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Central nervous system regeneration approach in the toxic cuprizone model of de- and remyelination: application of mesenchymal stem cells.

THESIS

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY
(PhD)

Awarded by the University of Veterinary Medicine Hannover

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Hannover, Germany 2015

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Date of final exam: 6th November 2015

Parts of this thesis have been submitted or accepted previously in:

Salinas Tejedor, L., Gudi, V., Kucman, V., Pul, R., Gingele, S., Sühs, W., Stangel, M., Skripuletz, T., 2015. Oligodendroglial markers in the cuprizone model of CNS de- and remyelination. Histol. Histopathol. Histol Histopathol. 2015 Dec; 30(12):1455-64.

Salinas Tejedor, L., Berner, G., Jacobsen, K., Gudi, V., Jungwirth, N., Gingele, S., Prajeeth, C.K., Hansmann, F., Baumgärtner, W., Hoffmann, A., Skripuletz, T., Stangel, M., 2015. Mesenchymal stem cells do not exert direct beneficial effects on CNS remyelination in the absence of the peripheral immune system. Brain. Behav. Immun. Brain Behav Immun. 2015 Nov; 50:155-65.

During her PhD, Laura Salinas Tejedor has also contributed to the following manuscripts:

Nessler, J., Bénardais, K., Gudi, V., Hoffmann, A., **Salinas Tejedor, L**., Janßen, S., Prajeeth, C.K., Baumgärtner, W., Kavelaars, A., Heijnen, C.J., van Velthoven, C., Hansmann, F., Skripuletz, T., Stangel, M., 2013. Effects of murine and human bone marrow-derived mesenchymal stem cells on cuprizone induced demyelination. PLoS One 8, e69795.

Skripuletz T, Manzel A, Gropengießer K, Schäfer N, Gudi V, Singh V, **Salinas Tejedor L**, Jörg S, Hammer A, Voss E, Vulinovic F, Degen D, Wolf R, Lee DH, Pul R, Moharregh-Khiabani D, Baumgärtner W, Gold R, Linker RA, Stangel M. Pivotal role of choline metabolites in remyelination. Brain. 2015 Feb;138(Pt 2):398-413

Skripuletz, T.*, **Salinas Tejedor, L.***, Prajeeth, C.K., Hansmann, F., Chhatbar, C., Kucman, V., Zhang, N., Raddatz, B.B., Detje, C.N., Sühs,W., Pul, R., Gudi, V., Kalinke, U.,

Baumgärtner, W., Stangel, M., 2015. The antiviral drug ganciclovir does not inhibit microglial proliferation and activation. Sci Rep. 2015 Oct 8;5:14935.

*equal contribution

Results of this thesis were presented in the following scientific meetings:

Salinas Tejedor L, Berner G, Gudi V, Hoffmann A, Jungwirth N, Hansmann F, Baumgärtner W, Skripuletz T, Stangel M. Influence of mesenchymal stem cells on the remyelination process in the cuprizone murine model. Hannover Graduate School for Veterinary Pathobiology, Neuroinfectiology and Translational Medicine (HGNI), GS-Day, Hannover, Germany, 29.-30.11.2013.

Salinas Tejedor L, Berner G, Gudi V, Hoffmann A, Jungwirth N, Hansmann F, Baumgärtner W, Skripuletz T, Stangel M. Influence of mesenchymal stem cells on the remyelination process in the cuprizone murine model. 2nd International Workshop of Veterinary Neuroscience, Hannover, Gemany, 20.-22.03.2014.

Skripuletz T, **Salinas Tejedor L**, Berner G, Nessler J, Benardais K, Gudi V, Hoffmann A, Janßen S, Prajeeth CK, Baumgärtner W, Kavelaars A, Heijnen CJ, van Velthoven C, Hansmann F, Stangel S. MSC – a potential treatment for demyelinating disease in the CNS? 2nd International Workshop of Veterinary Neuroscience, Hannover, Gemany, 20.-22.03.2014.

Skripuletz T, Manzel A, Gropengießer K, Schäfer N, Gudi V, Singh V, **Salinas Tejedor L**, Voss E, Vulinovic F, Hackstette D, Wolf R, Lee DH, Pul R, Moharregh-Khiabani D, Baumgärtner W, Gold R, Linker RA, Stangel M. The role of CDP-choline in CNS remyelination. 2014 Joint ACTRIMS-ECTRIMS Meeting, Boston, USA, 10.-13.09.2014. Mult Scler 2014;20:(S1)383.

Salinas Tejedor, L., Jacobsen, K., Gudi, V., Jungwirth, N., Hansmann, F., Baumgärtner, W., Hoffmann, A., Skripuletz, T., Stangel, M. Intralesional transplantation of mesenchymal stem cells in the toxic demyelinating cuprizone model. Hannover Graduate School for Veterinary Pathobiology, Neuroinfectiology and Translational Medicine (HGNI), Hannover, Germany, 28.-29.11.2014.

Salinas Tejedor, L., Jacobsen, K., Gudi, V., Jungwirth, N., Hansmann, F., Baumgärtner, W., Hoffmann, A., Skripuletz, T., Stangel, M. Intralesional transplantation of mesenchymal stem cells in the toxic demyelinating cuprizone model. Eleventh Göttingen Meeting of the German Neuroscience Society 18.-21.03.2015.

Salinas Tejedor, L., Jacobsen, K., Gudi, V., Jungwirth, N., Hansmann, F., Baumgärtner, W., Hoffmann, A., Skripuletz, T., Stangel, M. Intralesional transplantation of mesenchymal stem cells in the toxic demyelinating cuprizone model. XII European meeting on glial cells in health and disease, July, 14th-18th 2015, Bilbao, Spain

To my family

Table of contents

List of Abbreviations

ANOVA	Analysis of Variance
APC	Adenomatous Polyposis Coli
BBB	Blood Brain Barrier
CNPase	2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CNS	Central Nervous System,
CNTF	Ciliary Neurotrophic Factor
CSF	Cerebrospinal Fluid
CXCL10	C-X-C Motif Chemokine Ligand 10
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-Activated Cell Sorting
FGF 2	Fibroblast Growth Factor 2
GDNF	Glial cell-Derived Neurotrophic Factor
GFAP	Glial fibrillary acidic protein
GVDH	Graft Versus Host Disease
IDO	Indoleamine 2,3-dioxygenase
IFN γ	Interferon γ
IFN β	Interferon ß
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin 6
LIF	Leukemia inhibitory factor

LPS	Lipopolysaccharides
MAG	Myelin-Associated Glycoprotein
MBP	Myelin Basic Protein
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
MSC	Mesenchymal Stem Cells
NK cells	Natural Killers Cells
Nogo A	Neurite Outgrowth inhibitor A
OPC	Oligodendrocyte Precursors Cell
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PDGF α	Platelet-derived growth factor α
PFA	Paraformaldehyde
PLP	Proteolipid Protein
RFP	Red Fluorescence Protein
SEM	Standard Error of the Mean
TGF ß1	Tumor Growth Factor ß1
TNF α	Tumor Necrosis Factor α

Central nervous system regeneration approach in the toxic cuprizone model of de- and remyelination: application of mesenchymal stem cells

Laura Salinas Tejedor

1. Summary

Multiple Sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) characterized by neuroinflammation, neurodenegeration and functional disability. Available drugs reduce the inflammatory condition and thus protect from demyelination and axonal damage. However, treatments to enhance remyelination are not available. Among the new therapies that are being investigated, mesenchymal stem cell (MSC) therapy has emerged as a promising alternative for enhancing endogenous remyelinating processes. Beneficial effects have already been obtained from MSC therapy in the experimental autoimmune encephalomyelitis model, but its mechanism of action is still not well understood.

To provide a better understanding of the influence of MSC in the CNS, we transplanted MSC from different origins (human, murine and canine) directly into the ventricles or into the lesions in the corpus callosum during cuprizone induced demyelination. Evaluation of the remyelination process was conducted through immunohistochemical analysis of the myelin content and the glial cell populations. Additionally, we first characterized several oligodendroglial markers (APC and Olig2) for the unambiguous identification of oligodendroglial cells during our major experiments.

Our results show that MSC neither influenced remyelination nor glial reactions. Therefore, we conclude that MSC do not have a direct beneficial impact on CNS remyelination in the toxic cuprizone model, in which peripheral immune cells do not play an important role.

Consequently, we suggest that positive MSC effects might be depended on the presence of the peripheral immune system.

Regenerationsannäherung des zentralen Nervensystems im toxischen Cuprizone-Modell: Einsatz von mesenchymalen Stammzellen

Laura Salinas Tejedor

2. Zusammenfassung

Die Multiple Sklerose (MS) ist eine Autoimmunerkrankung des zentralen Nervensystems (ZNS), in deren Verlauf die Myelinschicht betroffener Nervenzellen geschädigt wird und neurologische Symptome auftreten. Verfügbare medikamentöse Therapien wirken entzündungshemmend mit dem Ziel, den entstandenen Schaden auf ein Minimum zu reduzieren. Behandlungen, die Remyelinisierung fördern, liegen nicht vor. Neue Therapien, die auf dem Einsatz mesenchymaler Stammzellen (MSC) beruhen, stellen eine vielversprechende Alternative dar und haben als Ziel die endogenen Remyelisinierungsprozessen zu verstärken. Erste therapeutische Erfolge konnten im Mausmodell der experimentellen autoimmunen Enzephalomyelitis bereits verbucht werden. Die unterliegenden Mechanismen sind weiterhin nicht geklärt.

Mit dem Ziel, die Wirkungsweise transplantierter MSCs besser zu verstehen, haben wir MSCs unterschiedlicher Herkunft (human, murin, canin) in die Ventrikel oder in die durch vorangegangene Cuprizone-Behandlung entstandenen Läsionen im Corpus callosum transplantiert. Die Effekte auf die Remyelinisierung und gliale Reaktionen wurden mittels immunohistochemischer Färbungen untersucht. Im ersten Schritt wurden außerdem zwei oligodendrogliale Antikörper (APC, Olig2) auf ihre Spezifizität getestet.

Unsere Ergebnisse zeigen, dass MSC Zellen weder die Remyelinisierung noch Glia-Zell-Reaktionen beeinflussen. Dadurch, stellten wir fest, dass MSC Zellen keinen direkten

förderlichen Effekt auf den Remyelinierungsprozess im ZNS im toxischen Cuprizone Modell, bei dem periphere Immunzellen keinen Einfluss haben, entfalten. Somit könnte ein möglicher positiver Effekt von MSC vom peripheren Immunsystem abhängig sein.

3. Introduction

Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune demyelinating chronic disease of the central nervous system (CNS) that leads to myelin loss resulting in degeneration of axons and neurons (Keough et al., 2015). The onset of this disease occurs in an early age, affecting predominantly young adults between 20 to 40 years old. Additionally, women are twice more susceptible in developing MS as men. In 2008, it was estimated that approximately between 2 to 2.5 million people in the world suffer from MS (Lassmann, 2014).

The pathogenesis is mediated by an autoimmune attack of autoreactive T cells against myelin proteins, such as myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte olygoprotein (MOG), or myelin associated glycoprotein (MAG), which infiltrate the CNS through the damaged blood brain barrier (BBB) (Kotter et al., 2001). Besides, not only complement, antibodies, macrophages, NK cells participate in the direct immune attack, but also toxic substances, such as nitric oxide and other oxygen reactive species secreted by activated microglia, contribute as well (Esiri, 2007). Demyelinated axons have a redistribution of ion channels, and begin to accumulate sodium and calcium ions, leading to swelling of the axolema, wallerian degeneration, axonal loss and finally, to neurodegeneration (Dutta and Trapp, 2007). Therefore, once demyelination takes place, remyelination is a very important process necessary to restore neurological functions and prevent neurodegeneration (Franklin and Ffrench-Constant, 2008).

Remyelination

Remyelination is a regenerative process that occurs spontaneously after demyelination. It involves the generation of new mature oligodendrocytes, which produce new myelin sheaths around the axons (Franklin and Ffrench-Constant, 2008). This process is important to restore

the fast saltatory nerve conduction. In addition, it might be neuroprotective due to the fact that oligodendrocytes offer trophic support and myelin protects the axons of further damage. Unfortunately, often in MS patients, this process is incomplete (Jarjour et al., 2015). Several studies have suggested that the main reason of remyelination failure could be attributed to a problem during oligodendrocytes precursor cells (OPC) proliferation, differentiation into mature oligodendrocytes, or a problem of the mature oligondendrocyte to wrap correctly the axons (Jarjour et al., 2015). Additionally, other studies have suggested that inflammation may be closely linked to remyelination (Franklin and Hinks, 1999) due to the observation that new myelin formation usually starts in MS during the acute inflammatory phase rather than in chronic stages. Therefore, the correct environmental signaling in a specific timing may be crucial for remyelination. Unfortunately, the role of glial cells in this process is controversial and not fully understood (Gudi et al., 2014).

The role of glial cells in de- and remyelination

Glial cells (oligodendrocytes, microglia, and astrocytes) have gained interest in neuroscience because they give support to neurons and play a crucial role in the maintenance of homeostasis during injury and repair. Oligodendrocytes, which are the myelinating cells of the CNS, originate from OPC. Myelin is a phospholipid rich layer around the axon that allows fast saltatory nerve conduction (Keirstead et al., 1999) and offers axonal protection by providing trophic support to the axon. During an inflammatory condition like in MS, oligodendrocytes are damaged. This leads to demyelination and consequently, neurological disability. In this pathological context, microglia and astrocytes are recruited to injured areas.

Microglia are the innate immune cells of the CNS. In their resting status they use their processes to survey the environment for hazardous signals that trigger their activation (Gudi et al., 2014). Activated microglia have different phenotypes and functions depending on mediators in the microenvironment. While the M1-like pro-inflammatory phenotype is

neurotoxic and damages oligodendrocytes trough a pro-inflammatory cytokine secretion and nitric oxide production (Giunti et al., 2012), The M2-like anti-inflammatory phenotype enhances remyelination by phagocyting myelin debris and contributing to degeneration and tissue remodeling (Perry et al., 2010). It was shown that macrophage/microglia depletion is unfavorable for remyelination, because myelin debris is slowly removed and this hinders the new formation of myelin fibers (Kotter et al., 2001). Additionally, microglia may also secrete soluble factors such as IGF-1 and TGF-β1, which promote OPC differentiation (Franklin and Hinks, 1999).

Astrocytes are glial cells of the CNS that provide trophic support to neurons and surrounding cells. They maintain the extracellular ion balance and have an important role during repair, as well. During a demyelinating insult, their role is controversial. On one hand, they are the main source of chemokines, such as TNFα, involved in the pathogenesis of MS. However, since they form astrogliosis, it was suggested it might be a physical barrier around demyelinated areas, which might hinder OPC to enter and remyelinate MS lesions (Fitch and Silver, 2008). On the other hand, a recent astrocyte ablation study reported their importance for remyelination by recruiting microglia and OPC to the lesion through chemokines such as CXCL10 (Skripuletz et al., 2013). Furthermore, among astrocyte secreted factors anti-inflammatory cytokines, growth and transcription factors, such as PDGF α, FGF2, LIF, CNTF, IGF-1, which promote myelination and oligodendrocyte and neuron survival have been found (Moore et al., 2011)

Animal models to study de- and remyelination

Animal models represent a useful tool to investigate de- and remyelination and the cellular and molecular mechanisms behind these processes. Indeed, different animal models for MS are known which mimic different aspects of the complex pathophysiology in MS.

1) Experimental autoimmune encephalomyelitis (EAE) is the most studied inflammatory animal model for MS. This model can be either induced by active or passive immunization. In the active EAE, autoreactive T cells proliferate and migrate towards the CNS upon immunization with purified myelin or myelin proteins (e.g. MGP, PLP or MOG) (Kipp et al., 2012). In the passive EAE, immunization is conducted by transfer of activated myelin specific T cells (McPherson et al., 2014). In both modalities, activated T cells, after encountering the specific myelin antigen, induce an immune reaction which leads to demyelination and disease development. Unfortunately, remyelination takes place simultaneously with demyelination, and peripheral immune cells infiltration in the CNS complicates the analysis (Tanaka and Yoshida, 2014).

2) The cuprizone model is a toxic demyelinating model, where the blood brain barrier remains intact. Upon cuprizone feeding, oligodendrocytes undergo apoptosis and myelin is degraded and consequently removed by activated microglia, which are recruited to injured areas. De- and remyelination is well characterized, especially in the corpus callosum and cerebral cortex (Skripuletz et al., 2011). After complete demyelination, cuprizone is removed allowing spontaneous remyelination. Although the demyelination induction is artificial, this model allows studying remyelination in the absence of the influence from the peripheral immune system. Additionally, there are other toxic models of demyelination. Direct injections of lysolecithin or ethidium bromide into the white matter lead to a local demyelination (van der Star et al., 2012; Kipp et al., 2012).

3) Theiler´s murine encephalomyelitis virus presents a virus induced model of demyelination. This virus produces a persisting infection in oligodendrocytes, causing their damage and consequently, demyelination (Ulrich et al., 2006).

4) Genetic models originate from a mutation in a gene encoding for a myelin protein such as PLP (Hudson et al., 1989) or MBP (Roach et al., 1985). Indeed, they are less suitable for MS studies because the lesion formation approach is different.

Remyelinating strategies

Current therapeutics in MS are based on the use of immunomodulatory drugs, which aim to reduce CNS inflammation and thus demyelination and axonal loss. However remyelination is not influenced by current drugs. Therefore, novel approaches for MS therapeutics aim to enhance remyelination and neuroregeneration.

Cell replacement therapy is one approach to achieve remyelination, in which cells are exogenously administered. Several cell types have been reported to achieve remyelination e.g. oligodendrocytes, OPC, Schwann cells (Bachelin et al., 2005), Schwann precursor cells and olfactory ensheathing cells (Franklin et al., 1996). However, this approach encounters a main problem, which is that their transplantation into a non-permissive remyelinative environment may not foster remyelination

For that reason, other approaches that modulate the endogenous mechanisms to boost remyelination could be more appealing. For this purpose several mechanisms could be used. The application of growth factors, such as PDGF2 and FGF2, could mediate the recruitment and proliferation of OPC (Franklin and Hinks, 1999). Other growth factors, such as IGF1 and TGFß1, could be applied to promote OPC differentiation into mature oligodendrocytes (Franklin and Hinks, 1999). In contrast, the removal or neutralization of inhibitory signals may also achieve remyelination. Additionally, transplantation of non-myelinating cells or other factors, which could provide a suitable environment, could promote remyelination. Recently, the transplantation of exogenous stem cells, such as mesenchymal stem cells (MSC) has been proposed to enhance repair processes in animal models of MS. MSC are multipotent

stem cells that can differentiate into different cells from the mesodermal lineage, such as osteoblast, adipocytes and chondrocytes (Uccelli et al., 2008). Although recent studies have observed its transdifferentiation into neuron-like cells (Zhang et al., 2005), their use was not meant for a cell replacement therapy. MSC solved the above mention problems from previous approach, because they may modulate the microenvironment. Moreover, they can be isolated from both adult and fetal tissues and they can be easily expanded to larger quantities ex vivo for transplantation. In addition, they might not need to migrate to the lesion areas to produce their effect and it is speculated that they may have a 'touch and go' mechanism. As a consequence it is likely that engraftment would not be required (Uccelli and Prockop, 2010). Furthermore, the growing interest around MSC relies on its immunoregulatory properties and its immunopriviledged state (Hass et al., 2011). MSC efficiency have been proved in several preclinical and clinical studies for several autoimmune diseases such as graft versus host disease (GVHD) (Prasad et al., 2011) and systemic lupus erythematosus (Sun et al., 2010).

The underlying mechanism of MSC has been widely discussed in the EAE model. MSC injection seems to suppress the innate and adaptative immune system through direct cellular interactions in secondary lymph nodes in lungs and by releasing soluble molecules, such as prostaglandin E2, tumor growth factor ß (TGFß), hepatocyte growth factor, human leukocyte antigen G isoform, indoleamine2,3-dioxygenase (IDO), interleukin 10, and metalloproteinases (Di Nicola et al., 2002; Krampera et al., 2003). Consequently, the priming process is modified, CD4+ T, CD8+ T, NK and B cells anergy is induced, B and T cells are arrested in the G0/G1 phase inhibiting proliferation and metalloproteinases secreted by MSC cleave chemochines required for T cell migration towards the CNS (Uccelli et al., 2008). Furthermore, MSC reduce the ongoing inflammation by down regulating the production of IFNγ, interleukin 2, and TNF α. Nonetheless, local and direct effects of MSC are not well understood because all reported studies are performed in the EAE model, where local effect

cannot be differentiated from the influence of the peripheral immune system (Morando et al., 2012; Uccelli and Prockop, 2010)

4. Aim of the study

The main goal of this thesis was to analyze possible beneficial effects of MSC on CNS remyelination. For this purpose, we have used the cuprizone model in which the interference of peripheral immune system does not play a role.

Our first attempt was to introduce human MSC intravenously and intranasally. This prior study from our group showed that MSC did not infiltrate into the brain because they were not able to cross the blood brain barrier when applied in the periphery (Nessler et al., 2013). For this reason, in this study we transplanted MSC from three different origins (human, murine, and canine) into the CNS (ventricle or demyelinating lesion in the corpus callosum) to overcome that limitation. Thereafter, we prepared mouse brain sections and we examined them through immunohistochemical analyses to follow the effects on myelination

To examine de- and remyelination processes by using immunohistochemistry, reliable markers are needed. Therefore, in the first part of the project, we aimed to characterize the adenomatous polyposis coli (APC) (monoclonal CC-1) and anti Olig2 antibody as valid oligodendrocytic markers to examine murine CNS tissue under normal and de- and remyelinating conditions.

5 . Manuscript I

Published in Histology and Histopathology 2015 Dec, Volume 30 (12): 1455-64

doi: 10.14670/HH-11-640.

Oligodendroglial markers in the cuprizone model of CNS de-and remyelination

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Abstract

Oligodendrocytes are the myelinating cells of the central nervous system. Since many studies of demyelinating diseases focus their research on this cell type, there is growing interest for obtaining reliable markers that can specifically recognize oligodendroglia. Established markers are the myelin-associated neurite outgrowth inhibitor (NogoA), the transcription factor Olig2, and the antibody CC-1, the latter being directed against the protein adenomatous polyposis coli (APC). Unfortunately, it has been discussed whether APC and Olig2 could recognize astrocytes under pathological conditions as well. Hence, we performed immunohistochemical studies using the oligodendroglial markers NogoA, APC, and Olig2 in a murine model of cuprizone induced demyelination. We have found that APC co-localizes with NogoA and the myelin protein CNPase and does not co-localize with the astrocytic marker GFAP. Olig2 shows co-localization with APC but there is also a small population of Olig2/GFAP double positive cells. In conclusion, our results underline that APC and NogoA are reliable markers for detection of mature oligodendrocytes. The use of the Olig2 marker should be combined with GFAP to exclude the GFAP positive population of cells from the quantification of oligodendroglia.

6 . Manuscript II

Published in Brain, Behavior and Immunity 2015 Nov, Volume 50:155-65

doi: 10.1016/j.bbi.2015.06.024.

Mesenchymal stem cells do not exert direct beneficial effects on CNS remyelination in the absence of the peripheral immune system

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Abstract

Remyelination is the natural repair mechanism in demyelinating disorders such as multiple sclerosis (MS) and it was proposed that it might protect from axonal loss. For unknown reasons, remyelination is often incomplete or fails in MS lesions and therapeutic treatments to enhance remyelination are not available. Recently, the transplantation of exogenous mesenchymal stem cells (MSC) has emerged as a promising tool to enhance repair processes. This included the animal model experimental autoimmune encephalomyelitis (EAE), a commonly used model for the autoimmune mechanisms of MS. However, in EAE it is not clear if the beneficial effect of MSC derives from a direct influence on brain resident cells or if this is an indirect phenomenon via modulation of the peripheral immune system. The aim of this study was to determine potential regenerative functions of MSC in the toxic cuprizone model of demyelination that allows studying direct effects on de- and remyelination without the influence of the peripheral immune system. MSC from three different species (human, murine, canine) were transplanted either intraventricularly into the cerebrospinal fluid or directly into the lesion of the corpus callosum at two time points: at the onset of oligodendrocyte progenitor cell (OPC) proliferation or the peak of OPC proliferation during cuprizone induced demyelination. Our results show that MSC did not exert any regenerative effects after cuprizone induced demyelination and oligodendrocyte loss. During remyelination, MSC did not influence the dynamics of OPC proliferation and myelin formation. In conclusion, MSC did not exert direct regenerative functions in a mouse model where peripheral immune cells and especially T lymphocytes do not play a role. We thus suggest that the peripheral immune system is required for MSC to exert their effects and this is independent from a direct influence of the central nervous system.

7. General discussion

Central nervous system regeneration might be a promising solution for several neurodegenerative and autoimmune diseases. In the case of multiple sclerosis, remyelination would not only protect axons from further damage, it could also slow down or stop disease progression. Neuroprotective strategies are continuously being studied in preclinical and clinical phases, but so far, no successful remyelinating approaches can be applied in the human disease.

During the evaluation of neuroregenerative therapies, oligodendroglial cells are in the focus of interest, because the failure in the remyeliantion process of demyelinated intact axons is mainly attributed to a defect in the regeneration process of new myelinating oligodendrocytes (Kotter et al., 2011; de Castro et al., 2013). Since the assessment of any new therapy requires the unequivocal recognition of oligodendrocytes (e.g. in immunohistochemical staining), in our first study we analyzed the adenomatous polyposis coli (APC) protein (monoclonal CC-1) and Olig2 surface markers as reliable indicators of oligodendroglial cells to examine murine CNS tissue in health and disease. The interest of the first part of this thesis raised after several studies suggested that APC and Olig2 could be expressed by astrocytes in pathological conditions (Leroy et al., 2001; Cai et al., 2007). In this study, we demonstrated that APC does not co-localize with the astrocytic marker GFAP, but with the established oligodendroglial markers NogoA and CNPase. Therefore, we conclude that APC can be used as a reliable marker for the detection of mature oligodendrocytes in both untreated control mice and in mice during de- and remyelination in the white and grey matter areas of the brain.

Regarding to the marker Olig2, we could confirm that Olig2 is not only expressed in OPC, but also in mature oligodendrocytes. However, we also observed co-expression of Olig2 with the astrocytic marker GFAP in a small cell population during cuprizone induced demyelination at the peak of OPC regeneration. We speculate that those cells are more likely to be OPC, since

several studies also showed oligodendrogenesis or Schwann cell differentiation from $GFAP^+/Olig2^+$ cells (Blakemore, 2005; Mecha et al., 2013).

In the second chapter, we investigated the use of mesenchymal stem cell as a remyelinating therapy to treat demyelinating disorders of the central nervous system. Several preclinical studies in the EAE model showed that this cellular therapy ameliorates the disease progression by inducing T cell anergy (Zappia et al., 2005; Kassis et al., 2013). However, it is not clear whether neuroprotection with MSC require the presence of the peripheral immune system for its effect. In order to address this question we used the advantages offered by the toxic demyelinating cuprizone model.

Unlike EAE or other models used for studying demyelinating diseases, the cuprizone model allows us to study demyelination and remyelination independently, because after complete demyelination is reached, cuprizone is removed from the diet and demyelinated areas start spontaneously to remyelinate (Skripuletz et al., 2011). In addition, the peripheral immune system takes no part in the process of demyelination, which is an important feature that makes this model appropriate to address our scientific question. Do MSC need the peripheral immune system to exert their beneficial effect?

First, we examined in vitro the change of growth factor expression of MSC in an inflammatory environment. This condition emulated the milieu found in the transplantation area at the injection time. As a consequence, we observed that the mRNA for IL-6 and GDNF was significantly upregulated. Since the cells reacted in that specific environment, we expected an analogous response after its transplantation.

However, it could be argued that while the cells are injected stereotactically directly into the ventricles or into the corpus callosum, the BBB is partially disrupted, at least, at the level of injection. For this reason, we evaluated the effect of the stereotactic procedure in the

cuprizone model. Although we did find a few CD3+ cells near the cannula´s track, we did not observe that this administration route boosted remyelination in the evaluated regions (cortex and corpus callosum) because comparative analysis between animals treated with cuprizone and animals that received cuprizone and underwent the stereotactic procedure confirmed that remyelination was not enhanced due to the inflammatory event at the injection site.

Regarding the results obtained of transplanted animals with MSC of different animal species and obtained from bone marrow (human and murine MSC) and adipose tissue (canine MSC), we did not observe any difference among all studied groups. Therefore, we concluded that MSC therapy did not have a beneficial effect in the cuprizone model. Moreover, since similar studies performed in the EAE model, where the peripheral immune system plays an important role, resulted in a beneficial effect, we drew the conclusion that MSC may not have a direct influence on the CNS, but they could exert their properties by modulating the peripheral immune system.

After intravenous transplantation, MSC mostly become trapped in lungs (Wagner et al., 2009) and just a few of them escape to the peripheral blood stream. For that reason, we speculate that whatever mechanism of action they have, it might take place in the lungs. Recently, a new study suggested that effector and memory T cells are primed the lungs before entering the central nervous system (Odoardi et al., 2012). This could explain where the MSC would establish contact with effector T cells to exert their immunoregulatory properties. Additionally, in a recent study MSC were directly injected into the CNS in the EAE model. In this case, MSC were able to stop the disease course and obtained similar results from intravenously injected cells in EAE, but they did not considered that in that model the BBB is not intact and there is infiltration of peripheral immune cells, growth factors and cytokines from the periphery. Therefore, they were not able to assure that cells have a direct effect in CNS. However, our results from this second study confirmed that MSC had no direct impact

in the CNS, consequently, there would not be any need to inject them directly into demyelinated areas, but they could be delivered intravenously (see Fig 1.).

Nevertheless, the use of any MSC therapy raises several concerns that should be addressed in order to approve them for therapeutic use. Safety is a major problem to resolve because until now it is still controversial whether MSC could generate or stimulate growth of existing tumors by promoting angiogenesis (Haarer et al., 2015). Furthermore, MSC are immunopriviledged, this means that the immune system of the host is not able to react against them. The advantage of this condition is that patients receiving MSC transplantation will not need the use of immunosuppressive drugs, but this could be problematic if they turned into precancerous MSC. In that case, the immune system of the patient would not be able to recognize them as a threat and they would not be rejected. Moreover, it is unclear whether this immunopriviledged state remains after stem cell differentiation (Haarer et al., 2015). If not, patients might need to use immunosuppressive drugs, as well.

Another aspect to determine is the quantity necessary to administrate for its effectiveness. Intravenous injections result in rapid reduction in cell number. Therefore, administration of large amounts of cells might be required, especially when MSC engraftment is scarce. In addition, the unclear mechanism of action and the lack of standardized trials complicate its study.

Future experiments should be focused to solve some of the afore-mentioned concerns in order to develop different measures that would improve MSC therapy. For instance, a recent study suggested to irradiate MSC at 80Gy before transplantation in order to avoid its malignant transformation into precancerous cells. This method neither reduces their viability nor interferes with their properties (Wang et al., 2014). Furthermore, Hoffman et al. suggested the use of hydrogels as a measure to solve the high doses necessary to inject MSC for therapeutic

use (Hoffman et al., 2014). Another approach to increase its effectiveness could be to transgenically engineer MSC for delivering known therapeutic genes. For example, they could overexpress BDNF or CNTF (Lu et al., 2009) or produce recombinant IFNß (Ryu et al., 2013).

Nevertheless, extrapolation from animal models to human therapy cannot be easily done, because they usually do not represent all the pathological aspects of human disease (Tanaka et al., 2014). However, we can take benefit of them to shed some light on MSC therapy and about the process of remyelination.

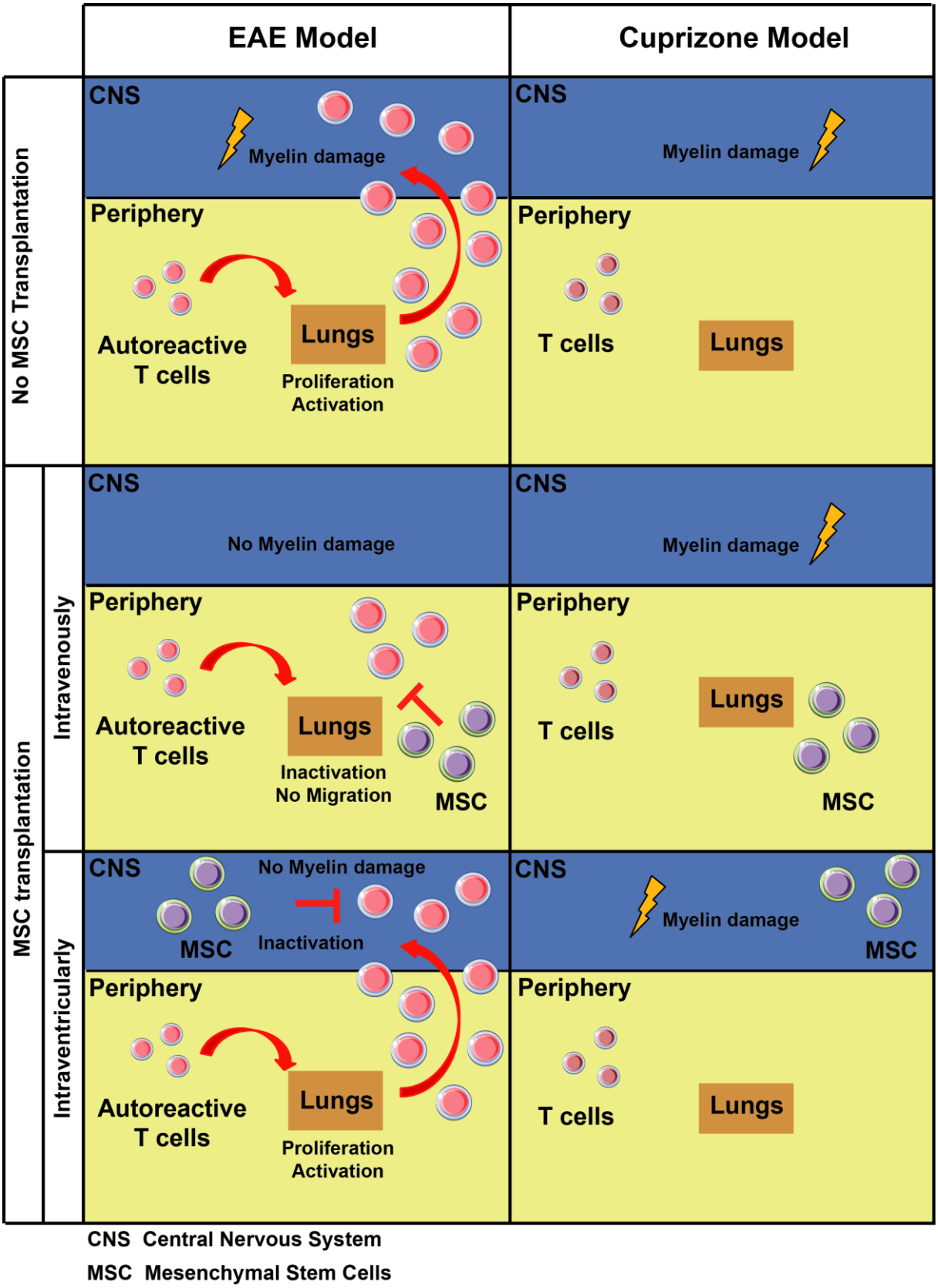

Fig. 1 Esquematic representation of MSC transplantation effect by two administration routes (intravenous and intraventricular) in the EAE and cuprizone model.

8. References

Bachelin, C., Lachapelle, F., Girard, C., Moissonnier, P., Serguera-Lagache, C., Mallet, J., Fontaine, D., Chojnowski, A., Le Guern, E., Nait-Oumesmar, B., Baron-Van, Evercooren, A., 2005. Efficient myelin repair in the macaque spinal cord by autologous grafts of Schwann cells. Brain 128, 540-549.

Blakemore, W.F., 2005. The case for a central nervous system (CNS) origin for the Schwann cells that remyelinate CNS axons following concurrent loss of oligodendrocytes and astrocytes. Neuropathol. Appl. Neurobiol. 3, 1-10.

Cai, J., Chen, Y., Cai, W.H., Hurlock, E.C., Wu, H., Kernie, S.G., Parada, L.F., Lu, Q.R., 2007. A crucial role for Olig2 in white matter astrocyte development. Development 134, 1887-1899.

de Castro, F., Bribián, A., Ortega, M.C., 2013. Regulation of oligodendrocyte precursor migration during development, in adulthood and in pathology. Cell Mol. Life Sci. 70, 4355-4368.

Di Nicola, M., Carlo-Stella, C., Magni, M., Milanesi, M., Longoni, P.D., Matteucci, P., Grisanti, S., Gianni, A.M., 2002. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99, 3838-3843.

Dutta, R. and Trapp, B.D., 2007. Pathogenesis of axonal and neuronal damage in multiple sclerosis. Neurology 68, S22-31; discussion S43-54.

Esiri, M. M., 2007. The interplay between inflammation and neurodegeneration in CNS disease. J. Neuroimmunol. 184, 4-16.

Fitch, M. T. and Silver, J., 2008. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. Exp. Neurol. 209, 294-301.

Franklin, R. J. and Ffrench-Constant, C., 2008. Remyelination in the CNS: from biology to therapy. Nat. Rev. Neurosci. 9, 839-855.

Franklin, R.J., Gilson, J.M., Franceschini, I.A., Barnett, S.C., 1996. Schwann cell-like myelination following transplantation of an olfactory bulb-ensheathing cell line into areas of demyelination in the adult CNS. Glia 17, 217-224.

Franklin, R. J. and Hinks, G.L., 1999. Understanding CNS remyelination: clues from developmental and regeneration biology. J. Neurosci. Res. 58, 207-213.

Giunti, D., Parodi, B., Usai, C., Vergani, L., Casazza, S., Bruzzone, S., Mancardi, G., Uccelli, A., 2012. Mesenchymal stem cells shape microglia effector functions through the release of CX3CL1. Stem Cells 30, 2044-2053.

Gudi, V., Gingele, S., Skripuletz, T., Stangel, M., 2014. Glial response during cuprizone-induced de- and remyelination in the CNS: lessons learned. Front Cell Neurosci. 8, 73.

Haarer, J., Johnson, C.L., Soeder, Y., Dahlke, M.H., 2015. Caveats of mesenchymal stem cell therapy in solid organ transplantation. Transpl. Int. 28, 1-9.

Hass, R., Kasper, C., Böhm, S., Jacobs, R., 2011. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. Cell Commun. Signal 9, 12.

Hoffman, M.D., Van Hove, A.H., Benoit, D.S., 2014. Degradable hydrogels for spatiotemporal control of mesenchymal stem cells localized at decellularized bone allografts. Acta Biomater. 10, 3431-3441.

Hudson, L.D., Puckett, C., Berndt, J., Chan, J., Gencic, S., 1989. Mutation of the proteolipid protein gene PLP in a human X chromosome-linked myelin disorder. Proc. Natl. Acad. Sci. U S A. 86, 8128-8131.

Jarjour, A.A., Boyd, A., Dow, L.E., Holloway, R.K., Goebbels, S., Humbert, P.O., Williams, A., Ffrench-Constant, C., 2015. The polarity protein Scribble regulates myelination and remyelination in the central nervous system. PLoS Biol. 13, e1002107.

Kassis, I., Petrou, P., Halimi, M., Karussis, D., 2013. Mesenchymal stem cells (MSC) derived from mice with experimental autoimmune encephalomyelitis (EAE) suppress EAE and have similar biological properties with MSC from healthy donors. Immunol. Lett. 154, 70-76.

Keirstead, H.S., Ben-Hur, T., Rogister, B., O'Leary, M.T., Dubois-Dalcq, M., Blakemore, W.F., 1999. Polysialylated neural cell adhesion molecule-positive CNS precursors generate both oligodendrocytes and Schwann cells to remyelinate the CNS after transplantation. J. Neurosci. 19, 7529-7536.

Keough, M.B., Jensen, S.K., Yong, V.W., 2015. Experimental demyelination and remyelination of murine spinal cord by focal injection of lysolecithin. J. Vis. Exp.97

Kipp, M., van der Star, B., Vogel, D.Y., Puentes, F., van der Valk, P., Baker, D., Amor, S., 2012. Experimental in vivo and in vitro models of multiple sclerosis: EAE and beyond. Mult. Scler. Relat. Disord. 1, 15-28.

Kotter, M.R., Setzu, A., Sim, F.J., Van Rooijen, N., Franklin, R.J., 2001. Macrophage depletion impairs oligodendrocyte remyelination following lysolecithin-induced demyelination. Glia 35, 204-212.

Kotter, M.R., Stadelmann, C., Hartung, H.P., 2011. Enhancing remyelination in disease--can we wrap it up?. Brain 134, 1882-1900.

Krampera, M., Glennie, S., Dyson, J., Scott, D., Laylor, R., Simpson, E., Dazzi, F., 2003. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 101, 3722-3729.

Lassmann, H., 2014. Mechanisms of white matter damage in multiple sclerosis. Glia 62, 1816-1830.

Leroy, K.., Duyckaerts, C., Bovekamp, L., Müller, O., Anderton, B.H., Brion, J.P., 2001. Increase of adenomatous polyposis coli immunoreactivity is a marker of reactive astrocytes in Alzheimer's disease and in other pathological conditions. Acta Neuropathol. 102, 1-10.

Lu, Z., Hu, X., Zhu, C., Wang, D., Zheng, X., Liu, Q., 2009. Overexpression of CNTF in Mesenchymal Stem Cells reduces demyelination and induces clinical recovery in experimental autoimmune encephalomyelitis mice. J. Neuroimmunol. 206, 58-69.

McPherson, R.C., Cambrook, H.E., O'Connor, R.A., Anderton, S.M., 2014. Induction of passive EAE using myelin-reactive CD4+ T cells. Methods Mol. Biol. 1193, 187-198.

Mecha, M., Feliú, A., Carrillo-Salinas, L., Mestre, L., Guaza, C., 2013. Mobilization of progenitors in the subventricular zone to undergo oligodendrogenesis in the Theiler's virus model of multiple sclerosis: implications for remyelination at lesions sites. Exp. Neurol. 250, 348-352.

Moore, C.S., Abdullah, S.L., Brown, A., Arulpragasam, A., Crocker, S.J., 2011. How factors secreted from astrocytes impact myelin repair. J. Neurosci. Res. 89, 13-21.

Morando, S., Vigo, T., Esposito, M., Casazza, S., Novi, G., Principato, M.C., Furlan, R., Uccelli, A., 2012. The therapeutic effect of mesenchymal stem cell transplantation in experimental autoimmune encephalomyelitis is mediated by peripheral and central mechanisms. Stem Cell Res. Ther. 3, 3.

Nessler, J., Bénardais, K., Gudi, V., Hoffmann, A., Salinas Tejedor, L., Janßen, S., Prajeeth, C.K., Baumgärtner, W., Kavelaars, A., Heijnen, C.J., van Velthoven, C., Hansmann, F., Skripuletz, T., Stangel, M., 2013. Effects of murine and human bone marrow-derived mesenchymal stem cells on cuprizone induced demyelination. PLoS One 8, e69795.

Odoardi, F., Sie, C., Streyl, K., Ulaganathan, V.K., Schläger, C., Lodygin, D., Heckelsmiller, K., Nietfeld, W., Ellwart, J., Klinkert, W.E., Lottaz, C., Nosov, M., Brinkmann, V., Spang, R., Lehrach, H., Vingron, M., Wekerle, H., Flügel-Koch, C., Flügel, A., 2012. T cells become licensed in the lung to enter the central nervous system. Nature 488, 675-679.

Perry, V.H., Nicoll, J.A., Holmes, C., 2010. Microglia in neurodegenerative disease. Nat. Rev. Neurol. 6, 193-201.

Prasad, V.K., Lucas, K.G., Kleiner, G.I., Talano, J.A., Jacobsohn, D., Broadwater, G., Monroy, R., Kurtzberg, J., 2011. Efficacy and safety of ex vivo cultured adult human mesenchymal stem cells (Prochymal) in pediatric patients with severe refractory acute graft-versus-host disease in a compassionate use study. Biol. Blood Marrow Transplant. 17, 534-541.

Roach, A., Takahashi, N., Pravtcheva, D., Ruddle, F., Hood, L., 1985. Chromosomal mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in shiverer mutant mice. Cell 42, 149-155.

Ryu, C.H., Park, K.Y., Hou, Y., Jeong, C.H., Kim, S.M., Jeun, S.S., 2013. Gene therapy of multiple sclerosis using interferon beta-secreting human bone marrow mesenchymal stem cells. Biomed. Res. Int. 2013, 696738.

Skripuletz, T., Gudi, V., Hackstette, D., Stangel, M., 2011. De- and remyelination in the CNS white and grey matter induced by cuprizone: the old, the new, and the unexpected. Histol. Histopathol. 26, 1585-1597.

Skripuletz, T., Hackstette, D., Bauer, K., Gudi, V., Pul, R., Voss, E., Berger, K., Kipp, M., Baumgärtner, W., Stangel, M., 2013. Astrocytes regulate myelin clearance through recruitment of microglia during cuprizone-induced demyelination. Brain 136, 147-167.

Sun, L., Wang, D., Liang, J., Zhang, H., Feng, X., Wang, H., Hua, B., Liu, B., Ye, S., Hu, X., Xu, W., Zeng, X., Hou, Y., Gilkeson, G.S., Silver, R.M., Lu, L., Shi, S., 2010. Umbilical cord mesenchymal stem cell transplantation in severe and refractory systemic lupus erythematosus. Arthritis Rheum. 62, 2467-2475.

Tanaka, T. and Yoshida, S., 2014. Mechanisms of remyelination: recent insight from experimental models. Biomol. Concepts 5, 289-298.

Uccelli, A., Moretta, L., Pistoia, V., 2008. Mesenchymal stem cells in health and disease. Nat Rev. Immunol. 8, 726-736.

Uccelli, A., and Prockop, D.J., 2010. Why should mesenchymal stem cells (MSCs) cure autoimmune diseases? Curr. Opin. Immunol. 22, 768-774.

Ulrich, R., Baumgärtner, W., Gerhauser, I., Seeliger, F., Haist, V., Deschl, U., Alldinger, S., 2006. MMP-12, MMP-3, and TIMP-1 are markedly upregulated in chronic demyelinating theiler murine encephalomyelitis. J. Neuropathol. Exp. Neurol. 65, 783-793.

van der Star, B.J., Vogel, D.Y., Kipp, M., Puentes, F., Baker, D., Amor, S., 2012. In vitro and in vivo models of multiple sclerosis. CNS Neurol. Disord. Drug Targets 11, 570-588.

Wagner, J., Kean, T., Young, R., Dennis, J.E., Caplan, A.I., 2009. Optimizing mesenchymal stem cell-based therapeutics. Curr. Opin. Biotechnol. 20, 531-536.

Wang, X., Kimbrel, E.A., Ijichi, K., Paul, D., Lazorchak, A.S., Chu, J., Kouris, N.A., Yavanian, G.J., Lu, S.J., Pachter, J.S., Crocker, S.J., Lanza, R., Xu, R.H., 2014. Human ESC-derived MSCs outperform bone marrow MSCs in the treatment of an EAE model of multiple sclerosis. Stem Cell Reports 3, 115-130.

Zappia, E., Casazza, S., Pedemonte, E., Benvenuto, F., Bonanni, I., Gerdoni, E., Giunti, D., Ceravolo, A., Cazzanti, F., Frassoni, F., Mancardi, G., Uccelli, A., 2005. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood 106, 1755-1761.

Zhang, J., Li, Y., Chen, J., Cui, Y., Lu, M., Elias, S.B., Mitchell, J.B., Hammill, L., Vanguri, P., 2005. Human bone marrow stromal cell treatment improves neurological functional recovery in EAE mice. Exp. Neurol. 195, 16-26.

9. Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled

“Central nervous system regeneration approach in the toxic cuprizone model of de- and remyelization: application of mesenchymal stem cells”

No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institution:

Department of Neurology, Hannover Medical School

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

_____

Date, Signature

10. Acknowledgements

After these three years of hard work, it is time to look back and thank to all the people that made the writing of this thesis possible.

First and foremost I would like to express my gratitude to Prof. Dr. Stangel for giving me the opportunity to conduct my PhD thesis in his group at the department of Neurology, and for his advice and support throughout the entire duration of the project.

I would also like to thank my co-supervisors Prof. Dr. Baumgärtner and Prof. Dr. Tipold for their guidance and useful comments during our annual meetings.

A special thanks to PD Dr. Skripuletz for his scientific support, everlasting help and supervision as well as for making my PhD productive and interesting.

I am also very thankful to all current and past members of the AG Stangel, but especially to Dr. Viktoria Gudi and Dr. Prajeeth Chittappen for sharing their experience and knowledge with me and providing a great work atmosphere.

I am very grateful to Ilona Cierpka-Leja and Sabine Lang for their remarkable personal and technical support in the lab.

I would also like to thank Andy Niesel for being ready to help us at any time with everything and to Gabriel Berner, Kristin Jakobsen, Nicole Jungwith and Florian Hansmann for their great work and contribution to this project.

Furthermore, I am very thankful to all the people of the PhD room for their help and the nice environment.

Finally, I would like to thank my family, especially to my parents, for their unconditional love and support and to Juan for showing me the way to happiness.