibodies could not clearly be shown, even though they could be considered a threat for developing further secondary brain pathology (124).

3.1.5.2 The complement system

The other major part of the humoral immune system is the complement system, an intricate biological system composed of numerous different proteins that act together in various conformations which can lead to an array of actions. This provides basis for flexibility, but also complex regulation (125). Many complement components are present at high levels in serum, and produced mainly in the liver, although synthesis is widespread also within the CNS (126, 127). The complement system is extensively studied in the current thesis and will therefore be further described in chapter 5.

4 GENES- THE BACKBONE OF PHYSIOLOGY

4.1 GENES, PROTEINS AND DISEASE

The central dogma of biology is that DNA is transcribed to RNA which is translated to proteins that in turn carry out the functions in the organism, including DNA replication, thus closing the circle (128). In this way genetic information can be safely and stably stored as DNA that is passed along over generations. The information stored in the DNA can after the right signal be made accessible and transcribed into the unstable, short-lived RNA at the time-point needed. The RNA is then translated into the desired effector protein that hopefully will correspond/respond to the signal that induced the DNA transcription, and the RNA will be rapidly broken down, so that the signal will terminate. Proteins are also steadily degraded, in the proteasome, in a little more complex process, not described further here, into amino acids that are recycled and put together into new proteins. The flow of genetic information is evidently not always this stream-lined. For instance, new knowledge has rapidly accumulated regarding mechanisms, such as modifications of histones and the structures surrounding the DNA that can have durative effect on gene expression, without changing the DNA sequence, a concept known as epigenetics (129). These signals are often believed to be environmentally triggered, making this an exciting interface between gene-environment interactions. But basically, by identifying a gene that is linked to a disease it also often means identifying a protein that is involved somewhere in the process of that disease. Pin-pointing this gene may therefore contribute to increased understanding of disease pathophysiology. Furthermore, as the gene codes for a protein and proteins are what perform the actions in the organism, the detection of a target gene associated with a disease can be the first step towards developing a directed pharmacological treatment.

4.1.1 Complex diseases

Most common diseases are what we call complex diseases, which means that the etiology is a combination of genetic predisposition and environmental influence. Thus, the genetic constitution of some individuals will make them more susceptible than others to develop disease, however there often needs to be a trigger (environment) for disease to develop. This partly holds true also for cancer, even though loss of control of cell proliferation is due to new mutations, some of the DNA changes in the cancer cells could be attributable to underlying genetic susceptibility to develop mutations, a phenomena well-known in several of the strongly hereditary cancer forms (130). However mutations are also caused by non-genetic, i.e. environmental factors, like for instance viral infections, toxins or radiation. The ever present role of genetics could in a more radical view perhaps be argued to be relevant even in trauma as hypothetically, some individuals are more prone to be exposed to trauma due to behavioral changes influenced by genetic predisposition.

The genetic heterogeneity within a population not only explains susceptibility to disease, but also some of the differences in the pathophysiological processes that occur during disease course. The impact of genetic variability manifests clearly in patients affected by TBI or MS, which are highly heterogeneous disease states, both with

respect to natural disease progression and also regarding the responses to medical treatments (131, 132).

4.2 GENETICS AS A TOOL TO DECIPHER DISEASE MECHANSIMS

Although the genetic heterogeneity makes diseases difficult to understand, the differences can also be exploited. For instance, we know that the severity of traumatic injury to the brain will differ between individuals in part due to genetic variability (131). If we start from two animal strains that are genetically different, and expose them to a standardized injury in an environment where we hypothetically can control all parameters, the genes are the only entity that varies and will thus be what govern outcome. If it were possible to identify the underlying genes, it would also mean a step forward in deciphering disease pathology.

A simple example of this is transgenic technology, where one gene is knocked out, and physiological differences between the knock-out (KO) and wild-type (WT) can be attributed to the missing gene. This is sometimes referred to as backward genetics. However, this may not be the most physiological way to study genetic influence, as in practice, genes are rarely knocked out, but rather mutated or slightly altered, i.e. they differ in their DNA sequence or expression dosage between individuals.

Another genetic tool commonly applied is the use of inbred strains. An inbred strain is created by consecutive brother to sister mating for at least 20 generations, with all offspring derived from a single breeding pair. After 20 generations all offspring are considered genetically identical (133). The inbred strains are genetically stable as they do not respond to selection and the DNA cannot change more, as long as continued brother-sister mating is performed, as they in theory are homozygous at every position. The inbred strains are also more uniform in their responses to for instance injury than outbred strains, meaning that smaller number of groups can be considered to achieve statistical significance (133). The main strength with inbred strains is however that the phenotypic differences observed between strains can be exploited. Two inbred strains that differ in outcome (phenotype) after injury, for instance nerve cell death after TBI, can then be bred, with mother from one strain and father from the other, to create an F1 (Table 1 and Figure 6). The first generation, F1, will all be genetically identical, at least the nuclear DNA sequence, and heterozygotes in every locus, as one chromosome is inherited from each parent/strain (Figure 6). An F2 generation is created by breeding F1 animals with each other. Due to the random recombination of DNA from one chromosome to the other during meiosis all individuals in the F2 generation will be unique, with randomly sized and placed segments of DNA from the original strains (Figure 6). In each locus 25% of the animals will be homozygous for strain A, 25% homozygous for strain B and 50% heterozygotes, on average. By genotyping the animals in the F2 generation, and linking genotypic and phenotypic variation with each other, gene regions can be identified that regulate phenotype, for instance motor neuron death after nerve injury (134). This is the principle of Quantitative Trait Loci (QTL) mapping and is sometimes called forward genetics. The advantage of this approach is that it is unbiased, as opposed to hypothesize driven research, and enables the discovery of new pathways, which could otherwise have been overlooked, if one instead chose to

knock-out a specific gene and then study effect. The disadvantage is that is very work intensive, due to large-scale breeding and tedious genotyping. However, there may be additional influence from for instance mitochondrial DNA, which is inherited from the maternal side, and also from which parent each chromosome is inherited due to genetic mechanisms such as imprinting, which has to be taken into account when creating the F2 cross, so that equal numbers of the four possible groups of F2 are bred (Table 1). To study mechanisms such as imprinting and parent-of-origin effects, backcrosses can be created, instead of the F2, where the F1 offspring instead are bred with one of the parental strains (Figure 6).

Parent	AA (f)	BB (m)	BB (f)	AA (m)
Parent breeding couples	AA (f) x BB (m)		BB (f) x AA (m)	
F1	AB (f)	AB (m)	BA (f)	BA (m)
F1 Breeding couples	AB (f) x AB (m)	AB (f) x BA (m)	BA (f) x AB (m)	BA (f) x BA (m)
F2	Group 1 (m/f)	Group 2 (m/f)	Group 3 (m/f)	Group 4 (m/f)

Table 1. Breeding setup when creating an F2 intercross.
A is a chromosome from strain "A", B is a chromosome from strain "B". Two groups of F1 animals are created ("AB" and "BA"), all genetically identical, as they all carry one chromosome from each strain, although the parent of origin is different. Four groups of F2 are created, depending on the combination of F1 female and F1 male. Furthermore, there exist males and females in each group. (f= females, m= males).

Another weakness with the F2 crosses is that since only 1-2 recombinations occur on each chromosome during meiosis the genetic fragments in each chromosome from respective strain are large, meaning that the genetic resolution is poor. Continued intercrossing by controlled random mating of the F2 individuals with each other can circumvent this problem and create Advanced Intercross Lines (AIL). By breeding two F2s with each other a G3 generation is created and by breeding G3 with G3 a G4 is created and so on. The F changes to G after F2 as before the F2 generation, siblings are mated, but from the F2 and forward, care is made not to mate siblings with another to ensure that the maximum number of recombinations occur. G3 is thus the first non-sibling cross (Guidelines for Nomenclature of Mouse and Rat Strains, Rat genome Database, http://rgd.mcw.edu). For each generation more and more recombination's occur and the genetic fragments from each parental strain will become smaller and smaller, giving an increased genetic resolution (135), but also the need for denser genotyping when performing QTL mapping.

A further development of the inbred strategy is to use congenic strains, where a genomic segment from one strain is bred onto the background of another strain, by repeatedly back-crossing one strain to another (Figure 6). This needs to be continued for at least 10 generations in order to decrease the amount of DNA from the original strain to a minimum and only have the desired gene fragment from that strain on the "pure" background of the recipient strain. On average, only half the background DNA will be transferred on each generation, this means that after 10 generations (0.5^{10} <0.001) 99.9% of the background DNA will be of the desired strain. After each generation, it is necessary to genotype the offspring to see that the desired DNA

fragment is still there. The use of congenic strains is a powerful method, likely more physiological than knocking one gene out, as it enables to study the function of genetic variation, as often is the case in the real life, rather than total lack of a specific gene. However, this is a labor intense method and it is very difficult to create a one-gene congenic, since one is dependent on the stochastic meiotic recombination to occur exactly at the congenic fragment. And in practice, most congenics carry gene fragments that contain multiple genes, and it can thus be hard to exactly prove which gene is responsible for the effect.

An interesting phenomenon commonly seen in congenics is epistasis, which implies that there are gene-gene interactions between the genes in the congenic fragment and the genes residing in the background genome where the fragment is integrated. This could give rise to unexpected effects, such as higher or lower expression of certain genes than in either of the parental strains (136, 137). Since the genes come from different strains, one can imagine that the unique interactions created in the congenic cause the balance to somehow become a little altered. This illustrates the complexity of gene interactions, and perhaps also suggests the imbalance that could potentially be expected to be created in a knock-out animal.

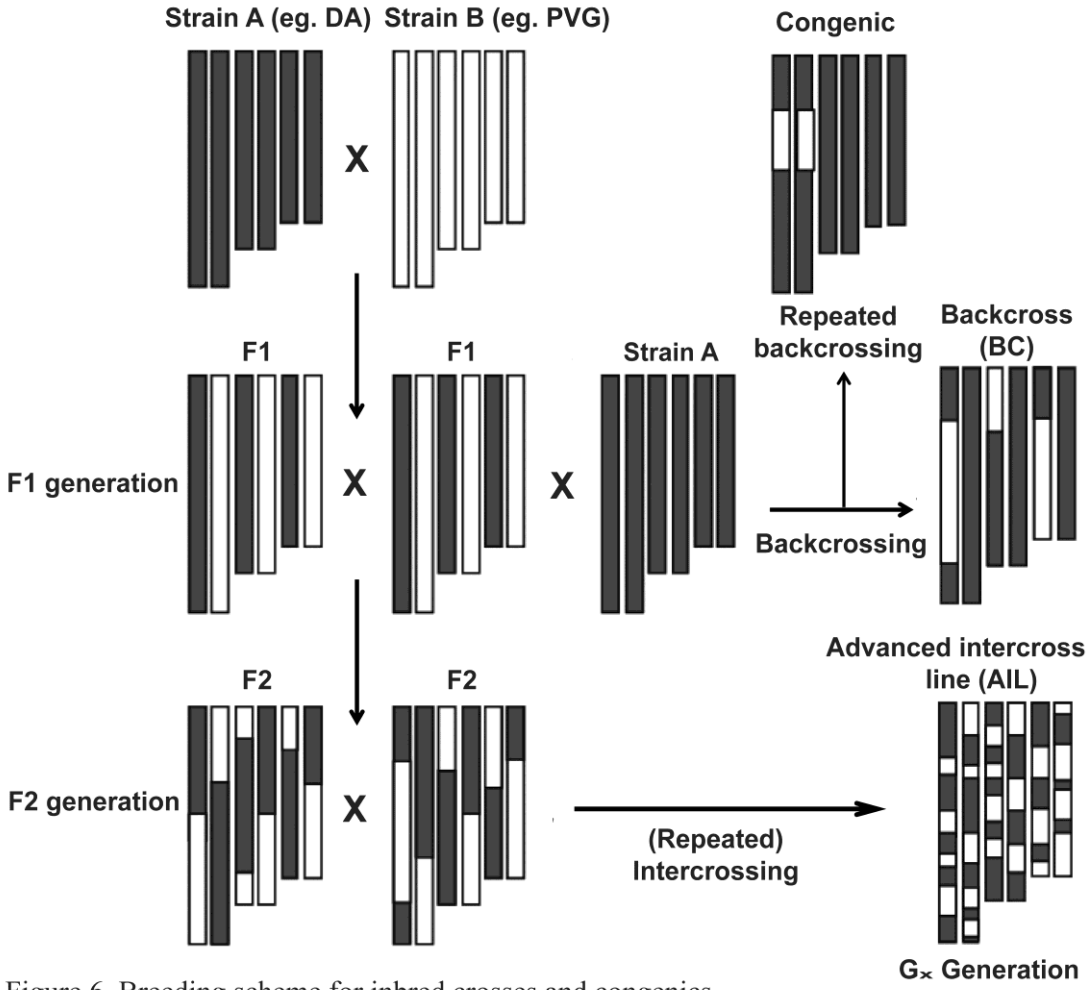

Figure 6. Breeding scheme for inbred crosses and congenics.
The inbred rat strains “A” and “B” are crossed which creates the F1 generation. All individuals in the F1 generation are heterozygotes, but genetically identical. By intercrossing the F1 siblings with each other an F2(AxB) intercross is created. The F2s are all genetically different due to the random exchange of DNA segments that occurs between chromosomes during meiosis (this also occurs when creating the F1s, but is in this case irrelevant as the parentals are homozygous at every locus). By further intercrossing the F2s with each other an AIL is created, with higher genomic resolution. By backcrossing the F1s with one of the parental strains a backcross is created, from which congenic strains can be developed by further intense backcrossing and genotyping.

5 DISEASE SPECIFIC MECHANISMS

Resting microglia or inactivated T cells do not readily cause disease. Instead, excessive tissue injury stems from misdirected actions that the cells perform. These actions are in turn the result of complex signaling pathways turned on at the right (or wrong!) time after a specific trigger, like trauma. And at every turning point in the long chain of events the genetic disposition of the individual controls, or at least fine-tunes the outcome. The following chapter will in more detail explain mechanisms and pathways explored or touched upon in the studies of this thesis.

5.1 THE COMPLEMENT SYSTEM IN DISEASE

The complement system is an important part of the innate immune system, composed of around 60 different proteins. The most acknowledged effector function of complement activation is the downstream assembly of the membrane attack complex (MAC). The MAC is a protein aggregate composed of the complement components C5b-C6-C7-C8 and C9 that together form a pore in the membrane of the target cell which ultimately leads to lysis of the cell (125, 138). However, many of the complement components do not only contribute to the formation of MAC, but also have discrete signaling functions and can bridge innate and adaptive immune responses (138-140). Especially C3, the key protein of the complement cascade has a wide array of functions with many active cleavage products/subcomponents (Figure 7) (141).

Complement activation can occur in three principally different ways, the classical pathway where C1q is activated by clusters of antibodies (IgG or IgM) bound to a surface, for instance a bacterial cell wall. The proteases C1s and C1r bind to C1q which starts a cascade that leads to cleavage of C4 and C2 to form C4b2b, which is a C3 convertase. The C3 convertase in turn cleaves C3, into C3a which is chemotactic, and C3b, which propagates the cascade towards MAC activation (Figure 7) (141, 142). The lectin pathway is activated by mannose binding lectin (MBL), a C-type lectin structurally similar to C1q, which binds to carbohydrates, abundant in for instance the capsule of many bacteria. To MBL binds a number of proteases (MASPs) which in turn cleave C4 and C2 to generate the C4b2b, the C3 convertase, that next activates C3, as above (Figure 7) (142, 143). The last pathway, the alternative pathway can in simple be said to be activated naturally, by the constant, slow spontaneous hydrolization of C3 that occurs in the body. This leads to the deposition of C3b fragments on cell surfaces, to which factor B, a plasma protein, binds which generates C3bBb, which also is a C3 convertase that cleaves C3 and propagates the cascade as above. However, whereas healthy cells protect themselves from complement activation by expressing complement inhibitors (125, 142), sick, dying or infected cells stop doing this and are thus targeted for removal (144). In this ingenious way homeostasis is kept and “bad” cells are removed, which perhaps also protects from autoimmunity (145).

The complement components in turn exert their actions via complement receptors (CR), some of the most common are CR1, CR2, CR3, CR4, C1qR and C3aR. The effect the complement component has upon binding its receptor depends on the cell type which expresses the receptor. For instance CR1 expressed on a B cell contributes to cell activation, whereas CR1 expressed on a macrophage will instead mediate

phagocytosis (146). Also, some of the receptors exist in both membrane bound and soluble forms; this constitutes a regulatory mechanism, as for instance soluble CR1 can bind and neutralize complement thus acting as a complement inhibitor (147).

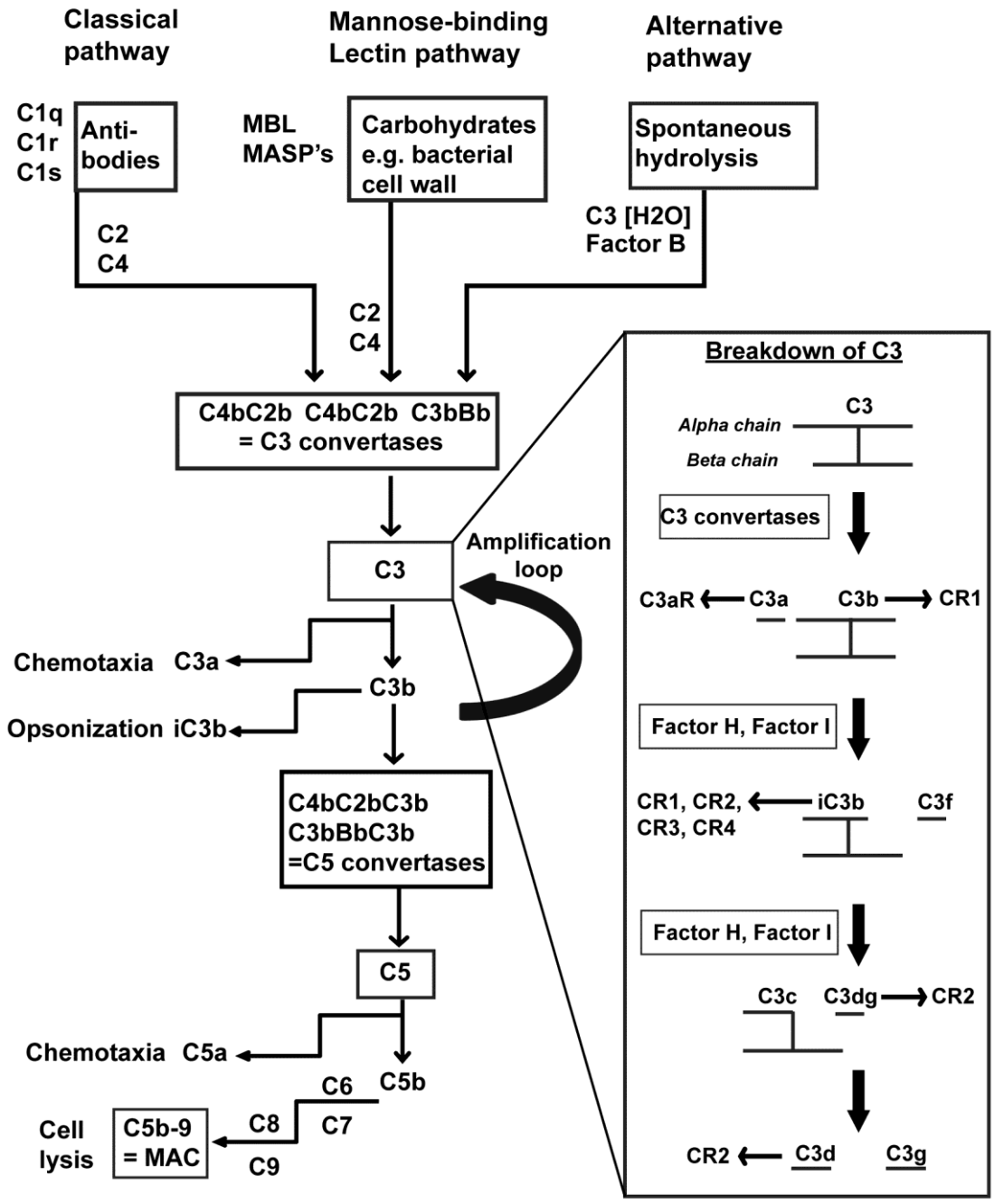

Figure 7. The complement cascade.
A simplified schedule demonstrating the three pathways that can activate the complement cascade. A few of the most important complement proteins are illustrated and some of the many functions the different components exert described. C3, the key component of the cascade, is broken down in multiple steps, and many of the components have active functions. A selection of the receptors that can bind the different C3 fragments are named.

Complement activation is implicated in a wide range of CNS diseases including acute conditions like TBI (148), SCI (147) and after stroke (149), as well as in states of chronic disease like AD (150), PD (150) and MS (151, 152). It has also been suggested

that complement is involved in the elimination of synaptic terminals, an important process during development, as an excess of synapses are formed in the immature brain (153), but this process can also occur in disease (154, 155). Especially C1q has been shown to increase the phagocytic capacity microglia leading to removal of apoptotic cells and debris (156).

But the role of complement in these disparate conditions is likely different. Following TBI there occurs both influx of complement from the systemic circulation secondary to the BBB defect caused by the injury, as well as local activation of complement in the brain (148, 157-159). This sudden increase in complement could overwhelm the MAC sensitive neurons (160) and possibly contribute to secondary injury. But is still not clear why local complement activation occurs, both after TBI as well as in the chronic CNS disease, nor the exact mechanisms by which complement exerts its function in the CNS. Perhaps the purpose of the local complement activation is to clear the microenvironment of dysfunctional cells and waste material in order to maintain a trimmed and functional CNS. But this balance is somehow lost in for instance progressive MS and AD, as well as in the secondary events after TBI, which leads to more degradation than initially intended and neurodegeneration as a consequence.

5.2 OXIDATIVE STRESS

Oxidative stress is a term frequently encountered in pathology, and means that the organism cannot detoxify the free oxidative radicals created after a certain event, which can be both endogenous (see below) or exogenous, like for instance UV-radiation (161). The free radicals constitute imbalanced molecules, with an unpaired electron, and are therefore highly reactive with almost any type of molecule in the cell including sugars, amino acids, lipids and nucleotides. The free radicals start chain reactions, as the unbound electron will continue to react until it is detoxified (161). Reactive oxygen species (ROS) constitute the largest and perhaps most known family of free radicals, although also reactive nitrogen species (RNS), like nitric oxide (NO) exist. Emergence of ROS or RNS does not naturally have to be pathologic, as ROS are created physiologically in the mitochondria as a part of the electron transport chain and energy metabolism and NO has a wide range of important physiological effects (162). The ROS family includes a number of derivatives of oxygen, including superoxide anion ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$) and several more (161). However, both ROS and RNS have been linked to a plethora of disease states, from cancer to neurodegeneration (163), not surprisingly as the radicals cause both immediate damage, like cell death, and also have long-term effects as they can affect the DNA, in turn of relevance for cancer development.

To protect the organism from the harm of radicals there exist a wide-array of anti-oxidants (161). One of the most potent anti-oxidants that exists in most cell types of the body is glutathione (GSH), which is capable of scavenging a number of both ROS and RNS (164). The glutathione-S-transferases (Gst) are a group of enzymes that conjugate toxic products to GSH (165). Notably, we found significant inherent differences in expression of Glutathione-S-transferase-alpha 4 (Gsta4) after both experimental nerve and brain injury (166, 167), where increased expression of Gsta4 was neuroprotective.

This demonstrates the important role of a functional response to oxidative injury triggered by insults to the CNS. Also, we saw that Gsta4 was expressed almost exclusively in the neurons whereas 4-HNE, a toxic aldehyde resulting from increased oxidative stress, which is effectively conjugated by Gsta4 to GSH, was abundant in both neurons and glia. This suggests that oxidative stress following CNS injury arises in both glia and neurons, but the neurons are equipped with an extra potent anti-oxidative defense.

5.3 T CELLS IN CNS PATHOLOGY

T cells are only rarely encountered in the intact CNS and infiltration is a tightly regulated process depending on recruitment/signaling events occurring in the tissue (83, 84). But, for example after a peripheral nerve injury, it is well established that T cells are recruited to the axotomized motor neuron pools (168), even though the molecular pathways behind this are not entirely understood. Distinct proof of the beneficial role of T cells after axonal motor neuron injury was elegantly demonstrated by using Scid mice, which lack an adaptive immune system, where replenishment of T cells increased motor neuron survival after injury (169). Experimental evidence also suggests that that T cells can mediate neuroprotective effects in models of chronic neurodegeneration (170).

There are many possible mechanisms by which T cells could confer neuroprotection, for instance that the T cells alter microglia phenotype towards a more protective M2 phenotype (171) or to a more DC-like profile (170). A more speculative mechanism could be that Tc's deplete auto-reactive/harmful microglia, as NK cells have been shown to do (109). A support for the protective role of Tc's was recently demonstrated when transfer of MOG-specific CD8 cells suppressed encephalitogenic CD4 cells and attenuated EAE (172). On the other hand it is important to acknowledge the well-established detrimental effect T cells can have for nerve cells in neuroinflammatory conditions like EAE (173). Also in MS, T cells are pivotal in driving the inflammation, at least in its relapsing-remitting form, which ultimately leads to myelin loss and subsequent axonal damage and nerve injury (61). An interesting twist to this was observed in rats exposed to spinal cord injury, where concomitant EAE was induced, which paradoxically led to increased survival of axotomized cells, associated with the infiltration of large numbers non-encephalitogenic T and NK cells found to express neurotrophic factors (174).

In summary- it is hard to give an unambiguous answer as to what the role of T cells are in different settings of CNS disease. This, as an array of both protective and detrimental effects has been demonstrated, and arguments for both sides can be found. Lastly, it should be emphasized the T cells are a very heterogeneous population, which should be taken into account when interpreting T cell data.

5.4 CHOLINERGIC SIGNALING

ACh is the most classic neurotransmitter and is fundamental in the CNS, PNS and autonomic nervous system (ANS), and has been an object for the Nobel Prize in

Physiology or Medicine when Henry Hallett Dale and Otto Loewi shared the prize in 1936 for their discoveries. ACh can be found in most living organisms, from algae and bacteria to humans (175), demonstrating that it also exists outside of the nervous systems. The action of ACh is mediated through nicotinic (nAChR) or muscarinic acetylcholine receptors (mAChR) and the effect ACh exerts differs depending on receptor type and cellular distribution. For example, ACh secretion at the neuromuscular junction from the motor neuron to the nAChRs on the muscle side starts a signaling cascade which leads to muscle activation (176). Whereas ACh release from autonomic nerve fibers regulates glandular secretion, sweating, blood-pressure and heart rate through binding mAChRs (177). Due to the potency of ACh to mediate signals it is a prerequisite to be able to terminate the action and regulate the activity of ACh. There is therefore a need for an efficient machinery to remove ACh from the synapses. Rapid hydrolysis of ACh is performed by AChE and also by BChE, two highly efficient enzymes present in high levels (178, 179) in both the blood and the CNS. ACh is thus very short-lived and has for long provided the neurobiological society with difficulties in assessing its exact localization and quantities (178). To restore the levels of ACh in the synaptic terminals and enable new signaling events, ACh is (re-) synthetized by acetylation of choline by the enzyme choline-acetyltransferase (ChAT) (178). There thus exists a fine balance between the cholinesterases (ChE) and ChAT.

But apart from its role as neurotransmitter, it has been known for some time that ACh also has immunomodulatory properties. These actions are mediated by ACh binding to a subclass of nAChRs, the alpha-7-nAChR (180), which apart from being expressed in the CNS also is expressed on multiple types of immune cells, endothelia and epithelia (181). By binding the alpha-7-nAChR ACh starts an intracellular signaling cascade that leads to modulation of TNF-α levels which in turn leads to reduced inflammatory activity (73). This is known as the neuroimmune reflex and is a topic of active research and an interesting bridge between the nervous and immune systems (182).

6 MATERIALS AND METHODS

6.1 ANIMAL MODELS

6.1.1 The rationale for using animals

In this thesis we have studied the perhaps two most sophisticated biological systems in life, the CNS and the immune system. Since complex disease arise and develop as a consequence from the interaction between multiple genes, each with a small impact, in concert with environmental factors it is necessary to be able control for as much as possible in the experimental setting. Because of this complexity it is also essential to be able to reproduce the experiments so as to obtain reliable and understandable results. Experimental animals living in a controlled environment allow us to control for a number of factors. The use of inbred strains reduces the impact of genetic heterogeneity, which further improves reproducibility and also facilitates genetic mapping efforts.

All animal experiments were approved by the regional ethical committee under the following ethical permit numbers (VRA; N42/06, N32/09 and N122/11, SNT; N343/10 and 225/08, TBI; N122/11 and N365/08, EAE; N298/11 and N478/12). All experiments were performed under deep isoflurane anesthesia, with subcutaneous injections of Temgesic 3 days post-operatively as analgesia, as requested by the university veterinaries. The animals were at the end of the experiment euthanized by CO_2 and perfused with either PBS or a fixative depending on subsequent experiments. Details can be found in respective study (I-IV).

6.1.1.1 VRA- Ventral Root Avulsion

Through a skin incision and dorsal laminectomy at the levels of L3-L5 the left ventral roots of L3, L4 and L5 are severed from the surface of the spinal cord, at interface between the peripheral and central nervous systems (Figure 8). This reproducible axotomy specifically targets the pool of motor neurons in the ventral horn of the cord (183, 184) and leads to motor neuron death in this pool. VRA also induces a localized inflammation in the spinal cord, characterized mainly by microglia and astrocyte activation but also limited lymphocyte infiltration. There are multiple advantages with the model, not least a high degree of reproducibility for a CNS lesion model. A disadvantage is that it is technically rather challenging and cannot be performed in mice. Nerve avulsion injuries occur in the clinical setting, most often in the brachial plexus, and can be a result of traumatic delivery at birth (185) or following high energy trauma in the adult setting (186).

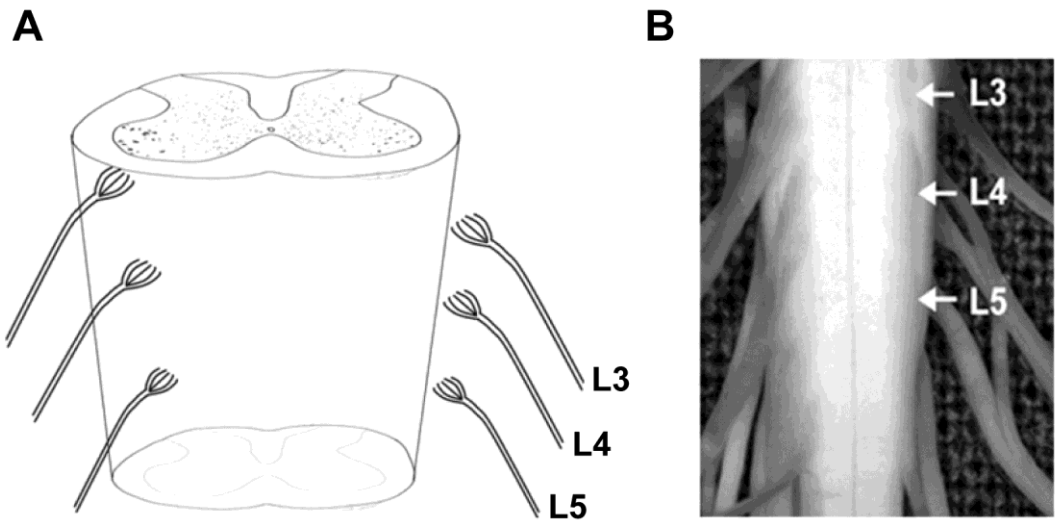

Figure 8. Ventral root avulsion- VRA.
Schematic picture demonstrating the ventral roots being avulsed from the anterior border of the lumbar spinal cord (A). Micrograph depicting the spinal cord of a rat five days after VRA showing the scar at the surface of the spinal cord where the ventral roots of L3, L4 and L5 have been (B).

6.1.1.2 SNT- Sciatic Nerve Transection

SNT is a lesion of the peripheral nervous system, where the sciatic nerve is transected below the obturator tendon on the thigh of the animal. This also induces a localized reaction in the spinal cord at levels L3/4-L6. As the sciatic nerve contains both efferent and afferent nerve fibres, the injury creates a response in both the ventral and dorsal part of the spinal cord. Activation of both microglia and astrocytes occur, and although the motor neurons are axotomized there is limited loss of motor neurons compared to VRA (187-189). SNT is easily performed in both rats and mice.

6.1.1.3 TBI- Traumatic Brain Injury

A contusion injury was performed using a weight drop injury model, as described previously (190, 191). In brief, the rats were placed in a stereotaxic frame and after skin incision the skull bone was removed 3 mm behind and 2.3 mm lateral to the bregma. A standardized brain contusion was performed by the releasing a weight (24 gram) from a fixed height (7cm) that slides in a tube and hits a piston (1,8mm in diameter) resting against the dura. The piston compresses the brain, with the dura intact, to a maximum depth of 3 mm. The animals show limited clinical symptoms after the injury.

6.1.1.4 EAE- Experimental Autoimmune Encephalomyelitis

EAE is an animal model widely used to study inflammatory demyelinating disease, i.e. MS. Animals are immunized using a CNS antigen, most often one of the following; purified myelin proteins such as myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) or peptides from these, even though whole spinal cord homogenate is also sometimes used. This is the to-date best, or at least most used experimental model of MS (192). However, it can be rightfully argued that there are considerable differences between MS and EAE especially regarding the time-phase of onset and the cellular subsets and humoral factors involved (193) as well as severity, as many of the animals die in the acute phase of EAE. Instead, EAE may perhaps be more

similar to acute disseminated encephalomyelitis (ADEM). Induction of EAE was used in study III, but only as a model of immune activation, to study T cells in lymph nodes.

6.2 CLINICAL DATA AND SAMPLING

In study II CSF and clinical data is analyzed. Both are obtained from patients attending the Neurology Clinic, Karolinska University Hospital, Solna, Stockholm. Written informed consent was obtained from all patients and the study was approved by the regional ethical committee. Clinical examinations were performed by a board certified specialist in neurology, and all patients diagnosed with MS fulfilled the McDonald criteria (194). An Expanded Disability Status Scale (EDSS) score (195) was determined at time of sampling by a certified rater. A control group consisting of patients with other non-inflammatory neurological/psychiatric conditions (OND) was also included. The patients in the OND group had normal MRI scans and no signs of inflammatory activity in CSF in terms of pleocytosis or intrathecal IgG production. In the MS group there were patients with RRMS, in both relapse and remission and SPMS. CSF was drawn at the time of initial examination and diagnosis, centrifuged, aliquoted and stored in our local biobank until further analysis.

6.3 EXPERIMENTAL METHODS AND METHODOLOGICAL CONSIDERATIONS

6.3.1 RT-PCR- reverse transcriptase polymerase chain reaction

RT-PCR, also called quantitative real-time PCR (qPCR), is a standard method used to quantify levels of mRNA and thereby transcriptional activity within the studied tissue. Detailed protocols and primer sequences can be found in the publications and manuscripts. The method is used in all the manuscripts and has the advantage that it is very sensitive and also very flexible, as primers for most targets can be designed. In general, mRNA levels correlate well with those of the corresponding protein (196, 197) however, the levels of the protein should preferably also be measured in the sample for exactness. A drawback with the method is that it does not provide information about the cellular localization of the expression.

Another drawback is that it is difficult to determine absolute mRNA copy numbers. Here, and in most other settings, relative levels of mRNA have been determined. This is done by comparing the target of interest with one or more housekeeping genes (HKG), i.e. transcripts that ideally are expressed at relatively constant levels in the tissue with low degree of regulation. Unfortunately, there is no perfect HKG since also expression of HKG's can be influenced by the intervention intended to study, and basal HKG transcription can also vary between strains. Therefore several different housekeeping transcripts may be used to increase the reliability of the assay. We have used at least two HKG in our experiments, hypoxanthine guanine phosphoribosyl transferase (HPRT), an enzyme involved in the metabolism of nucleotides and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) an enzyme involved in the glycolysis, i.e. metabolism of carbohydrates. Another commonly used HKG is beta-actin, which codes for a cytoskeletal protein involved in cell structure and also motility;

but as there is a high degree of cell migration and also damage to the cytoskeleton of the neurons in the models applied we chose not to use beta-actin.

6.3.2 IHC- Immunohistochemistry

Antibodies directed towards a specific epitope within a target protein structure that are equipped with a suitable detection system have revolutionized the way protein distribution within tissue can be assessed. The simplest technique is to use antibodies that are labeled with a fluorophore, which then can be directly visualized in a microscope. However, this technique is not very sensitive and a commonly applied method used to amplify the signal is to first use a primary antibody directed towards the target of interest, made in one species, and next a secondary antibody labeled with a fluorophore directed towards the antibodies from the first species. In this way the signal is amplified as several secondary antibodies can bind to the end of one of the primary antibodies. There are also more refined techniques, for instance the use of antibodies labeled with biotin. One molecule of biotin is covalently bound by multiple molecules avidin or streptavidin and each molecule of avidin/streptavidin can in turn be linked to different enzymatic systems which can amplify the signal many times.

A range of antibodies are used in this thesis and the details are described in the studies. In study I and II specialized software has been used to quantify synaptic density, after labeling a synaptic protein with an antibody. Immunohistochemistry (IHC) is otherwise rather a poor method for quantification and is preferably used for qualitative purposes. IHC provides a good mean of visualizing the spatial relationship between structures in tissue, and also for demonstrating the presence of a certain target. However, it is extremely important to include both positive and negative controls, as the absence of a presumed target in a tissue may indicate that the antibody/detection method is not sensitive enough, and equally to include negative controls as a positive signal from the tissue could instead implicate that the antibody, or detection system labels structures unspecifically.

6.3.3 FC- Flow cytometry

Cells in solution are labeled with antibodies, usually directed towards surface antigens, even though staining of intracellular antigens is possible. The labeled cells are then passed through a flow cytometer where laser technology is used to count, and sometimes also sort, the labeled cells. This is a routine method in immunology and hematology, since it is easily performed in cells not firmly attached in a tissue, which unfortunately is the case for CNS cells. It is therefore more difficult to extract cells from CNS tissue and there is also often disturbances from sticky myelin debris, although techniques are emerging to improve this (25). This method is used in studies III and IV and protocols are described therein. It is the gold standard to quantify cell numbers, but can also detect cell sizes and, depending on the labeling protocol, the technique has ever expanding applications. There are some disadvantages with the method, as for instance it is not always possible to have completely specific markers for the cell (sub-)population intended to study, as cells rarely demonstrate a “one or zero” expression pattern of surface receptors but more often display a sliding scale. This means that within the cell population intended to study there could hide other cell populations, for instance macrophages among the cells sorted as dendritic cells.

However, this can often be controlled for by adding additional markers, enabling gating of populations to smaller subgroups. Lastly, it is also rather an expensive method, at least the acquisition of the flow cytometer.

6.3.4 ISH- In situ hybridization

Labeled ribo- or oligonucleotide probes are hybridized with mRNA in tissues. The ribonucleotide probes (riboprobes) are more similar to the mRNA, as they are based on the correct sugar moiety, compared to the oligonucleotide probes (oligoprobes) that instead are DNA based, and the riboprobes therefore give a stronger binding. Riboprobes are however more unstable in themselves than oligoprobes and also more complex to construct and we have therefore used oligoprobes of between 40-48 bases in our studies. It is important to make the probe highly specific, both in order to make it sensitive enough to bind, and also to make it specific and avoid picking up other targets. The method can be used both to localize and quantify, based on signal intensity, mRNA in tissue. It is thus a good complement to RT-PCR. To detect the probes, they are coupled to either fluorophores or radioactive isotopes. The radioactive method has obvious disadvantages with radiation; however it is in general more sensitive than fluorescent methods. Although relatively simple in theory, it can be quite a difficult method in practice, in part because the relative instability of the mRNA makes it difficult to get the probes to hybridize in the tissue. The sensitivity is therefore significantly inferior to RT-PCR. The method is used in study I.

6.3.5 Elisa- Enzyme-linked immunosorbent assay

Also a standard method in molecular biology that uses antibody-based technology to quantify protein levels in solutions. The reliability and specificity of the assay thus depends on the antibodies used. The sensitivity is in general very good, as the signal can be amplified many times by coupling the antibodies to different substances. The method is a good complement to RT-PCR, as it enables quantitation also at protein level. The method is used in studies I, II and III.

6.3.6 Nerve cell counts

Motor neurons in the ventral horn are due to their morphology easily recognized and have in our studies been manually counted in tissue sections by a person blinded for strain or intervention. This method to quantify motor neurons is less sensitive than more modern stereologically based methods. Although stereology is more stringent, it also very resource demanding and time-consuming. Manual nerve cell counts were used in study IV, however, importantly in a recent study we performed (166) both methods were used and correlated very well, although variability was less when using stereology.

6.3.7 Western blot

This method is performed in study II and was carried out by our collaborators Kerstin Sandholm (Linnæus University, Kalmar) and professors Bo Nilsson and Kristina Nilsson Ekdahl (Department of Immunology, Genetics and Pathology, Uppsala University). The method is used to detect proteins in solutions or tissue homogenate using electrophoresis, where the sample is applied to a gel that is strongly reducing so

that the proteins remain denatured. Via electrophoresis the proteins in the sample can be separated due to their differences in molecular weight and electric charge. Subsequently the proteins are blotted from the gel onto a membrane to allow for antibodies that are coupled to some kind of reporter system to provide a measure of signal intensity, in turn corresponding to protein quantity. The signal intensity is normalized to that of a structural or house-keeping protein. Western blot has higher specificity than ELISA, since it also provides information about the size of the protein (a ladder with several proteins of known sizes is run alongside the sample), and can also show for instance multimers of the protein. The technique can however be technically demanding. It should also be noted that the proteins are denatured in the gel, whereas in staining of tissues the proteins are in their three dimensional shape, which can be of relevance when choosing your antibodies.

6.3.8 Genotyping

The rats in the F2(DAxPVG) intercross were all genotyped. This was performed by extracting genomic DNA from the tail tips and analyzing simple sequence length polymorphisms (SSLP) using microsatellite markers, a method previously used also in human genetics (198). Microsatellites are simple sequence repeats of 2-6 nucleotides repeated up to 50 times that occur in the genome, often in non-coding regions (199). By PCR the fragment is amplified and the size of the product analyzed with gel electrophoresis, and as the microsatellites are highly polymorphic in length between individuals (and rat strains) they can be used to determine from which individual/strain the genomic fragment is inherited. But new methods have emerged for genotyping, more often using Single Nucleotide Polymorphisms (SNPs), which occur much denser in the genome (1 per 900bp) (200) than microsatellites (1 per 16000bp) (201) and in humans, this method has replaced the use of microsatellites. But there have not been sufficiently dense SNP maps in the rat so far to allow for this method to be used here. Thus, in order to genotype the F2 intercross a panel of polymorphic microsatellite markers was selected from the Rat Genome Database (http://rgd.mcw.edu) and the Ensembl database (www.ensembl.org) and spaced evenly throughout the genome with an average distance of 20cM based on previous knowledge of optimum spacing in an F2 intercross (202). Since only 1-2 recombinations occur per chromosome the spacing in the initial screen does not have to be denser. In an AIL however, the genotyping has to be very dense, and the higher the generation of the cross the denser the genotyping, as there are more recombinations. The F2 intercross was genotyped with 113 microsatellite markers. In the initial part of the studies we used a radioactive based manual gel electrophoresis methodology but at later stages a more modern fluorescent based method was applied. The genotyping data was used in studies I, II and IV.

6.3.9 Microarray and bioinformatics

Global expressional gene profiling using microarrays is a powerful method that enables analysis of the global transcriptome. mRNA from the tissue of interest is prepared, reverse transcribed, amplified and labeled to cRNA and then hybridized to special gene chips covered with oligonucleotide probes complementary to the cRNA. The abundance of mRNA of a special target is correlated with the signal intensity, and the stronger the signal from a specific probe, the more RNA. The chips covered 27000 genes, and thus enabled to assess the expression of most known genes. The technique

therefore offers a good approach when trying to understand complex diseases as identification of gene activation patterns and networks is made possible. However, analysis of microarray data is complex due to the vast amount of generated information. Interpretation of the data is in turn complicated by possible/probable interconnectivity/dependency between genes. This type of global expression scanning consequently needs to be paired with good expertise in bioinformatics and often also by the use of specialized software. But the methodology has provided valuable information regarding complex disease regulation and patophysiology (203, 204). We have used the technique in all four studies included in this thesis.

6.3.10 eQTL mapping

Gene expression is considered a phenotype, which can be linked with the genotype acquired from genotyping all individuals/animals in the studied cohort- this is an application of classical Quantative Trait Loci (QTL) mapping. In this way a genetic region that regulates expression of the studied gene can be identified, an expression QTL (eQTL). This is fairly easy when studying expression of one or several genes. However, when this is done using microarrays, all 27000 transcripts can be linked with all the genetic markers in the cross (in our case 113 microsatellite markers), of all the animals (in our case 144 animals) which creates an enormous three dimensional data bank. To analyse this requires solid expertise in bioinformatics and powerful hardware. These analyses were performed in collaboration with Dr Matthias Heinig and professor Norbert Hübner (Max-Delbrück Center for Molecular Medicine, Berlin). The method thus makes it theoretically possible to identify gene regions that regulate expression of every single one of the 27000 transcripts. By further advanced bioinformatical analysis using custom made programming (for details see study I and II) and simpler analysis using softwares like Partek Express® (Partek Inc., St. Louis, MO, USA) and Ingenuity Pathway Analysis software (IPA 9.0, http://www.ingenuity.com), a web-based software, it is possible to identify pathways and clusters of co-regulated genes. This methodology is unbiased, and acts like a screening, and thus enables the identification of new pathways that may not have been found in pure hypothesis drive research. The drawback is of course that it is technically demanding and also expensive. eQTL mapping is applied in studies I, II and IV.

7 RESULTS AND DISCUSSION

7.1 EQTL MAPPING FOLLOWING VRA

The DA and PVG strains have previously been shown to differ in neuronal survival following both VRA (205, 206) and TBI (167). However, in these studies nerve cell death was explored at later time points following injury (21 and 18 days respectively), and it is likely that events occurring early after injury will effect downstream events, like death of neurons. We therefore performed a large-scale attempt in characterizing the global spinal cord response 5 days after VRA. Five days was chosen as time-point since there is an intense, localized inflammation in the spinal cord around the harnessed neurons, but the neurons have not yet died. An F2(DAxPVG) intercross that consisted of genetically heterogeneous animals which all carry randomly distributed DA or PVG DNA at every locus (see chapter 4) was created. The F2 rats were operated with VRA and 5 days after injury the injured ventral quadrant of the spinal cord taken for global expression profiling analysis using microarrays. The animals were also genotyped and eQTL mapping performed. Almost 3500 eQTLs at $p<0.01$ for genome-wide significance level, out of which around 800 regulated in *cis* and the remaining in *trans*, were identified. This enormous data set constitutes the fundament of studies I, II and IV, and the methodology and experience drawn from this are also applied in study III.

7.2 STUDY I- SPECIAL FOCUS ON THE COMPLEMENT SYSTEM

Prior work in the group had identified C1q and C3 to be among the most differentially regulated genes between DA and PVG using a simpler form of microarrays, and expression of C1q correlated with loss of nerve cells (207). There is also clinical data suggesting that complement is involved in neurodegenerative processes. Thus, the gradual neurologic worsening that occurs in progressive MS, in relative absence of relapses and radiological signs of inflammation, in combination with the lack of response to immunomodulatory treatment, suggests that other disease mechanisms than a T cell driven immune response become more important (208-210). Components of the complement system are strong candidates for these mechanisms, as they have been linked to multiple neurodegenerative disease (150). There is also an increasing body of evidence indicating the complement system to be dysregulated in MS (211). We therefore focused on the regulation of complement expression following VRA in the F2s, with the aim of identifying new pathways explaining the activation and role of complement following injury.

While no genetic linkage could be detected for expression of most complement components, expression of C1qa, C1qb, C3 and C9 all displayed regulation from distinct gene regions. Expression of C1qa, C1qb and C9 were all *trans*-regulated from distinct eQTLs that all contained different *cis*-regulated C-type lectins (CLECs), but without clear inter-connectivity expression patterns of the transcripts within the gene clusters. On the contrary C3 expression was co-regulated with 47 other genes out of which a majority of the transcripts were interconnected. None of the genes in the cluster were CLECs. An expression network identified a link between C3 and BChE expresssion. BChE has multiple functions in the CNS, not least degradation of ACh (179). BChE seemed a highly interesting candidate for further studies as altered BChE

activity has been associated with both AD (212) and MS (213) and ACh can regulate inflammation.

The link between ACh-inflammation-C3 was further explored *in vitro* where glial cells from rat and mice were exposed to TNF-α stimulation and found to up regulate C3 expression, which in turn was abrogated by concomitant ACh stimulation.

We also explored if the link between C3 and BChE could be relevant in the clinical setting and therefore analyzed C3 and BChE levels in the CSF of MS patients. We correlated these levels with degree of clinical disability and levels of Neurofilament-light (NFL), an established marker of neurodegeneration in MS (64, 214) in a well-defined cohort of MS patients and controls. Interestingly, C3 levels correlated strongly with those of BChE, and also with NFL when including controls in the analysis, as well as to clinical disability. Also, C3 levels were highest in patients with progressive disease. The clinical data will be summarized in a separate manuscript, currently under preparation (Aeinehband et al).

In summary, the results demonstrated two major findings- partly a link between the complement system and the CLECs, and also a connection between C3 and the cholinergic system, via BChE. The identified pathways could also be involved in regulating complement in MS, especially in the progressive/degenerative phase.

7.3 STUDY II- A NOVEL ROLE FOR COMPLEMENT RECEPTOR 2 IN THE CNS

Since complement proteins mediate their actions via receptors a natural continuation from the first study was to explore the role of complement receptors following nerve injury. We therefore studied four of the most common complement receptors, Cr1-4, in the eQTL material and found an exceptionally strong genetic regulation acting on Cr2 expression, *p*-value $<10^{-6}$, with PVG alleles driving higher expression. This motivated further studies of Cr2, a gene otherwise most studied in the context of B cell immunobiology and with poorly known function in the CNS.

First, the *cis*-regulation identified in the F2 intercross was confirmed in a G12(DAxPVG) AIL, pin-pointing the differential regulation of gene expression to the gene itself, located at the end of rat chromosome 13. Secondly, a kinetic study of Cr2 expression following VRA was performed demonstrating that Cr2 is not expressed in the unperturbed spinal cord, only after injury, with peak expression occurring 7 days post-injury and the highest levels in PVG. B cells are very rare after VRA, and Cd19 expression was not measurable after VRA, arguing against B cell influx/activation as a source of Cr2. To confirm that Cr2 expression was of general interest in nerve injury/inflammation we also exposed DA and PVG rats to another type of injury, SNT, and also here saw a strong up regulation of Cr2 after injury, especially in PVG rats. The PVG rats are protected from nerve cell death as compared to DA following VRA, and here we show that the up regulation of Cr2 in PVG is associated with less drastic loss of synapses, an early neurodegenerative event, following SNT. As VRA is not

technically possible in mice, but VRA and SNT in rats yielded similar results we could bridge over and work with the SNT model in mice.

To assess whether Cr2 could confer part of the protection from synapse loss observed in the PVG compared to DA rats, $Cr2^{-/-}$ mice were exposed to SNT. The Cr2 knock-out (KO) was found to suffer from more pronounced loss of synapses compared to wild-type (WT). The pattern of Cr2 up regulation resembled the pattern of Gfap expression suggesting that astrocytes could be the source of Cr2; this was confirmed in cell cultures and via IHC. But since Cr2 is known to exist in a soluble form, and the IHC stainings were a diffuse in character we assessed the potential presence of soluble Cr2 (sCR2) in the CSF. Indeed, this turned out to be the case, with clearly induced levels after VRA in rats. The finding of sCr2 in the CSF of rats prompted us to investigate whether this could be the case also in human disease; sCR2 was therefore measured in a cohort of MS patients and controls, which revealed increased levels of sCR2 in the patients as compared to non-inflammatory controls.

There are a number of new findings in this study- not only that CR2 exists/can be up regulated in the adult CNS but also plays an important role as it protects from loss of synapses following spinal inflammation in rodents. Also, CR2 is shown to exist in a soluble form in CSF and is increased following injury in rats and in patients with MS, thus possibly adding to the bank of biomarkers reflecting neuroinflammation. The most established role of CR2 is to bind different fragments of C3. This has been functionally exploited in experimental therapies used to treat complement related pathology, which have greatly enhanced efficacy when linked to CR2 (215, 216). It is thus tempting to believe that elevated levels of sCR2 can balance the increased complement activation occurring after injury/inflammation, in analogy with sCR1 treatment which is protective after SCI (147). However, further studies, for example using in vivo cell type specific knock-down, are needed to demonstrate more in detail the role of locally expressed CR2 in the CNS.

7.4 STUDY III- IMMUNE CELL INFLUX AND COMPLEMENT ACTIVATION FOLLOWING TRAUMATIC BRAIN INJURY

TBI is a serious condition. The term TBI is however rather unspecific as traumatic brain injuries display large differences in severity. Furthermore, the processes that occur after a TBI are complex and dynamic and many continue for long time after initial injury. However, inflammation is increasingly recognized as an important component of the TBI response, with many different cell types being involved. Based on this, we were prompted to undertake a broad study that aimed at screening both the expressional machinery and also the cell types involved in the TBI response. As in the previous studies, we worked with the rat strains DA and PVG, that we previously demonstrated to differ in both neuronal survival (167) and T cell responses (217) after TBI.

We first performed a microarray expressional screening of the injured area of the brains from DA and PVG rats exposed to a standardized experimental brain injury model, as well as from healthy control brains from both strains. TBI induced large changes in

expression between injured and control animals, but there was also considerable influence of the strain where approximately 34% of the variability was affected by strain or strain in combination with injury and 60% by injury (and 6% noise). Pathway analysis of the data revealed that the inflammatory response was one of the most differentially regulated pathways between DA and PVG, both in naïve animals and after injury. Since inflammation is a wide concept, further analysis of which inflammatory pathways were the most important, or at least most strongly regulated, revealed that the complement system not only was the most differentially regulated pathway between DA and PVG rats both in naïve and after TBI, but also one of the most regulated pathways when comparing healthy to injured animals in both strains. When analysing the pathways that were the most differentially regulated between injured and naïve animals in general, i.e. from both strains, multiple processes linked to immune cell trafficking were identified. This first screen thus pointed to immune cell trafficking and complement activation as highly relevant to explore more in depth.

We established a new protocol for flow cytometry of cells from rat brains, which enabled a robust characterization of most cell populations, as well as to discriminate between macrophages and microglia, which is very difficult using IHC. We found, not surprisingly that microglia were the largest immune cell population, but it was interesting to see that the microglia outnumber the macrophages almost 10 times, and at later time-points almost 20-fold. We also found quite a large number of NK cells, with considerable strain differences in numbers and composition of different NK cell subsets, with more NK cells in DA, especially those that were CD161(bright), as compared to CD161(dim). This is interesting as the CD161(dim) have been demonstrated to have anti-inflammatory properties that are lost in the CD161(bright) (218), which perhaps contributes to the increased inflammatory milieu in the DA.

We also performed a detailed analysis of complement activity, regarding kinetics of expression and localization, at both mRNA and protein level, which revealed up regulation of complement, mostly in microglia, during the days following TBI. This could contribute to accentuated secondary nerve cell loss, since the neurons are in a susceptible state after injury. This was supported by the findings that both C3 and MAC labeled both neuronal cell bodies and axons. Also, the degree of complement activation correlated with increased levels of NFL in the CSF.

7.5 STUDY IV- THE ROLE OF C-TYPE LECTINS AFTER INJURY INDUCED SPINAL CORD INFLAMMATION

In study I we identified a genetic link between the complement system and different CLECs. As we previously have found an association between complement expression and survival of axotomized motor neurons following VRA (207) we were prompted to explore the role of C-type lectins after VRA further. Also, in the F2(DAxPVG) intercross one of the strongest regulated eQTLs in the whole data set, comprising more than 27000 transcripts, was the eQTL regulating expression of Clec4a3, also called Dendritic Cell Immunoreceptor 3 (Dcir3). Clec4a3 is localized on chromosome 4, in a segment that contains a cluster of C-type lectin genes. Fortunately, we had access to a congenic rat strain, Aplec, which has a small PVG segment containing only seven

genes, all CLECs and including Clec4a3, onto DA background. A comparison of motor neuron survival in Aplec and DA following VRA revealed significantly improved survival in Aplec. To assess the mechanisms behind this, global expression profiling of spinal cords in naïve and injured Aplec and DA rats was performed. This revealed that around 230 genes ($p<0.01$) were differentially expressed in the injured Aplec compared to DA. These genes were grouped into clusters using bioinformatical software which, apart from differential expression of CLECs, suggested involvement of T cells. Flow cytometry of injured spinal cords from Aplec, and the parental strains DA and PVG was performed, which revealed that the Aplec strain had significantly more infiltrating T cells than the other strains. In summary this study demonstrated that genetic variability in C-type lectins can have large impact on survival of nerve cells following injury, and also that T cells could be involved in this process. The CLECs are expressed by microglia, which suggests that the microglia in the strains (DA compared to Aplec), could be different from each other regarding their reactivity and signaling following nerve injury, which subsequently affects the recruitment of T cells to the site of injury that in turn affects the injured motor neurons.

7.5.1 T cell depletion studies in Aplec

To further explore the role of T cells in Aplec we performed an additional set of experiments where we attempted to deplete T cells using a monoclonal antibody directed towards the TCR. Aplec rats were treated either with R73 mouse hybridoma IgG1 antibody (Mabtech, Stockholm, Sweden) directed against the alpha-beta T cell receptor, TCRαβ, by intraperitoneal injection 1 day before surgery and with an additional injection at 3 days after surgery (5 days survival) or 4 days and 11 days after surgery (21 days survival). Control animals were injected with an IgG1 isotype control antibody, Ly128 (Mabtech), towards an irrelevant bacterial antigen (219). Motor neuron survival assessed at 21 days after VRA revealed that the animals that had received active treatment with R73 had worse survival of motor neurons compared to controls (Figure 9).

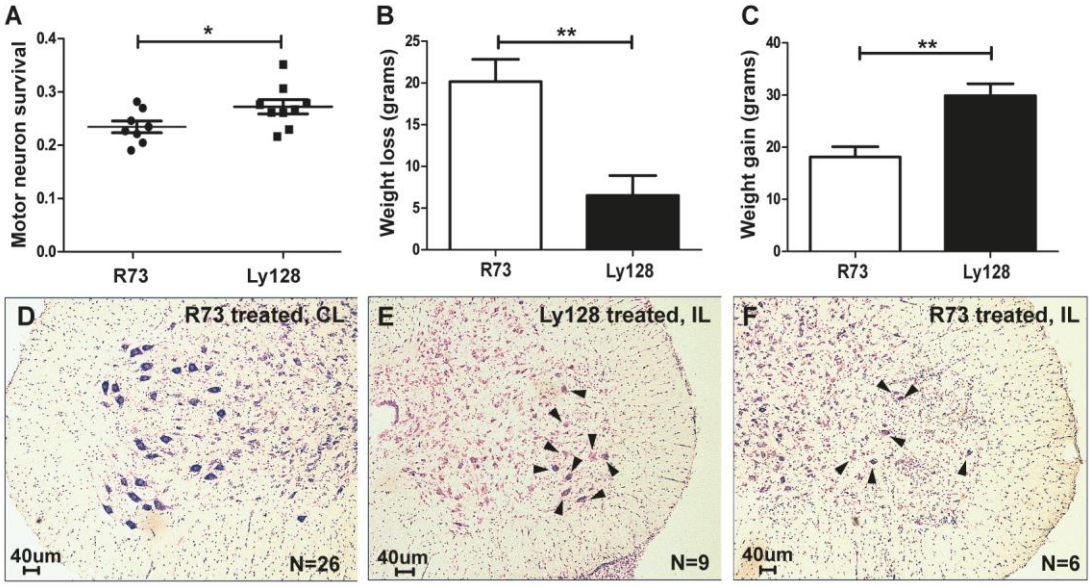

Figure 9. T cell depletion in Aplec leads to worse neuronal survival.
Treatment with a monoclonal antibody (R73), directed towards the T cell receptor, compared to treatment with an isotype control (Ly128), towards an irrelevant antigen, leads to worse neuronal survival at 21 days following VRA (A). The treatment also has systemic effects with more pronounced weight loss at 5 days (B) and less weight gain at 21 days (C) after VRA. Significant loss of motor neurons occurs in both groups in the ispilateral (IL) compared to contralateral side (CL) but is more pronounced in the R73 group (D-F). (* = $p<0.05$; ** = $p<0.01$).

However the animals that received the TCR antibody also lost more weight at 5 days after injury and had gained less weight at 21 days after injury which suggested that they in general were worse off. Therefore we could not conclude that the T cell depletion by this treatment directly affected the neurons, but that the worse neuronal survival could be a consequence of systemic effects.

To confirm that T cell depletion actually occurred following the antibody treatment, both in the systemic circulation and the spinal cord, we assessed this using flow cytometry, RT-PCR and IHC.

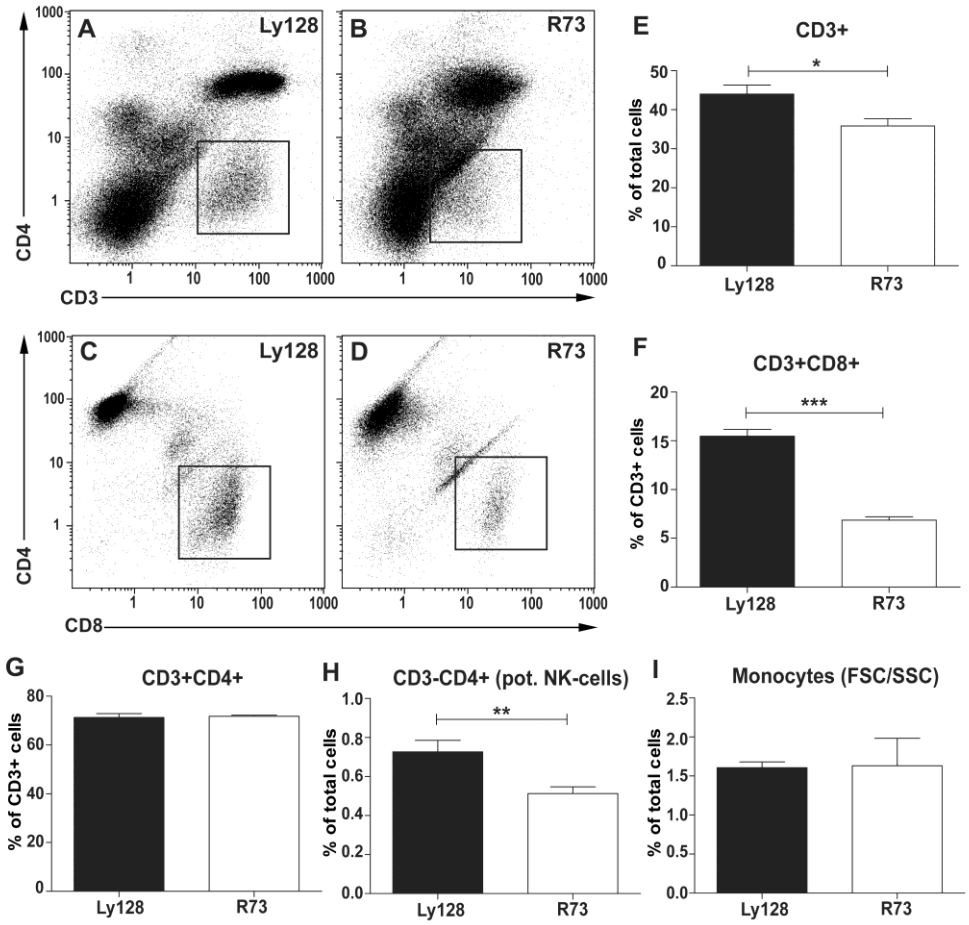

Figure 10. Flow cytometrical analysis of spleens at 5 days after VRA.
Analysis of animals treated either with the R73 or the control Ly128 antibody demonstrates that the R73 treatment depletes T cells as assessed with CD3 (A,B,E), but when studying specific T cell populations it can be seen that only CD8 cells are depleted (C,D,F), as the number of CD4 cells is unaffected (G). There also seems to be a depletion in CD3-CD4+ cells, which could constitute NK cells in the R73 treated group (H). Monocytes were unaffected (I). (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

The treatment with the R73 antibody unexpectedly depleted only CD8 T cells, whereas the CD4 population remained unaffected (Figure 10), as assessed with flow cytometry of spleens 5 days after VRA. This was verified also in the spinal cord as assessed using RT-PCR and IHC (Figure 11).

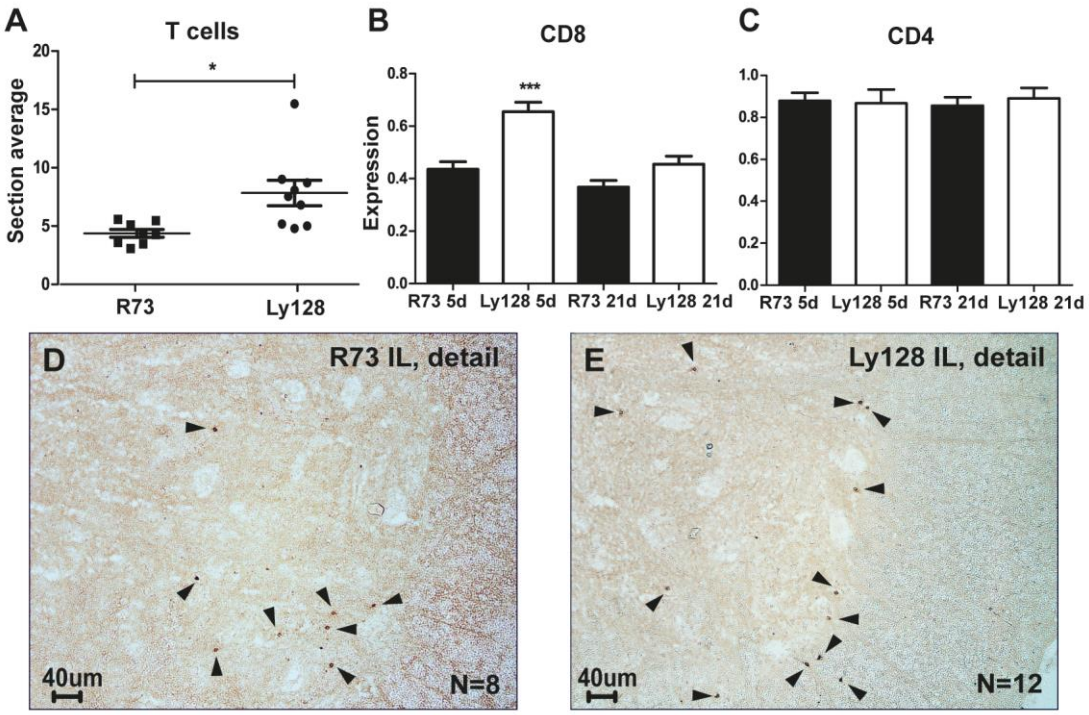

Figure 11. T cell depletion in the spinal cord.
Treatment with the R73 compared to with the Ly128 antibody depletes T cells in the spinal cord, assessed 21 days after VRA with immunohistochemistry (A, D, E). But, as in the spleen, only CD8 cells seem to be affected, since expression of CD4 is constant (B, C). (* = $p<0.05$; *** = $p<0.001$).

In summary, the T cell depletion experiments reduced the number of T cells, although far from completely and mainly affected the CD8 T cell pool. Also, the treatment had systemic effects as the animals treated with R73 compared to the controls lost more weight initially after injury and failed to regain as much weight as the controls at the end of the experiment. We could therefore not draw a firm conclusion as to the role of T cells in the spinal cord, but nevertheless the findings lend some support for the notion that T cells contribute to improved neuronal survival in the Aplec rats following VRA.

8 CONCLUSIONS AND FUTURE DIRECTIONS

8.1 THE LINK BETWEEN THE CHOLINERGIC AND THE COMPLEMENT SYSTEMS- AN IMPORTANT NEUROIMMUNE AXIS?

The acknowledged link between cholinergic signaling and immune regulation has definitely furthered understanding of how the CNS and immune system interact. This interplay perhaps also constitutes a key to explaining some of the mechanisms behind development of CNS disease. In a recent publication a correlation between levels of several complement components and ChAT was identified in in the CSF patients with MS (220) and in a related study the levels of multiple complement proteins correlated with activity of BChE in patients with AD (221). Together with our findings this suggests that an imbalanced cholinergic system could affect the complement system, or possibly vice-versa, and this in turn contributes to the progressive neurodegeneration that occurs in both AD and MS. I think this is a very interesting lead to follow up on, as MS lacks an effective treatment in its progressive forms, contrary to its relapsing-remitting phase. Also, curative treatments for AD are still lacking, and the best currently available target the cholinergic system. A plausible scenario that could occur in the progressive neurodegenerative diseases is the following; an altered balance with relatively less ACh, which could result from increased AChE/BChE or decreased ChAT activity emerges. This leads to insufficient inhibition of the low-grade complement expression that occurs constantly in the CNS as a way of maintaining homeostasis, i.e. preserving functional synapses and healthy cells while removing excess tissue and debris. When the chronic background complement activity is insufficiently inhibited a gradual increase in complement deposition occurs, first on areas distal from the neuronal soma, like synapses, and then on axons, which become degraded. This leads to slow but progressive impairment of neurological function but it is only late in the process that the neurons start to die, in accordance with findings which have demonstrated that synaptic degeneration often precedes nerve cell death (222, 223). However, even if this very simplified hypothesis would hold true, it does not explain how the process starts, or whether this is an intrinsic fault in the neurons or the result of an external signal.

Further data implicating that signaling through cholinergic pathways could mediate neuroprotection has been found in epidemiological studies, which identified smoking, as a protective factor against developing PD (76). These results suggested that nicotine, a nAChR agonist, could be involved in conveying the protective effects, which was confirmed in animal models (224). Nicotine can stimulate several of the nAChRs, but given the aforementioned anti-inflammatory effect of signaling through alpha-7-nAChR it would be interesting to study the role of agonists specific to this receptor in neurodegenerative diseases. Perhaps this treatment should be extended also to acute CNS diseases like trauma and ischemia?

8.1.1 Systemic inflammation and neurodegeneration

Also after cardiovascular surgery neurological injuries are among the most feared complications. Severe injuries like stroke occur in around 3% of the coronary artery by-pass grafting (CABG) operations (225) and are most often caused by emboli, due to

manipulation of the heart and major vessels. This is quite easy to understand from pathophysiologic point-of-view, however, the post-operative confusion without focal neurologic impairment, which occurs in up to 50% of the patients undergoing heart surgery, is less well understood (226). The confusion can cause significant morbidity as it sometimes can be long-lasting; in up to 25% of all the patients the impairment can remain even 6 months after surgery (226). Although some of the cases can be accounted for by silent emboli as detected on MRI (226), this does not explain everything, and often, no pathology can be detected radiologically, suggesting that the injury could be at microscopic level, perhaps even at synaptic. However, it is not only cardiovascular surgery that is complicated by neurological insults, as also other types of major, non-cardiac surgeries are associated with around 30% post-operative confusion rates, even in young patients (227). Perhaps the general state of inflammation caused by tissue damage from the surgery contributes to the cognitive effects.

A related, well-known phenomenon is that systemic inflammation aggravates neurodegeneration, for instance in MS, where bouts can be triggered by non-CNS infections (228). This was also demonstrated experimentally by challenging rats that were in the remission phase of EAE with systematically delivered endotoxin. The systemic inflammation caused the rats to worsen in neurological function as a consequence of up regulation of inflammatory cytokines in the CNS (229). The systemic increase in pro-inflammatory cytokines stimulated microglia to switch phenotype which mediated the CNS tissue damage, including axonal injury (229). But this being said, it is not certain that anti-inflammatory medications after major surgery or systemic disease is the best way to go, as this inflammation perhaps serves its purpose in the periphery to defend against pathogens. Ideally, one should perhaps target the inflammatory response in the CNS specifically; again, nAChR agonists would be an interesting therapy.

8.2 THE IMPORTANCE OF GENETIC BACKGROUND, WITH SPECIAL RELEVANCE FOR T CELLS

I also believe that the findings in study IV, where genetic variability in C-type lectins was shown to affect neuronal survival after injury, presumably through T cell related pathways, are of importance. One of the main implications of this study is that the genetic background of the experimental strain has large impact, which perhaps is not always accounted for. This could possibly contribute to explaining why there are so many contradictive results regarding T cells and neuroinflammation/degeneration/protection. Antigen presentation and subsequent T cell activation are highly complex processes, dependent not only on the stochastic assembly of DNA that forms the MHC and TCR molecules, but also on an array of co-stimulatory molecules, ligands and receptors, of which most I have not mentioned. If one takes into account that all of the above processes can be influenced by genetic variability then it is possible that T cells could behave differently in different strains. It is therefore not surprising that we still do not fully understand the role of T cells following CNS injury.

8.3 ESTABLISHMENT OF A REPRODUCIBLE FLOW CYTOMETRY PROTOCOL FOR TBI TISSUE

Working with CNS tissue in flow cytometry is often complicated by the stickiness of myelin, the relatively sensitive cells and the rather long sorting and separation processes that need to be performed as compared to for instance working with blood, lymphatic or spleen tissue. I therefore believe that establishing a reproducible and robust protocol for flow cytometry of brain tissue contributed to important and novel knowledge regarding the sizes and relative proportions of the different cellular populations involved in the brain injury response. Especially as it by for instance IHC is difficult to distinguish macrophages from microglia, and many studies therefore cluster these populations into one. Our current study convincingly demonstrated that the microglia outnumbered the macrophages almost 20-fold and I think that this is important to bear in mind when discussing the differential, and potential roles of macrophages and microglia following CNS injury (97).

8.4 A UNBIASED APPROACH REVEALS A NOVEL ROLE FOR CR2 IN THE CNS

The unbiased approach using linkage analysis in intercrosses has already been praised. However, I still want to emphasize the strength of this methodology, as this lead to the discovery of CR2, which probably was the last of the complement receptors we would have thought to study in the CNS. Instead, CR2 emerged as the strongest candidate, which we probably would have missed had we instead chosen a strict hypothesis driven approach. We would likely have focused on CD11b/CR3 instead, as this is the perhaps most established and studied complement receptor, readily expressed on microglia.

The CR2 study (study II) is the most extensive study in the thesis and is still not completely finished. A lot of work has been done, from characterization of CR2 expression kinetics, to cellular localization and also functional outcome of knocking CR2 out. Our first hypothesis was that the inflammation that occurs following VRA, and also in MS, leads to the shedding of the ectodomain of CR2, sCR2, into the CSF where it neutralizes complement released both after VRA and in MS. But it is also possible that sCR2 instead works in an indirect fashion, as a signaling molecule to down regulate or reprogram microglia, as sCD21 has been shown to do with monocytes (230). It is interesting that the shedding of the ectodomain of CR2 is a consequence of oxidative stress (231), which again links two systems together- oxidative stress and the complement system. The last experiments have aimed at deciphering the mechanisms by which sCR2 works although we are not quite there yet.

8.5 LAST WORDS

Personally, I will continue with research, but slightly change direction and instead focus on translational research at the intersection between the circulation and the nervous system. The unresolved questions regarding postoperative confusion after cardiovascular surgery are certainly interesting and warrant further exploration, especially if it were possibly to develop methods of examining synaptic pathology. Perhaps all these patients should be treated with nicotine patches to stimulate the alpha-7-nAChRs in an attempt to dampen the cerebral inflammation, down regulate local complement activation, stimulate cognition and in the end spare the synapses? Lastly, I am especially fascinated by the findings of Allen and Buckberg (52-54), who presented the remarkable result of next to complete neurological recovery following 30 minutes of global cerebral ischemia given a strict reperfusion protocol. But I believe that there still are some questions to answer before the cardiothoracic surgeons become the next generation neurologists!

9 ACKNOWLEDGEMENTS

This work was performed at the Department of Clinical Neuroscience, in the Neuroimmunology Unit, at the Center for Molecular Medicine, Karolinska Institutet, Stockholm between the years 2007 and 2013. Many people have been directly or indirectly involved, below are just a selection of those I would like to thank, and I apologize to those I have unintentionally failed to mention.

My supervisor professor **Fredrik Piehl**, I realized directly that you were intelligent and sympathetic; a good choice of supervisor for a PhD education. But I underestimated you- since from then on, for every month that passes my respect for you grows- you are not only very clever, but also an outstanding clinician, (I know how all neurologists at the clinic if in doubt say; ask Fredrik) a great researcher and a fantastic person.

My co-supervisor Dr **Margarita Diez**, you not only taught me how to breed F2's and congenics, perfuse, dissect and perform high-quality lab-work, but you have also throughout the years been ever enthusiastic and encouraging about both research as well as life's general questions.

My co-supervisor professor **Tomas Olsson**; for creating the formidable scientific and social atmosphere in the neuroimmunology unit! This provides the fundaments for great thoughts, work and well-being. I admire your capability to within minutes grasp a 5-year project and directly come up with suggestions for future research directions and writing- I guess that explains your success as a researcher!

Group members
Mikael for always keeping spirits up during the good (and not so good) times in the lab and animal house, during endless dissections and genotyping bouts that paved the way for the F2. And of course for your friendship, technical expertise and good scientific input… and all the great home-made brews! **Faiez**; your work discipline is impressing- who else has 200kg of sections- and knows exactly where to find every one of them? Thank you for teaching me all about DAB-staining and perfecting IHC, all the great co-operations, for your loyalty and help in various projects, for the long and hard FACS-days and your unbeatable sense of humor! **Shahin;** I don't think I could have finished this thesis without you- thank you for all the help during the last years- it has been invaluable! It is great to see your progress, from the first shaky in situ set-ups to the now advanced multi-plex-MACS-FACS experiments! I wish you all the best for your coming thesis and hope I can return some favors if you need! **Cecilia** for expanding our common knowledge regarding C-type lectins, your friendly and helpful attitude and great work with the glial cultures, which were essential for wrapping up the C3 story- thank you indeed! **Karl**, I believe you are of the right stuff to continue the proud VRA tradition and bring it into the future. Thank you for the help with various bits and pieces and the nice chats in the writing room.

Collaborators
Alexander for the excellent co-operation regarding the SNT animals and the work with the synapses both *in* and *ex vivo*. I always enjoy coming over to your lab with real coffee to discuss why the *in situ* just won't work. **Johan Z** for taking the initiative to start the co-operation between our groups and for being such a high achiever yet still so friendly and humble! Professor **Staffan Cullheim**, for providing the time and resources that enabled the successful co-operations between our labs.

Matthias- the best and brightest bioinformatician, with whom I have had lots of nice times in Berlin, and on multiple EuRATools/Trans meetings, for the help with analysing the F2 data. **Norbert Hübner** for providing the time and place for parts of the analysis of the F2.

Taher Darreh-Shori with group member **Swetha Vijayaraghavan,** for your positive attitude, your great knowledge regarding Acetylcholine pathways and the fruitful collaborations- I hope we can continue! To **Bo Nilsson, Kerstin Sandholm** and **Kristina Nilsson Ekdahl** for good collaborations regarding complement analysis.

Past and present lab colleagues
Mohsen, the ever smiling bank-director, teaching me great Persian jokes, keeping perfect order in the invaluable bio bank, never hesitating in looking up data on clinical samples, for remembering everyone's birthday and managing the lab with a firm and friendly hand. I will miss you!

Mélanie for your positive energy and contagious perfectionism, as well as being the one in the lab who I first learnt to know- and like- in our first summer of exhilarating RA-genotyping (and still they couldn't shake us off…). **Nada** for always stopping up to say hello and give a hug, for the supporting discussions during frustrated periods after rejected manuscripts and various help in the projects. **Ammenai**- it is totally understandable that the whole of CMM adores you- always helpful and very knowledgeable about every method and lab-problem available! **Petra** for having such a warm heart and taking responsibility for everyone- be it on the social or work level; you always provide that little extra! **Roham**- you have been an important addition to the unit with your genuine burning interest for immunology and of new techniques, as well as being essential for our FACS-studies you are a great guy!

To **Maja** for all the great JC's in your home, your intelligence and scientific advice and for being a good-role model in the lab when it comes to passion for science and hard work! **Bob** for always aiming at creating a good scientific discussion at every lab-meeting and helping me with student practicalities.

Past members in Fredrik's group; **Karin** for help with genotyping and teaching me lab-basics and your great humor and **Maria** for good discussions and inspirational scientific stringency and **Olle** for paving the VRA ground.

Xing-Mei for having to perfection developed the rat and mouse glial cultures, a very valuable addition to our studies, and also for spreading your lovely laughter on the floor! To **Sevi** for the collaboration with the Aplec work- keep me updated! The "new"

PhD-student talents in the group, **Hannes**, **Rasmus**, **Sohel**, **Marie**, **Sabrina**, **Andreas** and **Tojo**, who by now probably all are close to finishing, or at least half-time, who added musicality, dance-moves, neatness and order (some more than others) and other international qualities. To **Harald** for saving so many good antibodies from the fall of Astra. My multi-lingual room-mates; **André**- hard-working with outstanding knowledge regarding both science, clothes and restaurants and **Patrick** for combining the best of French and German attributes! **Pernilla** for your expertise on breeding speed- congenics and analysing crosses. **Cynthia**, **Rux** for being angry cell-room police and mail-writers but great fun IRL instead of the alternate, to **Maria** for the nice TF-talks and teaching me basics of ChIP and to sympathetic **Sreeni** and **Lou** who also is an inspiration in combining clinics and research. To **Clas** for nice discussions concerning science, medicine and microscopes! To **Venus** for helping me handle all kind of difficult lab practicalities. **Brinda**- I only wish you had joined the group earlier, when we weren't finished genotyping the F2! To **Ann-Marie**, **Maine** and **Britt** for great lab administration. **Magda**, **Emilie**, **Alexandra**, **Magnus** and **Ingrid** in the human genetics group, for sharing your knowledge and widening the genetic horizon from rodents!

Former group members; **Johan Ö**, a good example of how a senior PhD-student should be- knowledgeable, helpful and ambitious. **Alan** for being action-packed, energetic and getting-the job-done attitude regarding everything from freezer cleaning to sports. To **Rita** and **Biborka** for lively discussions regarding small and large issues.

The rheumatology people
Ferdinand you are such a nice and fun guy- you will be a great doctor- be it in the lab or clinic! **Nånnis-** you are a truly impressive bank of knowledge, be it regarding medicine or the life of others! **Gustavo** for your social skills, and your incomparable interest and devotion to muscle physiology. **Maria S, Marcus**, **Emelie, Patrick** and **Heidi** for nice times and chats. **Eva** for all the help with the sterotax, **Marianne** for all the help with the microscope, **Eva**, **Lotta**, **Lena**, **Gull-Britt** for always being friendly, helpful, kind and of course excellent at baking!

Colleagues from the clinic
Thanks to **all colleagues** in the Department of Cardiothoracic Surgery and Anesthesia in Uppsala for supporting my clinical development and continually asking how the research is coming along. Special thanks to **Peter Holm** for employing me repeatedly as a physician in the Cardiothoracic unit, first two times at Karolinska and then in Uppsala. Also special thanks to the following: **Rafael Astudillo** for taking on the task as head of surgical education, to **Anders Albåge** for giving me time-off (with salary!) for research during your time as head of department, to professor **Elisabeth Ståhle** for providing funds for and feed-back regarding new research projects and to my fellow resident colleague **Mikael Janiec** for maintaining a fair, good and open relationship between us during the various challenges of our clinical training. Lastly, I want to explicitly thank professor **Stefan Thelin**, not only for having agreed to be my clinical mentor and with great patience and confidence teaching me to operate, but also for encouraging and helping me start up new research in Uppsala.

Others
Åke Olssons Stiftelse för Utbildning through **Lil Ryott** for economic support. The animal staff at AKM for help with breeding and keeping the rats, especially **Velina** but also **Elisabeth**, **Jessica** and **Selam**.

Family
Mahin, **Mojtaba** and **Anahita** for being such a warm and loving extra family, for providing lots of Persian food to take me through the long evenings and always showing interest in my research.

My mother **Kristina** and father **Lars** for always encouraging, supporting and believing in me whatever the challenge, whatever the time of night. Thank you also for all your help during the years regarding practicalities and everyday problems which without things would not have worked. To my brother **Jonas** and sister **Susanna**, for being the best siblings one could want and always asking and showing much more interest and enthusiasm than could be expected on such a strange subject, so distant from your worlds!

Nathalie for everything we share and all the fun we have, for all your care and humour, and for helping me to carry the load in hard times. I cannot express enough how much I love us.

10 REFERENCES

1. Bear, M. C., B. Paradiso, M. 2006. *Neuroscience: Exploring the brain*. Lippincott Williams & Wilkins.
2. Kandel, E. S., JH. Jessel, TM. Siegelbaum, SA. Hudspeth, AJ. 2012. *Principles of Neural Science*. McGraw-Hill Medical.
3. Hilgetag, C. C., and H. Barbas. 2009. Are there ten times more glia than neurons in the brain? *Brain Struct Funct* 213: 365-366.
4. Azevedo, F. A., L. R. Carvalho, L. T. Grinberg, J. M. Farfel, R. E. Ferretti, R. E. Leite, W. Jacob Filho, R. Lent, and S. Herculano-Houzel. 2009. Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *The Journal of comparative neurology* 513: 532-541.
5. Pelvig, D. P., H. Pakkenberg, A. K. Stark, and B. Pakkenberg. 2008. Neocortical glial cell numbers in human brains. *Neurobiology of Aging* 29: 1754-1762.
6. Burish, M. J., J. K. Peebles, M. K. Baldwin, L. Tavares, J. H. Kaas, and S. Herculano-Houzel. 2010. Cellular scaling rules for primate spinal cords. *Brain Behav Evol* 76: 45-59.
7. Nedergaard, M., B. Ransom, and S. A. Goldman. 2003. New roles for astrocytes: redefining the functional architecture of the brain. *Trends Neurosci* 26: 523-530.
8. Farina, C., F. Aloisi, and E. Meinl. 2007. Astrocytes are active players in cerebral innate immunity. *Trends in immunology* 28: 138-145.
9. Aloisi, F. 2001. Immune function of microglia. *Glia* 36: 165-179.
10. Beauvillain, C., S. Donnou, U. Jarry, M. Scotet, H. Gascan, Y. Delneste, P. Guermonprez, P. Jeannin, and D. Couez. 2008. Neonatal and adult microglia cross-present exogenous antigens. *Glia* 56: 69-77.
11. Kettenmann, H., U. K. Hanisch, M. Noda, and A. Verkhratsky. 2011. Physiology of microglia. *Physiol Rev* 91: 461-553.
12. Ginhoux, F., S. Lim, G. Hoeffel, D. Low, and T. Huber. 2013. Origin and differentiation of microglia. *Front Cell Neurosci* 7: 45.
13. Aggarwal, S., L. Yurlova, and M. Simons. 2011. Central nervous system myelin: structure, synthesis and assembly. *Trends Cell Biol* 21: 585-593.
14. Hosokawa, M., A. Klegeris, J. Maguire, and P. L. McGeer. 2003. Expression of complement messenger RNAs and proteins by human oligodendroglial cells. *Glia* 42: 417-423.
15. Gaudet, A. D., P. G. Popovich, and M. S. Ramer. 2011. Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. *Journal of neuroinflammation* 8: 110.
16. Buchser, W. J., R. P. Smith, J. R. Pardinas, C. L. Haddox, T. Hutson, L. Moon, S. R. Hoffman, J. L. Bixby, and V. P. Lemmon. 2012. Peripheral nervous system genes expressed in central neurons induce growth on inhibitory substrates. *PLoS One* 7: e38101.
17. Chew, D. J., J. W. Fawcett, and M. R. Andrews. 2012. The challenges of long-distance axon regeneration in the injured CNS. *Progress in brain research* 201: 253-294.
18. Dancause, N., and R. J. Nudo. 2011. Shaping plasticity to enhance recovery after injury. *Progress in brain research* 192: 273-295.

19. Weidner, N., A. Ner, N. Salimi, and M. H. Tuszynski. 2001. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. *Proceedings of the National Academy of Sciences of the United States of America* 98: 3513-3518.
20. Ferguson, A. R., J. R. Huie, E. D. Crown, K. M. Baumbauer, M. A. Hook, S. M. Garraway, K. H. Lee, K. C. Hoy, and J. W. Grau. 2012. Maladaptive spinal plasticity opposes spinal learning and recovery in spinal cord injury. *Front Physiol* 3: 399.
21. Paz, J. T., C. A. Christian, I. Parada, D. A. Prince, and J. R. Huguenard. 2010. Focal cortical infarcts alter intrinsic excitability and synaptic excitation in the reticular thalamic nucleus. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30: 5465-5479.
22. Schnell, L., S. Fearn, H. Klassen, M. E. Schwab, and V. H. Perry. 1999. Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord. *Eur J Neurosci* 11: 3648-3658.
23. Batchelor, P. E., S. Tan, T. E. Wills, M. J. Porritt, and D. W. Howells. 2008. Comparison of inflammation in the brain and spinal cord following mechanical injury. *Journal of neurotrauma* 25: 1217-1225.
24. Ramlackhansingh, A. F., D. J. Brooks, R. J. Greenwood, S. K. Bose, F. E. Turkheimer, K. M. Kinnunen, S. Gentleman, R. A. Heckemann, K. Gunanayagam, G. Gelosa, and D. J. Sharp. 2011. Inflammation after trauma: microglial activation and traumatic brain injury. *Ann Neurol* 70: 374-383.
25. Beck, K. D., H. X. Nguyen, M. D. Galvan, D. L. Salazar, T. M. Woodruff, and A. J. Anderson. 2010. Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment. *Brain* 133: 433-447.
26. Olesen, J., A. Gustavsson, M. Svensson, H. U. Wittchen, and B. Jonsson. 2012. The economic cost of brain disorders in Europe. *Eur J Neurol* 19: 155-162.
27. Maas, A. I., B. Roozenbeek, and G. T. Manley. 2010. Clinical trials in traumatic brain injury: past experience and current developments. *Neurotherapeutics* 7: 115-126.
28. Bragge, P., M. Chau, V. J. Pitt, M. T. Bayley, J. J. Eng, R. W. Teasell, D. L. Wolfe, and R. L. Gruen. 2012. An overview of published research about the acute care and rehabilitation of traumatic brain injured and spinal cord injured patients. *Journal of neurotrauma* 29: 1539-1547.
29. Gunnarsson, T., and M. G. Fehlings. 2003. Acute neurosurgical management of traumatic brain injury and spinal cord injury. *Curr Opin Neurol* 16: 717-723.
30. Bains, M., and E. D. Hall. 2012. Antioxidant therapies in traumatic brain and spinal cord injury. *Biochim Biophys Acta* 1822: 675-684.
31. Lee, P. C., Y. Bordelon, J. Bronstein, and B. Ritz. 2012. Traumatic brain injury, paraquat exposure, and their relationship to Parkinson disease. *Neurology* 79: 2061-2066.
32. Sivanandam, T. M., and M. K. Thakur. 2012. Traumatic brain injury: a risk factor for Alzheimer's disease. *Neurosci Biobehav Rev* 36: 1376-1381.
33. Novy, J. 2012. Spinal cord syndromes. *Front Neurol Neurosci* 30: 195-198.
34. Kirshblum, S. C., S. P. Burns, F. Biering-Sorensen, W. Donovan, D. E. Graves, A. Jha, M. Johansen, L. Jones, A. Krassioukov, M. J. Mulcahey, M. Schmidt-Read, and W. Waring. 2011. International standards for neurological classification of spinal cord injury (revised 2011). *J Spinal Cord Med* 34: 535-546.
35. Burns, A. S., R. J. Marino, A. E. Flanders, and H. Flett. 2012. Clinical diagnosis and prognosis following spinal cord injury. *Handb Clin Neurol* 109: 47-62.

36. Bracken, M. B. 2012. Steroids for acute spinal cord injury. *Cochrane Database Syst Rev* 1: CD001046.
37. Lu, P., Y. Wang, L. Graham, K. McHale, M. Gao, D. Wu, J. Brock, A. Blesch, E. S. Rosenzweig, L. A. Havton, B. Zheng, J. M. Conner, M. Marsala, and M. H. Tuszynski. 2012. Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. *Cell* 150: 1264-1273.
38. Alper, J. 2009. Geron gets green light for human trial of ES cell-derived product. *Nature biotechnology* 27: 213-214.
39. 2009. World's first clinical trial of human embryonic stem cell therapy cleared. *Regen Med* 4: 161.
40. Edgerton, V. R., N. J. Tillakaratne, A. J. Bigbee, R. D. de Leon, and R. R. Roy. 2004. Plasticity of the spinal neural circuitry after injury. *Annu Rev Neurosci* 27: 145-167.
41. Donnan, G. A., M. Fisher, M. Macleod, and S. M. Davis. 2008. Stroke. *Lancet* 371: 1612-1623.
42. Prabhakaran, S., and A. M. Naidech. 2012. Ischemic brain injury after intracerebral hemorrhage: a critical review. *Stroke; a journal of cerebral circulation* 43: 2258-2263.
43. Hata, M., A. Sezai, I. Yoshitake, S. Wakui, A. Takasaka, K. Minami, and M. Shiono. 2010. Clinical trends in optimal treatment strategy for type A acute aortic dissection. *Ann Thorac Cardiovasc Surg* 16: 228-235.
44. Menon, R. K., and J. W. Norris. 2008. Cervical arterial dissection: current concepts. *Ann N Y Acad Sci* 1142: 200-217.
45. Pacini, D., L. Di Marco, and R. Di Bartolomeo. 2006. Methods of cerebral protection in surgery of the thoracic aorta. *Expert Rev Cardiovasc Ther* 4: 71-82.
46. Liu, Z. J., W. G. Fu, Z. Y. Guo, L. G. Shen, Z. Y. Shi, and J. H. Li. 2012. Updated systematic review and meta-analysis of randomized clinical trials comparing carotid artery stenting and carotid endarterectomy in the treatment of carotid stenosis. *Ann Vasc Surg* 26: 576-590.
47. Bicknell, C. D., C. V. Riga, and J. H. Wolfe. 2009. Prevention of paraplegia during thoracoabdominal aortic aneurysm repair. *Eur J Vasc Endovasc Surg* 37: 654-660.
48. Sinha, A. C., and A. T. Cheung. 2010. Spinal cord protection and thoracic aortic surgery. *Curr Opin Anaesthesiol* 23: 95-102.
49. Naess, H., and F. Romi. 2011. Comparing patients with spinal cord infarction and cerebral infarction: clinical characteristics, and short-term outcome. *Vasc Health Risk Manag* 7: 497-502.
50. Spence, J. D. 2010. Secondary stroke prevention. *Nat Rev Neurol* 6: 477-486.
51. Chalkias, A., and T. Xanthos. 2012. Post-cardiac arrest brain injury: pathophysiology and treatment. *J Neurol Sci* 315: 1-8.
52. Allen, B. S., and G. D. Buckberg. 2012. Studies of isolated global brain ischaemia: I. Overview of irreversible brain injury and evolution of a new concept - redefining the time of brain death. *Eur J Cardiothorac Surg* 41: 1132-1137.
53. Allen, B. S., Y. Ko, G. D. Buckberg, S. Sakhai, and Z. Tan. 2012. Studies of isolated global brain ischaemia: I. A new large animal model of global brain ischaemia and its baseline perfusion studies. *Eur J Cardiothorac Surg* 41: 1138-1146.
54. Allen, B. S., Y. Ko, G. D. Buckberg, and Z. Tan. 2012. Studies of isolated global brain ischaemia: II. Controlled reperfusion provides complete neurologic

recovery following 30 min of warm ischaemia - the importance of perfusion pressure. *Eur J Cardiothorac Surg* 41: 1147-1154.
55. Loisance, D. 2012. Save the brain: a new option! *Eur J Cardiothorac Surg* 41: 1163-1165.
56. Lambert, J. C., S. Heath, G. Even, D. Campion, K. Sleegers, M. Hiltunen, O. Combarros, D. Zelenika, M. J. Bullido, B. Tavernier, L. Letenneur, K. Bettens, C. Berr, F. Pasquier, N. Fievet, P. Barberger-Gateau, S. Engelborghs, P. De Deyn, I. Mateo, A. Franck, S. Helisalmi, E. Porcellini, O. Hanon, M. M. de Pancorbo, C. Lendon, C. Dufouil, C. Jaillard, T. Leveillard, V. Alvarez, P. Bosco, M. Mancuso, F. Panza, B. Nacmias, P. Bossu, P. Piccardi, G. Annoni, D. Seripa, D. Galimberti, D. Hannequin, F. Licastro, H. Soininen, K. Ritchie, H. Blanche, J. F. Dartigues, C. Tzourio, I. Gut, C. Van Broeckhoven, A. Alperovitch, M. Lathrop, and P. Amouyel. 2009. Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. *Nat Genet* 41: 1094-1099.
57. Brouwers, N., C. V. Cauwenberghe, S. Engelborghs, J. C. Lambert, K. Bettens, N. L. Bastard, F. Pasquier, A. G. Montoya, K. Peeters, M. Mattheijssens, R. Vandenberghe, P. P. Deyn, M. Cruts, P. Amouyel, K. Sleegers, and C. V. Broeckhoven. 2012. Alzheimer risk associated with a copy number variation in the complement receptor 1 increasing C3b/C4b binding sites. *Mol Psychiatry* 17: 223-233.
58. Nylander, A., and D. A. Hafler. 2012. Multiple sclerosis. *J Clin Invest* 122: 1180-1188.
59. Chard, D. T., C. M. Griffin, G. J. Parker, R. Kapoor, A. J. Thompson, and D. H. Miller. 2002. Brain atrophy in clinically early relapsing-remitting multiple sclerosis. *Brain* 125: 327-337.
60. Geurts, J. J., and F. Barkhof. 2008. Grey matter pathology in multiple sclerosis. *Lancet Neurol* 7: 841-851.
61. Compston, A., and A. Coles. 2008. Multiple sclerosis. *Lancet* 372: 1502-1517.
62. Glass, C. K., K. Saijo, B. Winner, M. C. Marchetto, and F. H. Gage. 2010. Mechanisms underlying inflammation in neurodegeneration. *Cell* 140: 918-934.
63. Hafler, D. A., A. Compston, S. Sawcer, E. S. Lander, M. J. Daly, P. L. De Jager, P. I. de Bakker, S. B. Gabriel, D. B. Mirel, A. J. Ivinson, M. A. Pericak-Vance, S. G. Gregory, J. D. Rioux, J. L. McCauley, J. L. Haines, L. F. Barcellos, B. Cree, J. R. Oksenberg, and S. L. Hauser. 2007. Risk alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med* 357: 851-862.
64. Gunnarsson, M., C. Malmestrom, M. Axelsson, P. Sundstrom, C. Dahle, M. Vrethem, T. Olsson, F. Piehl, N. Norgren, L. Rosengren, A. Svenningsson, and J. Lycke. 2011. Axonal damage in relapsing multiple sclerosis is markedly reduced by natalizumab. *Annals of neurology* 69: 83-89.
65. Aktas, O., P. Kury, B. Kieseier, and H. P. Hartung. 2010. Fingolimod is a potential novel therapy for multiple sclerosis. *Nat Rev Neurol* 6: 373-382.
66. Brookmeyer, R., E. Johnson, K. Ziegler-Graham, and H. M. Arrighi. 2007. Forecasting the global burden of Alzheimer's disease. *Alzheimers Dement* 3: 186-191.
67. Herrup, K. 2010. Reimagining Alzheimer's disease--an age-based hypothesis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30: 16755-16762.
68. Selkoe, D. J. 2002. Alzheimer's disease is a synaptic failure. *Science* 298: 789-791.

69. Bertram, L. 2009. Alzheimer's disease genetics current status and future perspectives. *International review of neurobiology* 84: 167-184.
70. Bertram, L., M. B. McQueen, K. Mullin, D. Blacker, and R. E. Tanzi. 2007. Systematic meta-analyses of Alzheimer disease genetic association studies: the AlzGene database. *Nature genetics* 39: 17-23.
71. Ferris, S., A. Nordberg, H. Soininen, T. Darreh-Shori, and R. Lane. 2009. Progression from mild cognitive impairment to Alzheimer's disease: effects of sex, butyrylcholinesterase genotype, and rivastigmine treatment. *Pharmacogenet Genomics* 19: 635-646.
72. de los Rios, C. 2012. Cholinesterase inhibitors: a patent review (2007 - 2011). *Expert Opin Ther Pat* 22: 853-869.
73. Tracey, K. J. 2009. Reflex control of immunity. *Nature reviews. Immunology* 9: 418-428.
74. Ferraiuolo, L., J. Kirby, A. J. Grierson, M. Sendtner, and P. J. Shaw. 2011. Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. *Nat Rev Neurol* 7: 616-630.
75. Scarmeas, N., T. Shih, Y. Stern, R. Ottman, and L. P. Rowland. 2002. Premorbid weight, body mass, and varsity athletics in ALS. *Neurology* 59: 773-775.
76. de Lau, L. M., and M. M. Breteler. 2006. Epidemiology of Parkinson's disease. *Lancet Neurol* 5: 525-535.
77. Lesage, S., and A. Brice. 2009. Parkinson's disease: from monogenic forms to genetic susceptibility factors. *Human molecular genetics* 18: R48-59.
78. National Collaborating Centre for Chronic Conditions, U. 2006. *Parkinson's Disease: National clinical guideline for diagnosis and management in primary and secondary care* National Institute for Health and Clinical Excellence, London.
79. Obeso, J. A., M. C. Rodriguez-Oroz, C. G. Goetz, C. Marin, J. H. Kordower, M. Rodriguez, E. C. Hirsch, M. Farrer, A. H. Schapira, and G. Halliday. 2010. Missing pieces in the Parkinson's disease puzzle. *Nature medicine* 16: 653-661.
80. Meisel, C., J. M. Schwab, K. Prass, A. Meisel, and U. Dirnagl. 2005. Central nervous system injury-induced immune deficiency syndrome. *Nature reviews. Neuroscience* 6: 775-786.
81. Kwan, J., R. M. Pickering, D. Kunkel, C. Fitton, D. Jenkinson, V. H. Perry, and A. M. Ashburn. 2013. Impact of stroke-associated infection on long-term survival: a cohort study. *J Neurol Neurosurg Psychiatry* 84: 297-304.
82. Iversen, P. O., N. Hjeltnes, B. Holm, T. Flatebo, I. Strom-Gundersen, W. Ronning, J. Stanghelle, and H. B. Benestad. 2000. Depressed immunity and impaired proliferation of hematopoietic progenitor cells in patients with complete spinal cord injury. *Blood* 96: 2081-2083.
83. Ransohoff, R. M., P. Kivisakk, and G. Kidd. 2003. Three or more routes for leukocyte migration into the central nervous system. *Nature reviews. Immunology* 3: 569-581.
84. Engelhardt, B. 2006. Molecular mechanisms involved in T cell migration across the blood-brain barrier. *J Neural Transm* 113: 477-485.
85. Rosenberg, G. A. 2012. Neurological diseases in relation to the blood-brain barrier. *J Cereb Blood Flow Metab* 32: 1139-1151.
86. Griffiths, M., J. W. Neal, and P. Gasque. 2007. Innate immunity and protective neuroinflammation: new emphasis on the role of neuroimmune regulatory proteins. *Int Rev Neurobiol* 82: 29-55.
87. Yong, V. W., and S. Marks. 2010. The interplay between the immune and central nervous systems in neuronal injury. *Neurology* 74 Suppl 1: S9-S16.

88. Abbas, A., Lichtman, AH., Pillai, S. 2010. *Cellular and Molecular Immunology*. Saunders
89. Villadangos, J. A., and P. Schnorrer. 2007. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nature reviews. Immunology* 7: 543-555.
90. Yewdell, J. W., E. Reits, and J. Neefjes. 2003. Making sense of mass destruction: quantitating MHC class I antigen presentation. *Nature reviews. Immunology* 3: 952-961.
91. Cho, J. H., and P. K. Gregersen. 2011. Genomics and the multifactorial nature of human autoimmune disease. *N Engl J Med* 365: 1612-1623.
92. Mangalam, A. K., V. Taneja, and C. S. David. 2013. HLA class II molecules influence susceptibility versus protection in inflammatory diseases by determining the cytokine profile. *Journal of immunology* 190: 513-518.
93. Zachary, A. A., D. Kopchaliiska, A. M. Jackson, and M. S. Leffell. 2010. Immunogenetics and immunology in transplantation. *Immunol Res* 47: 232-239.
94. Fleming, J. C., M. D. Norenberg, D. A. Ramsay, G. A. Dekaban, A. E. Marcillo, A. D. Saenz, M. Pasquale-Styles, W. D. Dietrich, and L. C. Weaver. 2006. The cellular inflammatory response in human spinal cords after injury. *Brain* 129: 3249-3269.
95. Nguyen, H. X., T. J. O'Barr, and A. J. Anderson. 2007. Polymorphonuclear leukocytes promote neurotoxicity through release of matrix metalloproteinases, reactive oxygen species, and TNF-alpha. *Journal of neurochemistry* 102: 900-912.
96. Barone, F. C., L. M. Hillegass, W. J. Price, R. F. White, E. V. Lee, G. Z. Feuerstein, H. M. Sarau, R. K. Clark, and D. E. Griswold. 1991. Polymorphonuclear leukocyte infiltration into cerebral focal ischemic tissue: myeloperoxidase activity assay and histologic verification. *Journal of neuroscience research* 29: 336-345.
97. Shechter, R., and M. Schwartz. 2013. Harnessing monocyte-derived macrophages to control central nervous system pathologies: no longer 'if' but 'how'. *J Pathol* 229: 332-346.
98. Valledor, A. F., M. Comalada, L. F. Santamaria-Babi, J. Lloberas, and A. Celada. 2010. Macrophage proinflammatory activation and deactivation: a question of balance. *Adv Immunol* 108: 1-20.
99. Horn, K. P., S. A. Busch, A. L. Hawthorne, N. van Rooijen, and J. Silver. 2008. Another barrier to regeneration in the CNS: activated macrophages induce extensive retraction of dystrophic axons through direct physical interactions. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 28: 9330-9341.
100. Popovich, P. G., Z. Guan, P. Wei, I. Huitinga, N. van Rooijen, and B. T. Stokes. 1999. Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. *Exp Neurol* 158: 351-365.
101. Shechter, R., A. London, C. Varol, C. Raposo, M. Cusimano, G. Yovel, A. Rolls, M. Mack, S. Pluchino, G. Martino, S. Jung, and M. Schwartz. 2009. Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. *PLoS Med* 6: e1000113.
102. Kigerl, K. A., J. C. Gensel, D. P. Ankeny, J. K. Alexander, D. J. Donnelly, and P. G. Popovich. 2009. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured

mouse spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29: 13435-13444.
103. Ransohoff, R. M., and V. H. Perry. 2009. Microglial physiology: unique stimuli, specialized responses. *Annu Rev Immunol* 27: 119-145.
104. Perry, V. H., J. A. Nicoll, and C. Holmes. 2010. Microglia in neurodegenerative disease. *Nat Rev Neurol* 6: 193-201.
105. Gasque, P., P. Chan, C. Mauger, M. T. Schouft, S. Singhrao, M. P. Dierich, B. P. Morgan, and M. Fontaine. 1996. Identification and characterization of complement C3 receptors on human astrocytes. *Journal of immunology* 156: 2247-2255.
106. Chastain, E. M., D. S. Duncan, J. M. Rodgers, and S. D. Miller. 2011. The role of antigen presenting cells in multiple sclerosis. *Biochim Biophys Acta* 1812: 265-274.
107. Sun, J. C., and L. L. Lanier. 2011. NK cell development, homeostasis and function: parallels with CD8(+) T cells. *Nature reviews. Immunology* 11: 645-657.
108. Raulet, D. H., and R. E. Vance. 2006. Self-tolerance of natural killer cells. *Nature reviews. Immunology* 6: 520-531.
109. Hao, J., R. Liu, W. Piao, Q. Zhou, T. L. Vollmer, D. I. Campagnolo, R. Xiang, A. La Cava, L. Van Kaer, and F. D. Shi. 2010. Central nervous system (CNS)-resident natural killer cells suppress Th17 responses and CNS autoimmune pathology. *The Journal of experimental medicine* 207: 1907-1921.
110. Morandi, B., P. Bramanti, I. Bonaccorsi, E. Montalto, D. Oliveri, G. Pezzino, M. Navarra, and G. Ferlazzo. 2008. Role of natural killer cells in the pathogenesis and progression of multiple sclerosis. *Pharmacol Res* 57: 1-5.
111. Poli, A., J. Kmiecik, O. Domingues, F. Hentges, M. Blery, M. Chekenya, J. Boucraut, and J. Zimmer. 2013. NK cells in central nervous system disorders. *Journal of immunology* 190: 5355-5362.
112. Ousman, S. S., and P. Kubes. 2012. Immune surveillance in the central nervous system. *Nat Neurosci* 15: 1096-1101.
113. Liu, M., J. Zhao, H. Liang, and X. Bian. 2009. Vaccination with dendritic cells pulsed with homogenate protein of spinal cord promotes functional recovery from spinal cord injury in mice. *Spinal Cord* 47: 360-366.
114. Yaguchi, M., M. Tabuse, S. Ohta, K. Ohkusu-Tsukada, T. Takeuchi, J. Yamane, H. Katoh, M. Nakamura, Y. Matsuzaki, M. Yamada, T. Itoh, T. Nomura, Y. Toyama, H. Okano, and M. Toda. 2009. Transplantation of dendritic cells promotes functional recovery from spinal cord injury in common marmoset. *Neurosci Res* 65: 384-392.
115. Ankeny, D. P., and P. G. Popovich. 2009. Mechanisms and implications of adaptive immune responses after traumatic spinal cord injury. *Neuroscience* 158: 1112-1121.
116. Yoles, E., E. Hauben, O. Palgi, E. Agranov, A. Gothilf, A. Cohen, V. Kuchroo, I. R. Cohen, H. Weiner, and M. Schwartz. 2001. Protective autoimmunity is a physiological response to CNS trauma. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21: 3740-3748.
117. Yamane, H., and W. E. Paul. 2013. Early signaling events that underlie fate decisions of naive CD4(+) T cells toward distinct T-helper cell subsets. *Immunol Rev* 252: 12-23.
118. Shachar, I., and N. Karin. 2013. The dual roles of inflammatory cytokines and chemokines in the regulation of autoimmune diseases and their clinical implications. *Journal of leukocyte biology* 93: 51-61.

119. Erta, M., A. Quintana, and J. Hidalgo. 2012. Interleukin-6, a major cytokine in the central nervous system. *Int J Biol Sci* 8: 1254-1266.
120. Croft, M., C. A. Benedict, and C. F. Ware. 2013. Clinical targeting of the TNF and TNFR superfamilies. *Nat Rev Drug Discov* 12: 147-168.
121. Jain, A., and J. A. Singh. 2013. Harms of TNF inhibitors in rheumatic diseases: a focused review of the literature. *Immunotherapy* 5: 265-299.
122. Caminero, A., M. Comabella, and X. Montalban. 2011. Tumor necrosis factor alpha (TNF-alpha), anti-TNF-alpha and demyelination revisited: an ongoing story. *Journal of neuroimmunology* 234: 1-6.
123. Disanto, G., J. M. Morahan, M. H. Barnett, G. Giovannoni, and S. V. Ramagopalan. 2012. The evidence for a role of B cells in multiple sclerosis. *Neurology* 78: 823-832.
124. Rudehill, S., S. Muhallab, A. Wennersten, C. von Gertten, F. Al Nimer, A. C. Sandberg-Nordqvist, S. Holmin, and T. Mathiesen. 2006. Autoreactive antibodies against neurons and basal lamina found in serum following experimental brain contusion in rats. *Acta Neurochir (Wien)* 148: 199-205; discussion 205.
125. Zipfel, P. F., and C. Skerka. 2009. Complement regulators and inhibitory proteins. *Nat Rev Immunol* 9: 729-740.
126. Gasque, P., M. Fontaine, and B. P. Morgan. 1995. Complement expression in human brain. Biosynthesis of terminal pathway components and regulators in human glial cells and cell lines. *J Immunol* 154: 4726-4733.
127. Thomas, A., P. Gasque, D. Vaudry, B. Gonzalez, and M. Fontaine. 2000. Expression of a complete and functional complement system by human neuronal cells in vitro. *Int Immunol* 12: 1015-1023.
128. Crick, F. 1970. Central dogma of molecular biology. *Nature* 227: 561-563.
129. Handel, A. E., G. C. Ebers, and S. V. Ramagopalan. 2010. Epigenetics: molecular mechanisms and implications for disease. *Trends Mol Med* 16: 7-16.
130. Daly, P. A. 2004. Hereditary cancer: guidelines in clinical practice--general overview. *Ann Oncol* 15 Suppl 4: iv121-125.
131. McAllister, T. W. 2010. Genetic factors modulating outcome after neurotrauma. *PM R* 2: S241-252.
132. Comabella, M., and K. Vandenbroeck. 2011. Pharmacogenomics and multiple sclerosis: moving toward individualized medicine. *Curr Neurol Neurosci Rep* 11: 484-491.
133. Festing, M. F. 1981. Inbred strains of rats. *Behav Genet* 11: 431-435.
134. Lidman, O., M. Swanberg, L. Horvath, K. W. Broman, T. Olsson, and F. Piehl. 2003. Discrete gene loci regulate neurodegeneration, lymphocyte infiltration, and major histocompatibility complex class II expression in the CNS. *J Neurosci* 23: 9817-9823.
135. Darvasi, A., and M. Soller. 1995. Advanced intercross lines, an experimental population for fine genetic mapping. *Genetics* 141: 1199-1207.
136. Bryant, C. D., L. A. Kole, M. A. Guido, G. Sokoloff, and A. A. Palmer. 2012. Congenic dissection of a major QTL for methamphetamine sensitivity implicates epistasis. *Genes Brain Behav* 11: 623-632.
137. Gale, G. D., R. D. Yazdi, A. H. Khan, A. J. Lusis, R. C. Davis, and D. J. Smith. 2009. A genome-wide panel of congenic mice reveals widespread epistasis of behavior quantitative trait loci. *Mol Psychiatry* 14: 631-645.
138. Gasque, P. 2004. Complement: a unique innate immune sensor for danger signals. *Mol Immunol* 41: 1089-1098.
139. Dunkelberger, J. R., and W. C. Song. 2010. Complement and its role in innate and adaptive immune responses. *Cell Res* 20: 34-50.

140. Carroll, M. C. 2004. The complement system in regulation of adaptive immunity. *Nat Immunol* 5: 981-986.
141. Sahu, A., and J. D. Lambris. 2001. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. *Immunol Rev* 180: 35-48.
142. Ricklin, D., G. Hajishengallis, K. Yang, and J. D. Lambris. 2010. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 11: 785-797.
143. Hajishengallis, G. 2010. Complement and periodontitis. *Biochem Pharmacol* 80: 1992-2001.
144. Cole, D. S., T. R. Hughes, P. Gasque, and B. P. Morgan. 2006. Complement regulator loss on apoptotic neuronal cells causes increased complement activation and promotes both phagocytosis and cell lysis. *Mol Immunol* 43: 1953-1964.
145. Alexander, J. J., A. J. Anderson, S. R. Barnum, B. Stevens, and A. J. Tenner. 2008. The complement cascade: Yin-Yang in neuroinflammation--neuro-protection and -degeneration. *J Neurochem* 107: 1169-1187.
146. Khera, R., and N. Das. 2009. Complement Receptor 1: disease associations and therapeutic implications. *Molecular immunology* 46: 761-772.
147. Li, L. M., J. B. Li, Y. Zhu, and G. Y. Fan. 2010. Soluble complement receptor type 1 inhibits complement system activation and improves motor function in acute spinal cord injury. *Spinal Cord* 48: 105-111.
148. Bellander, B. M., S. K. Singhrao, M. Ohlsson, P. Mattsson, and M. Svensson. 2001. Complement activation in the human brain after traumatic head injury. *J Neurotrauma* 18: 1295-1311.
149. Szeplaki, G., R. Szegedi, K. Hirschberg, T. Gombos, L. Varga, I. Karadi, L. Entz, Z. Szeplaki, P. Garred, Z. Prohaszka, and G. Fust. 2009. Strong complement activation after acute ischemic stroke is associated with unfavorable outcomes. *Atherosclerosis* 204: 315-320.
150. Wang, Y., A. M. Hancock, J. Bradner, K. A. Chung, J. F. Quinn, E. R. Peskind, D. Galasko, J. Jankovic, C. P. Zabetian, H. M. Kim, J. B. Leverenz, T. J. Montine, C. Ginghina, K. L. Edwards, K. W. Snapinn, D. S. Goldstein, M. Shi, and J. Zhang. 2011. Complement 3 and factor h in human cerebrospinal fluid in Parkinson's disease, Alzheimer's disease, and multiple-system atrophy. *The American journal of pathology* 178: 1509-1516.
151. Ingram, G., S. Hakobyan, C. L. Hirst, C. L. Harris, T. P. Pickersgill, M. D. Cossburn, S. Loveless, N. P. Robertson, and B. P. Morgan. 2010. Complement regulator factor H as a serum biomarker of multiple sclerosis disease state. *Brain* 133: 1602-1611.
152. Li, Y., Z. Qin, M. Yang, Y. Qin, C. Lin, and S. Liu. 2011. Differential expression of complement proteins in cerebrospinal fluid from active multiple sclerosis patients. *J Cell Biochem* 112: 1930-1937.
153. Perry, V. H., and V. O'Connor. 2008. C1q: the perfect complement for a synaptic feast? *Nat Rev Neurosci* 9: 807-811.
154. Stevens, B., N. J. Allen, L. E. Vazquez, G. R. Howell, K. S. Christopherson, N. Nouri, K. D. Micheva, A. K. Mehalow, A. D. Huberman, B. Stafford, A. Sher, A. M. Litke, J. D. Lambris, S. J. Smith, S. W. John, and B. A. Barres. 2007. The classical complement cascade mediates CNS synapse elimination. *Cell* 131: 1164-1178.
155. Berg, A., J. Zelano, A. Stephan, S. Thams, B. A. Barres, M. Pekny, M. Pekna, and S. Cullheim. 2012. Reduced removal of synaptic terminals from

axotomized spinal motoneurons in the absence of complement C3. *Exp Neurol* 237: 8-17.
156. Fraser, D. A., K. Pisalyaput, and A. J. Tenner. 2010. C1q enhances microglial clearance of apoptotic neurons and neuronal blebs, and modulates subsequent inflammatory cytokine production. *J Neurochem* 112: 733-743.
157. Kossmann, T., P. F. Stahel, M. C. Morganti-Kossmann, J. L. Jones, and S. R. Barnum. 1997. Elevated levels of the complement components C3 and factor B in ventricular cerebrospinal fluid of patients with traumatic brain injury. *J Neuroimmunol* 73: 63-69.
158. Stahel, P. F., M. C. Morganti-Kossmann, and T. Kossmann. 1998. The role of the complement system in traumatic brain injury. *Brain Res Brain Res Rev* 27: 243-256.
159. Nimer, F. A., R. Lindblom, M. Strom, A. O. Guerreiro-Cacais, R. Parsa, S. Aeinehband, T. Mathiesen, O. Lidman, and F. Piehl. 2012. Strain influences on inflammatory pathway activation, cell infiltration and complement cascade after traumatic brain injury in the rat. *Brain Behav Immun*.
160. Singhrao, S. K., J. W. Neal, N. K. Rushmere, B. P. Morgan, and P. Gasque. 2000. Spontaneous classical pathway activation and deficiency of membrane regulators render human neurons susceptible to complement lysis. *Am J Pathol* 157: 905-918.
161. Young, I. S., and J. V. Woodside. 2001. Antioxidants in health and disease. *J Clin Pathol* 54: 176-186.
162. Ricciardolo, F. L., P. J. Sterk, B. Gaston, and G. Folkerts. 2004. Nitric oxide in health and disease of the respiratory system. *Physiol Rev* 84: 731-765.
163. Roberts, R. A., D. L. Laskin, C. V. Smith, F. M. Robertson, E. M. Allen, J. A. Doorn, and W. Slikker. 2009. Nitrative and oxidative stress in toxicology and disease. *Toxicol Sci* 112: 4-16.
164. Wu, G., Y. Z. Fang, S. Yang, J. R. Lupton, and N. D. Turner. 2004. Glutathione metabolism and its implications for health. *J Nutr* 134: 489-492.
165. Hayes, J. D., J. U. Flanagan, and I. R. Jowsey. 2005. Glutathione transferases. *Annu Rev Pharmacol Toxicol* 45: 51-88.
166. Strom, M., F. Al Nimer, R. Lindblom, J. R. Nyengaard, and F. Piehl. 2012. Naturally occurring genetic variability in expression of Gsta4 is associated with differential survival of axotomized rat motoneurons. *Neuromolecular Medicine* 14: 15-29.
167. Al Nimer, F., M. Strom, R. Lindblom, S. Aeinehband, B. M. Bellander, J. R. Nyengaard, O. Lidman, and F. Piehl. 2012. Naturally occurring variation in the glutathione-S-transferase 4 gene determines neurodegeneration after traumatic brain injury. *Antioxid Redox Signal*.
168. Raivich, G., L. L. Jones, C. U. Kloss, A. Werner, H. Neumann, and G. W. Kreutzberg. 1998. Immune surveillance in the injured nervous system: T-lymphocytes invade the axotomized mouse facial motor nucleus and aggregate around sites of neuronal degeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18: 5804-5816.
169. Serpe, C. J., S. Coers, V. M. Sanders, and K. J. Jones. 2003. CD4+ T, but not CD8+ or B, lymphocytes mediate facial motoneuron survival after facial nerve transection. *Brain Behav Immun* 17: 393-402.
170. Chiu, I. M., A. Chen, Y. Zheng, B. Kosaras, S. A. Tsiftsoglou, T. K. Vartanian, R. H. Brown, Jr., and M. C. Carroll. 2008. T lymphocytes potentiate endogenous neuroprotective inflammation in a mouse model of ALS. *Proc Natl Acad Sci U S A* 105: 17913-17918.

171. Beers, D. R., J. S. Henkel, W. Zhao, J. Wang, A. Huang, S. Wen, B. Liao, and S. H. Appel. 2011. Endogenous regulatory T lymphocytes ameliorate amyotrophic lateral sclerosis in mice and correlate with disease progression in patients with amyotrophic lateral sclerosis. *Brain* 134: 1293-1314.
172. Ortega, S. B., V. P. Kashi, A. F. Tyler, K. Cunnusamy, J. P. Mendoza, and N. J. Karandikar. 2013. The disease-ameliorating function of autoregulatory CD8 T cells is mediated by targeting of encephalitogenic CD4 T cells in experimental autoimmune encephalomyelitis. *Journal of immunology* 191: 117-126.
173. Goverman, J. 2009. Autoimmune T cell responses in the central nervous system. *Nature reviews. Immunology* 9: 393-407.
174. Hammarberg, H., O. Lidman, C. Lundberg, S. Y. Eltayeb, A. W. Gielen, S. Muhallab, A. Svenningsson, H. Linda, P. H. van Der Meide, S. Cullheim, T. Olsson, and F. Piehl. 2000. Neuroprotection by encephalomyelitis: rescue of mechanically injured neurons and neurotrophin production by CNS-infiltrating T and natural killer cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20: 5283-5291.
175. Wessler, I., C. J. Kirkpatrick, and K. Racke. 1999. The cholinergic 'pitfall': acetylcholine, a universal cell molecule in biological systems, including humans. *Clin Exp Pharmacol Physiol* 26: 198-205.
176. Ruff, R. L. 1998. Electrophysiology of postsynaptic activation. *Ann N Y Acad Sci* 841: 57-70.
177. Boron, W. F., Boulpaep, E.L. 2003. *Medical Physiology*. Elsevier Science.
178. Anglade, P., and Y. Larabi-Godinot. 2010. Historical landmarks in the histochemistry of the cholinergic synapse: Perspectives for future researches. *Biomed Res* 31: 1-12.
179. Darvesh, S., D. A. Hopkins, and C. Geula. 2003. Neurobiology of butyrylcholinesterase. *Nat Rev Neurosci* 4: 131-138.
180. Parrish, W. R., M. Rosas-Ballina, M. Gallowitsch-Puerta, M. Ochani, K. Ochani, L. H. Yang, L. Hudson, X. Lin, N. Patel, S. M. Johnson, S. Chavan, R. S. Goldstein, C. J. Czura, E. J. Miller, Y. Al-Abed, K. J. Tracey, and V. A. Pavlov. 2008. Modulation of TNF release by choline requires alpha7 subunit nicotinic acetylcholine receptor-mediated signaling. *Molecular medicine* 14: 567-574.
181. Grando, S. A., K. Kawashima, C. J. Kirkpatrick, and I. Wessler. 2007. Recent progress in understanding the non-neuronal cholinergic system in humans. *Life Sci* 80: 2181-2185.
182. Rosas-Ballina, M., P. S. Olofsson, M. Ochani, S. I. Valdes-Ferrer, Y. A. Levine, C. Reardon, M. W. Tusche, V. A. Pavlov, U. Andersson, S. Chavan, T. W. Mak, and K. J. Tracey. 2011. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science* 334: 98-101.
183. Koliatsos, V. E., W. L. Price, C. A. Pardo, and D. L. Price. 1994. Ventral root avulsion: an experimental model of death of adult motor neurons. *The Journal of comparative neurology* 342: 35-44.
184. Piehl, F., C. Lundberg, M. Khademi, A. Bucht, I. Dahlman, J. C. Lorentzen, and T. Olsson. 1999. Non-MHC gene regulation of nerve root injury induced spinal cord inflammation and neuron death. *Journal of neuroimmunology* 101: 87-97.
185. Gilbert, A., G. Pivato, and T. Kheiralla. 2006. Long-term results of primary repair of brachial plexus lesions in children. *Microsurgery* 26: 334-342.
186. Kachramanoglou, C., D. Li, P. Andrews, C. East, T. Carlstedt, G. Raisman, and D. Choi. 2011. Novel strategies in brachial plexus repair after traumatic avulsion. *Br J Neurosurg* 25: 16-27.

187. Blinzinger, K., and G. Kreutzberg. 1968. Displacement of synaptic terminals from regenerating motoneurons by microglial cells. *Z Zellforsch Mikrosk Anat* 85: 145-157.
188. Oliveira, A. L., S. Thams, O. Lidman, F. Piehl, T. Hokfelt, K. Karre, H. Linda, and S. Cullheim. 2004. A role for MHC class I molecules in synaptic plasticity and regeneration of neurons after axotomy. *Proc Natl Acad Sci U S A* 101: 17843-17848.
189. Coggeshall, R. E., H. A. Lekan, F. A. White, and C. J. Woolf. 2001. A-fiber sensory input induces neuronal cell death in the dorsal horn of the adult rat spinal cord. *J Comp Neurol* 435: 276-282.
190. Al Nimer, F., A. D. Beyeen, R. Lindblom, M. Strom, S. Aeinehband, O. Lidman, and F. Piehl. 2011. Both MHC and non-MHC genes regulate inflammation and T-cell response after traumatic brain injury. *Brain Behav Immun* 25: 981-990.
191. Feeney, D. M., M. G. Boyeson, R. T. Linn, H. M. Murray, and W. G. Dail. 1981. Responses to cortical injury: I. Methodology and local effects of contusions in the rat. *Brain Res* 211: 67-77.
192. Constantinescu, C. S., N. Farooqi, K. O'Brien, and B. Gran. 2011. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol* 164: 1079-1106.
193. Sriram, S., and I. Steiner. 2005. Experimental allergic encephalomyelitis: a misleading model of multiple sclerosis. *Annals of neurology* 58: 939-945.
194. McDonald, W. I., A. Compston, G. Edan, D. Goodkin, H. P. Hartung, F. D. Lublin, H. F. McFarland, D. W. Paty, C. H. Polman, S. C. Reingold, M. Sandberg-Wollheim, W. Sibley, A. Thompson, S. van den Noort, B. Y. Weinshenker, and J. S. Wolinsky. 2001. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Annals of neurology* 50: 121-127.
195. Kurtzke, J. F. 1983. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* 33: 1444-1452.
196. Greenbaum, D., R. Jansen, and M. Gerstein. 2002. Analysis of mRNA expression and protein abundance data: an approach for the comparison of the enrichment of features in the cellular population of proteins and transcripts. *Bioinformatics* 18: 585-596.
197. Washburn, M. P., A. Koller, G. Oshiro, R. R. Ulaszek, D. Plouffe, C. Deciu, E. Winzeler, and J. R. Yates, 3rd. 2003. Protein pathway and complex clustering of correlated mRNA and protein expression analyses in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences of the United States of America* 100: 3107-3112.
198. Laird, P. W., A. Zijderveld, K. Linders, M. A. Rudnicki, R. Jaenisch, and A. Berns. 1991. Simplified mammalian DNA isolation procedure. *Nucleic Acids Res* 19: 4293.
199. Weissenbach, J. 1993. Microsatellite polymorphisms and the genetic linkage map of the human genome. *Curr Opin Genet Dev* 3: 414-417.
200. Frazer, K. A., D. G. Ballinger, D. R. Cox, D. A. Hinds, L. L. Stuve, R. A. Gibbs, J. W. Belmont, A. Boudreau, P. Hardenbol, S. M. Leal, S. Pasternak, D. A. Wheeler, T. D. Willis, F. Yu, H. Yang, C. Zeng, Y. Gao, H. Hu, W. Hu, C. Li, W. Lin, S. Liu, H. Pan, X. Tang, J. Wang, W. Wang, J. Yu, B. Zhang, Q. Zhang, H. Zhao, J. Zhou, S. B. Gabriel, R. Barry, B. Blumenstiel, A. Camargo, M. Defelice, M. Faggart, M. Goyette, S. Gupta, J. Moore, H. Nguyen, R. C. Onofrio, M. Parkin, J. Roy, E. Stahl, E. Winchester, L. Ziaugra, D. Altshuler, Y. Shen, Z. Yao, W. Huang, X. Chu, Y. He, L. Jin, Y. Liu, W. Sun, H. Wang,

Y. Wang, X. Xiong, L. Xu, M. M. Waye, S. K. Tsui, H. Xue, J. T. Wong, L. M. Galver, J. B. Fan, K. Gunderson, S. S. Murray, A. R. Oliphant, M. S. Chee, A. Montpetit, F. Chagnon, V. Ferretti, M. Leboeuf, J. F. Olivier, M. S. Phillips, S. Roumy, C. Sallee, A. Verner, T. J. Hudson, P. Y. Kwok, D. Cai, D. C. Koboldt, R. D. Miller, L. Pawlikowska, P. Taillon-Miller, M. Xiao, L. C. Tsui, W. Mak, Y. Q. Song, P. K. Tam, Y. Nakamura, T. Kawaguchi, T. Kitamoto, T. Morizono, A. Nagashima, Y. Ohnishi, A. Sekine, T. Tanaka, T. Tsunoda, P. Deloukas, C. P. Bird, M. Delgado, E. T. Dermitzakis, R. Gwilliam, S. Hunt, J. Morrison, D. Powell, B. E. Stranger, P. Whittaker, D. R. Bentley, M. J. Daly, P. I. de Bakker, J. Barrett, Y. R. Chretien, J. Maller, S. McCarroll, N. Patterson, I. Pe'er, A. Price, S. Purcell, D. J. Richter, P. Sabeti, R. Saxena, S. F. Schaffner, P. C. Sham, P. Varilly, L. D. Stein, L. Krishnan, A. V. Smith, M. K. Tello-Ruiz, G. A. Thorisson, A. Chakravarti, P. E. Chen, D. J. Cutler, C. S. Kashuk, S. Lin, G. R. Abecasis, W. Guan, Y. Li, H. M. Munro, Z. S. Qin, D. J. Thomas, G. McVean, A. Auton, L. Bottolo, N. Cardin, S. Eyheramendy, C. Freeman, J. Marchini, S. Myers, C. Spencer, M. Stephens, P. Donnelly, L. R. Cardon, G. Clarke, D. M. Evans, A. P. Morris, B. S. Weir, J. C. Mullikin, S. T. Sherry, M. Feolo, A. Skol, H. Zhang, I. Matsuda, Y. Fukushima, D. R. Macer, E. Suda, C. N. Rotimi, C. A. Adebamowo, I. Ajayi, T. Aniagwu, P. A. Marshall, C. Nkwodimmah, C. D. Royal, M. F. Leppert, M. Dixon, A. Peiffer, R. Qiu, A. Kent, K. Kato, N. Niikawa, I. F. Adewole, B. M. Knoppers, M. W. Foster, E. W. Clayton, J. Watkin, D. Muzny, L. Nazareth, E. Sodergren, G. M. Weinstock, I. Yakub, B. W. Birren, R. K. Wilson, L. L. Fulton, J. Rogers, J. Burton, N. P. Carter, C. M. Clee, M. Griffiths, M. C. Jones, K. McLay, R. W. Plumb, M. T. Ross, S. K. Sims, D. L. Willey, Z. Chen, H. Han, L. Kang, M. Godbout, J. C. Wallenburg, P. L'Archeveque, G. Bellemare, K. Saeki, D. An, H. Fu, Q. Li, Z. Wang, R. Wang, A. L. Holden, L. D. Brooks, J. E. McEwen, M. S. Guyer, V. O. Wang, J. L. Peterson, M. Shi, J. Spiegel, L. M. Sung, L. F. Zacharia, F. S. Collins, K. Kennedy, R. Jamieson, and J. Stewart. 2007. A second generation human haplotype map of over 3.1 million SNPs. *Nature* 449: 851-861.

201. Subramanian, S., R. K. Mishra, and L. Singh. 2003. Genome-wide analysis of microsatellite repeats in humans: their abundance and density in specific genomic regions. *Genome biology* 4: R13.
202. Darvasi A., S. M. 1994. Optimum spacing of genetic markers for determining linkage between marker loci and quantative trait loci. *Theoretical and Applied Genetics*: 351-357.
203. Barabasi, A. L., and Z. N. Oltvai. 2004. Network biology: understanding the cell's functional organization. *Nat Rev Genet* 5: 101-113.
204. Schadt, E. E., S. A. Monks, T. A. Drake, A. J. Lusis, N. Che, V. Colinayo, T. G. Ruff, S. B. Milligan, J. R. Lamb, G. Cavet, P. S. Linsley, M. Mao, R. B. Stoughton, and S. H. Friend. 2003. Genetics of gene expression surveyed in maize, mouse and man. *Nature* 422: 297-302.
205. Strom, M., F. Al Nimer, R. Lindblom, J. R. Nyengaard, and F. Piehl. 2011. Naturally Occurring Genetic Variability in Expression of Gsta4 is Associated with Differential Survival of Axotomized Rat Motoneurons. *Neuromolecular Medicine*.
206. Lundberg, C., O. Lidman, R. Holmdahl, T. Olsson, and F. Piehl. 2001. Neurodegeneration and glial activation patterns after mechanical nerve injury are differentially regulated by non-MHC genes in congenic inbred rat strains. *J Comp Neurol* 431: 75-87.

207. Swanberg, M., K. Duvefelt, M. Diez, J. Hillert, T. Olsson, F. Piehl, and O. Lidman. 2006. Genetically determined susceptibility to neurodegeneration is associated with expression of inflammatory genes. *Neurobiol Dis* 24: 67-88.
208. Weiner, H. L. 2008. A shift from adaptive to innate immunity: a potential mechanism of disease progression in multiple sclerosis. *J Neurol* 255 Suppl 1: 3-11.
209. Iacobaeus, E., P. Amoudruz, M. Strom, M. Khademi, L. Brundin, J. Hillert, I. Kockum, V. Malmstrom, T. Olsson, E. Tham, and F. Piehl. 2011. The expression of VEGF-A is down regulated in peripheral blood mononuclear cells of patients with secondary progressive multiple sclerosis. *PLoS One* 6: e19138.
210. Serafini, B., B. Rosicarelli, R. Magliozzi, E. Stigliano, and F. Aloisi. 2004. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. *Brain Pathol* 14: 164-174.
211. Ingram, G., S. Hakobyan, N. P. Robertson, and B. P. Morgan. 2009. Complement in multiple sclerosis: its role in disease and potential as a biomarker. *Clin Exp Immunol* 155: 128-139.
212. Mesulam, M. M., and C. Geula. 1994. Butyrylcholinesterase reactivity differentiates the amyloid plaques of aging from those of dementia. *Ann Neurol* 36: 722-727.
213. Darvesh, S., A. M. Leblanc, I. R. Macdonald, G. A. Reid, V. Bhan, R. J. Macaulay, and J. D. Fisk. 2010. Butyrylcholinesterase activity in multiple sclerosis neuropathology. *Chem Biol Interact* 187: 425-431.
214. Teunissen, C. E., C. Dijkstra, and C. Polman. 2005. Biological markers in CSF and blood for axonal degeneration in multiple sclerosis. *Lancet Neurol* 4: 32-41.
215. Atkinson, C., H. Song, B. Lu, F. Qiao, T. A. Burns, V. M. Holers, G. C. Tsokos, and S. Tomlinson. 2005. Targeted complement inhibition by C3d recognition ameliorates tissue injury without apparent increase in susceptibility to infection. *J Clin Invest* 115: 2444-2453.
216. Song, H., C. He, C. Knaak, J. M. Guthridge, V. M. Holers, and S. Tomlinson. 2003. Complement receptor 2-mediated targeting of complement inhibitors to sites of complement activation. *J Clin Invest* 111: 1875-1885.
217. Al Nimer, F., A. D. Beyeen, R. Lindblom, M. Strom, S. Aeinehband, O. Lidman, and F. Piehl. 2011. Both MHC and non-MHC genes regulate inflammation and T-cell response after traumatic brain injury. *Brain Behav Immun* 25: 981-990.
218. Kheradmand, T., P. P. Trivedi, N. A. Wolf, P. C. Roberts, and R. H. Swanborg. 2008. Characterization of a subset of bone marrow-derived natural killer cells that regulates T cell activation in rats. *Journal of leukocyte biology* 83: 1128-1135.
219. Lord, A., A. Gumucio, H. Englund, D. Sehlin, V. S. Sundquist, L. Soderberg, C. Moller, P. Gellerfors, L. Lannfelt, F. E. Pettersson, and L. N. Nilsson. 2009. An amyloid-beta protofibril-selective antibody prevents amyloid formation in a mouse model of Alzheimer's disease. *Neurobiology of disease* 36: 425-434.
220. Vijayaraghavan, S., A. Karami, S. Aeinehband, H. Behbahani, A. Grandien, B. Nilsson, K. N. Ekdahl, R. P. Lindblom, F. Piehl, and T. Darreh-Shori. 2013. Regulated Extracellular Choline Acetyltransferase Activity- The Plausible Missing Link of the Distant Action of Acetylcholine in the Cholinergic Anti-Inflammatory Pathway. *PLoS One* 8: e65936.
221. Darreh-Shori, T., S. Vijayaraghavan, S. Aeinehband, F. Piehl, R. P. Lindblom, B. Nilsson, K. N. Ekdahl, B. Langstrom, O. Almkvist, and A. Nordberg. 2013. Functional variability in butyrylcholinesterase activity regulates intrathecal

cytokine and astroglial biomarker profiles in patients with Alzheimer's disease. *Neurobiology of Aging*.
222. Centonze, D., L. Muzio, S. Rossi, F. Cavasinni, V. De Chiara, A. Bergami, A. Musella, M. D'Amelio, V. Cavallucci, A. Martorana, A. Bergamaschi, M. T. Cencioni, A. Diamantini, E. Butti, G. Comi, G. Bernardi, F. Cecconi, L. Battistini, R. Furlan, and G. Martino. 2009. Inflammation triggers synaptic alteration and degeneration in experimental autoimmune encephalomyelitis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29: 3442-3452.
223. Hilton, K. J., C. Cunningham, R. A. Reynolds, and V. H. Perry. 2013. Early Hippocampal Synaptic Loss Precedes Neuronal Loss and Associates with Early Behavioural Deficits in Three Distinct Strains of Prion Disease. *PLoS One* 8: e68062.
224. Quik, M., X. A. Perez, and T. Bordia. 2012. Nicotine as a potential neuroprotective agent for Parkinson's disease. *Mov Disord* 27: 947-957.
225. Tarakji, K. G., J. F. Sabik, 3rd, S. K. Bhudia, L. H. Batizy, and E. H. Blackstone. 2011. Temporal onset, risk factors, and outcomes associated with stroke after coronary artery bypass grafting. *Jama* 305: 381-390.
226. Sun, X., J. Lindsay, L. H. Monsein, P. C. Hill, and P. J. Corso. 2012. Silent brain injury after cardiac surgery: a review: cognitive dysfunction and magnetic resonance imaging diffusion-weighted imaging findings. *J Am Coll Cardiol* 60: 791-797.
227. Monk, T. G., B. C. Weldon, C. W. Garvan, D. E. Dede, M. T. van der Aa, K. M. Heilman, and J. S. Gravenstein. 2008. Predictors of cognitive dysfunction after major noncardiac surgery. *Anesthesiology* 108: 18-30.
228. Buljevac, D., H. Z. Flach, W. C. Hop, D. Hijdra, J. D. Laman, H. F. Savelkoul, F. G. van Der Meche, P. A. van Doorn, and R. Q. Hintzen. 2002. Prospective study on the relationship between infections and multiple sclerosis exacerbations. *Brain* 125: 952-960.
229. Moreno, B., J. P. Jukes, N. Vergara-Irigaray, O. Errea, P. Villoslada, V. H. Perry, and T. A. Newman. 2011. Systemic inflammation induces axon injury during brain inflammation. *Annals of neurology* 70: 932-942.
230. Fremeaux-Bacchi, V., J. P. Aubry, J. Y. Bonnefoy, M. D. Kazatchkine, J. P. Kolb, and E. M. Fischer. 1998. Soluble CD21 induces activation and differentiation of human monocytes through binding to membrane CD23. *Eur J Immunol* 28: 4268-4274.
231. Aichem, A., M. Masilamani, and H. Illges. 2006. Redox regulation of CD21 shedding involves signaling via PKC and indicates the formation of a juxtamembrane stalk. *J Cell Sci* 119: 2892-2902.