# Therapeutic Capacity of Apoptotic Mononuclear Cell Secretome in Experimental Spinal Cord Injury

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**Doctor of Philosophy**

Submitted by

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*Dedicated to my father Helmut.*

*I miss you every day.*

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# Declaration

This work was conducted at the Department of Thoracic Surgery of the Medical University of Vienna under the supervision of Hendrik Jan Ankersmit in cooperation with the Institute for Neurology (Medical University of Vienna), the Department of Transfusion Medicine (Medical University of Vienna), the Department for Dermatology (Medical University of Vienna), the Center for Anatomy and Cell Biology (Medical University of Vienna), the Department of Pediatrics (Medical University of Vienna), the Center for Biomedical Research (Medical University of Vienna), the Center for Brain Research (Medical University of Vienna), the Ludwig Boltzmann Institute for Traumatology in Vienna and the Red Cross Blood Bank in Linz. Performance of the animal model was trained at the Ludwig Boltzmann Institute for Traumatology under the supervision of Heinz Redl. All animal experiments were carried out at the Center for Biomedical Research of the Medical University of Vienna with assistance from Andreas Mitterbauer (Department of Thoracic Surgery, Medical University of Vienna). Production of secretome was realized in cooperation with the Red Cross Blood Bank in Linz under the direction of Christian Gabriel. Tissue preparation and histological evaluation were conducted in cooperation with Romana Höftberger, Irene Leisser and Gerda Ricken affiliated with the Institute for Neurology of the Medical University of Vienna. In-vitro and ex-vivo experiments were performed in cooperation with Beate Rüger, Tanja Buchacher (both Department of Transfusion Medicine, Medical University of Vienna), Michael Mildner and Bahar Golabi (both Department of Dermatology, Medical University of Vienna). Data evaluation, interpretation of results and manuscript preparation was performed under the supervision of Hendrik Jan Ankersmit (Department of Thoracic Surgery, Medical University of Vienna) and Romana Höftberger (Institute for Neurology, Medical University of Vienna) with support of Fritz Leutmezer (University Clinic for Neurology, Medical University of Vienna).

# Table of Contents

# List of Figures

# Abstracts in English and German

## Abstract

Spinal cord injuries (SCI) represent a severe form of trauma affecting mainly young patients during their most active years in life. In most cases, injuries to the spinal cord are associated with severe and irreversible neurological impairment causing individual and socio-economic burden. After the initial trauma, secondary mechanisms like inflammation, oxidative stress, glial scarring, thrombocyte activation, and apoptosis come into action and promote aggravation of the lesion further deteriorating functional outcome after SCI. Modulation of this secondary cascade was the main focus of pre-clinical and clinical trials investigating potential novel therapies for patients suffering from SCI. Within this secondary cascade of endogenous response to SCI, aside from mainly deleterious mechanisms, some pathways were shown to be beneficial and vital for regeneration of the spinal cord. Macrophages recruited from the systemic circulation to the spinal cord were reported to be essential for regeneration and restoration of neurologic function after SCI. This peculiarity of double-edged characteristics of the secondary response to SCI potentiates the complexity of this secondary response and complicates the endeavour for novel treatment options with no satisfying candidate at hand up until today.

Recently, the secretome (=conditioned medium) of apoptotic peripheral blood mononuclear cells (PBMCs; MNC-secretome) was shown to exhibit therapeutic capacity in numerous pre-clinical trials by modulating multiple molecular and cellular targets. Most of these reported mechanisms targeted were also shown to be involved in the secondary endogenous detrimental response to SCI. Therefore, this thesis aimed to address the question whether treatment with the secretome derived from apoptotic PBMCs is also capable of interfering with the endogenous response after SCI. A commonly used experimental contusion model in rats, histological and immunohistochemical evaluations, ex-vivo angiogenesis assays, enzyme-linked immunosorbent assays (ELISA) among other techniques were used for this purpose. PBMCs were obtained from healthy human donors, lethally irradiated to induce apoptosis and cultured for 24 hours using standard cell culture

methods. After discarding the cells, only the conditioned medium was further used for experiments.

Systemic administration of MNC-secretome lead to improved recovery of motor function compared to untreated animals. Modulation of the inflammatory response was confirmed histologically. We found augmented recruitment of macrophages to the perilesional area with concomitant reduction of pro-inflammatory markers. In-vitro assays revealed angiogenic potential in vascular and spinal cord tissue. Treatment with MNC-secretome lead to reduced lesion volumes and amelioration of axonal damage.

Efficacy of systemic treatment with conditioned medium derived from apoptotic PBMCs on multiple targets involved in the secondary cascade after SCI was successfully demonstrated in this study. This modulation was associated with improved neurological outcome and inhibition of secondary damage following SCI. We hypothesize that the combination of postulated characteristics of MNC-secretome resulted in improvement of neurological outcome and reduced spinal cord damage. Results from this thesis may provide the basis for translation to clinical application of MNC-secretome.

## Zusammenfassung

Traumatische Verletzungen des Rückenmarks betreffen in den meisten Fällen junge Patienten in ihren aktivsten Lebensjahren. Diese Art von Verletzung geht häufig mit irreversiblen neurologischen Defiziten und schweren persönlichen Einschränkungen einher. Nach dem initialen Trauma führen sekundäre Mechanismen wie Inflammation, oxidativer Stress, gliale Narbenbildung, Thrombozyten-Aktivierung und Induktion von Apoptose zu einer Zunahme des Schadenausmaßes, was eine weitere Verschlechterung des funktionellen und neurologischen Ausgangs verursacht. Diese, aus mehreren molekularen und zellulären Mechanismen aufgebaute, endogene Reaktion auf Rückenmarksverletzungen bietet eine Vielzahl an Zielen für mögliche therapeutische Modulierung. Der Umstand, dass innerhalb dieser sekundären Kaskade neben unerwünschten Mechanismen auch Signalwege aktiviert werden, die für die Regeneration und Wiederherstellung der neurologischen Funktion notwendig sind, erschwert die Suche nach einer kausalen Therapie. Insbesondere Makrophagen wird im Zusammenhang mit Regeneration des Rückenmarks und Inhibierung der überschießenden pro-inflammatorischen Antwort eine zentrale Rolle zugeschrieben. Dies führte unter anderem auch dazu, dass bis heute keine zufriedenstellende systemische Therapie für Patienten mit Rückenmarksverletzungen gefunden werden konnte.

Die systemische Therapie mit dem Sekretom von apoptotischen peripheren mononukleären Zellen (PBMCs) wurde bereits in mehreren Arbeiten als wirkungsvoll und vielversprechend beschrieben. Der Wirkmechanismus wurde mit Modulation multipler

molekularer und zellulärer Ziele in Verbindung gebracht. Einige dieser angesteuerten Signalwege spielen auch im Rahmen des sekundären Schadens nach Rückenmarksverletzungen eine zentrale Rolle. Aus diesem Grund war das Ziel dieser These, eine mögliche therapeutische Wirksamkeit des Sekretoms nach Rückenmarksverletzungen zu untersuchen und gegebenenfalls involvierte Signalwege und Wirkmechanismen aufzuzeigen. Ein mehrfach publiziertes Modell des Rückenmarktraumas in Ratten, histologische und immunhistochemische Methoden, ex-vivo Angiogenese-Experimente und ELISA (enzyme-linked immunosorbent assay) wurden unter anderem verwendet, um diese Fragestellung zu beantworten. PBMCs wurden von gesunden Probanden durch periphere venöse Blutabnahme entnommen und anschließend bestrahlt, um so Apoptose zu induzieren. Danach wurden die bestrahlten PBMCs für 24 Stunden kultiviert und ausschließlich das konditionierte Medium ohne zelluläre Anteile für weitere Experimente verwendet.

Systemische Applikation von Sekretomen von apoptotischen PBMCs führte zu signifikant besserem neurologischen und funktionellen Outcome nach Rückenmarkstrauma. Die histologische Auswertung ergab, dass die Therapie zu einer Modulation der immunologischen Antwort führte. Es kam, in Übereinstimmung mit früheren Ergebnissen, zu einer erhöhten Rekrutierung von Makrophagen bei gleichzeitig reduzierten pro-inflammatorischen Markern im verletzten Rückenmark. Ex-vivo Versuche bestätigten das Angiogenese-Potential des Sekretoms in vaskulären Strukturen, als auch in Rückenmarksgewebe. Die Summe dieser und anderer, in dieser Arbeit dargestellten, Wirkmechanismen des Sekretoms führte zu einem reduzierten Schadensausmaß und Reduzierung der axonalen Schädigung in der histologischen Auswertung.

In dieser These konnte die therapeutische Kapazität der Behandlung mit Sekretomen von apoptotischen PBMCs in einem experimentellen Modell des Rückenmarktraumas aufgezeigt werden. Die therapeutische Wirkung basiert auf mehreren Wirkmechanismen, welche im Zusammenspiel eine Abschwächung des sekundären Schadens verursachen. Die Ergebnisse dieser These könnten als Grundlage für klinische Studien dienen.

## Publication arising from the thesis

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# Abbreviations

AIS… American Spinal Cord Injury Association Impairment Scale

AMI…Acute Myocardial Infarction

AANS… American Association of Neurological Surgeons

ASIA…American Spinal Injury Association

BBB…Blood-Brain Barrier

BBB-score… Basso, Beattie, and Bresnahan Score

BCSFB…Blood-Cerebrospinal Fluid Barrier

BSCB…Blood-Spinal Cord Barrier

βAPP… β-Amyloid Precursor Protein

BDNF…Brain-Derived Neurotrophic Factor

cAMP…3'5' Cyclic Adenosine Monophosphate

CCL20…Chemokine Ligand 20

CD…Cluster of Differentiation

CNS…Central Nervous System

CREB…cAMP Response Element-Binding Protein

CSF…Cerebrospinal Fluid

DNA…Desoxyribonucleic Acid

ELISA…Enzyme-Linked Immunosorbent Assay

EPO…Erythropoietin

Erk 1/2…Extracellular Signal-Regulated Kinase 1/2

ESC…Embryonic Stem Cell

GDNF…Glial Cell Line-Derived Neurotrophic Factor

GMP…Good Manufacturing Practice

GRO-α…Growth related oncogene-α

Gy…Gray

H&E… Hematoxylin & Eosin

HLF… Hepatic Leukaemia Factor

HSP…Heat Shock Protein

ICAM-1…Intercellular Adhesion Molecule - 1

IFN-γ…Interferon-γ

IL-4…Interleukin-4

IL-10…Interleukin-10

IF…Immunofluorescence

iNOS…Inducible Nitric Oxide Synthase

iPS…Induced Pluripotent Stem Cell

i.p.…Intraperitoneally

ISNCSCI… International Standards for Neurological Classification of Spinal Cord Injury

KLB…Klüver-Barrera Staining

LFB… Luxol Fast Blue Staining

LPS …Lipopolysaccharide

MB…Methylene Blue

MCI…Myocardial Infarction

MHC-I …Major Histocompatibility Complex I

MMP…Matrix Metalloproteinase

MNC…Mononuclear Cell

MP…Methylprednisolone

MRI…Magnetic Resonance Imaging

MSC…Mesenchymal Stem Cell

NGF…Nerve Growth Factor

NLI…Neurological Level of Injury

NSC…Neural Stem Cell

NSCISC…The National Spinal Cord Injury Statistical Center

NSAIDs… Nonsteroidal Anti-Inflammatory Drugs

OEC…Olfactory Ensheathing Cell

PBMCs… Peripheral Blood Mononuclear Cells

PBS… Phosphate-Buffered Saline

PDGF…Platelet-Derived Growth Factor

RECA-1…Rat Endothelial Cell Antigen-1

RNA…Ribonucleic Acid

ROM…Range of Motion

ROS…Reactive Oxygen Species

SCI…Spinal Cord Injury

SEM…Standard Error of the Mean

TBB… Turnbull Blue

TGF-β…Transforming Growth Factor-β

TNF-α…Tumor Necrosis Factor-α

TLR-4…Toll-like Receptor 4

TUNEL…Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling

VCAM-1… Vascular Cell Adhesion Protein 1

VEGF…Vascular Endothelial Growth Factor

vWF… von-Willebrand Factor

# CHAPTER ONE: Introduction

## 1.1 Spinal Cord Injury

### 1.1.1 Definition

The term spinal cord injury (SCI) summarizes alterations to the structural integrity of the spinal cord. This includes damage originating from different forms of trauma, disruption of vascular supply, diseases affecting the spinal cord or other mechanisms narrowing the spinal canal resulting in damage to structures within the canal followed by impaired neurological function. Clinical presentation offers a broad spectrum of symptoms depending on the extent of injury and level of affected spinal cord segment. Besides motor pathways also sensory and autonomous nervous system functions can be affected by trauma to the spinal cord, thus resulting in impaired bowel and bladder function.[1-11] Pain, a common aftermath of SCI, further complicates the management of patients suffering from injuries to the spinal cord.[6,10,12,13] Two terms describing the course of SCI need to be further defined, namely acute and chronic SCI. Acute SCI delineates the injury observable immediately after the onset, while chronic SCI describes the long-lasting neurological deficits following spinal cord trauma.[1-11] If not stated otherwise, SCI caused by acute trauma (=traumatic SCI) will be discussed in the text below.

### 1.1.2 Spine and Spinal Cord – Anatomy and Physiology

The central nervous system (CNS) comprises the brain and the spinal cord. The spinal cord enables afferent and efferent transduction of information in form of electrical activity. Furthermore, neuron circuitry within the spinal cord provides the anatomical and physiological basis for pivotal reflexes. Fibers carrying information regarding motor, sensory and autonomous function run within the spinal cord.[14-18]

The spinal cord is enveloped in a rigid bony cage, the vertebral column or spine. Besides absorption of axial blows and protection of the spinal cord, its unique structure allows passage of nerves from and to the spinal cord. The spine also forms the central point of the trunk transferring weight of head, neck and upper extremity to pelvis and lower extremity. Its anatomical design represents a compromise between stability and flexibility. Observing the spine from a lateral view one can appreciate its double S shape.[14] The 4 consecutive bendings absorb axial shocks and ensure that axial blows are not passed on to the brain. Twenty-four vertebrae, the sacral bone, which is the results of bone fusion of 5 vertebral bodies, and the tailbone, form the vertebral column. In between 2 vertebrae bodies intervertebral discs ensure uniform pressure distribution as well as absorption and movement

limitation of adjacent vertebrae.[14-16] The intervertebral discs comprise a gelatin-like nucleus and a rigid outer layer of connective tissue.[14] Prolapse of the inner mass, also known as disc hernia, can cause narrowing of the spinal canal and often results in neurological impairment often necessitating surgical decompression.[19] Seven cervical vertebrae form the cervical spine and create a connection between cranium and vertebral column. The cervical spine is characterized by the highest range of motion allowing adjustment of the head in the environment.[14] This high mobility explains increased susceptibility for SCI of the cervical segment with up to 76% of all SCI affecting the cervical spinal cord.[10] The cervical vertebrae are the smallest among all vertebrae and feature a foramen in each transverse process to form a canal for the vertebral artery bilaterally.[14,16] This allows neck movement without disturbing the blood supply of posterior intracranial aspects. The first, second and seventh cervical have unique characteristics. The most cranial or first cervical, also called Atlas, lacks a solid centrum and forms a boney ring supporting the cranium.[14,20] The dens, an osseous protuberance of the second cervical or Axis, occupies the anterior part of the Atlas. This enables rotation of the head against the cervical spine.[14,17,21,22] The seventh cervical features a prominent spinous process creating a visible and palpable heightening at the caudal end of the nuchal groove. The thoracic segment of the vertebral column is characterized by lower flexibility due to its tight connection to the rib cage.[14] All 12 thoracic vertebrae show similar characteristics while increasing in height in caudal direction. Five lumbar vertebrae make up the lumbar spine. Higher mechanical load in this area demands these vertebrae to be generally larger in size. The sacral bone is made up of originally five sacral bones that underwent bone fusion. It is an integral part of the pelvic girdle and connects the spine with the lower extremity. The tailbone represents a rudimentary structure at the caudal end of the spine with variable number of bones.[14-16] A vast repertoire of ligaments and muscles increase stability of the vertebral column but will not be discussed in detail in this thesis.

The spinal cord, part of the CNS, is covered on its entire course by the same three meningeal layers ensheathing also the brain. The dura mater forms an outmost rigid layer from the foramen magnum to the second sacral vertebra. Underneath, the arachnoid and pia mater form the remaining two layers.[14] The dura mater runs downwards on the entire course of the spinal canal until reaching the coccygeal bone as the so-called filum terminale durae matris realizing the caudal attachment of the dura mater. On top of the dura mater and underneath the vertebral canal fat tissue, blood and lymphatic vessels occupy the epi-/extradural space. The subarachnoid space contains cerebrospinal fluid (CSF) supplying the CNS with important nutrients also adding another buffer zone.[14,15] The spinal cord itself is shorter than the spine due to the relatively lower growth rate during development. It originates caudally to the medulla oblongata and extends to the level of the lumbar vertebra 1 or 2 to reach its caudal end, the conus medullaris.[14,18] The height of the lower end of the

spinal cord varies individually and was reported to be associated with body height.[14,15] The cauda equina, a loose batch of spinal nerves and spinal nerve roots also covered by the three meningeal layers, follows the conus medullaris caudally and occupies the remaining spinal canal. The spinal cord possesses two enlargements, one cervical and one lumbar enlargement. These represent sources of spinal nerves forming the brachial and lumbosacral plexus responsible for innervation of the upper and lower extremity, respectively. Each segment features one corresponding spinal nerve occupying foramina between two vertebrae, also called intervertebral foramina.[14] The nomenclature of spinal nerves is in accordance with their passage through the intervertebral foramina being named corresponding to the vertebrae above their respective outlet.[14,18] Spinal nerves contain both afferent and efferent neurons coming from the ventral and dorsal root of the spinal cord. While efferent neurons arising from the ventral root of the spinal cord are responsible for innervation of muscles, afferent neurons entering the dorsal root transmit information back to the CNS.[2,14,18] Each spinal nerve innervates a certain area of skin, referred to as dermatome, and a specific set of muscles, known as myotome.[14,18] The course of spinal nerves varies depending on the spinal segment. Spinal nerves located more caudally travel a longer distance to reach their respective intervertebral foramen due to the relatively smaller size of the spinal cord compared to the spine.[2,14] One important spinal segment to mention is the fourth cervical segment (C4) since it represents the origin of the phrenic nerve in most indivduals.[14] Complete lesions above this level lead to total loss of respiratory function necessitating artificial ventilation.[14] The internal organization bears white matter containing mainly axons connecting different parts of the CNS surrounding an area of dense accumulation of neuron cell bodies, called the grey matter.[14,15,18,23,24] This pattern represents the opposite to the organization found in the brain where grey matter is aligned on the surface for the most part. Cross sections of different segments reveal that the ratio of white to grey matter decreases in caudal direction. Reaching caudal segments more and more axons leave the spinal cord to reach their target explaining the observed decrease of white to grey matter ratio.[14,15] White matter is divided into different anatomical regions each bearing different tracts with different functions. The dorsal aspect in between the dorsal horns of the grey matter comprises large ascending tracts, fasciculus gracilis and fasciculus cuneatus are also known as dorsal columns. These axons transmit information regarding proprioception, vibration and touch.[2,14,15] In between the dorsal and lateral part of spinal cord white matter lays the dorsolateral tract, also called the tract of Lissauer, filling up the space between the dorsal horn and the spinal cord margin. Lateral aspects of the spinal cord bear spinocerebellar, spinothalamic, spino-olivary rubrospinal and corticospinal tracts among other smaller fibers and pathways.[25] In general, ascending pathways are located more laterally than descending pathways in the lateral aspect of the spinal cord. Spinocerebellar

tracts, as the name suggest, deliver information from the periphery to the cerebellum for processing of movement and coordination. Medial and ventral to the spinocerebellar tracts run the spinothalamic tracts delivering somatosensory information to the thalamus. Spino-olivary tracts project to the olivary nuclei in the medulla oblongata and convey signals from muscle and tendon proprioceptors.[14,23] The lateral corticospinal tract, also located in the lateral aspect of the spinal cord, represents a major descending pathway, projecting from the motor cortex somatotopically organized to motor neurons located contralaterally for the most part on different levels of the spinal cord. The rubrospinal tract projects from the red nucleus located in the midbrain to neurons on all levels on the spinal cord.[14,26] In contrast to many animals, including rats, the rubrospinal tract is thought to be of ancillary importance in humans.[26] In other species, recovery of motor function was shown to be a result of increased activity in the rubrospinal tract.[27] A smaller number of corticospinal fibers, the ventral corticospinal tract, are also located in in the ventral white matter of the spinal cord. Further located in the ventral part of spinal cord white matter are the tectospinal tract, vestibulospinal tracts, and the ventral spinothalamic tract.[14] The grey matter of the spinal tract can be divided in four areas, the ventral horns, the dorsal horns, and the dorsal and ventral commissure. Centrally, surrounded by grey matter, the central canal is located bearing CSF.[14,23,24] This surrounding grey matter comprises the ventral and dorsal commissure, respectively. Two ventral horns contain neurons with axons mainly leaving the spinal cord via ventral nerve roots. On the posterior aspect of the spinal cord two dorsal horns are present and represent the target of primary afferent neurons.[14,24] Bror Rexed, a Swedish neuroscientist, defined ten layers or laminae of spinal cord grey matter in 1952.[23,24] In contrast to the six layers in the cerebral cortex postulated by Brodmann in 1909, Rexed distinguished ten different laminae.[14,23,24,28] This classification was initially based on morphology of neurons and density in the respective layers and was later also shown to be valid for their different physiologic function.[14,23,24] Lamina I is located at the posterior end of the dorsal horn, followed by lamina II-V anteriorly. The dorsal and ventral commissures contain lamina VI and VII, respectively. Lamina VIII and IX are located within the ventral horn and lamina X surrounds the central canal. The grey matter of the spinal cord furthermore contains the intermediolateral nucleus, the dorsal nucleus and the nucleus proprius. While the nucleus proprius can be found on all levels of the spinal cord, the nucleus dorsalis is present on level T1 to L3-4. The intermediolateral nucleus occupies part of the grey matter between T1 and L2. These nuclei are responsible for intraspinal coordination, relay sympathetic fibers, pain and temperature processing or transmission of proprioception.[14,23,24]

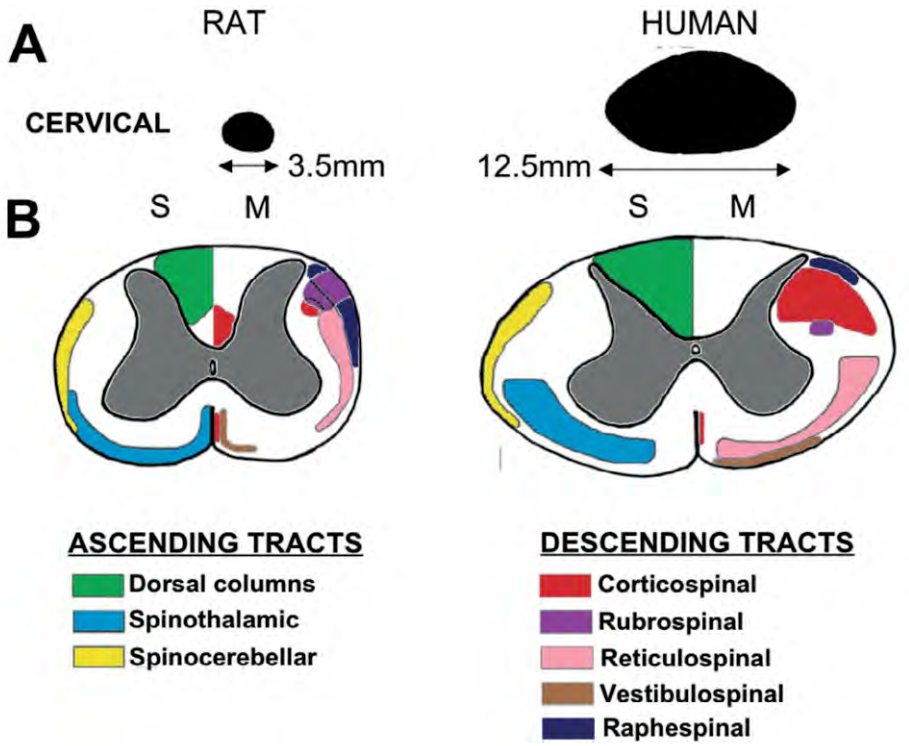


*Figure 1: Schematic illustration of organization of pathways within the white matter of the spinal cord in rats and humans. (Adapted from Watson et al.)*[25]

Blood supply of the spinal cord is delicately organized. Disturbances in the blood supply resulting in ischemia of the spinal cord can also lead to SCI.[14,16] Ventrally, one artery descends on top of the spinal cord´s anterior median fissure, the anterior spinal artery. It originates from both anterior spinal branches coming from the vertebral artery. On its course, various segmental medullary arteries feed the anterior spinal artery including the most prominent feeder, the major anterior segmental medullary artery or artery of Adamkiewicz. The artery of Adamkiewicz originates from either one of the lower intercostal arteries, from the subcostal artery or rarely from the one of the upper lumbar arteries and often carries the major blood supply for the lower two-thirds of the spinal cord.[14,16] The posterior view of the spinal cord reveals two major longitudinal arteries descending on the posterolateral sulci. These two posterior spinal arteries come either directly from the vertebral arteries or from the posterior inferior cerebellar artery. Again, segmental arteries anastomose with the two posterior spinal arteries on its path. The three longitudinal vessels meld in a plexus situated around the conus medullaris, the caudal end of the spinal cord.[14,16] Veins draining the spinal cord ensue a concomitant pattern to that of the arteries.[14,29] There are six main longitudinal veins, namely the anterior spinal vein, the posterior spinal vein, two anterolateral veins and to lateral veins forming a plexus, also called coronal plexus, located on the surface of the spinal cord within the subarachnoid space. Blood in the longitudinal part of the venous plexus

reaches the cranial sinuses and cerebellar veins, while segmental veins drain into caval and azygous veins via the intervertebral veins.[14,29]

As discussed in this section, the genuine structure of the spinal cord and its boney cage, the spine, allows flexibility needed for movement of the whole body and passage of neuronal structures while protecting the CNS from mechanical impacts. On the other hand, its sophisticated organization creates anatomical bottlenecks. If present trauma exceeds the protective capacity of the spine, damage to the spinal cord or to the spinal nerve roots is the consequence. But also other factors, like ischemia or lesions with mass effect can cause damage to the spinal cord resulting in neurological deficits.

### 1.1.3 Epidemiology and Economics of Traumatic Spinal Cord Injuries

An important review from 1995 estimated the worldwide annual incidence of traumatic SCI to range from 13 to 33 cases per million inhabitants.[30] Thereafter, studies reported an even higher variability with an incidence ranging from 10.4 to 83 cases per million people annually.[10,31-33] One explanation for this observed margin of deviation in the reported incidence of SCI is the different quantity and quality of data available for evaluation.[10,33] While some studies include patients with spinal cord trauma who died before admission to a hospital, other authors did not have access to data involving this particular patient collective.[10,33] Diverging volume of traffic and diverse recreational habits between countries might also add to discrepancies in incidence of injuries to the spinal cord.[10,31-33] Furthermore, most studies report numbers from developed countries, so estimations of worldwide incidence rates naturally underestimate the situation in undeveloped countries.[33] In 2005, the National Spinal Cord Injury Statistical Center (NSCISC) estimated 11,000 new patients with traumatic SCI per year in the USA, which corresponds to 40 cases per million persons.[34] Worldwide, estimates suggest 130,000 new SCI patients each year.[35,36] These numbers, however, do not include cases with pre-hospital mortality implying an overall higher incidence of spinal cord trauma.[33] Wyndaele et al. reported an estimated number of 15-56% pre-hospital mortality indicating a considerable higher overall incidence of spinal cord injuries.[33] All numbers mentioned above are related to SCI caused by any kind of trauma. Detailed reports or registries are missing for non-traumatic forms of SCI. If non-traumatic SCI was also included, total numbers were estimated to quadruple.[11]

Understanding the etiology of traumatic SCI is important in order to propagate the most effective form of treatment, which is prevention.[10] Different causes of injuries of the spinal cord have been reported with the leading cause being motor vehicle accidents.[10] Traffic accidents caused up to 58% of SCI.[10,31] Other etiological factors including falls, sport-related injuries, and violence among others have been reported.[10] Exact numbers of various causes of SCI are lacking due to different reporting standards including variable classification

systems and nomenclature.[10] Also, there are ethno-specific risk factors like carrying heavy loads on the head especially in third-world countries.[37,38] Etiology also shows a vast difference between age groups. While motor vehicle accidents represent the largest group in younger patients, the predominant cause of SCI in elder patients is falls.[10,39,40] Non-traumatic forms of SCI offer a broad spectrum of causes including developmental disorders, infectious diseases, ischemia, toxins and tumors among others. As mentioned above concrete numbers for these cases do not exist.[11]

The incidence peak of SCI was reported to range from the age of 20 to 40 years with a mean age of 31 years.[10] During past years the age of patients suffering from spinal cord trauma tended to increase. One possible explanation for this observed trend is the demographic development towards a more aged population in developed countries.[10,39,41-44] A lower mean age of patients with acute SCI in third-world countries, where an increase of the older population still holds off, confirms this suggestion.[10,37] Also, differences considering epidemiology of spinal cord trauma can be observed between female and male.[10] Several studies report a higher incidence among men compared to women.[10,45-47] The reports suggest a male-to-female ratio of approximately 3.5 to 1 with an increasing number of female patients during recent years.[10,33] Higher disposition to risks and exposure to violence could serve as an explanation for the higher numbers among the male population.[10,33] The observed gender spread even increases further in undeveloped countries to approximately 8 to 1.[10] More consolidated traditional roles in third-world country societies might lead to the observed higher spread in these countries.[10]

During recent years, novel therapeutic modalities and strategies lead to improved outcome and survival of patients affected by spinal cord trauma resulting in an increase of prevalence of chronic SCI. A recent review reported an annual prevalence of 485 per million inhabitants worldwide. This leads to 270,000 chronic SCI patients currently living in the United States and approximately 2.5 million worldwide.[33-36,48] It further shows that prevalence of SCI in Europe, North America and Australia was rising during past years.[33]

Spinal cord traumas cause tragedies for individuals and result in substantial emotional and social suffering for affected patients and their relatives.[49] Because SCI affects mainly young active persons during their most productive years, the increasing prevalence also represents a substantial economic strain.[10,11,33,48,50] Incurring costs for lifetime treatment of one individual suffering from SCI were reported to be as high as US$ 500,000 to $2 million dependent on the extent and location of injury.[11,50] This is also caused by often necessary long-term hospitalization at an average of 171 days within the first two years following SCI.[46] The total cost for SCI was estimated to be approximately $7 billion each year alone in the USA.[11,50] Besides treatment costs other factors need to be considered. Due to the low mean age of onset, SCI patients suffer from long-term disabilities and inability to work causing a

substantial tax deficit.[10,33] In the United States estimates for these additional indirect costs range from US$ 2.5 billion to 5.5 billion.[34,48]

#### 1.1.4 Pathophysiology of Spinal Cord Injuries

Injuries to the spinal cord are followed by a characteristic and complex pathophysiologic sequence. Initially, different forms of etiologies lead to damage and integrity loss of spinal cord tissue, also referred to as primary phase. Exogenous but also endogenous conditions can cause the primary insult.[35,51,52]

Different forms of trauma can cause a defective position of the spine and its enclosed spinal canal resulting in indirect damage to the spinal cord. But also direct damage to the spinal cord caused by trauma is possible.[35,36,51-53] Mechanics of trauma include rotation, acceleration and deceleration, stretching, compression, contusion, shear stress, penetration, maceration and laceration, each resulting in characteristic injury patterns and symptom complexes. The primary hit can further be distinguished by diverse morphologies of external trauma. The first possibility involves impact leading to a persistent compression of the spinal cord, e.g. caused by fracture of a vertebra.[35,36,51-53] This also represents the most common mechanism.[51] Secondly, temporary compression, with immediate return to regular alignment of the spine can also cause SCI. This mechanism is often causal in patients suffering from spinal cord injury with lack of detectable radiologic abnormalities of the spine. Distortion of the spinal cord represents the third possible morphology.[35,51-53] This is mainly present in children due to their still cartilaginous vertebrae and loose spinal ligaments, often without radiologic abnormalities.[51,52,54] Besides children, adults with degenerative spine disease are exposed to distortion and subsequent SCI.[51] Finally, direct discontinuation of spinal cord tissue via laceration or transection can lead to SCI representing the fourth possible morphology.[35,51-53] All possible trauma morphologies affect the grey matter to a larger extent.[51] Besides its central alignment, its softer texture, also caused by greater vascularity, predisposes the grey matter to larger extents of damage after trauma.[51,55] Disruption of vascularity leads to ischemia, again affecting grey matter to a larger extent due to its higher metabolic turnover.[51] This leads to a primarily vertical progression of the lesion site within the grey matter in the first 72 hours post injury.[32,51]

Aside from trauma, other endogenous and non-traumatic conditions can account for damage to the spinal cord. These causes include spinal cord infarction, mass lesions like tumors narrowing the spinal canal, toxins and abscesses amongst others.[11,35,56-62] These causes with their distinct characteristics will not be subject to detailed discussion in this thesis.

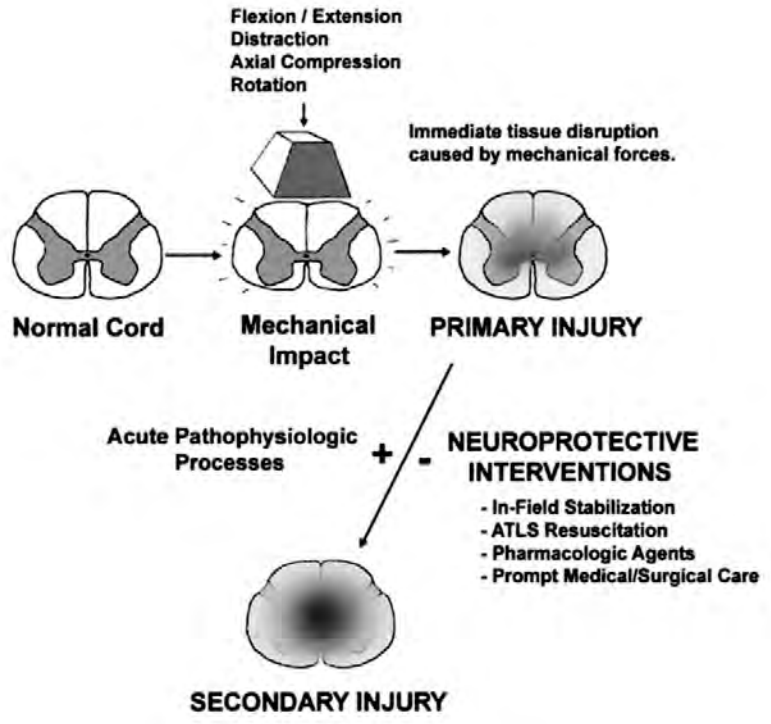


*Figure 2: Spinal cord injury and its pathophysiologic sequence. (Adapted from Kwon et al.)*[52]

Subsequent to the primary insult a secondary cascade, first postulated by Allen and colleagues in 1911, results in aggravation of the initial damage.[63,64] Morphology of initial trauma influences this secondary cascade, each mode of trauma creating unique patterns of secondary injury.[53] The myriad of mechanisms associated with the secondary phase in combination with the primary injury, leads to the final, at least up until today irreversible, extent of SCI.[35,51] Within this cascade, individual mechanisms do not act consecutively but rather simultaneously, influencing each other to form a vicious circle.[52] This downstream network offers starting points for possible therapeutic interventions for SCI patients, on the other hand. Secondary mechanisms occur within minutes after the primary event and last up to weeks or months.[35,65] The initial impact causes disruption of vasculature, mainly microvasculature, creating an ischemic environment within the spinal cord. Ongoing ischemia results in hypoxia leading to further cell-death.[35,52] Prior dying from hypoxia, cells are prone to swelling, thus further deteriorating perfusion of adjacent tissue by increasing pressure.[51] Ischemia following SCI aggravates also due to other reasons, such as edema formation, thrombosis, obstruction of venous drainage and vasospasms.[51,66-73] These local alterations are accompanied by systemic complications of SCI. Blood loss with subsequent hypovolemia due to concomitant injuries and loss of auto-regulatory homeostasis of systemic blood pressure. The latter is summarized under the term neurogenic shock and arguably caused by the loss of sympathetic tone with bradycardia and reduced peripheral vascular tonus.[35,52,74] This potentially causes cardiovascular and respiratory complications. Alterations of regulation mechanisms of peripheral blood flow can lead to potentially life-threatening venous and

pulmonary thromboembolic events.[51,52] The failure of systemic blood pressure regulation can also lead to reduction of cerebral blood flow, initiating cellular damage in the brain remote to the actual lesion site.[75] Besides the ischemic aftermath, vessel disruption results in micro-hemorrhages causing local necrosis over time.[51,52] Interestingly, this period of scarce blood supply is followed by a phase of increased perfusion.[35,51,52] The mechanisms behind this sudden increase in blood flow remain poorly understood but it is speculated that pH increase due to lactate accumulation plays a pivotal role.[51,76] This reperfusion phase causes production of reactive oxygen species (ROS). ROS are capable of damaging various cellular compartments including lipid membranes, mitochondrial respiratory chain proteins, metabolic enzymes and DNA (desoxyribonucleic acid) to name a few. Oxidative stress disrupts endothelium and thus aggravates the already precarious vascular situation following SCI.[35,51,52,77-80] A further complication of this endothelial damage is disturbance of the blood-brain barrier (BBB).[81,82] Most of mechanisms involved in the secondary cascade after SCI are interconnected with the production of and damage dealt by ROS, such as apoptosis, calcium overload and excitotoxicity.[51,79] Excitotoxicity, first postulated by Olney in 1978, refers to excessive secretion of glutamate, the most important excitatory transmitter in the CNS, and its deteriorate effect on neurons.[51,83] Toxic concentrations of glutamate are already present 15 minutes post SCI.[51,84] This high glutamate concentration causes uncontrolled firing of neurons with increased demand on nutrients and oxygen, intracellular accumulation of sodium with cellular edema and acidosis.[51,52] The electrolyte imbalance across neuronal cell membranes leads to uncontrolled influx of calcium ions ($Ca^{2+}$) persisting up to weeks following spinal cord trauma.[35,51,52] High levels of intracellular $Ca^{2+}$ is known to activate various proteases and kinases able to control gene expression, as well as to interfere with mitochondrial respiratory chain function.[35,51,52,80,85,86] Exemplarily, calpain is one the calcium-dependent proteases reported to be associated with breakdown of neuronal and glial structures after SCI.[51,85] Apoptosis or programmed cells death of cells building up the spinal cord can be a result of aforementioned insults coming from ROS or excitotoxicity. But, induction of apoptosis is also observable in areas distant to the injury up to weeks after the initial damage.[51,52,87-99] This represents an interesting and potentially preventable mechanism within the secondary cascade after SCI.[52]

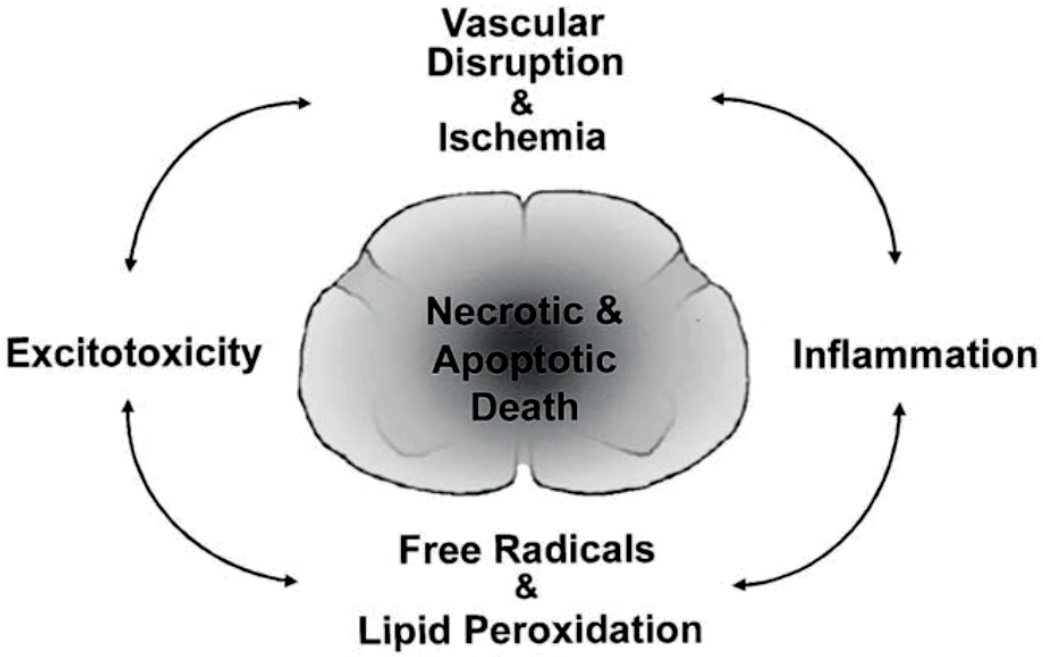


*Figure 3: Secondary mechanisms involved in damage progression following spinal cord injury. (Adapted from Kwon et al.)*[52]

The chronic phase after SCI represents the period of months to years after the primary injury. It is characterized by continued apoptosis leading to demyelination, as well as scar formation within the lesion site orchestrated by glial cells. This scar represents an obstacle for possible neuronal regeneration also by releasing factors inhibiting axonal growth, like proteogylcans and arachidonic acid derivatives.[35,100-102] Because of the naturally low regenerative capacity of neurons, already mediocre levels of inhibitory factors sufficiently suppress axonal sprouting.[100,103,104]

The primary and secondary phase of SCI, as described above, lead to final and irreversible spinal cord damage. This final stage is often referred to as chronic stage of SCI.[52,105] While mechanisms in the primary phase depend largely on trauma morphology and extent of input energy, the secondary phase is characterized by endogenous reaction to the injury leading to aggravation of spinal cord damage. Mechanisms involved in this secondary phase form a complex network of pathways perpetuating each other.[35,51,52] Besides abovementioned mechanisms, also the inflammatory response to SCI plays a pivotal role in this secondary cascade and will be discussed in the following section.

### 1.1.5 Blood-Brain Barrier, Immune Privilege of the CNS and Inflammatory Response to SCI

Besides the above-mentioned myriad of mechanisms, inflammation plays a central role in secondary damage to the spinal cord after spinal cord injury. The CNS in general represents an immune-privileged compartement.[106-110] Work done by Paul Ehrlich and his associate

Edwin E. Goldmann in the beginning of the 20th century already demonstrated that an anatomical barrier, known today as blood-brain barrier, prohibits unrestricted diffusion between the blood and the CNS.[108,109,111,112] In 1948, Paul Medawar was able to show that the CNS is an immune-privileged but not immune-isolated site in transplantation experiments using homologous skin grafts.[109,113] When homologous skin grafts were primarily transplanted into the CNS of recipient animals no rejection was observed. On the other hand, initial peripherally transplanted skin graft followed by transplantation into the CNS resulted in rejection.[109,110,113]

The blood-brain barrier, within the spinal cord sometimes referred to as blood-spinal cord barrier (BSCB), is realized by a complex composition of various cellular layers and intercellular constituents. The anatomical basis is an endothelial layer linked together with tight junctions denying paracellular passage of water-soluble molecules, including antibodies. Also, endothelial cells within the BBB demonstrate comparably low pinocytic activity and transcellular transit.[107-109,114-117] To meet the metabolic needs of CNS structures, these endothelial cells express specific abluminal and luminal transporters on their cell surfaces. The layer of specialized endothelial cells is mounted on a unique basement membrane harbouring a myriad of pericytes, regulating capillary blood flow and maturation of endothelial cells, as well as clearing cellular waste products, and antigen-presenting cells. This cellular-rich membrane is in close contact with feet processes coming from astrocytes, part of the glia-cell family and responsible for electrolyte homeostasis and structural support among other responsibilities.[107-109,114-116,118] A second, characteristic barrier can be distinguished, namely the blood-cerebrospinal fluid barrier (BCSFB) found in capillaries of the choroid plexus, the production site of CSF. Here, the endothelial layer lacks tight junctions allowing filtration of blood plasma for CSF production. On the abluminal side, choroid plexus epithelial cells (Kolmer cells) interconnected with tight junctions are responsible for CSF production. To accomplish this task, these cells express specialized transport molecules to realize secretory function and homeostasis of CSF electrolytes and nutrients.[107-109,114-116,118]

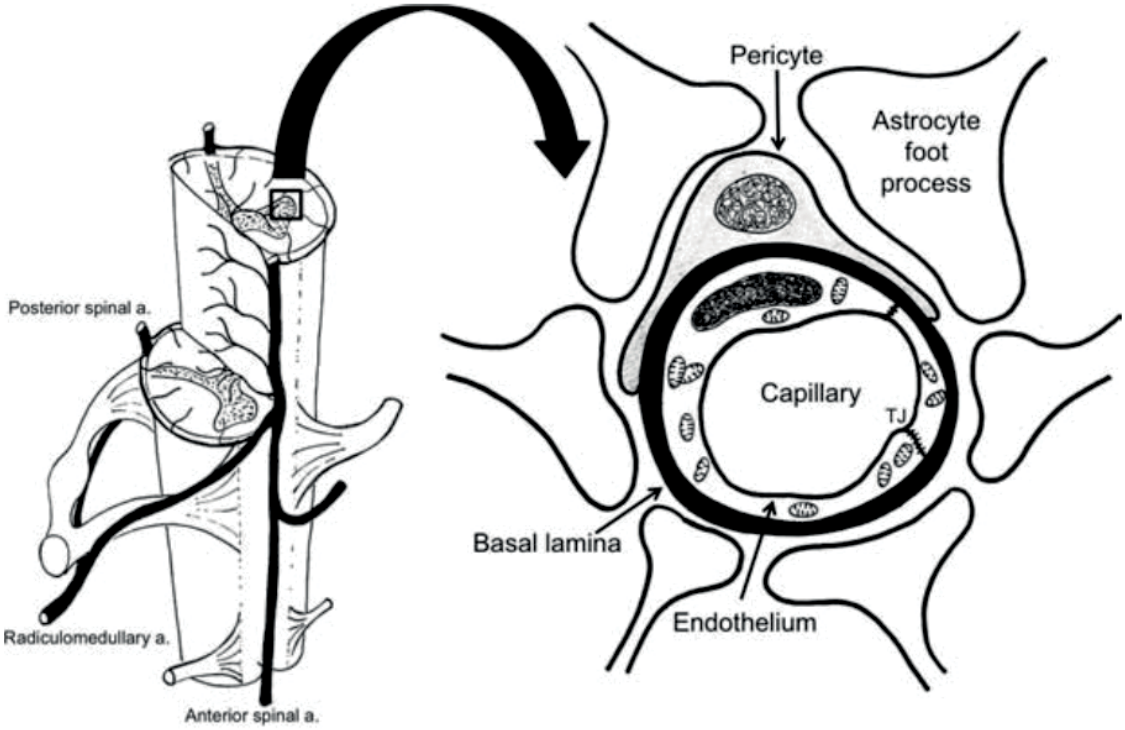


*Figure 4: Architecture of the Blood-Spinal Cord Barrier under physiological conditions. (Adapted from Bartanusz et al.)*[117]

Lacking of lymphatic drainage was thought to be characteristic for the CNS immune privilege.[107,108,114,115] A recent study reversed this dogma and described CNS lymphatic vessels for the first time.[119] The authors of this study localized these vessels in close proximity to the venous sinuses within the dura mater draining lymphatic fluid from the CNS towards deep cervical lymph nodes.[119] The best characterized route for this lymphatic transport is along the basis of the frontal lobe along olfactory rootlets through the cribriform plate of the ethmoidal bone.[114,119] These novel findings pave the way for new insights into neuroimmunology and pathophysiology of neurodegenerative and neuroinflammatory diseases.[119]

The BBB and its above-mentioned composition accomplished establishment of a stable environment for demanding CNS structures. Besides creating a specific surrounding in terms of nutrients, ions and osmotic characteristics, also cellular trafficking underlies strict regulation maintained by the BBB and BCSFB. Under physiological conditions, the primary entry site for leukocytes is thought to be across the BCSFB located within the choroid plexus.[115,120-124] As mentioned above, this barrier lacks tight junctions between endothelial cells, predestined to allow leukocyte diapedesis.[108,115] Several membrane proteins, including members of the Selectin-family, members of the Integrin-family, vascular cell adhesion protein 1 (VCAM-1) and chemokine ligand 20 (CCL20) among others are involved in this active cell passage. Around 175,000 to 500,000 cells are present in the CSF of healthy

individuals (around 1,000 to 3,000 cells per ml compared to 5,000,000 leukocytes per ml in the peripheral blood).[125] Besides the comparatively low cell count, also the composition of cell types varies. While neutrophils represent the largest leukocyte compartment in the peripheral blood, almost only lymphocytes can be found in the CSF, further indicating well-orchestrated and selective trafficking mechanisms.[114,125] In 2003, Kivisäkk et al. postulated that predominately $CD4^+/CD27^+$ memory T-cells are present in the CSF under physiological conditions.[116] These cells are not capable to exhibit immediate effector function, but play a crucial role in immunosurveillance.[116,126]

Besides above-mentioned rigid compartmentalization, specific attributes of structures within the CNS add to immunologic peculiarities. Brain-specific mechanisms to induce apoptosis deny auto-aggressive lymphocytes from attacking healthy CNS structures.[127] Furthermore, neurons lack expression of MHC-I (Major Histocompatibility Complex 1) under physiological conditions orchestrated by various neurothrophins. In case of impediment of electrical activity caused by disease, neurons start to express MHC-I on their surface and become a target for cytotoxic t-cells.[106]

This balance of immune-surveillance and quiescence in the CNS is disturbed after injuries to the spinal cord. The initial damage to the spinal cord is followed by release of pro-inflammatory cytokines including Interleukin-1, Interleukin-6 and TNF-α (Tumor Necrosis Factor-α) and IFN-γ (Interferon-γ) among others produced by resident cells of the CNS including astrocytes, oligodendroglia and neurons.[128,129] Besides activating microglia, pro-inflammatory cytokines in and around the lesion site induce up-regulation of adhesion molecules, like ICAM-1 (intercellular adhesion molecule-1) and VCAM-1, on the BBB allowing peripheral leukocytes to enter the spinal cord.[129,130] The physical disturbance of BBB integrity further benefits cellular infilatration.[35,131] The resident macrophages of the CNS, microglia, start to migrate to the lesion site within the first minutes after injury.[131-134] After activation, these cells cannot be distinguished histologically from macrophages derived from peripheral blood monocytes.[132,135] Blood-derived monocytes infiltrate the lesion site around 2 days after trauma with density peaks within the first week and can be detectable for weeks up to months.[130,133,136] Research in the area of monocytes and macrophages have lead to widely accepted opinion of 2 polarization states, namely M1 and M2. Pro-inflammatory signals like IFN-γ and the Toll-like receptor 4 (TLR-4) ligand, LPS (lipopolysaccharide), are known for their capacity to induce M1 polarization. On the other hand, Interleukin-4 (IL-4) and Interleukin-10 (IL-10) drive M2 polarization.[129,137-141] While M1 macrophages with their pro-inflammatory repertoire are thought to be detrimental for recovery after SCI, M2 macrophages deliver anti-inflammatory factors containing inflammation and promoting regeneration.[128,129,137-141] Until recently, it was unclear why predominantly M1-macrophages persist for weeks after injury to spinal cord since up-take of myelin in-vitro leads to an M2-

polarization and increase in anti-inflammatory cytokines.[128,142,143] Kroner and colleagues demonstrated that phagocytosis of iron and TNF-α is capable not only of inducing M1-polaization but is also able to reverse M2-polaization after phagocytosis of myelin.[141] These findings explain the high density of M1-macrophages within the spinal cord despite high myelin-turnover within the spinal cord after SCI.[141] Neuronal regeneration following SCI was recently reported to be tightly interconnected with macrophages in proximity to the lesion site. The authors of this recent study reported that macrophages sense hypoxia and induce angiogenesis. Newly formed vessels guide migration of oligodendrocytes and subsequently neuronal growth after SCI.[144] Recent studies have postulated that also the origin is crucial for the polarization and function of macrophages during the event of SCI.[124] While microglia-derived macrophages are thought to promote tissue damage via the production of ROS and other mechanisms, presence of blood-derived macrophages at the lesion site was associated with improved regeneration and motor recovery after SCI.[124,135,137] One study reported that monocytes mainly enter the CNS by the choroid plexus rather than in proximity to the lesion, which also underlines that SCI causes systemic inflammation.[124] Enlarging the pool of peripheral blood monocytes improved motor recovery while monocyte depletion lead to a worse neurological outcome in a SCI rodent model.[135] Interestingly, monocytes obtained from IL-10 knockout mice were not able to improve motor outcome.[135,145] These findings suggest a central role of IL-10 in polarization of macrophages during the event of SCI.[145] Clinical trials using IL-10 as therapeutic agent lead to inconsistent results due varying protocols including dosage, administration routes and time of treatment. Furthermore, IL-10 does not cross the intact BBB and undergoes rapid renal clearance complicating systemic therapy.[145]

Neutrophils are the first peripheral cell-type to enter the injury site approximately 3 to 4 hours after trauma and produce proteolytic and oxidative enzyme to prepare the lesion site for repair mechanisms but also causing further damage by lipid peroxidation and protein nitration.[131,133,136,146-150] Matrix-metalloproteinases (MMPs) secreted mainly by neutrophils cause aggravation of damage to the BBB by degradation of the basal membrane.[151-153] Pre-clinical studies showed that reduction of neutrophil influx is associated with improved motor function and reduced lesion extent after SCI.[129,146-150]

Migration of lymphocytes following injury to the spinal cord varies among different species.[129,136] In humans, T-cells, mainly $CD8^+$, infiltrate the lesion site within 3-7 days after trauma with increasing density over time while $CD20^+$ B-cells remain absent.[136] The specific role of lymphocytes in the inflammatory response to SCI is not yet fully grasped.[154] Evidence suggest involvement of lymphocytes in autoimmunity observed after SCI causing damage progression.[155]

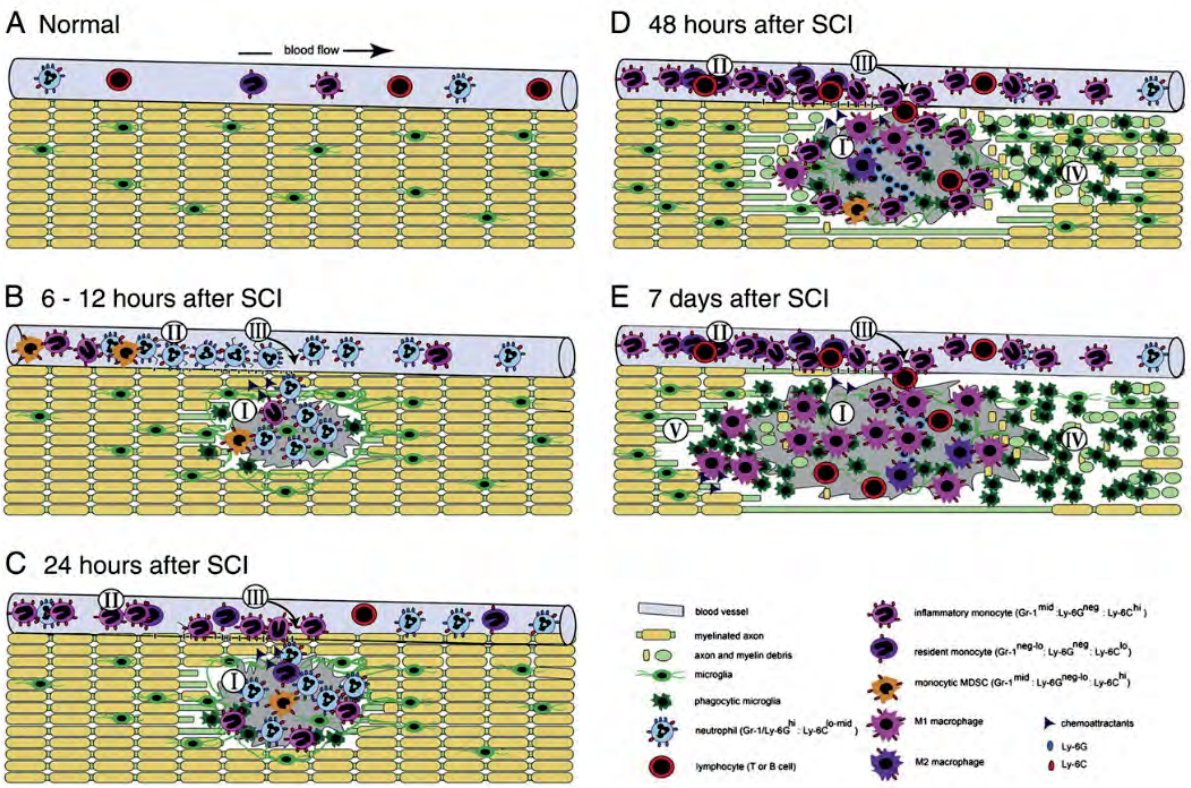


*Figure 5: Time course of cellular immune response following spinal cord injury (SCI). (Adapted from Plemel et al.)*[156]

### 1.1.6 Evaluation and Classification of Patients with SCI

Different causes, areas of damage and accident courses create unique forms of SCI leading to a vast spectrum of possible clinical signs and symptoms. Required therapy strongly depends on underlying pathomechanisms and injury extent. Therefore detailed classification of SCI is required to direct therapy and improve outcome of these patients.[9,18,157-160] The American Spinal Injury Association (ASIA) publishes the International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) to guide and standardize diagnosis and treatment of SCI.[18] This guideline allows assessment of SCI in all clinical environments and clinical phases utilizing only a fixing pin and a cotton swab.[18] The following section is primarily based on the latest version of the ISNCSCI.[18]

In suspicion of SCI a thorough neurological examination is indicated.[18,158] In order to determine the level of spinal cord lesion two systems, namely the motor and the sensory system, respectively, need to be investigated separately. The sensory examination requires bilateral evaluation of all 28 dermatomes from the second cervical segment (C2) to the forth and fifth sacral segment (S4-S5).[18] Crude touch and pain need to be evaluated separately on each dermatome to discriminate affected somatosensory pathways.[14,26] The ISNCSCI defines three possible scores, 0 being absent sensation, 1 meaning altered sensation and 2 representing normal sensation.[18] For evaluation of motor function the ASIA recommends

evaluation of 10 bilateral myotomes using a six-point scale.[18] To further delineate the level of SCI examiners describe a sensory, a motor and a neurological level of injury. The sensory level of injury describes the most caudal dermatome with intact sensation, while the motor level defines the most caudal key muscle within a myotome with full range of motion (ROM) against gravity. The neurological level of injury (NLI) represents the lowest area of intact sensation and antigravity muscle function. All three levels need to be evaluated separately for both sides after SCI since differences occur frequently.[18]

To classify patients with SCI the ASIA published the ASIA impairment scale (AIS) with five grades ranging from A to E. To discriminate between complete and incomplete lesions the sensory and motor function of level S4-S5 need to be examined. If neither motor nor sensory function is present at this segment the lesion is considered complete and represented by grade A. If one of these systems is still intact the term sacral sparing is used. Absent motor function at S4-S5, meaning no voluntary anal sphincter contraction possible, with intact sensation at this segment leads to grade B, also referred to as “sensory incomplete”. Grade C and D are used for patients with intact motor function but loss of sensation at level S4-S5. C represents motor incomplete injury with more than half of muscles below the NLI cannot be elevated against gravity and D also describes motor incomplete lesions but with more than 50% of muscles can be contracted against gravity, respectively. The highest grade, grade E, is used for SCI patients during follow-up with prior deficits and full recovery of both motor and sensory functions.[18] Many studies have shown that severity and extent of spinal cord trauma with the concomitant neurological grade are the strongest prognostic parameter.[51,161-168] Further clinical syndromes are described separately by the ASIA, namely central cord syndrome, Brown-Sequard syndrome, anterior cord syndrome, cauda equina syndrome and conus medullaris syndrome since patients suffering from these lesions experience a characteristic set of symptoms and deficits.[18] Besides motor and sensory deficits, SCI patients often suffer from concomitant dysfunction of vegetative systems, like bowel and bladder failure.[1-11] It was shown that neurological examination and grading of SCI patients requires intensive training for physicians to reduce inter-observer variability.[159] Magnet-resonance imagining (MRI) and electro-physical studies can contribute additional information about the injury extent and location, as well as required treatment management.[160,169-172] Other diagnostic tools and potential prognostic parameters for patients with SCI like molecular blood markers or genetic screening are still under evaluation.[160,173]

SCI patients can also be classified in a simplified manner by distinguishing between tetra- and paraplegia, meaning loss of motor function of all four extremities and remaining function in at least one of the extremities, respectively.[33] This simple differentiation is important since patients with tetraplegia experience a 6.6 times higher mortality.[11,33] The

proportion of patients with tetraplegia after SCI increased during past years according to published numbers.[33,174] Today, approximations indicate that up to 50% of SCI patients experience tetraplegia.[33] Higher lesions were reported to be associated with younger age of patients at time of injury.[175] Ackery et al. reported a high number of up to 77% of SCI patients suffering from pain.[10] These patients experience neuropathic pain in most cases, a condition with limited and often ineffective treatment options.[6,10,12,13]

Classification of spinal cord injury (SCI) severity using the American Spinal Injury Association (ASIA) Impairment Scale. The main categories of the Impairment Scale are as follows:

- A (complete): No motor or sensory function is preserved in the sacral segments S4–S5.
- B (incomplete): Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4–S5.
- C (incomplete): Motor function is preserved below the neurological level, and more than a half of key muscles below the neurological level have a muscle grade of <3.
- D (incomplete): Motor function is preserved below the neurological level, and at least a half of key muscles below the neurological level have a muscle grade of ≥3.
- E (normal): Motor and sensory functions are normal.

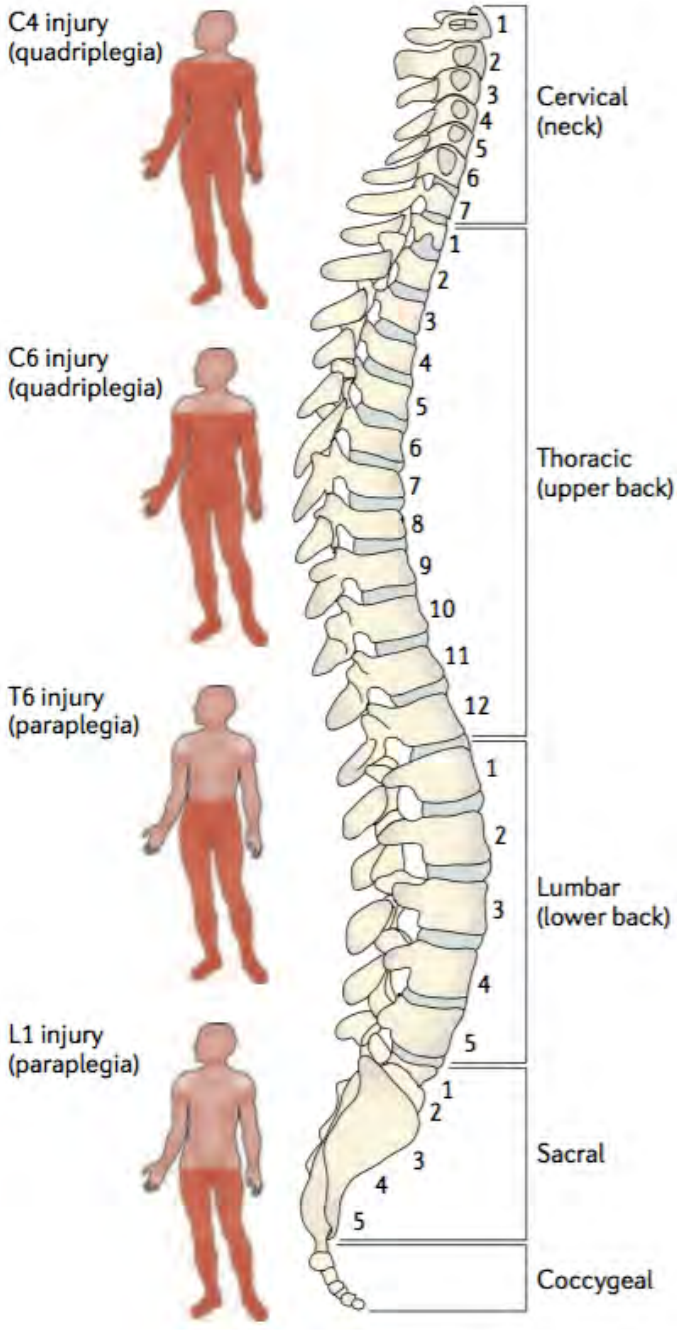


*Figure 6: Classification and Impairment Scale according to the American Spinal Injury Association (ASIA). (Adapted from Thuret et al.)*[36]

### 1.1.7 Management, Treatment and Regeneration of SCI – Today and Tomorrow

Today's treatment of acute SCI is based on surgical decompression at the level of injury. This aims to reduce secondary damage caused by oedema formation with subsequent increase of intraspinal pressure and reduced perfusion.[176-180] In case of accompanied vertebral instability, reposition, surgical stabilization and fusion of affected spine segments represent the therapy of choice. The purpose of this is to reduce dislocation of vertebral bodies narrowing the spinal canal with concomitant persisting compression of the spinal cord. Recently published data and common sense suggest decompression as early as possible.[176-180] Benefits of surgical intervention were reported for up to 72 hours post injury, although best outcome was achieved when patients underwent decompression within the first 24 hours post trauma.[176] Treatment with corticoids represents the only approved treatment of SCI besides surgery up until today. Despite its widespread clinical use in SCI patients, cortisone treatment remains an issue of controversial discussion.[181,182] In 2013, the American Association of Neurological Surgeons (AANS) changed their guidelines for treatment of acute SCI. They released a level I recommendation stating that "administration of MP (methylprednisolone) for the treatment of acute SCI is not recommended", which is in stark contrast to their recommendation pro MP-treatment after traumatic SCI released in 2002.[181,183] A series of reported complications and mediocre benefits regarding this treatment regime lead to the decision of changing their guidelines.[181,182] SCI are often accompanied by multiple injuries necessitating laborious polytrauma management in specialized level I trauma centres.[11] This circumstance further complicates and delays early specific SCI treament.[11,51]

Establishing new therapeutic approaches for the treatment of SCI represents a particularly challenging task due to following reasons. SCI injuries represent a comparable rare entity with heterogeneous clinical presentation including extent of neurological deficits and accompanying injuries. This complicates feasibility of prospective randomized clinical trials resulting in subprime study designs nurturing controversial discussion after completion of these trials.[184] SCI patients require, even without being enrolled in a clinical study, intensive monitoring and treatment resulting in high costs for clinical trials with cost estimates ranging from 50,000 to 100,000$ per patient.[35,184] Despite extensive efforts, only 1 treatment regime, intravenous therapy with methylprednisolone, was more or less successfully translated from bench to bedside within the last 37 years.[185] Besides these organizational and financial issues, the complex biology of SCI impedes discovery of potent therapies.

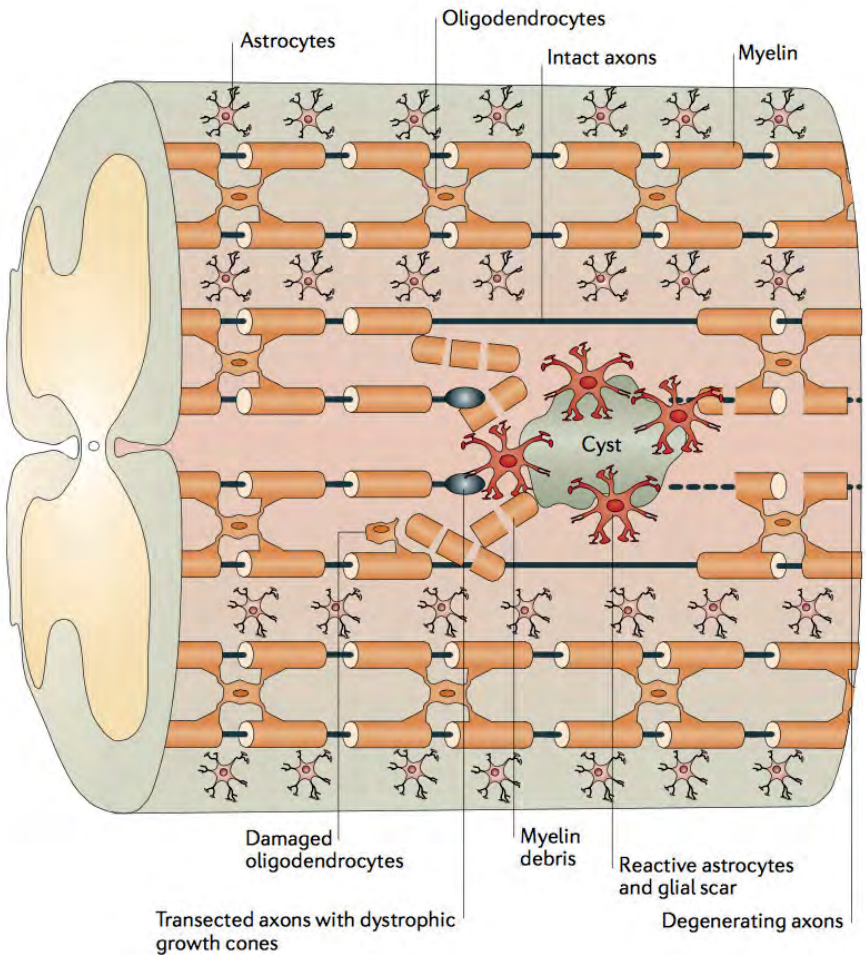


*Figure 7: Schematic illustration of spinal cord injury. (Adapted from Yiu and He)*[186]

Regeneration of injuries to structures of the CNS remains a challenging task due to various reasons. Growth and sprouting of neurons in the CNS is physiologically and after trauma inhibited mainly by 2 groups of molecules, namely myelin-associated molecules and proteoglycans, respectively. Damage to the spinal cord leads to myelin debris caused by neuronal and oligodendroglia cell-death leading to accumulation of molecules found on the surface of these cells.[63,186,187] A representative of the group of myelin-associated molecules is Nogo-A. It is expressed mainly by oligodendrocytes and inhibits axonal growth and collapse of growth cones when binding its receptor, NgR1. Another member of this group is the myelin-associated glycoprotein (MAG), also inhibiting neuronal growth.[63,186,187] Both molecules involve Rho, a small guanosine triphosphatase important for regulation of cell motility among others, in their intracellular signal transduction.[63,188,189] Blockade of either molecule to reverse their inhibitory capacity failed to deliver beneficial results in pre-clinical trials suggesting a more complex network of factors causing the inhibitory environment.[63] The second group, proteoglycans, is known for its role in formation of glial scar after trauma to the spinal cord injury.[63,190-192] Astrocytes, oligodendrocytes and meningeal cells express proteoglycans under physiological conditions primarily during development to guide axonal

growth and stabilize synapses.[63,193,194] The intracellular signal transduction is similar to that of myelin-associated molecules.[63,191,192] Another limiting factor for axonal regeneration after SCI represents glial scar formation. Following trauma, primarily astrocytes form the glial scar by changing their morphology and modifying the extracellular matrix. Other components of the glial scar are microglia, endothelial cells and fibroblasts. This fibrous remodelling strongly inhibits axonal growth and regeneration.[63,195,196] On the other hand, it was shown that glial scar formation delivers beneficial assets to the traumatized spinal cord like containment of inflammation and rebuilding of the BBB. Depletion of reactive astrocytes leads to worse clinical outcome in experimental SCI studies.[63,197,198] Therefore, the role of glial scar and possible modification by intervention remains controversial.[63] Despite abovementioned restrictive circumstances, neuronal regeneration takes place to a certain amount after CNS trauma combined with reorganization known as neuronal plasticity.[199,200] This, per se desirable partial regeneration of neuronal networks and endogenous attempt to restore spinal cord function, is often accompanied by deteriorating effects like central pain sensation, spasticity and aberrant autonomic regulation due to formation of new disorganized neuronal networks.[63,201] Also, apoptosis of oligodendrocytes after SCI leads to demyelination of spinal cord tracts associated with impaired neurological recovery.[202]

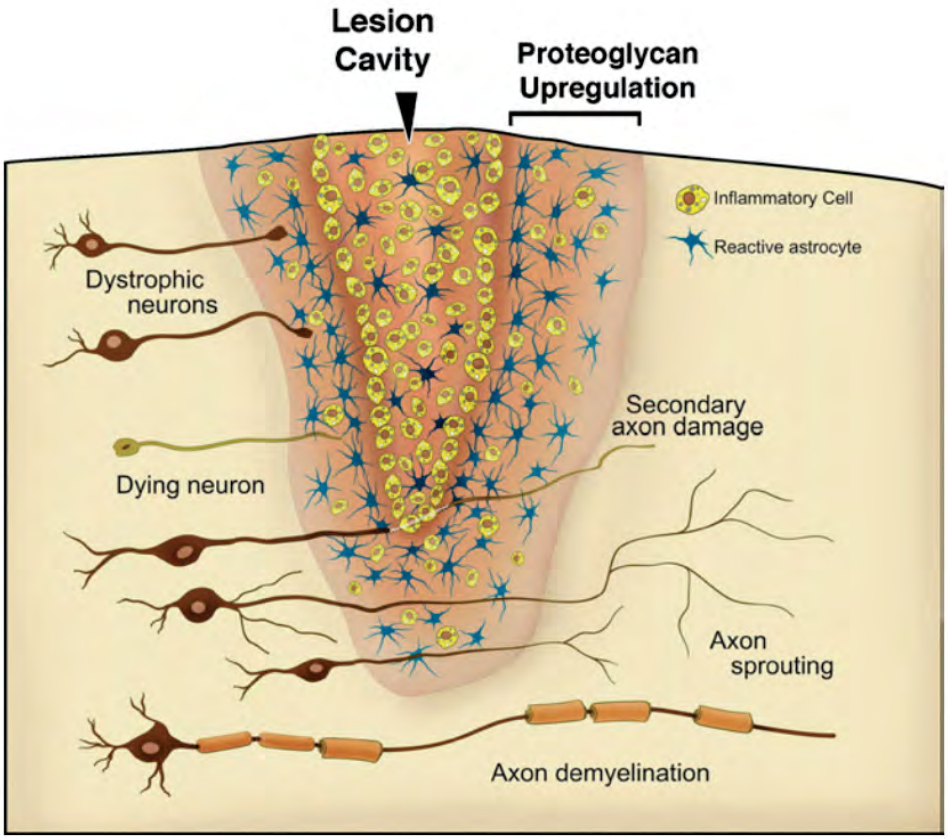


*Figure 8: Mechanisms involved in impeded neuronal regeneration after spinal cord injury. (Adapted from Fitch and Silver)*[101]

Despite this grim outlook, substantial effort has been undertaken to regenerate damaged spinal cord tissue and improve neurological outcome after SCI. Many potential novel treatment regimes with a broad spectrum of molecular and cellular targets were investigated during past years. Like in many other fields of regenerative medicine, cell therapy represents also a hot topic in SCI research.[35,52,63,203,204] Different cell sources as regenerative treatment after SCI were investigated including neural stem cells (NSCs) obtained from developing or adult CNS and expanded in vitro, mesenchymal stem cells (MSCs), olfactory ensheathing cells (OECs), Schwann cells embryonic stem cells (ESCs), activated macrophages and induced pluripotent stem cells (iPS).[63,203,205-256] All treatment protocols aimed to increase the concentration of potentially beneficial cell types at the lesion site to induce and promote regeneration after SCI. Although promising experimental data were reported, cell-based therapy failed to provide convincing and consistent results in clinical studies up until today.[63,255,257] Aside from poorly designed clinical trials, other specific problems like lacking cell-differentiation and depletion of administered cells are held responsible for previous failures.[63,255,257] Also ethical and safety issues have been addressed, especially in context of the use of embryonic and neural stem cells.[63,258-260] Another limitation is caused by often difficult and expensive handling methods to obtain, cultivate and expand cells for therapy in laboratories. Novel approaches, like the use of iPS cells, need to undergo further safety and feasibility studies before clinical trials can be initiated.[63] More and more studies investigated the therapeutic capacity of secretomes obtained from different cell types.[238,261-278] This issue shall be discussed in detail in chapter 1.2.4.

Aside from cellular therapy, approaches utilizing single molecules represent another treatment strategy to improve neurological outcome after SCI. Since the secondary damage following SCI summarizes a myriad of pathomechanisms and intracellular pathways a large number of potential molecular targets exists.[63,156] Prominent examples for the attempt to attenuate inflammation and reducing secondary damage following trauma to the spinal cord includes administration of IL-10, minocycline, non-steroidal anti-inflammatory drugs (NSAIDs) like indomethacin and ibuprofen, immunoglobulin G, atorvastatin, erythropoietin (EPO), riluzole and magnesium.[63,156,279-310] In 2012, a phase II-trial proved the safety of minocycline in SCI patients with a tendency towards improvement in some clinical parameters compared to the placebo group paving the way for an on-going prospective randomized clinical trial with minocycline with results expected in 2018.[311] Riluzole, a sodium-channel inhibitor, is an established treatment for patients suffering from amyotrophic lateral sclerosis (ALS) and its safety was already published in SCI patients.[312,313] First data considering efficacy of this treatment are expected for the end of 2015.[313] Despite promising data unsolved issues remain. Besides lack of reproducibility and contradictory publications, different dosage protocols and safety concerns resulted in low number of clinical trials up until today.[63,314]

More recent experiments utilized fenretinide, a vitamin A analog, triarylmethane-34, a selective inhibitor of the calcium-dependent potassium channel inhibitor, and recombinant secretory leukocyte protease inhibitor to dampen the secondary injury after SCI in a pre-clinical setting with promising results.[63,315-317] Another strategy to treat spinal cord injury by single molecules is to stimulate axonal regeneration and restore integrity of spinal cord cellular structure. To accomplish this task neurotrophic factors like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) among others were tested in pre-clinical studies.[52,63,204,318-327] Interestingly, experimental studies reported that only the combination of factors showed beneficial effects, while treatment with single factors was even associated with worse outcome.[326,327] One explanation for this paradox seems to be the complex architecture of the spinal cord requiring stimulation on multiple levels, while stimulation of a single target might cause even further disorganization like leaky BBB with subsequent increased oedema formation.[52,63,204,326] Being the downstream effector molecule for most growth factors, cAMP (3'5' cyclic adenosine monophosphate) and its direct intracellular accumulation was shown to exert therapeutic capacity.[63,234,304,328-337] For instance, inhibition of hydrolysis of cAMP with rolipram delivered promising results in pre-clinical experiments.[234,335,336]

During the event of SCI disruption of the complex microvascular structure within the spinal cord initiates and maintains substantial parts of secondary events aggravating the injury extent. This includes hypoxia, oedema formation, acidosis, excitatory neurotoxicity, activation of blood coagulation and increased extravasation of inflammatory cells among others.[35,52,151] Therefore many studies aimed to restore vascularization and protect remaining vessels after SCI. Various approaches were and still are under evaluation including cellular therapy, nitric oxide (NO), sildenafil, tissue engineering techniques, as wells as growth and angiogenic factors.[256,263,264,338,339] Due to the complex communication and interaction with glial cells to form the BBB, vessels in the spinal cord incorporate special characteristics and their regeneration remains a difficult task.[151,340]

Further strategies to promote regeneration and inhibit secondary damage after SCI were reported in literature, one example being tissue engineering. Efforts in this area aim to improve survival of grafted cells, to provide a microenvironment for enhancement of endogenous repair and to modulate the inflammatory response.[63,341] This area of regenerative medicine is still in its infancy but is already tainted with high expectations not only in spinal cord repair.[63,342,343] Also, hypothermia in the acute stage of SCI was associated with improved outcome and might enter clinical routine in the near future.[344-346]

Above mentioned strategies aim to modulate events occurring in the acute to sub-acute phase after SCI. Also chronic SCI is associated with limited treatment options due to various reasons. Therapy includes control of symptoms like neuropathic pain and autonomic

dysregulation, psychological support, rehabilitation and walking aids up to exoskeletons but will not be discussed in detail in this thesis.

### 1.1.8 Experimental Models of Spinal Cord Injury

Over past decades a multitude of diverse animal models was used for studying SCI and investigating efficacy of potential novel therapeutic approaches. Besides the use of different species, different approaches to experimentally damage the spinal cord have been postulated. In general, models for SCI can be divided in contusion and transection models.[105] The most commonly used model is the contusion model since it resembles trauma mechanism most frequently seen in humans. Contusion to the spinal cord can be applied via surgical clips, balloon-compression, weight-drop or computer-controlled devices.[3,105,347-351] Besides high-costs and elaborate experimental setup, models utilizing computer-controlled contusion feature many significant advantages including high reproducibility, comparable trauma morphology and variable injury extent.[3,351] Also, this experimental setting is thought to be most applicable to study pathophysiology and potential therapy approaches in the acute phase of SCI.[105] Most contusion models require laminectomy of the target spinal segments to ensure proper and reproducible alignment of the used contusion device with one exemplary exemption being the balloon compression model. In this particular model, the required instrumentation can be performed percutaneously.[352,353] On the other hand, for studying axonal regeneration and tissue engineering strategies transection models are thought to be more applicable. Different levels and extent of transection can be utilized.[105]

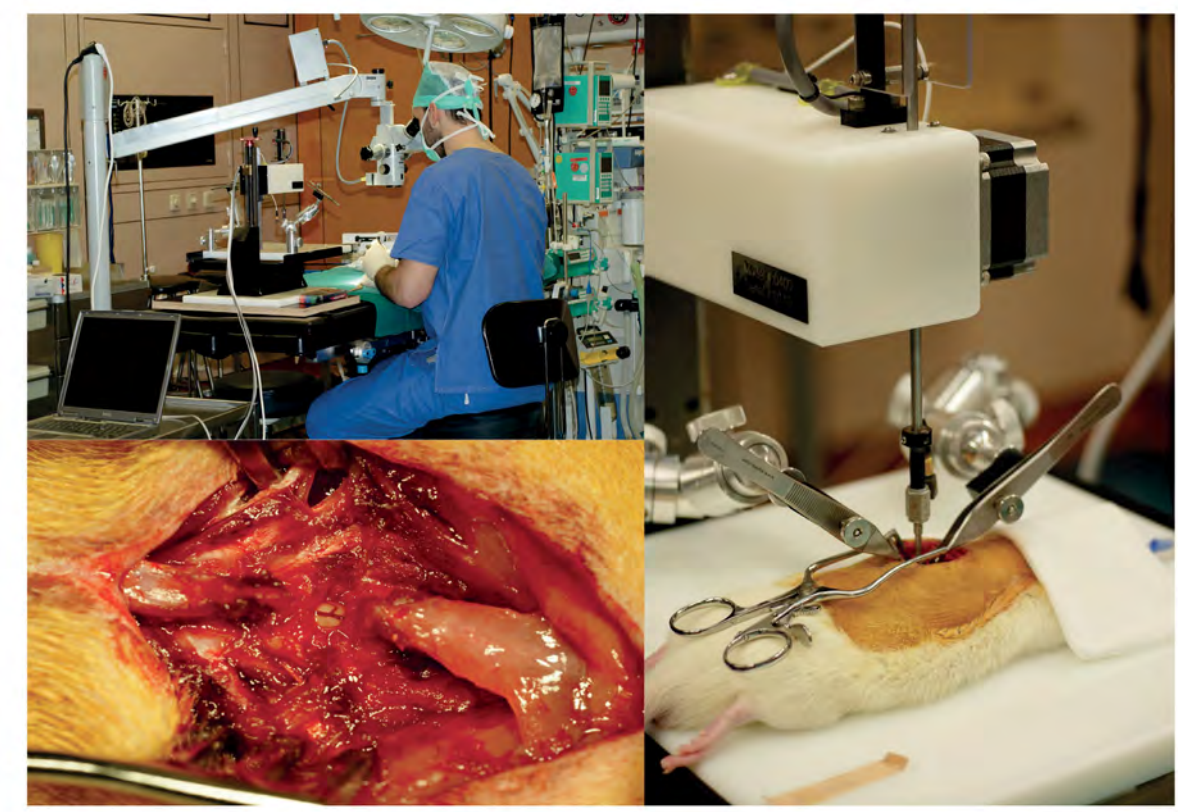

*Figure 9: Experimental setup of SCI contusion model used in this thesis. Upper left: Surgical microscope is used for exposing the spinal cord, computer is connected to the contusion device; lower left: exposed spinal cord with the posterior spinal vein visible; right: alignment of the rat in the contusion device.*

## 1.2 Cell-Based Therapy

### 1.2.1 Definition

Injuries and diseases often lead to celluar loss with subsequent functional deficits. For instance, hypoxia in the event of myocardial infarction (MCI) causes death of myocytes followed by reduced left ventricular ejection fraction or, as already mentioned above, injuries to the spinal cord lead to irreversible cellular loss accompanied by persisting neurological deficits. One concept of cellular therapy is to replace these lost cells lost after disease or injury to regenerate affected tissue and restore functionality.[354] Another possible implementation of cell therapy is application of immune cell with the intent to activate cell-mediated immunity against cancer cells.[355] Kick-off for utilizing cells as treatment in humans was pioneer work done by Mathé in 1959. He performed bone marrow transplantation in six patients, who were previously being exposed to high radiation doses.[63] Following his publication a multitude of studies investigated the applicability of cell therapy in various clinical settings.[354] As addressed in chapter 1.1.7 different cellular sources can be utilized and have been investigated for safety and efficacy in SCI.

### 1.2.2 Clinical Application

In the early 2000s, first randomized clinical trials utilizing cell therapy in patients suffering from acute myocardial infarction (AMI) were published.[356-358] Short-term results showed that patients after application of autologous progenitor cells experienced improved left ventricular function compared to control groups.[356-360] However, initial enthusiasm was followed by disenchantment due to negative long-term results showing no observable benefit of cellular treatment.[359,360] Meta-analyses further supported disappointing long-term results of this therapeutic intervention in AMI.[361-363]

Besides AMI, other diseases and their pathophysiology implicate the potential benefit of regeneration with cellular therapy and replacement. For instance, neurological disorders like stroke, Parkinson's disease, ALS, Huntington's disease and multiple sclerosis were and currently are under investigation for responsiveness to cellular therapy. Up until today, all studies failed to provide convincing data to support cellular treatment in abovementioned diseases.[364] Trials investigating cellular treatment in SCI are the topic of chapter 1.1.7.

### 1.2.3 The Dying Stem Cell Hypothesis

Initial positive results from trials investigating stem cell therapy in AMI patients were not able to fully elucidate the mode of action. Since only a minority of administered cells was found within the heart, direct mechanisms accomplished by transplanted cells seemed unlikely.[365] In 2005, Anker and his co-workers picked up that these studies reported a viability of injected cells ranging from 75% to 95%.[366] Some protocols even used irradiation of BMSCs prior autologous transfer.[367] Therefore, a significant amount of cells undergoing apoptosis was administered in these trials. Apoptotic cells are known for their capability to induce immunosuppression. For example, after phagocytosis of apoptotic cells macrophages start to secrete anti-inflammatory cytokines like IL-10 and transforming growth factor-β (TGF-β). AMI causes systemic inflammation leading to oxidative stress and other deteriorating factors.[366,368,369] Taken together, these findings lead to the hypothesis that therapeutic effects observed after stem cell therapy in these studies was a consequence of administration of apoptotic rather than viable, potentially trans-differentiable stem cells.[366]

Around 200 to 300 billion cells undergo apoptosis and subsequent clearance by the immune system physiologically every day. This vigorous process is accomplished without initiating an inflammatory response by up-regulation of immunosuppressive and inhibition of pro-inflammatory pathways mediated by apoptotic cells.[370] Further attributes have been accredited to apoptotic cells. For instance, regeneration can be induced by interaction of apoptotic cells with surrounding tissue in a process called apoptosis-induced compensatory proliferation.[370,371] Also, up-regulation of cytokines associated with angiogenesis upon co-incubation with apoptotic cells was reported.[372,373] This broad-spectrum of attributes is most

likely a tribute to the central role of apoptosis during development of multi-cellular organisms.[370]

### 1.2.4 From Cellcentric towards Secretome-Based Therapy

Therapeutic capacity of cell therapy in preclinical and clinical studies is thought to act through two possible principles of actions. Firstly, administered cells settle at the diseased organ and replace injured tissue. This possibility, however, can account if at all only in part for observed effects due to abovementioned reasons.[365,366] Secondly, applied cells act via intercellular communication causing local or systemic endogenous reactions like mobilization of resident pluripotent cells or immunomodulation.[265-267] To accomplish information exchange between cells either direct cell-cell interaction or paracrine signalling can be utilized. While the first requires specific ligand-receptor interaction, cell junctions and/or adhesion molecules, the latter can be conducted via secreted molecules.[267,374] While proteins are well-known paracrine mediators, other molecules like lipids, vesicles and RNA (ribonucleic acid) have attracted attention during recent years as further carriers for intercellular information.[375-378]

In 2005, the Italian researcher Gnecchi and his colleagues postulated that the therapeutic effect of stem cell therapy in AMI is a result of paracrine factors released by administered cells.[265] They found that administration of MSCs overexpressing the survival gene Akt1 achieves superior results compared to unmodified MSCs in a model of acute AMI. Beneficial effects were observed already 72 hours after administration suggesting a mechanism different from cardiomyogenic transdifferentiation of applied MSCs. Results were comparable when only conditioned medium of hypoxic MSCs without the cells themselves was used. Gnecchi and his colleagues concluded that secreted paracrine factors rather than implantation and transdifferentiation of applied cells account for the observed therapeutic capacity in AMI.[265]

During recent years, acceptance for the paracrine paradigm increased leading to a large number of pre-clinical trials in different animal models.[267,374,379,380] Postulated modes of action of conditioned medium include angiogenesis, up-regulation of anti-apoptotic pathways, immunomodulation, induction of tissue repair and remodelling and recruitment of endogenous progenitor cells.[379,380] Also, a recently published study utilized conditioned medium obtained from MSCs in a model of SCI as promising therapeutic agent.[261]

Treatment utilizing cell transplantation is accompanied by various difficulties. Extraction, production and preparation of cell suspensions represent time- and resource-consuming tasks, all of which have to be conducted under strict protocols meeting requirements of responsible authorities in specialized facilities. Further challenges include potential tumorigenicity, immune-compatibility and infection with intracellular pathogens.[380] Also, ethical issues, especially in connection with utilizing ESCs for regenerative treatment

have been addressed.[63] These difficulties can be avoided or at least be facilitated using conditioned medium alone without the cellular fraction.[380] Other potential advantages include off-the-shelf treatment, especially in acute conditions like AMI, stroke or SCI.[276,381] Besides these benefits, challenges for secretome-based therapy remain. Due to countless factors within the secretome, only fragmentary predictions of possible interaction during physiological and pathological conditions can be accomplished. Therefore, finding of optimal treatment protocols including timing, dosage and indication represents a difficult task. Lack of one effector molecular complicates the definition of exact storage conditions and quality parameters.[379,380]

In summary, initial cellcentric view shifted towards interest in secretome-based therapies in regenerative medicine. Despite mentioned challenges, promising pre-clinical results promote efforts being made to translate this therapeutic approach from bench to bedside in the near future.

## 1.3 Apoptotic PBMCs and their Secretome

### 1.3.1 PBMCs

All circulating nucleated blood cells are summarized under the term peripheral blood mononuclear cells (PBMCs) and can be isolated by venous blood-withdrawal with subsequent density gradient centrifugation. Lymphocytes, mainly T-cells, and monocytes represent the predominant fraction of PBMCs.[382]

### 1.3.2 Apoptotic PBMCs, their Secretome and Pre-Clinical Results

The hypothesis postulated by the group around Anker in 2005 implied that apoptosis rather than pluripotency is the difference-maker and key to therapeutic capacity in cell-based therapy without restriction to a certain cell-type.[366] Gnecchi and co-workers postulated secretory factors as mediators of cellular treatment.[265] Taken together, both hypotheses suggest secretory molecules by apoptotic cells exhibit therapeutic capacity in AMI.[267,366] Previous published data by Wollert and his group showed that expression profiles and secretory capacity of PBMCs and MSCs are much more comparable than initially thought. Analysis and comparison of both conditioned media revealed significant differences in only 35 out of 174 investigated factors. They furthermore showed that PBMCs and their secretome exhibit comparable biological activity. [367]

In 2009, these ideas were picked up and brought together by Ankersmit et al. using apoptotic PBMCs instead of progenitor cells in an acute model of AMI in rodents. Apoptosis was induced via caesium irradiation with 60 Gray (Gy) with reported apoptosis rates of over 90%. Injection of apoptotic PBMCs lead to smaller infarction area and improved left

ventricular parameters compared to the control group. Suggested underlying mechanisms included up-regulation of pro-angiogenic factors, dense CD68-positve cell infiltrate, homing of cells expressing progenitor markers, up-regulation of elastin expression in scar tissue, inhibition of ventricular remodelling and immunosuppressive features of apoptotic PBMCs.[383,384] Administered cells were found only in the liver and spleen but not in the heart.[384] Both human and rodent PBMCs were used with no reported difference in efficacy and therapeutic capacity.[383,384] This lead to further experiments utilizing conditioned medium/secretome of apoptotic PBMCs without the cellular fraction. Application of only this MNC(mononuclear cell)-secretome showed comparable results with decreased infarction areas and improved left ventricular function compared to the control group.[381] Another possible mechanism involved in beneficial attributes of the secretome is reported inhibition of thrombocyte aggregation and vasodilation leading to lower levels of micro-vascular obstruction (MVO).[276] Furthermore, up-regulation of anti-apoptotic and survival pathways in primary human cardiac myocytes was shown. The authors reported higher phosphorylation levels of intracellular factors involved in the so-called RISK pathway. This pathway is associated with attenuation of ischemia reperfusion injury and involves AKT, Erk 1/2, p38 and MAPK. Also expression of BAG1 and Bcl-2, genes counteracting apoptosis, was found to up-regulated in cardiomyocytes upon incubation with MNC-secretome.[381] Analysis of the secretome showed a myriad of present factors including VEGF (vascular endothelial growth factor), Interleukin-8 (IL-8), GRO-α (growth related oncogene-α) among others. Inhibition of various factors did not decrease capacity of the secretome to induce pro-survival and anti-apoptotic pathways. The authors concluded that the unique composition and interaction of components leads to observed therapeutic capacity rather than a single factor.[381] Therapeutic capacity was further shown in various pre-clinical studies. MNC-secretome was shown to exhibit immunosuppressive characteristics via induction of apoptosis in T-cells and therapeutic capacity in an experimental myocarditis model. Histological analysis revealed that myocardial inflammation was absent in animals receiving MNC-secretome.[277] Topical application of secretome obtained from apoptotic PBMCs lead to improved wound healing in an acute wound model in mice. The authors found improved re-epithelialization rates in animals treated with MNC-secretome.[275] Migration capacity of dermal fibroblasts and epidermal keratinocytes was increased after incubation with MNC-secretome in an in-vitro scratch assay. In-vitro and in-vivo observed angiogenesis after treatment with conditioned medium obtained from apoptotic PBMCs also served as an explanation for improved wound healing.[275] Furthermore, therapeutic capacity of MNC-secretome was observed in a burn model in large animals. Treatment lead to increased vessel density within the burn wound and improved scar quality (Hacker et al., unpublished data). Recently, reduced infarct volume and improved neurological outcome after treatment with MNC-secretome was

postulated in an experimental stroke model.[278] Increased phosphorylation levels in-vitro of CREB (cAMP response element-binding protein), cJun, Akt, Erk1/2 (extracellular signal-regulated kinase 1/2) and HSP27 (heat shock protein 27) were observed in primary human neurons and glial cells. These pathways were previously associated with cytoprotection and cell-survival.[278] In case of stroke, induced overexpression of HSP27 in rats showed reduction of infarct size of up to 30% after occlusion of the middle cerebral artery.[385] The transcription factor CREB plays a central role in orchestrating cellular response and gene expression in presence of cellular stress like hypoxia and oxidative stress. Phosphorylation of CREB was associated with cell survival and neuronal growth in acute neurologic conditions.[386] Up-regulation of the CREB pathway was also reported to be associated with increased BDNF production. This growth factor showed capacity to regulate and promote axonal growth, re-myelinisation and synaptic plasticity leading to improved functional outcome after SCI.[325,387] Upon administration of MNC-secretome in healthy rats, increased systemic plasma levels of BDNF were found and might be a direct consequence of up-regulation of the CREB pathway.[278] Phosphorylation of CREB was shown to increase after incubation with MNC-secretome in a dose dependent manner. Functionally, this up-regulation resulted in promotion of neurite growth in-vitro.[278]

To further dissect the underlying mechanisms involved in therapeutic capacity of MNC-secretome treatment in various pre-clinical settings, cellular adaptation to ionizing radiation and detailed description of secretome composition was warranted. Two recent studies aimed to investigate intracellular adaption to ionizing radiation on RNA and microRNA level, as well as to describe molecular composition of the secretory product of PBMCs in response to lethal irradiation.[388,389] In response to ionizing radiation characteristic apoptosis marker were found 2 hours after irradiation in PBMCs increasing in a time-dependent manner and peaking 20 hours after irradiation. Cell lysates of irradiated PBMCs contained high levels of Bad, BAX, BCL-2, ciap-1, ciap-2, XIAP, and survivin, all of which have been linked to induction and execution of programmed cell death.[388] Also, activation of caspase-3 with concomitant increased activation levels of p53, p21 and Rad17 was found. Levels of IL-16 served as surrogate parameter for caspase-3 activity in this study. Interestingly, the caspase-3 pathway was recently associated with angiogenic activity mediated by paracrine signalling.[390] Proteins involved in protection from oxidative stress, like PON2, HO-1, and HO-2 were also induced in PBMCs by irradiation. Chaperons like HSP27, HSP60 and HSP70 were up-regulated as well.[388] Enrichment and induction of these factors in PBMCs following irradiation are realized by phosphorylation, cleavage of inactive precursor molecules (i.e. pro-enzymes), or de-novo synthesis. Aside from apoptosis-related genes, especially genes associated with expression and release of secretory proteins were found to be up-regulated by lethal irradiation. Compared to non-irradiated PBMCs, lethally

irradiated PBMCs up-regulate synthesis of more than 40 proteins associated with secretory activity.[389] After irradiation of PBMCs, profound changes were also found on the mRNA and microRNA level. In total, irradiation induces alterations of 177 microRNAs in PBMCs targeting various intracellular pathways, including pathways involved in vesicular transport and mediation of apoptosis.[388] Computational analysis revealed hepatic leukaemia factor (HLF), a factor known for its anti-apoptotic characteristics, as a central target of microRNA up-regulated in response to irradiation in PBMCs. Targeting of HLF by microRNA results in up-regulation of pro-apoptotic and inhibition of anti-apoptotic pathways and was identified as key step in regulation of apoptosis following irradiation in PBMCs.[388] In another study, the aim was to describe the composition of the secretome derived from lethally irradiated PBMCs. Beer et al. were able to show that besides proteins, also lipids, microparticles and exosomes are present and biologically active.[389] For instance, irradiation of PBMCs resulted in higher contents of oxidized lipids.[389] Certain lipids were previously shown to promote neuroprotection and favourable immunomodulation in experimental SCI.[4,391,392] Microparticles and exosomes, more or less secreted cellular vesicles, contain proteins, RNA and lipids and are capable of conducting paracrine signalling. Microparticles and exosomes were shown to be capable of exhibiting regenerative potential. However, exact mode of action and molecular mechanisms remain to be elucidated.[393] A possible pathway involved in this regenerative capacity of exosomes is the activation of the Erk 1/2 pathway.[394] This pathway was also shown to be involved in protection from apoptosis in cardiomyocytes when incubated with secretome derived from irradiated PBMCs.[381] Irradiation of PBMCs results in increased release of exosomes most likely via the p53 pathway resulting in a high exosome concentration within the MNC-secretome.[389] These exosomes are pivotal for biological activity of the secretome with depletion of exosomes leading to attenuated in-vitro activity. Exosomes derived from PBMCs lead to induction of CXCL1 and CXCL8 expression and increased migration activity of primary human fibroblasts and keratinocytes. These results suggest that the combination of proteins and exosomes within the secretome exhibits synergistic effects and is responsible for biological activity and regenerative capacity.[389] Aside from increased exosome release upon lethal irradiation of PBMCs, also the protein content of exosomes is affected by irradiation. The authors speculated that this represents a consequence of activation of the p53 pathway.[389] Further analysis of secretome derived from apoptotic PBMCs lead to identification of more factors, all of which have been previously associated with regenerative potential including adrenomedullin, growth-differentiation factor 15. Adrenomedullin was shown to exert angiogenic characteristics, while growth-differentiation factor 15 is capable of dampening ischemia-reperfusion injury.[389,395,396] These findings further indicate that multiple components inducing pleiotropic effects are responsible for observed therapeutic capacity of secretome obtained from apoptotic PBMCs.

During recent years substantial effort was made to establish a production process of secretome obtained from apoptotic PBMCs according to GMP (good manufacturing practice) guidelines in order to promote translation towards clinical application. Detailed description of the production process can be found in the results section and in chapter 4.1 of this thesis. Among other steps, a two-step viral clearance is mandatory for using allogeneic MNC-secretome in human studies. Arguably, these intensive processing steps might interfere with several biological compounds potentially responsible for biological activity of secretome derived from apoptotic PBMCs. Therefore, a recent study aimed to investigate whether this GMP-compliant processing interferes with regenerative capacity of MNC-secretome. Large animal experiments, as well as in-vitro studies showed that processing of the secretome including viral clearance did not interfere with biological activity and did not impede regenerative capacity in-vitro and in-vivo.[389,397]

## 1.4 Aims of this Thesis

Spinal cord injury, like myocardial infarction or stroke, is caused by an initial event followed by secondary endogenous mechanisms causing amplification of tissue damage and deteriorated outcome. This secondary cascade is characterized by a multitude of cellular and molecular cascades including inflammation, thrombosis, ischemia, and neuronal and glial apoptosis. Modulation of this second hit is the aim of experimental treatment strategies, most of which lack potential to interact with more than one pathway. Up until today, no satisfying treatment for patients suffering from acute SCI is known.

Efficacy of MNC-secretome treatment was previously published in various pre-clinical settings. Postulated mechanisms include attenuation of MVO, inhibition of thrombocyte aggregation, immunomodulation, activation of survival and anti-apoptotic pathways, and angiogenesis among others with the advantage of acting simultaneously.

The aim of this thesis was to investigate whether previously published results concerning MNC-secretome and its therapeutic effects are translatable to spinal cord injury. Furthermore, the aim was to delineate potential underlying mechanisms and possible novel modes of action of treatment with conditioned medium obtained from apoptotic PBMCs. For this purpose the aim was also to modify an existing model of angiogenesis and investigate the efficacy of MNC-secretome in this in-vitro model.

# CHAPTER TWO: Results

## 2.1 Prologue

Secondary mechanisms involved in progression of damage after SCI represent an interesting starting-point for potential therapy strategies. However, due to complex underlying mechanisms, some of which also pivotal for regeneration after SCI, a treatment regime capable of beneficially modulating this secondary response is not at hand up until today. Secretome derived from apoptotic PBMCs (=MNC-secretome) was shown to interact with various molecular and cellular pathways, most of which also involved in the endogenous response to SCI. In the following publication, improvement of motor function after SCI upon MNC-secretome treatment was observed. Modulation of the secondary response capable of improving neurologic outcome is demonstrated. This includes modulation of the immune response, improvement of post-traumatic vascularity and up-regulation of pathways associated with cell-survival and anti-apoptosis.

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Regular Article

# The secretome of apoptotic human peripheral blood mononuclear cells attenuates secondary damage following spinal cord injury in rats

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ABSTRACT

After spinal cord injury (SCI), secondary damage caused by oxidative stress, inflammation, and ischemia leads to neurological deterioration. In recent years, therapeutic approaches to trauma have focused on modulating this secondary cascade. There is increasing evidence that the success of cell-based SCI therapy is due mainly to secreted factors rather than to cell implantation per se. This study investigated peripheral blood mononuclear cells as a source of factors for secretome- (MNC-secretome-) based therapy. Specifically, we investigated whether MNC-secretome had therapeutic effects in a rat SCI contusion model and its possible underlying mechanisms. Rats treated with MNC-secretome showed substantially improved functional recovery, attenuated cavity formation, and reduced acute axonal injury compared to control animals. Histological evaluation revealed higher vascular density in the spinal cords of treated animals. Immunohistochemistry showed that MNC-secretome treatment increased the recruitment of $CD68^+$ cells with concomitant reduction of oxidative stress as reflected by lower expression of inducible nitric oxide synthase. Notably, MNC-secretome showed angiogenic properties ex vivo in aortic rings and spinal cord tissue, and experiments showed that the angiogenic potential of MNC-secretome may be regulated by CXCL-1 upregulation in vivo. Moreover, systemic application of MNC-secretome activated the ERK1/2 pathway in the spinal cord. Taken together, these results indicate that factors in MNC-secretome can mitigate the pathophysiological processes of secondary damage after SCI and improve functional outcomes in rats.



## Introduction

Injuries to the spinal cord are a devastating form of trauma that mainly affect young active patients and often result in permanent neurologic deficits, including tetraplegy (Ackery et al., 2004; Cadotte and Fehlings, 2011; Filli and Schwab, 2012). Treatment of spinal cord injury (SCI) remains challenging due to factors such as the limited potential of axonal regeneration in the human central nervous system and secondary mechanisms of injury that can last months after the initial trauma (Hall and Springer, 2004; Hausmann, 2003; Oyinbo, 2011; Popovich et al., 1997; Shechter et al., 2009; Tuszynski and Steward, 2012; Wright et al., 2011). This secondary cascade involves inflammation, neurotoxicity, glial scarring, and thrombocyte activation that, lead to micro-vascular obstruction (MVO), to injury progression, and to increased neurological impairment (Bowes and Yip, 2014; Hall and Springer, 2004; Hausmann, 2003; Kwon et al., 2004; Oyinbo, 2011;

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0014-4886/

Popovich et al., 1997; Rowland et al., 2008; Wright et al., 2011). Furthermore, thrombocyte activation directly promotes neuroinflammation and is involved in aggravation of neuronal damage (Joseph et al., 1991, 1992; Sotnikov et al., 2013; Thornton et al., 2010).

Despite ongoing efforts, a therapy that attenuates secondary damage after SCI has proven elusive (Cadotte and Fehlings, 2011; Filli and Schwab, 2012). Cell-based therapies using different types of stem cells, e.g. bone marrow-derived stem cells (BMSCs), looked promising in preclinical settings, but the clinical findings were not convincing (Filli and Schwab, 2012; Wright et al., 2011). In recent years, researchers have found that paracrine factors secreted by stem cells mediate the observed beneficial effects of cell therapy (Gnecchi et al., 2005; Mirotsou et al., 2011; Teixeira et al., 2013). Cantinieaux et al. reported that treatment with conditioned medium from BMSCs improves motor function and reduces spinal cord damage after SCI in rats (Cantinieaux et al., 2013). They showed that the observed therapeutic effect was due mainly to induction of angiogenesis and modulation of the inflammatory response of macrophages (Cantinieaux et al., 2013). More recently, a study reported the first use of allogeneic apoptotic cord blood MNCs in humans; these cells show regenerative potential in critical hind limb ischemia (Perotti et al., 2013). In contrast to many studies that use mesenchymal stem cells (MSCs) or other cell types, here we focused on conditioned medium from apoptotic peripheral blood mononuclear cells (PBMCs), which we term MNC-secretome. Previously we showed that MNC-secretome has regenerative effects in that it attenuates microvascular obstruction, inhibits platelets, induces vasodilation, and has immunomodulatory properties (Ankersmit et al., 2009; Hoetzenecker et al., 2012, 2015; Lichtenauer et al., 2011b; Mildner et al., 2013; Pavo et al., 2014). More recently, we demonstrated that MNC-secretome leads to reduction of infarction area in a preclinical stroke model (Altmann et al., 2014). In addition, the MNC secretome upregulates pathways associated with cytoprotection in primary neural crest-derived human cells and induces neuronal sprouting in vitro (Altmann et al., 2014). Most surprisingly, when applied intraperitoneally, the human MNC-secretome causes a huge increase in the expression of rat brain-derived neurotrophic factor (BDNF) in healthy rodents (Altmann et al., 2014). Prompted by these encouraging findings, we became interested in investigating whether MNC-secretome treatment had similar effects in a rat model of neurotrauma. Accordingly, the aim of this study was to investigate the effects of MNC-secretome treatment after SCI. For this purpose, we used a well established rat model of spinal cord contusion (Scheff et al., 2003).

## Materials & methods

### *Ethics statement*

All animal experiments were approved by the Animal Research Committee of the Medical University of Vienna (Protocol No. 66.009/0299-II/3b/2011) and met the Austrian guidelines for the use and care of laboratory animals. The local ethics committee at the Medical University of Vienna (EK2010/034) approved blood donation by healthy volunteers. All donors provided written informed consent.

### *Preparation of MNC-secretome*

In anticipation of possible future clinical applications, we produced human apoptotic MNC-secretome according to good manufacturing practice (GMP) guidelines for all of our experiments (xenogeneic human MNCs in a rodent experimental model). Viral clearance was performed in order to comply with virus safety requirements set by national regulatory authorities.

Human MNCs were isolated from the whole blood of healthy donors by density gradient centrifugation. After 60-Gy irradiation, the cells were cultivated for 24 h with CellGro® serum-free medium without phenol red (Cellgenix, Freiburg, Germany) at a concentration of $25 \times 10^6$ cells/ml under sterile conditions. After centrifugation, the cells were discarded and the supernatant was collected. The supernatant was treated with methylene blue (MB) plus light treatment using the Theraflex MB-Plasma system (MacoPharma), the Theraflex MB-Plasma bag system (REF SDV 0001XQ), and an LED-based illumination device (MacoTronic B2, Maco-Pharma). This process is also used for production of units of fresh frozen plasma under GMP conditions (Seghatchian et al., 2011). The light energy was monitored and reached 180 J/cm$^2$ within 20 min of illumination time. A pill containing 85 mg of MB is integrated into the bag system, yielding a concentration range of 0.8–1.2 mmol/L MB per unit. MB and photoproducts are removed by consecutive Blueflex filtration steps; notably, a two-step filtration removes over 90% of MB (Seghatchian et al., 2011). The air was removed from the plasma units before illumination. After lyophilization of the viral-inactivated cell culture supernatant, the lyophilized powder was treated with gamma irradiation to further reduce the risk of viruses. Gamma irradiation was performed with a radioactive decay of Cobalt 60 (Gammatron 1500, Mediscan, Seibersdorf, Austria), leading to the desired sterility of the product. For this purpose, the MNC-secretome was transferred to metal sterilization boxes that pass on a meandering path through the irradiation vault around the emitting center in five layers. The cobalt unit emits photons that are almost isotropic. The dose is distributed consistently on the complex path (280 positions in 5 layers). The dose rate is recorded by a PMMA dosimeter and was determined to be 25,000 Gy after 23 h of irradiation. The lyophilized supernatant of apoptotic MNCs was considered pathogen-free after this two-step process and was stored at −80 °C until re-suspension as needed using sterile water (Aqua ad injectabilia, B Braun, Melsungen, Germany) at a concentration equivalent to $25 \times 10^6$ cells/ml. As a control, we processed CellGro® serum-free medium without phenol red in the same fashion as the cell culture supernatant. This process of producing human autologous and allogeneic MNCs was recently approved by the Austrian Health Authority.

### *Rats and the SCI model*

Adult male Sprague Dawley rats (Department of Biomedical Research, Medical University of Vienna, Himberg, Austria) weighing 300–350 g were obtained for our experiments and housed under standard conditions with alternating 12 h light and dark cycles at the Department of Biomedical Research at the Medical University of Vienna. Standard lab chow and water was provided ad libitum. In experiments, the animals were anesthetized with 1.5% isoflurane to assure proper intraperitoneal (i.p.) application of either medium or MNC-secretome.

Contusion of the spinal cord was performed using a commercially available spinal cord impactor (Infinite Horizon Impactor, Precision System and Instrumentation, Lexington, KY) as described previously (Scheff et al., 2003). Briefly, animals were anesthetized with i.p. application of xylazine (10 mg/kg) and ketamine (100 mg/kg); anesthesia was maintained by a continuous flow of 1.5% isoflurane. Partial laminectomy to expose the spinal cord was performed at T11 under sterile conditions. The vertebral column was then stabilized by clamping the rostral and caudal vertebral bodies with Adson micro forceps (Fine Science Tools, Heidelberg, Germany). Epidural fat tissue was removed carefully while leaving the dura intact. The 2.5-mm diameter stainless steel tip was placed 1 mm above the exposed spinal cord, and a preset force of 150 kDyne was applied with instantaneous retraction of the tip, leading to a moderate contusion (Scheff et al., 2003). The exposed spinal cord was examined visually to ensure successful symmetric impact. The surgical field was irrigated with saline, and the muscle and skin openings were sutured together in layers. Animals received 10 ml of physiological saline subcutaneously (s.c.), Carprofen (10 mg/kg) s.c., and Enrofloxacin (10 mg/kg) s.c. for three consecutive days after surgery and were placed under a heating lamp during recovery. Manual bladder evacuation was performed twice daily until micturition was present. Animals were randomly divided into two groups, the Medium group and the MNC-secretome group, and received either 1 ml of processed MNC-secretome

(the equivalent of 25 × $10^6$ PBMCs) or 1 ml of processed medium i.p. 40 min and 24 h after trauma. The treatment timing, frequency, and dose were determined in preliminary experiments (Altmann et al., 2014; Hoetzenecker et al., 2012; Lichtenauer et al., 2011b).

For evaluation of cytokine plasma levels 12 rats received a single i.p. dose of 1 ml containing MNC-secretome (the equivalent of 25 × $10^6$ PBMCs). Plasma was taken from three rats per group sacrificed 12 h or 24 h after administration, respectively. Plasma obtained from further three untreated rats served as negative control ("0 h after treatment" in Fig. 8). (See Fig. 1.)

*Functional assessment*

To investigate the possible therapeutic effects of MNC-supernatant, the overground hind limb locomotion of injured rats was assessed using the Basso, Beattie, and Bresnahan 21-point open field locomotor rating scale (BBB score) (Basso et al., 1995). The score was determined before surgery (to exclude preoperative hind limb motor deficits), and 1, 3, 7, 14, 21, and 28 days after surgery. Observers were blinded with respect to treatment and previous scores. Each hind limb was scored separately, and the mean value of the scores of both limbs for each evaluation was used for statistical evaluation.

*Preparation of tissue for histological and immunochemical assessment*

Animals were anesthetized deeply with xylazine (10 mg/kg) i.p. and ketamine (100 mg/kg) i.p. This was followed by puncture of the inferior vena cava to draw 4 ml of blood and by euthanasia, including deep liver incision and perfusion through the heart with 20 ml of 4% paraformaldehyde in PBS. Heparin was added to the blood samples, and the samples were centrifuged at 3500*g* for 15 min. Plasma was then removed and stored at −80 °C. For histological evaluation, the vertebral column, including the spinal cord, was excised and fixed for 24 h. The spinal cord was then separated from the vertebral column, and ink marks were applied 4-mm cranial and 4-mm caudal from the injury epicenter followed by dissection of the spinal cord into an average of 10 sections. For Western blot analyses, laminectomy was performed on levels T10–T12, and around 5mm of spinal cord tissue was removed, shock frozen, and stored at −80 °C until subsequent protein extraction.

*Histology and immunohistochemistry*

Histological evaluation was performed on paraffin-embedded sections of spinal cords. Hematoxylin & eosin (H&E) and Luxol Fast Blue staining were performed to assess inflammation, myelin breakdown, and axonal pathology. The following were stained using immunohistochemistry: T cells (CD43/W3/13; Harlan Laboratories, Indianapolis, IN), β-amyloid precursor protein (βAPP; Chemicon International, CA), microglia/macrophages (CD68, AbDSerotec, Duesseldorf, Germany), inducible nitric oxide synthase/iNOS (Anti-NOS II, Millipore, MA), and blood vessels (anti-von Willebrand factor/vWF, Abcam, Cambridge, UK). For staining, paraffin sections were pretreated with a steamer for 60 min. The bound primary antibody was detected with a biotin–avidin technique as described in detail previously (Kerlero de Rosbo et al., 1997). To determine axonal pathology using anti-βAPP staining, comparable 0.25 $mm^2$ areas in the lateral column of the injured spinal cord were defined 4-mm rostral and 4-mm caudal to the injury epicenter. In this field, βAPP-positive fibers were counted using ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health; Bethesda, MD). The area of cavity formation was measured in axial sections 4-mm cranial and 4-mm caudal to the injury epicenter using ImageJ planimetry software. The areas of the spinal cord section and cavity formation were measured, and their ratio was calculated. The mean values of the cranial and caudal measurements were then calculated and used for statistical evaluation. We quantified CD68 (activated microglia and macrophages), iNOS, vWF (endothelium), and CD43 (T-cells) as follows. The dorsal column and the anterolateral white matter were evaluated separately to distinguish between directly damaged and indirectly damaged spinal cord pathways. The areas and mean number of positive cells of at least 6 cross sections were counted for each animal and plotted as cell number/area. Observers blinded to treatment conducted all histological evaluations and measurements. All measurements were carried out using ImageJ.

We utilized the diaminobenzidine-enhanced Turnbull blue staining method (TBB) for the detection of non-heme iron. Ferritin expression was visualized by immunohistochemistry using rabbit anti-ferritin antibody (Sigma, St Louis, MO; dilution: 1:1000, 60 min steaming with EDTA pH 8.5). Slides were then evaluated using light microscopy. Iron accumulation was quantified by a blinded observer with the following scoring system: 0 = negative, 1 = low staining intensity, 2 = intermediate staining intensity, high staining intensity. Microglia and macrophages were distinguished by morphological appearance.

*Aortic ring assay and spinal cord assay*

Angiogenic activity was studied using the aortic ring assay based on the method published by Nicosia and Ottinetti (1990). Ring segments of rat aorta were sandwiched between two fibrin gels in individual wells of a 24-well culture plate (Corning, Corning, NY) and overlayed with M199 medium (Invitrogen, Carlsbad, CA) supplemented with 4 mM L-glutamine (Invitrogen), antibiotic antimycotic solution (100 U penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, Sigma, St. Louis, MO) and 10% FCS (Invitrogen). Fibrin matrices were prepared as described previously with some modifications (Ruger et al., 2008). In brief, human fibrinogen (2 mg/ml, Calbiochem, Darmstadt, Germany) was dissolved in PBS supplemented with 200 U/ml aprotinin (Gerot Pharmaceutica, Vienna, Austria) to prevent fibrinolysis. Clotting was initiated by adding human plasma thrombin (0.6 U/ml, Sigma, St. Louis, MO). After equilibration, the culture medium was removed and exchanged with complete M199 without or with the addition of MNC-

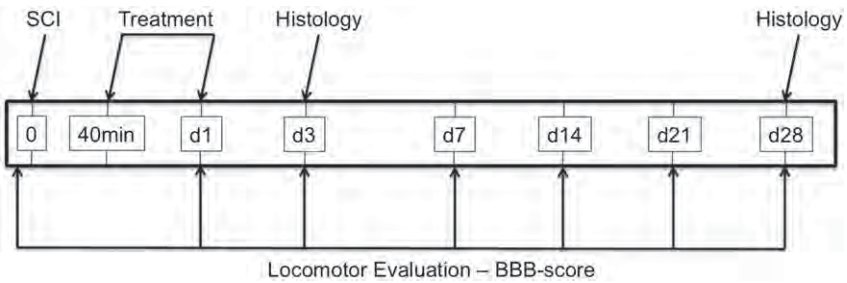


**Fig. 1.** Experimental timeline. Evaluation of motor function was conducted before and on days 1, 3, 7, 14, 21, and 28 after spinal cord injury (SCI). MNC-secretome or control medium was administered intraperitoneally 40 min and 24 h after SCI. Spinal cords were harvested 3 or 28 days after SCI and histological evaluation was performed. BBB score, Basso, Beattie, and Bresnahan score.

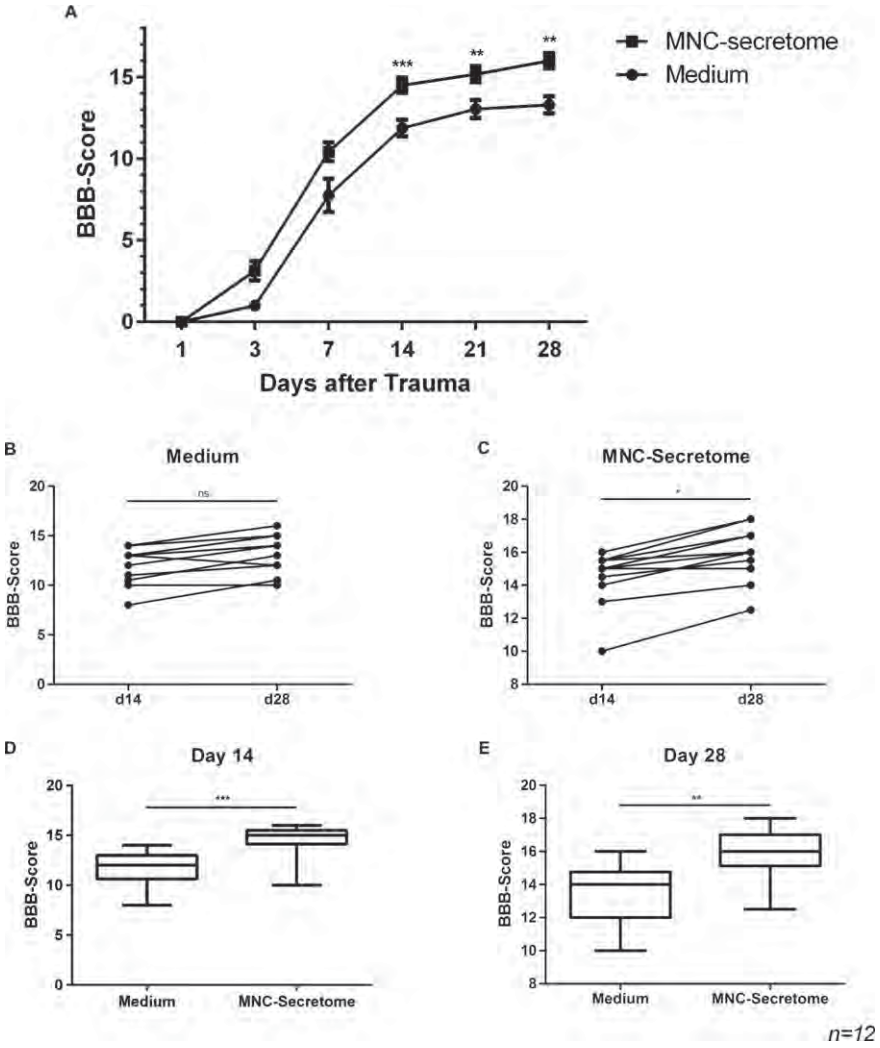


**Fig. 2.** Improved hind limb motor function after treatment with MNC-secretome. (A) Neurologic evaluation using the Basso, Beattie, and Bresnahan (BBB) scale revealed improved hind limb motor function at the indicated times after spinal cord injury (SCI) in animals treated with MNC-secretome as shown by the higher BBB scores (mean ± SD; Medium: d7: 7.75 ± 3.52, d14: 11.88 ± 1.76, d21: 13.04 ± 1.94, d28: 13.29 ± 1.84, MNC-secretome: d7: 10.42 ± 1.99, d14: 14.5 ± 1.62, d21: 15.17 ± 1.71, d28: 16.0 ± 1.61; Medium vs. MNC-secretome: d7: $p = 0.068$, d14: $p < 0.001$, d21: $p < 0.01$, d28: $p < 0.01$). (B,C) Improvement between day 14 and day 28 was not significant in the control group (B) (mean ± SD ; Day 14: 11.88 ± 1.76, day 28: 13.29 ± 1.84, $p = 0.07$), while in the same time period MNC-secretome treated animals experienced significant improvement in motor function (C) (mean ± SD ; Day 14: 14.50 ± 1.62, day 28: 16.00 ± 1.61, $^{*}p < 0.05$). (D, E) Comparison of day 14 (D) and day 28 (E) BBB-scores in both groups (mean ± SD; Medium vs. MNC-secretome: Day 14: 11.88 ± 1.76 vs. 14.50 ± 1.62, $^{***}p < 0.001$, day 28: 13.29 ± 1.84 vs. 16.0 ± 1.61 , $p < {}^{**}0{,}01$). n = 12 animals per group.

secretome (secretome corresponding to $4 \times 10^6$ PBMCs), MNC-secretome + anti-VEGF (300 ng/ml), recombinant rat VEGF (rrVEGF, 100 ng/ml), or rrVEGF + anti-VEGF (100 ng/ml and 300 ng/ml, respectively). The plate was incubated at 37 °C in a 5% CO2/97% humidified environment. Cultures were maintained for 4–7 days with medium changes every 2 days. The formation of tubular structures was monitored using a phase contrast microscope (Olympus IMT-2, Tokyo, Japan) on day 2, day 4, and day 6 using a digital camera (Olympus DP50). Outgrowth length was measured using ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health; Bethesda, MD).

In order to quantify the cellular outgrowth from the aortic rings, the fibrin gels were dissolved as described previously (Carrion et al., 2014). The aortic rings were removed, and the remaining cells were washed with PBS and counted in a Bürker–Türk counting chamber.

To assess angiogenic capacity in the central nervous system, we used spinal cord tissue instead of aortic rings. Spinal cord segments (T1–L3) from healthy rats were embedded in 3D fibrin gels in 24-well culture plates using the same method as for the rat aortic rings. In some experiments, imaging chambers for high-resolution microscopy (ibidi, Martinsried, Germany) were used for immunofluorescence analyses and subsequent confocal laser scanning microscopy of the 3D cultures.

For flow cytometry experiments human MNCs were isolated by density gradient centrifugation and seeded in 3D fibrin cultures as described above. After six days cultures were again dissolved and processed for flow cytometry experiments.

*Flow cytometry*

$100 \times 10^5$ cells per 100 µl PBS were stained with 10 µl PE-conjugated CD14 (clone RMO52), CD163 (clone GHI/61) and FITC-conjugated CD206 (clone 19.2) or with the appropriate isotype control antibodies (BD Biosciences) at 4 °C for 15 min. After one washing step, marker expression was analyzed on an FC 500 flow cytometer (Beckman Coulter), and data were analyzed using the FlowJo software (Tree Star Inc, Ashland, OR). Living macrophages were gated according to their forward- and side scatter characteristics and apoptotic or dead cells were excluded using the Annexin V-FITC/PI apoptosis detection kit (BD Biosciences).

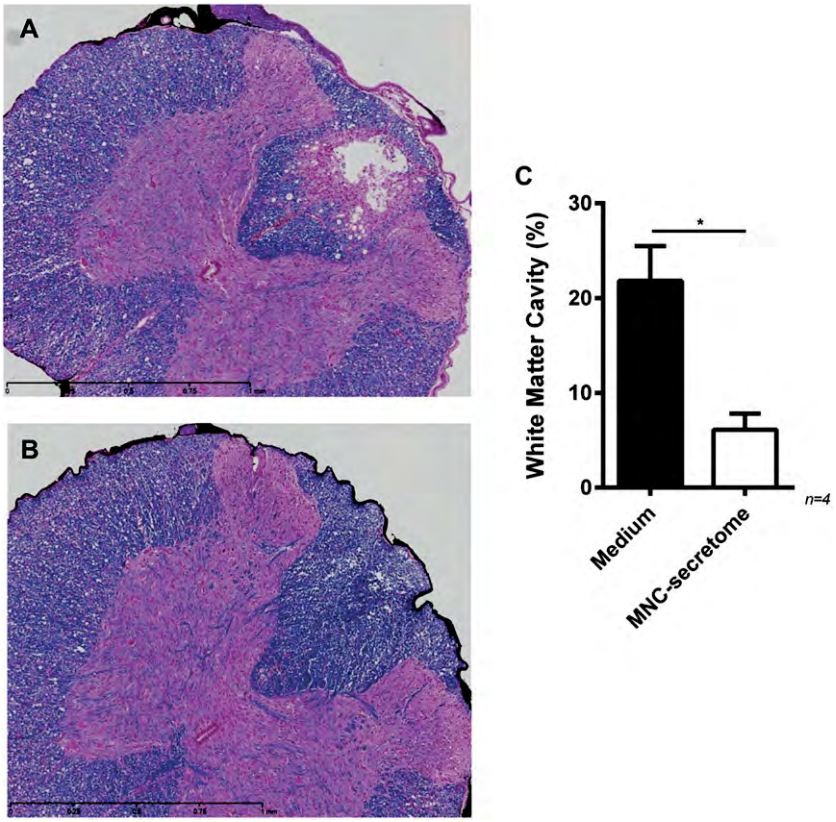


**Fig. 3.** Planimetric evaluation of cavity formation. Representative images of Luxol Fast Blue staining of spinal cords from (A) medium treated and (B) MNC-secretome treated animals 28 days after spinal cord injury (SCI). We marked cross sections 4-mm cranial and 4-mm caudal of the macroscopic lesion epicenter and used these for planimetric measurement. We also measured the cavity formation relative to the total dorsal column for each cross section. Then, the mean of both sections, 4-mm caudal and 4-mm cranial, was calculated for each rat and used for statistical evaluation and comparison (C; Medium vs. MNC-secretome: 21.81 ± 7.34% vs. 6.1 ± 3.4%, $^{*}p < 0.05$). n = 4 animals per group.

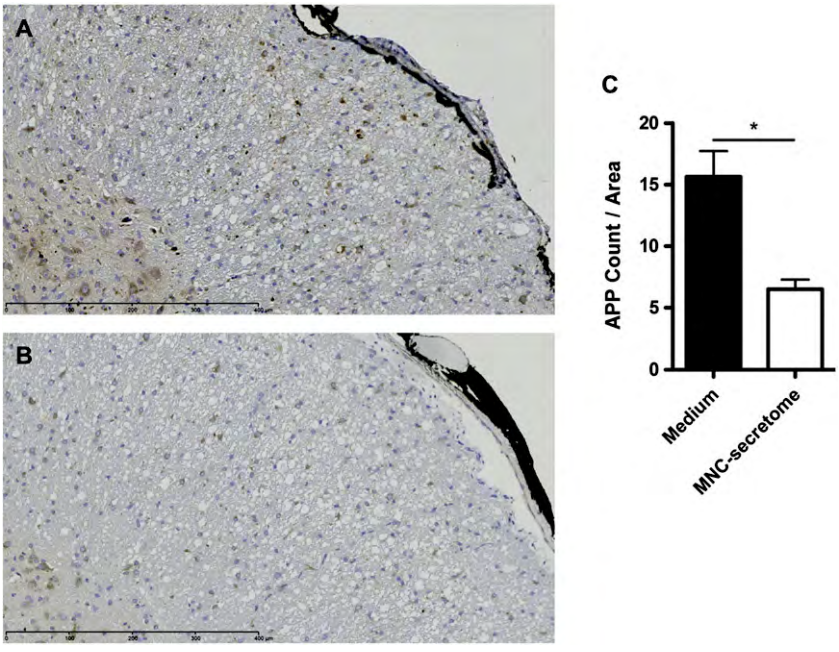


**Fig. 4.** Axonal damage was reduced in MNC-secretome treated animals. Immunohistochemical staining for β-amyloid precursor protein showed progressed axonal damage in spinal cord injury (SCI) animals treated with medium (A) than in animals treated with MNC-secretome (B). Similar to the method used for planimetric evaluation of lesion volume, cross sections 4-mm caudal and 4-mm cranial to the lesion epicenter were used for assessment of axonal pathology. Statistical analysis revealed significantly less in MNC-secretome treated animals, indicating reduced acute axonal injury (C; Medium vs. MNC-secretome: 16.73 ± 8.67/mm$^2$ vs. 6.5 ± 0.77/mm$^2$, $p < 0.05$). n = 5 animals per group.

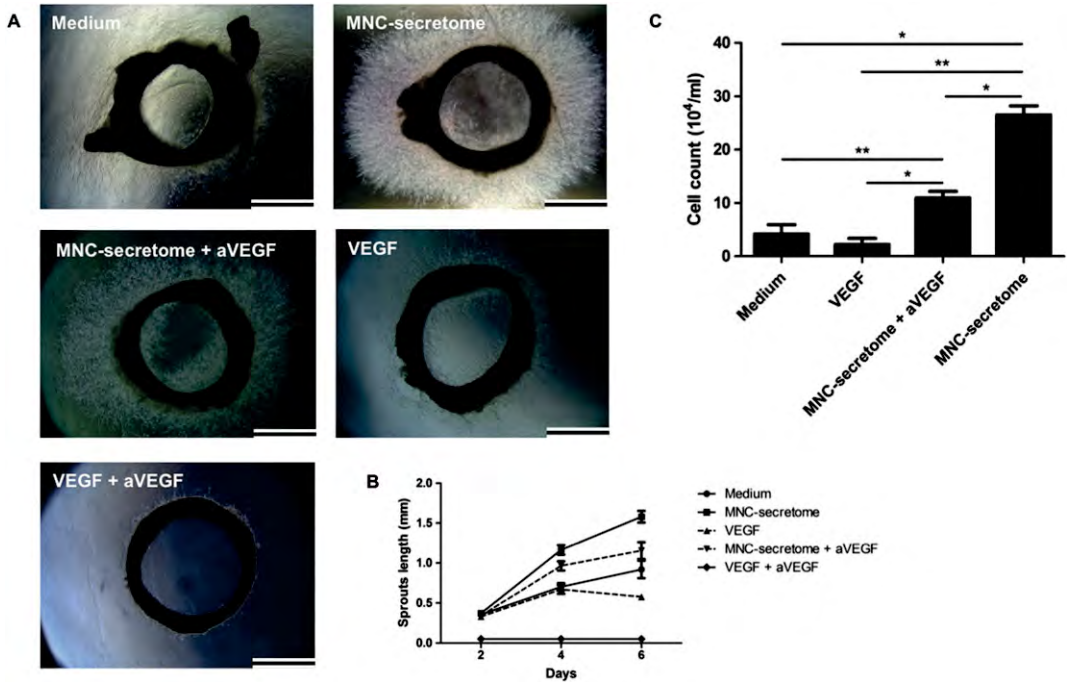


**Fig. 5.** Evaluation of angiogenic properties in a rat aortic ring model. (A) Representative phase contrast images of aortic rings cultured for four days in the indicated conditions: Medium, MNC-secretome, MNC-secretome + anti-VEGF, recombinant VEGF, and recombinant VEGF + anti-VEGF. (B) Graph showing the lengths of vessel sprouts on days 2, 4, and 6 after initiation of experiments. Aortic rings cultured with MNC-secretome showed the strongest angiogenic response of all groups (2-way ANOVA: MNC-secretome vs. Medium: $p < 0.001$; MNC-secretome vs. VEGF: $p < 0.001$; MNC-secretome vs. MNC-secretome + anti-VEGF: $p < 0.001$; MNC-secretome vs. recombinant VEGF + anti-VEGF: $p < 0.001$). The lengths of the 3 longest vessel sprouts were measured, and the mean length was calculated for each aortic ring and used for statistical calculations. (Mean ± SEM in mm, Day 2: MNC-secretome: 0.371 ± 0.023; Medium: 0.356 ± 0.039; VEGF: 0.328 ± 0.014; MNC-secretome + anti-VEGF: 0.340 ± 0.011; recombinant VEGF + anti-VEGF: 0.000 ± 0.000; Day 4: MNC-secretome: 1.164 ± 0.059; Medium: 0.703 ± 0.044; VEGF: 0.668 ± 0.052; MNC-secretome + anti-VEGF: 0.963 ± 0.057; recombinant VEGF + anti-VEGF: 0.000 ± 0.000; Day 6: MNC-secretome: 1.579 ± 0.073; Medium: 0.919 ± 0.109; VEGF: 0.578 ± 0.027; MNC-secretome + anti-VEGF: 1.155 ± 0.106; recombinant VEGF + anti-VEGF: 0.000 ± 0.000). (C) After ending the experiment and dissolving the assay matrix, there were significantly more cells in the MNC-secretome group compared to the other groups (MNC-secretome vs. Medium: $p < 0.05$; MNC-secretome vs. VEGF: $p < 0.01$; MNC-secretome vs. MNC-secretome + anti-VEGF: $p < 0.05$); (Means ± SEM in $10^4$ cells/ml: MNC-secretome: 26.50 ± 1.71; Medium: 4.17 ± 1.75; VEGF: 2.21 ± 1.14; MNC-secretome + anti-VEGF: 10.96 ± 1.22). $^*p < 0.05$, $^{**}p < 0.01$. Scale bars, 1 mm. n = 3 per group.

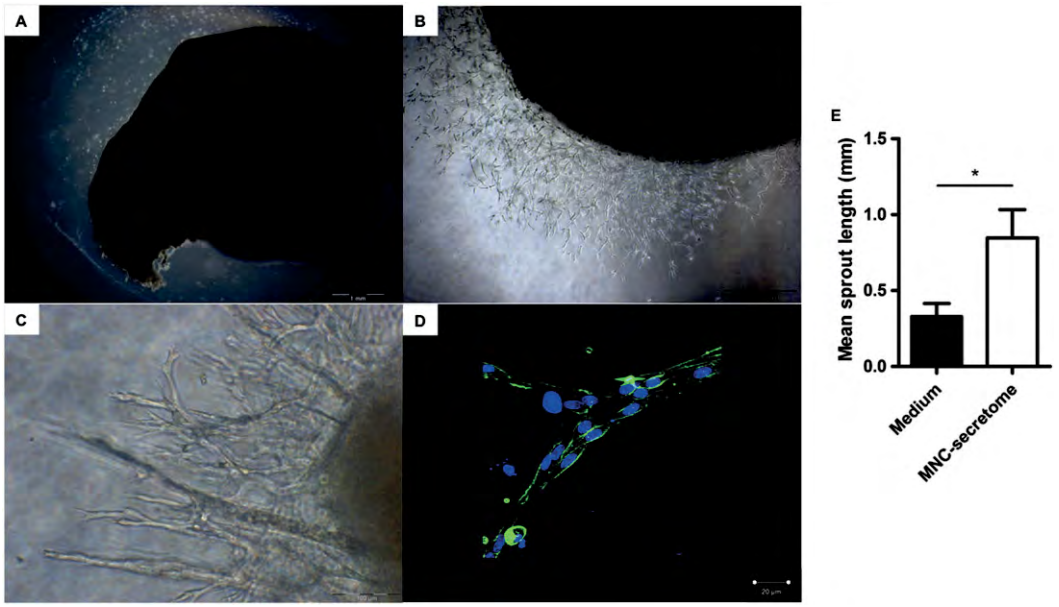


**Fig. 6.** Angiogenic potential of MNC-secretome in rat spinal cord tissue. (A) Representative phase contrast image of spinal cord tissue in the 3D fibrin matrix on day 6 of culture with control medium. (B, C) Representative phase contrast images of spinal cord tissue in 3D fibrin matrix on day 6 of culture with MNC-secretome. (D) Confocal laser scanning microscopy 3D-reconstruction (z-stack) of newly formed vascular sprouts in 3D fibrin matrix on day 6 of culture with MNC-secretome. The vascular structures expressed the endothelial marker RECA-1 (green). Nuclei are stained with DAPI (blue). (E): Graph showing sprout length on day 6 after initiation of 3D culture with either medium or MNC-secretome (Medium: 0.330 ± 0.084, MNC-secretome: 0.842 ± 0.186, $^*p < 0.05$). Scale bars, as indicated. n = 3 per group.

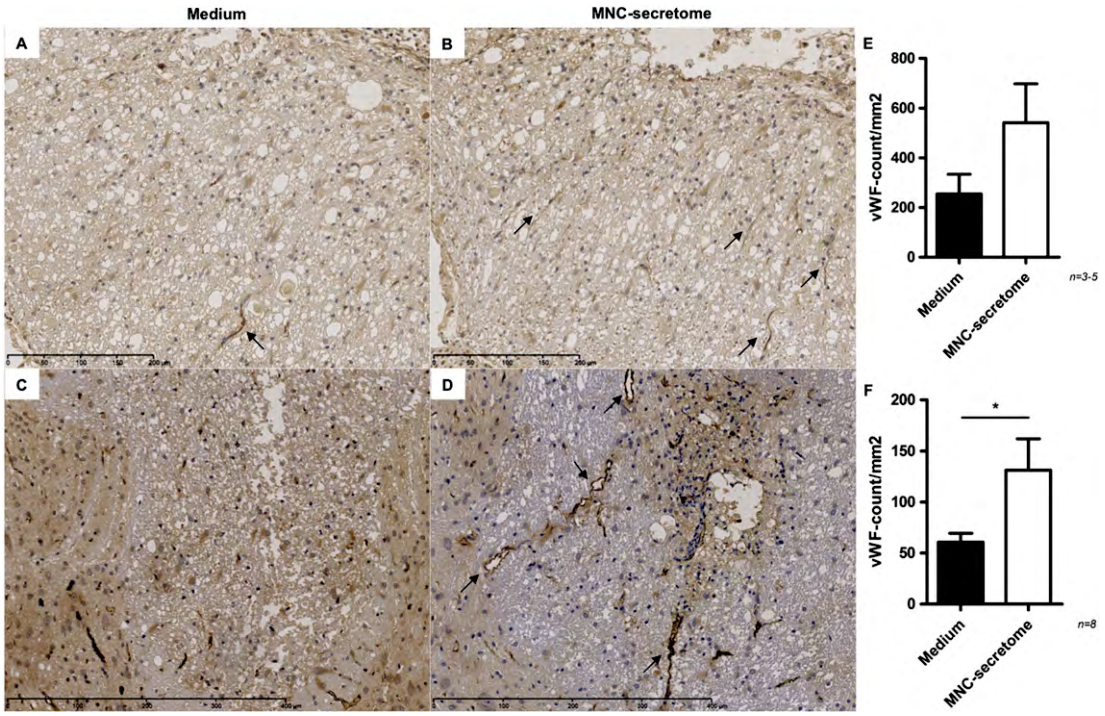


**Fig. 7.** MNC-secretome treatment leads to elevated expression of von Willebrand factor (vWF) in contused spinal cords. (A, B, C, D) Representative images of vWF-staining in the lesion epicenter (A, B) and in the dorsal column (C, D) 28 days after spinal cord injury (SCI) in animals treated with medium (A, C) or MNC-secretome (B, D). Arrows indicate vasculature expressing vWF. (E, F) Graphs showing the quantification of cells expressing vWF in the lesion epicenter (E) and dorsal column (F) 28 days after SCI. (Epicenter: Medium vs. MNC-secretome: 254.9 ± 78.99/mm$^2$ vs. 541.5 ± 156.5/mm$^2$, $p = 0.11$; Dorsal column: Medium vs. MNC-secretome: 60.74 ± 8.69/mm$^2$ vs. 131.2 ± 30.84/mm$^2$, $^*p < 0.05$).

*Immunofluorescence and confocal laser scanning microscopy of rat spinal cord 3D cultures*

To investigate the vascular phenotype of the cellular outgrowth from the rat spinal cord segments embedded in the 3D fibrin matrix, whole cultures were immunostained with RECA-1 (Abcam, Cambridge, UK), a monoclonal antibody to rat endothelial cells. 3D cultures were fixed with 4% paraformaldehyde followed by incubation with PBS/0.1 M glycine. After blocking with 5% donkey serum in immunofluorescence (IF) buffer containing 0.2% Triton X-100, 0.1% BSA, and 0.05% Tween 20 in PBS, the fibrin gel cultures were incubated with RECA-1 antibody (2 μg/ml) for 5 h at RT followed by an extended washing step with IF buffer. Visualization of the bound antibody was achieved by incubation with Alexa Fluor 488-labeled donkey anti-mouse antibody (2.5 μg/ml, Invitrogen). Cultures were washed with IF buffer, the nuclei were stained with DAPI, and the fibrin gels were stored in PBS at +4 °C until confocal laser scanning microscopy analysis. The 3D cultures were evaluated using a LSM 700 confocal laser scanning microscope (Carl Zeiss, Jena, Germany), and the acquired images were analyzed with the ZEN image processing and analysis software program (Zeiss).

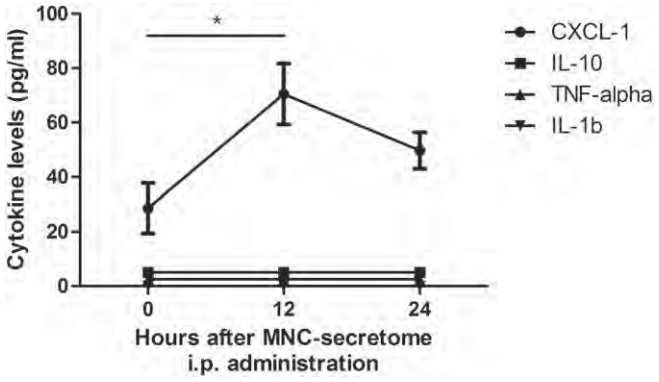


**Fig. 8.** Upregulation of CXCL-1 in-vivo after intraperitoneal (i.p.) administration of MNC-secretome. I.p. application of MNC-secretome increased the serum levels of CXCL-1 in healthy rats 12 h after administration compared to control animals (Medium vs. MNC-secretome: 28.63 ± 9.23 pg/ml vs. 70.5 ± 11.22 pg/ml, $^*p < 0.05$). After 24 h, the difference was not significant (Medium vs. MNC-secretome: 28.63 ± 9.23 pg/ml vs. 49.69 ± 6.65 pg/ml, $p = 0.138$). The other measured cytokines, i.e. IL-10, TNF-α, and IL-1β, were not detectable either 12 or 24 h after administration. n = 3 animals per group.

*Western blot analysis*

To analyze the phosphorylation state of intracellular signaling molecules in the spinal cord, we euthanized healthy rats 2 h after i.p. administration of either MNC-secretome or medium (control), removed the T10–T12 segments, and flash froze the segments in liquid nitrogen. Sample processing and Western blot analysis were carried out as described previously (Gschwandtner et al., 2014). Briefly, the samples were lysed in buffer containing 50 mM Tris (pH 7.4) and 2% SDS followed by sonication, centrifugation, protein concentration measurement by BCA assay (Pierce, Rockford, IL), and denaturation with 0.1 M DL-dithiothreitol (Sigma-Aldrich). We used 8–18% gradient gels for SDS-PAGE (GE-Amersham Pharmacia Biotech, Uppsala, Switzerland) for size fractionation. After electrotransferring the proteins onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and subsequent Ponceau S staining, we used the following primary antibodies for immunodetection: anti-phospho-Erk 1/2 (1:1000), anti-phospho-CREB (1:1000), anti-phopsho-Hsp27 (Ser15, 1:1000), and anti-phopsho-Akt (Ser473, 1:1000) (Cell Signalling Technology, Cambridge, UK); HRP-conjugated goat anti-mouse IgG (1:10,000, Amersham, Buckinghamshire, UK); and goat anti-rabbit IgG (1:10,000, Thermo Fisher, Rockford, IL). For detecting reaction products with chemiluminescence, we used the Immun-Star Western C Substrate kit (Bio-Rad).

Quantification of band density was carried out with ImageJ and was calculated relative to specimens from untreated healthy rats.

*ELISA analysis*

Plasma obtained from rats was analyzed with commercially available ELISA systems (DuoSet, R&D Systems, Minneapolis). These kits specifically detect rat cytokines. All assays were performed according to the reference manual, and samples were measured in technical duplicates. We analyzed the following cytokines: CXCL-1/CINC-1, IL-10, IL-1β, and TNF-α. Optical density values were measured at 450 nm on an ELISA plate reader (Victor3 Multilabel plate reader, PerkinElmer).

*Statistical methods*

If not stated otherwise, data are presented as mean ± SEM. We compared motor function and data from histological evaluations (area of cavity formation, APP, CD43, and CD68) between the two groups using the nonparametric Mann–Whitney *U*-test. We used the student's *t*-test for all other statistical calculations. SPSS software (Version 21, IBM, NY) was used for all statistical calculations. Graphs were calculated and plotted with GraphPadPrism 5 (GraphPad, CA). A p-value $< 0.05$ was defined as the level of significance for all experiments.

## Results

*Treatment with MNC-secretome improves motor function after SCI*

To evaluate whether application of MNC-secretome could improve motor function after SCI, the frequently used BBB-score was utilized (Basso et al., 1995). Indeed, treatment with MNC-secretome led to significant improvement of motor function compared to treatment with medium. The differences became statistically significant 14 days after SCI and remained significant until the end of the observation period on day 28 ($n = 12$; d14: $p < 0.001$, d21: $p < 0.01$, d28: $p < 0.01$; Fig. 2). A BBB score of 13 on the 21-point scale indicates that coordinated locomotion of fore and hind limbs can be frequently observed. This represents a important milestone in regeneration after SCI (Basso et al., 1995). Fourteen days after trauma, animals in the MNC-secretome treatment group exceeded this score (mean ± SD: 14.50 ± 1.62), while medium-treated animals had a score of 11.88 ± 1.76 (mean ± SD) two weeks after SCI. This difference was highly significant with a p-value $< 0.001$. Contrary to animals in the control group, we found significant improvement of motor function of MNC-treated animals between day 14 and day 28 post trauma ($p < 0.05$) (Figs. 2B and C).

*Animals treated with MNC-secretome show reduced cavity formation and axonal damage compared to controls*

To determine whether treatment with MNC-secretome resulted in reduced secondary damage after SCI, we determined the extent of injury 28 days after trauma. Specifically, we evaluated the extent of the lesion using planimetric measurements of the cavity formation relative to the total dorsal column 4-mm caudal and 4-mm cranial to the epicenter. This revealed a significantly smaller area of white matter cavity formation 28 days post-trauma in animals treated with MNC-secretome (Medium vs. MNC-secretome: 21.81 ± 7.34% vs. 6.1 ± 3.4%, $p < 0.05$) (Fig. 3).

To further evaluate the extent of acute axonal injury of motor pathways, we quantified βAPP positive axonal spheroids within the lateral column of the spinal cord. This histological evaluation showed a significant decrease in the lateral column in animals treated with MNC-secretome compared to control animals (Medium vs. MNC-secretome: 16.73 ± 8.67/mm$^2$ vs. 6.5 ± 0.77/mm$^2$, $p < 0.05$) (Fig. 4).

Finally, we investigated the state of α-motoneurons both 3 and 28 days after SCI (Supp. Figs. 4, 5). CHaT is an enzyme involved in the synthesis of the neurotransmitter acetylcholine. CHaT staining revealed pronounced damage around the lesion 3 days after injury in both groups (Supp. Fig. 4B). Interestingly, on day 28, the spinal cord appeared to be almost totally restored except for small areas of the ventral roots in both groups (Supp. Figs. 5C, D). We did not detect any differences between the two groups in terms of CHaT staining at either time point.

*MNC-secretome shows angiogenic properties in aortic rings and in ex vivo spinal cord tissue*

The aortic ring model is a common model for studying the angiogenic properties of drugs (Aplin et al., 2008; Nicosia and Ottinetti, 1990). In this model, we found increased vessel formation and sprouting after incubation with MNC-secretome compared to the control group (Figs. 5A, B). Furthermore, after removing the aortic ring and dissolving the matrix, cellularity was significantly higher in samples incubated with MNC-secretome (Fig. 5C). These observed effects were not VEGF-dependent; however, we noticed destabilization of the newly formed vessels on day 6 in the groups treated with anti-hVEGF (Fig. 5A and Suppl. Fig. 1). Recombinant human VEGF served as the positive control and showed less angiogenic capacity than MNC-secretome. The angiogenic capacity of rrVEGF was completely inhibited by adding the anti-VEGF antibody (Figs. 5A, B).

We designed a new assay to investigate whether MNC-secretome also exhibits these angiogenic properties in spinal cord tissue. Briefly, instead of aortic rings, we used spinal cord tissue from healthy rats in the assay. We confirmed the angiogenic effects of MNC-secretome in spinal cord tissue, similar to our findings in the aortic ring assay. Most importantly, endothelial cell outgrowth was positive for RECA-1, an endothelial cell marker (Fig. 6).

*Elevated expression of vWF in injured spinal cord following treatment with MNC-secretome*

These ex vivo data suggested the pro-angiogenic properties of MNC-secretome. Previous work by our group showed that MNC-secretome has other effects that can reduce damage after myocardial infarction, like vasodilation, inhibition of platelet activation, and obviation of MVO (Hoetzenecker et al., 2012). We therefore examined whether MNC-secretome might have these effects in an SCI model. We performed immunohistochemical staining against vWF to assess the microvascular density of the spinal cord (Tseliou et al., 2014; Wei et al., 2014). The vascular density was significantly increased in the entire dorsal column of the injured spinal cords of animals treated with MNC-secretome (Medium vs. MNC-secretome: 60.74 ± 8.69/mm$^2$ vs. 131.2 ± 30.84/mm$^2$, $p < 0.05$) (Figs. 7C, D). There was also increased vascular density at the lesion site, although it did not reach a statistically significant level (Medium vs. MNC-secretome: 254.9 ± 78.99/mm$^2$ vs. 541.5 ± 156.5/mm$^2$, $p = 0.11$) (Figs. 7A, B).

*MNC-secretome treatment increases CXCL-1 plasma levels in vivo*

To further elucidate the MNC-secretome mechanisms of action in vivo, we investigated whether plasma levels of pro-angiogenic CXCL-1, immune modulatory IL-10, and pro-inflammatory TNF-α and IL-1β were altered in healthy animals treated with MNC-secretome. We found elevated CXCL-1 plasma levels 12 h after i.p. administration, but the levels of the other cytokines were unchanged (Fig. 8). However, in the acute phase of SCI (3 days post-SCI) CXCl-1, TNF-α, and IL-1βplasma levels showed no differences compared to controls (Supp. Fig. 6).

*The immune response after SCI is modulated by MNC-secretome*

Monocytes and macrophages are key players in inflammatory modulation and in the resolution of inflammation after SCI (London et al., 2013;

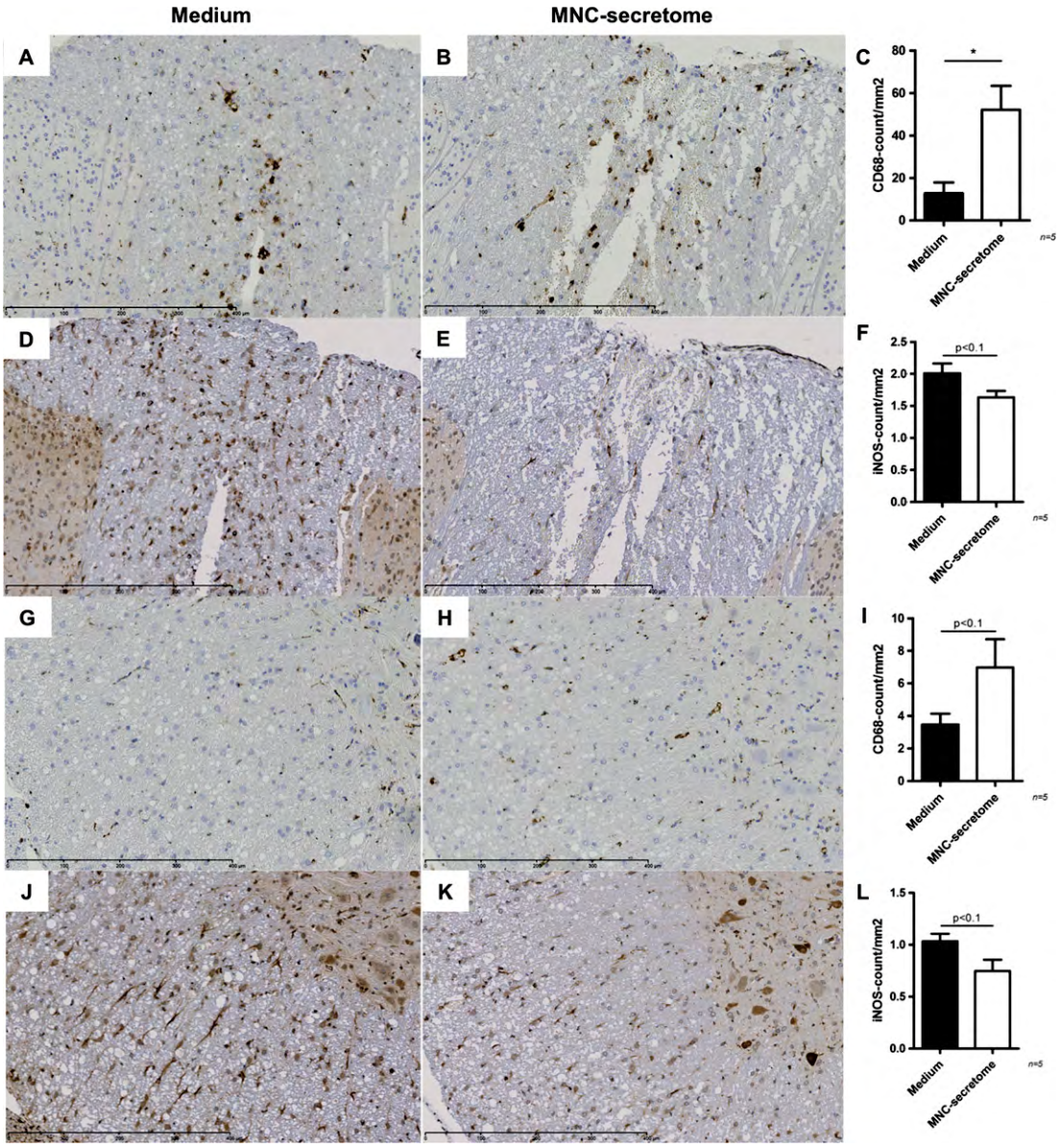


**Fig. 9.** Immune response after spinal cord injury is modulated by MNC-secretome. Immunohistochemical staining of the spinal cord dorsal and anterolateral columns (A, B, G, H) revealed that more cells expressed CD68 in animals treated with MNC-secretome 3 days after spinal cord injury (SCI; B, H) compared to animals treated with control medium (A, G). Despite having higher levels of $CD68^+$, tissue that was analyzed by immunohistochemistry showed lower expression levels of iNOS after treatment with MNC-secretome (E, K) compared to the spinal cord dorsal and anterolateral columns of control animals (D, J). I, J, K, L: Graphs show the number of cells expressing CD68 (C, I) and iNOS (F, L) in the dorsal and anterolateral columns of spinal cord sections (Medium vs. MNC-secretome: C: $12.7 \pm 5.2/mm^2$ vs. $52.1 \pm 11.2/mm^2$, $p < 0.05$; F: $2.0 \pm 0.2/mm^2$ vs. $1.6 \pm 0.1/mm^2$, $p = 0.072$; I: $3.5 \pm 0.7/mm^2$ vs. $6.9 \pm 1.7/mm^2$, $p = 0.097$; L: $1.0 \pm 0.1/mm^2$ vs. $0.7 \pm 0.1/mm^2$, $p = 0.061$).

Shechter et al., 2009). We were therefore interested to see whether MNC-secretome treatment modulated the immune response after SCI. Animals treated with MNC-secretome showed significantly increased $CD68^+$ cells in the dorsal column 3 days after SCI compared to controls (Figs. 9A, B, I). To assess the extent of nitric oxide radical production, we performed immunohistological staining of iNOS (London et al., 2013; Shechter et al., 2009). Despite elevated levels of $CD68^+$ cells, we found decreased iNOS expression (Figs. 9C, D, J). We observed the same trend in the remaining white matter (Figs. 9E, F, G, H, K, L). Furthermore, we detected a trend towards higher plasma levels of IL-10 on day 3 after SCI in the MNC-secretome-treated group (Supp. Fig. 2). In contrast, there were significantly higher levels of $CD68^+$ cells in the white matter of animals in the control group 28 days after SCI, while no significant differences in $CD68^+$ cell number were observed within the dorsal column (Fig. 10). We did not observe any differences in iNOS expression between the two groups 28 days after trauma (data not shown). Expression of CD43 was unaltered by MNC-secretome treatment both on day 3 and on day 28 after trauma (Supp. Fig. 3).

### *Augmented phosphorylation of ERK 1/2 in spinal cord tissue in response to MNC-secretome treatment*

In addition to analyzing the plasma levels of a number of cytokines involved in inflammation, repair, and angiogenesis, we also investigated the activation of relevant intracellular pathways in the spinal cord after MNC-secretome treatment in healthy animals without SCI. We found a significant 2.5-fold increase in the phosphorylation of Erk 1/2 in tissue from these rats compared to tissue from untreated rats (Fig. 11B). Other pathways that we examined, including the CREB, p38, and HSP27 pathways, showed no differences in phosphorylation levels between animals treated with MNC-secretome or with medium (Fig. 11).

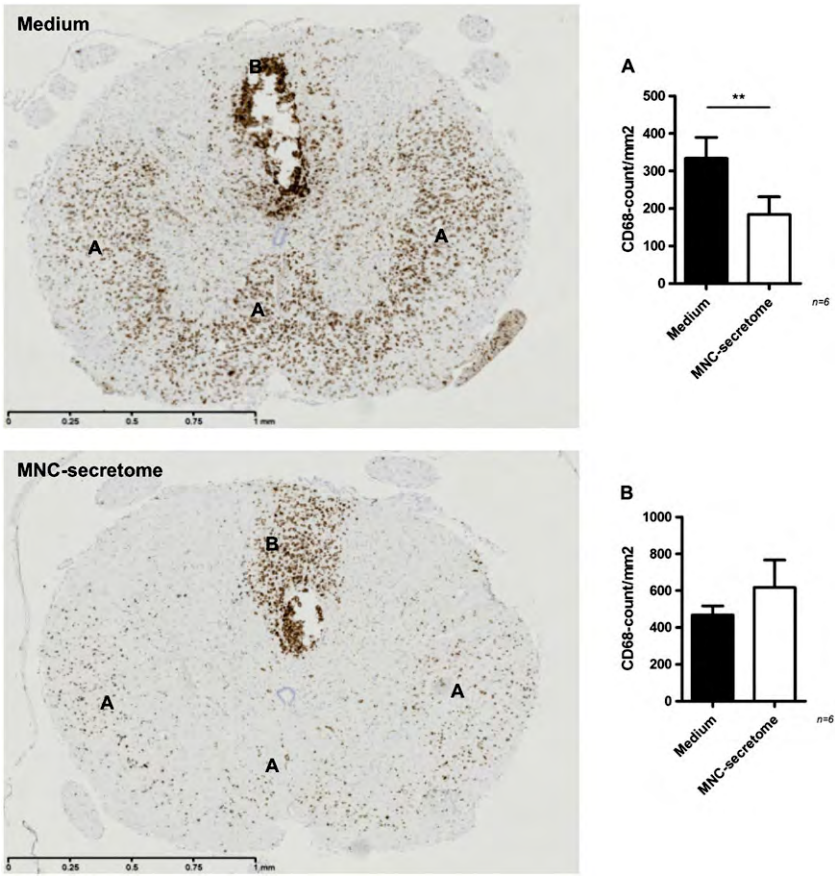


**Fig. 10.** CD68 expression 28 days after spinal cord injury (SCI). Representative images of immunohistological staining of CD68 in spinal cord sections 28 days after SCI. We found significantly lower expression levels of CD68 in the side and anterior columns of spinal cords after application of MNC-secretome (A; Medium vs. MNC-secretome: 333.8 ± 55.75/mm$^2$ vs. 183.7 ± 47.72/mm$^2$, $^{**}p < 0.01$). The expression levels of CD68 were slightly higher in the dorsal column in the treatment group, although this difference was not significant (B; Medium vs. MNC-secretome: 467.1 ± 49.4/mm$^2$ vs. 616.9 ± 149.2/mm$^2$, $p = 0.21$).

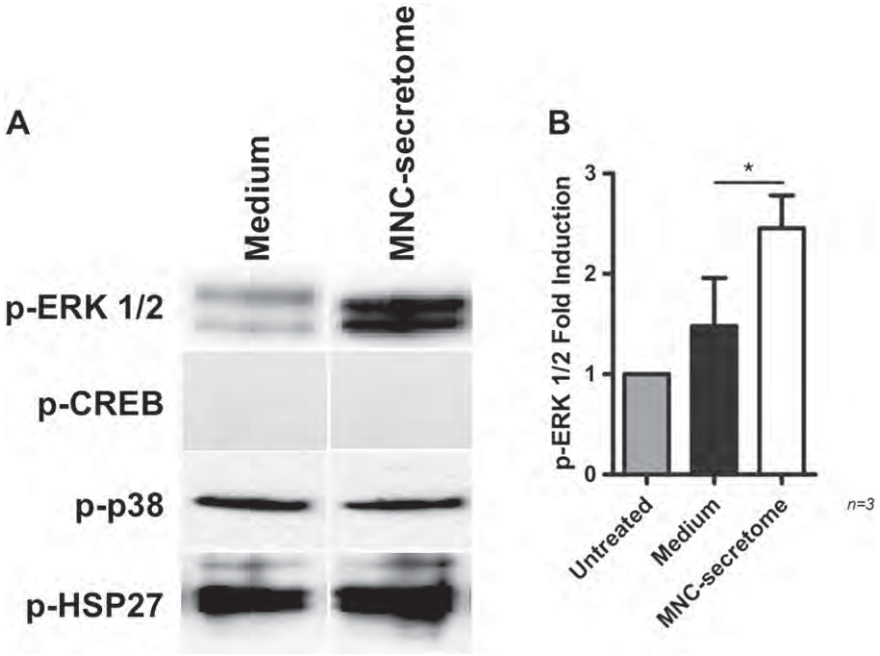


**Fig. 11.** MNC-secretome treatment leads to activation of the Erk-signaling cascade. Spinal cord tissue was obtained from healthy animals 2 h after i.p. treatment with either Medium or MNC-secretome. (A) Western blot analysis of the phosphorylated signaling factors pErk 1/2, pCREB, p-p38, and p-HSP27. (B) Fold increase of pErk 1/2 two hours after intraperitoneal treatment with either control medium or MNC-secretome compared to the spinal cord tissue of healthy untreated rats without spinal cord injury.

## Discussion

Damage to the spinal cord following trauma can lead to permanent neurological deficits and to ongoing pain. The secondary cascade following spinal cord injury aggravates the primary lesion and exacerbates neurological impairment. This cascade elicits inflammation and ischemia, with subsequent edema formation, closing the vicious cycle (Filli and Schwab, 2012; Fleming et al., 2006; Hall and Springer, 2004; Oyinbo, 2011). Until recently, researchers in the field focused mainly on therapies that targeted mechanisms involved in this secondary cascade.

In recent years, an increasing number of studies have suggested that the immune response following SCI—apart from its detrimental effects—positively influences the outcome of this type of trauma (Bowes and Yip, 2014; London et al., 2013; Rapalino et al., 1998; Shechter et al., 2009). Suggested mechanisms include the induction of neutrophil apoptosis, the release of neurotropic factors, and axonal regrowth (Bowes and Yip, 2014; Gordon and Taylor, 2005). Improved understanding of these mechanisms made the pursuit of an effective therapy that attenuates secondary damage after SCI even more complicated and there is still a need for a multimodal therapeutic strategy.

Previously published data from our group demonstrated the regenerative properties of MNC-secretome (Hoetzenecker et al., 2012; Lichtenauer et al., 2011b; Mildner et al., 2013). The therapeutic effects of MNC-secretome include upregulation of anti-apoptotic pathways, immune system modulation, inhibition of MVO, vasodilation, and inhibition of platelet activation, all of which play major roles in secondary injury mechanisms (Filli and Schwab, 2012; Fleming et al., 2006; Hall and Springer, 2004; Hoetzenecker et al., 2012, 2015; Joseph et al., 1991, 1992; Sotnikov et al., 2013; Thornton et al., 2010). Here we utilized a commonly used spinal cord contusion injury model in rats to investigate whether the previously published characteristics of MNC-secretome are also seen in this type of injury.

The data presented here show that MNC-secretome has angiogenic potential ex vivo (Figs. 5, 6). The observed angiogenic effects were not dependent on VEGF; however, we observed destabilized vessel structures on day 6 when anti-VEGF antibody was applied along with MNC-secretome. The stabilizing and anti-apoptotic effects of VEGF on endothelial cells have been described previously and might explain vessel degradation in the absence of VEGF (Folkman and Shing, 1992; Meeson et al., 1999; Thurston and Gale, 2004). These in vitro data were corroborated by histological and morphological data evidencing increased expression of vWF in injured spinal cords after treatment with MNC-secretome compared to control 28 days after trauma (Fig. 7). We hypothesize that in addition to possible neo-angiogenesis, inhibition of MVO and subsequent reduced degradation of obliterated vessels contribute to this result. These findings confirmed published data from our group in which MNC-secretome application led to increased vessel density in a murine wound model (Mildner et al., 2013). In addition, we found increased plasma levels of pro-angiogenic rat CXCL-1 after MNC-secretome treatment of rodents (Fig. 8). CXCL-1, also known as GROα, is known for its role in angiogenesis, among other processes (Fuhler et al., 2005; Lichtenauer et al., 2012; Miyake et al., 2013). Interestingly, some data indicate that overexpression of CXCL-1 has neuroprotective effects and causes remyelination (Omari et al., 2009). A previous study reported increased plasma levels of BDNF, a key modulator in neuronal survival and differentiation, after MNC-secretome application. This mechanism was very likely involved in the therapeutic effects observed in the present study (Altmann et al., 2014; Boyd and Gordon, 2003; Song et al., 2008). Both factors, i.e. CXCL-1 and BDNF, are reported to act via the Erk 1/2 pathway (Boyd and Gordon, 2003; Fuhler et al., 2005; Miyake et al., 2013; Song et al., 2008; Wenjin et al., 2011). I.p. administration of MNC-secretome resulted in increased phosphorylation of Erk 1/2 in spinal cord tissue just one hour after application, further suggesting direct activation of this pathway by factors in the secretome (Fig. 11). Activation of the Erk 1/2 pathway was reported previously to be involved in neuroprotection (Fu et al., 2014; Toborek et al., 2007).

Because it has both detrimental and beneficial effects, the immune response following spinal cord trauma remains a difficult therapeutic target. Monocyte-derived macrophages are reported to play a central role in orchestrating the resolution of inflammation and the initiation of regenerative processes, while activation of resident microglia is thought to aggravate the injury (Rapalino et al., 1998; Shechter et al., 2009). However, due to their overlapping expression profiles, distinguishing between microglia and monocyte-derived macrophages remains challenging (London et al., 2013; Popovich and Hickey, 2001).

Consistent with previous studies, we found increased recruitment of $CD68^{+}$ cells to the site of injury 3 days after trauma in the MNC-secretome treated group (Fig. 9) (Lichtenauer et al., 2011b). Upregulation of iNOS, triggered by pro-inflammatory cytokines or iron accumulation via phagocytosis of erythrocytes, is thought to reflect a pro-inflammatory M1 polarization of macrophages. This M1 polarization was mainly driven and maintained by TNF expression and iron accumulation of macrophages and was detrimental to neuronal viability and neurite growth in-vitro (Bao and Liu, 2002; Hall et al., 2010; Kroner et al., 2014; Lee et al., 2014; Yune et al., 2003). To evaluate the extent of M1 macrophage polarization in our study, we evaluated the expression levels of iNOS (Gehrmann et al., 1995; Han et al., 2013). Immunostaining revealed that MNC-secretome treatment led to lower expression levels of iNOS in injured spinal cords, indicating ameliorated oxidative stress on day 3 after spinal trauma (Fig. 6). We concluded that increased CD68 expression plus concomitant reduced iNOS expression in the treatment group indicates increased recruitment of monocyte-derived macrophages. If M1 polarization of macrophages is partially driven by phagocytosis of erythrocytes and subsequent iron accumulation, presumably comparable amounts of erythrocytes in the SCI lesions phagocytized by elevated numbers of CD68-positive macrophages in the MNC-secretome-treated animals could be responsible for decreased iNOS expression and M1 polarization in individual macrophages. Evaluation of iron staining revealed lower levels of iron accumulation in animals treated with MNC-secretome supporting this hypothesis (Supp. Fig. 7). Conducting flow cytometry, we found that upon incubation with MNC-secretome human CD14 positive cells up-regulated markers associated with the immunosuppressive M2-polarization (Supp. Fig. 8) (Biswas and Mantovani, 2010; Murray and Wynn, 2011). However, this view has to be substantiated by a more detailed characterization of the possible change of macrophage phenotypes related to MNC-secretome treatment. Shechter et al. reported that the expression of IL-10 is essential for the regenerative potential of monocyte-derived macrophages after SCI (Shechter et al., 2009). Further supporting our hypothesis, we found a trend towards higher IL-10 plasma levels in MNC-secretome treated animals 3 days after spinal cord contusion (Supp. Fig. 2). On post-injury day 28, the number of CD68-positive cells was significantly lower in the MNC-secretome treatment group compared to control animals, suggesting more advanced resolution of the inflammatory response in animals treated with MNC-secretome (Fig. 10).

Taken together, these results imply that the combination of effects observed on vascularization and modulation of inflammation lead to improved motor function in MNC-secretome treated animals after SCI (Fig. 2). Surprisingly, histological evaluation revealed decreased levels of βAPP, indicating reduced axonal pathology (Fig. 4); further, lesion volume was attenuated in the treatment group, indicating that MNC-secretome reduced secondary damage after SCI (Fig. 3).

CD43 is expressed by all types of T cells, irrespective of their activation state (Clark and Baum, 2012). Previous work from our group showed that MNC-secretome has inhibitory and immune-suppressive properties and is protective against experimentally induced T-cell dependent autoimmune disease (Hoetzenecker et al., 2015). However, in this study, we did not observe any differences in CD43 expression between the MNC-secretome group and the Medium control group (Supp. Fig. 3), indicating an unaltered total number of T-cells in injured spinal cord after MNC-

secretome. This, however, does not exclude the possibility of effects of MNC-secretome treatment on the phenotype of T-cells in the setting of experimental SCI but was not further evaluated in this study.

We extended our findings by investigating differences in the ChaT staining in the treatment and control groups 3 and 28 days after trauma (Supp. Figs. 4, 5). We hypothesized that functional preservation in treated animals correlated with less acute axonal injury, as reflected by lower βAPP levels, rather than morphological recovery observable by day 28 in the ChaT staining.

Multiple previously studies have examined treatment with a single factor for complex models of disease. A study published by Lutton et al. reported the beneficial effects of the growth factors PDGF and VEGF after spinal cord hemisection in rats (Lutton et al., 2012). Interestingly, only the use of both factors together proved efficacious, while either PDGF or VEGF alone had deleterious effects that were recently confirmed in a contusion injury model (Chehrehasa et al., 2014; Lutton et al., 2012).

Here we present compelling evidence that MNC-secretome can affect multiple biological mechanisms involved in attenuating secondary damage after SCI. Both PDGF and VEGF are present in MNC-secretome and might be involved in its observed therapeutic effects (Lichtenauer et al., 2011a).

Over the years, the focus of the field of cell-based therapy has moved away from a "cell-centric" view and towards soluble paracrine factors that have observed effects in multiple pre- and clinical studies (Cantinieaux et al., 2013; Quertainmont et al., 2012; Teixeira et al., 2013; Wright et al., 2011). The first publication in the literature that supported the "paracrine hypothesis" was published in 2005 by Gnecchi et al. (2005). A recent study described the regenerative and immunomodulatory effects of the application of BMSC-conditioned medium in a SCI rat model, findings that are in accordance with our results (Cantinieaux et al., 2013).

In contrast to obtaining bone marrow stem cells and their paracrine factors, the method used to obtain MNC-secretome is simple. In previous work we showed that MNC-secretome could modulate internal inflammatory reactions in experimental stroke, acute myocardial infarction, and myocarditis (Altmann et al., 2014; Hoetzenecker et al., 2012; Hoetzenecker et al., 2015; Lichtenauer et al., 2011b).

A recent study compared the expression profiles of stem cells and MNCs (Korf-Klingebiel et al., 2008). The authors found similar cytokine concentrations in the supernatants of both cell types, with small differences in 35 of the 174 investigated secreted factors. MNCs have advantages compared to stem cells as "bioreactors" because (a) MNCs are an underutilized raw material in that currently MNCs are a waste product of the blood product generation process; (b) the secretome derived from MNCs shows minimal or no antigenicity owing to the absence of cellular epitopes; (c) MNC-secretome is easy to produce; (d) lyophilizing the conditioned medium allows "off the shelf" utilization in a clinical setting; (e) the steps involved in MNC-secretome production, including viral clearance, can be adapted to the setting of a GMP production line (which is a mandatory regulatory requirement for later clinical use in humans).

In an experimental setting, we administered the xenogenic human MNC-secretome i.p. rather than applying it locally in order to determine whether systemic application produces the desired effects found previously in experimental SCI (Abrams et al., 2012; Lee et al., 2014; Ravikumar et al., 2007). There are two possible explanations for the efficacy of systemic treatment. First, the breakdown of the blood–brain barrier after SCI allows penetration of systemic therapies; second, the systemic inflammatory response to trauma is decisive in terms of the extent of second injury (Abrams et al., 2012; Lee et al., 2014; Ravikumar et al., 2007).

Our data confirmed that the human-derived MNC-secretome attenuates secondary damage after experimental SCI. Currently we have approval from the Austrian Health Authority (AGES) to produce human allogeneic MNC secretome according to GMP guidelines, including mandatory viral clearance methods. This study provides a sound basis for the use of MNC-secretome in future SCI clinical trials.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.expneurol.2015.03.013.

## Author disclosure statement

The Christian Doppler Research Association, APOSCIENCE AG, and the Medical University of Vienna funded this study. The Medical University of Vienna has claimed financial interest (Patent number: EP2201954, WO2010070105-A1, filed Dec 18th 2008). H.J.A. is a shareholder of APOSCIENCE AG, which owns the rights to commercialize MNC-secretome for therapeutic use. All other authors declare that they have no competing financial interest.

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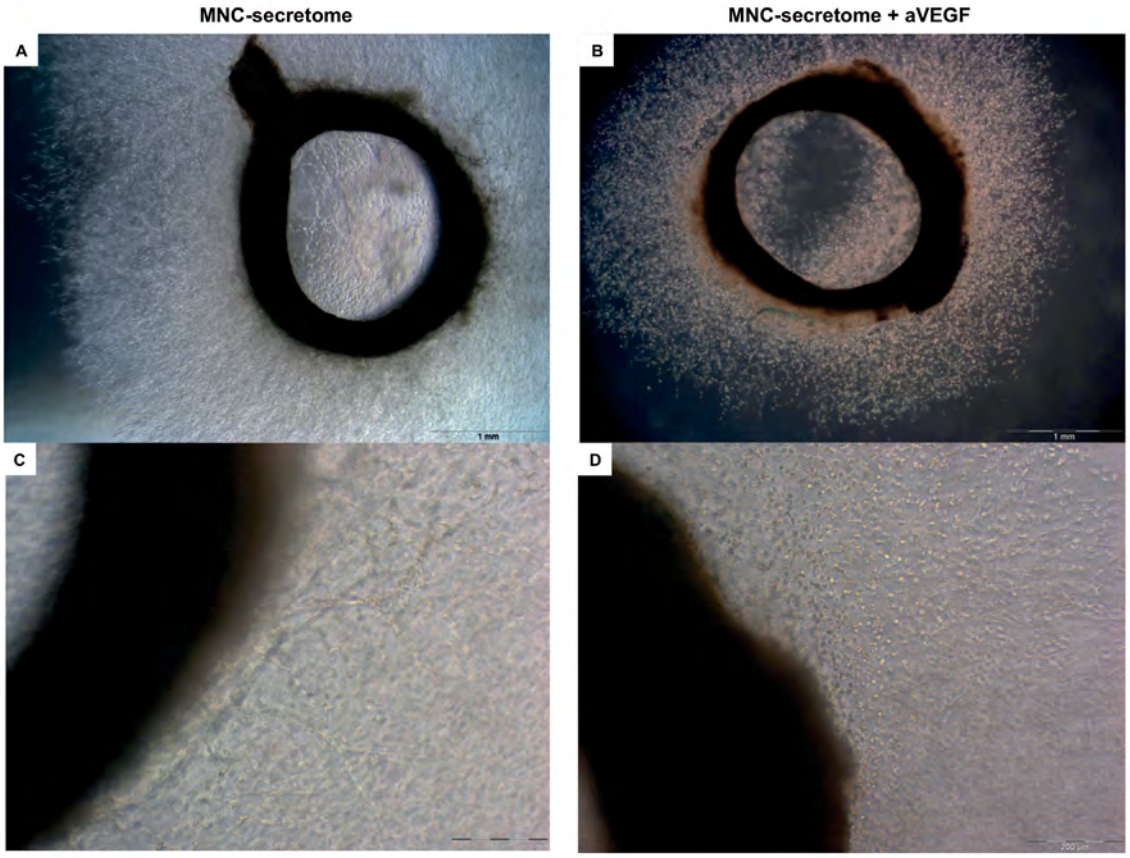


*Figure 10: Effects of MNC-secretome and anti-VEGF antibody in the aortic ring assay. Representative phase contrast images of aortic ring cultures with MNC-secretome show stable vascular structures (A, C). Addition of anti-VEGF antibody to aortic rings exposed to MNC secretome leads to destabilization of the preformed vessel structures after six days of 3D culture (B, D). (Supplementary figure from [397])*

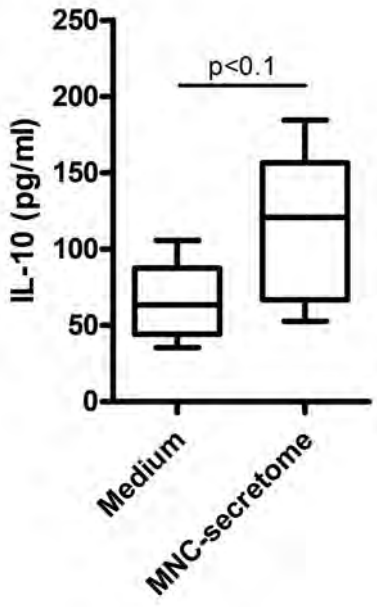


*Figure 11: Elevated plasma levels of IL-10 after MNC-secretome treatment 3 days after spinal cord injury (SCI). Compared to the Medium control group, there was a trend towards higher plasma levels of IL-10 in animals treated with MNC-secretome on day 3 after SCI (Medium vs. MNC-secretome: 65.39 ± 11.6 pg/ml vs. 113.4 ± 22.43 pg/ml, p = 0.094). n = 5 animals per group. (Supplementary figure from [397])*

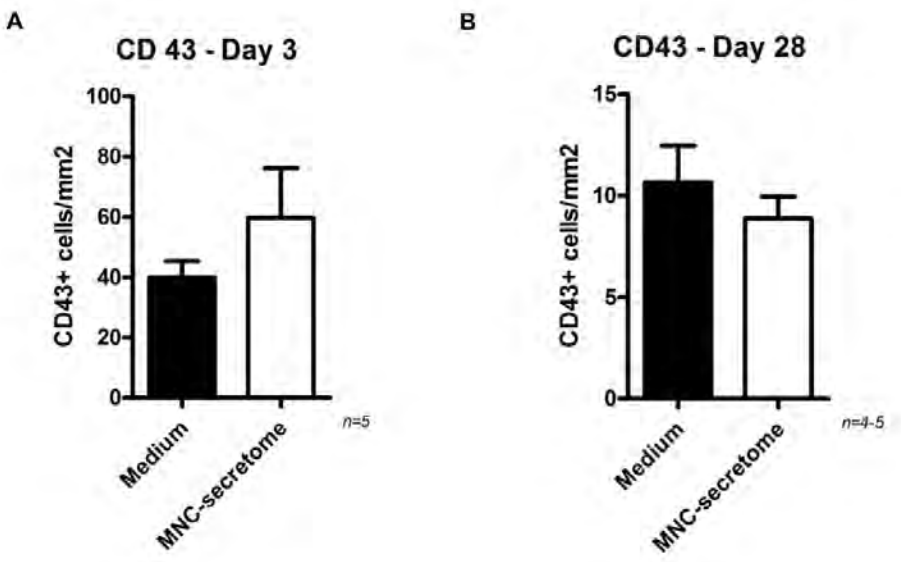


*Figure 12: CD43 expression in spinal cord 3 and 28 days after spinal cord injury (SCI). Immunohistochemistry showed no significant differences in the number of CD43-positive cells on day 3 (A; Medium vs. MNC-secretome: 40.03.1 ± 5.27/mm$^2$ vs. 59.68 ± 16.46/mm$^2$, p = 0.21) or on day 28 (B; Medium vs. MNC-secretome: 10.65 ± 1.82/mm$^2$ vs. 8.87 ± 1.08/mm$^2$, p = 0.29) after spinal cord injury. (Supplementary figure from [397])*

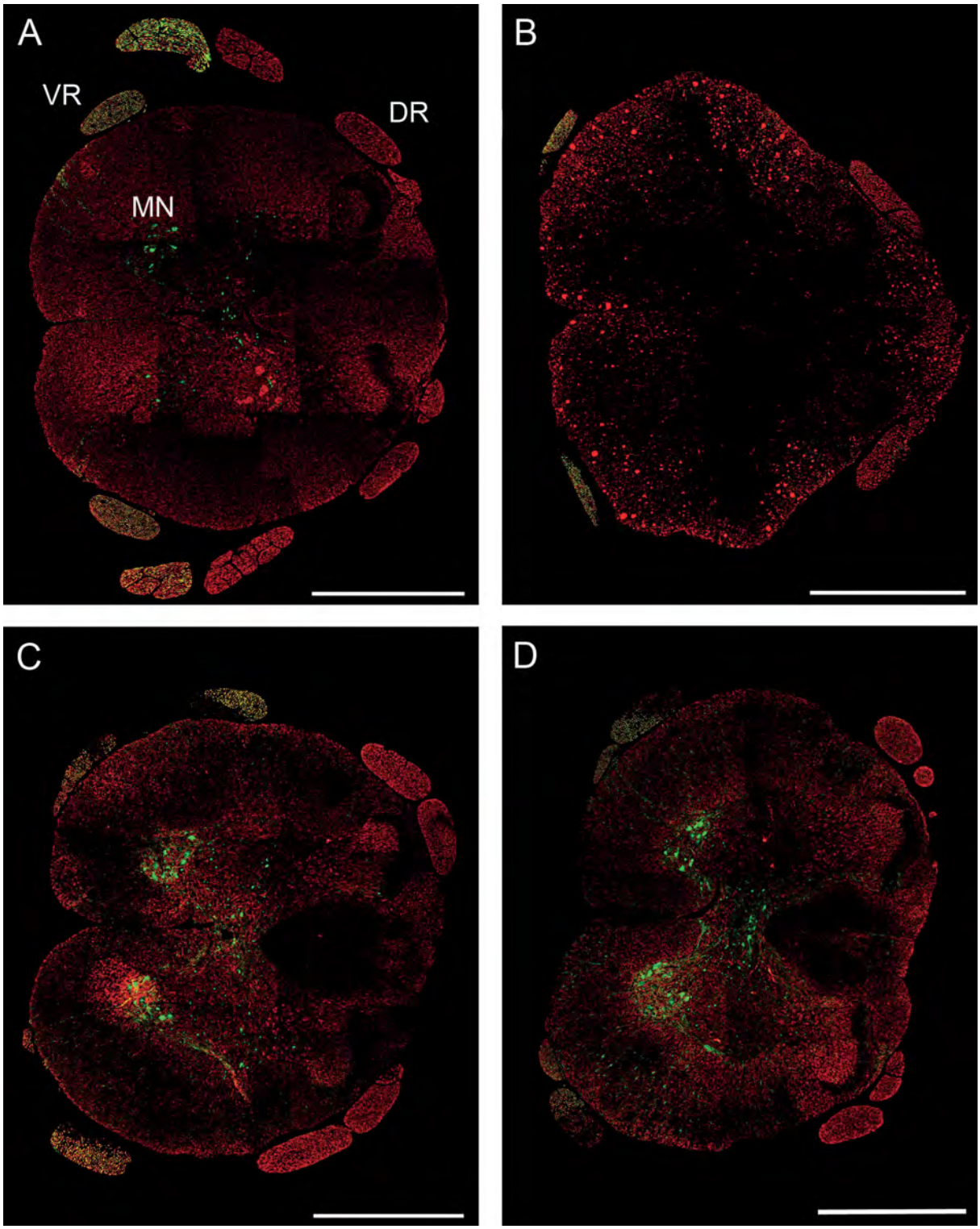


*Figure 13: Expression of choline acetyl transferase (ChAT) and neurofilament protein in the spinal cord as determined using immunohistochemistry. (A) A representative image of a cross section through the spinal cord of a healthy control animal at the level of thoracic segment T11. The spinal cord shows normal morphology. The ventral roots (VR) are positive for ChAT (green) and for neurofilament protein (red); dual-positivity is yellow. Motoneurons (MN) of the ventral horn exhibit ChAT immunoreactivity as well. The dorsal roots (DR) exhibit no ChAT immunoreactivity and appear red. (B) A representative image of a spinal cord 3 days after spinal cord injury (SCI). The cross section is positioned 4-mm caudal to the lesion. The spinal cord shows no boundary between the gray and white matter and shows a large cavitation. The*

*ventral roots exhibit ChAT/neurofilaments protein staining. No motoneurons were detected in the ventral horn. C, D A representative image of a spinal cord 28 day after SCI (C) with medium control treatment and (D) with MNC-secretome treatment. The cross section is positioned 4-mm caudal to the lesion. In both animal groups, the spinal cord recovered 28 days after SCI. Motoneurons are visible in the ventral horn. Numerous axons in the ventral roots appear damaged and lacked ChAT/neurofilament immunoreactivity. Based on immunohistochemistry, there was no difference at 28 days between medium and MNC-secretome treated animals. Scale bars, 1 mm. (Supplementary figure from [397])*

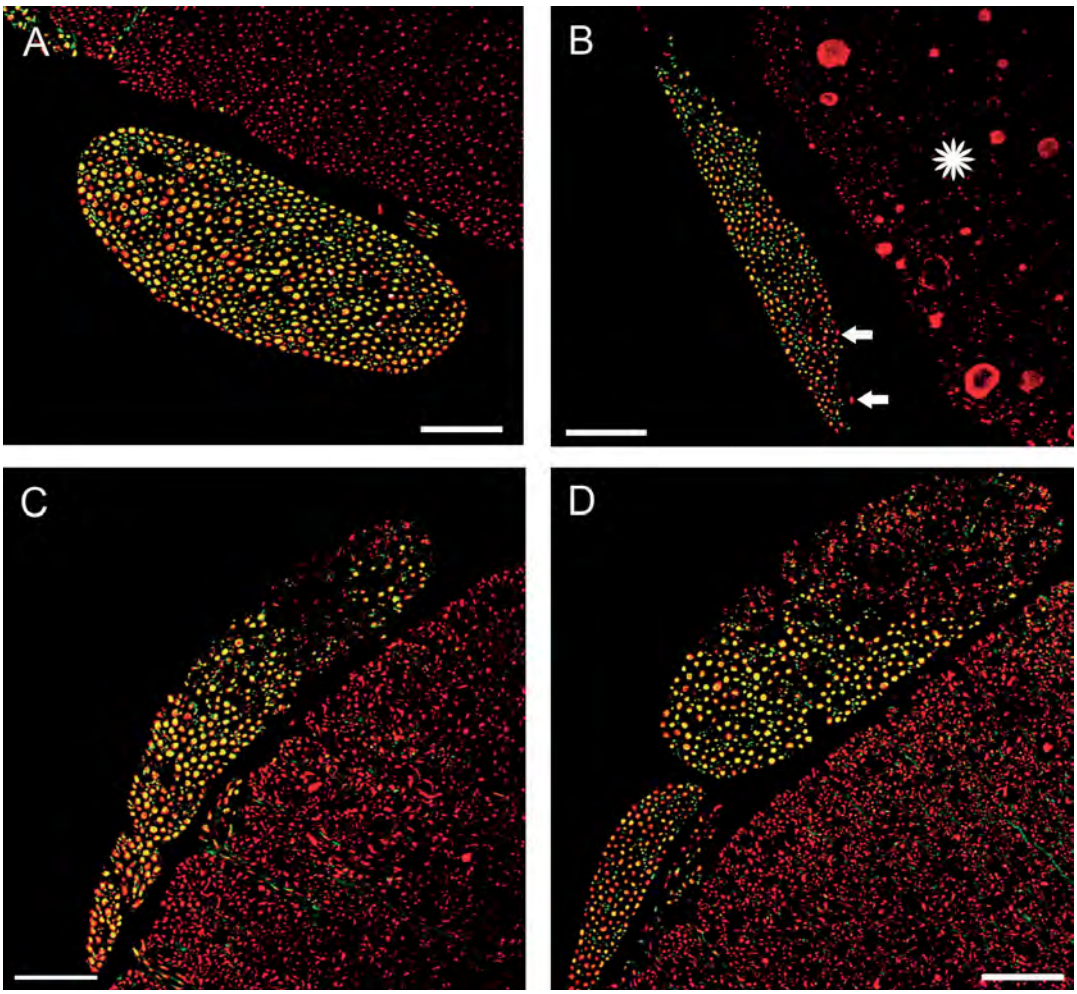


*Figure 14: Staining of the ventral root of the spinal cord with antibodies against choline acetyltransferase (ChAT) and neurofilament protein. (A) A cross section through the ventral root of a healthy control animal. All axons showed both ChAT (green) and neurofilament protein (red) immunoreactivity. In the overlay, double-positive axons appear yellow or show a mix of colors. (B) A cross section through the ventral root of an animal 3 days after spinal cord injury (SCI). Almost all axons were positive for ChAT/neurofilaments protein. Very few axons (arrows) lacked ChAT staining. In the white matter of the spinal cord (asterisk), some axons had a swollen appearance, and there were areas between the axons without a neurofilament protein signal. (C), (D) Cross sections through the ventral roots of 2 animals 28 days after spinal cord injury (SCI): (C) medium control treatment, (D) treatment with MNC-secretome. In both cases, many axons showed ChAT/neurofilament protein immunoreactivity. Other axons showed no ChAT signals. Scale bars, 100 µm. (Supplementary figure from [397])*

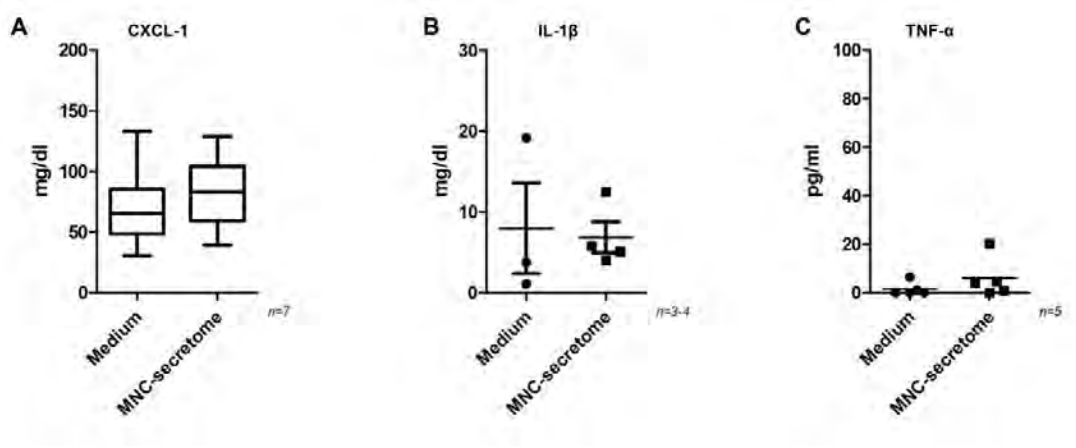


*Figure 15: Cytokine levels on day 3 after spinal cord injury (SCI). There were no differences in the plasma levels of the following cytokines in animals in the medium treatment control group versus the MNC-secretome treatment group on day 3 after spinal cord injury: (A) CXCL-1 (Medium vs. MNC-secretome: 70.85 ± 12.25 pg/ml vs. 85.43 ± 11.26 pg/ml, p = 0.39); (B) IL-1β (Medium vs. MNC-secretome: 7.992 ± 5.611 pg/ml vs. 6.871 ± 1.91 pg/ml, p = 0.84); (C) TNF-α (Medium vs. MNC-secretome: 1.519 ± 1.249 pg/ml vs. 6.017 ± 3.695 pg/ml, p = 0.28). (Supplementary figure from [397])*

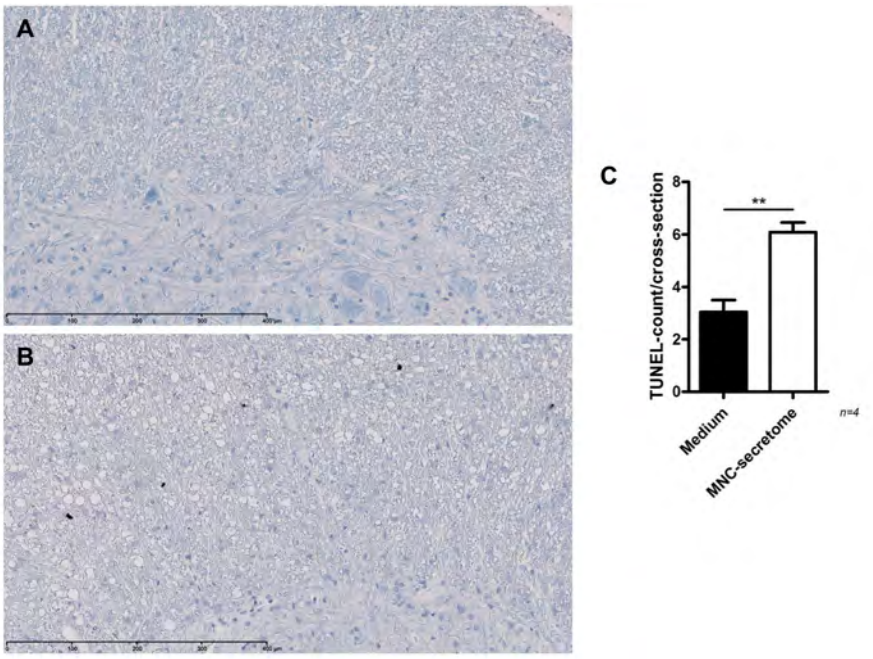


*Figure 16: Number of TUNEL-positive cells in the spinal cord was increased in MNC-secretome treated animals. A higher number of TUNEL-positive cells were found in the treatment group (B) 3 days after injury compared to the control group (A). By 28 days post injury, no difference in the number of TUNEL-positive cells between the two groups was observed (data not shown).*

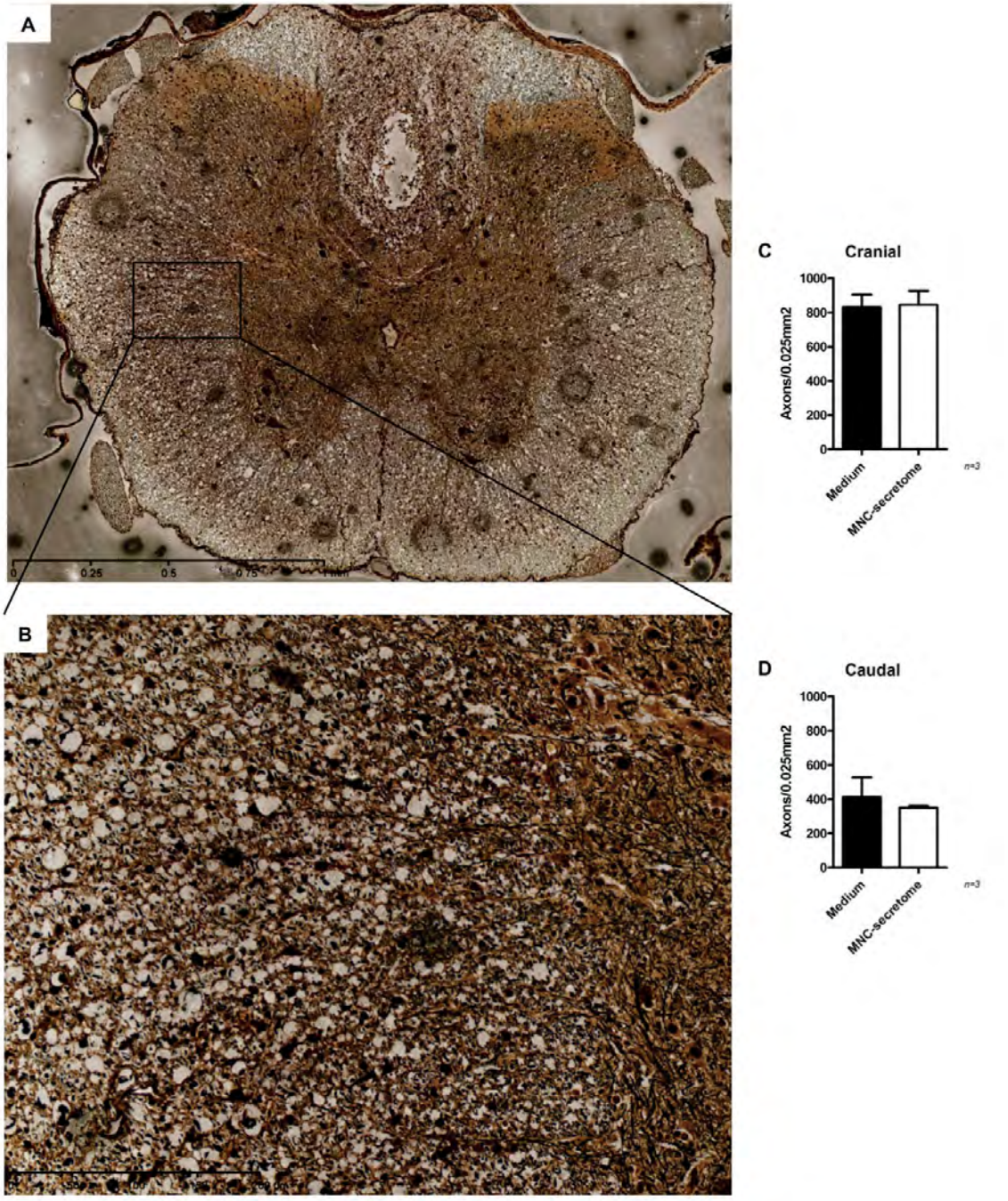


*Figure 17: Quantity of axons was not influenced by treatment with MNC-secretome. Bielschowsky silverstaining showed that there was no difference in axons count in the side column of spinal cords of the two groups cranial (C; Medium vs. MNC-secretome: 829.3±75.52/0.025mm2 vs. 844.7±82.19/mm2, p=0.89) and caudal to the lesion (D; Medium vs. MNC-secretome: 412.0±114.4/0.025mm2 vs. 350.0±11.93/mm2, p=0.62).*

# CHAPTER THREE: Discussion

## 3.1 General Discussion

The spinal cord represents a data highway allowing transportation of information from the brain to peripheral structures and back regulating and sustaining neurological function of the organism. To meet high demands structures within the spinal cord from a tightly regulated complex network of various cell types able to process vast amount of information at the same time.[15] Sophisticated and complex mechanisms are required to maintain homoeostasis within the spinal cord. Injuries to the spinal cord interfere with this delicate equilibrium and cause permanent impairment and neurological deficits accompanied by severe pain.[11,398] In most cases, SCI affects young adults leading to long time spans of impairment with devastating individual and economic burden.[30,166]

Due the complex multilayered architecture of the spinal cord, injures of this delicate structure lead to a multitude of alterations and complications on a cellular and molecular level. After the primary hit endogenous response to the trauma is initiated. Aside from potentially desirable molecular and cellular responses, endogenous answers to the trauma also cause aggravation of spinal cord damage, referred to as secondary damage.[52,53,349] This secondary damage is also present in other forms of medical conditions and trauma like myocardial infarction, stroke and burn injuries among others.[399-401] Due to this equivocal, partially beneficial partially deteriorating response, interference with the secondary response of therapeutic strategies has proven to be a challenging task with no satisfying candidate at hand up until today.[63]

Substantial effort was undertaken to discover therapy strategies capable of improving the outcome of SCI patients. But still, administration of high-dose glucocorticoids, mainly in the form of methylprednisolone, represents the only approved non-surgical treatment in SCI up until today.[181] The rationale behind using methylprednisolone was reduction of edema formation within the spinal cord after trauma and attenuation of the inflammatory response.[283] Despite being approved for several decades, treatment with glucocorticoids remains controversial. Contradicting reports on efficacy, major side effects and lack of alternatives resulted in an ongoing discussion. In 2013, the AANS revoked its 2002 recommendation for the use of methylprednisolone due to the increasing number of reported side effects and lack of evidence for its efficacy in improving neurological outcome after SCI.[181,182] Cellular treatment especially received a lot of attention in the pursuit of discovering novel therapeutic approaches.[63] The principal idea was to administer pluripotent progenitor cells differentiating at the site of injury thereby replacing damaged or dead cells and restoring spinal cord function. Initial euphoria due to positive pre-clinical and short-term clinical data stagnated

after studies reported lacking long-term efficacy.[63] Also, it became clear that injected cells are not capable of settling at the point of interest, the damaged spinal cord area for example. Already hours after administration, most transplanted cells were found in the spleen or liver.[224,227,361,364,384] Furthermore, studies reported that certain cell types used for transplantation were not able to transdifferentiate into needed cell types.[220] Besides technical issues, also ethical concerns considering the use of progenitor cells, especially those derived from embryonic origin, were addressed.[63,260]

In 2005, two major findings paved the way for a novel approach in the field of regenerative medicine. Thum et al. reported that most of administered cells in stem cell studies undergo apoptosis at the time being infused. They argued that therapeutic effects observed might be a consequence of apoptotic cells inducing anti-inflammation and cytoprotection.[366] Gnecchi and co-workers further postulated that observed effects after cellular therapy in myocardial infarction was a consequence of paracrine factors released by transplanted cells rather than homing and cardiomyogenic differentiation. Their hypothesis was based on 3 observations. Firstly, therapeutic effects were reported to occur already within 72 hours after administration suggestive of mechanisms other than direct cellular actions. Secondly, therapeutic efficacy increased when cells were provided with improved secretory capacity by overexpression of Akt1. And thirdly, conditioned medium alone exhibited equal therapeutic capacity in-vitro and in-vivo.[265-268] Paracrine factors produced by these cells are thought to exert pleiotropic effects.[267] Besides up-regulation of anti-apoptotic pathways improving cellular survival, angiogenesis, immunomodulation and regenerative properties are thought to be involved mechanisms. These effects are not restricted to a specific cell type, which is of utmost importance in regeneration of complex tissue and organs, respectively.[267]

Cellular stress causes profound alteration of expression profiles with concomitant increase in secretory capacity. Intentionally applied cellular stress is referred to as preconditioning. Several preconditioning methods have been described including hypoxia, starvation, various molecules such as TNF-α or LPS, physical stimuli and irradiation. Irradiation effectively causes apoptosis, can be applied comparatively easy, is readily available and introduction of agents into the cell culture is not required.[402] A study conducted by our group demonstrated that irradiation leads to expression of 213 genes associated with secretion of proteins compared to 167 in non-irradiated PBMCs.[388] Higher expression levels of these genes were associated with increased biological activity of secretome obtained from irradiated PBMCs in comparison to conditioned medium obtained from non-irradiated PBMCs.[388]

Expression profiles of stem cells and PBMCs are much more comparable than initially expected. This was one of the findings in a study published in 2008 conducted by Wollert

and colleagues. Using ProteinChip arrays the authors found quantitative differences of secreted factors in conditioned medium from either PBMCs or BMSCs in only 35 of 174 investigated factors.[367] Compared to stem cells PBMCs feature some favorable characteristics. Acquisition and handling in the cell culture is comparatively easy. Also, peripheral blood being the source of PBMCs, a larger pool of cells for utilization is available and easier to exploit. Today, PBMCs are considered only a waste product in the production of erythrocyte concentrates and discarded during the process. And, ethical concerns regarding the use of PBMCs can be neglected. Over the past year, research conducted by our group focused on PBMCs as source of paracrine factors. Picking up and combining the postulations made by Anker and Gnecchi in 2005, we were able to show that administration of apoptotic PBMCs exhibits therapeutic potential in a pre-clinical model of AMI. Compared to control group, animals receiving apoptotic PBMCs had improved left ventricular function and smaller infarction areas histologically. Dyeing of cells revealed that most cells can be found in the liver and spleen 24 hours after administration without any observable homing to the myocard.[384] This finding lead to studies investigating the efficacy of the secretome or conditioned medium obtained from apoptotic PBMCs alone by discarding the cellular part after cultivation and utilizing the supernatant for further processing. Subsequently, therapeutic efficacy of this secretome was reported in various pre-clinical models of disease including an acute wound model, myocardial infarction, stroke, experimental myocarditis and experimental burn wounds (Hacker et al., unpublished data).[275-278,383,384] Efficacy of the secretome was explained by modulation of multiple targets exerting pleiotropic effects. It was shown that co-incubation of primary human cardiomyocytes, keratinocytes, fibroblasts, endothelial cells, glial cells, and neurons with the secretome leads to up-regulation of pathways associated with anti-apoptosis and pro-survival genes like CREB, Akt or Erk 1/2. In-vitro studies showed capability of conditioned medium from apoptotic PBMCs to induce angiogenesis, fibroblast and keratinocyte migration, neuronal sprouting and neurite growth, as well as inhibition of thrombocyte activation and vasodilation.[275,276,278,381] Also, immunomodulatory characteristics were reported.[277,381] These findings were confirmed in-vivo using various, above-mentioned experimental models, which lead to improved clinical and functional outcome.[275-278,381]

At first glance, the above-mentioned clinical entities addressed experimentally might seem to feature rather heterogeneous pathophysiology, but share common pathways in disease development and progression. For instance, inflammation is a dominant endogenous reaction to internal and external trauma like skin lesions or myocardial infarction. Its purpose is to eliminate potential pathogens and preparing the injury site for repair and regeneration. Prolonged inflammatory response, on the other hand, causes further tissue damage and worse clinical outcome.[403,404] Micro-vascular obstruction summarizes the phenomenon of

reduced blood flow in small vessel around the injury site caused by activation of thrombocytes and vasoconstriction. This leads to a larger areas at risk in terms of ischemic damage, for instance in myocardial infarction, stroke and cutaneous wounds.[276,403-406] Also, induction of apoptosis in primarily unaffected cells caused by paracrine signaling adjacent to the damaged area leads to lesion progression in stroke and myocardial infarction.[369,401,407] These pathways also play a role in the secondary cascade following SCI and cause aggravation of the lesion and worse neurological outcome. Besides its detrimental nature, this secondary damage offers targets for treatment strategies. Although numerous pre-clinical and several clinical studies were conducted with the aim to counteract this secondary damage a convincing candidate is still not at hand up until today.[63] Publications in the recent past postulating partially beneficial attributes of this secondary cascade demonstrated impressively the complexity of the endogenous response to spinal cord trauma. Also, timing of activated pathways within this cascade seems to play a pivotal role further complicating the pursuit of discovering treatment strategies.[408]

The secretome obtained from apoptotic PBMCs, as mentioned above, is capable of interacting with multiple molecular pathways, many of which were previously described to play a role in the secondary cascade following traumatic SCI.[35,51,52,349] Therefore, the aim of this study was to assess whether this secretome is capable of interacting with the secondary cascade after the event of SCI and delineate possible involved mechanisms. For this purpose, we utilized a commonly used impaction model in rodents with the advantage of resembling trauma morphology most frequently present in human SCI patients.[3,351]

In recent years, studies reported that the endogenous secondary answer to SCI not only causes further damage but rather also represents a crucial step in recovery and restoration of spinal cord function. Especially in the context of immunological response more and more data suggests a pivotal role of contained inflammation for recovery after trauma to the spinal cord. Mechanisms involved in this favorable immunological response are stimulation of axonal regeneration and secretion of neurotropic factors.[138,408] This might also explain the inefficacy of causal immunosuppression with cortisone treatment.[181] Most notably monocytes and accordingly macrophages and their subpopulations within the immunological response to SCI were of interest. After trauma to spinal cord recruitment of monocyte from the peripheral blood causes accumulation of macrophages around the lesion. While monocyte-derived macrophages are thought to initiate resolution of inflammation and prepare the tissue for endogenous regenerative efforts, the resident macrophages of the CNS, microglia, are associated with nourishing of inflammation and exacerbation of tissue damage by expression of iNOS resulting in higher levels of oxidative stress among other mechanisms.[135,409] Common expression patterns among microglia and monocyte-derived macrophages however make it difficult to distinguish between these two cell types of distinct

origin.[134,410] Furthermore, discrimination between M1- and M2-polarization of macrophages seems to be important. While pro-inflammatory M1-macrophages are thought to promote further tissue damage, while anti-inflammatory M2-macrophages seem to contain excessive inflammation and prepare the lesion site for reparation. M1-polarization was shown to interfere with neurite growth and to be detrimental for neuronal survival.[141,411-414] Previous studies reported that TNF-α and accumulation of iron from bleedings within the lesion site promote and maintain M1-polarization and is linked to increased expression of iNOS.[141]

Histopathological analysis showed increased levels of $CD68^+$ cells adjacent to the lesion following treatment with MNC-secretome 3 days after experimental SCI. This finding is consistent with previous studies in experimental myocardial infarction.[381] We also found an increased number of apoptotic cells in the spinal cord of animals in the treatment group. Our interpretation of this result is that it represents a consequence of increased recruitment of $CD68^+$ cells to the spinal cord caused by the treatment and subsequent apoptotic clearance. CD68 is a marker both expressed on monocyte-derived macrophages and microglia and can therefore not be used to differentiate between these two cell types. Also, M1- and M2-polarization cannot be depicted using this marker. Staining against iNOS revealed lower expression levels with concomitant ameliorated oxidative stress in the treatment group indicating a higher prevalence of M2-macrophages of peripheral blood origin. According to previous published data, iron phagocytosis and intracellular accumulation drives M1-polarization.[141] We assume that higher presence of macrophages around the lesion results in lower individual cellular iron phagocytosis thereby decreasing tendency towards M1-polarization and iNOS expression. This hypothesis is further supported by results obtained from iron stainings revealing lower levels of iron accumulation in MNC-secretome treated animals. Flow cytometry experiments suggested also a direct influence of the secretome on M2-polarization. Incubation of human $CD14^+$ cells with the secretome resulted in up-regulation of markers associated with M2-polarization.[415,416] Further studies are warranted to validate and substantiate these findings of possible effects of MNC-secretome on macrophage polarization. Interestingly, 28 days after SCI we found significantly lower levels of CD68-expression in the treatment group suggestive of faster clearance and resolution of inflammation. In 2009, a study reported that expression of IL-10 is pivotal for beneficial potential of macrophages after trauma to the SCI. In animals treated with MNC-secretome 3 days post-injury we found a tendency towards higher systemic plasma levels of IL-10. Also, activation of thrombocytes follofiwng CNS damage was reported to promote neuroinflammation with aggravation of neuronal damage.[417-420] As mentioned above, MNC-secretome was recently shown to inhibit thrombocyte activation. It is tempting to argue that MNC-secretome also caused indirect inhibition of detrimental neuroinflammation via inhibition of thrombocyte activation in this study. However, this possible mode of action of

secretome treatment was not further investigated in this thesis and will be the subject of future studies.

To evaluate angiogenic capacity of the secretome we utilized an ex-vivo aortic ring model and found significantly increased formation of vessels and sprouting compared to control medium. The angiogenic potential was independent of VEGF in inhibition assays but we observed destabilization of vessels after 6 days in absence of VEGF. This suggests that factors other than VEGF within the secretome are capable of inducing angiogenesis but are not capable to maintain the structure of newly formed vessels. This finding is in accordance with previously published data ascribing anti-apoptotic effects on endothelial cells and vessel stabilizing properties to VEGF.[421-423] To evaluate whether observed angiogenic capacity is also present in CNS-derived tissue we modified the angiogenesis assay and used spinal cord tissue of healthy, uninjured rats instead of aortic rings within the same experimental setup. Indeed, MNC-secretome unfolded angiogenic potential also in this setting. Vessel formation was confirmed using confocal microscopy and staining against a specific rat endothelial marker. These results were substantiated by in-vivo results showing increased vessel density in animals treated with MNC-secretome compared to control group. Higher vessel density after secretome treatment was also observed in an experimental wound model and corroborates this finding.[275] We hypothesize that aside from neo-formation of vessels inhibition of MVO and subsequent reduced amount of vessel degradation after SCI might further explain this result.[276] Plasma levels of CXCL-1/GROα, a cytokine known to promote angiogenesis, neuroprotection and re-myelination were increased in animals after MNC-secretome administration.[424,425] Previously, our group reported increased BDNF plasma levels upon MNC-secretome treatment.[278] This growth factor efficiently promotes differentiation and survival of neuronal cells.[233,324] Both CXCL-1 and BDNF activate the intracellular Erk 1/2-pathway.[233,324,424,426,427] Higher phosphorylation levels of Erk 1/2 were found in the spinal cord of animals 24 hours after systemic secretome treatment. We speculated that both higher levels of plasma CXCL-1 and BDNF levels, as wells as direct effects of the secretome contributed to this result. Fu et al. and Toborek et al. reported independently that activation of the Erk 1/2-pathway results in neuroprotection.[428,429]

Neurological evaluation revealed that treatment with secretome derived from apoptotic PBMCs was accompanied by significantly improved recovery of motor function with higher BBB-scores from day 14 after injury on. Histological assessment showed attenuated axonal pathology as well as reduced extent of lesion volume in the MNC-secretome treatment group compared to control medium. We were not able to observe differences in quantity of axons or motor neurons in ChaT or Bielschowsky stainings between both groups. We concluded that in our acute experimental setting with the endpoint being 28 days after injury significant axonal and neuronal regeneration couldn't be observed. Rather modulation

of secondary mechanisms as described above attenuated damage after the initial impact and might have caused the observed improvement in functional outcome.

MNC-secretome was reported to induce apoptosis of T-cells thereby leading to immunosuppression. It was also shown that this treatment is capable of preventing experimental myocarditis, a T-cell dependent disease. However, in this study expression of CD43, a T-cell marker expressed irrespective of their activation state, within the spinal cord was not altered after MNC-secretome treatment.[430] This result is suggestive of comparable T-cell counts in injured spinal cords between both groups. Further experiments in this regard were not conducted in this study, therefore possible effect on T-cells of MNC-secretome can not be excluded, e.g. modulation of T-cell phenotypes.

In concordance with here presented results, recently published data suggested that paracrine factors from cultured MSCs also show therapeutic efficacy in SCI. Conditioned medium obtained from cultured MSCs lead to improved neurologic outcome and exerted angiogenic capacity in-vitro and in-vivo.[264]

We were not able to identify a single factor within the used secretome capable of orchestrating all of the observed effects and therefore concluded that observed pleiotropic effects are caused by the combination of various factors. Complex diseases and multilayered endogenous responses to trauma in general seem unlikely to respond to treatment targeting a single factor or manipulating one pathway. A “golden bullet” in form of a single molecule treatment most likely does not exist.[185,204] Treatment with a single factor might even have deleterious effects on the outcome after spinal cord injury. In previously published study, Lutton et al. showed that the combination of the growth factors PDGF (platelet-derived growth factor) and VEGF exerted therapeutic capacity in experimental SCI. Controversially at first glance, the sole administration of either PDGF or VEGF worsened the functional outcome.[326,327] A possible explanation might be neo-formation of incomplete, leaky vessels due to one-sided and insufficient growth stimulus and increased permeability of already present vessels.[431] This further highlights the incapability of a single factor treatment after SCI and even demonstrates possible hazard arising from punctual modulation of the secondary cascade following SCI.

We decided to apply MNC-secretome systemically because it is more feasible in the clinical setting especially in terms of fast and safe administration in acute clinical entities. We suggest that efficacy of this treatment despite being applied systemically can be explained as follows. Firstly, injury to spinal cord causes disruption of the BBB not only at the site of lesion. Also distant to the injury more traffic of cells and molecules across the BBB has been described.[109,114] This should allow factors of the secretome reach the CNS more readily. Increased phosphorylation levels of Erk 1/2 in spinal cords of healthy rats upon MNC-secretome application are further suggestive of diffusion or active transport across the BBB

even under physiological conditions. However, this hypothesis needs to be confirmed in further experiments, e.g. via direct CSF measurement of known factors present in the secretome after treatment, which will be one of the aims of future studies planned by our group. Secondly, the endogenous response to SCI involves not only local but rather systemic mechanisms, which can be targeted via systemically administered therapy.[63,257]

Cellular-based treatment strategies without the cellular fraction offer important advantages, like lacking cellular epitopes with reduced antigenicity and easier handlings in terms of storability and durability. Furthermore, viral clearance steps as demanded by regulating authorities can be performed without possible deleterious effects on cells. This potentially allows allogeneic production and administration, whereas cellular treatment is limited to autologous uses. Recent efforts lead to more profound understanding of molecular composition of the conditioned medium of apoptotic PBMCs. Conditioned media represent a rich source of various factors. Besides proteins, many other molecules like lipids, microparticles, exosomes or RNA among others can be expected to be included since apoptosis is known to cause the release of these molecules. Beer et al. were recently able to show that, indeed, these molecules are present within the secretome and capable of exerting biological activity.[389] Certain Lipids are known for their potential neuroprotective and immunomodulatory characteristics and were already successfully tested in pre-clinical settings.[4,428] Also, microparticles and exosomes exhibit regenerative potential and promote angiogenesis.[377,393] It is tempting to suggest that also these biological compounds within the secretome add to the therapeutic capacity observed in our experiments. However, this hypothesis needs to be verified in upcoming studies.

Secretome used in all of the experiments within this thesis was produced under conditions meeting GMP guidelines and was recently approved by the Austrian Health Authority (AGES) for the use in human patients.

## 3.2 Conclusion and Outlook

We applied xenogenic secretome dervided from apoptotic human PBMCs in a well-established pre-clinical model of SCI. Treatment with MNC-secretome was associated with improved functional recovery after trauma to the spinal cord. We suggest that secretome treatment modulates the secondary endogenous response to SCI on multiple levels with subsequent amelioration of secondary damage, thereby exerting observed therapeutic effects. Possible involved mechanisms include modulation of the immune response, improvement of post-traumatic vascularization and up-regulation of pathways associated with neuronal and glial survival. We postulate that a single factor within the compound cannot be held responsible for all observed effects and that only the unique mix of various factors within the secretome leads to reported therapeutic capacity.

Combination with up-coming strategies like tissue engineering is conceivable and might further improve therapeutic capacity of MNC-secretome treatment after SCI. For instance, local therapy brought in by local scaffolds combined with systemic application. Aside from extending utilization of the treatment, description of further possible mechanisms and detailed elucidation of molecular pathways of already described modes of action represent the principal aim of future studies. Results reported in this thesis represent an encouraging foundation for potential future clinical trials.

# CHAPTER FOUR: Materials and Methods

## 4.1 Preparation of Secretome derived from Apoptotic PBMCs

The local ethics committee at the Medical University of Vienna (EK2010/034) approved Blood donation of healthy volunteers. All production steps meet GMP guidelines. A xenogeneic approach (secretome obtained from human PBMCs in rodent SCI model) was chosen to evaluate efficacy of the definitive treatment. Whole blood samples were taken by peripheral blood withdrawal after written informed consent was obtained.

After peripheral blood withdrawal, density gradient centrifugation with Ficoll density centrifugation medium was used to isolate human PBMCs. To induce apoptosis irradiation using a caesium source with a total dosage of 60Gy was applied. Then, irradiated cells were cultivated in a phenol-red free and serum-free cell culture medium (CellGro®, Cellgenix, Freiburg, Germany) at a concentration of $25 \times 10^6$ cells per ml for 24 hours. Following cultivation, another centrifugation step was used to separate the conditioned medium (=secretome) from the cellular fraction. Cells were then discarded and the secretome underwent the first viral clearance step in the form of treatment with methylene blue (MB, concentration range: 0.8–1.2 mmol/L per unit) plus light treatment utilizing a Theraflex MB-Plasma system (MacoPharma), as well as an LED-based lighting device (MacoTronic B2, Maco-Pharma). The illumination process was monitored and stopped after reaching 180 $J/cm^2$ within 20 minutes. Usually this process is suitable for the preparation and production of fresh frozen plasma used in clinical routine under GMP concordant conditions.[432] Remnants of MB and photoproducts were removed with a two-step filtration utilizing the Blueflex filtration system. Following this first step of viral clearance, the secretome was lyophilized and forwarded to the second viral clearance with gamma irradiation obtained from a Cobalt 60-source (Gammatron 1500, Mediscan, Seibersdorf, Austria). Dosage was measured with a PMMA (Polymethylemethacrylat) dosimeter and irradiation was terminated when a total dosage of 25,000 Gy was reached. This was the case after 23 hours of irradiation. Upon this two-step clearance the supernatant was contemplated free of pathogens and stored in a freezer at −80 °C.

Prior application of the secretome re-suspension with sterile water (Aqua ad injectabilia, B Braun, Melsungen, Germany) was performed to reach a concentration equivalent to $25 \times 10^6$ cells/ml. For all conducted experiments equally processed CellGro® serum-free medium without phenol red served as control arm. All included steps and the final product were approved by the Austrian Health Authority and deemed fit for clinical application in humans.

## 4.2 Experimental Model of SCI

Prior execution of animal experiments approval of the animal research committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (Protocol No. 66.009/0299-II/ 3b/2011) was obtained.

A commonly used contusion model was chosen since it resembles the most common trauma mechanism observed in humans.[3] Adult male Sprague Dawley rats (Department of Biomedical Research, Medical University of Vienna, Himberg, Austria) weighing 300–350 grams were used for conducted animal experiments. Housing of laboratory animals was maintained within standard protocols of the Department of Biomedical Research at the Medical University of Vienna with alternating 12hour light and dark cycles and freely accessible standard lab chow and water. Before first experiments animals were allowed to acclimatise for at least 1 week. Prior surgical manipulation animals were initially anesthetized with intraperitoneal (i.p.) application of xylazine (10 mg/kg) and ketamine (100 mg/kg). Animals were then intubated, ventilated and anaesthesia was maintained with 1.5% isoflurane.

The experimental contusion model was conducted with an Infinite Horizon Impactor® (Precision System and Instrumentation, Lexington, KY) and as previously described by Scheff and colleagues.[351] Following anaesthetic procedure animals transferred to the surgery table sited in a prone position and the operation site was surgically washed and sterile covered. Under the surgical microscope the spinous processes of T9 to T13 were exposed and a partial laminectomy of T11 was carried out. Two Adson micro-forceps (Fine Science Tools, Heidelberg, Germany) were then applied at T10 and T12 to stabilize the vertebral column before impact to avoid yielding. To ensure reproducible impacts blunt resection of epidural fat tissue was conducted meticulously while leaving the dura intact. A stainless steel tip with 2.5 mm in diameter was then placed 1 mm above the T11 spinal segement. A defined force of 150 kDyne with immediate retraction of the tip was used to create a moderate contusion.[351] Visual control ensured success and proper alignment of the impact. Irrigation with saline and wound closure in layers completed the surgical procedure. A heating lamp for the first hour after surgery supported the intial recovery. During the postoperative course animals subcutaneously (s.c.) received 10 ml of physiological saline, painkillers and antibiotic treatment for a total of 3 days. Until micturition was observed manual bladder evacuation was conducted twice a day. After randomization, 40 minutes and 24 hours after impact animals received either MNC-secretome containing the equivalent of $25 \times 10^6$ PBMCs in 1 ml volume or 1 ml of equally processed medium i.p. This treatment regime was chosen due to experience gained from previously conducted experiments.[276,278,381]

To evaluate plasma levels of different cytokines upon MNC-secretome animals received a single treatment dose i.p. Plasma was taken 12 or 24 hours after administration by terminal puncture of the inferior vena cava.

## 4.3 Neurological Assessment

The well-established and accepted Basso, Beattie, and Bresnahan-score (BBB-score) represents the gold standard for functional assessment after experimental spinal cord injury in rats.[433] Locomotion is observed in an open field and evaluated. The scale comprises a 21-point scale with 0 representing no observable hind limb function and 21 describes regular hind limb function. Before surgery, all animals were evaluated for functional hind limb movements to exclude pre-existing deficits. Observers blinded with respect to prior BBB scores and treatment graded hind limb function on day 1, day 3, day 7 and from then on weekly until 4 weeks postoperative. Mean value of both limbs was calculated and utilized for statistical evaluation.

## 4.4 Histology, Immunohistochemistry and Immunochemistry

To evaluate spinal cord tissue by means of histology, immunohistochemistry and immunochemistry animals were deeply anesthetized followed by sternotomy. Then, the inferior vena cava was punctured and around 4 ml of blood were withdrawn, transferred to heparinized tubes, centrifuged and plasma was stored at −80 °C upon further experiments. This was followed by euthanasia with intravenous potassium chloride, deep liver incision to allow proper passage of neutral buffered 4% paraformaldehyde, which was injected into the left ventricle. Simultaneous depolarization of muscles gave positive feedback of successful perfusion. Subsequent crude preparation of the vertebral column was performed and the whole spine surrounding the spinal cord was transferred to a vial containing neutral buffered 4% paraformaldehyde and fixed for 24 hours followed by storage in phosphate-buffered saline (PBS) at room temperature for at further least 24 hours. Then the vertebral column was separated carefully from the spinal cord. The lesion epicentre was identified and marks were placed 4mm caudally and cranially, respectively. On average, 10 axial cross-sections were excised from spinal cords of each animal and embedded in paraffin. Animals subjected to immunoblot analyses underwent the same preparation steps as described above but instead of total removal of spine including spinal cord only another approach was applied. After laminectomy ranging from T10 to T12, 5mm of spinal cord tissue was excised and immediately flash frozen using liquid nitrogen and stored at −80 °C upon further experiments.

For evaluation of inflammation Hematoxylin & eosin (H&E) was used. Luxol Fast Blue Staining (LFB) or Klüver-Barrera Staining (KLB) was performed to determine white matter

pathology. Bielschowsky staining was used for quantification of axons. Following immunohistological stainings were conducted: T cells (CD43/W3/13; Harlan Laboratories, Indianapolis, IN), β-amyloid precursor protein (βAPP; Chemicon International, CA) to address axonal pathology, microglia/macrophage marker CD68 (AbDSerotec, Duesseldorf, Germany), inducible nitric oxide synthase (iNOS) (Anti-NOS II, Millipore, MA) as indirect marker for oxidative stress, and von-Willebrand factor (vWF) (anti-von-Willebrand factor/vWF, Abcam, Cambridge, UK) to delineate blood vessels. Diaminobenzidine-enhanced Turnbull blue (TBB) staining method was utilized for staining of iron from non-heme sources and ferritin expression was visualized (rabbit anti-ferritin antibody, Sigma, St Louis, MO). Slides were then evaluated using light microscopy and digitalization with a tissue scanner (Hamatsu Photonics, Japan). Area measurements if necessary and cell counts were conducted using open-access ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD) by blinded observers.

To assess motor neuron damage, we performed immunofluorescence staining against choline acetyltranserferase (ChaT). The spinal cord was cryoprotected, shock-frozen and 10 µm sections were cut transversally with a microtome (CM1950; Leica, Heidelberg, Germany) and mounted onto glass slides (SuperFrost Ultra Plus, Fisher Scientific). Sections were blocked for two hours with 10% donkey serum in sodium phosphate-buffered saline (PBS) containing 0.1% Triton (PBS-T) followed by the concomitant incubation with the primary antibodies goat anti-CHAT (1:100, Millipore) and chicken anti-neurofilament (1:5000, Millipore) for 48 hours. After a washing step with PBS-T, sections were consecutively incubated with donkey anti-chicken AF 568, (1:500, Invitrogen) and donkey anti-goat AF488, (1:500, Invitrogen) for two hours each, with a washing step between the two secondary antibodies. Finally, the sections were rinsed again, mounted with fluorescent mounting medium (Dako) and analyzed using a confocal laser scanning microscope (CLSM 510, Zeiss, Germany).

## 4.5 Angiogenesis Assays

To evaluate angiogenesis in-vitro a previously published aortic ring assay with slight modifications was used. [434] Briefly, after deep anaesthesia and euthanasia as described above thoracic and abdominal aorta was dissected and removed from untreated healthy animals. Then aortic ring segments were prepared and placed between two layers of fibrin gel whereas attention was paid to correct orientation of aortic rings. Preparation of fibrin layers was accomplished as previously described in detail.[435] Lyophilized MNC-secretome or lyophilized control medium was dissolved in complete M199 medium (Invitrogen, Carlsbad, CA) and added to the gels. To investigate the role of VEGF this setting anti-VEGF antibody (R&D-systems, Minneapolis, USA) and recombinant rat VEGF (rrVEGF, R&D-systems,

Minneapolis, USA) was added in different combinations. Subsequently, plates were incubated in standard conditions (37 °C, 5% $CO_2$ and 97% humidity). Medium was changed every second day and photographs were taken with a digital camera (Olympus DP50, Tokyo, Japan) mounted on a phase contrast microscope (Olympus IMT-2). Images were evaluated using ImageJ software. Same experiments were undertaken using spinal cord tissue instead of aortic rings to address angiogenesis within the CNS in-vitro in a novel assay. After preparation of spinal cords as described above, spinal cord tissue was embedded in between two fibrin layers followed by identical protocol as described for aortic rings. Detailed description can be found in the publication in the results section.[397] Cellular density in vascular outgrowth was quantified in a Burker-Turk counting chamber after dissolving fibrin gels according to previously published protocol.[436]

To define sprouting observed in the spinal cord assay and determine vascular phenotype cultures were immunostained with a mouse monoclonal antibody against RECA-1 (rat endothelial cell antigen-1, Abcam, Cambridge, UK). RECA-1 is a specific marker for rat endothelial cells.[437] Detailed description of following steps can be found in publication in the results section.[397] In brief, cultures were fixed using 4% paraformaldehyde followed by blocking with immunofluorescence (IF) buffer containing 5% donkey serum. Incubation with RECA-1 antibody for 5 hours, washing with IF buffer, addition of Alexa Fluor 488-labeled donkey anti-mouse antibody (Invitrogen, CA, USA) and another wash step, DAPI was used for staining of nuclei and cultures were stored at +4 °C until further evaluation under the confocal laser scanning microscope (LSM 700 confocal laser scanning microscope, Carl Zeiss, Jena, Germany). Obtained images were analysed using the ZEN image processing and analysis software program (Zeiss). For cultures allotted to confocal laser scanning microscopy imaging chambers specifically created for this type of microscopy were used (ibidi-plates, Martinsried, Germany).

Human PBMCs from healthy donors were also cultured in identical prepared 3D fibrin cultures for flow cytometry analysis. After six days of cultivation cultures were dissolved and prepared for flow cytometry.

## 4.6 Flow Cytometry

After dissolving 3D fibrin cultures PBMCs were stained against the following markers: PE-conjugated CD14 (clone RMO52), CD163 (clone GHI/61) and FITC-conjugated CD206 (clone 19.2) or with the appropriate isotype control antibodies (BD Biosciences). Incubation time was 15 min at +4 °C. A concentration of 100 x $10^5$ cells per 100 µl PBS was chosen for all flow cytometry experiments. Expression was visualized using a FC 500 flow cytometer (Beckman Coulter) after a washing step. FlowJo software (Tree Star Inc, Ashland, OR) was used for analysing and depicting obtained flow cytometry data. The Annexin V-FITC/PI

apoptosis detection kit (BD Biosciences) was used to exclude apoptotic/dead cells from analysis. Gating of viable macrophages was accomplished corresponding to characteristics in the forward- and side scatter.

## 4.7 Immunoblot Analysis

For evaluation of intracellular signalling in the spinal cord after administration of MNC-secretome Western Blot analysis were carried out. Healthy rats, 2 h after receiving MNC-secretome or control medium, were sacrificed as described above. Spinal cord segments T10 to T12 were removed and flash frozen in liquid nitrogen also according to above described procedure. Further preparation of tissue samples and western blot analysis was realized as previously published.[438] Detailed description can also be found in the results section.[397] The following antibodies were used: anti-phospho-Erk 1/2 (1:1000), anti-phospho-CREB (1:1000), anti-phopsho-HSP27 (Ser15, 1:1000), and anti-phopsho-Akt (Ser473, 1:1000) (Cell Signalling Technology, Cambridge, UK); HRP-conjugated goat anti-mouse IgG (1:10,000, Amersham, Buckinghamshire, UK); and goat anti-rabbit IgG (1:10,000, Thermo Fisher, Rockford, IL).

## 4.8 Enzyme-linked Immunosorbent Assay

ELISA (enzyme-linked immunosorbent assay) analysis was used for evaluation of systemic cytokines. For this purpose kits specifically developed for detection of rat cytokines were purchased and used (DuoSet, R&D Systems, Minneapolis). Experiments were conducted following the instruction manual. All samples were measured in technical duplicates. The following molecules were evaluated: CXCL-1/CINC-1, IL-10, IL-1β, and TNF-α. After finalization of assays plates were analysed using an ELISA plate reader (Victor3 Multilabel plate reader, PerkinElmer) at 450 nm wavelengths.

## 4.9 TUNEL assay

For detection of apoptotic cells we used a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (In Situ Cell Death Detection Kit, Roche Applied Sciences, Indianapolis, USA). Apoptotic cells were counted on at least 5 total cross-sections and results are expressed as count of positive cells per cross-section.

## 4.10 Statistical Methods

Data are given as mean ± SEM (standard error of the mean) if not stated otherwise. For evaluation and comparison of neurological scores and histological comparison the

nonparametric Mann–Whitney U-test was used. All other calculations were realized by using student's t-test. Visualization and presentation of data was accomplished by using GraphPadPrism 5 (GraphPad, CA, USA). Statistical calculation were carried out with SPSS 21 software (IBM, NY, USA). Statistical significance was defined as a p-value $< 0.05$ for all experiments.

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# CURRICULUM VITAE

## Dr. med. univ. Thomas Haider

### PERSONAL BACKGROUND

Date of Birth: May 7th, 1988
Nationality: Austrian
Marital Status: Single

### EDUCATION

| | |
|---|---|
| 2015/02 – present | Resident at the Department of Trauma Surgery, Medical University of Vienna, Austria |
| 2012/08 – 2015/10 | M.D./Ph.D.-Student at the Department of Surgery, Medical University of Vienna, Austria |
| 2014/07 | Graduation Dr. med. univ. (M.D.), Medical University of Vienna, Austria |
| 2008/10 – 2014/07 | M.D.-Student, Medical University of Vienna, Austria |
| 2013/09 – 2013/12 | Semester abroad, Karl-Ruprechts-University Heidelberg, Germany |
| 2008/01 – 2008/07 | Military Service (mandatory), Vienna, Austria |
| 2007/06 | Matura (High-school graduation) with distinction |
| 2002/09 – 2007/06 | High School (Höher Technische Lehranstalt, Technisches Gewerbemuseum, TGM), Vienna, Austria |
| 1994/09 – 2002/07 | Primary and Junior High School, Vienna, Austria |

## CLINICAL TRAINING

| | |
|---|---|
| 2014/07 | Clinical Clerkship in Trauma Surgery, University Hospital Tulln, Austria |
| 2014/02 – 2014/03 | Clinical Clerkship in Neurology, Wilhelminen Hospital Vienna, Austria |
| 2013/07 | Clinical Clerkship in Psychiatry, Medical University of Vienna, Austria |
| 2013/09 – 2013/12 | Clinical Clerkship in Dermatology, Infectiology, Pediatrics, Gynecology and Obstetrics, ENT and Ophthalmology, Medical School Heidelberg, Germany |
| 2013/04 – 2013/06 | Clinical Clerkship in Emergency and Intensive Care Medicine, Medical University of Vienna, Austria |
| 2013/02 | Clinical Clerkship – Department of Pathology, Donauspital Vienna, Austria |
| 2012/12 – 2013/04 | Clinical Clerkship – Department of Cardiology, Donauspital Vienna, Austria |
| 2012/09 – 2012/12 | Clinical Clerkship in General Surgery, Cardiac Surgery and Orthopedic Surgery, Donauspital Vienna, Medical University of Vienna and Orthopedic Hospital Vienna, Austria |
| 2012/06 | Clinical Clerkship in Trauma Surgery, Emergency Hospital Lorenz Böhler, Vienna, Austria |
| 2011/09 | Clinical Clerkship – Department of Thoracic Surgery, Medical University of Vienna |
| 2011/08 | Clinical Clerkship in General Surgery, State Hospital Korneuburg, Austria |
| 2010/09 | Clinical Clerkship – Department of Cardiology, Medical University of Vienna, Austria |

## CONGRESSES

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|---|---|
| 2015/09 | 4th European Congress of Immunology, Vienna, Austria |
| 2014/12 | 4th EACTS Meeting on Cardiac and Pulmonary Regeneration, Berne, Switzerland |
| 2014/10 | 50th Annual Meeting of the Austrian Society of Neurosurgery, Vienna, Austria |
| 2014/07 | 9th FENS – Forum of Neuroscience, Milan, Italy |
| 2014/06 | 55th Congress of the Austrian Society of Surgery, Graz, Austria |
| 2014/05 | 10th YSA PhD Symposium, Vienna, Austria |
| 2014/02 | IAI-CCHD PhD Symposium 2014, Vienna, Austria |
| 2014/01 | 2nd Vascular Biology Meeting, Vienna, Austria |
| 2013/10 | 49th Annual Meeting of the Austrian Society of Neurosurgery, Innsbruck, Austria |
| 2013/08 | 15th European Burn Association Congress, Vienna, Austria |
| 2013/05 | 54th Congress of the Austrian Society of Surgery, Vienna, Austria |
| 2013/01 | 1st Vascular Biology Meeting, Vienna, Austria |
| 2013/01 | 31st Annual Meeting of the German Society for Burn Medicine – DAV, Mayrhofen, Austria |
| 2012/10 | 50th Annual Meeting of the Austrian Society of Plastic, Reconstructive and Aesthetic Surgery, Linz, Austria |
| 2012/10 | 48th Annual Meeting of the Austrian Society of Neurosurgery, Graz, Austria |
| 2012/09 | 3rd TERMIS World Congress, Tissue Engineering and Regenerative Medicine, Vienna, Austria |
| 2012/03 | 5th World Congress for Endoscopic Surgery of the Brain, Skull Base & Spine, First Global Update on FESS, the Sinuses & the Nose, Vienna, Austria |
| 2011/10 | 47th Annual Meeting of the Austrian Society of Neurosurgery, Vienna, Austria |
| 2011/04 | 7th Vienna Interdisciplinary Symposium on Aortic Repair, Vienna, Austria |
| 2010/12 | 2nd EACTS Meeting on Cardiac and Pulmonary Regeneration, Vienna, Austria |
| 2010/10 | 24th Annual Meeting of the Austrian Society for Transplantation, Transfusion and Genetics, Austrotransplant, Villach, Austria |

## CONTRIBUTIONS

35 Abstracts at national and international meetings
11 Oral presentations
4 Poster presentations

Diploma Thesis

*Specification of Soluble ST2 Serum Levels in Burn Patients*, 2012, Dr. Hacker, Department of Plastic and Reconstructive Surgery, Medical University of Vienna

Oral Presentations

*"Supernatant of Apoptotic Leukocytes Improves Motor Function after Spinal Cord Injury in Rats"*
4th EACTS Meeting on Cardiac and Pulmonary Regeneration, Berne, Switzerland, 2014

*„The secretome of apoptotic PBMCs induces angiogenesis ex-vivo and in the injured spinal cord"*
50th Annual Meeting of the Austrian Society of Neurosurgery, Vienna, Austria, 2014

*"Soluble ST2 serum concentrations are increased in burn patients and predict mortality"*
55th Congress of the Austrian Society of Surgery, Graz, Austria, 2014

*"Supernatant of Apoptotic Leukocytes Improves Motor Function after Spinal Cord Injury in Rats"*
55th Congress of the Austrian Society of Surgery, Graz, Austria, 2014

*"The Secretomes of Apoptotic White Blood Cells Ameliorate Neurological Damage in Rats with Focal Ischemia"*
55th Congress of the Austrian Society of Surgery, Graz, Austria, 2014

*"The Secretomes of Apoptotic White Blood Cells Ameliorate Neurological Damage in Rats with Focal Ischemia"*

49th Annual Meeting of the Austrian Society of Neurosurgery, Innsbruck, Austria, 2013

*"Supernatant of Apoptotic Leukocytes Improves Motor Function after Spinal Cord Injury in Rats"*
49th Annual Meeting of the Austrian Society of Neurosurgery, Innsbruck, Austria, 2013

*"Soluble ST2 serum concentrations are increased in burn patients and predict mortality"*
15th European Burn Association Congress, Vienna, Austria, 2013

*"Soluble ST2 in the serum of burn patients as marker for immunosuppression and mortality"*
31st Annual Meeting of the German Society for Burn Medicine, Mayrhofen, Austria, 2013

*"Increased Concentrations of sST2 in the serum of burn patients as marker for immunosuppression and mortality"*
50th Annual Meeting of the Austrian Society of Plastic, Reconstructive and Aesthetic Surgery, Linz, Austria, 2012

*"Very large and giant arterial bifurcation aneurysms in the rabbit model: Proof of feasibility and comparability using CFD simulations"*
48th Annual Meeting of the Austrian Society of Neurosurgery, Graz, Austria, 2012

## CONTINUING EDUCATION

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|---|---|
| 2015/09 | 36th European Workshop on Basic Techniques of Microsurgery and Cerebral Revascularization, Prof. Bavinzski, Medical University of Vienna, Austria |
| 2014/12 | 57th Microsurgical training Emergency hospital Meidling, Dr. Russe, Dr. Rois, Vienna, Austria |
| 2014/11 | Experimental biomedical studies in animals (equates to FELASA-B), Medical University of Vienna, Austria |

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| 2014/05 | Intellectual Property Rights and Project Management, Medical University of Vienna, Austria |
| 2014/05 | Clinical Studies, Prof. Singer, Medical University of Vienna, Austria |
| 2014/04 | Methods in Cellular Biology, Prof. Schmid, Medical Univeristy of Vienna, Austria |
| 2014/03 | Ethics in Medicine and Good Scientific Practice, Prof. Singer, Medical University of Vienna |
| 2014/03 | Spring School Neurology, Medical School Mainz, Medical School Frankfurt, Germany |
| 2013/11 | Road Show Orthopaedics and Trauma surgery – Hands on, BGU Ludwigshafen, Germany |
| 2013/03 | Hands-On Infiltration-Workshop, Prof. Dunky, Medical University of Vienna, Austria |
| 2012/12 | Methods Seminar: “Statistics”, Prof. Mittlböck, Medical University of Vienna |
| 2012/11 | Microsurgical Training, “Rookie-Course”, MAZ Linz, Austria |
| 2012/10 | Anatomy for Neurosurgery, Dr. Sherif, Medical University of Vienna, Austria |
| 2012/05 | Methods Seminar: “Designing Clinical Trials”, Prof. Blöchl-Daum, Medical University of Vienna, Austria |
| 2012/03 | Anatomy Basics for Neurosurgery, Prof. Winkler, Salzburg, Austria |
| 2012/02 | Biometry I: Description and Visualization of Medical Data, Medical University of Vienna, Austria |
| 2011/10 | Anatomy for Neurosurgery, Prof. Di Ieva, Medical University of Vienna, Austria |
| 2010/2011 | Anatomica practica – Advanced course in anatomy, Prof. Weninger, Medical University of Vienna, Austria |
| 2010 | Applied Immunology and Tissue Regeneration, Medical University of Vienna, Austria |
| 2010 – 2014 | Current Topics in Applied Immunology, Medical University of Vienna, Austria |
| 2010 – 2014 | AHA Guidelines in Cardiovascular Surgery, Medical University of Vienna, Austria |
| 2010 – 2013 | English for Medical Professionals – Continuing Education, Christian Doppler Laboratory, Vienna, Austria |

## TEACHING ACTIVITY

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| 2015/09 – present | Lecturer at the Medical University of Vienna, TUT-1, Austria |
| 2015/02 – 2015/07 | Lecturer at the Center for Anatomy and Cellular Biology, Organ-morphology I and III, Medical University of Vienna, Austria |
| 2011 – 2014 | Teaching Assistant at the Center for Anatomy and Cellular Biology, Organ-morphology I, II, III and Organ-morphology for dentists, Prof. Traxler, Medical University of Vienna, Austria |

## AWARDS AND GRANTS

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| 2015 | Erwin-Domanig-Price awarded by the Austrian Society of Serology, Transfusion, Regenerative Medicine and Immunogenetics for outstanding scientific work |
| 2014 | Scholarship for outstanding academic achievements, Medical University of Vienna, Austria |
| 2014 | Scientific Scholarship, Medical University of Vienna, Austria |
| 2013 | Scholarship for outstanding academic achievements, Medical University of Vienna, Austria |
| 2012/09 – 2014/07 | M.D./Ph.D. Excellence Program, Medical University of Vienna, Austria |
| 2012 | Scientific Scholarship, Medical University of Vienna, Austria |
| 2011/09 – 2012/08 | Student Scholarship, Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Medical University of Vienna, Austria |
| 2011 | Scientific Scholarship, Medical University of Vienna, Austria |

## LANGUAGE SKILLS

Native German Speaker
Proficient in English

## EXTRACURRICULAR ACTIVITES

American Football (former member of the Austrian Nationalteam), Tennis, Squash and Snowboarding

## PUBLICATION LIST

Thell K, Hellinger R, Sahin E, Michenthaler P, Gold-Binder M, **Haider T**, Göransson U, Gründemann C, Schabbauer G, Gruber CW.: **Oral activity of nature-derived cyclic peptide for the treatment of multiple sclerosis.** (submitted)

Janik S, Schiefer A, Bekos C, Hacker P, **Haider T**, Klepetko W, Müllauer L, Ankersmit HJ and Moser B.: **Thymic Epithelial Tumors, Thymic Neuroendocrine Tumors and Thymic Hyperplasia, Thymic Cysts and Regular Thymuses of foetuses, infants and adults: Heat shock proteins 27 and 70.** (submitted)

Hacker S, Mittermayr R, Nickl S, **Haider T**, Lebherz-Eichinger D, Mitterbauer A, Leiss H, Zimmermann M, Schweiger T, Keibl C, Gabriel C, Pavone-Gyöngyösi M, Redl H, Tschachler E, Mildner M, Ankersmit HJ.: **Topical Application of Paracrine Factors from Peripheral Blood Mononuclear Cells Improves Wound Healing, Scar Quality and Angiogenesis in a Porcine Full-Thickness Burn and Skin Grafting Model.** (submitted)

Sherif C, Herbich E, Plasenzotti R, Bergmeister H, Windberger U, Mach G, Sommer G, Holzapfel GA, **Haider T**, Krssak M, Kleinpeter G.: **Very large and giant microsurgical bifurcation aneurysms in rabbits: proof of feasibility and comparability using computational fluid dynamics and biomechanical testing.** (submitted)

Sahin E, Brunner JS, Kral JB, Kuttke M, Hanzl L, Datler H, Paar H, Neuwinger N, Saferding V, Zinser E, Halfmann A, Soukup K, Hainzl E, Lohmeyer T, Niederreiter B, **Haider T**, Dohnal AM, Krönke G, Blüml S, Schabbauer G.: **Loss of Phosphatase and Tensin Homolog in APCs Impedes Th17-Mediated Autoimmune Encephalomyelitis.** J Immunol. 2015 Aug 5.

Soukup K, Halfmann A, Le Bras M, Sahin E, Vittori S, Poyer F, Schuh C, Luger R, Niederreiter B, **Haider T**, Stoiber D, Blüml S, Schabbauer G, Kotlyarov A, Gaestel M, Felzmann T, Dohnal AM.: **The MAPK-Activated Kinase MK2 Attenuates Dendritic Cell-Mediated Th1 Differentiation and Autoimmune Encephalomyelitis.** J Immunol. 2015 Jul 15;195(2):541-52.

**Haider T,** Höftberger R, Rüger B, Mildner M, Blumer R, Mitterbauer A, Buchacher T., Sherif C, Altmann P, Redl H, Gabriel C, Gyöngyösi M, Fischer MB, Lubec G, Ankersmit HJ.: **The Secretome of Apoptotic Human Peripheral Blood Mononuclear Cells Attenuates Secondary Damage Following Spinal Cord Injury in Rats.** Experimental Neurology. 2015 Mar 19;267:230-242.

Sherif C, Kleinpeter G, Loyoddin M, Mach G, Plasenzotti R, **Haider T**, Herbich E, Krssak M.: **Aneurysm Wall Thickness Measurements of Experimental Aneurysms: In Vivo High-Field MR Imaging Versus Direct Microscopy.** Acta Neurochir Suppl. 2015; 120:17-20.

Altmann P, Mildner M, **Haider T**, Traxler D, Beer L, Ristl R, Golabi B, Gabriel C, Leutmezer F, Ankersmit HJ.: **Secretomes of apoptotic mononuclear cells ameliorate neurological damage in rats with focal ischemia.** F1000 Research. 2014 Oct 28.

Di Ieva A, Bruner E, **Haider T**, Rodella LF, Lee JM, Cusimano MD, Tschabitscher M.: **Skull base embryology: a multidisciplinary review.** Childs Nerv Syst. 2014 Apr 17

Sherif C, Kleinpeter G, Mach G, Loyoddin M, **Haider T,** Plasenzotti R, Bergmeister H, Di Ieva A, Gibson D, Krssak M.: **Evaluation of cerebral aneurysm wall thickness in experimental aneurysms: Comparison of 3T-MR imaging with direct microscopic measurements.** Acta Neurochir 2013, Oct 30.

Mildner M, Hacker S, **Haider T**, Gschwandtner M, Werba G, Barresi C, Zimmermann M, Golabi B, Tschachler E, Ankersmit HJ.: **Secretome of peripheral blood mononuclear cells enhances wound healing.** PloS One 2013, Mar 22.

Di Ieva A, Bruner E, Davidson J, Pisano P, **Haider T**, Stone SS, Cusimano MD, Tschabitscher M, Grizzi F.: **Cranial sutures: a multidisciplinary review.** Child Nerv Syst. 2013, Mar 8.