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YURIY POMESHCHIK

Novel strategies for spinal cord injury repair

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ABSTRACT

Traumatic spinal cord injury (SCI) is a catastrophic condition affecting primarily young males. The insult to the spinal cord results in functional impairment and loss of sensation and autonomic function below the injury level, often leaving patients bound to a wheelchair. Treatment options for SCI are limited to high doses of methylprednisolone (MP), the side effects of which overpower relatively modest neurological improvements.

SCI is biphasic in nature with the primary trauma triggering the multicomponent secondary injury cascade that greatly augments initial damage. While the primary damage is irreversible the secondary injury is an attractive target for therapeutic interventions. Oxidative stress and inflammation are interrelated key components of the secondary injury cascade. The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is important for endogenous protection from oxidative stress, whereas interleukin-33 (IL-33) has potential to modulate adaptive and innate immunity. Induced pluripotent stem cells (iPSCs) lack many drawbacks of other stem cell types and are a novel, promising cell source for SCI regeneration. In the present study we aimed to investigate whether Nrf2 gene transfer, pharmacological IL-33 treatment and iPSCs transplantations alleviate secondary injury and improve functional recovery after contusion SCI.

Our results demonstrated the sustained nature of Nrf2 and IL-33 activation after contusion SCI. We identified astrocytes as the main reservoir of intracellular IL-33. Using mice deficient for Nrf2 we confirmed the essential role of Nrf2 in SCI pathophysiology and discovered new mechanisms of Nrf2-mediated protection. Transfer of the Nrf2 gene into the spinal cord with a lentivirus vector resulted in overexpression of Nrf2 in astrocytes and neurons. Gene therapy resulted in a number of side effects and did not provide extra benefit over natural Nrf2 induction. In contrast, recombinant IL-33 showed great promise as a novel treatment for SCI. IL-33 modulated inflammation in the spinal cord and in the periphery, leading to attenuated secondary injury and improved functional recovery. Despite iPSC therapy being safe and transplanted cells having had the potential to differentiate into neurons *in vivo* they neither survived nor improved functional recovery when transplanted into contused spinal cord. Scarce survival of transplanted cells may be attributed to insufficient levels of immunosuppression provided by monotherapy with calcineurin inhibitor Tacrolimus.

The results of this thesis provided new insights into the complex pathophysiology of secondary damage after traumatic SCI and highlighted the importance of its proper regulation. Modulation of the inflammatory response with IL-33 treatment represents a promising therapeutic approach for patients with acute contusion SCI. Although Nrf2 gene transfer and iPSCs transplantation did not provide the expected benefits, our results are important for the further, successful development of these treatments in the future.

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TIIVISTELMÄ

Traumaattinen selkäydinvaurio on laaja-alainen kehon toimintaa vaikeuttava vamma, jonka riskiryhmään kuuluvat nuoret miehet. Selkäydinvaurio johtaa toiminta- ja liikuntakyvyn menetykseen ja autonomisen hermoston toiminnan häiriöihin. Koska selkäydinvammoihin ei ole olemassa tehokasta hoitoa, on uusien hoitomuotojen kehittämisen tarve suuri.

Selkäydinvaurion jälkeiset sekundaariset patologiat, kuten tulehdusreaktio ja hapettumisvauriot voimistavat selkäydinvamman aiheuttamaa ensivauriota.

Nrf2-transkriptiotekijä säätelee tärkeää solujen puolustuskokonaisuutta, joka koostuu sadoista hapettumisvaurioilta suojaavista geeneistä. Interleukiini-33 (IL-33) on elimistön oma sytokiini, joka säätelee immuunivastetta. Indusoidut pluripotentit (iPSCs) kantasolut ovat erilaistuneista soluista ohjelmoituja kantasoluja, joilla on kyky erilaistua miksi tahansa elimistön soluksi. Niiden käyttöön liittyvien vähäisten eettisten ongelmien ja olemattomien yhteensopivuusongelmien vuoksi iPSC-solut ovat mullistaneet kantasoluterapian.

Tämän työn tavoitteena oli tutkia kolmea uutta hoitomuotoa, joiden ajateltiin vaikuttavan selkäydinvaurion sekundaarista patologiaa lieventävästi ja toiminnallista palautumista edistävästi: Nrf2 geeniterapia, IL-33 hoito ja kantasoluterapia.

Tutkimustulokset osoittavat, että lentivirusvälitteinen Nrf2-geeninsiirto hiirten selkäytimeen ei vähennä selkäydinvammaan liittyviä oireita. Sen sijaan Nrf2-tekijän poistaminen lisää selkäydinvammaan liittyvää toiminnallista heikentymistä, osoittaen siten Nrf2-tekijän olevan erittäin tärkeä selkäydinvauriosta toipumisessa.

IL-33-hoidolla aikaansaatiin tehokas, solujen luonnollista tulehdusvastetta säätelevä hoitovaste selkäydinvauriossa. Hoito vähensi selkäydinvamman jälkeistä sekundaarista patologiaa ja paransi vaurioon liittyvää toiminnallista heikentymistä hiirissä.

Lääkinnällinen immuunivasteen heikentäminen ei parantanut ihmisperäisten kantasolujen selviytymistä hiiren vaurioituneessa selkäytimessä. Siten soluterapia ihmisen iPSC-soluista erilaistetuilla aivosoluilla ei lievittänyt selkäydinvaurioon liittyvää toiminnallista heikentymistä hiirissä. Tutkimustulokset korostavat immuunivasteen huomioimisen tärkeyttä kantasoluterapiassa.

Tämän väitöskirjatutkimuksen perusteella todettiin, että sekundaarista patologiaa säätelevä IL-33-terapia on uusi ja lupaava hoitomuoto selkäydinvauriossa. Lisäksi havainnot kantasoluterapian ja Nrf2-tekijän vaikutuksista lisäävät ymmärrystämme näihin kohdistuvien terapiamuotojen edellytyksistä ja tulevat tulevaisuudessa lisäämään uusien, tehokkaiden hoitomuotojen kehittämisen mahdollisuuksia selkäydinvauriossa.

Yleinen Suomalainen asiasanasto: autonominen hermosto; eläinkokeet; geenitekniikka; geeniterapia; hoitomenetelmät; hoitovaste; immuunivaste; kantasolujen siirto; kehon hallinta; selkäydinvammat; selkäydin; sytokiinit; toimintahäiriöt; toipuminen; tulehdus; vammat; vauriot

I'm an idealist. I don't know where I'm going, but I'm on my way.

- Carl Sandburg

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Yuriy Pomeshchik

List of the original publications

This dissertation is based on the following original publications:

I. **Pomeshchik Y**, Kidin I, Savchenko E, Rolova T, Yamamoto M, Levonen AL, Ylä-Herttuala S, Malm T, Kanninen K, and Koistinaho J. Does Nrf2 Gene Transfer Facilitate Recovery After Contusion Spinal Cord Injury? *Antioxidants & Redox Signaling 20:1313-23, 2014*

II. **Pomeshchik Y***, Kidin I*, Korhonen P, Savchenko E, Jaronen M, Lehtonen S, Wojciechowski S, Kanninen K, Koistinaho J, and Malm T. Interleukin-33 treatment reduces secondary injury and improves functional recovery after spinal cord injury. *Brain Behavior and Immunity. Accepted manuscript, 2014*

III. **Pomeshchik Y,** Puttonen K.A, Kidin I, Ruponen M, Lehtonen S, Malm T, Åkesson E, Hovatta O, and Koistinaho J. Transplanted human iPSC-derived neural progenitor cells do not promote functional recovery of pharmacologically immunosuppressed mice with contusion spinal cord injury. *Cell Transplantation. Accepted manuscript, 2014*

*Authors with equal contribution.

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Contents

Abbreviations

ALS	amyotrophic lateral sclerosis
AMPA	alpha-amino-3-hydroxy-5-methyl-isoxazolepropionate
ARE	antioxidant response element
BBB	blood-brain barrier
BDNF	brain derived neurotrophic factor
BMS	Basso mouse scale
BSA	bovine serum albumin
BSB	blood-spinal barrier
CBA	cytometric bead array
ChABC	chondroitinase ABC
CNS	the central nervous system
CSPG	chondroitin sulfate proteoglycan
DCX	doublecortin
EAE	experimental autoimmune encephalomyelitis
EMA	European Medicines Agency
EPO	erythropoietin
ESCs	embryonic stem cells
FBS	fetal bovine serum
FGF	fibroblast growth factor
FSCs	fetal stem cells
GCL	glutamate–cysteine ligase
GDNF	glial cell-line derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSH	Glutathione
GSK-3β	glycogen synthase kinase 3
GST	glutathione-S-transferases
HO-1	heme oxygenase 1
HuNu	human nuclei antigen
Iba-1	Ionized calcium-binding adapter molecule 1
ICC	immunocytochemistry
IFN-γ	interferon-gamma
IHC	immunohistochemistry
IL	interleukin
IL-1RAcP	IL-1 receptor accessory protein
iPSCs	induced pluripotent stem cells
JAK2	Janus kinase 2
Keap1	Kelch-like ECH-associated protein 1
LFB	luxol fast blue
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
LV	lentivirus
MAC	membrane attack complex
Maf	masculoaponeurotic fibrosarcoma
MAP-2	microtube-associated protein
MAPK	mitogen-activated protein kinase

MMP	matrix metalloproteinase
MP	methylprednisolone
MRI	magnetic resonance imaging
MSCs	mesenchymal stem cells
NeuN	neuronal nuclear antigen
NBQX	2,3-dihydro-6-nitro-7-sulfamoyl-benzo(f)quinoxaline
NF-κB	nuclear factor κB
NGF	nerve growth factor
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
NQO1	NAD(P)H:(quinine acceptor) oxidoreductase 1
Nrf2	nuclear factor (erythroid-derived 2)-like 2
NS/PCs	neural stem/progenitor cells
NSAIDs	nonsteroidal anti-inflammatory drugs
Oct-4	octamer-binding transcription factor
OECs	olfactory ensheathing cells
PAX-6	paired box protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
RI	regulatory index
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
SCI	spinal cord injury
SCs	Schwann cells
SOD	Cu, Zn-superoxide dismutase
TBI	traumatic brain injury
TGF-β	transforming growth factor beta
Th1	type 1 helper T-cell
Th2	type 2 helper T-cell
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

1 Introduction

The oldest known trauma textbook in history, the Edwin Smith papyrus (1700 BCE), described spinal cord injury (SCI) as a ''medical condition that cannot be healed" (van Middendorp et al., 2010). Now, more than 3700 years later, despite enormous progress in clinical medicine and preclinical research, damage from SCI is largely irreversible and SCI remains a catastrophic condition for which treatment is mostly palliative (Kwon et al., 2013; Mothe and Tator, 2013; Varma et al., 2013; Silva et al., 2014).

SCI is a sudden and unexpected condition affecting primarily young males and resulting in early invalidization. Motor function impairments up to tetraplegia (Figure 1) and/or loss of sensation and autonomic function developing after SCI seriously diminish the quality of life of injured individual and lead to social isolation (Rooney et al., 2009; Chen Y et al., 2013). Currently the management of acute SCI involves surgical decompression followed by physical therapy and rehabilitation. Pharmacological interventions are limited to high doses of synthetic glycocorticoid methylprednisolone (MP) (Hurlbert et al., 2013; Varma et al., 2013). However, the advantages of high-dose MP for SCI treatment are very controversial due to serious adverse effects and relatively modest neurological improvements (Bracken et al., 1990; Hurlbert et al., 2013). Therefore, there is urgent need to explore new therapeutic strategies for restoring the neurological function after SCI and also to expand the knowledge on the cellular and molecular mechanisms of this devastating condition.

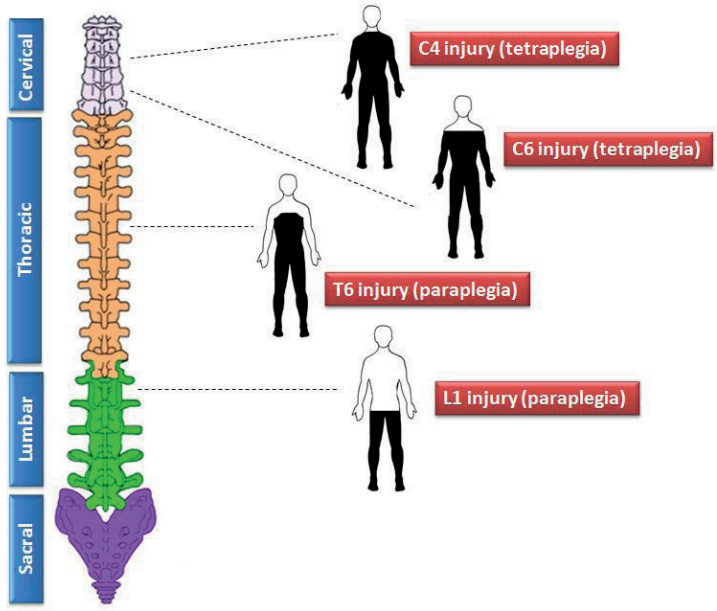

Figure 1. Extent of injury due to damage of specific spinal segments (modified from Thuret et al., 2006).

Primary mechanical injury destroys glial and neural elements and triggers the secondary injury cascade that induces the delayed death of cells surviving after initial trauma. Secondary injury greatly increases the neurological deficit and complicates the restoration of spinal cord function (Tator, 1995; Kwon et al., 2004; Oudega, 2013). Multiple mechanisms participate in the complex secondary injury cascade and amplify the initial damage.

Oxidative stress and the inflammatory response are well-established critical components of the secondary injury cascade and probably occupy a central place in pathophysiology of the secondary damage. Importantly, these processes are closely interrelated and augment each other (Rowland et al., 2008).

Oxidative stress resulting from the excessive production of reactive oxygen species (ROS) and exhaustion of the endogenous antioxidant system (Halliwell et al., 2007) induces a number of detrimental processes such as lipid peroxidation, protein oxidation, and DNA damage (Smith et al., 2013). At the front line of defence against oxidative stress is the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Under oxidative and electrophilic stresses, Nrf2 dissociates from its cytosolic negative regulator Kelch-like ECH-associated protein 1 (Keap1) and translocates to the nucleus to induce gene expression of hundreds cytoprotective genes that contain an antioxidant response element (ARE) in their promoter region (Itoh et al., 1997, 1999, 2010; Kobayashi and Yamamoto, 2006; Baird and Dinkova-Kostova, 2011). Therefore, activation of the Nrf2-ARE pathway represents an endogenous compensatory response to oxidative stress in order to restore impaired redox balance (Zhang et al., 2013). While the importance of the Nrf2-ARE system has been proven in brain injury models such as stroke and traumatic brain injury (TBI) (Chen and Regan, 2007; Tanaka et al., 2011; Shang et al., 2013), the contribution of Nrf2-ARE in protection from SCI is scarcely investigated and mainly limited to compression SCI model (Mao et al., 2010, 2011, 2012), warranting the need for comprehensive research of Nrf2 functions in more relevant SCI models.

Gene therapy approaches hold great promise for the treatment of different incurable diseases. Gene transfer with viral vectors allows the delivery of therapeutic genes to target cells. While gene therapy has already entered into clinical trials for several neurodegenerative diseases (Mandel, 2010; LeWitt et al., 2011; Palfi et al., 2014) its advantages are largely unexplored in SCI models. Gene delivery of transcription factors is especially attractive as it enables simultaneous induction or repression of several genes sharing a common regulatory pathway. Therefore, Nrf2 gene transfer after SCI may provide induction of numerous cytoprotective genes helping the cells to cope with secondary injury.

Various cells types and mediators participate in the highly complex inflammatory response after SCI (Rowland et al., 2008; Plemel et al., 2014). Primary injury disrupts the blood-spinal barrier (BSB) and triggers the release of pro-inflammatory mediators from the activated resident central nervous system (CNS) cells, resulting in influx of neutrophils, monocytes and lymphocytes from the blood stream (Hausmann, 2003; Pineau and Lacroix, 2007; Donnelly and Popovich, 2008; Beck et al., 2010). For a long time inflammation has been considered to be absolutely deleterious for regeneration and functional recovery (Taoka et al., 1997; Popovich et al., 1999; Young, 2002). However, gradually it became clear that immune cells are highly heterogeneous and not all immune subsets are detrimental for SCI recovery. Some are even beneficial and essential. The scarce effect of traditional anti-inflammatory drugs further supported the view that abolishing inflammation is not a feasible therapeutic strategy for SCI. Instead, immunomodulatory therapy that promotes the skewing of pro-inflammatory cells into an anti-inflammatory phenotype is believed to allow the harnessing of beneficial components of inflammation and facilitation of functional recovery (Rossignol et al., 2007; David et al., 2012; Plemel et al., 2014). Interleukin-33 (IL-33), a member of the IL-1 cytokine family, is known to possess immunomodulatory properties and therefore represents an attractive tool for SCI repair.

Stem cell -based approach for SCI repair emerged in the middle of 1990s. Since that time various types of cells have been tested in preclinical studies and some even moved to the clinical trial phase (Fehlings and Vawda, 2011; Mothe and Tator, 2012, 2013; Silva et al., 2014). Transplanted stem cells are believed to replace damaged neuronal and glial cells, provide trophic support for the surviving cells, facilitate remyelination and create an environment favorable for regeneration (Fehlings and Vawda, 2011; Mothe and Tator, 2012,

2013). Inherently specified to differentiate along the neural lineage neural stem/progenitor cells (NS/PCs) may be derived from the adult and fetal CNS, pluripotent embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). While autologous derivation of adult CNS-derived NS/PCs is almost impossible in clinical settings (Fehlings and Vawda, 2011; Faulkner et al., 2014) and the use of ESCs-derived NS/PCs is very limited by ethical, safety (potential tumorigenesis) and immunological (allogeneic nature) reasons (Mothe and Tator, 2013), iPSC-NS/PCs derived directly from a patient's own somatic cells almost do not have these limitations (Fehlings and Vawda, 2011; Kramer et al., 2013; Nakamura and Okano, 2013). The existing preclinical studies estimating the efficiency of iPSC-derived NS/PCs provided very promising preliminary results. However, they mainly utilized immunodeficient rodents to avoid the host immune response (Nori et al., 2011; Fujimoto et al., 2012; Lu et al., 2014; Sareen et al., 2014), which is not very relevant to clinical settings.

This study was carried out to estimate the effect of three novel therapeutic strategies on recovery from contusion SCI. First, we employed a gene therapy approach to deliver transcription factor Nrf2 into contused mouse spinal cord with the aim of boosting Nrf2-ARE pathway and enhancing endogenous defence in response to secondary injury. Prior to Nrf2 gene transfer we evaluated whether the Nrf2-ARE pathway responds to SCI and determined the magnitude and duration of this response. Furthermore, we investigated the role of the Nrf2-ARE system in secondary injury using mice deficient for the Nrf2 gene. Next, utilizing a pharmacological approach we assessed whether IL-33, a member of the IL-1 family, is able to beneficially modulate the inflammatory response after SCI by reducing secondary injury and promoting functional recovery. Both peripheral and central mechanisms of IL-33 action after contusion SCI were identified. Finally, we assessed the potential of human iPSC-derived NPCs subacutely transplanted into contused spinal cord to survive and promote functional recovery when pharmacological immunosuppression was used to avoid the host immune response.

2 Review of the literature

2.1 SPINAL CORD INJURY (SCI)

2.1.1 Definition and epidemiology of SCI

SCI is defined as any traumatic injury to the spinal cord. It demolishes neural and glial cells and interrupts pathways connecting the brain and the rest of the body. Disruption of nerve connections results in sensory loss, paralysis and loss of autonomic function (Rooney et al., 2009; Nutt et al., 2013; Olson, 2013).

Although reliable information on the epidemiology for traumatic SCI is unavailable for many countries, it is clear that incidence, prevalence, and injury etiology vary considerably from region to region (Burns and O'Connell, 2012). Worldwide, about 2.5 million people suffer from SCI, with more than 130,000 new cases reported each year (Thuret et al., 2006; Rossignol et al., 2007). In the United States, the incidence of SCI is approximately 40 cases per million individuals per year (Rosner et al., 2012), whereas the number of people who currently suffer from SCI is estimated to be around 253,000, with 11,000-12,000 new cases occurring every year (Rosner et al., 2012; Silva et al., 2014). Importantly, this amount does not include injuries that result in death prior to hospitalization and therefore the incidence of SCI is underestimated (Rosner et al., 2012). In Finland the incidence of SCI is about 14 cases per million individuals per year (Ahoniemi et al., 2008), resulting in about 60–70 new cases per year (Ahoniemi et al., 2011).

The leading cause of SCI is traffic accidents, causing up to 30-50% of all injuries. Among other reasons are falls, penetrating bullet wounds and other forms of violence, sport, and work-related accidents (Sekhon and Fehlings, 2001; Rosner et al., 2012; Chen et al., 2013; Silva et al., 2014). Interestingly, in Finland during 1976-2005 the majority of SCI cases were the result of falls (41,2%), whereas traffic accidents (39,5%) were only the second leading cause (Ahoniemi et al., 2011).

Approximately 80% of all SCI occur among males and the most common age of SCI is between the ages of 16 and 30 years (Chen et al., 2013). This young onset of lifelong invalidization results in particularly high personal and economic costs (Rosner et al., 2012). In the late 1990s the total direct costs of treating individuals with SCI exceeded $7 billion per year in the United States only (McDonald and Sadowsky, 2002).

Although the life expectancy after SCI is good overall, such important handicaps as paralysis, sensory loss, pain, pressure sores, urinary and other infections seriously diminish the quality of life for SCI patients (Rossignol et al., 2007).

2.1.2 Classification of SCI

SCI may be neurologically complete or incomplete based on sacral sparing, which is defined as the presence of sensory or motor function in the most caudal sacral segments as determined by the neurological examination. The presence of any sensory and/or motor function below the neurological level that includes the lowest sacral segments, S4-S5, indicates that the injury is incomplete. If no sensory and motor function is preserved in these most caudal sacral segments the injury is defined as complete (Waters et al., 1991; Kirshblum et al., 2011).

2.2 THE CONCEPT OF PRIMARY AND SECONDARY INJURY

Understanding the biochemical and cellular events composing SCI pathophysiology is extremely important for the development of effective therapeutic interventions. SCI pathogenesis is best described as a biphasic process consisting of an initial primary and a progressive secondary phase of injury (Tator, 1995; Kwon et al., 2004; Rowland et al., 2008; Oyinbo, 2011; Silva et al., 2014).

2.2.1 Primary injury

Although tumor growth or other disorders can lead to SCI, the initial mechanical damage more often is the result of a) contusion or compression by a blunt force, b) laceration or transection of the spinal cord caused by a sharp penetrating force, c) infarct which can be caused by a vascular insult (DeVivo et al., 2002; Silva et al., 2014). While the transection or full disrupture of the anatomical continuity of the spinal cord occurs relatively rarely (Rowland et al., 2008), the most common form of acute SCI in humans is a compressive-contusive trauma. In this type of injury displaced bone fragments exert force on the spinal cord, leading to immediate mechanical damage, which is often followed by persistent compression (Sekhon and Fehlings, 2001; DeVivo et al., 2002; Mekhail et al., 2012).

Primary damage is defined as the immediate effect of an injury to the spinal cord. Regardless of the cause, the initial impact leads to rapid cell necrosis in the immediate vicinity of the injury site, especially in the central gray matter. The death of endothelial cells and disruption of local blood vessels result in an intraparenchymal hemorrhage, BSB-dysfunction, and compromised oxygen and nutrient supply to the damaged area and its surroundings. Local neurons, astrocytes, oligodendrocytes and endothelial cells die due either to direct mechanical damage to the cell membranes or ischemia developing after the disruption of microvessels (Hausmann, 2003; Kwon et al., 2004; Hagg and Oudega, 2006; Rowland et al., 2008; Ek et al., 2010; Oyinbo, 2011). Immediately after impact the activation of microglia, the resident immune cells of the CNS (Yang et al., 2004; Donnelly and Popovich, 2008), and upregulation of tumor necrosis factor (TNF) and IL1-β are observed (Yang et al., 2004; Pineau and Lacroix, 2007). Activation of microglial cells has been detected around damaged axons as early as 30 minutes after human SCI. However, no influx of neutrophils and macrophages were observed at this timepoint (Yang et al., 2004).

In addition, the levels of extracellular glutamate reach excitotoxic levels already within minutes after injury (Wrathall et al., 1996). Vascular damage and developing inflammation lead to edema or swelling of spinal cord tissue soon after initial impact (Hagg and Oudega, 2006; Rowland et al., 2008). The primary phase lasts approximately 2 hours and results in instant impairment or even loss of functions at and below the level of injury (Norenberg et al., 2004; Hagg and Oudega, 2006).

Soon the primary damage is followed by spreading of secondary tissue damage from the injury core both horizontally into white matter and rostro-caudally into the gray matter, aggravating the spinal cord pathology (Tator, 1995; Kwon et al., 2004; Oyinbo, 2011; Oudega, 2013). This secondary injury occurs within minutes to weeks after initial impact and induces delayed damage and death to cells that survive the original trauma (Oyinbo, 2011; Silva et al., 2014). As the result of secondary injury the area of primary damage enlarges significantly.

Due to unexpectedness and short duration the primary injury to the spinal cord cannot be prevented or treated and therefore, treatment strategies are directed mainly to combating secondary injury mechanisms (Kwon et al., 2004; Stirling and Yong, 2008; Stirling et al., 2009; Fehlings and Nguyen, 2010; Oyinbo, 2011; Raslan and Nemecek, 2012).

2.2.2 Secondary injury

More than 100 years ago it was for the first time noted that secondary damage occurs after SCI. These observations were based on the fact that inflammatory fluid removal improved neurological functions in injured dogs (Allen, 1911). The observed improvement was associated with the presence of a harmful agent in the inflammatory fluid, exacerbating damage to the spinal cord (Allen, 1914).

As of today, more than 25 different secondary mechanisms have been described (Oyinbo, 2011). Importantly, these processes are interrelated and often positively influence one another to promote secondary damage. The most significant of them are vascular disturbances, ionic balance disturbances and excitoxicity, free radical formation and lipid peroxidation, inflammation, astroglial scar formation and demyelination.

2.3 VASCULAR EVENTS AFTER SCI

Vascular disruption, hemorrhage and ischemia are some of the most important aspects of the secondary injury cascade (Tator and Fehlings, 1991; Mautes et al., 2000b; Kwon et al., 2004; Oudega, 2012).

Within several hours after initial impact microvascular damage increases and spreads in all directions. Thus, intraparenchymal hemorrhage is initially localized in the highly vascularized gray matter and subsequently expands into the adjacent white matter (Noble and Wrathall, 1989; Tator and Koyanagi, 1997; Mautes et al., 2000b). Blood itself is harmful for the nervous tissue probably due to hemoglobin, which contains iron (Sadrzadeh et al., 1987). Recently, iron accumulating in macrophages due to phagocytosis of red blood cells was identified as one of the main reasons leading to polarization of macrophages to pro-inflammatory M1 phenotype and determining their chronic persistence in the injured spinal cord (Kroner et al., 2014). Secondly, in areas surrounding hemorrhages the blood supply is usually compromised, resulting in different grades of ischemia. Vasogenic edema, vasospasm, direct compression by adjacent tissue and thrombosis all may contribute to the posttraumatic ischemia (Tator and Fehlings, 1991; Mautes et al., 2000b). Ischemia, in turn, leads to a sharp decrease in oxygen and glucose required for cell metabolism with the subsequent accumulation of cytotoxic proteolytic enzymes and ROS resulting in cell death and tissue loss (Hagg and Oudega, 2006; Oudega, 2012). Importantly, the restoration of blood supply and return of oxygen to ischemic tissues causes an increase in free radicals and ROS, contributing to additional tissue damage known as reperfusion injury (Oudega, 2012).

Damage of blood vessels also leads to accumulation of interstitial fluid. Lack of drainage of interstitial fluid causes edema or swelling, resulting in the additional compression of nervous tissue (Oudega, 2012). Compression may additionally aggravate ischemia (Hagg and Oudega, 2006).

Another feature of vascular disturbances in the secondary injury phase is the breakdown of the BSB leading to the influx of blood cells, including neutrophils, T-lymphocytes and macrophages, which in their own way contribute to secondary damage (Popovich, 2000; Hausmann, 2003; Profyris et al., 2004; Hagg and Oudega, 2006; Donnelly and Popovich, 2008). In the secondary injury phase, a number of inflammatory mediators are capable of altering BSB permeability. The cytokines TNF and IL-1β, free radicals, and histamine are known to induce BSB damage in spinal cord (Schnell et al., 1999; Donnelly and Popovich, 2008; Rowland et al., 2008). In addition, the important roles of endothelin-1 and matrix metalloproteinase-9 (MMP-9) in BSB dysfunction after SCI have been reported (Mautes et al., 2000b; Noble et al., 2002). BSB permeability typically peaks at 24 hours following injury and lasts at least for 2-4 weeks (Noble and Wrathall, 1989; Popovich et al., 1996; Mautes et al., 2000b).

2.4 ELECTROLYTE IMBALANCE AND EXCITOTOXICITY AFTER SCI

Ionic imbalance and excitotoxicity are closely related processes contributing to the propagation of secondary injury after SCI (Rowland et al., 2008). The SCI-induced damage of plasma membranes leads to the disruption of physiological ion balance resulting in intracellular calcium and sodium rise (Hamann and Shi, 2009). An additional contribution to the rise in intracellular calcium is provided by glutamate excitotoxicity (Kwon et al., 2004; Hamann and Shi, 2009). Glutamate, the most prevalent excitatory neurotransmitter in the CNS (Choi, 1992; Kwon et al., 2004; Oyinbo, 2011), is released and accumulates rapidly within and around the injury site in response to ischemia and membrane depolarization (Wrathall et al., 1996; McAdoo et al., 1999). The increased extracellular glutamate levels lead to excessive stimulation of glutamate receptors, such as N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-isoxazolepropionate (AMPA)/kainate receptors through which ions, and particularly calcium and sodium, pass. Therefore, excessive activation of these receptors leads to the uncontrolled influx of extracellular calcium and sodium into the cell (Choi, 1992; Fehlings and Agrawal, 1995; Agrawal and Fehlings, 1996, 1997; Kwon et al., 2004). Calcium influx, in turn, leads to release of intracellular calcium stores (Sattler and Tymianski, 2000). Other factors contributing to calcium overload after SCI are free radicals and oxidative stress (Xiong et al., 2007) resulting in inhibition of two enzymes extremely sensitive to free radical damage, Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase. These enzymes are responsible for regulation of ionic homeostasis (Rohn et al., 1996; Hall, 2011). The rise in intracellular calcium causes the activation of proteases such as calpains, resulting in degradation of cytoskeletal proteins. In addition, high levels of intracellular calcium activate caspases and phospholipases, cause mitochondrial dysfunction and increased generation of ROS, ultimately leading to apoptotic death of the cells (Lu et al., 2000; Kwon et al., 2004; Park et al., 2004; Hamann and Shi, 2009).

2.5 OXIDATIVE STRESS AFTER SCI

Oxidative stress is a well-established critical component of the secondary SCI damage contributing to axonal disruption and the death of neuronal and glial cells (Hall and Braughler, 1989; Hamann et al., 2008; Rowland et al., 2008; Hamann and Shi, 2009; Hall, 2011; Jia et al., 2012). Recent clinical observations confirmed that SCI patients display increased oxidative stress and reduced antioxidant defence for at least one year after injury (Bastani et al., 2012).

2.5.1 Free radical production and their detrimental effects in SCI

Compared to other organs the CNS, including spinal cord, is particularly susceptible to oxidative stress and free radical damage due to its active oxygen metabolism and low antioxidant capacity (LeBel and Bondy, 1991; Andersen, 2004; Sayre et al., 2008; Li et al., 2013; Singhal et al., 2013). Moreover, the cell membranes in brain and spinal cord are highly rich in polyunsaturated fatty acids, such as linoleic acid and arachidonic acid, which are most vulnerable to oxidation (Mautes et al., 2000a; Sayre et al., 2008; Hamann and Shi, 2009; Singhal et al., 2013).

Free radicals, including ROS and reactive nitrogen species (RNS), are molecules that contain one or several unpaired electrons, making them highly reactive. Molecular oxygen has two unpaired electrons. The addition of one electron to molecular oxygen leads to formation of superoxide. If two electrons are transferred, the product is hydrogen peroxide. Although hydrogen peroxide is not a free radical, it is still a damaging ROS as it is able to penetrate biological membranes. Transferring a third electron to hydrogen peroxide produces a highly reactive hydroxyl radical. When superoxide interacts with nitric oxide it produces a highly reactive free radical called peroxynitrite. The oxidation process caused

by free radicals geometrically generates more ROS that promote the reaction across the cell surface (Lewen et al., 2000; Kwon et al., 2004; Jia et al., 2012)

There are several reasons leading to free radical production and oxidative stress in the secondary injury phase. ROS are rapidly generated in spinal cord tissue during ischemia and also upon subsequent reperfusion (Sakamoto et al., 1991; Mautes et al., 2000a; Oudega, 2012). Among other reasons are mitochondrial dysfunction, glutamate-mediated increase of intracellular calcium, release of iron from storage proteins due to acidosis, and liberation of catalytically active metal ions due to hemoglobin degradation following hemorrhage. Phagocytic cells, such as activated microglia and infiltrating neutrophils and macrophages provide additional sources of ROS (Mautes et al., 2000a; Hausmann, 2003; Bao et al., 2004; Xiong et al., 2007; Hamann and Shi, 2009; Hall, 2011; Jia et al., 2012).

Although the generation of ROS under physiological conditions is important for normal cellular redox reactions, the imbalance between ROS formation and antioxidant defence system under pathophysiological conditions, such as SCI, leads to excessive production of ROS, consequent oxidative stress and secondary cell death. The main damage to cells is caused by ROS-induced oxidation of polyunsaturated fatty acids in the plasma membranes (lipid peroxidation), oxidation of amino acids in proteins, DNA alteration, Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase inhibition, Ca2+ overload and enhancement of glutamate-mediated excitotoxicity (Rohn et al., 1996; Kwon et al., 2004; Park et al., 2004; Xiong et al., 2007; Hamann et al., 2008; Hall, 2011; Jia et al., 2012).

The byproducts of lipid peroxidation are aldehydes, such as malondialdehyde, 4-hydroxynonenal and α, β-unsaturated acrolein. Acrolein may play a particularly important role in SCI cell damage since it is the most reactive and toxic of all known unsaturated aldehydes (Esterbauer et al., 1991; Luo and Shi, 2004; Park et al., 2014). Acrolein has a significantly longer half-life than the transient ROS and rapidly accumulates in the spinal cord following injury, inducing severe membrane disruption through mechanisms including ROS generation and subsequent lipid peroxidation. Its formation may thus represent a bioamplification step. Importantly, acrolein has been shown to diffuse away from the injury site to neighboring healthy tissue and therefore further propagate secondary injury following initial mechanical trauma. In addition to membrane damage, acrolein can impair cellular glucose transport and glutamate uptake in surviving neurons and glia leading to cell death due to excitotoxicity. Moreover, acrolein readily forms conjugates with glutathione, resulting in glutathione depletion and compromise of the endogenous antioxidant system (Lovell et al., 2000; Luo and Shi, 2004; Luo et al., 2005; Hamann et al., 2008; Hamann and Shi, 2009). More recently, it has been shown that acrolein is also involved in myelin damage, suggesting its detrimental role in SCI-induced demyelination (Shi et al., 2011). Importantly, when injected into uninjured rat spinal cord acrolein induces motor deficits and tissue damage (Park et al., 2014).

In addition to ROS, peroxynitrite, generated by the interaction of nitric oxide and superoxide, and peroxynitrite-derived radicals, such as hydroxyl radical, nitrogen dioxide radical and carbonate radical, have been shown to play not less important, but maybe even more crucial role in oxidative damage after SCI (Liu et al., 2000, 2005; Bao and Liu, 2002, 2003; Xiong et al., 2007; Hall, 2011).

2.5.2 Extreme role of Nrf2-ARE pathway in endogenous protection from oxidative stress

During evolution cells have developed multiple defence mechanisms to protect themselves from oxidative stress. They range from free radical scavengers and antioxidant enzymes to sophisticated repair mechanisms (Kryston et al., 2011). These mechanisms also involve the activation of redox-sensitive endogenous inducible defence systems. The transcription factor Nrf2 is the major regulator of such systems in the body (Sandberg et al., 2014) and belongs to the basic leucine zipper transcription factor family, which also contains NF-E2, Nrf1, Nrf3, Bach1, and Bach2 (Motohashi et al., 2002). Since Nrf2 modulates expression of hundreds cytoprotective genes in response to changes in the redox

state of the cell it is often referred to as the ''master regulator'' of the antioxidant, detoxification, and cell defence response (Hybertson et al., 2011; Gan and Johnson, 2014; Gao et al., 2014). Nrf2 was initially identified about 20 years ago (Moi et al., 1994) and today it is known to regulate not only a series of phase II detoxification and antioxidant genes, but also cell survival, anti-inflammatory, energy metabolism, and other groups of genes that contain a *cis*-acting DNA sequence, termed the antioxidant response element (ARE), in their promoter region (Hybertson et al., 2011; Gao et al., 2014). It therefore plays an extreme role in endogenous cellular protection from oxidative damage.

Under basal conditions Nrf2 is bound in the cytoplasm to its negative regulator Keap1 which functions as a substrate adaptor protein for Cullin3/Rbx1 E3 ubiquitin ligase complex and continuously directs Nrf2 to ubiquitination and degradation by the 26S proteasome (Cullinan et al., 2004; Kobayashi et al., 2004; Zhang et al., 2004). Under normal physiological conditions the half-life of Nrf2 is only about 10-20 minutes (Stewart et al., 2003; Kobayashi and Yamamoto, 2006; Baird and Dinkova-Kostova, 2011). Additionally, studies from Keap1 deficient mice show that without Keap1, Nrf2 constitutively accumulates in the nucleus (Wakabayashi et al., 2003). Overall, the binding of Keap1 to Nrf2 is considered to be inhibitory to Nrf2 function because it effectively enhances degradation of Nrf2 and provides the low basal expression of cytoprotective genes under physiological conditions (Itoh et al., 1999, 2010; Wakabayashi et al., 2003; Zhang et al., 2005; Baird and Dinkova-Kostova, 2011; Zhang et al., 2013; Baird et al., 2014).

Keap1 is also the key signaling protein which functions as a molecular sensor for oxidants and electrophiles, which recognize and chemically modify specific cysteine residues of Keap1 (Dinkova-Kostova et al., 2002; Wakabayashi et al., 2004; Yamamoto et al., 2008; McMahon et al., 2010; Takaya et al., 2012). Upon exposure to oxidative stress the ability of Keap1 to deliver Nrf2 to proteasomal degradation becomes impaired resulting in stabilization of Nrf2 along with an extension in half-life to 100-200 minutes (Stewart et al., 2003; Kobayashi and Yamamoto, 2006; Baird and Dinkova-Kostova, 2011). The stabilized Nrf2 protein translocates to the nucleus, binds to the ARE as a heterodimer with one or several small masculoaponeurotic fibrosarcoma (Maf) proteins and regulates transcription of its downstream target genes that include antioxidant and phase II enzymes as well as other genes promoting cell survival (Figure 2) (Itoh et al., 1997, 1999, 2010; Kobayashi and Yamamoto, 2006; Kensler et al., 2007; Baird and Dinkova-Kostova, 2011; Zhang et al., 2013; Baird et al., 2014; Levonen et al., 2014). In this fashion, oxidants or electrophiles induce Nrf2 and upregulate cytoprotective genes in order to compensate their harmful effects (Baird and Dinkova-Kostova, 2011; Zhang et al., 2013).

Keap1-independent mechanisms of Nrf2-ARE regulation have also been reported. Phosphorylation is another important mechanism regulating expression of Nrf2 downstream genes. Several protein kinases, such as protein kinase C (PKC), protein kinase RNA-like endoplasmic reticulum kinase (PERK), mitogen-activated protein kinases (MAPK) and Fyn can phoshorylate Nrf2. In addition, phosphatidylinositol 3-kinase (PI3K) may regulate Nrf2 via an indirect mechanism. Because active glycogen synthase kinase 3 (GSK-3β) phosphorylates Nrf2 Neh6 domain binding β-transducin repeat-containing protein leading to Cullin1-dependent ubiquitination and degradation of Nrf2, inhibiting the activity of GSK-3β by PI3K results in an increase in Nrf2 (Jain and Jaiswal, 2006; Baird and Dinkova-Kostova, 2011; Rada et al., 2011, 2012; Bryan et al., 2013; Chowdhry et al., 2013; Zhang et al., 2013; Levonen et al., 2014). Importantly, peroxynitrite has been shown to activate Nrf2 via the PI3K-Akt pathway (Kang et al., 2002; Li et al., 2006). However, despite the fact that Keap1-independent mechanisms of Nrf2-ARE regulation are well established it is believed that Keap1 plays the greatest role in Nrf2 regulation (Baird and Dinkova-Kostova, 2011).

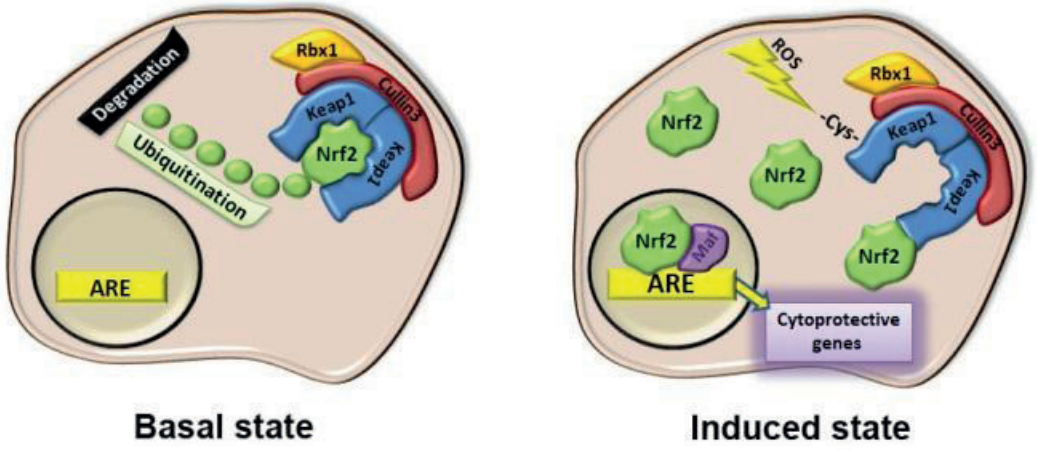

Figure 2. Regulation of Nrf2-ARE pathway. Under basal conditions Nrf2 binds to Keap1 homodimer allowing its ubiquitination and degradation. Inducers react with Keap1 cysteine residues which results in Nrf2 stabilization, translocation to the nucleus and activation of ARE-dependent transcription (modified from Baird and Dinkova-Kostova, 2011).

Following translocation to the nucleus, degradation of Nrf2 has been reported to occur either directly in the nucleus or in the cytosol, after export of Nrf2 out of the nucleus (Jain and Jaiswal 2006; Sun et al., 2007; Niture and Jaiswal, 2009; Kaspar et al., 2012). The latter mechanism involves tyrosine kinase Fyn, which phosphorylates Nrf2 and leads to its nuclear export, ubiquitination and degradation. The Fyn activity, in turn, is regulated by GSK-3β (Jain and Jaiswal, 2007). Regardless of the mechanism, when the inducers are removed, degradation of Nrf2 brings down levels of Nrf2 to those resembling basal conditions and prevents permanent induction of Nrf2 target genes, which is deleterious to the cell.

A number of biochemical, microarray and chromatin immunoprecipitation-sequencing (ChIP-Seq) analyses have demonstrated that Nrf2 regulates the gene expression of a battery of cytoprotective proteins that are characterized by extraordinary diversity (Thimmulappa et al., 2002; Malhotra et al., 2010; Chorley et al., 2012; Hirotsu et al., 2012, Hayes and Dinkova-Kostova, 2014). Approximately one-third of the Nrf2 target genes are involved in maintaining redox cellular homeostasis (Hayes et al., 2010; Gao et al., 2014). Among this group of Nrf2 downstream genes are heme oxygenase 1 (HO-1), NAD(P)H:(quinine acceptor) oxidoreductase 1 (NQO1), glutamate–cysteine ligase or γ-glutamylcysteine synthetase (GCL), glutathione-S-transferases (GSTs), catalase, Cu/Zn-superoxide dismutase (SOD), sulfotransferases, uridine diphosphoglucuronosyltransferases, thioredoxin and many other genes which protect the cell against electrophilic and oxidative stress (Hayes et al., 2010; Hayes and Dinkova-Kostova, 2014).

Inducible HO-1 catalyzes the first and rate-limiting step of heme degradation to carbon monoxide, biliverdin and iron (Maines, 1988; Ponka, 1999). Biliverdin is further metabolized by biliverdin reductase to bilirubine. HO-1 therefore confers a two-fold protection: it degrades toxic heme and generates the antioxidants biliverdin and bilirubin (Stocker et al., 1987; Stocker, 1990; Syapin, 2008; Ryter and Choi 2010). NQO1 is a flavoprotein catalyzing the two-electron reduction and detoxification of quinones and quinoneimines, preventing their participation in redox-cycling and subsequent generation of ROS (Prochaska et al. 1987; Bianchet et al., 2004; Dinkova-Kostova and Talalay 2010). In addition, NQO1 has also been shown to directly scavenge superoxide (Siegel et al., 2004). Glutathione (GSH) is a tripeptide comprised of glutamate, cysteine and glycine and is a major antioxidant in the brain. GSH exerts its functions via several mechanisms including scavenging of free radicals, especially the hydroxyl radical (Dringen, 2000; Aoyama et al.,

2008). In addition, it serves as an essential cofactor for a number of enzymes, as a cysteine storage form, the major redox buffer maintaining intracellular redox homeostasis and a neuromodulator (reviewed in Aoyama et al., 2008). GCL catalyses the first the rate-limiting step in the biosynthesis of GSH, mediating the reaction between glutamate and cysteine to form γ-glutamylcysteine, which, in turn, reacts with glycine in a reaction catalyzed by GSH synthetase to produce GSH (Dringen, 2000). The primary function of GSTs is to catalyze the conjugation of electrophilic substrates to GSH (Sheehan et al., 2001; Oakley, 2011; Wu and Dong, 2012).

HO-1 has been shown to be upregulated after SCI (Mautes et al., 2000a; Lin et al., 2007). In addition to modulation of oxidative stress, in the acutely injured spinal cord HO-1 stabilizes the BSB, limits neutrophil infiltration and white matter damage, therefore playing an important protective role (Yamauchi et al., 2004; Lin et al., 2007). Similarly to HO-1, NQO1 and GST are also induced after SCI (Jin et al., 2014a), suggesting the involvement of Nrf2 downstream genes in the protection from oxidative damage in the secondary SCI phase.

Approximately two-thirds of the genes regulated by Nrf2 are not involved in detoxification or antioxidant functions, but many of them are considered to be protective (Hayes et al., 2010). Thus, Nrf2 is also implicated in regulation of other protective mechanisms, such as inhibition of apoptosis (Kotlo et al., 2003; Vargas et al., 2006; Niture and Jaiswal, 2012, 2013; Pan et al., 2013) and attenuation of inflammation (Innamorato et al., 2008; Li et al., 2008; Koh et al., 2009; Piantadosi et al., 2011; Sandberg et al., 2014). Recently, Nrf2 and its downstream HO-1 were also shown to be involved in the regulation of neurotrophic factor expression (Hung et al., 2010; Sakata et al., 2012).

Although a deficiency in the Nrf2 gene does not impair normal development, viability and fertility of mice, the Nrf2-deficient mice are known to develop white matter leukoencephalopathy characterized by widespread astrogliosis and myelinopathy (Hubbs et al., 2007). In addition to spontaneous pathological changes in Nrf2-deficient mouse brains, suppression of the Nrf2-ARE system by genetic deletion results in decreased constitutive and inducible expression of detoxification enzymes and antioxidants and therefore, increased susceptibility of the body systems, including CNS, to oxidative stress and inflammation (Kobayashi and Yamamoto, 2006; Zhang et al., 2013; Sandberg et al., 2014). The crucial role of Nrf2 disruption has been demonstrated in various models of neurological diseases, such as Parkinson's disease (Burton et al., 2006; Jakel et al., 2007; Chen et al., 2009; Innamorato et al., 2010), ischemic and hemorrhagic stroke (Shih et al., 2005; Shah et al., 2007; Wang et al., 2007; Zhao et al., 2007; Srivastava et al., 2013), experimental autoimmune encephalomyelitis (EAE) (Johnson et al., 2010) and TBI (Jin et al., 2008, 2009; Hong et al., 2010). Recently, it has been shown that impairment of the Nrf2 gene also exacerbates the neurologic deficit and inflammation after compression mouse SCI (Mao et al., 2010, 2011, 2012). However, whether Nrf2 disruption has the same consequence after contusion SCI yet remains to be explored.

Activation of Nrf2 and its target genes has been reported in acute brain injuries models such as ischemic stroke (Tanaka et al., 2011), intracerebral hemorrhage (Chen and Regan, 2007; Shang et al., 2013) and TBI (Yan et al., 2008, 2009), suggesting that the upregulation of the Nrf2-ARE pathway is an endogenous compensatory attempt to enhance endogenous defence in response to these conditions (Zhang et al., 2013). Several studies also observed the activation of the Nrf2-ARE pathway within the first 72 h after either compression or contusion rat SCI (Wang X et al., 2012b; Jin et al., 2014a) and within the first 24 h after compression mouse SCI (Mao et al., 2012). However, the time-course expression of Nrf2 and its main downstream genes have not been studied in the injured spinal cord, especially after contusion injury. Because Nrf2 is known to also regulate inflammation, apoptosis and neurotrophic factor expression, investigating these aspects of SCI-induced Nrf2 response is also important for deep understanding of the role of the Nrf2-ARE system in traumatic spinal cord injuries.

2.6 INFLAMMATORY CELLS AND MEDIATORS IN SCI

Numerous cell types such as astrocytes, microglia, T-cells, neutrophils and monocytes take part in the highly complex inflammatory response after SCI (Rowland et al., 2008). This inflammatory process may be represented as a dual-edged sword, with both neuroprotective and neurotoxic properties. Immune cells residing or invading into spinal cord participate in cellular debris clearance and promote regeneration by secreting growth factors and protective cytokines. However, at the same time, inflammatory cells promote also the secondary damage via release of a cocktail of pro-inflammatory mediators. The most intriguing fact is that the same inflammatory elements have both beneficial and detrimental effects depending on time and target of their action. Despite the controversial role of the inflammatory response in SCI it is apparent that an uncontrolled immune response can damage healthy tissue and aggravate the injury. It therefore requires tight regulation (Kwon et al., 2004; Rossignol et al., 2007; Donnelly and Popovich, 2008; Rolls et al., 2009; Oyinbo, 2011; David et al., 2012)

Primary injured endothelial cells, as well as the activated resident glia and neurons release a number of proinflammatory mediators capable of recruiting neutrophils and monocytes/macrophages to the site of injury. Disruption of the BSB additionally facilitates the influx of immune cells from the blood and therefore triggers the secondary inflammatory response (Popovich, 2000; Hausmann, 2003; Profyris et al., 2004; Yang et al., 2005; Hagg and Oudega, 2006; Pineau and Lacroix, 2007; Donnelly and Popovich, 2008; Beck et al., 2010).

2.6.1 Neutrophils

Polymorphonuclear neutrophils are the first inflammatory cells arriving at the site of injury (Carlson et al., 1998; Taoka and Okajima, 2000; Hausmann, 2003; Fleming et al., 2006). These cells are particularly abundant in and around the areas with hemorrhage and necrosis (Taoka and Okajima, 2000; Hausmann, 2003; Fleming et al., 2006). Thus, already at 4-5 hours following human SCI neutrophils are localized in blood vessels adherent to endothelial cells and in perivascular spaces, but not yet found in the spinal cord parenchyma. At one day after human spinal cord trauma, neutrophils are widely spread throughout the damaged tissue and their number reaches a peak at this time (Yang et al., 2004; Fleming et al., 2006). Later than one day after injury, the number of neutrophils dramatically declines and only occasional cells persist in the lesion by the third day (Norenberg et al., 2004; Yang et al., 2004), although there are evidence to suggest that neutrophil infiltration lasts for up to 10 days after human SCI (Fleming et al., 2006).

In rodent models of SCI a generally similar pattern of neutrophil infiltration is observed. Neutrophils appear at the lesion site 4-6 hours after injury, peak in number at 12-24 hours and disappear within 5-7 days (Carlson et al., 1998; Hausmann, 2003; Bao et al., 2004; Yang et al., 2005; Donnelly and Popovich, 2008; Stirling and Yong, 2008; Stirling et al., 2009). Interestingly, in mice at 12 hours after SCI neutrophils were the predominant cell type within the cellular infiltrates (Stirling and Yong, 2008). In some cases in mice, a secondary peak of neutrophil infiltration is detected at 2 weeks after SCI and these cells persist in spinal cord for up to 6 weeks post injury (Kigerl et al., 2006). Beck et al., 2010 observed the chronic persistence of neutrophils for even up to 90 days in the contused rat spinal cord. Importantly, the number of neutrophils migrated into the spinal cord mostly depends on the time after injury and does not depend on the injury type, regardless if it is laceration, contusion or compression (Fleming et al., 2006).

Neutrophils are able to clear tissue debris and restore homeostasis due to their phagocytic properties. However, they are also involved in the modulation of the secondary injury by generation of ROS and RNS and release of neutrophil proteases (Carlson et al., 1998; Taoka and Okajima, 2000; Hausmann, 2003; Stirling and Yong, 2008). One such protease, neutrophil elastase, has been shown to increase vascular permeability and

damage endothelial cells leading to secondary haemorrhage (Taoka and Okajima, 2000; Hausmann, 2003). MMP-9, released by neutrophils, is known to participate in BSB impairment and vascular dysfunction and also to cleave myelin basic protein and therefore to contribute to demyelination of healthy axons (Noble et al., 2002). Myeloperoxidase abundantly expressed in neutrophils exacerbates secondary injury and impairs the functional recovery after SCI by generating extremely toxic hypochloric acid and enhancing neutrophil infiltration (Kubota et al., 2012). Since neutrophils are able to release proteases and other cytotoxic factors, such as myeloperoxidase, the general view is that they have a detrimental effect after SCI (David et al., 2012). In support of this, a number of studies have shown the beneficial effect of reduction of neutrophil infiltration on SCI recovery (Taoka et al., 1997; Naruo et al., 2003; Bao et al., 2004, 2008, 2012; Gris et al., 2004; Fleming et al., 2008, 2009; Nguyen et al., 2012). However, since these treatments do not selectively reduce neutrophils but also macrophages, it is difficult to address the observed beneficial effect solely to neutrophils (David et al., 2012). Interestingly, in contrast to the general view, one recent study showed that selective depletion of neutrophils by >90% without obvious alteration of monocyte or lymphocyte numbers resulted in a worsened neurological outcome after SCI (Stirling et al., 2009). Even though the obvious discrepancy seen in the results may be explained by insufficient specificity and different mechanisms of the antibodies used for depletion as well as different types of injuries involved (Bao et al., 2012), these findings raise cautionary thoughts against potential therapeutic strategies that inhibit infiltration of neutrophils into lesioned spinal cord. Of note is the fact that the positive effect of neutrophil depletion manifested only when the treatment was initiated 2 hours after injury, whereas delaying treatment by 4 hours yielded no functional recovery (Fleming et al., 2009).

2.6.2 Microglia and macrophages

Macrophages observed in spinal cord lesions are derived from haematogenous monocytes and resident microglia (Popovich et al., 1997, 1999; Fleming et al., 2006; David and Kroner, 2011). Microglial cells, the resident immune cells of the CNS, are diffusely distributed throughout the brain and spinal cord. They comprise approximately 13% of the entire glial cell population in the CNS (Watanabe et al., 1999). Haematogenous or circulating monocytes are mobilized into the blood stream from the bone marrow (van Furth and Cohn, 1968) or spleen (Swirski et al., 2009). Interestingly, recently it was demonstrated that monocytes infiltrating spinal cord lesions predominantly originate from the splenic reservoir (Blomster et al., 2013).

Already in the primary injury phase, as early as 30 minutes after initial damage, activation of resident microglia has been reported in human SCI (Yang et al., 2004). This rapid microglial response occurs due to release from the damaged tissue of extracellular ATP, which upregulates the microglial allograft inflammatory factor-1 and thereby causes microglial activation (Schwab et al., 2001; Davalos et al., 2005). At 1-3 days after trauma, the number of activated microglial cells is greatly increased and are observed in patches around the margin of lesions. Within this period the activated non-phagocytic microglia start to change their phenotype and transform into the reactive phagocytic type, also referred to as microglial-derived brain macrophages (Popovich et al., 1997; Fleming et al., 2006). In contrast to abundant activated resident microglia, at this time only a small number of circulating monocytes are found adherent to the endothelium of blood vessels and in the perivascular space, suggesting the beginning of monocyte infiltration (Fleming et al., 2006). At 5-10 days after injury, a great number of phagocytic ''foamy'' macrophages are detected in the necrotic areas, whereas early activated microglia are widely present outside areas of necrosis in the white matter regions adjacent to the lesion (Yang et al., 2004; Fleming et al., 2006). As soon as the macrophages establish a ''foamy'' morphology the microglial or monocytic origin of these cells cannot be any more distinguished by their morphology or antigenic markers. From this time onwards they are referred to as microglia/macrophages

(Fleming et al., 2006; David and Kroner, 2011; David et al., 2012; Shechter and Schwartz, 2013; Kroner et al., 2014). At this time the microglia/macrophages are the predominant type of immune cell in the injured human spinal cord (Fleming et al., 2006) and most of them within the lesion are thought to be derived from circulating monocytes (Norenberg et al., 2004). These cells persist in spinal cord tissue for weeks to months after injury (Norenberg et al., 2004; Fleming et al., 2006). Importantly, similarly to neutrophils, no differences in the pattern of microglial/macrophage activation are observed between the laceration, contusion and compression types of injuries (Fleming et al., 2006).

A similar rapid activation of resident microglia within the first few hours after SCI is observed also in rodents (Popovich et al., 1997; Carlson et al., 1998; Yang et al., 2005). Starting at 2-3 days after injury, circulating monocytes infiltrate the spinal cord and transform into macrophages in tissue. Within the first week after injury the activation of resident microglia increases and is followed by subsequent transformation of activated microglial cells into phagocytic macrophages. These microglia-derived macrophages resemble macrophages derived from circulating monocytes in their morphology and antigenic phenotype in histological sections. The numbers of activated microglia/macrophages reaches its peak at 5-7 days and they remain in the tissue for several weeks or months (Blight, 1992, Popovich et al., 1997; Sroga et al., 2003; Profyris et al., 2004; Stirling and Yong, 2008; Beck et al., 2010; David et al., 2012). The second peak of microglia/macrophage numbers is observed after rat SCI from 60 to 180 dpi, suggesting prolonged biphasic response of microglia/macrophage after spinal cord trauma (Beck et al., 2010). Although the reasons leading to the secondary microglia/macrophage peak and its role in SCI pathophysiology are not known, the second phase of macrophage/microglial response may contribute to prevention of further loss of function after SCI (Beck et al., 2010).

Early activated resident microglia are able to release pro-inflammatory cytokines and chemokines and together with neurons and astrocytes promote the recruitment of neutrophils and circulating monocytes from the blood stream into the injured tissue (Yang et al., 2004, 2005; David and Kroner, 2011). Moreover, activated microglial cells along with neutrophils are the major source of ROS/RNS in the injured spinal cord (Fleming et al., 2006). The close proximity of these cells to axons suggest that they are able to contribute to lipid peroxidation of the myelin sheath and therefore to demyelination (Fleming et al., 2006).

The role of activated macrophages after SCI (either derived from resident microglia or circulating monocytes) has been widely debated and is still far from clear (David et al., 2012). The primary and natural function of macrophages in SCI is to phagocytize cellular debris and degenerating myelin and therefore repair injured tissue (Blight, 1992; Popovich et al., 1999; Norenberg et al., 2004). Since the large amounts of lipids in necrotic tissues are a potential source of ROS and oxidative stress the phagocytic function of macrophages is crucial for SCI recovery (Norenberg et al., 2004). Macrophages also release a number of growth factors such as fibroblast growth factor (FGF), nerve growth factor (NGF), glial cell-line derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF) and neutrotrpin-3, which promote regeneration and tissue repair (Dougherty et al., 2000, Hashimoto et al., 2005; Donnelly and Popovich, 2008). However, in the process of tissue repairing the activated macrophages are able to release pro-inflammatory cytokines, proteases, glutamate and to produce ROS/RNS, thus contributing to secondary tissue damage and demyelination (Popovich et al., 1997, 1999; Carlson et al., 1998; Donnelly and Popovich, 2008; David and Kroner, 2011). Depleting macrophages derived from circulating monocytes improved functional recovery and tissue protection after SCI (Popovich et al., 1999; Horn et al., 2008; Ianotti et al., 2011), whereas macrophages activated *in vitro* and then transplanted into injured spinal cord in contrast were neuroprotective and improved functional recovery (Rapalino et al., 1998; Bomstein et al., 2003; Schwartz and Yoles, 2006; Shechter et al., 2009). The latter led to clinical studies of macrophage transplantation for SCI. A phase I study using autologous macrophages activated with autologous skin as treatment

for acute complete SCI provided some evidence that this treatment is well tolerated and has therapeutic potential in humans (Knoller et al., 2005), whereas a phase II study failed to show significant benefit (Lammertse et al., 2012). Thus it is clear that after SCI macrophages may promote both secondary injury and repair (Rapalino et al., 1998; Popovich et al., 1999; Schwartz and Yoles, 2006; Horn et al., 2008; Donnelly and Popovich, 2008; Busch et al., 2009; Gensel et al., 2009; Shechter et al., 2009).

The discrepancies regarding the effects of activated macrophages after SCI may be explained by previous findings from non-neural systems that macrophages exhibit great plasticity and can switch their phenotype in response to changes in the microenvironment. Macrophages can be polarized to phenotypically and functionally heterogeneous M1 (classically activated) or M2 (alternatively activated) types with either pro- or anti-inflammatory properties, respectively. The classically polarized pro-inflammatory M1 macrophages are activated through exposure to type 1 helper T-cell (Th1) cytokines such as interferon-gamma (IFN-γ) and TNF or by products such as lipopolysaccharide (LPS). These cells proficiently produce cytotoxic and pro-inflammatory substances and function as killers of intracellular pathogens and tumor cells. In contrast, the alternatively activated M2 macrophages are induced by type 2 helper T-cell (Th2) cytokines such as IL-4, IL-5, IL-13, and IL-10. M2 cells reduce inflammation, scavenge debris, promote angiogenesis and are involved in tissue remodeling and repair (Gordon, 2003; Mantovani et al., 2004; Schwartz and Yoles, 2006; Kigerl et al., 2009; Martinez et al., 2009; Cassetta et al., 2011; David and Kroner, 2011; Shechter and Schwartz, 2013). Interestingly, it has been proposed that in contrast to the infiltrating monocytes which can transform to anti-inflammatory macrophages, resident microglia are generally pro-inflammatory and do not acquire anti-inflammatory properties at least during the dynamic phase of the SCI repair (Shechter et al., 2009; Shechter and Schwartz, 2013).

Classically activated M1 macrophages possess neurotoxic properties as they produce pro-inflammatory cytokines (IL-12, IL-23, IL-1β and TNF), release ROS/RNS and proteolytic enzymes, thus leading to death of neurons and glia (Kigerl et al., 2009). Since the expression of chondroitin sulfate proteoglycan (CSPG), a potent inhibitor of axon growth and regeneration, is 16-fold higher in M1 than M2 type macrophages (Martinez et al., 2006), M1 cells are also implemented in inhibition of axonal regeneration. In addition, macrophages stimulated with IFN-γ and LPS facilitate retraction of dystrophic axons at the site of injury (Horn et al., 2008). Therefore, M1 cells are believed to be responsible for promoting secondary injury and impairing recovery from SCI (Kigerl et al., 2009; David and Kroner, 2011) and to play a role in axonal ''die-back'' and regeneration failure within the CNS (Horn et al., 2008; Busch et al., 2009).

In contrast, alternatively activated M2 macrophages possess anti-inflammatory properties as they express high levels of IL-10 and transforming growth factor beta (TGF-β), show impaired nuclear factor κB (NF-κB) activation, exhibit high expression of arginase 1 and low expression of pro-inflammatory cytokines (Gordon, 2003; Mantovani et al., 2004; Sica et al., 2008; Martinez et al., 2009; Sica and Mantovani, 2012). The abundant expression of BDNF and reduced release of TNF are thought to be mechanisms of neuroprotective action of alternatively activated macrophages preincubated with autologous skin and transplanted in contused spinal cord (Bomstein et al., 2003). Recently, it was shown that M2 macrophages, but not M1 macrophages, are a source of neurotrophic factors (BDNF, NGF) in the compressed spinal cord (Hirai et al., 2013). M2 macrophages express a high level of mannose receptor CD206 (Gordon, 2003), which is required for non-inflammatory sequestration of dying cells (Nauta et al., 2003). Therefore, M2 macrophages could enhance clearing of necrotic debris without causing neurotoxicity (Kigerl et al, 2009). Additionally, M2 cells promote axonal growth because macrophage-derived proteases are capable of degrading axon growth inhibitory molecules without causing overt toxicity. Therefore, M2 macrophages/microglia are also essential for efficient remyelination as their depletion impairs oligodendrocyte differentiation (Miron et al., 2013). These studies all provide

evidence that in the injured spinal cord M2 macrophages are essential in the amelioration of inflammation and facilitation of reparation (Kigerl et al, 2009).

Importantly, in contrast to lymphocytes, which have a rather fixed phenotype after exposure to polarizing cytokines, macrophages can reversibly shift their functional phenotype in response to changes in the cytokine environment. For example, M2 macrophages can be porarized to M1 phenotype by exposure to LPS or IFN-γ (Stout et al., 2005; Mylonas et al., 2009). In the injured spinal cord tissue both M1 and M2 macrophages are present (Kigerl et al, 2009). However, the microenvironment of the injured spinal cord, especially high levels of pro-inflammatory cytokines, such as TNF, that promote the activation of macrophages through the classic pathway and low or undetectable levels of IL-4, IL-13 and IL-10, favors polarization of macrophages to the M1 type. These pro-inflammatory macrophages outnumber M2 cells and persist in the spinal cord for weeks after injury, whereas M2 macrophages disappear within one week due to suppression or downregulation of the M2 phenotype by lesion-derived factors (Kigerl et al, 2009; David and Kroner, 2011; Guerrero et al., 2012; Hirai et al., 2013; Kroner et al., 2014). The low number of M2 macrophages and their transient appearance after SCI may be some of the reasons for the prolonged pro-inflammatory response leading to secondary tissue damage and impaired axon regeneration (David and Kroner, 2011; Shechter and Schwartz, 2013). Therefore, modulating the microenvironment of the injured spinal cord to reduce excessive and prolonged M1 polarization and enhance M2 polarization may be a desirable goal in SCI repair (David and Kroner, 2011; Guerrero et al., 2012).

2.6.3 Lymphocytes

Adaptive immune cells, such as T- and B-lymphocytes, are activated by SCI and play a significant role in secondary injury and repair (Ankeny and Popovich, 2009).

Interestingly, SCI in humans and rodents causes different patterns of lymphocyte infiltration. Only solitary, scattered cytotoxic $CD8^+$ T-lymphocytes are observed in human spinal cord within the first 10 days after injury. Weeks and months after injury the $CD8^+$ T-cells are encountered more often in the damaged area usually randomly distributed among macrophages. $CD4^+$ T-helper lymphocytes are fewer and follow the same pattern of distribution as $CD8^+$ T-cells, whereas B-cells are not detected in the injured human spinal cord (Fleming et al., 2006; Chang, 2007). In mice, T-cells are present in very small numbers within the first 96 hours after trauma (Stirling and Yong, 2008). They peak at 14 days after injury with a second peak at 6 weeks (Sroga et al., 2003; Kigerl et al., 2006). In contrast to mice, rats show a first peak of T-cell infiltration already within the first week (Sroga et al., 2003) or at 9 days (Beck et al., 2010) after SCI and a second peak between 3 and 4 weeks postinjury (Sroga et al., 2003). The number of T-cells remains elevated in the rat spinal cord for up to 90 days (Beck et al., 2010). Of note is the fact that in contrast to humans, CD4+ T-cells outnumber CD8+ T-cells in the rodent spinal cord at all times postinjury (Popovich et al., 1997; Kigerl et al., 2006).

In contrast to humans, B-cells are found in the injured mouse spinal cord. Similarly to T-cells they accumulate in the spinal cord tissue mainly 2 weeks after injury and form large B-cell clusters that are surrounded by T-cells and resemble lymphoid follicles in the spleen/lymph node (Ankeny et al., 2006; Ankeny and Popovich, 2010).

The role of infiltrating T-lymphocytes after SCI still remains ambiguous since both beneficial and detrimental effects have been reported (Schwartz and Kipnis, 2001; Hauben, 2000, 2001; Gonzalez et al., 2003, 2007; Jones et al., 2004; 2005; Ankeny and Popovich, 2009; Wang HJ et al., 2012; Laliberte and Fehlings, 2013). The primary injury initiates the release of neuroantigens into the blood and lymphatic vessels with drainage into spleen and lymph nodes where neuroantigens are processed and presented by antigen presenting cells to lymphocytes, triggering primary lymphocyte activation. Later lymphocytes activated in the periphery infiltrate to the injury site irrespective of their antigen specificity and persist there indefinitely (Ankeny and Popovich, 2009). Within the injured spinal cord T-

lymphocytes recognize host antigens, such as myelin basic protein. In response to these antigens T-cells proliferate and mediate a number of deleterious processes. Thus, activated T-lymphocytes destroy target cells, produce cytokines, augment microglia and macrophage recruitment and/or activation and promote tissue damage (Gonzalez et al., 2003, Jones et al., 2005). It has been shown that nude (athymic) rats have improved locomotor recovery following SCI (Potas et al., 2006). Additionally, blocking of the chemokine CXCL10, a potent T lymphocyte recruiter, reduces neuroinflammation and improves functional recovery (Gonzalez et al., 2003, 2007). Moreover, chronic T-cell activation can induce pathological fibrosis and scarring (Wynn, 2004). However, several studies demonstrate a neuroprotective role of T-cells in SCI, suggesting that autoimmune boosting by the administration of autoreactive T-cells (passive immunization) or a CNS antigen to expand autoreactive T cell clones (active immunization) may be beneficial. It is believed that autoreactive T-cells can serve as a source of neurotrophic factors and cytokines, which activate macrophages to ameliorate neurotoxicity, produce growth factors and remove inhibitors of axonal growth after SCI (Hauben, 2000, 2001; Schwartz and Kipnis, 2001; Schwartz, 2005). Nevertheless, the protective role of autoimmunity following SCI is still very questionable (Laliberte and Fehlings, 2013).

One possible explanation for the contradictory results may be that different subtypes of T-lymphocytes mediate distinct T-cell effects. Thus, Th2-lymphocytes or a systemic Th2 shift is believed to promote neuroprotection and regeneration of axons after CNS injury and therefore promoting Th2 shift is considered to be a promising strategy for CNS repair. However, the side effect of the systemic Th2 shift is increased susceptibility to infection (Hendrix and Nitsch, 2007). Interestingly, recent studies have shown that adoptive transfer of Th-1 conditioned lymphocytes, but not Th2 or Th17-conditioned cells activate anti-inflammatory M2 macrophages, facilitate myelination and improve functional recovery and tactile sensation after mouse SCI (Ishii et al., 2012, 2013).

Increased numbers of studies support a negative effect of humoral immunity after SCI (Ankeny and Popovich, 2010). Activated B-cells synthesize pathogenic autoantibodies that may augment neuroinflammation and cause neurotoxicity (Ankeny et al., 2006). Moreover, injured mice lacking B cells demonstrate improved neurological function compared with mice with normal B-function, suggesting a pathogenic role for B cells (Ankeny et al., 2009). Therefore, it is generally considered that B-lymphocytes and SCI-induced antibodies exacerbate tissue damage and impair neurological recovery after SCI (Ankeny et al., 2006, 2009; Ankeny and Popovich, 2010).

Finally, studies with RAG2-/- and NOD-SCID mice which lack both functional T- and B-lymphocytes show that activated T- and B- cells, as the effectors of the adaptive immune response, are harmful to the regenerating spinal cord and show evidence that immune suppression is beneficial for SCI recovery (Luchetti et al., 2010; Wu B et al., 2012).

2.6.4 Inflammatory mediators

Numerous pro- and anti-inflammatory mediators tune the cellular response after SCI. Among them TNF, IL-1β and IL-6 are considered to be key pro-inflammatory cytotoxic cytokines (Hausmann et al., 2003). TNF and IL-1β are produced in the injured spinal cord almost immediately (within 5-15 minutes) after initial impact. The upregulation of TNF and IL-1β is followed by elevation of IL-6 (Pineau and Lacroix, 2007). In human injured spinal cord upregulation of TNF, IL-1β and IL-6 was found as early as 30 minutes after injury (Yang et al., 2004). In mouse spinal cord the peak of TNF expression is observed at 1 hour, whereas IL-1β and IL-6 expression levels are highest at 12 hours after injury. After reaching maximal levels the expression of these cytokines decreases gradually and is then followed by a second prominent peak of TNF and IL-1β at 14 days (Yang et al., 2004, Pineau and Lacroix, 2007). Endogenous glia, neurons and endothelial cells are mainly responsible for the initial release of TNF, IL-1β and IL-6 resulting in increase of vascular permeability and facilitation of neutrophil and macrophage migration into the injured spinal cord. Therefore,

it is established that resident CNS cells, but not immune cells recruited from periphery, are responsible for the initiation of the inflammatory response following SCI (Yan et al., 2001; Hausmann et al., 2003; Yang et al., 2004, 2005; Pineau and Lacroix, 2007). The second peak of TNF and IL-1β production is mainly associated with microglia/macrophages and coincides with the entry of lymphocytes and glial scar formation (Pineau and Lacroix, 2007).

In addition to their significant role in recruitment and activation of peripheral leukocytes, TNF, IL-1β and IL-6 mediate a number of other processes following CNS injury. Thus, release of TNF and IL-1β precedes the activation of astrocytes and shares a similar distribution with astrocytic reaction. Therefore, rapid and widespread expression of TNF and IL-1β play a role in regulation of astrogliosis (Herx and Yong, 2001; Pineau and Lacroix, 2007). In support of this, it is known that astrogliosis is attenuated in the brain of adult IL-1β deficient mice (Herx and Yong, 2001). Another consequence of rapid production of TNF and IL-1β is the activation of resident microglia, which has been described both *in vitro* and *in vivo* (Pineau and Lacroix, 2007). Studies showing that microgliosis, astrogliosis and leukocyte infiltration are reduced in IL-6 deficient mice confirm that IL-6 also participates in activation of astrocytes and microglia as well as in regulation of leukocyte infiltration (Klein et al., 1997; Penkowa et al., 1999). The second peak of TNF and IL-1β expression observed 2 weeks after SCI may be important for lymphocyte recruitment as lymphocytes enter into mouse spinal cord about 14 days following spinal cord trauma (Sroga et al., 2003; Kigerl et al., 2006; Pineau and Lacroix, 2007).

TNF, IL-1β and IL-6 are thought to play mainly detrimental roles in SCI pathology. Thus, TNF is known to be specifically cytotoxic to oligodendrocytes, causing their apoptotic death and demyelination in the injured spinal cord (Hisahara et al., 1997; Yan et al., 2001; Yune et al., 2003; Genovese et al., 2008, 2009; Inukai et al., 2009). Additionally, TNF has been shown to contribute to excitotoxic cell death after SCI (Ferguson et al., 2008). Importantly, TNF is a cytokine mainly responsible for sustained M1 polarization of microglia/macrophages in the injured spinal cord (Kroner et al., 2014). Although it has been reported that mice deficient for TNF receptors exhibit locomotor impairment after SCI due to increased apoptosis (Kim et al., 2001), substantial numbers of studies have demonstrated that pharmacological or genetic inhibition of TNF or IL-1β, in contrast, reduce apoptosis and cell infiltration, decrease tissue damage and improves functional recovery after SCI (Bethea et al., 1999; Nesic et al., 2001; Genovese et al., 2006, 2008; Chen et al., 2011). Similarly, overexpression of IL-6 in the injured spinal cord enhances inflammation and impaires functional recovery (Lacroix et al., 2002), whereas temporal blockade of IL-6 signaling results in improved outcome after SCI (Okada et al., 2004; Mukaino et al., 2010; Guerrero et al., 2012). However, there is evidence from IL-6 deficient mice that certain levels of this cytokine are still required for regeneration within the CNS (Cafferty et al., 2004).

IL-10 is a potent anti-inflammatory cytokine known to suppress the activation of neutrophils, macrophages, eosinophils and T-lymphocytes and therefore limit the production of pro-inflammatory mediators. It is thought to play a protective role following SCI (Abraham et al., 2004; Genovese et al., 2009; David et al., 2012; Thompson et al., 2013). IL-10 deficient mice following SCI show an increased degree of inflammatory response, apoptosis and impaired locomotor recovery (Abraham et al., 2004; Genovese et al., 2009), whereas most of these effects are reversed with IL-10 treatment (Abraham et al., 2004). Administration of IL-10 to contused Sprague-Dawley rats is also beneficial for the functional recovery from SCI (Bethea et al., 1999), whereas in the Fisher rat strain IL-10, although it reduces spinal gray matter loss, surprisingly fails to facilitate locomotor recovery (Takami et al., 2002b). In addition to the anti-inflammatory effects of IL-10 it has been demonstrated that IL-10 activates signaling pathways involved in neuronal survival and growth and therefore exerts direct neuroprotective effects on neurons (Zhou et al., 2009b). Accordingly, local upregulation of IL-10 at the injury site with vector-mediated IL-10 gene transfer results in significant neuronal survival and improves functional outcome after a hemisection injury (Zhou et al., 2009a). Importantly, IL-10 is known to induce

polarization of macrophages to the anti-inflammatory M2 phenotype (Shechter et al., 2011; Deng et al., 2012; Sica and Mantovani, 2012), which may be one reason for the functional improvement observed after IL-10 treatment (Thompson et al., 2013).

In addition to TNF, IL-1β, IL-6 and IL-10, an enormous number of other inflammatory mediators such as granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), TGF-β, IFN-γ, chemokines (MIP-1, IP10, MCP-1) and proteinases, especially the MMPs, regulate inflammatory processes in the injured spinal cord and have been studied at different extent in various SCI models (Hausmann et al., 2003; Profyris et al., 2004; David et al., 2012). Many of these mediators have both beneficial and deleterious effects, depending on when and at which extent they are released, and on which cellular population they act upon.

Finally, several studies have shown that activation of the complement system, composed of over 30 cellular and plasma/serum proteins, negatively contribute to secondary injury following SCI (Anderson et al., 2004; Qiao et al., 2006; Galvan et al., 2008; David et al., 2012). Within one day of SCI classical (C1q and C4), alternative (Factor B) and terminal (C5b-9) complement pathways are strongly activated. Disruption of the BSB allows entry of the complement proteins into the spinal cord parenchyma in addition to induction of local synthesis of the complement proteins in oligodendrocytes and neurons. As a result, complement proteins are deposited on oligodendrocytes, neurons, and axons in injured spinal cords. The association of complement activation with axons suggests that the complement cascade plays an important role in demyelination or axonal degeneration (Anderson et al., 2004). The detrimental role of the complement system in SCI is confirmed by studies where mice deficient for the complement proteins show improved functional recovery and increased tissue sparing (Qiao et al., 2006, 2010; Galvan et al., 2008; Guo et al., 2010; Li et al., 2014).

Additionally, pharmacological inhibition of C3, C5a or Factor B components of the complement system result in improvements in neurological recovery (Qiao et al., 2006, 2010; Li et al., 2014). Since complement activation mediates the recruitment of inflammatory cells the protective effect of complement deficiency/inhibition may be associated with attenuation of neutrophil and monocyte infiltration, inhibition of microglia/macrophage activation and reduction of astrogliosis (Qiao et al., 2006; Li et al., 2014). In addition, complement inhibition leads to reduction of the membrane attack complex (MAC) formation. The MAC, the terminal product of the complement cascade, may contribute directly to neuronal and glial injury and demyelination but can also induce the production of pro-inflammatory mediators, thus augmenting the inflammatory response after SCI (Qiao et al., 2006).

2.6.5 Interleukin-33, a novel mediator of inflammation in CNS

IL-33, initially discovered as a nuclear factor from high endothelial venules (Baekkevold et al., 2003), later was identified as a member of the IL-1 cytokine family that is most closely related in structure to IL-18 and IL-1β (Schmitz et al., 2005). However, in contrast to highly inflammatory IL-1β and IL-18 promoting Th1 response, IL-33 has been shown to induce the production of Th2 cytokines (Schmitz et al., 2005). Additionally, in contrast to other IL-1 family members, IL-33 is not mainly produced by sentinel immune cells, but instead constitutively produced predominantly by tissue-related cell types such as endothelial cells, smooth muscle, fibroblasts, cardiomyocytes, keratinocytes, adipocytes and mucosal epithelial cells (Schmitz et al., 2005; Moussion et al., 2008; Smith, 2010).

IL-33 functions both as a classical cytokine by binding to its specific receptor on the plasma membrane and as an intracellular nuclear factor involved in transcriptional regulation of gene expression (Ali et al., 2007, 2011; Carriere et al., 2007; Chackerian et al., 2007; Moussion et al., 2008; Cayrol and Girard, 2009; Haraldsen et al., 2009; Lüthi et al., 2009) (Figure 3).

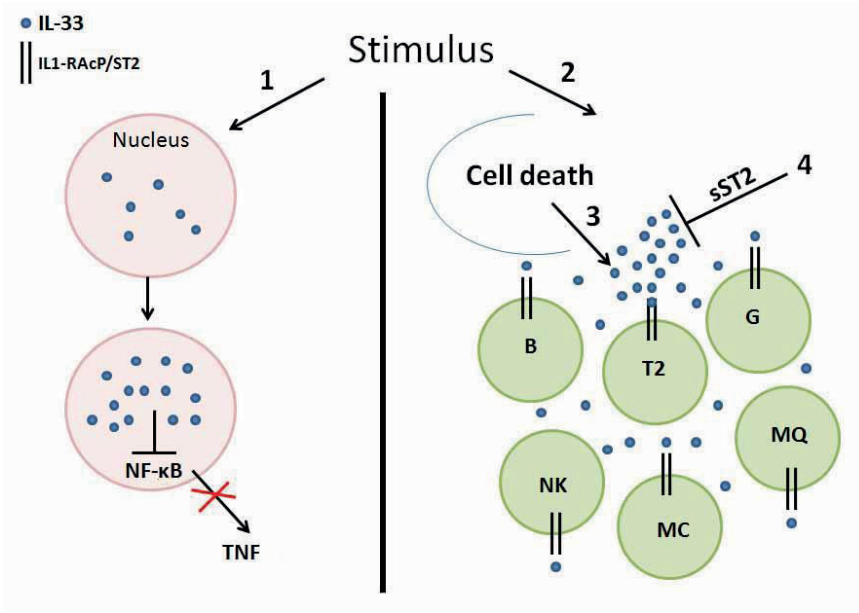

Figure 3. The dual role of IL-33 in inflammation. 1: In response to a pro-inflammatory stimulus IL-33 is upregulated in the nucleus. Upregulated IL-33 represses NF-κB and thereby prevents expression of NF-κB target genes. 2: Cell damage and necrosis result in release of intracellular IL-33 from the cells. 3: Released IL-33 binds to a variety of target cells expressing the IL-33 receptor (IL-1RacP/ST2) and induces their activation, differentiation and proliferation. 4: Soluble St2 form acts as a decoy receptor to bind and inhibit IL-33 activity. MQ - macrophages, G - granulocytes, B - B cells, T2 – T2 cells, NK - natural killer cells, MC - mast cells (modified from Sattler et al., 2013).

Intracellular IL-33 is localized in the nucleus associated with heterochromatin and negatively regulates gene transcription in a non–IL-33 receptor mediated fashion (Carriere et al., 2007; Roussel et al., 2008). Importantly, intracellular IL-33 interacts with p65 subunit of NF-κB repressing the activity of this transcription factor. NF-κB is a well-known trigger of pro-inflammatory cytokine expression and therefore IL-33-NF-κB interaction results in inhibition of NF-κB target genes, such as TNF (Ali et al., 2011). However, under pathological conditions, strong signals leading to activation of many p65 subunits can overcome the IL-33 mediated repression and result in induction of full NF-κB activity (Ali et al., 2011).

The IL-33 protein does not contain a classical secretory signal sequence that directs it for secretion via the ER–Golgi pathway (Schmitz et al., 2005). It is currently believed to be released mainly passively when cells undergo damage and necrotic cell death (Moussion et al., 2008; Lüthi et al., 2009; Smith, 2010). However, there are also reports indicating that release of IL-33 in the absence of cellular necrosis might also be possible (Kouzaki et al., 2011; Kakkar et al., 2012). Regardless of the mechanism, nuclear IL-33 when released from the cell in response to infection or tissue damage begins to function as an alarmin to alert and activate the immune system (Moussion et al., 2008; Cayrol and Girard, 2009; Lüthi et al., 2009; Haraldsen et al., 2009; Kakkar et al., 2012).

Extracellular IL-33 exerts cytokine activities and binds to its receptor consisting of a heterodimer between ST2 and IL-1 receptor accessory protein (IL-1RAcP) (Ali et al., 2007; Chackerian et al., 2007; Palmer et al., 2008; Liu et al., 2013). ST2 and IL-1RAcP recruit the adaptor molecules MyD88 and IL-1 receptor-associated kinases to induce activation of the transcription factor NF-κB as well as MAPKs in target cells (Schmitz et al., 2005). This results in expression of various downstream genes, leading to the release of various cytokines and chemokines or causing cell differentiation, polarization, and activation, depending on the target cell (Sattler et al., 2013; Villarreal and Weiner, 2014). In addition to

the transmembrane form of ST2, the soluble ST2 isoform (sST2) is produced and acts as a decoy receptor to bind and effectively inhibit IL-33 activity (Hayakawa et al., 2007), therefore playing an important role in regulation of IL-33 signaling (Smith, 2010).

Th2 lymphocytes, but not Th1 lymphocytes, have been shown to express ST2 (Xu et al., 1998), and were the first immune cells identified to respond to IL-33 stimulation (Schmitz et al., 2005). Further studies demonstrated that in addition to Th2 cells, the IL-33 receptor is expressed on a broad range of innate and adaptive immune cells, including neutrophils, basophils, eosinophils, NK and NKT cells, mast cells and macrophages and, therefore, IL-33 stimulates many immune cell types to exert their diverse effector functions (reviewed in Liew et al., 2010; Smith, 2010; Sattler et al., 2013; Villarreal and Weiner, 2014). Thus, IL-33 accelerates the production of pro-inflammatory leukotrienes and cytokines in mast cells (Allakhverdi et al., 2007; Moulin et al., 2007; Enoksson et al., 2011), promotes the production of Th2 cytokines in T-lymphocytes (Schmitz et al., 2005; Chackerian et al., 2007) and dendritic cells (Rank et al., 2009), and shifts the macrophage polarization from M1 to M2 type (Kurowska-Stolarska et al., 2009; Miller et al., 2010). IL-33 is also believed to act as selective Th2 chemoattractant (Komai-Koma et al., 2007), suggesting an important role for IL-33 in Th2 cell mobilization.

Initially IL-33 was thought to be mainly involved in initiating and expanding Th2-driven responses (Schmitz et al., 2005), however, recently it has been found that depending on the immune cells targeted by IL-33, it is able not only to promote Th2 expansion, but also to contribute to the development of the Th1 immune response (Smithgall et al., 2008; Bourgeois et al., 2009; Smith, 2010; Villarreal et al., 2014). In addition, IL-33 has been shown to amplify the CD8+ T cell response (Yang et al., 2011; Bonilla et al., 2012; Villarreal et al., 2014). Therefore, it is now believed that in different conditions, depending on the type of target cells and the microenvironment, IL-33 can exert different functions either associated in promoting Th2-immune or Th1-immune responses (Villarreal and Weiner, 2014).

IL-33 is a cytokine possessing pleiotropic activities which can regulate complex innate and adaptive immune responses in immunity and disease. Increased levels of IL-33 may have a dual function, as its intracellular form can negatively modulate NF-κB-mediated activity and dampen pro-inflammatory signaling, whereas its extracellular form stimulates adaptive and innate immune cells in order to clear the initial trigger and repair damaged tissues.

IL-33 is known to be highly expressed in the naïve mouse brain and spinal cord (Schmitz et al., 2005). However, it is only recently that the production and function of IL-33 in the CNS have attracted great attention (Han et al., 2011).

The cellular localization of IL-33 and its receptor in the CNS remains controversial. In glial cultures IL-33 has been shown to be inducible in astrocytes and upon induction by inflammatory stimuli it is localized to the nucleus (Hudson et al., 2008). In addition to astrocytes, IL-33 expression is also observed in brain endothelial cells, but in contrast to astrocytes, in endothelial cells it is not upregulated by inflammatory stimuli (Yasuoka et al., 2011). IL-33 expression has not been observed in microglia (Hudson et al., 2008; Yasuoka et al., 2011). *In vivo,* IL-33 has been shown to be upregulated primarily in astrocytes in spinal cords of mice with EAE (Yasuoka et al., 2011) and bone cancer-induced pain (Zhao et al., 2013), as well as in human brains from patients with multiple sclerosis (Christophi et al., 2012), indicating that IL-33-expressing astrocytes may act as a potentially critical regulator of immune responses in the CNS. However, in a recent study utilizing the mouse model of EAE the levels of IL-33 was shown to be increased in both neurons and astrocytes (Jiang HR et al., 2012). The expression and cellular localization of IL-33 in other neurological diseases and specifically CNS injuries have not been examined.

While neurons express only the IL-1RAcP subunit, astrocytes and microglia have been shown to express both ST2 and IL-1RAcP *in vitro*, suggesting that microglia and astrocytes may be a main target cell for IL-33 in CNS (Yasuoka et al., 2011). This was further confirmed by studies where IL-33 treatment was shown to induce a Th2 immune response

in IL-33-treated glia (Hudson et al., 2008), increase proliferation of microglia, induce production of TNF, IL-1β and IL-10 by microglial cells and enhance microglial phagocytosis (Yasuoka et al., 2011). Moreover, recently it has been shown that IL-33 is able to induce the release of pro-inflammatory mediators from mouse astrocytes and mediate neuronal damage and death in mixed glia-neuronal cultures (Kempuraj et al., 2013). Another study has demonstrated that ST2 is expressed in astrocytes, but not in neurons or microglia (Andre et al., 2005). Interestingly, in spinal cord tissue of mice with EAE the ST2 protein is detected in neurons, but not in astrocytes (Jiang HR et al., 2012).

The existing data as to whether IL-33 is beneficial or detrimental to CNS diseases is also controversial. Blockade of IL-33 inhibits the onset and severity of EAE, whereas when administered before the onset of clinical symptoms recombinant IL-33 augments the disease course of EAE (Li et al., 2012). In contrast, IL-33 treatment attenuates EAE when it is administered after EAE induction (Jiang HR et al., 2012). An increase in Th2-associated cytokine production together with polarization of macrophages towards M2 is proposed as the main mechanism of IL-33-mediated protection in EAE (Jiang HR et al., 2012). Given that Th2-expansion and M2-polarization are believed to be beneficial for SCI recovery (Schwartz, 2005; Kigerl et al., 2009; David and Kroner, 2011; Guerrero et al., 2012) IL-33 might be a promising candidate for SCI treatment.

2.7 ASTROGLIAL ACTIVATION AND GLIAL SCAR FORMATION AFTER SCI

Reactive astrogliosis is a prominent hallmark of CNS trauma, including SCI. It involves changes in astrocyte morphology and molecular expression. In severe cases, astroglial activation results in a formation of glial scar. In response to injury reactive astrogliosis is triggered by many different mediators including cytokines and growth factors such as IL-6, LIF, TNF, IFN-γ, IL-1, IL-10, TGF-β, FGF2, neurotransmitters such as glutamate and noradrenaline, LPS and Toll-like receptor ligands, ROS/RNS, endothelin-1, ATP, fibrinogen and many others (Faulkner et al., 2004; Silver and Miller, 2004; Fitch and Silver, 2008; Rowland et al., 2008; Sofroniew, 2009; Hamby and Sofroniew, 2010; Sofroniew and Vinters, 2010; Cregg et al., 2014). Reactive astroglia are heterogeneous in the injured spinal cord. Thus, a recent study demonstrated that following SCI mature scar borders are mainly consisting of newly proliferated, elongated astrocytes with overlapping processes, whereas at a distance from scar borders the reactive astrocytes are non-proliferative, hypertrophic stellate cells with non- or overlapping, far less extensive processes (Wanner et al., 2013).

It is well known that reactive astrogliosis and formation of astroglial scar consisting of glial (mainly astrocytic) and fibrotic components, create a physical barrier and inhibitory environment at the lesion site, leading to unsuccessful axonal regeneration (Silver and Miller, 2004; Fitch and Silver, 2008; Kawano et al., 2012; Cregg et al., 2014). Therefore, for a long time it was thought that reactive astrogliosis and scarring are the main obstacles on the way to functional recovery after SCI. Indeed, a number of studies have demonstrated that the molecular composition of the scar and the production of molecules inhibiting axonal growth, including tenascin, semaphorin 3A, ephrins and CSPGs family are the key factors contributing to failure of regeneration following SCI (McKeon et al., 1991, 1995; Fawcett and Asher, 1999; Rhodes and Fawcett, 2004; Silver and Miller, 2004; Fitch and Silver, 2008; Rolls et al., 2009; Kawano et al., 2012; Cregg et al., 2014). Elimination of these inhibitors or neutralization of their effect has been reported to enhance axonal regeneration in the injured spinal cord (Bradbury et al., 2002; Caggiano et al., 2005, Kaneko et al., 2006; Tan et al., 2006). These findings suggest that activation of astrocytes is detrimental for clinical outcome. However, the total inhibition of reactive astrogliosis is not a feasible therapeutic strategy for SCI (Sofroniew, 2009; Hamby and Sofroniew, 2010; Sofroniew and Vinters, 2010).

Numerous studies have demonstrated that the astroglial scar is much more than just a physical barrier impeding axonal regeneration and have provided compelling evidence that reactive astrocytes are able to protect adjacent tissue by different mechanisms including uptake of excitotoxic glutamate, free radical scavenging, facilitation of BSB repair, reduction of vasogenic edema and limiting the spread of inflammatory cells into healthy tissue (Faulkner et al., 2004; Rolls et al., 2009, Sofroniew, 2009; Hamby and Sofroniew, 2010; Sofroniew and Vinters, 2010; Kawano et al., 2012). In addition, astrocytes provide trophic support in the injured site by producing nutrients and growth factors (Rolls et al., 2009). Therefore, astrocytes have multiple responses to CNS trauma. Scar formation is one of them and represents the extreme form of reactive astrogliosis in response to overt tissue damage and inflammation (Sofroniew, 2009). The importance of reactive astrogliosis and glial scar for SCI repair is supported by experiments where ablation of reactive astrocytes and disruption of scar formation result in increased inflammatory cell infiltration, failure of BSB repair, increased demyelination and tissue damage leading to impaired functional recovery (Faulkner et al., 2004). Furthermore, conditional knockout of STAT3 (intracellular signal transducer for various cytokines including IL-6) from astrocytes markedly reduces astrogliosis and scar formation and result in aggravated neuropathology and reduced locomotor recovery (Okada et al., 2006; Herrmann et al., 2008). More recently, Wanner et al., 2013 demonstrated that following SCI scar forming, the astrocytes are able ''to corral inflammatory and fibrotic cells into discrete areas separated from adjacent tissue that contains viable neurons'' (Wanner et al., 2013). The inhibition of scar formation by selective deletion of STAT3 from astrocytes leads to increased inflammation and aggravated neuronal death in adjacent healthy tissue (Wanner et al., 2013). Importantly, genetic ablation of astrogliosis in addition to other harmful effects fails to promote desired axonal regeneration (Okada et al., 2006; Herrmann et al., 2008), which is possibly in part due to extended inflammation (Kawano et al., 2012). Taken together, these studies show that reactive astrogliosis, including scar formation, is an adaptive process, which exerts multiple beneficial functions in preventing the spread of inflammation and protecting intact neuronal elements from secondary damage (Faulkner et al., 2004; Rolls et al., 2009; Sofroniew, 2009; Hamby and Sofroniew, 2010; Cregg et al., 2014). Therefore, total suppression of glial scar formation cannot be a useful strategy for SCI treatment (Hamby and Sofroniew, 2010; Kawano et al., 2012).

However, under certain circumstances reactive astrocytes may play also a harmful role through several mechanisms including overproduction of ROS/RNS or/and pro-inflammatory cytokines (Sofroniew, 2009; Sofroniew and Vinters, 2010). Moreover, selective inhibition of certain signaling mechanisms in astrocytes, for example NF-κB, is associated with an improved outcome (Brambilla et al., 2005). Since different specific signaling mechanisms can induce reactive astrocytes to have different and sometimes opposite effects (pro-inflammatory or anti-inflammatory, increase or reduce oxidative stress), reactive astrocytes may exhibit both beneficial and detrimental effects depending on astrocytic location, severity or phase of response (Rolls et al., 2009; Sofroniew, 2009; Sofroniew and Vinters, 2010). Therefore, instead of total inhibition of reactive astrogliosis, novel therapeutic strategies should target specific signaling mechanisms in astrocytes with the aim to improve axon regrowth past the astroglial scar without altering its reparative and neuroprotective function (Hamby and Sofroniew, 2010; Wanner et al., 2013). Recently, it was demonstrated that transplantation of hESC-derived progenitors promotes astrogliosis through activation of signaling mechanisms that support axonal survival. The transplanted cells in synergism with reactive astrocytes created a permissive environment for axonal regeneration, inhibiting expression of detrimental genes such as CSPG and tenascins and increasing the expression of beneficial ones therefore resulting in improved functional recovery after spinal cord transection (Lukovic et al., 2014).

2.8 CELL DEATH AND DEMYELINATION AFTER SCI

Both necrotic and apoptotic mechanisms contribute to cell death after SCI. The initial mechanical damage results in haemorrhagic necrosis in gray matter involving the swelling of neuronal and glial cells, disruption of organelles and release of the intracellular contents through the ruptured membrane. The secondary injury cascade leads to further spread of the necrotic wave (Beattie et al., 2002; Hausmann et al., 2003; Kwon et al., 2004; Profyris et al., 2004).

Apoptosis, a form of programmed cell death, initially occurs in the lesion core 6 hours after injury (Crowe at al., 1997). At this moment, it accompanies necrosis in damaging multiple types of cells. By the end of the first week the level of apoptosis decreases and in several days the second wave of apoptotic death starts predominantly in white matter. Although apotosis also involves neuronal cells, mainly it affects oligodendrocytes and lasts for weeks, leading to persistent demyelination (Crowe at al., 1997; Profyris et al., 2004; Mekhail et al., 2012). Many of the secondary injury mechanisms contribute to apoptosis of oligodendrocytes. The most important ones are ischemia, oxidative stress, Ca^{2+} overload, excitotoxicity and release of extracellular ATP (Casha et al., 2001; Mekhail et al., 2012; Plemel et al., 2014). Another factor contributing to death of oligodendrocytes is the increased level of pro-inflammatory cytokines and particularly TNF, which is especially cytotoxic to oligodendrocytes (Hisahara et al., 1997; Yan et al., 2001; Yune et al., 2003; Genovese et al., 2008, 2009; Inukai et al., 2009; Mekhail et al., 2012; Plemel et al., 2014). After SCI, oligodendrocytes also overexpress the death receptors FAS and p75, which are via their respective ligands responsible for initiating of the apoptotic process (Casha et al., 2001; Beattie et al., 2002; Demjen et al., 2004; Keane et al., 2006). In addition to apoptosis, another form of programmed cell death, namely autophagy (Tsujimoto and Shimizu, 2005), was recently implemented in the death of oligodendrocytes (Kanno et al., 2009b, 2011).

As oligodendrocytes are responsible for myelinating neuronal axons their death results in widespread demyelination (Kwon et al., 2004; Mekhail et al., 2012; Plemel et al., 2014). Since one oligodendrocyte myelinates multiple axons, loss of a single cell might induce the demyelination of many axons surviving after initial trauma (Profyris et al., 2004). The loss of myelin sheaths results in impairment of axonal transmission and over time may result in degeneration of denuded axons (Irvine and Blakemore, 2008). It is thought that prominent early loss of oligodendrocytes which occurs by both necrosis and apoptosis within the first 24 hours after trauma underlies the loss of function observed after injury, while delayed apoptotic oligodendrocyte death results in impaired functional recovery (Plemel et al., 2014).

Although spontaneous remyelination is known to occur in the injured spinal cord, it is very often an incomplete and abnormal process (Totoiu and Keirstead, 2005; Mekhail et al., 2012; Powers et al., 2012). Therefore demyelination might be an attractive target for therapeutic intervention after SCI.

2.9 CURRENT CLINICAL STRATEGIES IN SCI MANAGEMENT

Although our understanding of pathological mechanisms involved in SCI has broadened significantly over the last decades, effective treatments for SCI are limited and there is no complete restorative therapy (Olson, 2013; Oudega, 2013; Varma et al., 2013).

Currently the management of acute SCI includes surgical and pharmacological intervention followed by physical therapy and rehabilitation (Raslan and Nemecek, 2012).

2.9.1 Surgical treatment

Surgical treatment involves the application of surgical decompression to remove the bones that compress the spinal cord as well as spinal stabilization (Wilson and Fehlings,

2011; Raslan and Nemecek, 2012). The timing of surgical intervention in SCI management for a long time was one of the most controversial topics (La Rosa et al., 2004; Fehlings and Perrin, 2006; Wilson and Fehlings, 2011; Raslan and Nemecek, 2012). However, the results of the recent Surgical Timing in Acute Spinal Cord Injury Study (STASCIS) show that decompression within 24 hours following SCI is safe and leads to improved neurologic outcome (Fehlings et al., 2012).

2.9.2 Pharmacological treatment

The main aim of pharmacological intervention after SCI is to reduce the secondary injury cascade and protect the cells that initially survived after mechanical injury and thus promote functional recovery (Kwon et al., 2004; Raslan and Nemecek, 2012). Although it is not approved by regulatory authorities (Hurlbert et al., 2013), synthetic glycocorticoid methylprednisolone (MP) remains the only drug used worldwide for the treatment of acute SCI in clinical practice (Varma et al., 2013; Chikuda et al., 2014). MP administration after acute SCI has been the subject of debate for more than 20 years. The beneficial effect of high-dose MP was initially reported in a series of National Acute Spinal Cord Injury Studies (NASCIS) in the 1990s. It has been demonstrated that MP administered systemically in very high doses can reduce human neurological deficits after SCI when the first dose is given within the first 8 hours after injury (Bracken et al., 1990; 1998). Inhibition of lipid peroxidation and inflammatory response are considered to be the main mechanisms of MP action after SCI (Bracken et al., 1990; Hall, 1992; 1993). However, the use of high-dose MP in treating acute SCI is controversial due to significant dose related adverse effects (gastric bleeding, hyperglycemia requiring insulin administration, sepsis, pneumonia, urinary tract infections, acute corticosteroid myopathy, wound infection) and relatively modest neurological improvement (Bracken et al., 1990; Gerndt et al., 1997; Qian et al., 2005; Suberviola et al., 2008; Ito et al., 2009; Hurlbert et al., 2013; Chikuda et al., 2014). As a result, many centers have ceased to use this drug. Moreover, as there is clear medical evidence suggesting that high-dose MP administration is associated with a variety of complications, there are recommendations that MP should not be routinely used in the treatment of patients with acute SCI (Hurlbert et al., 2013). Of note is the fact that it has been proposed that most of the adverse effects are due to toxicity associated with the high systemic dosage of MP, whereas the modest beneficial effects are due to insufficient dosing to the lesion site (Kim et al., 2009).

2.9.3 Rehabilitation

An additional component in SCI management is physical therapy and rehabilitation, which can make a significant impact on the overall recovery (Fouad and Tetzlaff, 2012; Raslan and Nemecek, 2012). After several studies showing that paralyzed cats with complete spinal cord transection could be trained to maintain weight supported stepping on a treadmill (Lovely et al., 1986, 1990; Barbeau and Rossignol, 1987; de Leon et al., 1998) the potential of the plasticity within the adult spinal cord became clear (Roy et al., 2012). The observations suggesting that plasticity after injury in the spinal cord is significantly influenced by physical activity led to novel rehabilitation strategies for SCI (Silva et al., 2014), including developing of robotic systems for automating locomotor training in patients (Wu M et al., 2012). However, although rehabilitative physiotherapy and training are nowadays routinely used in many centers around the world after SCI, the optimal use of rehabilitative training, the underlying mechanisms and possible side effects are still to be determined (Marsh et al., 2010; Onifer at al., 2011; Fouad and Tetzlaff, 2012).

Overall, the long-term outcome after SCI is a combination of the initial extent of the injury, natural recovery from it, surgical and pharmacological interventions, rehabilitation, and social/community reintegration (Raslan and Nemecek, 2012). However, even despite early medical care and specialized rehabilitation, SCI is associated with a dramatic decrease

in quality of life for affected individuals, resulting in lower life expectancy than that of the general population (Ahoniemi et al., 2011; Varma et al., 2013). Therefore, there is need to explore new therapeutic strategies for restoring neurological function after SCI and also to expand the knowledge on the cellular and molecular mechanisms of this devastating condition.

2.10 ANIMAL MODELS IN SCI RESEARCH

The use of animal models is crucial for studying SCI mechanisms and development of new SCI treatments (Silva et al., 2014). Rat and mouse are the most widely used species in SCI research (Silva et al., 2014). Despite the fact that SCI pathophysiology in mice is probably even further different from human SCI pathophysiology than in rats (Rosenzweig and McDonald, 2004), use of mouse SCI models has increased exponentially. This is mainly due to the availability of genetically modified mice, which allow elucidating the function of specific genes in molecular mechanisms of secondary injury and repair (Kundi et al., 2013; Zhang et al., 2014). The use of non-human primates, although is very important to prove safety and efficacy in the late stages of treatment development, is limited due to high costs and ethical reasons (Silva et al., 2014).

Three general classes of SCI experimental animal models are frequently used: transection, contusion and compression (Rosenzweig and McDonald, 2004).

The transection-based or laceration model requires the opening of the dura and cutting of spinal cord with a sharp instrument, usually microsurgical knife or spring scissors (Rosenzweig and McDonald, 2004, Zhang et al., 2014). This SCI model is useful primarily for assessment of axon regeneration after SCI. Because transection or full disruption of spinal cord is relatively rarely observed in human patients (Rowland et al., 2008) many researchers use compression and contusion injury models (Rosenzweig and McDonald, 2004; Silva et al., 2014).

The compression injury model is induced by compressing the spinal cord with a modified aneurysm clip (Rivlin and Tator, 1978), forceps, or a compression balloon (Borgens and Shi, 2000; Vanicky et al., 2001). The clip compression model allows precise control over the applied pressure and the time of compression, however, this model does not truly mimic human injury (Kundi et al., 2013). Insertion of balloons also produces adjustable compression lesions, however, this method is not optimal in rodents because of their small subarachnoid space (Vanicky et al., 2001).

The contusion models are currently believed to be the most clinically relevant injury models of SCI (Kwon et al., 2010; Zhang et al., 2014). This type of SCI model is induced by hitting the exposed spinal cord with a blunt contusion force without disruption of the dura. Several types of computer-controlled contusion devices are available that produce consistent and reproducible contusion injuries in rats and mice (Wrathall et al., 1985; Gruner, 1992; Jakeman et al., 2000; Young, 2002; Scheff et al., 2003; Ghasemlou et al., 2005). Currently the most widely used are Ohio State University's electromagnetic spinal cord injury device (ESCID), New York University's MASCIS Impactor (NYU Impactor) and Infinite Horizone Impactor (IH Impactor) (Ghasemlou et al., 2005). While ESCID uses electromagnetic force to create contusion of the spinal cord (Jakeman et al., 2000), NYU Impactor utilizes the dropping a rod of different weights from different heights (Gruner, 1992; Young, 2002). The NYU Impactor has been extensively utilized on rats but does not produce reproducible lesions in mice (Zhang et al., 2014). In contrast to the NYU Impactor, the IH Impactor allows the experimenter to determine the severity of injury by adjusting the impact force that is applied to the exposed spinal cord (Scheff et al., 2003). After laminectomy, the impactor tip rapidly displaces the exposed spinal cord tissue and when the desired force is obtained, it is withdrawn (Scheff et al., 2003). Importantly, recently, in addition to the widely used thoracic SCI models, contusion cervical SCI models were

developed in rats and mice using the IH Impactor (Aguilar and Steward, 2010; Lee et al., 2012; Streijger et al., 2013). This is of utmost importance since the majority of human spinal cord injuries occur in the cervical spinal cord (Norenberg et al., 2004; Lee et al., 2012; Streijger et al., 2013).

The main advantages and disadvantages of SCI models are summarized in Table 1.

*Table 1.*Summary of SCI models

Instrument	Mechanism	Advantages	Disadvantages	Animal
Knife, scissors	Transection	Easy to perfom, large animals, optimal for regeneration studies	Uncommon clinical SCI type, not suitable to study pathophysiology of secondary injury	Mice, rats, cats, dogs, primates
Clip	Compression	Widely used, reproducible, easy to perform, inexpensive, controlled force and duration	Injury parameters not recorded	Mice, rats
Forceps	Compression	Easy to perform, inexpensive	Injury parameters not recorded	Mice, rats
Baloon	Compression	Inexpensive, large animals	Not optimal in rodents, inconsistent injury, injury parameters not recorded	Dogs, rabbits, primates
ESCID	Contusion	Reproducible, consistent force and displacement, most clinically relevant	Not commercially available, requires vertebral stabilization, requires displacement calibration	Mice, rats
NYU Impactor	Contusion	Widely used, Reproducible, most clinically relevant	Requires vertebral stabilization, not optimal in mice	Mice, rats
IH Impactor	Contusion	Widely used, Reproducible, consistent force, most clinically relevant	Expensive, requires vertebral stabilization	Mice, rats

2.11 PRECLINICAL STRATEGIES FOR SCI TREATMENT

Although effective treatments for SCI remain limited, in recent years there have been many promising studies from the clinical translational perspective that are aimed at preventing secondary damage, promoting regeneration and replacing of destroyed spinal cord cells (Varma et al., 2013; Silva et al., 2014).

2.11.1 Targeting secondary injury following SCI

Given the crucial role of the secondary injury cascade in SCI pathogenesis a variety of therapies have been exploited to reduce excitotoxic damage to neurons, decrease oxidative stress and alter neuroinflammation (Hawryluk et al., 2008; Rowland et al., 2008; Kwon et al., 2011; Varma et al., 2013; Silva et al., 2014).

Targeting excitotoxicity following SCI

Since overstimulation of NMDA receptors due to high glutamate levels after SCI leads to the uncontrolled influx of extracellular calcium and sodium into the cell and results in neuronal and glial damage (Fehlings and Agrawal, 1995; Agrawal and Fehlings, 1996, 1997; Kwon et al., 2004; Rowland et al., 2008), the therapeutic potential of either sodium channel

blockers, such as riluzole, or NMDA receptor antagonists, such as magnesium sulfate, have been studied in SCI in order to protect the spinal cord tissue from excitotoxic injury.

Riluzole is a benzothiazole anticonvulsant, which has been approved for patients with amyotrophic lateral sclerosis (ALS) (Miller et al., 2007). The neuroprotective effects of riluzole result from blockade of voltage-activated sodium and calcium ion channels, inhibiting presynaptic glutamate release and activating potassium ion channels (Doble, 1996; Duprat et al., 2000; Wang et al., 2004). In animal models of SCI, Riluzole has been found to promote tissue sparing, decrease oxidative stress, reduce neuronal loss and promote functional recovery (Stutzmann et al., 1996; Mu et al., 2000; Schwartz and Fehlings, 2001; Ates et al., 2007; Nógrádi et al., 2007). Additionally, Riluzole may promote outgrowth of sensory neurons (Shortland et al., 2006). Based on these encouraging preclinical results two multicenter clinical trials for acute human SCI have been initiated (NCT00876889 and NCT01597518). The recently completed NCT00876889 Phase I study demonstrated that Riluzole treatment was well tolerated and the pilot data suggested that Riluzole may have a beneficial effect on motor outcome in cervical SCI that was manifest at 90 days postinjury (Grossman et al., 2014). Further studies of the pharmacokinetics, safety, and effects of Riluzole on neurological outcome in acute traumatic SCI will be carried out in a phase II trial (Grossman et al., 2014).

The potential mechanisms of magnesium sulfate action include the attenuation of excitotoxicity by blockage of NMDA receptors and of voltage-gated calcium channels resulting in reduction of intracellular calcium levels, the inhibition of free radical generation and glutamate release, as well as the reduction of apoptosis (Nowak et al., 1984; Lee et al., 2004; Kwon et al., 2009). In SCI animal models, magnesium sulfate administration has been shown to improve tissue sparing, reduce apoptosis and lipid peroxidation, restore BSB integrity, and improve functional recovery (Kaptanoglu et al., 2003; Ozdemir et al., 2005; Wiseman et al., 2009). More recently, it was demonstrated that magnesium formulated within a hydrophilic polymer, polyethylene glycol, resulted in improved tissue sparing at the lesion site and greater locomotor recovery after thoracic and cervical rodent SCI even when administered intravenously at a low doses (Ditor et al., 2007; Kwon et al., 2009; Lee JH et al., 2010a). Importantly, the formulation of magnesium within polyethylene glycol will be used in the upcoming Phase II multicenter clinical trial (Silva et al., 2014). Interestingly, memantine, a clinically used NMDA receptor antagonist, was not protective after experimental SCI probably due to insufficient affinity of memantine for spinal cord NMDA receptors (von Euler et al., 1997).

AMPA/kainic acid receptor antagonists have also been tested in preclinical SCI studies. It has been shown that 2,3-dihydro-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) promotes grey matter sparing and improves functional recovery after thoracic SCI (Wrathall et al., 1996, 1997), but not cervical SCI (Gensel et al., 2012). An AMPA-specific antagonist, Topiramate, which is clinically used for treatment of migraines and epileptic seizures, was recently reported to preserve oligodendrocytes and neurons after severe cervical SCI. However, in this study Topiramate did not promote functional recovery and its neuroprotective effect did not expand to moderate SCI (Gensel et al., 2012). Further studies are required to estimate the therapeutic potential of Topiramate for SCI treatment.

Targeting oxidative stress following SCI

As oxidative stress is strongly implicated in SCI pathogenesis, alleviating free radical damage is considered to be an effective strategy in SCI treatment (Hall, 2011; Jia et al., 2012). The assumption that antioxidants may minimize the degree of secondary damage has resulted in extensive research on the neuroprotective properties of different antioxidant compounds in SCI preclinical models (Jia et al., 2012). Probably one of the first antioxidants shown to be beneficial for SCI recovery was vitamin E. Supplementation with high-dose oral vitamin E attenuates posttraumatic ischemia in spinal cord white matter and significantly improves hind limb motor function compared to controls when it is

administered before injury (Hall and Wolf, 1986; Anderson et al., 1988; Iwasa et al., 1989). However, despite these beneficial effects, the use of vitamin E for treatment of acute SCI is limited due to the long time required to achieve a significant increase in CNS tissue levels (Machlin and Gabriel, 1982). Inhibition of lipid peroxidation is also one of the main mechanisms of MP action (Hall et al., 1992). However, the potential for adverse effects has resulted in the continued search for compounds with antioxidant properties that are safer and more effective than MP. Furthermore, given the complexity of the oxidant-antioxidant system, instead of attempting to restore the balance by the administration of one antioxidant or another, a promising alternative might be to enhance endogenous protection from oxidative stress by activating the Nrf2-ARE pathway (Hybertson et al., 2011). Importantly, while the Nrf2-ARE pathway is induced after SCI, it is obviously unable to provide adequate protection and therefore must be amplified and temporally accelerated in order to combat oxidative damage induced by SCI (Miller et al., 2014). Hence, induction of Nrf2-ARE pathway in order to augment endogenous antioxidant defences may be a useful approach to SCI treatment (Jia et al., 2012). Recently, the administration of a natural activator of Nrf2, sulforaphane, to injured rats has been shown to enhance Nrf2 expression and phase II detoxification enzyme production leading to neuroprotection with enhanced tissue sparing and improvement in coordination (Benedict et al., 2012; Wang X et al., 2012b). The potential of Nrf2 activators has been further confirmed as dimethyl fumarate, recently identified as a novel Nrf2 activator, has been shown to be beneficial in two Phase III clinical trials and has been approved as a first-line oral treatment of multiple sclerosis by FDA and European Medicines Agency (EMA) (Ruggieri et al., 2014). To date, there is no information available about clinical trials involving compounds activating Nrf2 in SCI.

Since α,β-unsaturated aldehyde acrolein, a reactive byproduct of lipid peroxidation, is likely responsible for continuously propagating degeneration in SCI (Lovell et al., 2000; Luo and Shi, 2004; Luo et al., 2005; Hamann et al., 2008; Hamann and Shi, 2009) suppressing acrolein might be a promising strategy for SCI treatment (Hamann and Shi, 2009; Park et al., 2014). Indeed, in a recent study hydralazine, a proven acrolein scavenger, was shown to ameliorate tissue damage, motor deficits, and neuropathic pain in the rat contusion SCI model (Park et al., 2014).

Targeting inflammation following SCI

Traditionally immune reactions following SCI have been considered to be unambiguously deleterious to axonal regeneration and functional recovery (Taoka et al., 1997; Popovich et al., 1999; Young, 2002; Profyris et al., 2004) and therefore the efforts of many researchers have been directed to limiting inflammation after SCI.

The nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and ibuprofen, which are known as potent inhibitors of endogenous prostaglandin synthesis, were used in early attempts to attenuate the inflammatory components of the secondary injury cascade after SCI (Priestley et al., 2012; Silva et al., 2014). Although indomethacin treatment in certain studies resulted in decreased edema and tissue damage as well as reduced microgliosis and astrogliosis (Winkler et al., 1993; Schwab et al., 2004; Pantovic et al., 2005), behavioral outcomes were rather contradictory (Simpson et al., 1991; Guth et al., 1994) and even adverse effects have been reported, suggesting that indomethacin treatment may be harmful in SCI (Guven et al., 1999). Interestingly, high doses of ibuprofen have been found to promote functional recovery when administrated 3 or 7 days post injury (Fu et al., 2007; Wang et al., 2009). These benefits might be in part attributed to the ability of ibuprofen to reduce RhoA activation (Zhou et al., 2003). Since small GTPase RhoA is a key molecule in the axonal re-growth inhibitory cascade (Kopp et al., 2012), ibuprofen in addition to attenuation of inflammation is believed to promote axonal sprouting (Fu et al., 2007; Wang et al., 2009; Xing et al., 2011), prevent loss of oligodendrocytes, and enhance myelination (Xing et al., 2011). However, re-assessment of Fu et al., 2007 data only partially replicated the observed behavioral benefits and failed to detect any effect of ibuprofen

treatment on axonal growth (Sharp et al., 2013). Moreover, in another recent study, chronic ibuprofen administration after rat contusion SCI, despite early positive effect on reducing microgliosis, failed to provide functional or histological evidence of neuroprotection. This suggests that early inhibition of inflammation may be detrimental for recovery from SCI (Redonda-Castro and Navarro, 2014). Nevertheless, in the beginning of 2014 a clinical trial in order to assess safety of ibuprofen to treat acute traumatic SCI has been initiated (NCT02096913).

Statins, which are commonly used for the treatment of high cholesterol levels and prevention of cardiovascular/cerebrovascular diseases, are additionally known to possess antioxidant and anti-inflammatory properties (Stuve et al., 2003; Farooqui et al., 2007) and were also examined for SCI treatment. Atorvastatin has been found to reduce apoptosis, astrogliosis and macrophage infiltration resulting in increased tissue sparing and improved functional recovery (Pannu et al., 2005, 2007; Dery et al., 2009). However, similarly to ibuprofen, a recent attempt to replicate these results failed to demonstrate any beneficial outcome (Mann et al., 2010) and therefore further preclinical investigation of statins in SCI is warranted.

Erythropoietin (EPO) is a cytokine stimulating proliferation and differentiation of erythroid precursor cells (Jelkmann, 1992). After SCI, EPO provided neuroprotection by reducing inflammation and oxidative stress, limiting neuronal apoptosis, and increasing tissue sparing, which resulted in improved locomotor recovery (Gorio et al., 2002; Boran et al., 2005; Yazihan et al., 2008; Huang et al., 2009; Ning et al., 2011; Wang S et al., 2012). Derivatives of EPO lacking hematopoietic effects, such as asialoerythropoietin and carbamylated erythropoietin, have also been shown to be neuroprotective in preclinical SCI models (Erbayraktar et al., 2003; Leist et al., 2004). Interestingly, recently EPO was shown to promote the induction of Nrf2 signaling pathway after SCI, suggesting a novel mechanism of EPO-mediated neuroprotection (Jin et al., 2014a). However, despite these promising results two studies were not able to confirm the efficacy of EPO in experimental SCI (Mann et al., 2008; Pinzon et al., 2008a). Furthermore, EPO has been also tested in patients with SCI. However, this clinical trial was suspended without public explanations (NCT00561067) and another clinical trial administrating EPO to patients with malignant spinal cord compression was also prematurely terminated due to insufficient accrual (NCT00220675).

The broad-spectrum antibiotic minocycline is known to reduce neural inflammation and prevent cell death in several animal models of neurodegenerative diseases and CNS trauma (Tikka et al., 2001; Stirling et al., 2005). Beneficial effects of minocycline for SCI treatment have been reported in a number of studies and include inhibition of lipid peroxidation, reduction of astrogliosis and microgliosis, enhancement of axonal sprouting, reduction of lesion size and improvement of functional recovery (Lee et al., 2003; Wells et al., 2003; Stirling et al., 2004; Teng et al., 2004; Festoff et al., 2006; Sonmez et al., 2013). However, similarly to the above-mentioned compounds, several studies have failed to demonstrate beneficial effects of minocycline in SCI models (Lee JH et al., 2010b; Pinzon et al., 2008b; Saganova et al., 2008). Despite this contradictory data, a human clinical trial was initiated in Canada (NCT05594494). Minocycline treatment was proven to be feasible and safe since no severe adverse effects were reported. Importantly, it showed a tendency towards improvement in several studied parameters (Casha et al., 2012). Although this study did not confirm the efficacy of minocycline for SCI treatment, the results were accepted as encouraging. In 2013, a Phase III Study of Minocycline in Acute Spinal Cord Injury was initiated in Calgary, Alberta and is currently recruiting participants (NCT01828203).

In addition, numerous studies utilizing agents that target neuroinflammation in general or its cellular effectors have reported improved functional recovery following SCI. However, even at the clinical trial phase, these strategies failed to prove the expected beneficial effect (reviewed in David et al., 2012; Priestley et al., 2012; Plemel et al., 2014; Silva et al., 2014). Given that there is now strong evidence that not all immune cell subsets are detrimental and some are even beneficial, probably the desirable therapeutic strategy

might be to modulate the inflammatory response in such a way that to harness its beneficial effects and minimize its harmful effects, rather than to fully abolish it (Rossignol et al., 2007; David et al., 2012; Plemel et al., 2014; Silva et al., 2014).

One of these attractive immunomodulatory strategies, which has emerged recently, is the manipulation on residing microglial cells or macrophages infiltrating the injured spinal cord in order to induce their polarization to the M2 state (David and Kroner, 2011; Guerrero et al., 2012; Jiang MH et al., 2012, 2013). Indeed, the introduction of substance P (Jiang MH et al., 2012, 2013) or temporal blockade of IL-6 (Guerrero et al., 2012) inhibits neutrophil recruitment, reduces TNF and IFN-γ levels, augments IL-4 and IL-13 expression and therefore promotes M2 macrophage polarization resulting in enhanced phagocytosis and axonal regeneration with subsequent improvement of locomotor recovery (Guerrero et al., 2012). Recently, Guo et al., 2013 showed that administration of G-CSF within 72 h after SCI can promote an anti-inflammatory response via inhibiting M1 activation and favoring M2 polarization. Interestingly, minocycline has recently been demonstrated to dampen selectively the M1 inflammatory response without altering M2 subsets (Kobayahi et al., 2013). In light of this, IL-33, which is known to inhibit the M1 and augment the M2 response, might be a promising candidate for immunomodulatory therapy following SCI.

2.11.2 Regenerative approaches for SCI treatment

As it was mentioned earlier in the chapter 2.4, the glial scar forms a physical barrier and by expressing inhibitory molecules including CSPGs and tenascin creates a microenvironment that is inhibitory to axonal regeneration within the injured spinal cord (McKeon et al., 1991; Silver and Miller, 2004; Fitch and Silver, 2008; Kawano et al., 2012; Cregg et al., 2014). However, not only glial scar and CSPGs are responsible for the lack of regeneration after SCI. For instance, myelin contains several molecules that inhibit axonal growth after trauma (Schwab and Caroni, 1988; Schnell and Schwab, 1990; Chen et al., 2000; Grandpre and Strittmatter, 2001; Fournier et al., 2001). One of the most potent of these is the 250-kDa glycoprotein Nogo-A. Interacting with its receptor Nogo-A activates RhoA, leading to growth cone collapse and axonal outgrowth inhibition (Chen et al., 2000; Grandpre and Strittmatter, 2001; Fournier et al., 2001; Schwab, 2004; Teusch and Kiefer, 2006). In addition, many ligands including myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein bind and activate Nogo receptors and, therefore, prevent regeneration after SCI (Barton et al., 2005; Lauren et al., 2007).

In order to overcome the inhibitory effect of myelin components on regeneration strategies directed to blockade of Nogo-A or its receptors have been proposed. Already prior to Nogo-A discovery, it had been shown that a blocking antibody (IN-1) raised against myelin-associated neurite growth inhibitory proteins induced axonal sprouting and long-distance corticospinal regeneration leading to improved behavioral outcome after rat SCI (Schnell and Schwab, 1990; Bregman et al., 1995). After Nogo-A was identified (Chen et al., 2000) the beneficial effect of anti-Nogo-A antibodies on regeneration after SCI have been demonstrated in rats (Liebscher et al., 2005; Atalay et al., 2007) and non-human primates (Fouad et al., 2004; Freund et al., 2006, 2007, 2009). Similarly, blocking Nogo receptors with the competitive antagonist NEP 1–40 enhances corticospinal tract growth and functional recovery after SCI (GrandPre at al., 2002; Li and Strittmatter, 2003). However, a more recent study failed to replicate the positive effect of NEP 1-40 (Steward et al., 2008). Nevertheless, the pre-clinical data supporting the beneficial effect of Nogo-A inhibition for SCI recovery resulted in a Phase I safety trial in patients with acute SCI (NCT00406016). Even though the trial was completed in 2011, no results have yet been reported.

Interestingly, genetic deletion of Nogo or the Nogo receptor do not necessarly improve regeneration (Zheng et al., 2003; Teng and Tang, 2005; Zheng et al., 2005; Steward et al., 2007; Young, 2014). Although it has been reported that Nogo knockout mice are able to regenerate (Kim et al., 2003), other studies were not able to confirm these results (Zheng et al., 2003, 2005; Steward et al., 2007) or reported only a modest increase of regenerative

capacity (Simonen et al., 2003; Lee JK et al., 2010). It is also worth mentioning that regeneration was also observed in the injured spinal cord even without eliminating Nogo or glial scar (Liu et al., 2010; Zukor et al., 2013; Lewandowski and Steward, 2014; Young, 2014). Therefore, it is still questionable whether Nogo, CSPGs and glial scar are sufficient to abolish spinal cord regeneration. Instead of limiting the inhibition, a useful approach may be to increase intrinsic regenerative mechanisms (Young, 2014).

2.11.3 Gene therapy approaches for SCI treatment

The idea to deliver therapeutic genes using viruses as vectors in order to treat human diseases first emerged more than 40 years ago (Friedmann and Roblin, 1972). Since then many viral systems, such as adenoviruses (AV), adeno-associated viruses (AAV), herpesviruses (HV) and retroviruses (RV) including lentiviruses (LV) have been developed as vectors for therapeutic gene delivery (Lim et al., 2010; Lentz et al., 2012). Each of them possess their own properties which determine specific advantages and disadvantages of every vector type. To estimate the potential of different viral vectors it is important to consider several aspects including specific tropism for target tissue, efficiency of transduction of the target cells, duration and level of transgene expression and side effects such as the host immune response (Lim et al., 2010; Lentz et al., 2012; Maguire et al., 2014).

Viral vectors are powerful tools for transgene delivery to specific regions of the brain. Due to the limitations imposed by the blood-brain barrier (BBB), the most common delivery route of viral vectors is direct injection into the brain parenchyma, which allows bypassing the BBB (Maguire et al., 2014). However, recently several AAV serotypes were shown to be able to cross the BBB and provide expression during systemic delivery (Zincarelli et al., 2008; Foust et al., 2009; Zhang et al., 2011; Bourdenx et al., 2014). LV vectors are believed to have low immunogenicity and an ability to transduce neuronal cells with high efficiency. Importantly, they possess higher packaging capacity than AAV vectors (Wong et al., 2006; Lentz et al., 2012; Palfi et al., 2014). The ability of lentiviruses to integrate into the host genomes allows stable transduction of the host cells (Wong et al., 2006; Lundberg et al., 2008; Lim et al., 2010). However, random integration may lead to insertional mutagenesis and is the primary safety concern for LV use in gene therapy (Dave et al., 2004; Bokhoven et al., 2009; Lim et al., 2010; Lentz et al., 2012). Substantial advancement in the field of gene delivery with lentiviruses was the development of non-integrating lentiviral vectors that are deficient in integrase activity (Apolonia et al., 2007; Sarkis et al., 2008). Random integration of AAV vectors associated with tumorigenesis in mice has also been reported (Deyle and Russell, 2009). However, in the case of AAV vectors the risk of insertional mutagenesis is very low (Lentz et al., 2012).

Gene therapy for several neurodegenerative diseases including AD and PD has already moved from preclinical studies to Phase I and Phase II clinical trials (Tuszynski et al., 2005; Kaplitt et al., 2007; Christine et al., 2009; Mandel et al., 2010; Marks et al., 2010; Muramatsu et al., 2010; LeWitt et al., 2011; Palfi et al., 2014) and offers a promising approach also for SCI treatment.

In SCI settings, gene delivery can provide long-term expression of therapeutic proteins in the injured spinal cord. For example, EPO delivery to the spinal cord by direct injection of HV vector in a model of cervical hemicord contusion resulted in robust and sustained EPO expression. Vector-mediated expression of EPO reduced the injury size and enhanced tissue sparing resulting in improved functional recovery (Wang S et al., 2012). Gene transfer of GDNF with adenovirus vector has been reported to preserve neuronal fibers and promote hind limb recovery following spinal cord contusion injury (Tai et al., 2003). Recently, chondroitinase ABC (ChABC) was delivered via LV vector in order to assess the efficacy of large-scale CSPG digestion after SCI. CSPGs are known to inhibit repair following SCI (Silver and Miller, 2004; Carulli et al., 2005; Bradbury and McMahon, 2006) and their large-scale digestion was found to result in significant neuroprotection and long-term functional improvement following spinal contusion injury. Interestingly, LV-ChABC

gene transfer induced polarization of macrophages to alternatively activated M2 phenotype in this study (Bartus et al., 2014).

In addition to single gene transfer, the delivery of different transcription factors to the injured spinal cord with gene therapy enables simultaneous induction or repression of various genes sharing a common regulatory pathway. For example, intraspinal delivery of AV-HIF-1α, a subunit of hypoxia-inducible factor 1, which is a transcription factor playing essential roles in the mammalian development and physiology, resulted in simultaneous induction of vascular endothelial growth factor (VEGF), antiapoptotic B-cell lymphoma 2 (Bcl-2) and repression of proapoptotic bcl-2-like protein 4 (BAX). Amelioration of neuronal apoptosis and promoting of angiogenesis with HIF-1α gene delivery led to enhanced functional recovery (Chen et al., 2013). In another study, the intraspinal delivery of transcription factor Sox11, which is proposed to be involved in the development and regeneration of the brain (Jankowski et al., 2006), enhanced neuronal regeneration, increased expression of BDNF and promoted locomotor function after SCI (Guo et al., 2014).

In oxidative stress conditions the simultaneous induction of antioxidant genes may be a better approach than administration of a single antioxidant, or gene transfer with individual antioxidant genes (Levonen et al., 2007, 2008). Since Nrf2 is orchestrating antioxidant and cytoprotective responses to oxidative and electrophilic stress (Hybertson et al., 2011; Gan and Johnson, 2013; Gao et al., 2014), overexpression of this transcription factor with gene delivery in the tissues affected by oxidative stress might be a promising therapeutic strategy. Recently, human Nrf2 was delivered into the mouse hippocampus, an area of the brain affected in AD. This resulted in sustained Nrf2 expression in the hippocampus and improved spatial learning and memory of transgenic AD mice (Kanninen et al., 2009). Whether Nrf2 gene transfer would be beneficial in SCI remains to be elucidated.

2.11.4 Cellular approaches for SCI treatment

Cell transplantation is a promising treatment strategy for promotion of repair in the injured spinal cord. A number of preclinical studies demonstrate that cell transplantation can provide neuroprotection by reducing inflammation and gliosis in the injured spinal cord and therefore ameliorate secondary injury enhancing preservation of host neuronal and glial cells. Cell therapy also restores lost tissue through replacement of damaged neuronal and glial cells and restoration of neuronal circuitry. Beneficial effects of cell therapy in SCI also include remyelination of spared axons, increased expression of beneficial neurotrophins/cytokines by transplanted or host cells, and stimulation of endogenous angiogenesis and neurogenesis. Importantly, transplanted cells create an environment favorable for plasticity and axonal regeneration (Mothe and Tator, 2012; 2013).

Various cell types have been tested for transplantation into the injured spinal cord, including olfactory ensheathing cells, Schwann cells, mesenchymal stem cells, oligodendrocyte and neural progenitor cells (reviewed in Sahni and Kessler, 2010; Fehlings and Vawda, 2011; Tetzlaff et al., 2011; Wright et al., 2011; Mothe and Tator, 2012, 2013; Vawda and Fehlings, 2013; Dasari et al., 2014; Roet and Verhaagen, 2014; Silva et al., 2014).

Olfactory ensheathing cells

Olfactory ensheathing cells (OECs) are a specialized type of glia that exist exclusively within the olfactory system in the olfactory nerves and olfactory nerve layer of the olfactory bulb. These unique cells throughout the whole life of adult mammals actively support regeneration of primary olfactory axons from the olflactory mucosa in periphery into the olflactory balb in CNS (Doucette, 1991; Boyd et al., 2005; Mackay-Sim and St John, 2011; Roet and Verhaagen, 2014) and therefore represent cells potentially capable of creating a microenvironment favorable for axonal regeneration across the injury site.

For clinical application, the OECs can be isolated through nasal biopsies (Feron et al., 1998; Bianco et al., 2004) and enough cells for autologous transplantation can be obtained

by culturing the cells for 4–6 weeks (Feron et al., 2005). Due to the time required to generate and culture the autologous cells their use in the acute stages of SCI is not possible (Feron et al., 2005; Rubio et al., 2008).

In 1990s, Ramón-Cueto and Nieto-Sampedro were the first to report the use of OECs to repair the injured rat spinal cord (Ramón-Cueto and Nieto-Sampedro, 1994). Since this pioneering study, many research groups have reported improved functional recovery after OECs transplantation into a wide range of SCI models, including transections and contusions, and several mechanisms underlying functional recovery after OECs transplantation have been identified (Li et al., 1997; Ramón-Cueto et al., 2000; Lu et al., 2002; Plant et al., 2003; Ramer et al., 2004; López-Vales et al., 2006; Muñoz-Quiles et al., 2009; Ziegler et al., 2011; Granger et al., 2012). Probably the main one is the promotion of axonal outgrowth and the creation of a permissive pathway for axonal growth (Gladwin and Choi, 2013). Other mechanisms include increased tissue sparing, reduction of astrogliosis and modulation of inflammation, remyelination and angiogenesis. In addition, the OECs produce and release neurotrophic factors, such as NGF and BDNF, and recruit Schwann cells to the injury site (Gladwin and Choi, 2013; Roet and Verhaagen, 2014; Silva et al., 2014).

Despite numerous successful reports of regeneration with OEC transplantation, negative studies also exist (Takami et al., 2002a; Riddell et al., 2004; Barakat et al., 2005; Steward et al., 2006; Pearse et al., 2007). The reasons for this are not very clear but may include variability of the cell sources, cell culture conditions or surgical techniques (Riddell et al., 2004; Tetzlaff et al., 2011; Gladwin and Choi, 2013).

Nevertheless, the potential of the OECs has led to their use in clinical trials. A phase I/IIa clinical study was initiated in Australia to examine the effects of autologous transplantation of human OECs for treatment of chronic SCI. This study included 6 patients with complete thoracic paraplegia. After three years no adverse effects were detected, however, no significant functional improvement was either reported (Feron et al., 2005; Mackay-Sim et al., 2008). More encouraging are the results of the Phase I clinical trial recently completed in Poland (Tabakow et al., 2013). At one year after OECs transplantation into three patients suffering from chronic thoracic paraplegia, neurological improvement was observed in transplant recipients, but not in non-operated controls. Importantly, no adverse effects related to transplantation of OECs at one year after surgery have been reported. Safety and possible efficacy of OECs transplantation were also shown in six patients with complete chronic spinal cord injuries which received fetal OECs transplants in China (Rao et al., 2013). These results show that implantation of OECs in humans appears safe and well-tolerated, however, to confirm the beneficial effect of the therapy future clinical trials should include larger numbers of patients (Roet and Verhaagen, 2014).

Schwann cells

Schwann cells (SCs) are the myelin-forming cells of the peripheral nervous system. Following SCI endogenous SCs are known to migrate from the periphery into the injury site and remyelinate the spared axons (Beattie et al., 1997; Oudega and Xu, 2006; Fehlings and Vawda, 2011). In addition, they provide injured axons with a permissive microenvironment for regeneration producing extracellular matrix molecules, such as laminin, fibronectin and collagen, which are known to support axon growth, and releasing of a variety of growth factors, such as NGF, BDNF, NT3, CNTF, GDNF and FGF. SCs also express on their surface cell adhesion molecules that support axon growth (reviewed in Oudega and Xu, 2006; Silva et al., 2014).

Transplantation of SCs for SCI repair is promising as it is possible to obtain them from an autologous source and these cells are not tumorigenic (Fehlings and Vawda, 2011). Although several studies have demonstrated the ability of transplanted SCs to promote axonal regeneration and remyelination in the injured spinal cord, which resulted in functional improvement (Takami et al., 2002a; Barakat et al., 2005; Firouzi et al., 2006; Schaal et al., 2007), it is generally thought that SCs alone are not sufficient to promote

regeneration of corticospinal tract axons. Because of this, SCs are often used in combination with other treatments, such as neuroprotective agents or other cell substrates (Fehlings and Vawda, 2011; Tetzlaff et al., 2011). For autologous SCs transplantation, the sacrificing of peripheral nerve is required. To overcome this limitation, SCs have been derived from skin or bone marrow. When transplanted into contused or transected spinal cord these treatments lead to functional improvement in preclinical models (Kamada et al., 2005; Biernaskie et al., 2007; Novikova et al., 2011). However, the time required for generation of amount of cells sufficient for transplantation is another limitation of this autologous approach for the acute SCI treatment (Tetzlaff et al., 2011).

Despite the lack of preclinical data a clinical trial utilizing SCs has been completed in Iran. In this study autologous SCs were transplanted into 33 patients with chronic thoracic and cervical SCI. After 2 years of follow-up no adverse effects were reported and neurological improvements have been observed in some of patients in terms of motor, sensory, and sphincter abilities (Saberi et al., 2008, 2011). Recently, a phase I clinical trial has been initiated in the USA which aims to assess the safety of autologous human SCs in subacute SCI patients (NCT01739023).

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent, tissue-specific stem cells that can be obtained from multiple easily accessible sources such as bone marrow, umbilical cord blood, Wharton's jelly, the placenta, dental pulp and adipose tissue (reviewed in Vawda and Fehlings, 2013; Dasari et al., 2014). The possibility to avoid the immunological and ethical problems together with low tumorigenesis of the transplanted cells make MSCs attractive for different regenerative applications including SCI. Importantly, transplanted MSCs display significant anti-inflammatory, antiapoptotic and immunosuppressive features, which might be useful for SCI repair (Uccelli et al., 2011; Vawda and Fehlings, 2013; Dasari et al., 2014; Silva et al., 2014).

The bone marrow derived MSCs (bmMSCs) are probably the most widely used stem cells in SCI experiments (Tetzlaff et al., 2011; Vawda and Fehlings, 2013; Silva et al., 2014). Although their neurogenic differentiation potential is controversial (Brazelton et al., 2000; Mezey et al., 2000; Woodbury et al., 2000; Castro et al., 2002; Lu et al., 2004), bmMSCs were successfully used in a number of preclinical studies utilizing rodent, porcine, canine and nonhuman primate SCI models (Cízková et al., 2006; Deng et al., 2006; Zurita and Vaquero, 2006; Lu et al., 2007; Zurita et al., 2008; Shin et al., 2013; Penha et al., 2014). The beneficial effect of bmMSCs transplantations mostly has been reported after intraspinal delivery, but has also been observed after intrathecal (Ohta et al., 2004; Urdzíková et al., 2014) or even intravenous transplantation (Urdzíková et al., 2006; Shin et al., 2013). However, some studies have reported no functional benefits from bmMSCs transplantation (Yano et al., 2006; Yoshihara et al., 2006; Sheth et al., 2008) or have detected highly variable outcomes (Neuhuber et al., 2005; Himes et al., 2006).

The exact mechanisms by which bmMSCs are able to provide neuroprotection and improve functional recovery are still debated. However, they apparently include secretion of trophic factors, modulation of inflammation, recruitment of endogenous stem and Schwann cells leading to reduced cell death, and increased tissue sparing (Tetzlaff et al., 2011). Interestingly, in a recent study bmMSCs transplanted in an acute SCI rat model modified the inflammatory environment in the lesion by macrophages polarization from M1 to M2 phenotype and therefore provided an environment favorable for axonal growth and functional recovery (Nakajima et al., 2012).

The relatively convincing preclinical data as well as the easy access to bmMSCs for autotranplantations explain their use in several clinical trials. However, these studies have enrolled small numbers of patients and mostly have used autologous BM-derived cells instead of purified stromal cells. Despite the fact that transplanted cells were safe and even provided modest improvement (Sykova et al., 2006; Yoon et al., 2007; Karamouzian et al.,

2012; Dai et al., 2013; Jiang PC et al., 2013) long-term and large scale multicenter clinical studies are required to determine the precise effect of MSCs therapy. Several clinical trials are currently active or recruiting patients in order to analyze the safety and efficacy of autologous MSCs transplantation in patients with SCI (NCT01694927, NCT01446640, NCT01676441 and NCT02152657).

MSCs from umbilical cord gelatinous matrix, known as Wharton's jelly reduce microgliosis and astrogliosis, and promote axonal regeneration associated with functional recovery when transplanted into a rat model of spinal cord transection (Yang et al., 2008). A similar beneficial outcome was reported after transplantation of MSCs derived from umbilical cord blood (Park et al., 2012; Cui et al., 2014), adipose tissue (Zhang et al., 2009; Zhou et al., 2013; Menezes et al., 2014) and dental pulp (de Almeida et al., 2011; Sakai et al., 2012). However, more preclinical data are required in order to move these types of cells to clinical trials (Silva et al., 2014).

Neural stem/progenitor cells

Neural stem/progenitor cells (NS/PCs) are multipotent cells that self-renew, readily expand *in vitro* and are able to differentiate into neurons, oligodendrocytes, and astrocytes. They can be obtained from adult and fetal brains/spinal cords, pluripotent ESCs or iPSCs (Figure 4). Transplantation of NS/PCs in SCI settings aims at the replacement of lost cells, as well as provision of trophic support to the survived tissues (Gage, 2000; Fehlings and Vawda, 2011; Mothe and Tator, 2013; Nakamura and Okano, 2013; Puttonen et al., 2013; Silva et al., 2014). The great advantage of NS/PCs for transplantation is that unlike other types of stem cells they are inherently specified along the neural lineage (Mothe and Tator, 2013).

Adult NS/PCs are typically harvested from the subventricular zone of the brain or ependymal region of rodent spinal cord, and then expanded as neurospheres or as an adherent monolayer prior to transplantation (Fehlings and Vawda, 2011; Mothe et al., 2011; Mothe and Tator, 2013; Faulkner et al., 2014). When transplanted into rat or mouse spinal cord, adult-derived rodent NS/PCs have been shown to promote functional recovery in several studies (Hofstetter et al., 2005; Karimi-Abdolrezaee et al., 2006; Parr et al., 2008; Bottai et al., 2008; Moreno-Manzano et al., 2009; Cusimano et al., 2012; Wilcox et al., 2014). Grafted NS/PCs mainly developed an oligodendroglial phenotype, while the expression of neuronal markers was generally rare (Vroemen et al., 2003; Karimi-Abdolrezaee et al., 2006; Parr et al., 2007, 2008; Wilcox et al., 2014). Recently, human NS/PCs isolated from the adult spinal cord of organ transplant donors have been shown to differentiate to both glia and neurons following transplantation into rats with SCI, although no behavioral data has been reported (Mothe et al., 2011). The key advantages of adult-derived NS/PCs are the lack of tumorigenicity and ethical controversy. However, the very limited neuronal differentiation using the current protocols is a potential problem. In addition, autologous derivation of these cells is almost unfeasible in clinical settings (Fehlings and Vawda, 2011; Faulkner et al., 2014).

Several studies have shown that NS/PCs generated from mouse ESCs and rat fetal stem cells (FSCs) give rise to neurons, astrocytes and oligodendrocytes, form functional synapses with host neurons, and promote functional recovery when transplanted into an injured rodent spinal cord (McDonald et al., 1999; Ogawa et al., 2002; Okada et al., 2005). Human fetal forebrain-derived NS/PCs transplanted into immunodeficient mice with subacute (Cummings et al., 2005,2006; Hooshmand et al., 2009) or chronic SCI (Salazar et al., 2010) similarly integrated into the injured spinal cord, provided remyelination and improved functional recovery. Importantly, when subacutely transplanted into a non-human primate SCI model, these cells exhibit trilineage neural differentiation and also improve functional recovery (Iwanami et al., 2005).

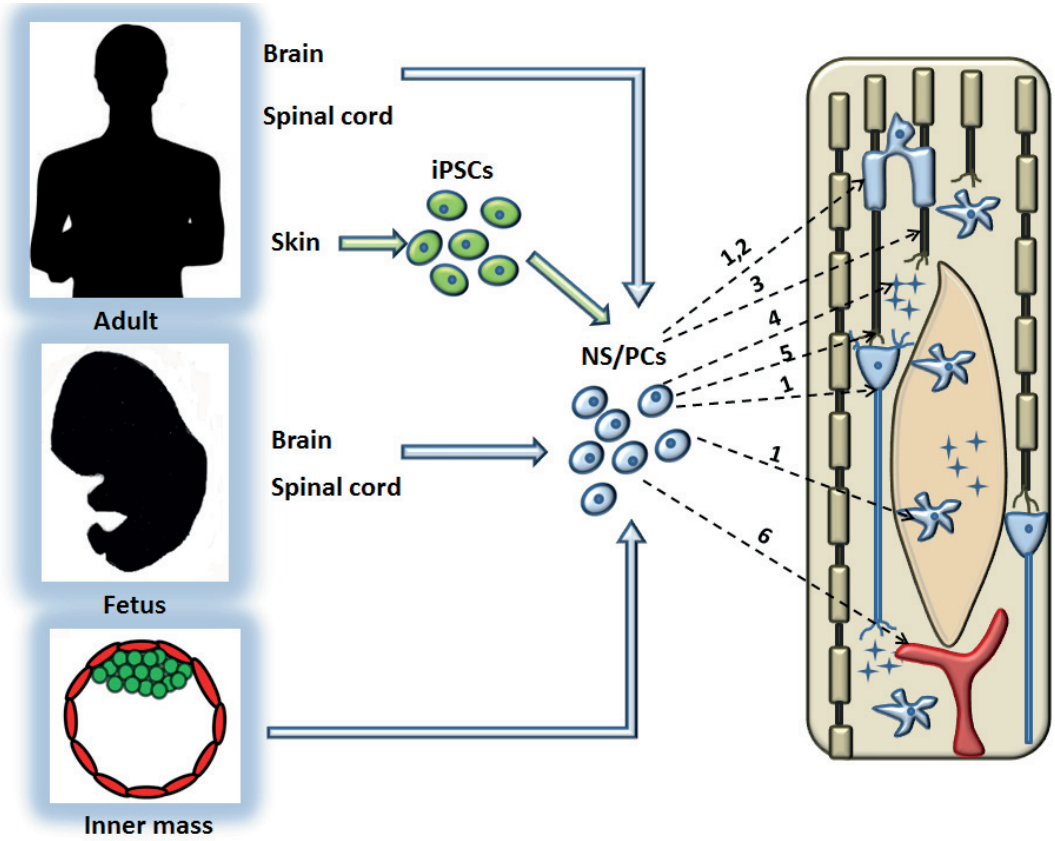

Figure 4. Sources of NS/PCs for transplantation after SCI and their proposed effects in the injured spinal cord. 1 – cell replacement, 2 – remyelination, 3 - promoting axonal sprouting, 4 – secretion of neurotrophic factors, 5 – synapse formation, 6 – angiogenesis (modified from Mothe and Tator, 2012).

In addition to ESCs-NS/PCs, oligodendrocyte progenitor cells derived from ESCs (ESC-OPCs) remyelinate spared axons and promote functional recovery after transplantation into injured rat spinal cord (Nistor et al., 2005; Sharp et al., 2010). These promising results have led into a phase I clinical trial, which was initiated by Geron in 2009 and was the first approved clinical trial that utilized ESC-derived cells. However, the trial was discontinued in 2011 for financial reasons. Currently, one phase I/II clinical trial evaluating the safety and therapeutic potential of human fetal central nervous system stem cells (HuCNS-SC) in patients with thoracic SCI is ongoing (NCT01321333) and two more are recruiting patients (NCT01772810, NCT01725880). Moreover, in 2014 another clinical trial was initiated, which will evaluate the safety and efficacy of HuCNS-SC transplantation into patients with cervical SCI (NCT02163876).

Despite the fact that preclinical studies overall show the beneficial potential and safety of ESCs/FSCs-derived progenitors for transplantations in SCI settings one of the potential concerns with transplantation of the cells from these sources is the possibility of tumorigenesis due to incomplete differentiation of pluripotent cells. In addition, the clinical applications of ESC/FSCs-derived NS/PCs are limited by ethical and immunological (allogeneic nature) reasons (Mothe and Tator, 2013). These limitations might be solved by using induced pluripotent cells (iPSCs) which are generated by reprogramming a specialized adult cells to an ESCs-like state (Tsuji et al., 2010; Fehlings and Vawda, 2011; Kobayashi et al., 2012; Kramer et al., 2013; Nakamura and Okano, 2013; Matsui et al., 2014). Reprogramming mouse fibroblasts by retroviral introduction of the transcription factors

Oct4, Sox2, c-Myc, and Klf4 was initially performed by Takahashi and Yamanaka in 2006 (Takahashi and Yamanaka, 2006). In 2007, the first human iPSCs were generated using either the original transcription factors (Takashi et al., 2007) or a combination of Oct4, Sox2, Nanog, and Lin28 (Yu et al., 2007). Since then enormous progress has been made in iPSCs research including the new, improved methods of iPSCs generation (Okano et al., 2013). iPSCs share many properties with ESCs. They express stem cell markers, are capable of forming teratomas containing cell types from all three germ layers and have the potential to differentiate into every cell type of the human body (Patel and Yang, 2010; Kramer et al., 2013; Nakamura and Okano, 2013; Okano et al., 2013). However, in contrast to ESCs, patient specific iPSCs do not have any ethical limitations. Importantly, being obtained from an autologous source, the iPSCs theoretically do not require immunosuppression, in contrast to allogeneic ESCs. Since SCI patients are prone to opportunistic infections (Nash et al., 2000) immunosuppressive therapy would compromise their resistance to infections and interfere with healing processes (Nutt et al., 2013). Therefore, the possibility to avoid immunosuppressive treatments after transplantation is doubtlessly an advantage of iPSC over ESCs. Although iPSCs also have some potential for tumorigenesis, the development of non-integrating reprogramming methods substantially reduces this risk (Gonzalez et al., 2011).

Although preclinical studies evaluating iPSC-derived NS/PCs in SCI settings are scarce, the existing preliminary results look very promising. Mouse (Tsuji et al., 2010) and human (Nori et al., 2011; Fujimoto et al., 2012) iPSC-derived NS/PCs delivered subacutely into mouse contused spinal cord differentiate into trineural lineage, expresse neurotrophic factors, stimulate angiogenesis and axonal regrowth, improve remyelination, form synapses with host neurons and improve functional recovery. Recently, the beneficial outcome of iPSC-derived NSCs transplantations was also demonstrated in the model of spinal cord transection (Lu et al., 2014). Human iPSC–derived NS/PCs were further evaluated in a non-human primate model of cervical injury. Subacutely intraspinally transplanted cells enhance axonal sparing/regrowth and angiogenesis, prevent demyelination and promote functional recovery after SCI (Kobayashi et al., 2012). However, studies showing negative outcome of iPSC therapy also exist. Undifferentiated human iPSCs transplanted subacutely into nude rats failed to provide any benefits by 5 weeks after SCI compared to human fibroblast controls (Kramer et al., 2013), whereas in another study caudalized human iPSC-derived NS/PCs did not improve functional recovery in an early chronic SCI model (Nutt et al., 2013).

Of note is the fact that most studies evaluating the efficacy of iPSC-derived cells for SCI transplantations have utilized immunodeficient rodents to avoid the host immune response. However, due to the long time required for generation of appropriately characterized and safe autologous iPSC lines, the first clinical trial with iPSCs at least in acute or subacute SCI would still require allogeneic cells and therefore immunosuppressive therapy (Fehlings and Vawda, 2011; Faulkner et al., 2014). Therefore, more preclinical studies, especially those including an immunosuppressive regimen, are required before iPSC-based clinical trials can be initiated.

3 Aims of the study

Direct mechanical trauma to the spinal cord causes primary damage, which is followed by a wave of complex progressive biochemical and cellular processes that make up the secondary injury. Several pathophysiological mechanisms including ionic balance disturbances, excitoxicity, oxidative stress and inflammation participate in the secondary injury cascade and greatly exacerbate neurological deficits and complicate recovery. In spite of almost 4000 years of history, treatment of SCI remains largely palliative. Deep understanding of mechanisms involved in the secondary injury cascade as well as the search for novel strategies efficiently targeting the secondary injury mechanisms are some of the main goals in SCI preclinical research.

The transcription factor Nrf2 is a unique ''master regulator'' of the antioxidant, detoxification, and cell defence response and extremely important for protection from oxidative stress. IL-33 is a novel cytokine possessing high potential to beneficially modify the CNS microenvironment in inflammatory settings. The impact of Nrf2 in protection from secondary injury requires further assessment, whereas IL-33 is a new player in the SCI field. Induced pluripotent cells are already at the threshold of translation into humans, however, available preclinical studies in SCI are scarce and results are controversial.

This study was carried out to exploit three novel preclinical strategies to SCI treatment: augmenting intrinsic protection against oxidative stress with Nrf2 gene transfer, IL-33-mediated modulation of inflammation, and adult stem cell therapy. Furthermore, it aims to increase our knowledge of Nrf2 and IL-33, both involved in key mechanisms of secondary injury, namely oxidative stress and inflammation.

The specific aims were the following:

1. Assess the function of the Nrf2-ARE pathway in the injured spinal cord and exploit the possibility to improve recovery following SCI by Nrf2 gene transfer into mouse contused spinal cord.

2. Study the cellular localization and expression of intracellular IL-33 in the injured spinal cord, evaluate the efficacy of recombinant IL-33 treatment for SCI recovery, and to clarify the mechanisms of recombinant IL-33 action in a mouse contusion SCI model.

3. Estimate the therapeutic potential of neural progenitors derived from human iPSCs for contusion SCI recovery when continuous pharmacological immunosuppression is used.

4 Materials and methods

4.1 ANIMALS (I - III)

All experimental procedures were approved by the Animal Experiment Committee in State Provincial Office of Southern Finland and carried out according to the national regulation of the usage and welfare of laboratory animals.

All mice used throughout these studies were at the age of 2-4 months and weighing 18-23 g at the time of surgery.

For studies I – III, female C57BL/6J mice from the National Laboratory Animal Center (NLAC), University of Eastern Finland, Kuopio, Finland were used. For study I, female mice lacking the Nrf2 gene ($Nrf2^{-/-}$) (Itoh et al., 1997) and their age- and weight-matched wild-type (WT) littermates in C57BL/6J background were also bred in the NLAC. $Nrf2^{-/-}$ mice and their WT littermates were randomly assigned to experimental groups subjected to SCI or only laminectomy as described below. The genotypes of the mice were determined by polymerase chain reaction (PCR).

The mice were housed in groups of three in cages under 12-hour light/dark cycle in temperature control environment with drinking water and standard rodent chow provided *ad libitum*. In order to provide easy access to food and water for the paralyzed mice, drinking water and powdered rodent chow were placed on the bottom of the cages for the first week after surgery.

4.2 ANESTHESIA (I - III)

For SCI induction, the mice were anesthetized with isoflurane (Baxter, Deerfield, IL) in a carrier gas mixture of 30% O_2, 70% N_2O (initial dose of 5% which was reduced to 1–1.5% for maintaince of surgical depth anesthesia) delivered through a nose mask during the surgery. In study III, for the cell transplantations the same anesthesia protocol was used.

For magnetic resonance imaging (MRI), anesthesia was induced with 5% isoflurane (Baxter) in 30% O_2/ 70% N_2O and maintained at 1% isoflurane during imaging.

For transcardiac perfusions, mice were terminally anesthetized (ip.) with an overdose of tribromethanol (Avertin, Sigma-Aldrich, St.Louis, MO).

4.3 INDUCTION OF SPINAL CORD CONTUSION INJURY (I - III)

4.3.1 Spinal cord contusion injury model

Midline incision of skin was done at the T8 -T12 level. Laminas and lateral processes of T9-T11 were cleared of muscles. The T10 lamina was carefully removed with small scissors and dura mater was exposed. Vertebraes adjacent to the laminectomy window were immobilized with Adson forceps and clinically relevant moderate contusion SCI was performed at the T10 level using the Infinite Horizons Spinal Cord Impactor (Precision Scientific Instrumentation, Lexington, KY) with a force of 60 kdyn. The special mouse tip with a diameter of 1.25 mm was aligned over the exposed spinal cord with intact dura, so as to be centered from right to left in order to produce a consistent bilateral injury. Several parameters including the actual impact force and spinal cord tissue displacement were recorded for each animal. Immediately after impact, the muscles were sutured and skin was

closed with wound clips. At the time of surgery the mouse body temperature was maintained at a constant level of 37 ± 1 °C with a controlled heating blanket.

In study I, the dura mater was exposed in a similar manner as above and moderate thoracic contusion injury was performed at the T10 level using the NYU Impactor (W.M. Keck Center for Collaborative Neuroscience) by dropping the 5.6 g impact head rod from a distance of 6.25 cm on exposed dura. The mice injured with NYU Impactor were used only in the experiments assessing time-course expression of the Nrf2-ARE pathway. In all other experiments throughout the studies I-III Infinite Horizons Impactor was used.

Laminectomized mice without impact served as sham controls.

In study II, the majority of the surgeries were performed by Iurii Kidin, MD at the A.I. Virtanen Insitute for Molecular Sciences, University of Eastern Finland, Finland.

4.3.2 Postoperative care procedures

Immediately after wound closing, 1ml of 0.9% NaCl was injected subcutaneously (s.c.) for compensation of blood lost during surgery. After SCI, the mice are unable to maintain their body temperature (Wells et al., 2003) and therefore to keep it at the constant level in the early postoperative period the mice were housed in cages placed on heating pads at 37°C for three days after surgery. For analgesia, buprenorfine (Temgesic®, Schering-Plough, Belgium) at 0.1 mg/kg was injected subcutaneously 30 min before surgery followed by the same dose every 12 hours for 3 days. Bladders were manually evacuated 2 times daily for approximately 2 weeks until mice were able to regain normal bladder function.

4.4 LENTIVIRUS-MEDIATED GENE TRANSFER (I)

4.4.1 Viral vectors

The viral vectors used in this study were produced by the laboratory of Professor Seppo Ylä-Herttuala at the A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Finland.

Lentiviral vectors expressing enhanced green fluorescent protein (LV-GFP) and human Nrf2 (LV-hNrf2) under the control of human phosphoglycerate kinase promoter (hPGK) were cloned as described in Kanninen et al., 2009. The lentiviruses were produced and titrated as described in Follenzi and Naldini, 2002. The lentiviral vectors encoding GFP were used as a control. Prior to intraspinal delivery, the lentiviral vectors were diluted with sterile phosphate buffered saline (PBS, Life Technologies) to achieve a titer of 1.88×10^6 TU/µl.

4.4.2 Intraspinal gene transfer

To deliver viral vectors into the injured spinal cord tissue, two stereotaxical injections were performed for each mouse. Immediately after impact (or laminectomy for sham controls) the dura matter was incised 0.5 mm from midline at both the rostral (left to the midline) and caudal (right to the midline) ends of the laminectomy window and a 33-gauge needle attached to a 5-µl Hamilton syringe was lowered 1.3 mm beneath the dorsal surface of the spinal cord. Two or three microliters (in the case of experiment with the increased amount of viral vector) of PBS, LV-GFP or LV-hNrf2 were delivered into the spinal cord tissue at a rate of 0.5 µl/min using the injecting minipump (Nanomite Injector Syringe Pump; Harvard Apparatus, Holliston, MA). 7 min after injection the needle was slowly raised and the wound was sutured as described above.

4.5 IL-33 TREATMENT (II)

The mice were randomly divided to receive IL-33 or vehicle. In the IL-33 treatment group, the mice received i.p 1 μg of IL-33 immediately after wound closing, followed by the same dose at 3 days post injury (dpi) and 0.5 μg at 7 and 10 dpi. Control and sham mice were injected similarly with 0.0025% bovine serum albumin (BSA) in PBS as a vehicle. Prior to administration, recombinant mouse IL-33 (Mouse IL-33 protein, Biorbyt, San Francisco, CA) was diluted in 0.0025% BSA in sterile PBS to achieve 5 μg/ml concentration. The IL-33 dose was selected based on a previous mouse study for treatment of atherosclerosis in $ApoE^{-/-}$ mice (Miller et al., 2008).

4.6 CELL TRANSPLANTATIONS (III)

Generation and differentiation of iPSC-derived neural progenitor cells (iPSC-NPCs) intended for transplantation studies were performed by the Stem Cell Unit, at the A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Finland.

4.6.1 Generation and differentiation of human iPSCs

Primary fibroblasts were obtained from a skin biopsy of a healthy human adult female volunteer (approved by the committee on Research Ethics of Northern Savo Hospital district). Cre-excisable constitutive polycistronic (Oct4, Klf4, Sox2 and c-Myc) lentivirus reprogramming kit (STEMCCA, Millipore, Temecula, CA, USA) was used to induce pluripotency and cell transduction and characterizations were performed as described in Qu et al., 2013.

The generated iPSCs line (UEFhfiPS 1.4) was maintained on the top of mitotically inactivated human foreskin fibroblasts (CRL-2429, ATCC, Manassas, VA). Neural differentiation was performed in a suspension of cell spheres (Puttonen et al., 2013).

The success of the neuralization process was assessed with a selected panel of pluripotency and neural markers. To confirm neural differentiation, mRNA expression of paired box protein 6 (PAX-6), doublecortin (DCX) and microtube-associated protein-2 (MAP-2) were assessed by quantitative real-time polymerase chain reaction (RT-PCR). The last two were also detected at protein level by immunocytochemistry (ICC). To exclude the presence of pluripotency, mRNA expression of Nanog was detected by RT-PCR. Octamer-binding transcription factor (Oct-4) protein expression was investigated by ICC. RT-PCR and ICC methodology were described earlier in detail in Puttonen et al., 2013. Analysis of disappearing pluripotency and development of neuronal properties was performed every 2 weeks during the differentiation period. As the result, expression of PAX-6, DCX and MAP-2 mRNA increased over time (III, Figure 2D - F) and prior to transplantation most of the NPCs were positive for DCX and MAP-2 (III, Figure 2A, B), thus proving that neuronal differentiation had been achieved. Meanwhile, the reduction of Nanog expression at the mRNA level (III, Figure 2C) and Oct-4 by ICC confirmed the disappearance of pluripotency in differentiated NPCs.

Seven to nine weeks after initiation of the differentiation small and homogenous spheres were collected and dissociated. Isolated, single NPCs were seeded on polyornithine - human laminin coated wells in neural proliferation medium (Puttonen et al., 2013). Two days later, these monolayers were transduced with lentiviruses encoding GFP at multiplicity of infection 5 for 18 hours to allow in vivo tracking. Just prior to the transplantation (at days 5-8 post-transduction) GFP-positive NPCs were collected and suspended into PBS (Life Technologies) at a concentration of 100 000 cells/μl.

4.6.2 Cell transplantation procedure

Human UEFhfiPS 1.4 - derived NPCs (UEFhfiPS1.4-NPCs) expressing GFP were intraspinally transplanted either into uninjured laminectomized mice immediately after T10 lamina removal or into injured mice 7 days after contusion. In the latter case, the injury site was re-exposed to provide access to the laminectomy window. Two small dura incisions were made 0.5 mm from midline rostrally (left to midline) and caudally (right to midline) to the lesion epicenter (III, Fig. 1A) and a total of two injections were stereotaxically performed for each mouse using a 5 µl Hamilton syringe and the Nanomite Injector Syringe Pump (Harvard Apparatus). A 33-gauge needle was lowered 1.3 mm beneath the dorsal surface of the spinal cord and 2 µl of PBS containing 200 000 NS/PCs were injected at a rate of 0.5 µl/min. The needle was left in situ for 7 min after injection before being slowly raised, and the wound was sutured. 2 µl of PBS were intraspinally injected into control mice with a similar technique.

4.7 IMMUNOSUPPRESSIVE THERAPY (III)

For immunosuppressive therapy, the transplanted and control mice received daily i.p. injections of Tacrolimus (2 mg/kg, Prograf^{R}, Astellas Pharma a/s, Glostrup, Denmark) until the end of the study.

The dose of Tacrolimus was selected based on several transplantation studies involving rodents or minipigs in which this immunosuppressant was administered i.p or s.c. at a dose range of 0.05 - 3 mg/kg/24h (Marsala et al., 2004; Cizkova et al., 2007; Usvald et al., 2010; Sevc et al., 2013). Additionally, prior to transplantation into SCI mice, we confirmed the efficacy of the selected immunosuppression regimen in other projects involving transplantations of human NPCs into the brains of uninjured mice, transgenic APdE9 mice or mice subjected to permanent focal brain ischemia. A high number of grafted cells were consistently detected in the brains of transplanted mice (III, Fig. 1B) at least 5-12 weeks after grafting, thus indicating that daily intraperitoneal injections of Tacrolimus at a dose of 2-3 mg/kg are sufficient to provide immunosuppression in the CNS under basal and pathologic conditions.

4.8 ANALYSIS OF FUNCTIONAL RECOVERY (I-III)

4.8.1 Basso Mouse Scale open field test (I-III)

The motor function recovery of hind limbs was evaluated using Basso Mouse Scale (BMS) (Basso et al., 2006) in a large Plexiglas field (diameter 1.5 meters) by two independent raters who were blinded to the experimental conditions. Each mouse was observed separately for 4 min in every session. BMS scores for left and right hind limbs were averaged to obtain a single value per mouse. The behavioral analysis was performed 24 h after injury, and then weekly for up to 28 dpi in study I and up to 42 dpi in studies II and III. Prior to testing, the mice were acclimated to the open field during three consecutive days. The mice with a BMS score higher than one at 24 h after injury were considered as outliers and were excluded from further studies.

The BMS is a sensitive and reliable scale specially developed and validated for assessment of the degree of hind limb locomotor recovery in mice after contusion SCI. It ranges from 0 (no ankle movement) to 9 (complete functional recovery) points and includes the evaluation of ankle movement at the early phase of recovery, plantar placement of the paw, weight support, dorsal and plantar stepping at the intermediate phase of recovery and coordination, paw position and trunk stability at the late stage of recovery (Basso et al., 2006).

4.8.2 CatWalk automated gait analysis (III)

In study III, the precise movements of the hind limbs were analyzed at 42 dpi with the CatWalk automated gait analysis system (Noldus, Wageningen, The Netherlands; software 7.1). The detailed technical description of the system can be found in Hamers et al., 2001 and Hamers et al., 2006.

All data acquisition was performed in a dark and silent room. Prior to the testing day, the mice were trained during three consecutive days to run without interruption along the runway towards the end zone. Since the CatWalk system requires that mice are able to run along the walkway with a constant speed only animals that showed at least occasional plantar stepping in the open field (BMS score ≥4) were included in the automatic gait evaluation. On the day of testing each mouse was placed in front of the start zone of the CatWalk runway and footprints of the animal crossing a glass walkway were recorded by the video camera positioned under the walkway. Data was collected from the three uninterrupted runs per animal. Several gait parameters such as base of support, intensity of the paws, print length, width and area, stand and swing speed and duration, max area and contact, stride length and regularity index (RI) were analyzed with the CatWalk software. The values for every paw were taken and averages between left and right hind paws were used for statistical analysis.

4.9 MAGNETIC RESONANCE IMAGING (MRI) (I-III)

In order to visualize the contused mouse spinal cord *in vivo*, MRI was performed two weeks after SCI in studies I and II, whereas in study III imaging was done 3 weeks after contusion. A 9.4 Tesla Varian scanner (Varian Inc, Palo Alto, CA) interfaced to a Varian DirectDrive console was used for imaging. Mice were placed in a custom-built plastic mouse holder providing careful and reproducible positioning of the mouse body allowing sagittal imaging of the spinal cord. The holder was equipped with a warm water heating system to keep the body temperature at a constant level during imaging. The holder was positioned within a volume coil (diameter 35mm) serving as transmitter and receiver. Lesion volumes were determined from T2-prepared fast spin echo images (minimum repetition time 1.5s, echo spacing 7.02ms, four echoes, TE for preparation 30ms, 16 averages, field-of-view 16 x 16 mm^2, slice thickness 0.5 mm, data matrix 128 x 128, 8 adjoining sagittal slices). Imaging data were analyzed using in-house-built Matlab software (Aedes, http://aedes.uef.fi) by an observer who was blinded to the experimental conditions. For each animal the lesion was manually outlined on each slice, areas were summed and converted to micrometers (μm).

In study II, the majority of the of image acquisition were performed by Iurii Kidin, MD at the A.I. Virtanen Insitute for Molecular Sciences, University of Eastern Finland, Finland.

4.10 BLOOD AND TISSUE COLLECTION AND PROCESSING (I-III)

4.10.1 Blood collection and processing (II)

The blood samples were collected from the heart at the time of sacrifice and mixed with 3.8% trisodium citrate (1:10, Sigma) as anticoagulant. Plasma was separated by centrifugation at 2000g for 15 min, and then the upper layer was further centrifuged at 12,000g for 3 min and stored at −70 °C for subsequent cytometric bead array (CBA). Pellets obtained at 8dpi were immediately used for flow cytometry.

4.10.2 Tissue collection and processing (I-III)

At the day of sacrifice, the mice were transcardially perfused with heparinized (2500 IU/L) (Leo Pharma A/S, Ballerup, Denmark) saline (Baxter) to remove blood from tissues. The spleens were snap-frozen in liquid nitrogen and stored at −70 °C until RNA isolation and CBA, or used immediately for flow cytometry. Collected inguinal, axillary and brachial lymph nodes were immediately processed for flow cytometry. Spinal cords were dissected and either 6-mm (study II) or 10-mm (study I) segments centered on the lesion epicenter (or respective area in shams) were stored at −70 °C until RNA isolation or CBA.

When the aim was to collect spinal cords for immunohistochemistry (IHC), transcardiac perfusion with heparinized saline was followed by perfusion with 50 ml of 4 % paraformaldehyde (PFA) (Sigma-Aldrich). The dissected spinal cords were post-fixed in the same fixative overnight at 4°C. After fixation, spinal cords were cryoprotected in 10% sucrose (VWR International, Leuven, Belgium) for 24 h followed by 20% sucrose (study II) or 30% sucrose (study III) for the next 24 h, or 30% sucrose for 48 h (study I). After cryoprotection, 6-mm (studies II and III) or 10-mm (study I) spinal cords segments centered on the lesion epicenter (or respective area in shams) were dissected and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Zouterwoude, the Netherlands), frozen on either dry ice (studies I, III) or liquid-nitrogen-supercooled isopentane (study II) and stored at −70 °C for transversal 20 μm thick cryostat serial sectioning (Leica Microsystems GmH, Wetzlar Germany).

4.11 CYTOKINE PROTEIN DETECTION (I,II)

Cytokine protein levels in spinal cord (study I), plasma and spleens (study II) were detected by cytometric bead array (CBA), which allows simultaneous determination of a wide range of cytokines in the same sample. The method is based on flow cytometric detection of microbeads with unique fluorescence intensity. Each microbead set is pre-coated with antibodies specific for different cytokines. These capture antibodies are recognized by specific labeled secondary antibodies. The cytokine concentration in the sample is proportional to the secondary antibody signal and is quantified from a calibration curve.

Frozen spinal cords and spleens were homogenized in buffer containing 10 mM Tris–HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 10 % glycerol, 0.1 % Nonidet P-40 and protease inhibitor cocktail (Complete; Roche Applied Science, Mannheim, Germany).

The levels of IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17 and IL-10 proteins in plasma, spleen and spinal cord homogenates were measured by using the Th1/Th2/Th17 kit (BD Biosciences, Franklin Lakes, NJ) according to manufacturer's instructions. Data were acquired using FACSCalibur (BD Biosciences, San Jose, CA) and analyzed by FCAP Array software (Soft Flow Inc., St. Louis Park, MN).

CBA were performed by Taisia Rolova, M.Sc. and Paula Korhonen, M.Sc., at the A.I. Virtanen Insitute for Molecular Sciences, University of Eastern Finland, Finland.

4.12 FLOW CYTOMETRY (II)

Single cell suspensions were prepared from the spleens and lymph nodes by crushing organs through 70-μm and 40-μm cell strainers (BD Biosciences), respectively. The resulting cells were washed in Hank's balanced salt solution (HBSS) (BioWhittaker Lonza, Basel, Switzerland) supplemented with 1% fetal bovine serum (FBS) (Life Technologies), centrifuged for 5 min at 400g, and resuspended in RPMI-1640 medium supplemented with 5% FBS (Sigma–Aldrich). Red blood cells were removed from blood and spleen cell

suspensions by lysis with BD Pharm Lyse (BD Biosciences). After erythrocyte lysis the white blood cells were counted on a Z2 Coulter Counter (3.5–10 μm).

For detection of CD4+ and CD8+ T-cells in blood, lymph nodes and spleen, cells were incubated with CD4-FITC (GK1.5, RM4-5) and CD8-PerCP eFluor710 (53–6.7) antibodies at +4°C for 45 min.

For cytokine enumeration, CD4+ cells were isolated from spleen cell suspension using magnetically labeled CD4 (L3T4) MicroBeads (Miltenyi Biotec, Lund, Sweden) according to manufacturer's instructions. Purity was confirmed (92.3 ± 0.93%) by flow cytometry. Cytokine stimulation was done as described by Foster et al., 2007. Briefly, $1x10^6$ cells were stimulated for 5 h at +37°C in 5% CO2 in the presence of Brefeldin A (10 μg/ml, Sigma–Aldrich), Ionomycin (500 ng/ml, Sigma–Aldrich) and phorbol 12-myristate 13-acetate (PMA) (10 ng/ml, Sigma–Aldrich). After cytokine stimulation the cells were incubated with CD4-FITC (GK1.5). Next, the cells were permeabilized with fix/perm buffer (eBioscience) overnight and next the day incubated with TNF-PE (MP6-XT22) and IFNγ-PE (XMG 1.2) antibodies for 30 min at +4°C.

In order to detect T regulatory Foxp3+ cells (Tregs) in blood and spleen, cells were incubated with CD4-FITC (GK1.5, RM4-5) and CD25-APC (PC61.5) antibodies followed by permeabilization and incubation with Foxp3-PE (FJK-16s) antibody for 80 min at +4°C.

For blocking of Fc receptors, anti-CD16/CD32 (24.G2) antibody was used. All used antibodies were from eBioscience.

Samples were resuspended in 1% FBS in HBSS and acquired on BD FACSCalibur equipped with a single 488 nm argon laser or BD FACSAriaIII equipped with 488 and 633 nm lasers with the standard detector configuration. Post-acquisition data analysis was performed using FCS Express4 (DeNovo, Los Angeles, CA) or Cellquest Pro™ software (BD Biosciences).

Flow cytometry analysis was performed by Sara Wojciechowski and Iurii Kidin, MD, at the A.I. Virtanen Insitute for Molecular Sciences, University of Eastern Finland, Finland.

4.13 HISTOLOGY AND IMMUNOHISTOCHEMISTRY (I-III)

4.13.1 Luxol fast blue staining (I,II)

In order to estimate the total spinal cord area (study I) and degree of white matter sparing (studies I, II) Luxol fast blue staining (LFB) staining for myelin was performed as described in Yune et al., 2007. Transversal spinal cord sections with an interval of 200 μm were incubated in 0.1% LFB (Sigma-Aldrich) in acidified 95% ethanol overnight at +60°C. Differentiation was carried out with 0.05% lithium carbonate (Sigma-Aldrich). The spinal cord sections were imaged with an Olympus AX70 microscope and photographed by a digital camera (Color View 12; SoftImaging Systems). The LFB-positive area was quantified by using ImagePro Plus software (Media Cybernetics, Rockville, MD) in a blinded fashion. In study I, LFB staining was used to determine the epicenter of injury. The spinal cord section containing the lowest percentage of spared white matter was considered as the lesion epicenter.

4.13.2 Immunohistochemical stainings (IHC) (I-III).

Frozen spinal cord sections spacing 200 μm apart were air-dried for 1 hour at room temperature (RT) and rehydrated in PBS, pH 7.4. Permeabilization and antigen retrieval were used when needed. Sections were washed 3 times in PBS with 0.5% Tween (PBST) pH 7.4. Blocking of unspecific antibody binding was performed with 10% normal goat serum (NGS, Chemicon International, Millipore) and sections were incubated with primary antibody prepared in 5% NGS in PBST at RT with slow agitation overnight. On the next day, sections were again washed in PBST before incubation with appropriate Alexa-fluor 488 or 568- conjugated secondary antibody (goat anti-mouse, goat anti-rat, goat anti-rabbit

or rabbit-anti-goat, 1:200, all from Life Technologies) in 5% NGS in PBST at RT for 2 hours. After washing and air-drying, the sections were mounted with Vectashield mounting media with Dapi (Vector Laboratories, INC. Burlingame, CA) or consequently processed for double-staining with compatible antibodies. All primary antibodies used throughout the study are listed in table 2.

For stainings detecting neuronal nuclear antigen (NeuN), GFP and human nuclei (HuNu), M.O.M.™ Mouse Ig Blocking Reagent (Vector Laboratories) was used to block endogenous mouse antibody in the spinal cord sections. Permeabilization with 0.4% Triton X-100 (Sigma-Aldrich) for 30 min was applied for IL-33, HuNu, DCX and Ki-67 stainings. For Arginase-1 staining, antigen retrieval was performed using 0.3% sodium citrate dehydrate aqueous solution (Sigma-Aldrich; pH 6.0) preheated to +92°C. For CD3 staining, after the incubation with the biotinylated secondary antibody (rabbit-anti-rat, 1:500, Vector Laboratories) tyramide-based signal amplification (TSA Biotin System; PerkinElmer, Waltham, MA) was used according to the manufacturer's instruction.

Table 2. List of primary antibodies used for Immunohistochemistry

Antibody	Manufacturer	Source	Dilution	Catalog ID	Study
GFAP	DAKO	Rabbit	1:200	Z0334	I-III
NeuN	Millipore	Mouse	1:200	MAB377	I
CD45	Serotec	Rat	1:100	MCA1388	I
Iba-1	Wako	Rabbit	1:250	019-19741	II, III
CD3	Serotec	Rat	1:5000	MCA5006A	II
Arginase-1	Santa Cruz	Goat	1:200	SC-18351	II
IL-33	R&D Systems	Goat	1:200	AF3626	II
DCX	Cell Signalling	Rabbit	1:200	4604	III
Ki-67	Abcam	Rabbit	1:500	Ab16667	III
Tuj-1	Abcam	Rabbit	1:300	Ab52901	III
Nanog	Abcam	Rabbit	1:200	Ab21624	III
GFP	Life Technologies	Mouse	1:200	A-11120	III
HuNu	Millipore	Mouse	1:250	MAB4383	III

Stained spinal cord sections were visualized using an Olympus AX70 microscope equipped with a digital camera (Color View 12 or F-Fiew; SoftImaging Systems) and the immunoreactive areas or number of cells profiles were quantified using ImagePro Plus (Media Cybernetics) or ImageJ (Wayne Rasband, National Institutes of Health, Betheda, MD) software.

In study II, the degree of tissue sparing was assessed by quantification of the total remaining area covered by glial fibrillary acidic protein (GFAP) as previously described (López-Vales et al., 2005; Klopstein et al., 2012). The spinal cord section with the lowest amount of spared tissue (GFAP sparing) was considered as the lesion epicenter.

Astrogliosis, microglial activation and Arginase-1 were assessed by calculating the immunopositive area which was defined as the area with the intensity of the staining exceeded the threshold set for the background staining. The results were expressed as the percentage of immunopositive area within the selected area of interest. GFAP immunoreactivity (astrogliosis assessment) was evaluated within the whole spinal cord

section at the lesion epicenter and 1 mm rostrally and caudally (in study I) or in $7.5x10^3$ μm^2 area in the lateral white matter at the lesion epicenter, as well as 0.6 and 1.2 mm rostrally and caudally (in study II). Ionized calcium-binding adapter molecule 1 (Iba-1) immunoreactivity (microgliosis assessment) was quantified in $3x10^4$ μm^2 area in the lateral white matter at the lesion epicenter, as well as 0.4 and 0.8 mm rostrally and caudally. Arginase-1 immunoreactivity was quantified within the whole spinal cord section at the lesion epicenter, 0.2, 0.6 and 1 mm rostrally and caudally.

Cells positive for IL-33 were manually counted in the lateral white matter ($3x10^4$ μm^2 area at the lesion epicenter, 0.4 and 0.8 mm rostrally and caudally) or in the ventral horns ($3x10^4$ μm^2 area 0.8 mm rostrally and caudally to the lesion epicenter). Results were expressed as number of cell profiles within the selected area. Cells expressing CD3 were counted in a similar way, but within 1 mm around the lesion epicenter in 5 sections per spinal cord 200 μm apart and expressed as average number of cell profiles per section. For all stainings where quantification was done within the preselected area in the lateral white matter or ventral horns, the images were obtained from the left and right sides of the spinal cord section and results are expressed as averages at the selected distance.

Several stainings were performed to investigate the cellular localization of the protein of interest. Thus, in study I the cellular distribution of lentivirus vector encoding GFP was assessed by the co-localization of GFP with NeuN, GFAP and CD45. In study II, cellular localization of IL-33 and Arginase-1 were examined by double-staining with anti-NeuN, anti-GFAP and anti-Iba-1 antibodies. In study III, stainings detecting HuNu and GFP were performed to track transplanted GFP-positive human cells *in vivo*, whereas stainings detecting Nanog, DCX, Ki-67, GFAP, Iba-1 and neuron-specific class III beta-tubulin (Tuj-1) were used for cell characterization based on co-localization of these markers with GFP. For all co-localization studies, Zeiss LSM 700 confocal microscope (Zeiss Inc., Maple Grove, USA) with an attached digital camera (Color View 12 or F-Fiew; SoftImaging Systems) running Zen 2012 Image analysis Software (Zeiss Inc.) was used. All IHC quantifications were done in a blinded fashion.

4.14 QUANTITATIVE RT-PCR (I,II)

Total RNA was extracted from the spinal cords (study I, II) and spleens (study II) with TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Frozen samples were homogenized in TRIzol reagent with 5 mm stainless steel beeds in TissueLyzer II homogenizator (Qiagen, Hilden, Germany). The final purity and concentration of total RNA were measured with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Five hundred nanograms of total RNA were used for cDNA synthesis using random hexamer primers (Promega, Madison, WI) as a template and Maxima reverse transcriptase (Thermo Fisher Scientific). The relative expression levels of mRNA encoding genes of interest (Table 3) were measured according to manufacturer's protocol by quantitative real-time polymerase chain reaction (qRT-PCR) (StepOnePlus; Life Technologies) using TaqMan chemistry and specific assays-on-demand target mixes (Life Technologies). The expression levels were obtained by normalizing the target gene to ribosomal RNA and are presented as fold change in the expression.

Table 3. List of genes of interest analyzed by RT-PCR

Gene*	Full name	Tissue	Time-point	Study
Nfe2l2	Nuclear factor, erythroid 2-like 2 (Nrf2)	Spinal cord (SC)	12h, 24h, 3, 7, 21, 42 dpi	I
Hmox1	Heme oxygenase (decycling) 1 (HO-1)	SC	12h, 24h, 3, 7, 21, 42 dpi	I
Nqo1	NAD(P)H dehydrogenase, quinone 1 (NQO1)	SC	12h, 24h, 3, 7, 21, 42 dpi	I
Gclc	Glutamate-cysteine ligase, catalytic subunit (GCLC)	SC	12h, 24h, 3, 7, 21, 42 dpi	I
Gclm	Glutamate-cysteine ligase, modifier subunit (GCLM)	SC	12h, 24h, 3, 7, 21, 42 dpi	I
Mgst1	Microsomal glutathione S-transferase 1 (MGST1)	SC	3, 7 dpi	I
Casp3	Caspase 3	SC	3, 7 dpi	I
Bcl2	B-cell CLL/lymphoma 2 (Bcl-2)	SC	3, 7 dpi	I
Bcl2l1	BCL2-like 1 (Bcl-xL)	SC	3, 7 dpi	I
Ngf	Nerve growth factor (beta polypeptide)	SC	3, 7 dpi	I
Gdnf	Glial cell derived neurotrophic factor	SC	3, 7 dpi	I
Bdnf	Brain-derived neurotrophic factor	SC	3, 7 dpi	I
Tnf	Tumor necrosis factor (TNF)	SC, Spleen	24h, 3, 7 dpi 24h	I,II II
Il1b	Interleukin 1, beta (IL-1β)	SC	24h, 3, 7 dpi	I,II
Il6	Interleukin 6 (IL-6)	SC, Spleen	24h, 3, 7 dpi 24h	I,II II
Il10	Interleukin 10 (IL-10)	SC, Spleen	24h, 3, 7 dpi 24h	I,II II
Il4	Interleukin 4 (IL-4)	SC, Spleen	24h	II
Ifng	Interferon, gamma (IFN-γ)	SC, Spleen	24h	II
Il33	Interleukin 33 (IL-33)	SC	24h	II
Cd3d	CD3d molecule, delta (CD3-TCR complex) (CD3)	SC	8 dpi	II
Cd4	CD4 molecule (CD4)	SC	8 dpi	II
Foxp3	Forkhead box P3 (Foxp3)	SC, Spleen	8 dpi, 24h, 8 dpi	II II
Nos2	Nitric oxide synthase 2, inducible (iNOS)	SC, Spleen	24h, 8 dpi 42 dpi	II II
Arg1	Arginase 1	SC, Spleen	24h, 8 dpi 42 dpi	II II
Chi3l3	Chitinase 3-like 3 (Ym-1)	SC	24h, 8 dpi	II

* Gene names are given in accordance with the HUGO Gene Nomenclature Committee (HGNC). http://www.genenames.org;

**For clarity, the full gene names or their abbreviations will be used further in the text.

4.15 STATISTICAL ANALYSES (I-III)

All data are shown as mean ± standard deviation (SD) (study III) or mean ± standard error of mean (SEM) (study I, II). Unpaired t-test was employed for comparing means of two treatment groups. For multiple comparisons, one-way ANOVA followed by Tukey's *post hoc* test or two-way ANOVA followed by Bonferroni's *post hoc* test were used. Behavioral data were analyzed using repeated measures two-way ANOVA followed by Bonferroni's *post hoc* test or non-parametric Mann-Whitney test (for two group comparison) and Kruskal-Wallis test followed by Dunn's *post hoc test* (for multiple comparisons). All statistical analyses were carried out by GraphPad Prism version 5.03 software (GraphPad Software, La Jolla, CA, http://www.graphpad.com). p values of less than 0.05 were considered to be significant.

5 Results

5.1 NRF2 WAS INVOLVED IN SCI PATHOGENESIS AND ITS GENE DELETION AFFECTED THE DEGREE OF SECONDARY DAMAGE AFTER CONTUSION SCI (I)

Oxidative stress is well known to augment the secondary damage after SCI (Hall and Braughler, 1989; Rowland et al., 2008). Upon oxidative stress transcription factor Nrf2 binds to the ARE and induces the transcription of a number of cytoprotective genes in order to compensate the deleterious effect of oxidants and electrophiles (Itoh et al., 1997, 1999; Kobayashi and Yamamoto, 2006; Baird and Dinkova-Kostova, 2011). Knowing the important role of the Nrf2-ARE system in cell defence, in study I, we aimed to determine whether the Nrf2-ARE pathway is involved in SCI pathophysiology. Next, we aimed to estimate the magnitude and duration of the Nrf2-ARE response and assess the effect of Nrf2 gene deletion on several components of secondary injury including inflammation and apoptosis and importantly, functional recovery from mouse contusion thoracic SCI, which is currently thought to be the most clinically relevant thoracic SCI model (Kwon et al., 2010; Zhang et al., 2014)

5.1.1 Contusion SCI induced sustained activation of Nrf2 and HO-1 gene expression

First, we estimated the way that the Nrf2-ARE system responds to the contusion at the thoracic level. Messenger RNA expression of Nrf2 and its main target genes were examined in acute, subchronic and early chronic phases of disease. We detected upregulation of Nrf2 and HO-1 genes (gene names according HUGO Gene Nomenclature Committee are given in table 3) with a synchronous peak at 7dpi (I, Figure 1A, B). Nrf2 upregulation was preceded by HO-1 elevation. Both genes remained elevated for up to at least 6 weeks indicating that after contusion lesions induction of Nrf2-ARE pathway has sustained nature. Expression of another Nrf2 inducible gene, NQO1, slowly increased simultaneously with Nrf2 upregulation and reached statistically significant levels at 42 dpi (I, Figure 1C), whereas no correlation was observed between Nrf2 and GCLM/GCLC expression (I, Figure 1D, E).

5.1.2 Nrf2 gene deletion altered the expression of genes involved in apoptosis, inflammation and neuroprotection in addition to classic Nrf2-regulated genes.

Next, we took advantage of $Nrf2^{-/-}$ mice to estimate whether deletion of the Nrf2 gene results in altered transcription of Nrf2 downstream genes after contusion injury. In addition, genes regulating such components of secondary injury as apoptosis and inflammation were included in the assessment and finally expression of growth factors was also examined. Cytokines were studied at both mRNA and protein levels. Since we observed maximal activation of the Nrf2-ARE from 3 to 7dpi these time-points were selected for gene expression analysis in $Nrf2^{-/-}$ mice.

Contusion injury in Nrf2 deficient mice resulted in impaired expression of important detoxifying enzyme NQO1 (I, Figure 4A,B) and anti-apoptotic Bcl-xL (I, Figure 5B). Both genes were significantly downregulated when compared to WT littermates at 3, 7 dpi (NQO1) and 7 dpi (Bcl-xL), respectively. Moreover, upregulation of NGF, a neurotrophin important for the neuronal growth and survival, was significantly reduced at 7dpi in mice lacking Nrf2 compared to WT (I, Figure 5C). In contrast, the gene expression of pro-inflammatory TNF and protein levels of IL6 were significantly higher in $Nrf2^{-/-}$ (I, Figure 5E,F) at this time-point. Additionally, in Nrf2-deficient mice after contusion injury, we

identified a clear trend toward delayed upregulation of MGST1, another detoxifying enzyme regulated by Nrf2 (I, Figure 4A,B), and GDNF, a growth factor known to promote neuronal survival (I, Figure 5D). A surprising result was that at 7 dpi HO-1 expression was significantly higher in Nrf2-deficient mice when compared with WT (I, Figure 4B), suggesting substantial impact of transcription factors other than Nrf2 in HO-1 regulation upon SCI.

5.1.3 Nrf2 gene deletion increased spinal cord atrophia, demyelination and astrogliosis

To investigate if alterations observed in Nrf2 deficient mice at acute and subacute SCI phases influence the outcome at later stages of recovery, we assessed the degree of spinal cord atrophia and demyelination at 28 dpi with LFB staining. The transverse area of the spinal cord and preserved white matter were significantly reduced in Nrf2-knockouts (I, Figure 3A-C). In addition to increased demyelination at 28 dpi, $Nrf2^{-/-}$ mice exhibited increased astrogliosis at regions caudally to the injury epicenter (I, Figure 3D,E).

5.1.4 Nrf2 gene deletion exacerbated functional recovery

To estimate whether the observed aggravation of the secondary injury in Nrf2-deficient mice was translated to the impairment in functional recovery after contusion SCI, we assessed locomotor function of hind limbs in open field using BMS on a weekly basis. Indeed, already at 7dpi WT mice significantly surpassed Nrf2 knockouts in the hind limb locomotor performance. The observed difference in functional recovery increased over time and at 4 weeks after injury resulted in almost 2-fold functional advancement in WT mice over Nrf2 deficient counterparts (score 4.7±0.6 in WT vs. 2.8±0.2 in $Nrf2^{-/-}$ mice) (I, Figure 2). The observed difference has important biological significance as on average the WT mice were able to perform plantar stepping, whereas mice lacking Nrf2 were able only to perform extensive ankle movements or plantar placement.

Taken together, these findings showed significant and broad involvement of Nrf2 in regulating the secondary injury cascade and indicated that impairment of the Nrf2-ARE response was detrimental for recovery from contusion SCI.

5.2 NRF2 OVEREXPRESSION IN THE SPINAL CORD PREVENTED FUNCTIONAL IMPAIRMENT CAUSED BY LENTIVIRAL TRANSDUCTION BUT FAILED TO PROMOTE FUNCTIONAL RECOVERY FROM SCI

As the protective role of Nrf2 in contusion SCI had been proved, in study I, we further exploited the possibility to enhance the SCI-induced Nrf2-ARE response with human Nrf2 gene delivered directly into the injured spinal cord.

5.2.1 The transgene was expressed in neurons and astrocytes for 4 weeks after gene transfer

Prior to human Nrf2 gene delivery in SCI, we assessed the distribution of transgene in the spinal cord and identified its main cellular targets. Seven days after intraspinal delivery of lentiviruses encoding GFP into uninjured tissue, we observed intensive transgene expression in the grey matter neurons and astrocytes, but not in microglia (I, Figure 6). Next, we confirmed the duration of transgene expression in the uninjured spinal cord. Intraspinal delivery of the human Nrf2 gene resulted in stable expression of its mRNA 4 weeks after gene transfer (350-fold higher than background signal in control).

5.2.2 Gene transfer with lentivirus vector impaired hind limb motor function

To assess whether Nrf2 overexpression improves functional recovery after contusion SCI, we assessed locomotor performance of hind limbs weekly for up to 28 days after gene transfer by the BMS scoring system.

Unexpectedly, intraspinal delivery of the transgene with lentivirus vector significantly impaired hind limb function compared to PBS injections. The observed impairment in functional recovery became evident 2 weeks after gene delivery and was not transgene dependent (I, Figure 7A). At this time-point, we also performed MRI to estimate whether the functional disturbances correlate with the lesion progression. However, imaging revealed that gene transfer did not affect the lesion size. Functional aggravation after lentivirus mediated gene transfer persisted up to the end of observation period in both LV-hNrf2 and LV-GFP treated groups.

The detrimental effect of gene therapy observed in contused mice prompted us to examine whether introduction of the lentivirus vector encoding GFP or human Nrf2 into spinal cord affects the locomotor performance of uninjured laminectomized mice in a similar way. Indeed, gene transfer significantly impaired locomotor function of sham mice (I, Figure 7B). Laminectomized mice injected with LV-GFP were almost paraplegic 14 days after gene delivery and were able to perform only ankle movements by the end of the observation period. Uninjured mice injected with LV-hNrf2, although they performed in BMS remarkably better than LV-GFP, exhibited significantly impaired locomotor performance when compared with untransduced mice.

5.2.3 Increased amount of intraspinally delivered Nrf2 transgene prevented functional impairment of hind limbs

In a subsequent experiment, we increased the total amount of LV-hNrf2, LV-GFP or PBS delivered into the spinal cord by 33%. Interestingly, while increasing the transgene amount resulted in significantly improved locomotor performance of the contused mice transduced with LV-hNrf2 compared to the LV-GFP treated group (I, Figure 7C), it did not provide any advancement in comparison with PBS treated animals.

In addition, an increased amount of LV-hNrf2 delivered to the spinal cords prevented impairment in functional recovery, which we observed in uninjured mice in the previous experiment. BMS scores of sham mice injected with LV-hNrf2 were not significantly different from the BMS scores of uninjured mice in the PBS treated group. At the same time, the mice injected with an increased amount of LV-GFP were significantly impaired in locomotor performance when compared to LV-hNrf2 and PBS treated groups (I, Figure 7D).

5.2.4 Gene transfer with lentivirus vector increased expression of pro-inflammatory cytokines and growth factors

Three days after the gene transfer, we assessed whether Nrf2 boosting modulates the expression of several groups of genes, namely Nrf2-regulated genes, apoptosis-related genes, cytokines and growth factors.

The successful rate of the gene transfer was confirmed by the 1300-fold higher expression of human Nrf2 transgene in the group transfected with LV-hNrf2 when compared with background signal in other studied groups. However, surprisingly, even such a high level of human Nrf2 expression did not result in upregulation of mouse Nrf2 or HO-1 genes. Similarly to our previous findings, Nrf2 or HO-1 were induced by injury, yet human Nrf2 gene transfer did not further amplify their expression (I, Figure 8A,B). Only NQO1 responded to LV-hNrf2 delivery accordingly to our expectance since its expression in LV-hNrf2 group was significantly higher than in controls (I, Figure 8C). Next we observed that delivery of either LV-GFP or LV-hNrf2 into the contused spinal cord significantly augmented the induction of pro-inflammatory cytokines such as TNF, IL-1β, and IL-6 and growth factor such as NGF and GDNF (I, Figure 9A-C, E, F). From all of them only GDNF expression was further significantly increased after human Nrf2 gene transfer (I, Figure 9F). Since there was no difference in NGF and pro-inflammatory cytokines gene expression between the transduced groups it appears that these genes were induced by the virus vector itself, but not the transgene. The gene therapy did not affect expression of apoptosis related genes (I, Figure 9D).

Collectively, these results revealed the inability of human Nrf2 gene transfer to remarkably enhance the mouse Nrf2-ARE pathway. Moreover, lentivirus vectors encoding GFP and Nrf2 appeared toxic in current titers. Augmenting the amount of introduced Nrf2 prevented impairment in functional recovery induced by viral vectors, but did not further facilitate recovery from contusion SCI.

5.3 IL-33-MEDIATED MODULATION OF INFLAMMATION PROMOTED RECOVERY FROM CONTUSION SCI (II)

Inflammation is a key component of the secondary injury cascade (Fleming et al., 2006; Rossignol et al., 2007; David et al., 2012). A growing number of evidence indicates that certain components of the inflammatory response might be protective in SCI (Rossignol et al., 2007; David et al., 2012; Plemel et al., 2014). Therefore, in study II, in order to facilitate recovery from contusion SCI, we exploited the possibility to regulate the inflammatory response following SCI with IL-33, a cytokine known for its ability to modulate inflammation. We examined the effect of treatment on functional recovery and investigated central and peripheral mechanisms of IL-33 action in SCI.

5.3.1 Contusion SCI induced sustained activation of intracellular IL-33 in astrocyte nuclei

The intracellular form of IL-33 is known to act as a transcription factor and negatively regulate the expression of p65 subunit of NF-κB (Ali et al., 2011). To estimate whether intracellular IL-33 is involved in secondary injury, we assessed IL-33 mRNA levels in the injured spinal cord 24 hours after spinal cord contusion and identified distribution and cellular localization of IL-33 protein 6 weeks after the insult.

In the acute SCI phase we observed more than a 3-fold elevation of IL-33 mRNA levels (II, Figure 1A), indicating involvement of IL-33 signaling in the secondary injury. Induction of intracellular IL-33 persisted for up to at least 42 dpi as we observed a significantly higher amount of IL-33 positive cell profiles within 1.6 mm around the lesion epicenter in injured group at this time-point when compared to sham mice (II, Figure 1B–E). IHC and confocal imaging revealed that IL-33 protein was localized to the nuclei of astrocytes distributed within white and grey matter of the spinal cord (II, Figure 1D-F). We did not observe IL-33 protein in neurons or microglia (II, Suppl. Figure 2).

5.3.2 Recombinant IL-33 treatment did not affect intracellular IL-33

To examine whether supplementary IL-33 affects intracellular IL-33 levels we treated contused mice (i.p.) with recombinant IL-33 immediately after wound closing. However, supplementary IL-33 did not further amplify IL-33 mRNA and IL-33 protein 24 hours and 6 weeks, respectively, after injury (II, Figure 1A-E).

5.3.3 Recombinant IL-33 treatment modulated peripheral immunity

Extracellular IL-33 is known to stimulate a broad range of innate and adaptive immune cells including T-lymphocytes and macrophages (Liew et al., 2010; Smith, 2010; Sattler et al., 2013). Th2 lymphocytes are one of the primary IL-33 cellular targets and promoting Th2 response was the first reported mechanism of IL-33 action (Schmitz et al., 2005), whereas IL-33 driven macrophage polarization from M1 to M2 type only recently was identified (Kurowska-Stolarska et al., 2009). Therefore, to assess the peripheral effects of recombinant IL-33 treatment on innate and adaptive immunity, we analyzed the effect of IL-33 treatment on T-cell population and Th1/Th2/Th17 cytokines in the blood, spleen and lymph nodes after contusion SCI. In addition, the effect of IL-33 on suppressive T regulatory cells (Tregs) in these important T-cell reservoirs was examined. Furthermore, we investigated whether IL-33 administration results in altered expression of M1/M2 markers in spleen.

Already 24 hours after contusion and first dose of IL-33, we observed an increased level of IL-10 protein in the spleen and decreased levels of IFN-γ and IL-17 in plasma when compared to the vehicle group (II, Figure 11D-F). Importantly, flow cytometry analysis at 8 dpi showed that mice treated with IL-33 had a lower percentage and absolute number of CD4+ T lymphocytes expressing TNF than vehicle-treated and sham mice (II, Figure 9A,B). We also noticed that at 24 hours after injury IL-33 treatment significantly elevated Foxp3 mRNA expression, a marker of Tregs, in spleen (II, Figure 10). However, increased Foxp3 expression did not translate into increased numbers of Treg+ (CD4+/CD25+/Foxp3+) lymphocytes either in spleen or in plasma at 8 dpi. Additionally, at 42 dpi in the spleen IL-33 treatment caused a significant reduction of mRNA expression of the iNOS gene, a marker of M1 macrophages (II, Fig. 12F). This was accompanied by more than a 30% increase in Arginase-1 expression, a marker of M2 macrophages, compared to the vehicle treated controls (II, Figure 12G). Notably, we did not observe a significant difference in any of above studied parameters when sham mice were compared with mice treated with vehicle.

Overall these results indicate that recombinant IL-33 modulated adaptive and innate immunity at the periphery, promoting the Th2/M2 and inhibiting the Th1/M1 response after spinal cord contusion.

5.3.4 Recombinant IL-33 treatment modulated inflammation in the injured spinal cord

To determine whether IL-33 modulates the inflammatory response in the injured spinal cord, we analyzed gene expression and protein levels of pro- and anti-inflammatory cytokines, assessed T-cell infiltration, and studied the microglia/macrophage response in the spinal cord tissue. To assess T-cell infiltration, we quantified CD3 positive T-lymphocytes and measured expression of CD3 and CD4 genes, markers of total T cell population and T helper cells, respectively. Microgliosis was assessed by Iba-1 staining and microglia/macrophage phenotypes were analyzed by mRNA expression of M1 (iNOS) and M2 (Arginase-1, Ym-1) markers and Arginase-1 immunohistochemistry.

SCI significantly elevated the expression of pro-inflammatory cytokines TNF, IL-6 and IL-1β at 24 hours after insult. Already the first dose of recombinant IL-33 significantly reduced TNF expression compared to the injured mice treated with vehicle, whereas mRNA levels of IL-6 and IL-1β were not affected by IL-33 treatment (II, Figure 11A-C). We did not detect the mRNA expression of IL-10, IL-4 and IFN-γ gene in the spinal cord at this timepoint.

At 8 dpi, SCI remarkably upregulated the expression of CD4 mRNA, whereas expression of CD3 remained unaltered. Furthermore, at 42 dpi, we observed a significant increase in the number CD3 positive cell profiles in the injured spinal cord compared to sham mice. Recombinant IL-33 treatment significantly reduced CD3 mRNA expression, decreased CD4 expression level by approximately 40%, and at 42 dpi caused a trend towards reduction in the number of CD3 positive cells compared to the vehicle treated group (II, Figure 8).

SCI significantly induced the expression of iNOS, Arginase-1 and Ym-1 in the contused spinal cord compared to the sham mice at 24 hours after trauma, whereas IL-33 did not alter the expression of the studied M1 and M2 markers at this time point (II, Figure 12A–C). However, already at 8 dpi, the M2 markers Arginase-1 and Ym-1, but not the M1 marker iNOS were significantly elevated after IL-33 treatment compared to vehicle (II, Figure 12D,E). Importantly, at 42 dpi, IL-33 administration resulted in a remarkable increase in Arginase-1 protein expression, which at the lesion epicenter was approximately 35-fold higher than in the vehicle treated group, whereas in the sham mice Arginase-1 immunoreactivity was not detected (II, Figure 13A,B). Confocal imaging revealed that Arginase-1 protein expression was predominantly localized to Iba-1 positive macrophages/microglia (II, Figure 13C,D). Additionally, at 42 dpi, we observed a significant, trauma-induced increase in Iba-1 protein expression. IL-33 treatment had no effect on the degree of microgliosis in the injured spinal cord tissue (II, Figure 7).

Collectively, these results provide evidence that recombinant IL-33 modified the inflammatory response in the injured spinal cord, affecting T-cell infiltration and promoting M2 polarization of microglia/macrophages.

5.3.5 Modulation of inflammation after recombinant IL-33 treatment attenuated spinal cord tissue loss, demyelination and astrogliosis, and facilitated functional recovery

To determine if modulation of the inflammatory response identified after recombinant IL-33 treatment resulted in reduced secondary tissue damage and improved functional recovery after contusion injury, we assessed the degree of astrogliosis, estimated the amount of spared spinal cord tissue and preserved myelin, and monitored locomotor performance in the open field.

As expected at 42 days after trauma, the density of GFAP staining was significantly reduced in the mice treated with IL-33 compared with vehicle, indicating reduced degree of astrogliosis after IL-33 treatment (II, Figure 6). This effect was observed within 2.4 mm around the lesion epicenter. Moreover, IL-33 treatment resulted in increased spinal cord tissue sparing compared to the vehicle treated controls (II, Figure 4). Similarly, areas of preserved myelin were consistently larger in IL-33-treated mice almost at all of the analyzed levels (II, Figure 5), thus indicating reduced demyelination after IL-33 treatment.

Finally, IL-33 treatment significantly facilitated motor recovery of hind limbs in injured mice. The improvement became evident at 21 dpi and at the end of the observation period mice treated with IL-33 attained an average BMS score of 3.6 ± 0.26 vs. 2.5 ± 0.14 in the vehicle treated group. The biological significance of the identified improvement is that the mice in the IL-33 treated group were already able to perform stepping or at least plantar placement, whereas vehicle-treated mice on average demonstrated only ankle movements.

The obtained results provide evidence to support the concept that IL-33 modulates inflammation and improves locomotor recovery after contusion SCI in mice.

5.4 HUMAN iPSC-NPCs FAILED TO IMPROVE FUCTIONAL RECOVERY AFTER TRANSPLANTATION INTO SPINAL CORD OF CONTUSED MICE IMMUNOSUPPRESSED WITH TACROLIMUS (III)

Transplantation of iPSC-derived neural progenitor cells is a novel and promising strategy for SCI repair. Several preclinical studies have demonstrated the beneficial effect of iPSC therapy in contusion and transection SCI models when immunodeficient rodents were used as the recipients of human cells (Nori et al., 2011; Fujimoto et al., 2012; Lu et al., 2014; Sareen et al., 2014). However, clinical translation of autologous iPSCs transplantations for the treatment of acute and subacute SCI is barely feasible due to the time required for generation of safe autologous iPSC lines (Fehlings et al., 2011; Faulkner et al., 2014). In study III we aimed to evaluate the therapeutic potential of human iPSC-NPCs subacutely transplanted into spinal cord of mice with contusion SCI when pharmacological immunosuppression was used. UEFhfiPS1.4-NPCs generated and characterized at the University of Eastern Finland were used for all transplantations. Daily intraperitoneal injections of Tacrolimus at a dose of 2 mg/kg were used in all transplantation studies to provide immunosuppression in grafted and control vehicle treated mice.

5.4.1 Human iPSC-NPCs transplanted into spinal cord of uninjured mice immunosuppressed with Tacrolimus were safe, survived at least one week after grafting and expressed markers of proliferating immature neuronal cells

First, we transplanted UEFhfiPS1.4-NPCs that had been prelabeled by lentivirus encoding GFP into uninjured spinal cord and assessed the efficiency of grafting 7 days after transplantation by GFP fluorescence. The GFP positive cells were detected in ventral horns

and adjacent white matter within the whole studied 6 mm segment of the spinal cord (III, Figure 3A). We also histologically confirmed that undifferentiated pluripotent cells were not present in the grafts by Nanog staining (III, Figure 3D). To assess the differentiation state of the transplanted cells we applied DCX, Ki-67, Tuj1, and GFAP labeling. Confocal imaging revealed that many of the GFP positive cells were positive for DCX and Ki-67, but not Tuj1 or GFAP, indicating that at 7 days after grafting most of the transplanted cells were still proliferating immature neuronal cells (III, Figure 3B,C). Finally, Iba-1 staining revealed accumulation of activated microglia/macrophages around the transplanted cells (III, Figure 3E), suggesting the presence of an immune response towards the transplanted cells.

Next, to estimate the safety of our stem cell therapy, we transplanted the donor human cells or injected vehicle into the spinal cord of uninjured mice seven days after thoracic laminectomy and monitored the locomotor performance weekly for the next 5 weeks. Within the first 3 weeks after grafting the identified locomotor disturbances included only instances of mild trunk instability (BMS score 8) registered in both studied groups, whereas starting from day 28 after transplantation all animals achieved the maximal BMS score of 9, indicating full functional recovery (III, Figure 4). In addition, at postmortem dissection, no tumor formation was observed in the spinal cord. These findings allowed us to consider UEFhfiPS1.4-NPCs to be safe for transplantations into the injured spinal cord.

Taken together, these results confirmed the safety of UEFhfiPS1.4-NPCs transplanted into the uninjured spinal cord of pharmacologically immunosuppressed mice and indicated that transplanted cells were able to survive for at least one week after grafting and had the potential to differentiate along the neuronal lineage.

5.4.2 Human iPSC-NPCs transplanted into spinal cord of contused mice immunosuppressed with Tacrolimus exhibited scarce survival and did not improve functional recovery

In order to determine whether the transplanted UEFhfiPS1.4-NPCs are able to promote functional recovery of injured mice, we produced contusion thoracic injury and 7 days later injected vehicle or UEFhfiPS1.4-NPCs into the spinal cord. On the day of the cell transplantation the BMS scores of mice injected with vehicle were 2.09 ± 0.94 vs. 2.00±0.87 in the group transplanted with UEFhfiPS1.4-NPCs ($p>0.05$) (III, Figure 6), indicating injury similarity in the control and transplanted groups prior to cell transplantation. The locomotor performance of the hind limbs was evaluated weekly for the next 5 weeks. Although we did not detect a statistically significant difference at any time-point between the studied groups, the BMS scores in mice transplanted with UEFhfiPS1.4-NPCs were constantly lower than in the vehicle treated group. Five weeks after grafting the vehicle treated mice attained a BMS score of 5.09±1.11 vs. 4.18±0.84 in the transplanted group. According to the BMS scoring system, vehicle treated mice on average were able to perform frequent or consistent plantar stepping without coordination or with some coordination, but with paws rotation, whereas mice transplanted with donor human cells on average demonstrated only occasional plantar stepping. No signs of tumor formation were observed in the spinal cords of mice transplanted with UEFhfiPS1.4-NPCs at the time of postmortem dissection.

To confirm the results obtained with BMS, 5 weeks after cell transplantation we used CatWalk automatic gait analysis system to evaluate the precise movements of the hind limbs in the uninjured sham group, injured vehicle treated group and NPC transplanted group. Out of the assessed gait parameters a) Regularity index (RI), reflecting the degree of inter-limb coordination during the gait cycle, b) Hind paws intensity, a measure of weight support, c) Maximal contact, the time at which the largest part of a paw contacts the ground, d) Swing speed, reflecting the velocity of the moving limb during the swing phase, and e) Stride length, the distance between successive placements of the same paw (Hamers et al., 2001), were all significantly higher in the sham group when compared to injured groups (III,

Figures 7 and 8). We did not observe significant differences in any of these gait parameters between groups treated with vehicle or transplanted with iPSC-derived NPCs.

In addition, we performed MRI at 2 weeks after cell transplantation to estimate whether the transplanted UEFhfiPS1.4-NPCs affect the lesion size. Stem cell transplantations had no effect on lesion volume compared to vehicle-treated controls (III, Figure 5).

In order to find an explanation for the observed poor therapeutic effect of iPSC therapy we assessed GFP fluorescence in spinal cord sections of 6 mice transplanted with UEFhfiPS1.4-NPCs at 42 days after the transplantation. As expected, no surviving GFP-positive cells were found in five out of six examined spinal cords, whereas only a few survived cells were detected in one sample (III, Figure 9). The scarce iPSCs survival in the injured spinal cords was additionally confirmed by anti-GFP and human specific anti-HuNu immunostaining.

The obtained results indicate that human iPSC-derived NPCs transplanted into the injured spinal cord of mice immunosuppressed with Tacrolimus exhibited very limited survival and failed to promote functional recovery.

6 Discussion

Traumatic SCI, leading to paralysis, sensory loss and autonomic nervous system dysfunction, even in the 21st century, remains one of the most devastating conditions across the globe. With more than 130,000 new cases each year worldwide and primarily young males as victims, SCI represents an important target for modern medicine. The initial mechanical trauma results in rapid cell necrosis. This is followed by a secondary injury cascade that leads to delayed neuronal and glial cell death and demyelination that substantially aggravate impairment in functional recovery. Nowadays more than 25 different mechanisms participating in the secondary injury cascade are identified, including oxidative stress and inflammation. Scientific discoveries that have been made during the last decades substantially broaden our understanding of these pathophysiological processes. Transcription factor Nrf2 has been identified as the main regulator of the expression of hundreds of cytoprotective genes in response to oxidative stress. The aim of the present study was first to increase knowledge regarding the role of Nrf2 in SCI. This was achieved in study I by assessing the SCI-induced response in Nrf2 deficient mice.

While the initial damage is incurable, secondary injury mechanisms represent potential therapeutic targets. Synthetic glycocorticoid Methylprednisolone (MP) administered in very high doses is the only drug used for the treatment of acute SCI in patients. However, the significant dose-related side effects and relatively modest benefits of therapy make the administration of MP unjustified for acute SCI. Therefore, it is of utmost importance to identify novel therapeutic strategies that can limit the secondary damage, preserve spinal cord tissue and restore impaired function. In the present *in vivo* preclinical study we probed three relatively recently emerged strategies for SCI treatment, namely a) gene transfer, b) pharmacological immunomodulation and c) stem cell transplantation. In study I, we aimed to enhance the Nrf2-driven response in order to more efficiently withstand oxidative stress. In study II, pharmacological immunomodulation was achieved with IL-33, a cytokine with pleiotropic activities, but known for initiating and expanding of the Th2/M2 response, which is believed to be beneficial for SCI recovery. Lastly in study III, neural progenitor cells derived from human iPSCs were used to assess the efficacy of iPSC therapy in pharmacologically immunosuppressed mice.

The main findings presented in chapter 5 will be discussed below.

6.1 METHODOLOGICAL CONSIDERATIONS AND LIMITATIONS (I-III)

The most commonly used SCI models are contusion, compression and transection. As most spinal cord injuries in humans occur as the result of blunt, non-penetrating trauma, models in which the spinal cord is rapidly contused are currently thought to be the most relevant way to model typical injuries in humans (Kwon et al., 2010; Zhang et al., 2014). Therefore, in the present study, we used a bilateral contusion SCI model. To produce the moderate contusion injury in mice, we used two commercially available devices: NYU impactor, where a 5.6 gram weight was dropped from the standardized height of 6.25 cm, and Infinite Horizon Impactor, where a hit with a force of 60 kDyn was applied to the spinal cord tissue. Both devices allow the creation of reproducible spinal cord contusion injuries of different severity in rodents. Since the NYU Impactor was originally designed for modeling contusion SCI in rats (Gruner, 1992), in the present study, we used the minimum available weight of the rod and the drop was done from the lowest possible height. Despite this, we noticed that in some cases the NYU Impactor disrupted the dura in mice. Therefore, this type of impactor was only used in the experiment assessing the time-

course expression of Nrf2 and its target genes in study I, whereas in all subsequent experiments we utilized the Infinite Horizons Impactor.

In the present study, we selected to induce contusions at the T10 thoracic level, which allowed us to study recovery of hind limb function using standardized behavioral tests, e.g. BMS scoring system. While most human injuries occur at the cervical level (Norenberg et al., 2004; Lee et al., 2012), cervical spinal cord models significantly increase the need for animal care. In addition, by the time most of the experiments were planned and performed no validated cervical contusion SCI mouse model had been reported.

Inherent strain differences in mice can significantly affect the response to SCI (Basso et al., 2006; Kigerl et al., 2006; Lee and Lee, 2013). Therefore, to minimize variability in the injury response, appropriate control mice were chosen to match the genetic background. For Nrf2-deficient mice, we used WT littermates as controls.

In the present study, the functional recovery of the hind limbs was evaluated by the BMS scale, a scoring system specially designed and validated for mouse SCI (Basso et al., 2006). The BMS is ordinal and the degree of difference between ranks does not necessarily equal to the degree in functional improvement. Therefore, while interpreting BMS results it is always important to consider the biological significance of the identified changes (Basso et al., 2006; Hodgetts et al., 2013). For instance, the change in the BMS score from 3 to 4 reflects a transition from no stepping to stepping, and would have higher biological significance, than a similar one-point increase from 1 to 2 (improvement from slight to extensive ankle movements).

In study I, human Nrf2 gene was delivered into mouse spinal cord with a lentivirus vector. Nrf2 is evolutionally conserved with high-sequence homology in many species, including mouse and human (Cho, 2013). Human Nrf2 delivered into mouse hippocampus was capable of inducing mouse Nrf2 downstream genes (Kanninen et al., 2009). Therefore, delivery of the human Nrf2 gene in a mouse SCI model in the present study was justified.

The lentivirus vector titer (study I) was chosen based on the previous study where lentiviruses at approximately at the same titer were used in hippocampus of a mouse model of Alzheimer's disease (Kanninen et al., 2009). The dosing scheme of IL-33 in study II and Tacrolimus in study III were selected according to the earlier published reports of rodents studies (Marsala et al., 2004; Cizkova et al., 2007; Miller et al., 2008; Usvald et al., 2010; Sevc et al., 2013) and also based on our preliminary data.

In studies I and II, the administration of LV-hNrf2 and IL-33 were initiated immediately after injury. However, to deliver the patient from the scene of an accident to the hospital, resuscitate and define diagnosis often requires several hours. In the present study, we did not aim to assess the effect of delayed administration of LV-hNrf2 and IL-33. This may be a subject of subsequent studies.

In study III, we transplanted iPSC-NPCs into spinal cord 7 days after trauma. This corresponds to the subacute phase of SCI and is considered as the optimal time window for NPCs transplantation (Nakamura and Okano, 2013). The acute phase of SCI is not suitable for transplantation due to the extensive inflammatory response developing at this time and the presence of an extremely hostile microenvironment, whereas in the chronic phase regeneration is prevented by glial scar formation.

In study III, we used PBS as a control for the transplanted cells. Although non-viable cells, such as fibroblasts, are generally considered to be a better control, PBS is also often used in cell transplantation studies. Notably, most studies where iPSC-NPCs were transplanted into SCI models have utilized PBS or medium as control (Tsuji et al., 2010; Nori et al., 2011; Fujimoto et al., 2012; Kobayashi et al., 2012).

6.2 ESSENTIAL ROLE OF NRF2 IN LIMITING SECONDARY TISSUE DAMAGE IN SCI (I)

Numerous preclinical and clinical studies have shown that oxidative stress is an important component of the secondary injury cascade, resulting in the delayed death of neuronal and glial elements. In the secondary injury phase, several processes, including ischemia, mitochondrial dysfunction, calcium overload and inflammation, lead to the imbalance between ROS production and antioxidant defence system resulting in the oxidative stress and secondary cell death (Hall and Braughler, 1989; Kwon et al., 2004; Rowland et al., 2008; Jia et al., 2012). Nrf2 is a redox-sensitive transcription factor, which is known to modulate the expression of hundreds cytoprotective genes containing the ARE in their promoter region (Hybertson et al., 2011; Gan and Johnson, 2013; Gao et al., 2014). Activation of Nrf2 and Nrf2-regulated genes has been reported in a number of neurological diseases, including stroke and TBI (Chen and Regan, 2007; Tanaka et al., 2011; Shang et al., 2013), suggesting an attempt of endogenous defence systems to protect brain cells from the oxidative stress in these conditions. Recently, induction of the Nrf2-ARE pathway has been reported in the early phase of acute SCI (Mao et al., 2012, Wang X et al., 2012b, Jin et al., 2014a). However, whether activation of the Nrf2-ARE system after SCI has a transient or sustained nature has not been investigated.

In study I, we for the first time determined the magnitude and duration of the Nrf2-ARE response in the mouse contusion thoracic SCI model. In the injured spinal cord, we observed remarkable sustained induction of the Nrf2 gene and its downstream target HO-1. After rat contusion SCI, Nrf2 is activated already within the first 6 hours (Wang X et al., 2012b). However, in mouse contusion SCI model, we observed that Nrf2 induction was delayed for 72 hours, suggesting a different pattern of Nrf2 response in mouse and rats. The upregulation of Nrf2 expression was preceded by even earlier induction of HO-1, which later showed a sharp peak simultaneously with Nrf2 at one week after injury. Although HO-1 is one of the main Nrf2-regulated genes, its expression can also be modulated by other transcriptional regulators (Schipper et al., 2009; Paine et al., 2010; Foresti et al., 2013). For instance, the early induction of HO-1 may be driven by NF-κB which is known to be activated shortly after SCI (Bethea et al., 1998; Paine et al., 2010). The important protective role of HO-1 in SCI has been previously established (Yamauchi et al., 2004; Lin et al., 2007; Kanno et al., 2009a). In our study the activation of both Nrf2 and HO-1 persisted for at least 42 days after contusion, indicating that the Nrf2-ARE pathway functions not only in acute and subacute phases of the SCI, but is also implicated in its chronic phase of recovery.

NQO1 is a redox-regulated flavoenzyme and one of the most robust responders to Nrf2 activation (Dinkova-Kostova and Talalay, 2010). Early upregulation of NQO1 has been shown after rat contusion/compression and mouse compression SCI (Wang X et al., 2012b; Mao et al., 2011, 2012; Jin et al., 2014b). However, in our study induction of NQO1 was observed only at 42 days post injury. In addition, we detected transient induction of the two GCL subunits GCLM and GCLC within the first 24 hours after injury, which shifted to downregulation several days after the injury. The reasons for the observed pattern of NQO1 and GCLC/GCLM gene expression are not clear, however, we could assume that the difference in model, species or strains used may have affected the results. Nevertheless, the observed changes indicate that NQO1 and GCL contribute to SCI at different stages of recovery.

Overall, our results provide evidence for the involvement of Nrf2 and its downstream genes in acute, subacute and chronic phases of SCI. While the genes tested in the current study do not represent the full scope of Nrf2-driven genes, it is well conceivable that the Nrf2-ARE response to contusion SCI is not limited to the studied genes. We also may assume that the remote activation of Nrf2 and its target genes in cells outside of the lesion area or even in the periphery contribute to the Nrf2-ARE response to SCI.

Although the induction of Nrf2-regulated genes is known to provide cell protection, chronic upregulation of some Nrf2 downstream genes may in contrast augment injury (Stahnke et al., 2007). Therefore, to estimate whether the observed Nrf2-ARE response confers protection from SCI, we assessed the effect of Nrf2 gene deletion on the outcome of contusion mouse SCI.

Our results showed dramatically impaired hind limb functional recovery in $Nrf2^{-/-}$ mice, which became apparent already seven days after contusion injury and preserved until the end of the observation. These findings are in line with the deficit in motor function reported after compression model in Nrf2-deficient mice (Mao et al., 2012). Delayed apoptotic death of oligodendrocytes results in demyelination of many spared axons and is believed to underlay the impaired locomotor recovery after SCI (Basso et al., 2006; Plemel et al., 2014). Indeed, in our study the genetic ablation of Nrf2 resulted in increased atrophy and demyelination in the injured spinal cord. The observed effect of Nrf2 deficiency on myelin sparing is consistent with the previous studies demonstrating that $Nrf2^{-/-}$ mice exhibit greater demyelination than WT mice in EAE (Johnson et al., 2010). As reactive astrogliosis is triggered by many factors, including the broad spectrum of pro-inflammatory mediators, aggravated astroglial reaction observed in the current study in $Nrf2^{-/-}$ mice may indicate the increased pro-inflammatory status in spinal cords of mice deficient for Nrf2. In support of this, pro-inflammatory cytokines such as TNF and IL-6 were strongly elevated in Nrf2 deficient mice compared to wildtypes at the time when the impairment in functional recovery became evident. On the other hand, activated astroglia are a well-known source of pro-inflammatory cytokines (Pineau and Lacroix, 2007) and Nrf2 is highly inducible in astrocytes (Bell et al., 2011; Dowell and Johnson, 2013). In the absence of Nrf2 primary cultured astrocytes expressed higher levels of pro-inflammatory cytokines than wildtype astrocytes in response to injury (Pan et al., 2012). Therefore, we cannot also exclude the possibility that the increased inflammatory response observed in Nrf2-deficient mice is secondary to activation of astrocytes.

It is thought that by decreasing oxidative stress Nrf2 inactivates redox-sensitive transcription factor NF-κB and thereby negatively regulates pro-inflammatory cytokines (Li et al., 2008). Indeed, the deletion of Nrf2 resulted in greatly increased NF-κB activity in the acute phase of SCI (Mao et al., 2010). Although in our study we did not provide evidence showing that Nrf2 activation directly inhibited NF-κB signaling, it is possible to assume that the increased levels of pro-inflammatory cytokines was mediated by insufficient NF-κB suppression in Nrf2-deficient mice. Both TNF and IL-6 are critical mediators of the post-traumatic inflammatory response and are believed to play a mainly deleterious role in SCI pathology (Hausmann et al., 2003; Okada et al., 2004; Genovese et al., 2008). Importantly, TNF is specifically cytotoxic to oligodendrocytes, mediating their apoptotic death (Hisahara et al., 1997; Yune et al., 2003; Genovese et al., 2008, 2009; Inukai et al., 2009). Therefore, elevated levels of TNF observed in Nrf2-deficient mice in the current study might be at least in part responsible for the increased demyelination. During the secondary injury phase oligodendrocytes die mainly through apoptosis (Crowe et al., 1997; Profyris et al., 2004; Mekhail et al., 2012). Despite the fact that there is little information available on whether Nrf2 affects apoptotic cell death after SCI, Nrf2 has been proposed to prevent apoptosis by regulating the expression of anti-apoptotic Bcl-2 and Bcl-xL *in vitro* (Niture and Jaiswal, 2012, 2013; Son et al., 2014). Recently curcumin and EPO were shown to reduce apoptosis after rat compression SCI and their effect probably was mediated through Nrf2 activation (Jin et al., 2014a,b). In line with these reports, we observed altered expression of Bcl-xL in $Nrf2^{-/-}$ mice at 7 days after insult, indicating that Nrf2 is involved in regulating apoptosis in the contused mouse spinal cord. Collectively, these data indicate that Nrf2 is important for prevention of demyelination and limiting of astrogliosis, inflammation and apoptosis after contusion SCI.

Surprisingly, the impaired motor recovery in $Nrf2^{-/-}$ mice was not associated with reduced expression of such an important Nrf2 downstream gene as HO-1, suggesting the

Nrf2 independent regulation of HO-1 in the injured spinal cord and supporting our previous observation that after contusion injury upregulation of HO-1 precedes the activation of Nrf2. However, Nrf2 deficient mice exhibited significantly diminished expression of NQO1 and a clear trend in reduced MGST1 expression, prior to and at the time of the onset of the motor impairment, indicating an altered ability of these mice to cope with oxidative stress in the absence of Nrf2.

Finally, Nrf2 deficiency resulted in diminished upregulation of NGF compared to wildtype mice. In addition, we observed a clear trend towards less GDNF expression in Nrf2 deficient mice, indicating a possible role of Nrf2 in regulation of this growth factor expression. Nrf2 has been reported to regulate NGF production in glioblastoma cells and astrocytes (Mimura et al., 2011). In line with this, our findings show that Nrf2 might be also involved in neurotrophic factor signalling *in vivo*. Growth factors, such as NGF and GDNF modulate neuronal survival, facilitate neurite outgrowth, improve synaptic plasticity and neurotransmission, and exogenous administration of growth factors represents a potential therapy for SCI (reviewed in Awad et al., 2013). Therefore, lack of NGF and GDNF upregulation in Nrf2 deficient mice may negatively affect the recovery from SCI.

Overall our results indicate that Nrf2 deficient mice exhibit significantly enhanced secondary damage leading to impairment in functional recovery after contusion SCI. Therefore, proper functioning of Nrf2 is crucial for SCI outcome. While our work was in progress, several studies reported that Nrf2 deletion impairs recovery from compression SCI in mice (Mao et al., 2010, 2011, 2012). These studies are in full agreement with our findings that Nrf2 is essential for limiting secondary tissue damage after contusion SCI. Furthermore, altered expression of genes regulating inflammation, apoptosis and neuronal survival observed after Nrf2 deletion in our study indicate an even broader role of this transcription factor in SCI.

6.3 NO ADDITIONAL BENEFIT OF LENTIVIRUS-MEDIATED NRF2 OVEREXPRESSION OVER ITS NORMAL INDUCTION IN THE INJURED SPINAL CORD (I)

While activation of Nrf2 and its downstream genes after SCI confers substantial protection from the secondary injury it is obviously not sufficient to fully abolish the pathologic process. Augmentation of Nrf2 activity via chemical inducers may be a feasible strategy to boost cellular defence mechanisms and thus to mitigate the secondary tissue damage. Indeed, chemical compounds such as sulforaphane, curcumin and more recently EPO have been reported to provide neuroprotection against SCI in mice and rats through activation of the Nrf2 pathway (Mao et al., 2011; Benedict et al., 2012; Wang X et al., 2012b; Jin et al., 2014a,b). However, the degree of impact of Nrf2 activation in the beneficial effect of these chemicals in SCI is not very clear. For example, the protective effect of sulforaphane is mediated at least in part through inhibition of the NF-κB pathway (Cheung and Kong, 2010). Mechanisms of EPO neuroprotection in addition to NF-κB inhibition engage activation of the Janus kinase 2 (JAK-2) signaling pathway (Carelli et al., 2011). In contrast, introduction of a certain transcription factor with a gene therapy approach allows specifically boosting its expression and dissecting associated effects. Therefore, to estimate whether Nrf2 enhancement confers additional protection against SCI, in study I, we examined the possibility to overexpress this transcription factor in the injured spinal cord by direct introduction of a lentivirus encoding human Nrf2.

Through intraspinal gene transfer, we achieved strong overexpression of the transgene mainly throughout the grey matter, including both neurons and astrocytes. Human Nrf2 expression was sustained in the spinal cord tissue at substantially high levels for at least one month after gene delivery. However, surprisingly, Nrf2 overexpression not only failed to promote functional recovery after contusion SCI, but even worsened locomotor function

compared to vehicle control. In addition, we observed that both LV-hNrf2 and LV-GFP delivered into spinal cords of uninjured mice severely impaired the motor function of hind limbs. Interestingly, introduction of an increased volume of LV-hNrf2 into the spinal cord of uninjured or contused mice significantly mitigated the observed toxicity, but still was not sufficient to provide additional benefit over vehicle.

The reasons for the toxic effect observed in the current study are not clear since approximately at the same titers lentiviral vectors have been successfully used for intraspinal delivery of ChABC (Bartus et al., 2014), and LV-hNrf2 was delivered in the hippocampus of a mouse model of Alzheimer's disease without apparent toxicity (Kanninen et al., 2009). Recently, successful transduction of neurons in cortex and hippocampus after intranasal treatment with LV-hNrf2 has been reported. No side effects or toxicity related to lentiviral transduction were observed compared with untreated control animals in this study (Wang et al., 2014). However, in our experiments both LV-hNrf2 and LV-GFP dramatically increased the expression of pro-inflammatory cytokines, such as TNF, IL6 and IL-1β over levels of vehicle treated controls. Although the increased inflammatory response did not result in extension of the lesion volume and was not associated with the altered expression of proapoptotic and anti-apoptotic genes, we may suppose that it underlaid the observed impairment in functional recovery. It is possible to assume that both transgenes at high expression rates induced toxicity in the spinal cord. Indeed, as an exogenous protein, GFP has been reported to be immunogenic and induce cytotoxicity promoting oxidative stress and apoptosis (Liu et al., 1999; Stripecke et al., 1999; Goto et al., 2003; Yang et al., 2014), whereas permanent induction of Nrf2 and its downstream genes might also be deleterious for the cells (Stahnke et al., 2007; Ni et al., 2014). However, increasing the amount of LV-hNrf2 delivered into the spinal cord did not further augment functional impairment, but instead prevented it. Therefore, it is more likely that at least in the case of the LV-hNrf2 gene transfer, the observed inflammatory response was triggered by the lentivirus itself, whereas an increased volume of delivered LV-hNrf2 protected the spinal cord from the lentivirus mediated toxicity.

The absence of the beneficial effect of Nrf2 gene transfer at least in part may be explained by the failure of the transgene to activate Nrf2-ARE pathway in the mouse spinal cord shortly after gene delivery, even in the experiment where the amount of LV-hNrf2 introduced into the spinal cord was increased by 33%. Indeed, 3 days after gene transfer, only NQO1 was further upregulated in the LV-Nrf2 group, whereas Nrf2, HO-1 and MGST-1 were not altered by human Nrf2 gene transfer. The increased expression of NQO1 was obviously not sufficient to provide improvement in the functional recovery over vehicle controls, especially during conditions of increased inflammatory response, which we observed after the gene transfer. However, together with increased GDNF expression, NQO1 may be involved in preventing functional impairment seen after the delivery of LV-hNrf2 at a relatively low amount.

The lack of functional improvement after Nrf2 overexpression observed in this study does not diminish the importance of Nrf2 for recovery from SCI, nor does it contradict the results obtained in experiments with Nrf2 deficient mice. In Nrf2-knockout animals the expression of Nrf2 is absent since the first days of embryonic development in all cell types of the body, whereas the LV-hNrf2 was introduced into the spinal cord only right after the contusion and its expression was limited only to neurons and astrocytes. Our current findings also do not contradict the previous studies showing the beneficial effect of chemical compounds activating Nrf2 for recovery from SCI (Mao et al., 2011; Benedict et al., 2012; Wang X et al., 2012b; Jin et al., 2014a,b). We can assume that these chemicals reach their targets in all cell types in the spinal cord and in periphery, and in addition, possess Nrf2-independent mechanisms of action. Therefore, the insufficient beneficial effect of LV-Nrf2 treatment on SCI functional recovery observed in the current study may be due to late administration or more likely restriction of its expression within certain cell types. Also, we cannot rule out the possibility that variations in virus vector preparation could affect the

outcome. Considering the possible role of Nrf2 in preventing apoptosis, the prominent role of apoptosis in delayed oligodendrocyte death, and the importance of oligodendrocytes for functional recovery after SCI it might be crucial to achieve stable Nrf2 overexpression in this specific cell type. Further efforts should be also made to estimate the optimal time of administration and titer of the virus vector to achieve functional improvement and avoid the toxicity associated with the gene transfer approach.

6.4 MODULATION OF INFLAMMATION WITH IL-33 AS A POTENTIAL THERAPEUTIC APPROACH FOR SCI (II)

It is now becoming increasingly clear that traditionally widely used anti-inflammatory strategies, such as high doses of steroids or NSAIDs, have yielded little success to improve functional recovery after SCI. Therefore, the traditional view that inflammation is absolutely harmful for the recovery from SCI requires re-assessment. It is obvious that immune cells are much more heterogeneous than it was previously thought and there are much more diverse functions of these immune cell subtypes. In light of this, modulation of inflammation in order to harness its components that are important for repair appears extremely attractive (Rossignol et al., 2007; David et al., 2012; Plemel et al., 2014). Macrophages are a highly heterogeneous group of effector cells of innate immunity known to exert both beneficial and detrimental effects in SCI (Kigerl et al., 2009; David et al., 2012; Plemel et al., 2014). The ability of macrophages to shift their functional phenotype in response to changes in the microenvironment makes them an attractive target for immunomodulatory therapy. Unfortunately, the microenvironment of the injured spinal cord is favorable to the neurotoxic M1 subtype, whereas the presence of the anti-inflammatory M2 subtype is transient (Kigerl et al., 2009). IL-33 is a cytokine that stimulates T-lymphocytes to produce cytokines required for the M2 shift and therefore create an environment favorable for M2 polarization (Schmitz et al., 2005; Chackerian et al., 2007; Kurowska-Stolarska et al., 2009). The potential of IL-33 to harness the immune response encouraged us, in study II, to explore whether IL-33 treatment could be beneficial in a mouse model of contusion SCI.

6.4.1 Recombinant IL-33 as a modulator of central and peripheral inflammation after SCI

Administration of recombinant IL-33 after contusion injury resulted in improved functional recovery when compared to vehicle treated mice. We observed that improved functional recovery in IL-33 treated mice was associated with increased tissue sparing, reduced demyelination and decreased astrogliosis in the spinal cord, indicating that IL-33 provided tissue protection and reduced secondary tissue damage. Although we did not observe a reduction in lesion volume with MRI, the contradictory results between IHC and imaging could be attributed to the different time-points. MRI was performed one week prior to the time when the improvement in functional recovery was identified, whereas tissue sparing was assessed at the endpoint when the improvement in functional recovery was obvious.

Although in the present study we did not specifically tag IL-33 we may assume that it penetrates into the injured spinal cord due to the damage to the BSB. Indeed, BSB permeability typically peaks at 24 hours following SCI and lasts at for least 2-4 weeks after trauma (Noble and Wrathall, 1989; Popovich et al., 1996; Mautes et al., 2000b). In support of this, a reduction of TNF expression in the spinal cord after IL-33 treatment was detected as early as 24 hours after the first dose. The exact mechanisms of TNF inhibition after IL-33 treatment remain unclear, however knowing that resident spinal cord cells are mainly responsible for the release of TNF at this time-point we may suppose that by binding to its receptor in these cells IL-33 was able to dampen TNF release. Since astrocytes and microglia express the full IL-33 receptor, whereas neurons lack the ST2 subunit (Andre et al., 2005;

Yasuoka et al., 2011) we may assume that the observed effect is mainly mediated by the interaction of IL-33 with glial cells. At the same time we did not observe altered expression of IL-6 and IL-1β after IL-33 treatment in the injured spinal cord. Interestingly, recent *in vitro* data shows that IL-33 induces the release of pro-inflammatory cytokines from glia (Yasuoka et al., 2011; Kempuraj et al., 2013). While these discrepancies should be resolved in future studies, we suppose that the early reduction of TNF detected in the current experiments was crucial for functional recovery improvement observed after IL-33 treatment. Our findings are in line with studies showing that pharmacological inhibition of TNF is protective in SCI. Thus, Etanercept, which is a clinically approved TNF antagonist, has been shown to reduce secondary injury, decrease tissue damage, and improve functional recovery after SCI (Genovese et al., 2006; Chen et al., 2011).

As it was previously discussed in chapter 6.2, TNF might be responsible for increased demyelination due to specific cytoxicity towards oligodendrocytes. Activation of astrocytes is also mediated by TNF signaling. Therefore, reduced demyelination and astrogliosis detected after IL-33 treatment might be at least partly attributed to TNF inhibition.

TNF plays a significant role in the activation of peripheral leukocytes and their recruitment to the injured spinal cord (Pineau and Lacroix, 2007) and therefore, reduced TNF expression after IL-33 treatment could mitigate T-cell infiltration. Indeed, in our study, IL-33 treatment reduced the expression of T-lymphocyte markers already 8 days after injury and induced a trend towards reduction in the number of infiltrating T-cells at 6 weeks post injury, the time when the second peak in the T-cell infiltration into the injured mouse spinal cord usually occurs (Sroga et al., 2003; Kigerl et al., 2006). These findings are well in line with the previously reported capacity of IL-33 to reduce T-cell infiltration *in vivo* (Turnquist et al., 2011). In the injured spinal cord, the infiltrating T-lymphocytes recognize specific antigens, such as myelin basic protein. Proliferating in response to these antigens, the T-cells promote axonal injury, demyelination, and functional loss (Gonzalez et al., 2003; Jones et al., 2005). However, activated T-cells can also provide neuroprotection and increase axonal regeneration (Schwartz and Kipnis, 2001; Hauben 2000, 2001; Schwartz, 2005). Th2-lymphocytes produce IL-4, IL-5, IL-13 and IL-10 required for polarization of macrophages to the beneficial anti-inflammatory M2 phenotype (Gordon, 2003; Mantovani et al., 2004; Martinez et al., 2009). IL-33 is known to stimulate T-lymphocytes to produce these cytokines (Schmitz et al., 2005; Chackerian et al., 2007) and therefore to promote the M2 shift. Since we did not specifically study the T-cell populations in the spinal cord tissue, we lack direct evidence to demonstrate an IL-33-induced Th2 response in the injured spinal cord. In addition, we did not observe an increased expression of Th2 cytokines in the spinal cord tissue after IL-33 treatment. However, it is worth noticing that in our study the expression of Th2 cytokines was assessed 24 hours after SCI, whereas T-cell infiltration into mouse spinal cord typically occurs 2 weeks after injury (Sroga et al., 2003; Kigerl et al., 2006). At the same time, the finding that IL-33 treatment decreased levels of Th1/Th17 cytokines, IFN-γ and IL-17, in plasma, elevated IL-10, and reduced the percentage and absolute number of CD4+ T helper cells expressing TNF in the spleen indicate the suppression of the Th1 response and induction of the Th2 response in the periphery. Future research may answer the question of whether beneficial effects of IL-33 treatment after SCI in addition to attenuation of T-cell infiltration involve skewing of T-lymphocytes into the Th2 phenotype in the injured spinal cord.

In the spinal cord lesions, M1 microglia/macrophages outnumber M2 cells and they are believed to be detrimental to recovery after SCI (Kigerl et al., 2009; David and Kroner, 2011). Recently, TNF was identified as the main cytokine responsible for prolonged M1-polarization of microglia/macrophages in the injured spinal cord (Kroner et al., 2014) and, therefore, reduced TNF levels might create an environment favorable for chronic persistence of beneficial M2 cells. We observed that IL-33 treatment induced a dramatic increase in the expression of Arginase-1 and Ym-1, two M2 markers, in the spinal cord 8 days after injury, i.e. at the time when the numbers of activated microglia/macrophages

reaches its peak (Sroga et al., 2003). The increased number of Arginase-1-positive M2 microglia/macrophages persisted in the injured spinal cord for at least up to 6 weeks after injury. Our results are in line with a previous study showing that IL-33 treatment induces a shift towards the M2 type macrophage activation in the lymph nodes and spleen of a mouse model of EAE (Jiang HR et al., 2012). However, the current study is the first showing the ability of IL-33 to induce a remarkable M2 response in the CNS, and specifically in the spinal cord. Recently, spleen has been identified as the main source of monocytes infiltrating the spinal cord after SCI (Blomster et al., 2013). Therefore, we may speculate that in our study recombinant IL-33 treatment targeted infiltrating macrophages, including those originating from the spleen. This view was further supported by the findings that IL-10 levels were elevated in spleen shortly after IL-33 treatment. The important functions of IL-10 are to increase arginase activity and inhibit pro-inflammatory cytokine production and NO release (Liu et al., 2011). As IL-10 is one of the main cytokines responsible for M2 polarization (Shechter et al., 2011; Deng et al., 2012), it might have induced a shift of splenic monocytes into the M2 type. In addition, CD4+CD25+ Treg cells express a unique transcription factor, Foxp3, (Fontenot et al., 2003) and are known to induce M2 macrophage differentiation *in vitro* (Tiemessen et al., 2007) and *in vivo* (Liu et al., 2011). In the present study, we observed increased Foxp3 expression in spleen at the same time when IL-33 was elevated, suggesting that shortly after injury IL-33 created an environment favorable for M2 polarization in the spleen. It is interesting to note that one week after injury we did not observe increased numbers of Tregs in spleen or plasma, indicating the transient nature of the Tregs response on IL-33 treatment. Finally, we observed a significant reduction of the M1 marker iNOS expression and a tendency towards upregulation of Arginase-1 expression in the spleen 6 weeks after IL-33 treatment. This further supports the skew to the M2 phenotype in the spleen after IL-33 treatment. Additional studies utilizing splenectomised mice are required to estimate the contribution of splenic macrophages in M2 polarization detected in injured spinal cord after IL-33 treatment. Since it is impossible to distinguish fully activated resident microglia and infiltrating macrophages by their morphology or antigenic markers (Fleming et al., 2006; David and Kroner, 2011), we also cannot exclude the possibility that resident microglia may have acquired the M2 phenotype in our experiments, and therefore contributed to the observed beneficial effects of IL-33 treatment. Importantly, the degree of microgliosis was not altered by the treatment, indicating that IL-33 induced a shift in microglia/macrophage phenotype without alteration of their activation.

6.4.2 Intracellular IL-33 is involved in SCI-induced inflammatory response

In addition to its extracellular cytokine function, IL-33 also acts as a nuclear transcription factor regulating gene expression in a non-IL-33 dependent manner (Carriere et al., 2007; Roussel et al., 2008). The cellular source of IL-33 in CNS is controversial (Yasuoka et al., 2011; Jiang HR et al., 2012) and whether it is involved in the SCI-induced response has not been investigated. Since in the current study endogenous IL-33 was induced in the spinal cord shortly after contusion, our results indicate that IL-33 as a transcription factor readily responds to the SCI. We also observed that IL-33 protein expression persists at high levels for at least 6 weeks after injury and its protein expression is restricted to astrocyte nuclei. Therefore, our findings are in line with the observations that astrocytes are the main cellular source of IL-33 in the CNS (Hudson et al., 2008; Yasuoka et al., 2011; Christophi et al., 2012; Zhao et al., 2013). Since we have not studied the time-course of IL-33 expression, we lack information about whether the rate of its activation changes with the progression of secondary injury. However, our results show that IL-33 remains activated also in the chronic phase of SCI.

Intracellular IL-33 is known to repress NF-κB activity and transcription of NF-κB downstream genes (Ali et al., 2011). Therefore, the induction of intracellular IL-33 after trauma may be an attempt to inhibit an excessive inflammatory response after SCI. Future

studies involving IL-33-knockout mice are required to estimate the precise role of IL-33 in the secondary injury response. It is important to notice that recombinant IL-33 did not affect the level of intracellular IL-33 expression. This indicates that the effects of recombinant IL-33 were independent from the function of nuclear IL-33.

Collectively, our results suggest that intracellular IL-33 contributes to the immune response after contusion SCI, whereas recombinant IL-33 treatment modulates both central and peripheral inflammation, leading to reduced secondary damage and improved functional recovery. Although more intensive research is needed to define the precise mechanisms of IL-33 action, our study has provided the first insights into the beneficial role of IL-33 in recovery after contusion SCI. We believe that optimization of IL-33 dosing and estimation of the optimal treatment regimen may produce even better results in future studies. The proposed mechanisms of IL-33 action in SCI are summarized in Figure 5.

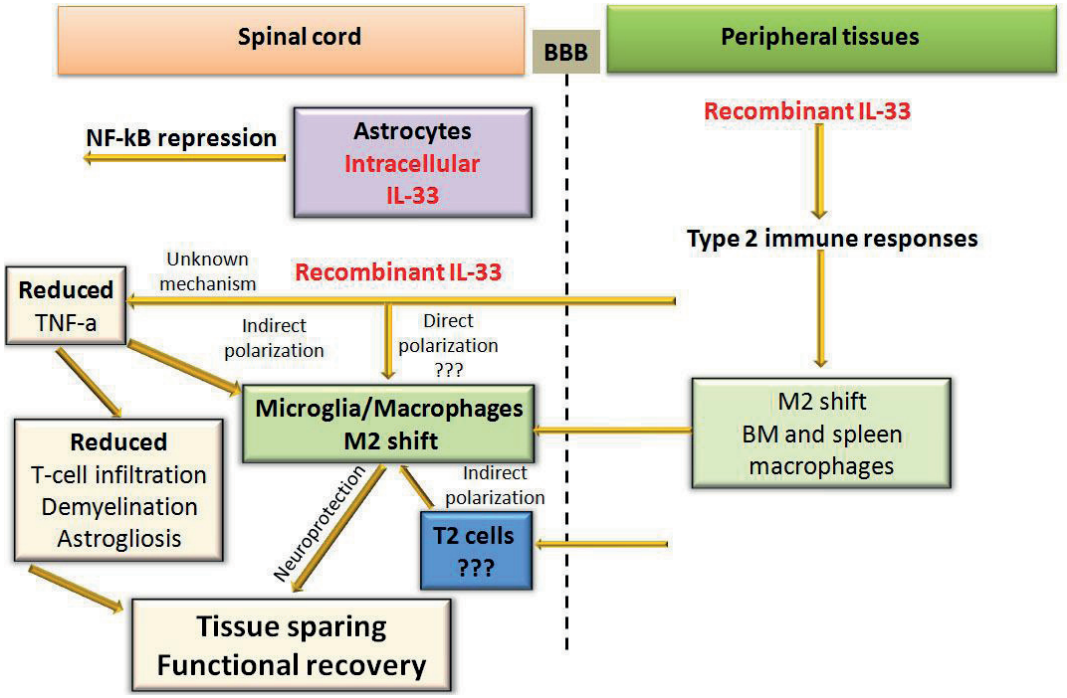

Figure 5. Proposed immunomodulatory mechanisms of IL-33 action resulting in neuroprotection after contusion SCI.

6.5 TRANSPLANTED HUMAN iPSC–DERIVED NEURAL PROGENITORS NEITHER SURVIVE NOR PROMOTE FUNCTIONAL RECOVERY OF CONTUSED MICE IMMUNOSUPPRESSED WITH TACROLIMUS (III)

Numerous preclinical studies have convincingly demonstrated that stem cell transplantation approaches have great potential to yield repair and recovery of lost function in different CNS diseases, including SCI. The patient-specific iPSCs, generated by the reprogramming of adult somatic cells, avoid ethical concerns common for ESCs and can be used for autologous transplantations without the risk of immune rejection (Kramer et al., 2013; Mothe and Tator, 2013). Differentiated along the neural lineage the iPSCs represent a potentially unlimited source of neural cells for cell replacement therapy. However, in the case of acute injuries, such as SCI, the translation of NPCs derived from patient-specific iPSCs in clinical practice is limited by the time required to properly characterize and

carefully validate autologous iPSCs in sufficient quantities. Therefore, carefully pre-evaluated allogeneic iPSCs lines are likely more suitable for transplantations into patients with acute SCI (Fehlings and Vawda, 2011; Kramer et al., 2013; Faulkner et al., 2014). In this situation immunosuppressive drugs will be required to blunt the recipient's immune response. Recently, human iPSC-derived NS/PCs transplanted into spinal cord have been shown to improve the outcome after SCI in several preclinical studies (Nori et al., 2011; Fujimoto et al., 2012; Kobayashi et al., 2012; Lu et al., 2014; Sareen et al., 2014). However, in most of these reports NS/PCs were transplanted into genetically immunocompromised rodents to avoid the host immune response (Nori et al., 2011; Fujimoto et al., 2012; Lu et al., 2014; Sareen et al., 2014). From these studies it is not clear whether the beneficial effect of iPSC therapy would be preserved in the more clinically relevant settings of pharmacological immunosuppression. In study III, we addressed this question by using the calcineurin inhibitor, Tacrolimus, which is widely used in clinical practice to prevent rejection after allograft transplantation (Fukudo et al., 2005; Fukuoka et al., 2010; Choi and Reddy, 2014).

In the present study, we used iPSC-derived NPCs (UEFhfiPS1.4) generated and characterized in our laboratory (Qu et al., 2013). Prior to grafting, we assured that cells lose pluripotency and express the neural markers PAX-6, MAP-2 and DCX. By transplanting the cells into spinal cords of uninjured laminectomized mice, we further confirmed the potential of iPSC-derived precursors to differentiate into neuronal lineage *in vivo.* Thus, one week after grafting confocal imaging revealed that many of the transplanted cells actively proliferated and were still at the stage of immature neuronal cells at that time.

Since iPSCs are potentially tumorigenic (Miura et al., 2009; Tsuji et al., 2010; Kramer et al., 2013), controlling the safety of the grafted iPSC-derived NPCs is of particular importance. The presence of even small amounts of undifferentiated cells after *in vitro* or *in vivo* differentiation may result in tumor formation in the recipient (Miura et al., 2009; Fu et al., 2012). Seven days after grafting into injured mice we did not observe any cells positive for Nanog, a marker detecting undifferentiated pluripotent cells. Usually, the tumor formation in the spinal cord results in substantial impairment in locomotor performance already within 10 days after stem cell grafting, which is followed by the full hind limb paralysis (Wang X et al., 2012a). However, in our study, uninjured transplanted mice did not exhibit functional impairment at least up to 5 weeks after transplantation. Furthermore, no signs of tumor formation were observed at postmortem dissection, indicating the safety of NPCs used in our studies.

These results encouraged us to further explore whether UEFhfiPS1.4-NPCs are able to improve the outcome of contusion SCI. We transplanted NPCs into the spinal cords one week after contusion injury, which is considered to be within the optimal time window for cell transplantations into injured spinal cord (Nakamura and Okano, 2013). However, transplanted cells failed to improve functional recovery and did not reduce the lesion size when compared to the vehicle-treated mice. Moreover, BMS scoring showed sustained impairment in recovery of motor function in the group transplanted with UEFhfiPS1.4-NPCs, which appeared already one week after grafting and at the end of the observation period resulted in occasional plantar stepping. Vehicle-treated mice performed frequent or constant stepping. Although the observed impairment was not statistically significant, given the ordinal nature of the BMS scale we may speculate that the transplanted cells possibly even worsened the behavioral outcome in our study.

Since any beneficial effect of transplanted cells on functional recovery is usually interrelated with good cell survival, we presumed that the poor effect of NPCs transplantation in our study was associated with limited cell survival in the injured spinal cord. Indeed, very scarce numbers of cells were found only in one sample at 5 weeks after the cell transplantation, whereas the others were devoid of viable transplanted cells. Interestingly, it has been reported that an 18-20% survival rate of transplanted mouse and human iPSC-derived progenitors into immunodeficient mice is enough to promote

functional improvement (Tsuji et al., 2010; Fujimoto et al., 2012). Clearly, the amount of survived cells in our study was insufficient to provide functional improvement.

While our results contradict the majority of studies reporting the beneficial effect of iPSC therapy in acute SCI models (Nori et al., 2011; Fujimoto et al., 2012; Kobayashi et al., 2012; Lu et al., 2014; Sareen et al., 2014), studies reporting no effect from iPSC therapy in subacute SCI settings also exist (Kramer et al., 2013). ESCs are known to induce a strong immune response involving both T-lymphocytes and NK cells (English and Wood, 2011; Kramer et al., 2013), whereas immunogenic properties of iPSCs have also been reported (Zhao et al., 2011; Cao et al., 2014). Throughout our study, to prevent the graft rejection, we used the same immunosuppressive regimen, which included daily administration of the calcineurin inhibitor, Tacrolimus, in a dosage commonly used in allogeneic cell transplantation studies (Marsala et al., 2004; Cizkova et al., 2007; Usvald et al., 2010; Sevc et al., 2013). Notably, most of the successful reports utilized genetically immunocompromised rodents including nonobese diabetic – severe combined immunodeficient mice (NOD-SCID). These mice have severe impairment in both innate and adaptive immunity, as they lack complement hemolytic activity and both mature T- and B-lymphocytes. In addition, the function of NK cells, dendritic cells and macrophages is altered in this mouse strain (Schultz et al., 1995; Greiner et al., 1998; Anderson et al., 2011). Importantly, while calcineurin inhibitors effectively target the T-cell response, these immunosuppressants do not affect NK cells or other rejection mechanisms (Anderson et al., 2011). As an immune response to transplanted cells may not only result in graft rejection, but also affect the cell fate (Sontag et al., 2013) it is not surprising that outcome of cell grafting in genetically and pharmacologically immunosuppressed rodents may be different. In the present study, we observed accumulation of microglia/macrophages around the grafts in uninjured mice already one week after transplantation, indicating the triggering of an immune response towards the transplanted allogeneic cells even in the uninjured mice. Together with the limited graft survival and absence of functional improvement in injured mice, our results suggest that monotherapy with Tacrolimus is not sufficient to provide an effective immunosuppression for successful iPSC grafting after contusion SCI.

The poor effect of pharmacological immunosuppression has been reported in the majority of preclinical allogeneic transplantation studies (Luchetti et al., 2010; Anderson et al., 2011). For example, Tacrolimus monotherapy fails to provide efficient immunosuppression in studies involving the G93A SOD1 animal model of ALS (Yan et al., 2006; Hefferan et al., 2011). In traumatic injury models, such as SCI, it may be even more challenging to achieve effective and long-term immunosuppression, due to the complex nature of the immune response involving numerous cell types and mediators, and especially BSB breakdown enhancing access of the immune cells to the injured spinal cord (Anderson et al., 2011). In line with this, in our experiments when human ESCs or iPSC-derived NPCs were transplanted into the brains of uninjured mice, transgenic APdE9 mice and mice subjected to permanent focal brain ischemia, we observed graft survival for 5-12 weeks despite the immunosuppressive regimen used in those experiments being similar to the current study. This indicates that the beneficial effect of immunosuppressants in promoting transplanted cell survival observed in intact animals or other models may be cancelled out in SCI by the complex and diverse immune response in the injured spinal cord. In support of this, recent studies have shown that immunosuppression is associated with increased efficacy in focal cerebral ischemia (Lees et al., 2012), whereas in SCI, in contrast, efficacy of immunosuppressants is reduced (Antonic et al., 2013).

The calcineurin inhibitors Cyclosporine and Tacrolimus are the most widely used immunosuppressants in the clinical practice (Choi and Reddy, 2014). Although both drugs act in a similar manner, there is substantial evidence that Tacrolimus exerts stronger immunosuppression than cyclosporine (Saudek et al., 2005; Grimm et al., 2006; Krämer et al., 2008; Fukuoka et al., 2010) and has a lower nephrotoxicity potential (Naesens et al., 2009). In light of this, it is interesting that recently a beneficial effect was reported after

transplantation of human iPSC-NS/PCs into a contused nonhuman primate spinal cord when monotherapy with Cyclosporine was used for immunosuppression (Kobayashi et al., 2012). Quite often in clinical settings calcineurin inhibitors are combined with other drugs to increase the efficacy of immunosuppression (Choi and Reddy, 2014). In preclinical studies the increased efficiency of combined immunosuppression (Tacrolimus + Sirolimus, Tacrolimus + Mycophenolate or Tacrolimus + anti-CD4 antibody) in comparison with Tacrolimus monotherapy has also been reported (Yan et al., 2006; Hefferan et al., 2011; Sontag et al., 2013). Of note, very recently the combination of Cyclosporine, Azathioprine and Methylprednisolone have been reported to provide efficient and long-term immunosuppression after transplantation of human iPSC-derived NPCs into spinal cords of rats with compression SCI (Romanyuk et al., 2014). While more future studies should determine whether combined immunosuppression would be efficient enough in acute SCI, our study is the first one where monotherapy with Tacrolimus was used for allogeneic stem cell transplantation in an acute SCI model.

Taken together, our results demonstrate that immunosuppressive monotherapy with Tacrolimus was not sufficient to provide long-term survival of human iPSC-derived NPCs transplanted into contused mouse spinal cord, and demonstrate the importance of studies involving combined immunosuppression or/and reduction of immunogenicity of transplanted cells prior to implementation of iPSC-based therapy into clinical practice.

7 Summary and Conclusions

In the present study, we investigated the effect of (i) Nrf2 gene transfer, (ii) IL-33 pharmacological treatment and (iii) transplantation of iPSC-derived neural progenitors on recovery from contusion SCI. Special focus was placed on assessing the immunomodulatory properties of recombinant IL-33 and role of Nrf2 in protection from secondary injury. In addition, we studied the response of endogenous Nrf2 and IL-33 to SCI. The main results of this thesis can be summarized as follows:

I
Contusion SCI induced sustained activation of the Nrf2-ARE pathway. Deletion of the Nrf2 gene substantially exacerbated secondary damage leading to impaired functional recovery. Proper Nrf2 function was important for regulation not only of classic target genes, but also genes involved in inflammation, apoptosis, and tissue repair. These results provide new insights into the essential role of Nrf2 in acute CNS injuries. Nrf2 gene transfer with a lentiviral vector resulted in strong, long-term overexpression of the transgene in astrocytes and neurons. The side effect of gene therapy manifested as impaired motor function. An increased amount of delivered Nrf2 gene prevented locomotor impairment but did not provide extra benefit compared to normal activation of Nrf2 after contusion SCI. These results suggest the development of an inflammatory response after gene transfer and underline the importance of the optimal titer of the virus vector used for gene therapy after SCI.

II
Contusion SCI induced endogenous IL-33 expression in astrocyte nuclei. The results suggest the involvement of intracellular IL-33 in the SCI-induced immune response. Recombinant IL-33 ameliorated secondary injury and dramatically improved functional recovery. The neuroprotective effect of IL-33 was mediated through the modulation of the inflammatory response in the spinal cord and in the periphery. Sustained polarization of microglia/macrophages to the anti-inflammatory M2 phenotype in spinal cord was associated with decreased expression of cytotoxic TNF and a tendency to reduced T-cell infiltration. The beneficial effects of IL-33 in spinal cord were accompanied by elevation of anti-inflammatory IL-10 and suppression of the Th1 response in the peripheral tissues. The results indicate that IL-33 is a novel promising treatment for SCI.

III
The transplantation of human iPSC-derived neural progenitor cells was safe and grafted precursors had the potential to differentiate into neuronal cells *in vivo*. Subacutely transplanted into the contused spinal cord, the human iPSC-NPCs exhibited limited survival and did not facilitate functional recovery when the calcineurin inhibitor Tacrolimus was used to prevent graft rejection in doses commonly used in preclinical transplantation studies. The results suggest that contusion SCI is a particularly challenging condition to achieve effective and long-term immunosuppression and highlight the importance of additional studies involving combined immunosuppressive strategies to prevent rejection of human iPSC-derived cells.

In conclusion, we have for the first time demonstrated that recombinant IL-33 is an attractive candidate to modulate inflammation and promote functional recovery after contusion SCI. Despite the fact that Nrf2 plays crucial and multifaceted roles in protection from secondary injury, even strong Nrf2 overexpression in neurons and astrocytes with gene transfer technology may not offer extra benefit. Special attention should be paid to vector-associated toxicity issues in the future. Tacrolimus treatment is not sufficient to promote survival of human iPSCs subacutely transplanted into contused spinal cord. Achieving efficient and long-term immunosuppression is especially challenging in SCI. Additional studies are warranted to find an optimal regimen of immunosuppression to gain the potential beneficial effect of iPSC therapy after contusion SCI.

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Yuriy Pomeshchik

Novel strategies for spinal cord injury repair

Spinal cord injury (SCI) is a catastrophic condition resulting in loss of sensation, motor, and autonomic function. There is no effective therapy for SCI. This thesis demonstrates that interleukin-33 is a promising novel therapeutic approach for acute SCI. Special attention is paid to the protective role of the transcription factor Nrf2 in SCI. The thesis also evaluates potential therapeutic avenues in the utilization of Nrf2 gene transfer and stem cell therapy for SCI.

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