Neural stem cells and interleukin-13 as a combination therapy for spinal cord injury

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List of abbreviations

BBB	Basso Beattie Bresnahan
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BMS	Basso mouse scale
BSA	bovine serum albumin
CNS	central nervous system
CNTF	ciliary neurotrophic factor
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
EAE	experimental autoimmune encephalomyelitis
EGF	epithelial growth factor
eGFP	enhanced green fluorescent protein
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
IBA-1	ionized calcium binding adaptor molecule
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon-γ
IL	interleukin
IL-4R	interleukin-4 receptor
iNOS	inducible nitric oxide synthase
IRS	insulin receptor substrate
JAK	janus kinase
LFA-1	lymphocyte function-associated antigen 1
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MS	multiple sclerosis
NGF	nerve growth factor
NO	nitric oxide
NPCs	neural precursor cells
NSCs	neural stem cells
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PK	protein kinase
PI3K	phosphatidylinositol-3’-kinase-Akt
SCI	spinal cord injury
SGZ	subgranular zone
STAT	signal transducer and activator of transcription
SVZ	subventricular zone
Th2	T helper 2

Summary

Spinal cord injury (SCI) is associated with damage to nervous tissue en consequently leads to loss of motor and sensory function. Until now, there is no curative therapy available for SCI. During the last decade there is a growing interest in the therapeutic potential of neural stem cell (NSC) transplantation into the damaged spinal cord, as these cells have the ability to replace damaged neural tissue, enhance endogenous regeneration and modulate the inflammatory response after trauma. However, NSC transplantation into the injured spinal cord will most likely not be fully successful without the application of additional factors which modulate the tissue environment to make it more supportive and permissive for endogenous neural regeneration. Therefore, we have combined the immunomodulatory and regenerative properties of NSCs with those of the anti-inflammatory cytokine interleukin-13 (IL-13). IL-13 is predominantly secreted by activated T helper 2 cells and is thought to have a major role in the pathogenesis of allergic inflammation.

In this study, our aim was to determine whether NSCs and IL-13, alone or in combination, induce a significant improvement in clinical and histological outcome after SCI by modulating the inflammatory response. Therefore, enhanced green fluorescent protein (eGFP)-positive NSCs were first transplanted into the lesion after spinal cord hemisection in mice. Grafting of NSCs improved functional recovery of the transplanted mice compared to the PBS injected control mice. This improvement could be the result of the immunosuppressive effect of the NSCs, as a reduction, however not significant, in lesion size and demyelinated area was measured. Three weeks after transplantation no differentiation of the NSCs into neurons was observed. Moreover, a number of NSCs were phagocytosed by microglia/macrophages after three weeks.

In the second *in vivo* experiment, NSCs were injected into the injured spinal cord, followed by the addition of an IL-13 containing gelfoam. IL-13, NSCs, or NSCs combined with IL-13 had no significant effect on the functional recovery of the mice after SCI, which was probably due to the low number of mice included in this experiment. A trend indicated that IL-13 impaired clinical outcome of the treated mice after SCI. Survival of the NSCs was evaluated 3, 7 and 28 days after grafting in the spinal cord lesion. The morphology of the NSCs suggested that these cells survived 3 and 7 days post transplantation, however, after 28 days they were probably phagocytosed.

This study revealed that allogeneic NSC transplantation improved the functional recovery of mice after SCI, whereas this seemed to be impaired by recombinant IL-13 treatment. These results give a first indication that IL-13 is not the ideal factor to combine with NSCs as treatment for SCI.

Samenvatting

Ruggenmergletsels zijn geassocieerd met beschadiging van het zenuwweefsel en leiden vervolgens tot verlies van motorische en sensorische functies. Tot op heden is er geen therapie die de functie van het beschadigd zenuwweefsel kan herstellen. Gedurende de laatste jaren, is er een stijgende interesse in de therapeutische toepassing van neurale stamcel (NSC) transplantatie in het beschadigd ruggenmerg, aangezien NSC beschadigd neuronaal weefsel kunnen vervangen, endogene regeneratie kunnen stimuleren en de inflammatoire respons kunnen moduleren. Toch zal NSC transplantatie in het beschadigd ruggenmerg waarschijnlijk niet succesvol zijn zonder de toediening van additionele factoren die zorgen voor een meer ondersteunend en stimulerend milieu voor endogene neuronale regeneratie. Om deze reden hebben we de immunomodulatoire en regeneratieve eigenschappen van NSC gecombineerd met deze van het anti-inflammatoire cytokine interleukin-13 (IL-13). IL-13 wordt voornamelijk gesecreteerd door geactiveerde T helper 2 cellen en wordt verondersteld een belangrijke rol te spelen in de pathogenese van allergische inflammatie.

Het doel van deze studie, was te bepalen of NSC en IL-13 de klinische en histologische uitkomst na ruggenmergschade kunnen verbeteren, door modulatie van de inflammatoire respons. Eerst werden *enhanced green fluorescent protein* (eGFP)-positieve NSC getransplanteerd in de lesie na hemisectie van het ruggenmerg in muizen. Transplantatie van NSC verbeterde het functioneel herstel van de getransplanteerde muizen vergeleken met de controle groep. Deze verbetering zou het gevolg kunnen zijn van het immunosuppresieve effect van de NSC, aangezien een dalende trend in lesiegrootte en demyelinisatie werd waargenomen. Drie weken na transplantatie werd geen differentiatie van de NSC in neuronen geobserveerd. Wel werd fagocytose waargenomen van een aantal NSC door microglia/macrofagen.

Vervolgens werden NSC geïnjecteerd in de ruggenmerglesie in combinatie met de toediening van recombinant IL-13. IL-13, NSC, of NSC gecombineerd met IL-13 hadden geen significant effect op het functioneel herstel van de muizen na ruggenmergschade. Dit was waarschijnlijk te wijten aan het lage aantal muizen dat gebruikt werd in dit experiment. Een trend gaf aan dat IL-13 het functioneel herstel van de behandelde muizen verminderde. Ook werd de overleving van de NSC 3, 7 en 28 dagen na de transplantatie geëvalueerd. De morfologie van de stamcellen suggereerde dat ze overleefde 3 en 7 dagen na injectie, maar na 28 dagen waren ze waarschijnlijk gefagocyteerd.

Uit deze studie kan geconcludeerd worden dat allogene NSC transplantatie het functioneel herstel van muizen na ruggenmergschade verbeterde, terwijl IL-13 dit net verslechterde. Deze resultaten geven een eerste indicatie dat IL-13 geen goede factor is om te combineren met NSC als behandeling voor ruggenmergletsels.

1. Introduction

Spinal cord injury (SCI) is associated with damage to nervous tissue and consequently results in loss of motor and sensory function. The mechanical trauma rapidly kills neurons and glia, while a delayed secondary response follows which causes additional tissue degeneration and may persist for months after the initial trauma. Until now there is no fully restorative therapy for SCI and research primarily focuses on reducing neural degeneration and promoting axonal outgrowth. A promising therapy may be the transplantation of neural stem cells (NSCs) that produce an anti-inflammatory cytokine, such as interleukin-13 (IL-13).

1.1 Spinal cord injury

Worldwide, approximately 2,5 million people suffer from SCI, with more than 130.000 new cases reported each year [1]. SCI has a major impact on patients and their families as well as on a broader socio-economical scale. This is due to the fact that most of these injuries cause a lifelong disability and that it affects many individuals, especially young ones. The mean age of patients is reported to be 33 years, with a predominantly male patient population (3,8:1 male/female ratio) [2]. SCI leads to fundamental changes of life, as patients are confronted with motor and sensory deficits as well as dysfunction of bladder and bowel. The additional live-long supportive care to prevent various complications, such as respiratory tract and urinary infections, are a substantial financial burden for patients as well as for the society [3].

1.1.1 Pathogenesis

SCI is characterized by marked neuropathology and limited functional recovery [4]. At the primary site of injury, SCI causes neural and axonal destruction as well as demyelination. The initial mechanical disruption or contusion of the nerve tracts is progressively extended by a second phase of tissue degeneration during the following hours, days and weeks. This phase consists of a massive inflammatory reaction, which involves the synthesis and release of cytokines and chemokines leading to the influx of peripheral inflammatory cells (T cells and macrophages) and the activation of resident microglia [3, 4]. Moreover, this phase includes glutamatergic excitotoxicity, free radical generation, lipid peroxidation and ischemia, which ultimately cause death of neurons, oligodendrocytes, astrocytes and precursor cells. The secondary response will finally result in the formation of a glial scar surrounding a central cavitation at the site of the initial trauma, which is considered as a major physical and chemical barrier for endogenous regeneration of nerve tracts [3]. Other factors that contribute to limited regenerative properties of the central nervous system (CNS) to recover from injury are inhibitory myelin debris [5], the inability of endogenous NSCs to induce neurogenesis upon injury [6] and the lack of sufficient trophic support [7]. The resulting cavities and cysts may interrupt descending and ascending axonal tracts, leading to loss of sensory and motor function (Figure 1).

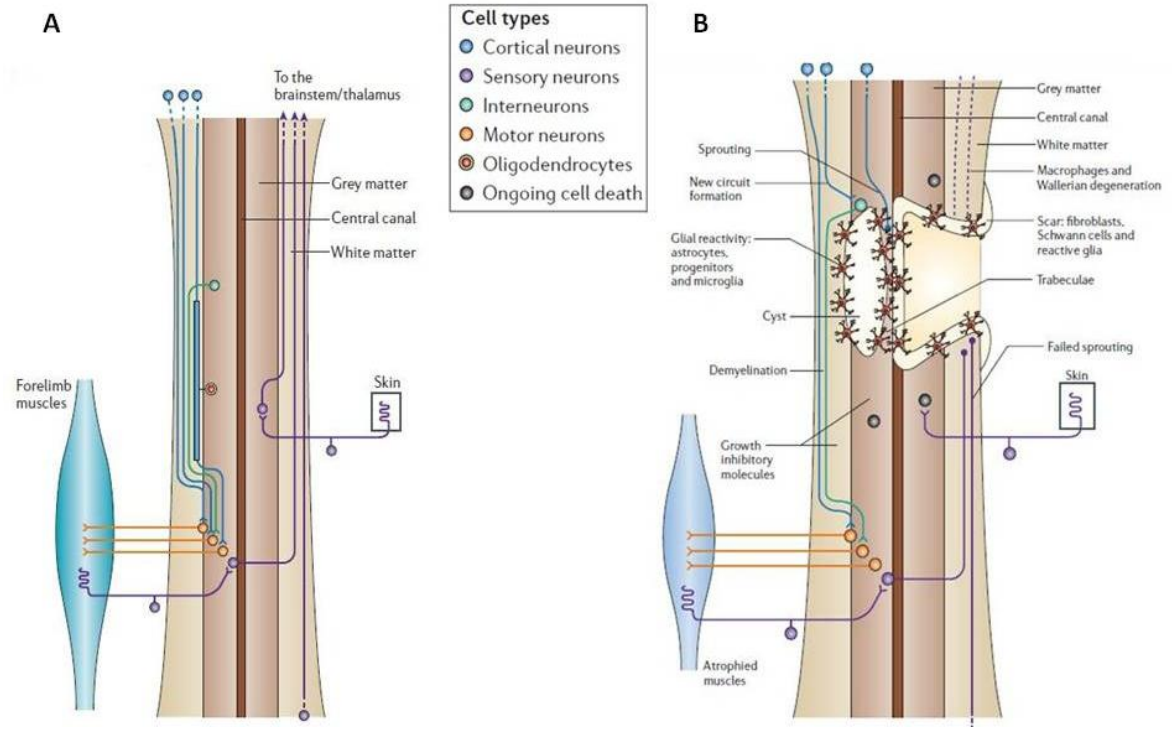


Figure 1: Schematic representation of sagittal sections through the human spinal cord, showing an intact spinal cord (A) and a spinal cord after injury (B). (A) The spinal cord consists of white matter in the peripheral region, containing myelin-sheathed nerve fibers. These nerve fibers conduct sensory information to the brain and motor information from the brain to the rest of the body. Internal to this region is the gray matter, which is mainly composed of nerve cell bodies. This central gray region surrounds the central canal, through which cerebrospinal fluid circulates and which is an extension of the ventricles of the brain. (B) SCI leads to infiltration of immune cells, death of neurons and glial cells, and formation of a glial scar. Many ascending and descending axons are interrupted and fail to regenerate, leading to sensory and motor deficits. Some axons however are able to form new circuits, for example via a connection with interneurons that synapse onto target motor neurons (figure adapted from [1]).

1.1.2 Therapy

Until now, there is no curative therapy available for SCI; current therapy primarily focuses on preventing and restricting the secondary inflammatory response. First, most patients undergo surgery, which is aimed at decompression of the spinal cord, by for example laminectomy or orthopaedic fixation of the involved vertebrae. A second widely used therapeutic intervention is the administration of high doses of steroids (methylprednisolone) in the acute phase after trauma (within 8 hours after injury) [3, 8]. This latter therapy is primarily based on the National Acute Spinal Cord Injury Studies I and II [8]. However, in several other studies methylprednisolone failed to demonstrate improvement and high doses of steroid administration increases the risk of various adverse effects, such as infections, respiratory complications and sepsis [9].

Due to the limited endogenous regeneration of the CNS after trauma, most research has focused upon reducing neural degeneration and promoting axonal outgrowth. Over the last decade there is a growing interest in the therapeutic potential of NSC transplantation into the damaged spinal cord. This strategy aims at (i) exogenous cell replacement, (ii) enhancement of endogenous neural regeneration and (iii) modulation of the inflammatory response after trauma [3, 10].

1.2 Neural stem cells

NSCs are undifferentiated cells of the nervous system that have the capacity for self-renewal and can differentiate into the three types of neural cells, neurons, astrocytes and oligodendrocytes (multipotency). The balance between self-renewal and differentiation is maintained by a process called asymmetrical cell division, in which a dividing NSC generates a daughter NSC and an intermediate progenitor. In the adult CNS, NSCs are present in the subventricular zone (SVZ) of the lateral wall of the lateral ventricle and in the subgranular zone (SGZ) of the dentate gyrus [10, 11]. NSCs of the SVZ (also known as B cells) have been demonstrated to give rise to transit-amplifying progenitor cells (or C cells), which can differentiate into neurons and oligodendrocytes [12]. Newborn neurons derived from NSCs of the SVZ integrate into the existing functional circuitry of the olfactory bulb. NSCs of the SGZ are classified depending on their morphological appearance and expression profile in type I or II hippocampal progenitors. These cells have been shown to give rise to neurons, which integrate in the dentate gyrus, and astrocytes [13]. Furthermore, recent studies have isolated cells with stem cell properties from various other CNS regions, including the subcallosal area [14] and the spinal cord [15]. *In vitro* these cells are multipotent and have the capacity to renew themselves; whether they also have these properties *in vivo* has to be further investigated.

A major advantage of the use of NSCs is that recent progress has made it possible to routinely expand NSCs obtained from a small amount of fetal CNS tissue *in vitro*. Furthermore, due to these selective culture techniques for NSCs, major progress has been made in elucidating the biological properties of NSCs. In response to CNS injury, it has been demonstrated that endogenous NSCs form astrocytes, and not neurons [6]. This is probably due to radical environment changes after injury, which are caused by the production of cytokines and growth factors by infiltrating immune cells. For example, expression of ciliary neurotrophic factor (CNTF), which induces astrocytic differentiation of NSCs *in vitro*, is induced in response to SCI [16, 17].

1.3 Neural stem cell transplantation after spinal cord injury

Over the last decade several studies have investigated the therapeutic potential of NSC transplantation into the damaged spinal cord. NSCs can induce recovery of function of the injured spinal cord by several mechanisms.

1.3.1 Replacement of damaged neural tissue

NSC transplantation could restore the function of the damaged spinal cord by replacement of damaged neural tissue. Differentiation of NSCs into neurons could result in restoration of neural circuits, whereas differentiation into oligodendrocytes could lead to remyelinisation of demyelinated axons. Differentiation into astrocytes could provide scaffolding and nutrition, however, astrocytes express several axon growth inhibitory molecules which could prevent axon regeneration and thereby limit overall recovery.

Several studies have successfully demonstrated the differentiation of NSCs after transplantation into the damaged spinal cord. For example, in vitro-expanded fetal NSCs were able to generate neurons in vivo as well as improve motor function after transplantation into a spinal cord contusion injury model in adult rats. The authors claimed that NSCs have to be transplanted at the appropriate time point, 9 days after SCI, in order to generate the ideal microenvironment for survival and differentiation [18]. Furthermore, transplantation of NSCs derived from adult brain into the demyelinated spinal cord of adult rats 3 days after induction of demyelination resulted in extensive remyelination, morphologically comparable with the myelination patterns of Schwann's cells in the peripheral nervous system. The remyelinated axons conducted impulses at near normal conduction velocities, indicating the functionality of the newly formed myelin [19]. Following homologous transplantation of embryonic NSCs immediately after SCI in mice, the cells remained undifferentiated or differentiated into the astrocytic phenotype. The authors stated that these NSC-derived astrocytes show features typical of the early phase after SCI when the glial scar is still permissive to regenerating axons and that these cells might support neurite outgrowth of the host axons [20].
These data indicate that the microenvironment after trauma has a major influence on the ability of the cells to differentiate into neurons, oligodendrocytes or astrocytes.

1.3.2 Enhancement of endogenous neural regeneration

A second mechanism by which NSC transplantation could result in functional recovery is the enhancement of endogenous neural regeneration, which can be achieved by, for instance, the production of a variety of neurotrophic factors. It has been demonstrated that NSCs constitutively secrete neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) [21]. Transplantation of these NSCs into the damaged spinal cord of adult rats resulted in extensive growth of host axons [21]. Moreover, grafting of adult neural precursor cells (NPCs) into the injured rat spinal cord resulted in enhanced axonal regeneration by providing permissive guiding substrate [22].

1.3.3 Modulation of inflammatory response

The secondary inflammatory response is a major cause of tissue degeneration after SCI. Growing evidence indicates that NSCs transplanted in the CNS could modulate inflammation. For example, following spinal cord contusion injury, transplanted NSCs were shown to interact closely with endogenous activated microglia cells, stimulating their production of neuroprotective factors and contributing to functional recovery [23]. Moreover, transplantation of NSCs into the ventricles of mice or rats after induction of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), resulted in a reduction of brain inflammation at the acute phase of the disease. This was indicated by a reduced number of perivascular infiltrates and brain $CD3^+$ T cells, an elevated number of regulatory T cells and a decreased expression of adhesion molecules, including

intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 1 (LFA-1), in the brain. Consequently, both the demyelinating process and axonal injury were reduced in the transplanted brains, resulting in a milder EAE disease course [24, 25]. The immunomodulatory properties of NSCs are further supported by the finding that, in EAE systemically injected NPCs enter the inflamed CNS and accumulate in the perivascular areas, where they induce apoptosis of infiltrating encephalitogenic T cells [26].

All together, these data suggest that functional recovery after SCI will not only result from the replacement of damaged cells or tissue, but mainly via stimulating endogenous repair. A major challenge is to modulate the tissue environment to make it more supportive and permissive for endogenous neural regeneration. NSCs have the ability to produce neurotrophic factors as well as modulate the immune response after trauma. However, cell transplantation alone will probably not be successful without the application of additional factors that modulate the hostile lesion area. Therefore, we want to combine the properties of NSCs with the beneficial effects of the anti-inflammatory cytokine IL-13, to provide a more permissive environment for endogenous regeneration.

1.4 Interleukin-13

IL-13 is a well-known anti-inflammatory cytokine predominantly secreted by activated T helper 2 (Th2) cells. Other T cell subsets as well as dendritic and microglial cells are also able to produce IL-13. Over the past years, evidence has indicated that IL-13 is involved in the pathogenesis of allergic inflammation [27].

IL-13 exerts its biological functions through interaction with cell surface receptors (Figure 2). IL-13 receptors are expressed on many different cell types, including B cells, basophils, eosinophils, mast cells, endothelial cells, fibroblasts, monocytes and macrophages [28]. The biological and immunoregulatory functions of IL-13 resemble mostly those of IL-4, particularly because both proteins have the IL-4 receptor α (IL-4Rα) chain in common [29]. There are two types of IL-4 receptors, type I consists of the IL-4Rα and γ chain (γc), whereas type II consists of the IL-4Rα and the IL-13Rα1. This type II receptor is also able to transmit IL-13 signals [29, 30]. Binding of IL-13 to IL-13Rα1 induces heterodimerization with IL-4Rα, forming an IL-13 type I or IL-4 type II receptor. This dimerization leads to phosphorylation of tyrosine residues present in the cytoplasmic tail of the receptors, which in turn act as docking site for different signaling molecules. After phosphorylation, STAT6 dimerizes and translocates to the nucleus, where it activates several IL-4 and IL-13 response genes. Moreover, activation of insulin receptor substrate (IRS) family members and other adaptor molecules can result in the induction of several signaling pathways, including protein kinase (PK) A, PKB, PKC, phosphatidylinositol-3'-kinase-Akt (PI3K) and mitogen-activated protein kinase (MAPK)

pathway. Activation of these pathways leads to a number of cellular events, including proliferation, differentiation, survival and protection from apoptosis [28, 30].

IL-13 also binds to an IL-13 specific type II receptor, consisting of the IL-13Rα2 subunit which shows a high-affinity binding of IL-13 [31]. However, the expression of IL-4Rα and IL-13Rα1 together seems to be sufficient to render cells responsive to IL-13, indicating that IL-13Rα2 is not required for IL-13 function [32]. Increasing evidence suggests that IL-13Rα2 is involved in a negative feedback loop on the IL-13 response by acting as a decoy receptor [28, 31]. This is supported by other studies that demonstrated that IL-13Rα2 exists in a soluble form *in vivo* [33] and that its overexpression diminishes IL-13 signaling [34, 35].

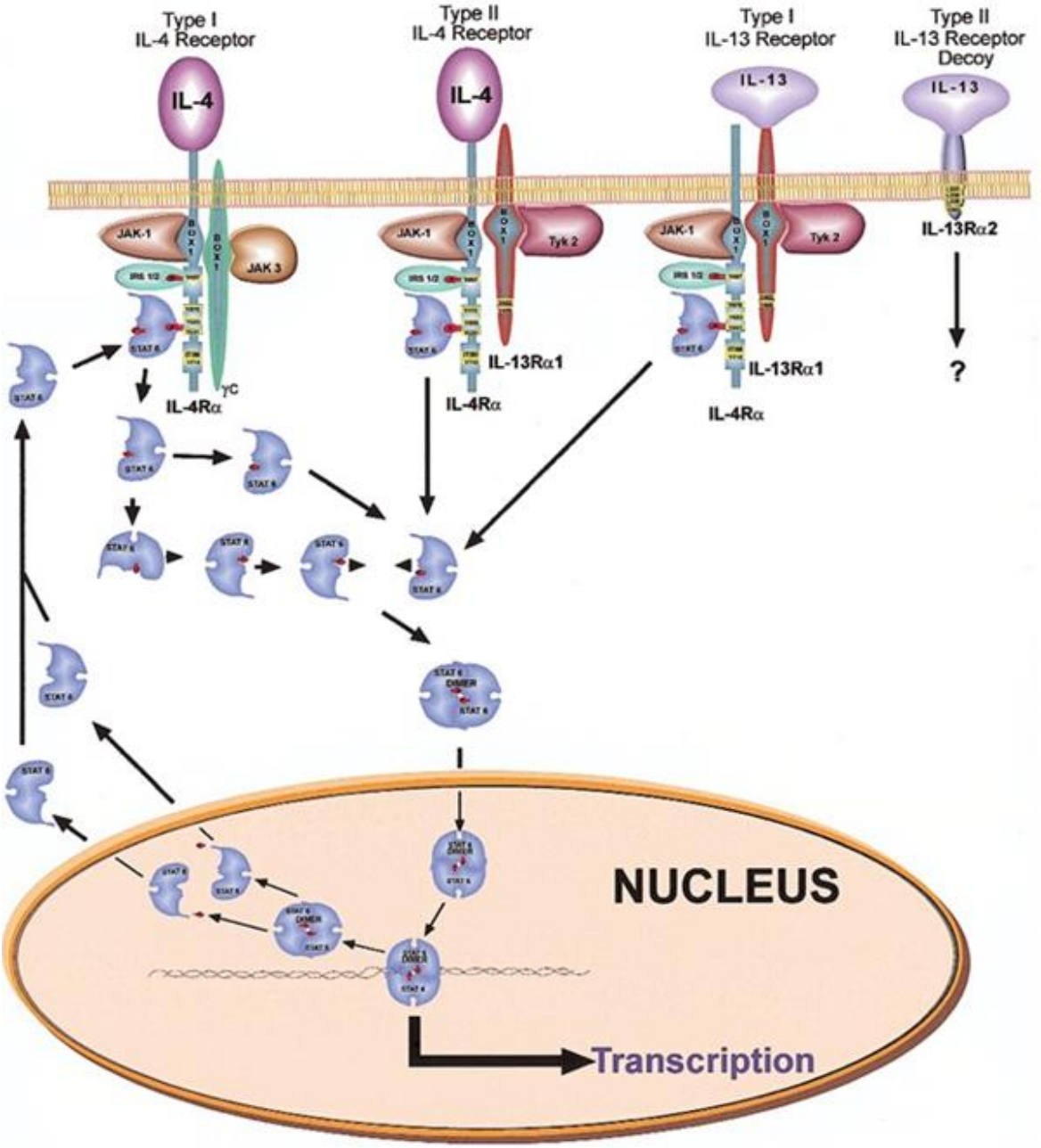


Figure 2: Schematic representation of IL-4 and IL-13 receptors and signaling pathways. The IL-4R type I, which consists of an IL-4Rα and γ chain, is specific for IL-4, whereas the IL-4R type II, consisting of IL-4Rα and IL-13Rα1, conducts signals of

both IL-4 and IL-13. Binding these cytokines results in dimerization of the two receptor chains, which activates members of the JAK family that initiate phosphorylation of the residues in the cytoplasmic tail of the receptor. These phosphorylated residues act as docking sites for adaptor and signaling molecules. After phosphorylation, STAT6 dimerizes, migrates to the nucleus and binds to different gene promoters. IL-13Rα2 binds IL-13 with a high affinity, however, it does not transduce signals, but is believed to act as a decoy receptor (IL-13R type II). IL, interleukin; JAK, janus kinase; STAT, signal transducer and activator of transcription, IRS, insulin receptor substrate (figure adapted from [28]).

1.5 The role of interleukin-13 in the central nervous system

To date, not much is known about the role of IL-13 in the CNS. IL-13 suppresses the production of pro-inflammatory cytokines, such as IL-6 and IL-1β, from activated microglial cells *in vitro* [36]. Moreover, IL-13 strongly reduces interferon (IFN)-γ or lipopolysaccharide (LPS)-induced nitric oxide (NO) production as well as inducible NO synthase (iNOS) mRNA and protein levels in macrophage cell lines and peritoneal macrophages [37, 38]. Over the last years it is thought that IL-13, as it is a Th2 cytokine, is involved in the phenotypic and functional changes of macrophages into alternatively activated or type II polarized macrophages (M2). These M2 macrophages have a more anti-inflammatory phenotype, with a different receptor expression, as well as an altered NO and cytokine production as the above mentioned studies indicate.

Furthermore, IL-13 selectively induces cell death of activated microglial cells *in vitro* [39]. Following injection of LPS into the cortex of rats, increased levels of IL-13 were measured, which was accompanied by substantial loss of microglial cells 3 days post injection. Death of these activated microglial cells *in vivo* resulted in an enhancement of neural survival [40]. These findings support the hypothesis that apoptotic cell death of activated microglial cells could act as an endogenous mechanism for the resolution of brain inflammation. In addition, this study indicates that IL-13 plays a key role in this process. A more recent study has shown that IL-13 induces this microglial cell death via the production of several cyclooxygenase-2 products [41].

Following myelin basic protein (MBP)-immunization, application of a human recombinant IL-13-secreting vector reduced the mean duration, severity and incidence of the EAE disease course [42]. This reduction was associated with only minimal decrease of MBP-directed T cell autoreactivity and no alteration in MBP-specific auto-antibody production, suggesting that IL-13 probably primarily targets cells of the macrophage/microglia lineage. In an experimental model of sepsis, IL-13 increased the survival of mice by modulating the inflammatory response via overall suppression of pro-inflammatory cytokine and chemokine production [43].

Previous data from our research group indicated that Th2 cells stimulate axon regeneration *in vitro* and *in vivo* by secreting IL-4. Following SCI in mice, injection of IL-4-producing T cells induced axonal outgrowth, accompanied by a significant improvement of clinical outcome. Moreover, IL-4 was found to be the key factor in this process since recombinant IL-4 was shown to be able to promote axonal regeneration *in vitro* and *in vivo* without the involvement of Th2 cells. Preliminary data suggested that IL-13 has an even higher pro-regenerative potential. Therefore, we have combined the immunomodulatory and regenerative properties of NSCs and IL-13 as a therapeutic strategy for SCI.

1.6 Experimental approach

We hypothesize that NSCs and IL-13, alone or in combination, induce a significant improvement in clinical and histological outcome after SCI by modulating the inflammatory response. Firstly, the enhanced green fluorescent protein (eGFP)-positive NSCs to be injected were characterized *in vitro* by immunohistochemistry, in order to determine whether they expressed a number of glial and neural markers. Secondly, we investigated whether the transplanted eGFP-positive NSCs survived after transplantation into the spinal cord lesion, as well as measuring the clinical and histological outcome. Clinical outcome was measured by performing locomotion tests on a regular basis. NSC survival and differentiation was investigated by means of immunohistochemistry. Moreover, to determine whether NSC transplantation resulted in a reduced inflammatory response, the lesion size and demyelinated area were evaluated. Finally, the combined immunomodulatory and regenerative properties of NSCs and IL-13 were investigated. Therefore, eGFP-positive NSCs were injected into the spinal cord lesion of mice, followed by the addition of an IL-13 or PBS containing gelfoam. Clinical outcome was again determined by locomotion tests and morphological characteristics of survival by using immunohistochemistry.

This study will provide new insights into the distinct and combined immunomodulatory and regenerative functions of NSCs and IL-13, and will elucidate whether NSCs combined with IL-13 can be used as a therapeutic strategy for SCI. This is of major importance as our final goal is to make IL-13-secreting NSCs, which can then be transplanted into the damaged spinal cord. Ultimately, this approach will lead to the development of new therapeutic strategies for CNS trauma. Given that within the next decades, injuries to the CNS will be a major cause of death and disability, and will have an enormous socio-economical impact on our society, this is of major relevance.

2. Materials and methods

In this project we investigated whether NSCs and IL-13 improve clinical and histological outcome after CNS injury by modulating the inflammatory response. Various methods that were applied during this project are explained in the following sections.

2.1 Animals

Experiments were performed with BALB/C and C57BL/6 mice (Harlan, the Netherlands) housed in a conventional animal facility at Hasselt University under regular conditions, i.e. in a temperature-controlled room (20±3°C) on a 12h light-dark schedule, and free access to food and water. All experiments were approved by the ethical committee of Hasselt University.

2.2 Neural stem cell culture

The eGFP-positive NSCs used were kindly provided by dr. Peter Ponsaerts from the laboratory of experimental hematology of the university of Antwerp. Briefly, the NSCs were isolated from embryonic day 14 (E14) C57BL/6 eGFP-positive transgenic mice. The cerebral cortices of these embryos were mechanically dissociated and enzymatically treated using collagenase. The resulting cell suspension was transferred to culture flasks pre-coated with fibronectin (5-10 µg/ml; R&D systems, Oxon, United Kingdom) and in the presence of 10 ng/ml epithelial growth factor (EGF; Immunotools, Friesoythe, Germany) and 10 ng/ml basic fibroblast growth factor (bFGF; Immunotools) in Neurobasal-A medium (Invitrogen, Merelbeke, Belgium) supplemented with 1% L-glutamine (Invitrogen), 1% penicillin-streptomycin (Invitrogen) and 1% N2 supplement [containing 6,88% Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen), 0,1% bovine serum albumin (BSA; Invitrogen), 1% insulin (Sigma, Bornem, Belgium), 1% Apo-transferrin (Sigma), 0,01% sodium selenite (Sigma), 0,1% putrescine (Sigma) and 0,033% progesterone (Sigma)]. The NSC cultures were maintained in a humidified atmosphere of 37°C and 5% CO_2. Every other day 10 ng/ml EGF and 10 ng/ml bFGF were added to the medium, and every fifth day the medium was replaced. NSCs were subcultivated at a ratio of 1:3, every 5-7 days when 90-95% confluence was reached, by using accutase treatment (Sigma) to dislodge the cells from the bottom of the flask.

2.3 Preparation of neural stem cells for transplantation

NSCs used for transplantation into the damaged spinal cord were harvested at passage 8-11. After harvesting the NSCs through accutase treatment (Sigma), cells were centrifuged, washed with medium and finally resuspended in 0,1 M phosphate-buffered saline (PBS; pH = 7,4) at a density of 100.000 cells/µl. Cell preparations were kept on ice until injection in the spinal cord lesion.

2.4 Spinal cord injury and transplantation of neural stem cells

Spinal cord hemisection was performed in 8 to 11 week old BALB/C and C57BL/6J mice (17-23g). The animals were anesthetized by intraperitoneal injection of ketamine (0,1 mg/g body weight; Ketalar®; Pfizer, Elsene, Belgium) and xylazine (0,02 mg/g body weight; Rompun®; Bayer, Gent, Belgium). Partial laminectomy was performed at thoracic level T8 and bilateral hemisection was carried out with iridectomy scissors, to transect the left and right dorsal funiculus, the dorsal horns and the ventral funiculus, which resulted in a complete transection of the dorsomedial and ventral corticospinal tract (Figure 3). NSCs ($5x10^5$ cells in 5 µl PBS) or PBS were injected in the lesion by means of a glass pipette (1 mm in diameter) with a sharp tip, which was connected to a Hamilton syringe. Following grafting, a gelfoam patch containing IL-13 (500 ng/ml; Pepro Tech Incorporated, New Jersey, USA) or PBS was placed on top of the lesion. The muscles were sutured using Vicryl stitches and the skin was closed with metallic wound clips. Postoperatively, the animals were injected subcutaneously with a painkiller, Buprenorphine (0,1 mg/kg body weigth; Temgesic®; Schering Plough, Heist-Op-Den-Berg, Belgium), and also received an intraperitoneal injection of 1 ml of 20% glucose (B. Braun, Diegem, Belgium) to compensate for possible loss of blood. In order to improve recovery from surgery and anesthesia, the mice were placed in an incubator at 32°C for 2h. Manual bladder emptying was performed daily until the voluntary micturition reflex returned.

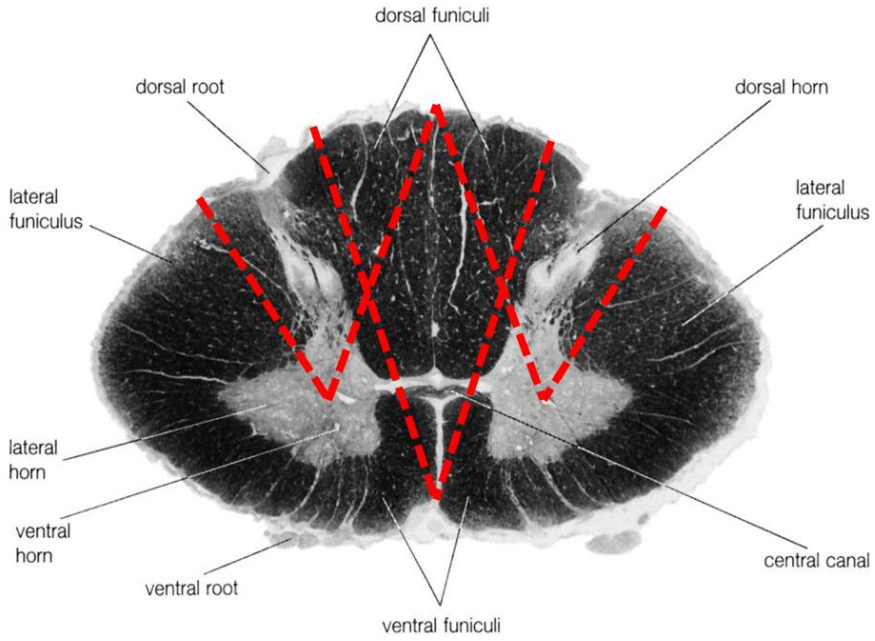


Figure 3: Representative picture of a coronal spinal cord section of a mouse indicating the bilateral hemisection performed in this study. Bilateral hemisection was carried out, to transect the left and right dorsal funiculus, the dorsal horns and the ventral funiculus, resulting in a complete transection of the dorsomedial and ventral corticospinal tract. The dashed lines indicate the hemisection.

2.5 Behavioral analysis

Functional recovery of the mice was analyzed by locomotion tests, including the Basso Mouse Scale (BMS) and Rotarod performance. The BMS is a 10-point scale, in which each category reflects a different stage of hind limb recovery [44]. Briefly, the early phase of recovery is based on resolution of paralysis and/or paresis progressed from no ankle movement to larger ankle movement (score 0-2). Plantar placing and the development of stepping is analyzed in the intermediate phase of recovery (score 3-4). In the late phase, hindlimb-forelimb coordination, paw position during stance, and trunk stability are scored (score 5-8). Normal locomotor mobility, with trunk stability and refined performance is indicated by a maximum score of 9 [44]. The mice were scored based on these hind limb movements made in an open field during a 4-minute interval. This test was performed starting the day after the surgery (day 1), followed by day 2, 3, 4, 5, 6, 7, 9, 12, 13, 14, 16, 19, 20 and 21. For the second *in vivo* experiment the mice were also scored on day 23, 26 and 27.

Rotarod performance, widely used to assess motor activity, was started at day 5 or 6 after the surgery in order to let the mice recover [45]. This test was performed at day 5, 6, 7, 9, 12, 13, 14, 16, 19, 20, 21 (23, 26, and 27). The mice were placed on an accelerated rolling rod (Ugo Basile, Comeris VA, Italy), and the period that a mouse runs on the rod was automatically recorded after the mouse jumped or fell off the rod onto a trigger plate.

2.6 Immunohistochemical analysis of cultured neural stem cells

NSCs were plated at a density of $2x10^4$ cells/well in a 24-well plate on glass coverslips pre-coated with fibronectin (5-10 µg/ml; R&D systems) in Neurobasal-A medium (Invitrogen), containing 1% L-glutamine (Invitrogen), 1% penicillin-streptomycin (Invitrogen), 1% N2 supplement, 10 ng/ml EGF (Immunotools) and 10 ng/ml bFGF (Immunotools). After 48h in culture, cells were fixed with 4% paraformaldehyde (PFA) in 0,1 M PBS (pH = 7,4), followed by protein blocking with 10% goat or monkey serum (Millipore, Brussels, Belgium) and 0,5 % Triton in 0,1 M PBS. Immunoreactivity against glial fibrillary acidic protein (GFAP; mouse monoclonal; 1/500; Sigma), MBP (rabbit polyclonal; 1/500; Millipore), neurofilament (mouse monoclonal; 1/500; Dako, Herverlee, Belgium), ionized calcium binding adaptor molecule 1 (IBA-1; rabbit polyclonal; 1/250; Wako, Neuss, Germany), MAC-3 (rat monoclonal; 1/200; BD Pharmingen, Erembodegem, Belgium) and F4/80 (rat monoclonal; 1/200; AbD Serotec, Düsseldorf, Germany) was determined (2h at room temperature) (Table 1). Binding of these primary antibodies was visualized with the appropriate Alexa 555 or 568-conjugated secondary antibody (1/400; Invitrogen) for 1h at room temperature (Table 1) and counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Between incubation steps, sections were washed with 0,1 M PBS. Finally, the sections were mounted with Shandon Immu-Mount (Thermo scientific, Cheshire, UK). Microscopical analysis was performed using an Eclipse 80i microscope (Nikon, Amstelveen, the Netherlands) and for image collection the Nis-Elements Basic Research version 2.3 microscopy software was used.

Table 1: Primary and secondary antibodies used for immunohistochemical analysis of the NSC cultures.

	Primary antibody		Secondary antibody	
GFAP	mouse anti-GFAP	1/500, Sigma	goat anti-mouse Alexa 568	1/400, Invitrogen
MBP	rabbit anti-MBP	1/500, Millipore	donkey anti-rabbit Alexa 555	1/400, Invitrogen
Neurofilament	mouse anti-neurofilament	1/500, Dako	goat anti-mouse Alexa 568	1/400, Invitrogen
IBA-1	rabbit anti-IBA-1	1/500, Wako	donkey anti-rabbit Alexa 555	1/400, Invitrogen
MAC-3	rat anti-MAC-3	1/200, BD Phamingen	goat anti-rat Alexa 568	1/400, Invitrogen
F4/80	rat anti-F4/80	1/200, Serotec	goat anti-rat Alexa 568	1/400, Invitrogen

GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; IBA-1, ionized calcium binding adaptor molecule 1.

2.7 Immunohistochemical analysis of spinal cord tissue sections

Three weeks after surgery, the animals were deeply anesthetized with an intraperitoneal injection of Nembutal® (90 mg/kg body weight; Ceva, Brussels, Belgium) and transcardially perfused with Ringer solution, followed by 4% PFA in 0,1 M PBS (pH = 7,4). Spinal cords were isolated and stored in 4% PFA in 0,1 M PBS with 5% sucrose at 4°C. After 24h, the spinal cords were transferred to 30% sucrose in 0,1 M PBS for 48h. The samples were embedded in Tissue-Tek (Sakura, Zoeterwoude, the Netherlands), slowly frozen down using liquid nitrogen-cooled isopentane (-50°C) and stored at -80°C until further processing. Sagittal serial sections of 16 µm were made by using a Cryocut 1800 cryostat (Reichtert-Jung, New York, USA). Following blocking with 10% goat or monkey serum (Millipore) and 0,5% Triton in 0,1 M PBS, spinal cord sections were incubated with antibodies against GFAP (mouse monoclonal; 1/500; Sigma), MBP (rabbit polyclonal; 1/500; Millipore), neurofilament (mouse monoclonal; 1/500; Dako) and IBA-1 (rabbit polyclonal; 1/250; Wako) for 2h at room temperature. After incubation with the primary antibody, immunodetection was performed by using the appropriate Alexa 555 or 568-conjugated secondary antibody (1/250; Invitrogen) for 1h at room temperature (Table 2). Counterstaining was performed with DAPI (Invitrogen) and between incubation steps sections were washed with 0,1 M PBS. The sections were mounted with Shandon Immu-Mount (Thermo scientific) and again an Eclipse 80i microscope (Nikon) equipped with Nis-Elements Basic Research version 2.3 microscopy software was used for microscopical analysis.

For measurement of lesion size and demyelinated area, 5-8 sections per animal (80µm apart) containing the lesion area were analyzed. Lesion size was evaluated by GFAP expression, with the lesion area defined as the region showing no GFAP staining, whereas the demyelinated area was defined as the area showing a significant loss of MBP expression. Both evaluations were performed by using image analysis software (Image J, NIH).

Table 2: Primary and secondary antibodies used for immunohistochemical analysis of the spinal cord sections.

	Primary antibody		Secondary antibody	
GFAP	mouse anti-GFAP	1/500, Sigma	goat anti-mouse Alexa 568	1/250, Invitrogen
MBP	rabbit anti-MBP	1/500, Millipore	donkey anti-rabbit Alexa 555	1/250, Invitrogen
Neurofilament	mouse anti-neurofilament	1/500, Dako	goat anti-mouse Alexa 568	1/250, Invitrogen
IBA-1	rabbit anti-IBA-1	1/250, Wako	donkey anti-rabbit Alexa 555	1/250, Invitrogen

GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; IBA-1, ionized calcium binding adaptor molecule 1.

2.8 Statistical analysis

Statistical analysis was performed with SPSS Statistics 17.0. Locomotion tests were analyzed using repeated measures ANOVA, whereas the lesion size and demyelinated area were analyzed by means of a Mann-Whitney U test. Data represent mean values ± standard error of the mean (SEM) and differences were considered to be significant when $P < 0,05$.

3. Results

In this study we wanted to determine whether NSCs and IL-13 improve clinical and histological outcome after SCI by modulating the inflammatory response. Firstly, the NSCs were characterized *in vitro* by means of immunohistochemistry. Secondly, NSCs were transplanted in the lesion after SCI, in order to investigate whether the transplanted NSCs survived, as well as measuring the clinical and histological outcome. Finally, NSCs were injected in the spinal cord lesion, in combination with an IL-13 containing gelfoam. In these mice clinical outcome and survival of the NSCs was again determined.

3.1 *In vitro* characterization of NSCs

In order to visualize the NSCs after transplantation in the spinal cord lesion, they were isolated from E14 C57BL/6 eGFP-positive transgenic mice. The eGFP-positive NSCs were cultured in flasks pre-coated with fibronectin, which resulted in an adherent cell culture (Figure 4).

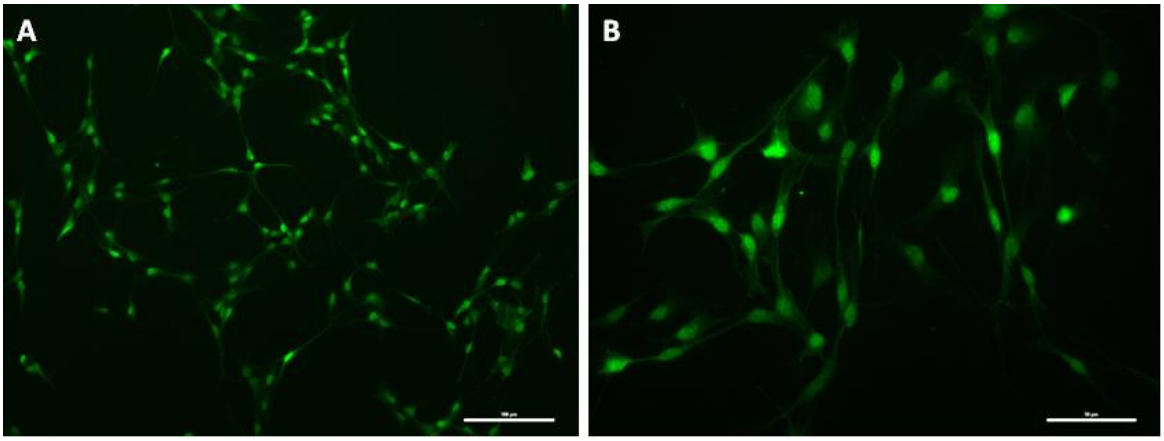


Figure 4: eGFP-positive NSCs in culture. eGFP-positive NSCs (green) were cultured in flasks pre-coated with fibronectin, which caused the cells to attach to the flasks. Scale bars represent 100 µm (A) and 50 µm (B).

Before the NSCs were transplanted in the spinal cord of mice after trauma, they were first characterized *in vitro* by immunohistochemistry. We investigated whether these cells expressed a number of glial and neural markers, as well as whether there were macrophages/microglia present in the culture.

3.1.1 NSCs expressed GFAP and MBP, whereas they were negative for neurofilament

To elucidate whether these NSCs expressed a number of glial and neural markers, immunoreactivity against GFAP, MBP and neurofilament was determined. GFAP is the principal intermediate filament of mature astrocytes and is thought to be important in astrocyte motility and shape by providing structural stability to astrocytic processes. MBP is one of the major proteins of the myelin sheath surrounding the axons in the CNS, and is a marker for mature oligodendrocytes, as they produce this

protein. Neurofilaments can be defined as intermediate or 10 nm filaments found specifically in neural cells. They provide structural support for neurons and support the large axon diameters required for rapid conduction of impulses. Immunohistochemical analysis of these NSCs revealed that after approximately 30 days in culture all cells were positive for GFAP and MBP, whereas they did not express neurofilament (Figure 5). Negative control sections were only incubated with the secondary antibodies and no nonspecific binding of these antibodies was observed (Figure 5).

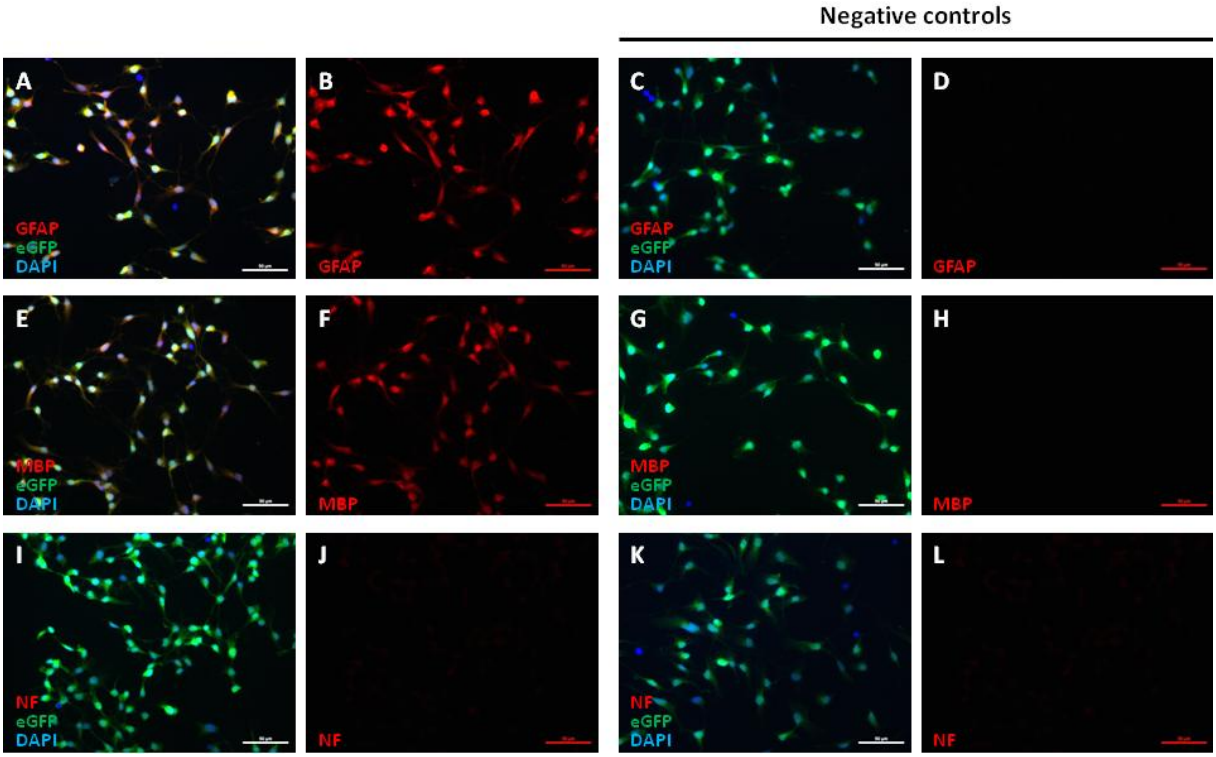


Figure 5: NSCs were positive for GFAP and MBP, however, they did not express neurofilament. Immunoreactivity of the NSCs (green) against GFAP (red; A-B), MBP (red; E-F) and neurofilament (red; I-J) was determined and counterstaining was performed with DAPI (blue). No nonspecific binding of the secondary antibodies for GFAP (C-D), MBP (G-H) and neurofilament (K-L) was observed in the negative controls. Scale bars represent 50 µm. GFAP, glial fibrillary protein; MBP, myelin basic protein; NF, neurofilament; eGFP, enhanced green fluorescent protein.

3.1.2 NSCs were positive for IBA-1, however, they did not express MAC-3 and F4/80

To investigate the purity of the NSC cultures, we determined whether there were IBA-1 positive cells in this culture by immunohistochemistry. IBA-1 is a calcium binding protein of which the expression is restricted to macrophages/microglia and which is upregulated during activation of these cells. All NSCs were positive for IBA-1, however, the pattern of expression is not in line with immunohistochemical analysis of IBA-1 expression in cell cultures found in the literature (Figure 6A-D). Therefore, additional stainings of the NSC cultures with other macrophage/microglia markers were performed, including MAC-3 and F4/80. MAC-3 is a membrane glycoprotein, also known as CD107b and LAMP-2, which is expressed on lysosomal membranes and the plasma membrane of macrophages and some myeloid cell lines. F4/80 is also a cell surface glycoprotein expressed on

macrophages. In contrast to MAC-3, expression of F4/80 is heterogeneous and is reported to vary during macrophage maturation and activation. Analysis revealed that there were no MAC-3 or F4/80 positive cells present in the NSC cultures (Figure 6E-L). Since the NSC cultures were negative for these 2 macrophage/microglia markers, we can conclude that there were no macrophages/microglia in this culture. Again, no nonspecific binding of the secondary antibodies was observed in the negative controls (Figure 5).

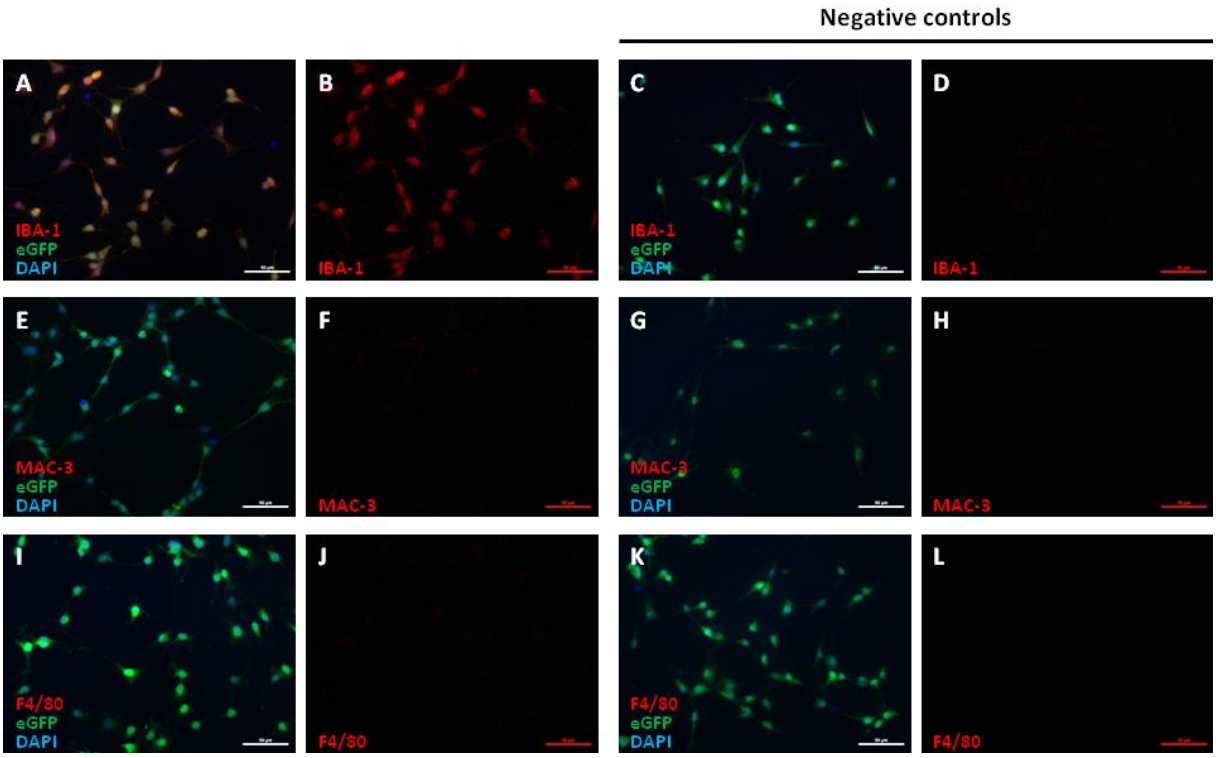


Figure 6: NSCs were positive for IBA-1, while they did not express MAC-3 and F4/80. NSC cultures were stained for IBA-1 (red; A-B), MAC-3 (red; E-F) and F4/80 (red; I-J) and counterstaining was performed with DAPI (blue). Negative controls showed no nonspecific binding of the secondary antibodies for IBA-1 (C-D), MAC-3 (G-H) and F4/80 (K-L). Scale bars represent 50 µm. IBA-1, ionized calcium binding adaptor molecule 1; eGFP, enhanced green fluorescent protein (green).

3.2 Transplantation of NSCs in the lesion after SCI

eGFP-positive NSCs were injected in the lesion after spinal cord hemisection in BALB/C mice, to investigate whether the mice and transplanted NSCs survived, as well as to determine the clinical and histological outcome. Clinical outcome was measured by performing locomotion tests, whereas survival and differentiation of the NSCs was investigated by means of immunohistochemistry. Moreover, lesion size and demyelinated area were evaluated, to determine whether NSC transplantation reduced the inflammatory response. Control mice received an injection of an equal volume of PBS in the spinal cord lesion. All mice, control and NSC transplanted, survived the surgery.

3.2.1 Transplantation of NSCs improved functional recovery after SCI

In order to determine the effect of NSC transplantation on the functional recovery of mice after SCI locomotion tests were performed, including the BMS and Rotarod performance. The BMS is a 10-point scale in which hind limb movements are scored in an open field. This test demonstrated that the locomotor function of the NSC injected mice was significantly improved compared to the control mice during the 3-week observation period (Figure 7A). A major hallmark of significant functional recovery scored in the BMS test is the development of plantar stepping. In the control group, none of the mice showed plantar stepping before day 21, and only 50% of the mice eventually did. In the NSC transplanted mice however, this occurred in some mice already early in the observation period (day 7) with eventually plantar stepping in all mice 21 days after transplantation (Figure 7B). One mouse from the NSC transplanted group was excluded from the experiment as it never reached a BMS score higher than 2.

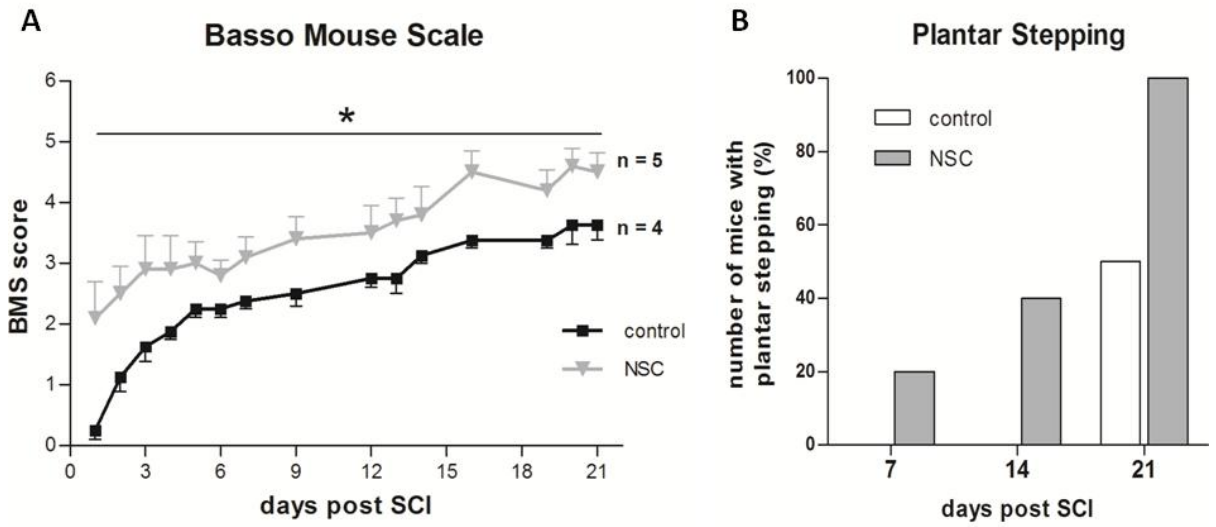


Figure 7: Transplantation of NSCs improved clinical outcome after SCI in the BMS test. (A) Mice grafted with NSCs (n = 5) after spinal cord hemisection had a significant improvement in their BMS score during the 3-week observation period compared to PBS injected control mice (n = 4) (*P* = 0,02). (B) Development of plantar stepping occurred earlier in the NSC injected mice and after 21 days all mice developed this, whereas only half of the control group developed plantar stepping by day 21. (A) Data represent mean values ± SEM, (B) data represent percentage of the mice with plantar stepping; **P* < 0,05. NSC, neural stem cell; SCI, spinal cord injury.

Furthermore, NSC transplantation increased the Rotarod performance compared to PBS injected control mice (Figure 8A). In this test, the time that the mice can run on an accelerating rolling rod is recorded. A representative picture indicates that the NSC transplanted mice had more efficient climbing behavior on the rolling rod compared to the control mice. The control mice lost grip already at early time points, when the rotation speed of the wheel was still low (Figure 8B).

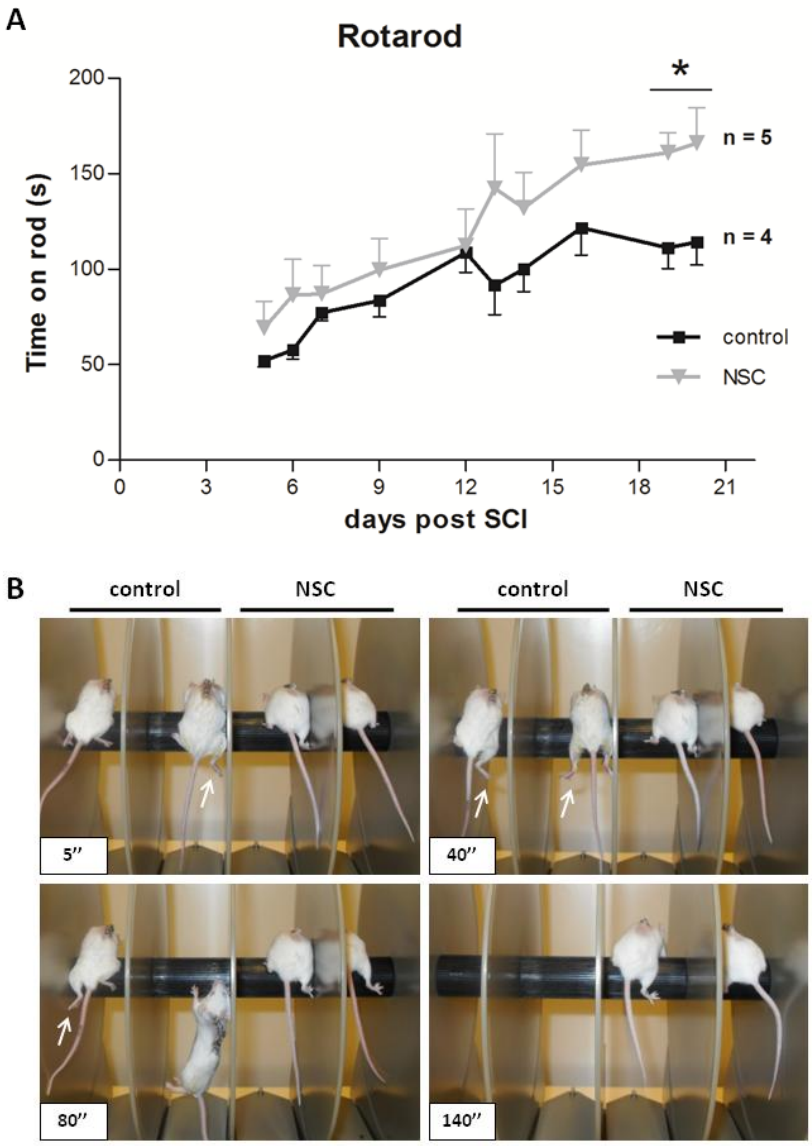


Figure 8: Improvement of locomotor performance on the Rotarod after transplantation of NSCs in the spinal cord lesion. (A) Transplantation of NSCs in the lesion after SCI increased the time that the mice (n = 5) can climb on an accelerating rolling rod compared to PBS injected control mice (n = 4) (*P* = 0,036). (B) Representative picture of improved locomotor function; the arrows indicate that the control mice were losing grip at early time points, whereas the NSC transplanted mice climbed on the wheel efficiently. After 140 seconds, the control mice had jumped of the wheel, while the NSC transplanted mice continued to perform well. Data represent mean values ± SEM; **P* < 0,05. NSC, neural stem cell; SCI, spinal cord injury.

3.2.2 NSCs were still present in the lesion area 3 weeks after transplantation, however, a number of cells were phagocytosed

Three weeks after surgery, the control and NSC transplanted mice were perfused and saggital sections of the spinal cords were made. In the grafted mice, eGFP-positive NSCs could still be identified in the lesion area at that time point (Figure 9).

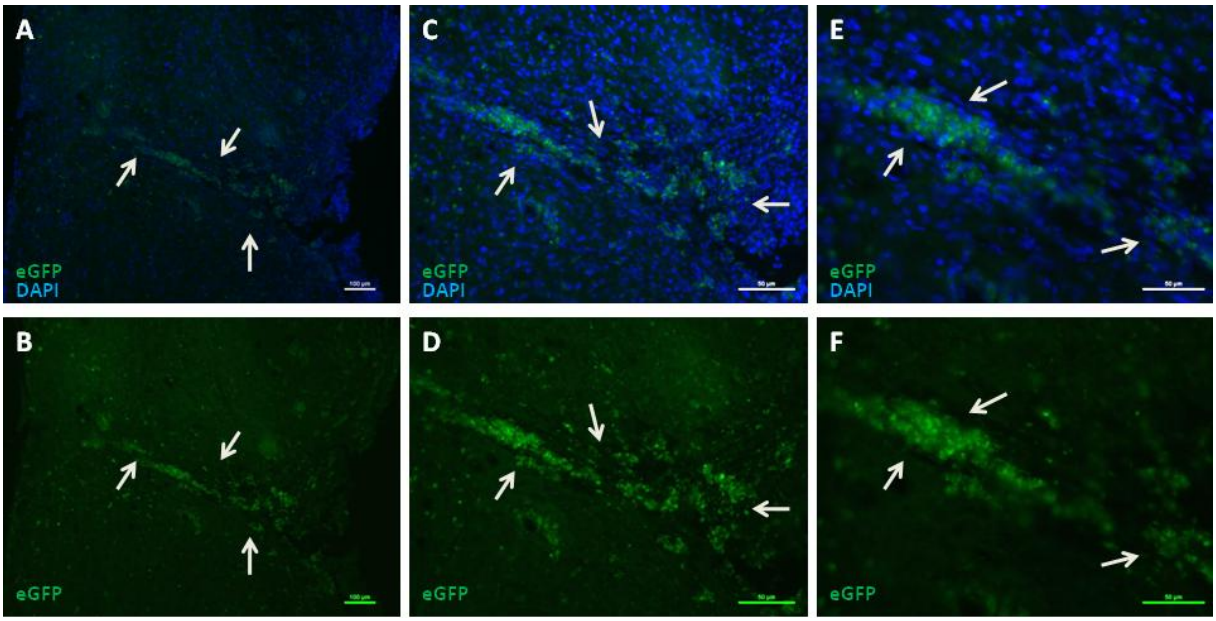


Figure 9: eGFP-positive NSCs were present in the lesion area 3 weeks after SCI and transplantation. Saggital sections of the spinal cords of the NSC transplanted group were made 21 days after NSC injection in the spinal cord lesion and DAPI staining (blue) was performed. Arrows indicate the eGFP-positive NSCs (green) that were present in the lesion area. Scale bars represent 100 µm (A-D) and 50 µm (E-F). eGFP, enhanced green fluorescent protein.

In order to check whether the NSCs survived or were phagocytosed by resident microglial cells and infiltrating macrophages during the 21 days after transplantation, saggital sections of the spinal cords of the NSC transplanted mice were stained for expression of IBA-1. There was a clear double-positive staining of some eGFP-positive NSCs and IBA-1, which most likely indicates that a number of NSCs were phagocytosed by macrophages/microglia (Figure 10).

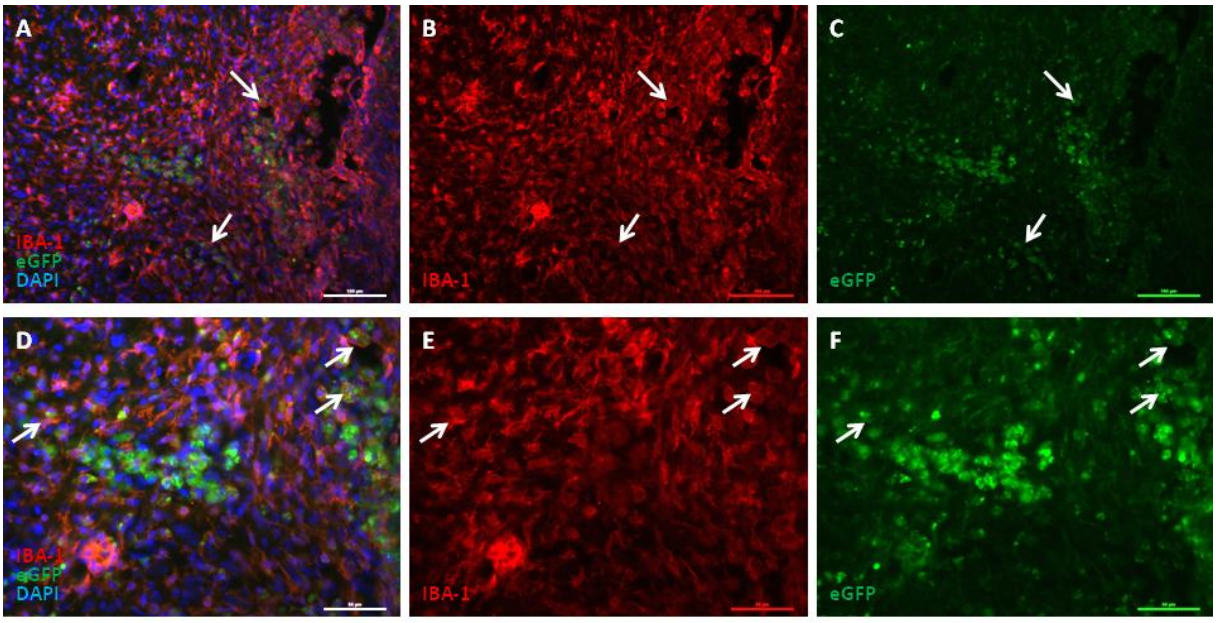


Figure 10: A number of NSCs were phagocytosed by microglial cells and/or infiltrating macrophages 3 weeks after grafting. Saggital spinal cord sections of NSC transplanted mice were stained for IBA-1 (red) and counterstained with DAPI (blue). Arrows indicate the cells that were double-positive for eGFP (green) and IBA-1, suggesting that these NSCs were

phagocytosed by macrophages/microglia. Scale bars represent 100 µm (A-C) and 50 µm (D-F). IBA-1, ionized calcium binding adaptor molecule 1; eGFP, enhanced green fluorescent protein.

3.2.3 NSCs did not differentiate into neurons after transplantation in the spinal cord lesion

After demonstrating that the NSCs were present in the lesion following transplantation, we wanted to determine whether they differentiated into neuronal or glial cells. To evaluate whether the NSCs differentiated into neurons, saggital spinal cord sections were stained for neurofilament 3 weeks after injection of the NSCs in the spinal cord lesion. In the spinal cord sections of the NSC transplanted mice, no double-positive staining of eGFP and neurofilament was detected, suggesting that the NSCs did not differentiate into neurons (Figure 11).

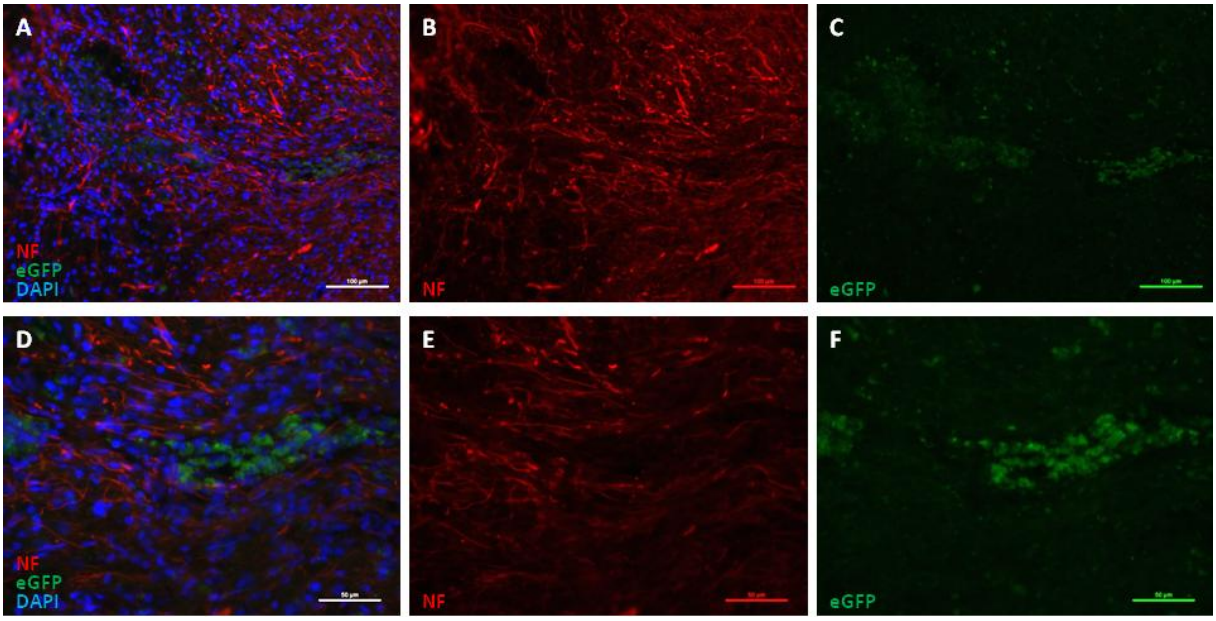


Figure 11: No differentiation of the NSCs into neurons 3 weeks after transplantation in the spinal cord lesion. Saggital spinal cord sections of the NSC transplanted mice were stained for neurofilament expression (red) and counterstaining was performed with DAPI (blue). Scale bars represent 100 µm (A-C) and 50 µm (D-F). NF, neurofilament; eGFP, enhanced green fluorescent protein (green).

Since all cells in the NSC cultures were positive for both GFAP and MBP, we could not evaluate whether these cells differentiated into respectively astrocytes or oligodendrocytes. However, immunohistological analysis of the GFAP expression in saggital spinal cord sections of NSC transplanted mice revealed that not all eGFP-positive cells expressed GFAP (Figure 12A-C). This was similar for MBP, as not all NSCs present in the lesion area of the grafted mice were positive for eGFP and MBP (Figure 12D-F).

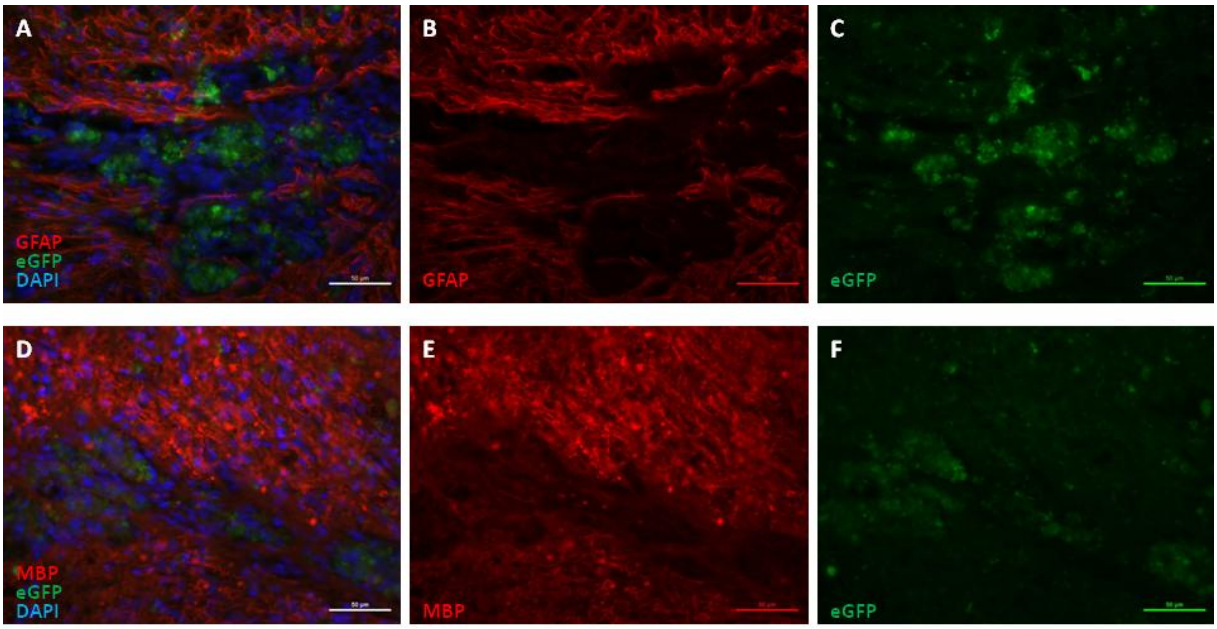


Figure 12: Three weeks after transplantation, not all NSCs present in the spinal cord lesion expressed GFAP and MBP. Saggital spinal cord sections of the NSC transplanted mice were stained for GFAP (red; A-C) and MBP (red; D-F) and counterstaining was performed with DAPI (blue). Scale bars represent 50 µm. GFAP, glial fibrillary protein; MBP, myelin basic protein; eGFP, enhanced green fluorescent protein (green).

3.2.4 NSC transplantation after SCI did not significantly reduce the lesion size and demyelinated area

To determine whether the improved functional recovery of the NSC transplanted mice after SCI resulted from a reduced inflammatory response, the lesion size and demyelinated area were evaluated. As GFAP-positive astrocytes delineate the lesion area after injury in the CNS, this was used to determine the lesion size, whereas the demyelinated area was evaluated by immunohistochemical analysis of MBP, as this is one of the major components of the myelin sheath.

No significant reduction was found in lesion size and demyelinated area in the NSC transplanted group compared to the control group (Figure 13). However, in both graphs there is a trend, that suggests that there was a reduction in demyelinated area and especially in lesion size after transplantation of NSCs in the spinal cord lesion. Probably, significance was not reached due to the low number of mice (n = 4 or 5) used in this experiment.

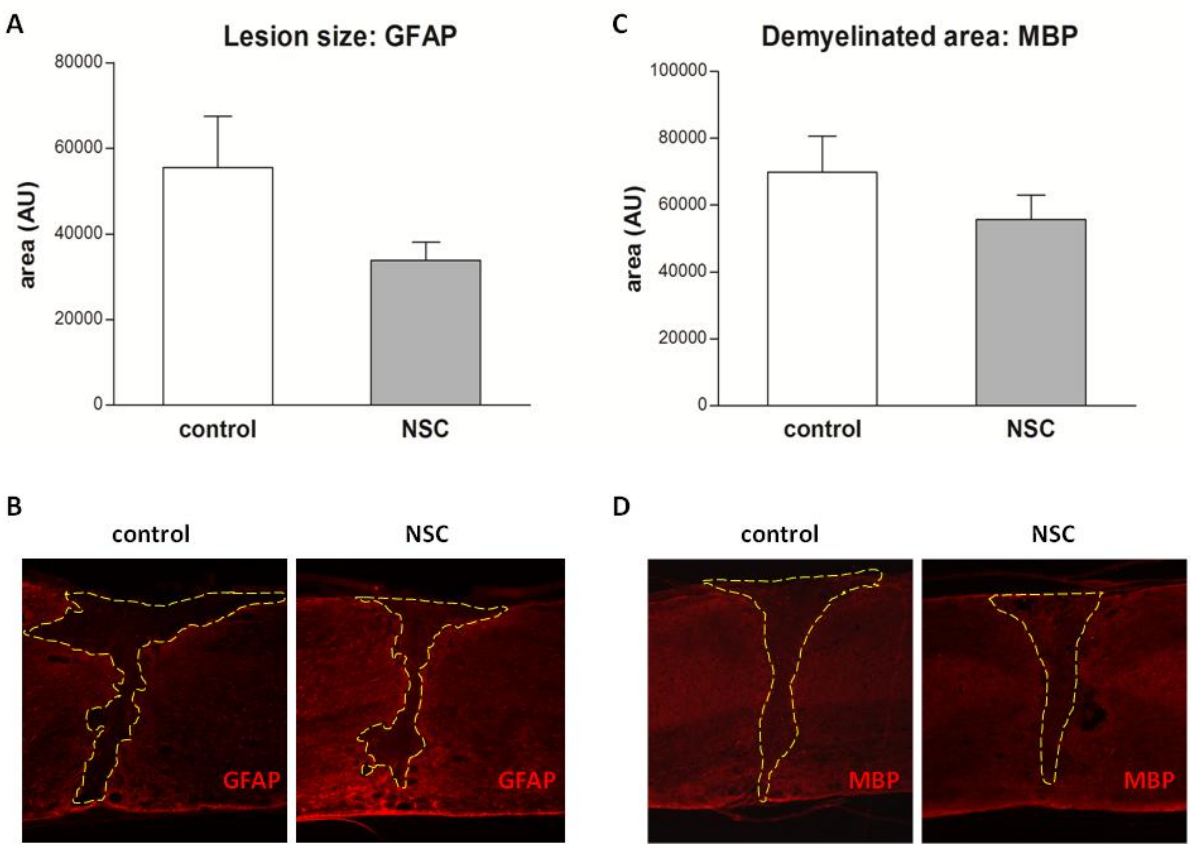


Figure 13: No significant reduction in lesion size and demyelinated area was observed between the control and NSC transplanted group. Three weeks after surgery, saggital spinal cord sections were stained for GFAP and MBP expression to evaluate respectively the lesion size and demyelinated area. (A, C) Quantification of the lesion size (A) and demyelinated area (C) demonstrated that there was no significant difference between the control (n = 4) and NSC transplanted mice (n = 5). (B, D) Representative pictures of saggital spinal cord sections of a control and NSC grafted mouse stained for GFAP (B) and MBP (D). The dashed lines delineate the lesion size (B) and demyelinated area (D). Data represent mean values ± SEM. NSC, neural stem cell; GFAP, glial fibrillary protein; MBP, myelin basic protein.

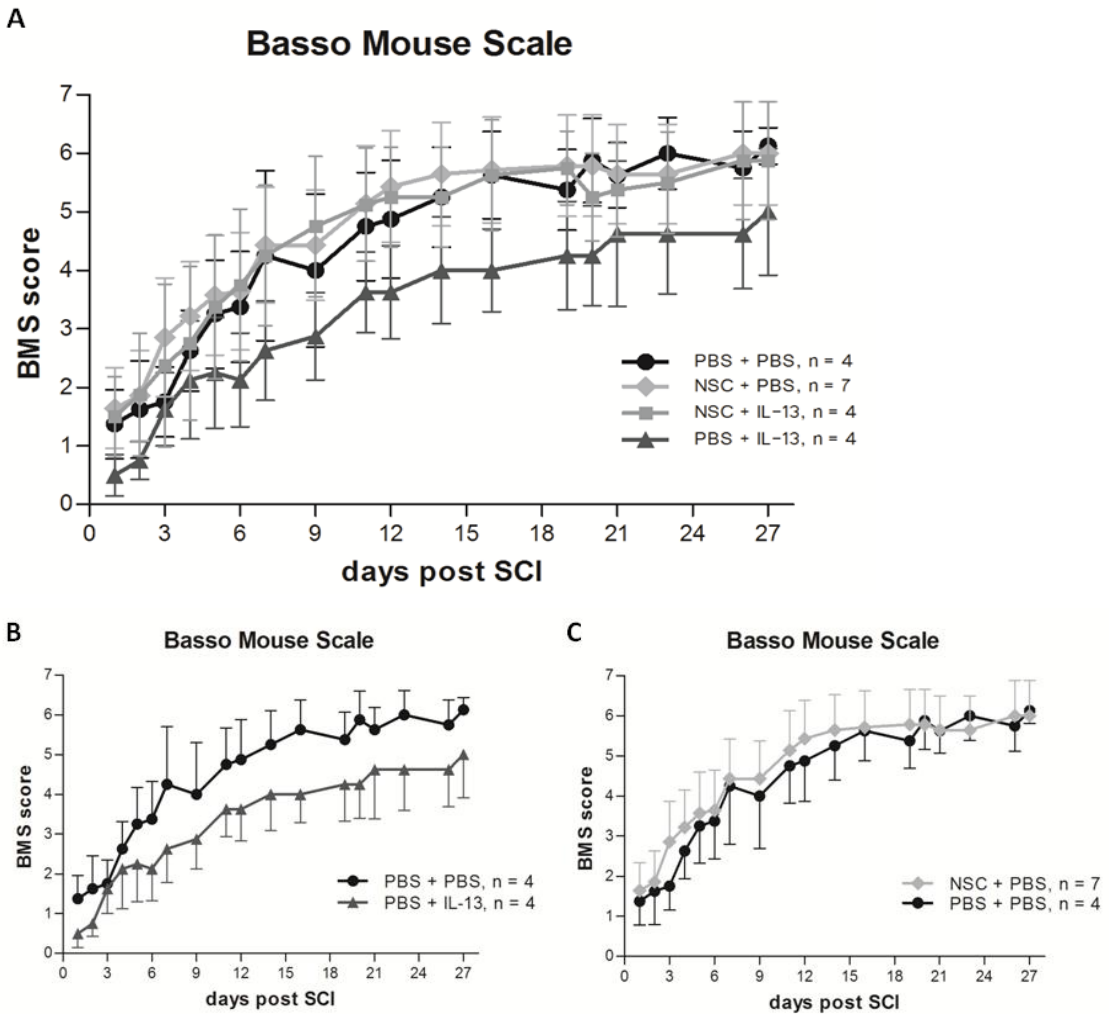
3.3 Transplantation of NSCs followed by addition of IL-13 after SCI

In order to investigate the combined immunomodulatory and regenerative properties of NSCs and IL-13, eGFP-positive NSCs were injected in the spinal cord lesion of C57BL/6 mice, followed by the addition of an IL-13 or PBS containing gelfoam. Functional recovery was again measured by BMS and Rotarod performance, additionally to the morphological characteristics of survival which were evaluated using immunohistochemistry. Four groups were included in this experiment, a control group that received an injection with PBS in the spinal cord lesion and a PBS containing gelfoam (PBS + PBS, n = 4). The second group was also injected with PBS, however, an IL-13 containing gelfoam was added, in order to determine the effect of IL-13 after SCI (PBS + IL-13, n = 4). The third group was injected with NSCs and received a PBS containing gelfoam, to investigate the effect of NSC transplantation following spinal cord hemisection (NSC + PBS, n = 7). Finally, to determine the combined effect of NSCs and IL-13 after SCI, the fourth group was injected with NSCs and an IL-13 containing gelfoam was added (NSC + IL-13, n = 4).

3.3.1 IL-13, NSCs, or NSCs combined with IL-13 had no significant effect on the functional recovery after SCI

BMS and Rotarod performance were again evaluated to determine the functional recovery of the mice after spinal cord hemisection. No significant effect was observed of IL-13, NSCs, or NSCs combined with IL-13 on the clinical outcome of the mice after SCI (Figure 14 & Figure 15). This was probably due to the low number of mice (n = 4 or 7 per group) that were used; if this experiment is again repeated significance will most likely be reached between several groups. In order to make the graphs more comprehensible, separate graphs were made for comparing selected groups (Figure 14B-E & Figure 15B-E).

In the BMS test there is a clear trend indicating that IL-13 impaired clinical recovery of mice after SCI compared to the control group (Figure 14B). In contrast, no effect was seen of NSC transplantation (Figure 14C), as well as NSC transplantation combined with IL-13 treatment following SCI compared to the control group (Figure 14D). Furthermore, IL-13 had no effect on the outcome after NSC grafting in the spinal cord lesion (Figure 14E).



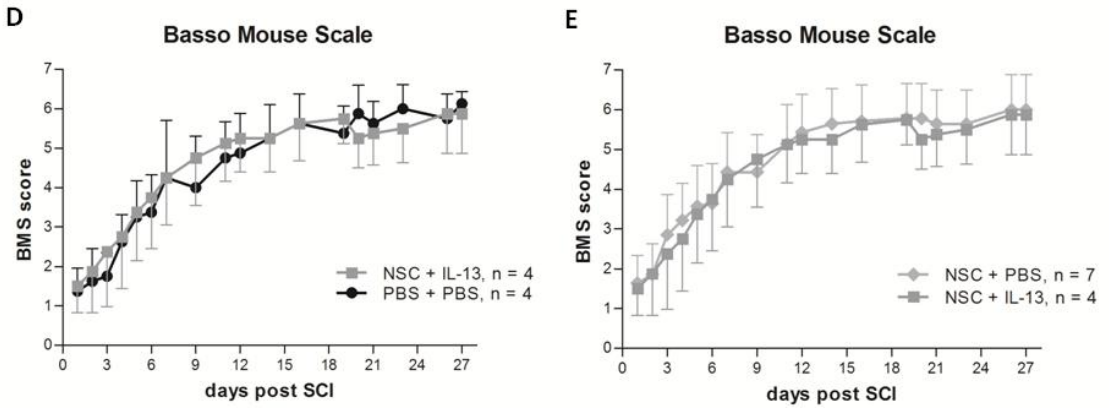
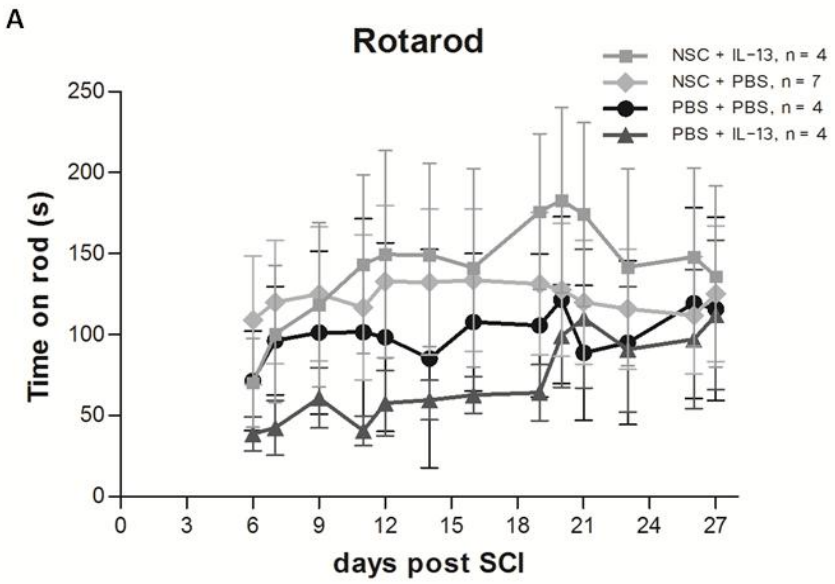


Figure 14: Treatment with IL-13, NSCs, or NSCs combined with IL-13 had no significant effect on clinical outcome as measured by the BMS test. In this experiment the BMS score was evaluated for 27 days and 4 groups were included, the control group received a PBS injection and PBS containing gelfoam (PBS + PBS, n = 4), the second group was injected with PBS and an IL-13 containing gelfoam was added (PBS + IL-13, n = 4), the third group received NSC transplantation and a PBS containing gelfoam (NSC + PBS, n = 7) and the fourth group was injected with NSCs and an IL-13 containing gelfoam was added (NSC + IL-13, n = 4). Separate graphs were made for comparing selected groups (B-E), to make the data more comprehensible. A trend suggests that IL-13 alone reduced functional recovery of the mice after SCI (B). No effect was observed in the NSC transplanted group (B), and the NSC transplanted group that received an IL-13 containing gelfoam (D) after SCI as compared to the control mice. In addition, IL-13 had no effect on the outcome after NSC transplantation in the spinal cord lesion (E). Data represent mean values ± SEM. NSC, neural stem cell; IL-13, interleukin-13; SCI, spinal cord injury.

Rotarod performance indicated a negative trend of IL-13 treatment compared to the control group, which lasted until day 21 (Figure 15B). In contrast, NSC transplantation in the spinal cord lesion had no effect on clinical outcome (Figure 15C). Grafting of NSCs combined with IL-13 treatment resulted in a positive trend on the Rotarod performance as compared to the control mice (Figure 15D). Finally, IL-13 had no effect on the functional recovery after NSC transplantation in the spinal cord lesion (Figure 15E).



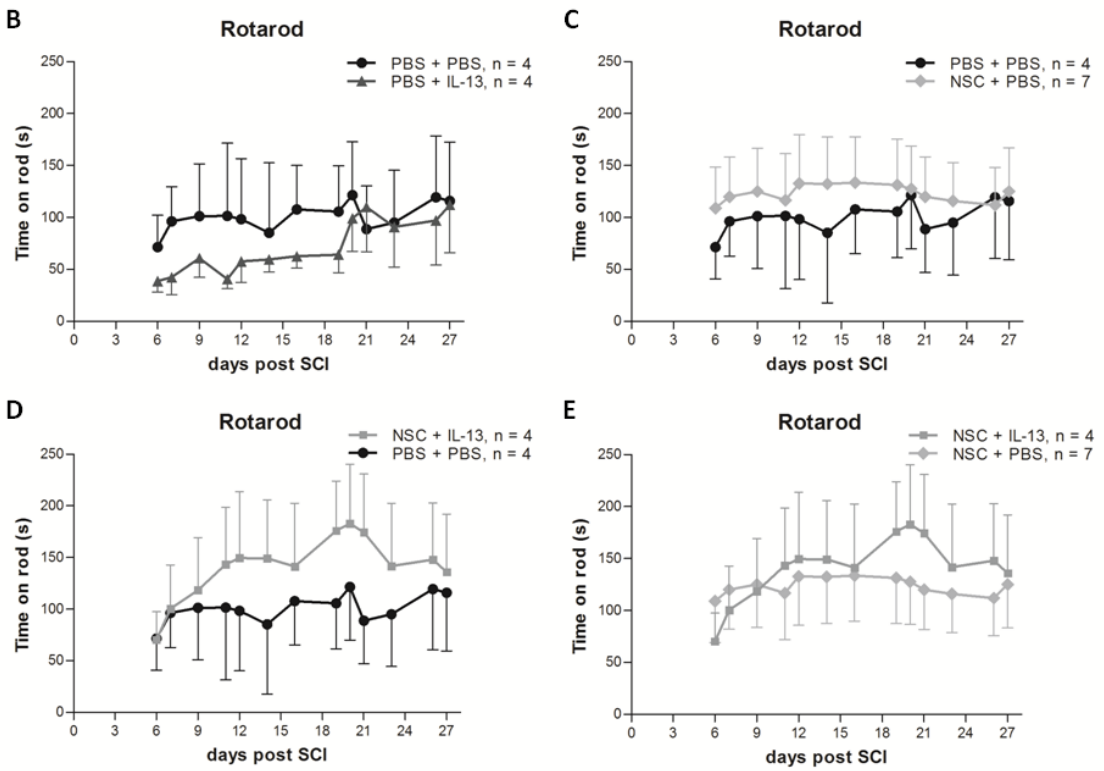


Figure 15: No significant effect of IL-13, NSCs, or NSCs combined with IL-13 on the functional recovery is demonstrated by the Rotarod performance. Rotarod performance was measured for 27 days in 4 groups, the control group was injected with PBS and received a PBS containing gelfoam (PBS + PBS, n = 4), the second group was injected with PBS and IL-13 containing gelfoam was added (PBS + IL-13, n = 4), the third group received an injection with NSCs and a PBS containing gelfoam (NSC + PBS, n = 7) and the fourth group was injected with NSCs and an IL-13 containing gelfoam was added (NSC + IL-13, n = 4). To make the graph more comprehensible (A), separate graphs were made for comparing selected groups (B-E). There is a trend which suggests a reduced functional recovery after IL-13 treatment (B), whereas following NSC transplantation combined with IL-13 treatment an improvement was observed (D) as compared to the control mice. There is no effect of NSC transplantation on Rotarod performance compared to the control group (C) and IL-13 has no effect on the outcome of NSC grafting (E). Data represent mean values ± SEM. NSC, neural stem cell; IL-13, interleukin-13; SCI, spinal cord injury.

3.3.2 NSCs probably survived for 3 and 7 days after transplantation, however, after 28 days their morphology suggests that they were phagocytosed

In the previous experiment, the NSCs did not all survive three weeks after transplantation in the injured spinal cord, therefore, at least one mouse was perfused on day 3, 7 and at the end of the observation period (day 28) in this experiment. Saggital spinal cord sections were prepared and a DAPI staining was performed.

The morphology of the NSCs indicates that these cells probably survived after 3 and 7 days in the spinal cord lesion (Figure 16A-F), whereas 28 days post SCI, the morphology of the stem cells suggests that they were phagocytosed (Figure 16G-I). This is concluded as the cells have a more round morphology, additionally to the fact that the eGFP expression is not homogenous throughout the cells and has a reduced intensity compared to the eGFP-positive NSCs 3 and 7 days post transplantation. Furthermore, the same morphology was seen in the previous experiment, when the mice were perfused 3 weeks after transplantation in the damaged spinal cord. In this experiment

immunoreactivity against IBA-1 was determined and revealed that a number of NSCs were phagocytosed by resident microglial cells and/or infiltrating macrophages.

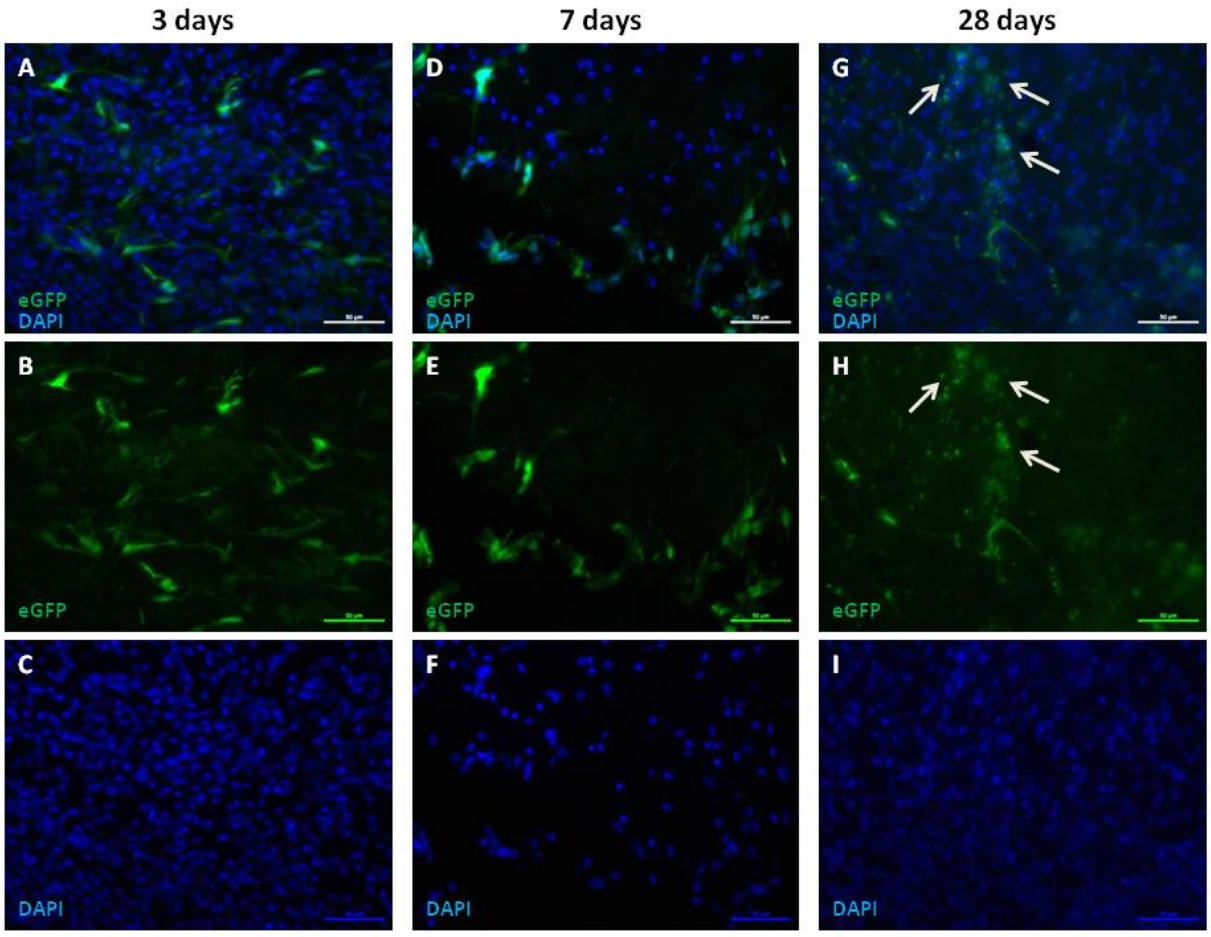


Figure 16: eGFP-positive NSCs probably survived 3 and 7 days after grafting in the injured spinal cord, however, after 28 days their morphology suggests that they were phagocytosed. To evaluate whether the NSCs survived 3, 7 and 28 days after grafting in the spinal cord lesion, at least one mouse was perfused at each time point, saggital spinal cord sections were prepared and a DAPI staining (blue) was performed. After 3 (A-C) and 7 (D-F) days, the morphology of the NSCs (green) indicates that they probably survived in the spinal cord lesion. While, 28 days post transplantation their morphology suggests that they were phagocytosed, as the cells appear more round, and the eGFP expression is not homogenous throughout the cell and has a lower intensity (G-I). Arrows indicate the fragmented morphology of the eGFP-positive NSCs, suggesting that they were phagocytosed (G-H). Scale bars represent 50 µm. eGFP, enhanced green fluorescent protein.

4. Discussion

A promising therapy for SCI could be the transplantation of NSCs into the lesion, as these cells have the ability to replace damaged neural tissue, enhance endogenous regeneration and modulate the inflammatory response after trauma. However, cell transplantation will probably not be fully successful without the application of additional factors, which modulate the tissue environment to make it more supportive and permissive for endogenous neural regeneration. Therefore, we have combined the immunomodulatory and regenerative properties of NSCs and IL-13 for the treatment of SCI.

4.1 *In vitro* characterization of NSCs

In this study, mouse NSCs were first characterized *in vitro* by immunohistochemistry, in order to evaluate whether they expressed a number of glial and neural markers. This revealed that all NSCs were positive for both GFAP and MBP, while they did not express neurofilament. GFAP is an astrocytic marker, while MBP is specific for oligodendrocytes and neurofilament for neurons. Probably there was nonspecific binding of the primary antibody to MBP, as the NSCs cannot be positive for both GFAP and MBP, and MBP is specific for mature oligodendrocytes [46]. In addition, preliminary results of the laboratory of experimental hematology of the university of Antwerp, that kindly provided us with these cells, indicated that the NSCs express GFAP, but not MBP (personal communication with dr. Peter Ponsaerts). Nonspecific binding of the secondary antibody for MBP can be excluded, as there was no staining in the negative control. To investigate whether the primary anti-MBP antibody binds nonspecifically, a control with cells that certainly do not express MBP, such as microglia or fibroblast, could be performed.

Furthermore, the purity of the NSC culture was analyzed by determining whether there were IBA-1 positive macrophages/microglia present. Analysis demonstrated that all NSCs were positive for IBA-1. However, although the secondary antibody showed no nonspecific binding, the expression pattern is not in line with immunohistological analysis of IBA-1 in cell cultures found in the literature. Therefore, we investigated the immunoreactivity of the NSCs against two other macrophage/microglia markers, MAC-3 and F4/80. The cells did not express MAC-3 and F4/80, indicating that there were most likely no macrophages/microglia present in the NSC culture.

4.2 Transplantation of NSCs in the lesion after SCI

Secondly, the eGFP-positive NSCs were transplanted in the spinal cord lesion to investigate whether the mice and transplanted NSCs survived, in addition to measure the clinical and histological outcome. In this experiment we performed an allogeneic transplantation, with NSCs derived from C57BL/6 mice being injected in the spinal cord lesion of BALB/C mice. This is of major importance as

it is likely that future cell therapy for CNS injury will be performed using allogeneic stem cell populations.

All mice survived the surgery. Moreover, transplantation of NSCs after spinal cord hemisection improved functional recovery of the transplanted mice compared to the control mice. This was demonstrated by two locomotion tests, BMS and Rotarod performance. Several other studies confirm these results, for example, transplantation of human NSCs after SCI in adult mice and rats resulted in a significant improvement of functional recovery, as measured by the Basso Beattie Bresnahan (BBB) locomotor rating scale [47, 48]. Furthermore, transplantation of stem cells derived from the rat embryonic spinal cord, also resulted in an improved motor function in adult rats after contusion injury [18]. In contrast to our study, these stem cells were transplanted 9 days after contusion injury. Transplantation of human NSCs 9 days after cervical contusion injury was also performed in nonhuman primates whereby motor function, measured by bar grip power and spontaneous motor activity, was found to be significantly higher in the NSC transplanted group as compared to the control group [49].

At 21 days post SCI, eGFP-positive NSCs could be identified in the spinal cord sections of the transplanted mice. However, not all grafted cells survived, since a number of NSCs appeared to be phagocytosed by resident microglial cells or infiltrating macrophages. As an allogenic transplantation was performed, it cannot be excluded that this is the result of a rejection of the transplant. However, the laboratory of experimental hematology of the university of Antwerp injected these NSCs derived from C57BL/6 mice intracranially in C57BL/6 mice and observed that these cells could survive for 2 weeks. After these 2 weeks the cells became gradually phagocytosed by macrophages/microglia (personal communication with dr. Peter Ponsaerts).

Differentiation of NSCs into glial and neuronal cells following transplantation in the injured spinal cord is still controversial [18, 50-52]. Evidence that NSCs can give rise to large numbers of properly functioning cells that integrate into the CNS network is still very limited. We have shown that the transplanted NSCs did not differentiate into neurons 3 weeks after transplantation in the spinal cord lesion. This is confirmed by several other studies; for instance, 2 months post engraftment of NSCs into the contused adult rat spinal cord, the majority of cells differentiated into astrocytes while no differentiation into neurons or oligodendrocytes was observed [50]. In contrast to these and our results, it has been shown that transplanted fetus-derived neurosphere cells were able to generate neurons after spinal cord contusion injury in adult rats. Five weeks after grafting, histological analysis revealed that these donor-derived neurons extended their processes into the host tissue and that the neurites formed synaptic structures [18]. In this study, the NSCs were transplanted 9 days after contusion injury. The authors claimed that this is the ideal time point for transplantation, as at this point there is less inflammation and thus a more supportive and permissive microenvironment for

survival and differentiation of the NSCs. Immediately following trauma to the CNS, the lesion area is probably not the ideal environment for differentiation into neurons, since there is a dramatic alteration in the expression of various neurotrophic factors [7, 53] and the levels of neurite growth-inhibitory molecules [5, 54]. For example, both CNTF [16, 17] and leukemia inhibitory factor (LIF) [55, 56] are markedly upregulated following trauma to the spinal cord. These factors prevent neural differentiation and induce differentiation along the astrocytic lineage. After SCI, these glial differentiation signals may dominate over the neural differentiation signals and thereby contribute to the inhibition of NSC differentiation into neurons.

Since all NSCs were *in vitro* positive for both GFAP and MBP, we could not demonstrate whether the transplanted NSCs differentiated into astrocytes or oligodendrocytes. In contrast to *in vitro,* in saggital spinal cord sections of the NSC transplanted mice 3 weeks after surgery, not all eGFP-positive cells expressed GFAP and MBP. This could be due to phagocytosis of the NSCs by microglia/macrophages, which has been demonstrated by IBA-1 expression 3 weeks after NSC grafting. Several other studies have shown that differentiation of NSCs in astrocytes and oligodendrocytes is possible, for example, Pallini et al has shown that after NSC transplantation in the injured spinal cord of mice, the cells remained undifferentiated or differentiated into the astrocytic phenotype [20]. As astrocytes are involved in the glial scar and produce several axon growth inhibitory molecules, they could prevent axon regeneration and thereby limit recovery. However, the authors of this paper stated that these NSC-derived astrocytes show features typical of the early phase after SCI when the glial scar is still permissive to regenerating axons and that these cells might support neurite outgrowth of the host axons [20]. Differentiation of NSCs into oligodendrocytes has also been demonstrated by some [52, 57], while other state that this inhibited in the injured spinal cord due to the unfavorable microenvironment [50].

These data indicate that differentiation of NSCs is largely dependent on the microenvironment and that the acute inflammatory response immediately following trauma to the CNS is probably not the ideal environment for differentiation.

To date, not much is known about the modulation of the inflammatory response by NSCs after injury to the spinal cord. However, in for example neurodegenerative diseases this is investigated more extensively. In EAE, transplantation of NSCs in the ventricles of mice or rats resulted in a reduced brain inflammation in the acute phase of the disease, attenuated demyelination and axonal injury, and reduction of the clinical severity of the disease [24, 25]. In this study, we investigated whether grafting of NSCs reduced the lesion size and demyelinated area following spinal cord hemisection. No significant reduction in lesion size as well as demyelinated area was detected 3 weeks after surgery in the NSC transplanted group compared to the control group. However, there is a trend which suggests that there was a decrease in demyelinated area and especially in lesion size after transplantation of

NSCs in the spinal cord lesion. Significance was not reached, probably due to the low number of mice, 4 or 5 per group, that was used in this experiment.

Together these data indicate that there was an improved functional recovery following transplantation of NSCs in the spinal cord lesion. This was probably not a result of replacement of damage tissue, as there was no differentiation of the NSCs found in the lesion area. Differentiation of the NSCs into oligodendrocytes and astrocytes cannot be excluded, since *in vitro* all NSCs were positive for respectively MBP and GFAP. Improvement of clinical outcome could result from the immunosuppressive effect of the NSCs, as a reduction, however not significant, was observed in lesion size and demyelinated area in the NSC transplanted group. The immunomodulatory properties of the NSCs could be further investigated by, for instance, quantification of the infiltrating T cells and macrophages.

In this experiment, it cannot be excluded that the improved functional recovery results from an intrinsic effect of the NSCs via for example the production of growth factors. Furthermore, given that in this experiment an allogeneic transplantation was performed, it can also not be excluded that the enhanced clinical outcome is the result of an immune response against the transplant. Further research has to focus on this potential graft rejection and the mechanisms that mediated this, as in the future stem cell therapy for SCI will be probably performed by using allogeneic transplantation. After trauma to the CNS it is important to immediately start the therapeutic intervention to reduce the secondary damage, therefore, in clinical applications allogeneic transplantation is most likely.

4.3 Transplantation of NSCs followed by addition of IL-13 after SCI

Finally, the combined immunomodulatory and regenerative properties of NSCs and IL-13 were investigated by transplantation of NSCs in the spinal cord lesion, followed by the addition of an IL-13 containing gelfoam. In this experiment NSCs derived from C57BL/6 mice were injected in the spinal cord lesion of C57BL/6 mice, as we could not exclude that the allogeneic transplantation in the previous experiment resulted in graft rejection. Functional recovery was again measured by locomotion tests and survival of the NSCs by means of immunohistochemistry.

BMS and Rotarod performance demonstrated no significant effect of IL-13, NSCs, or NSC combined with IL-13 on the functional recovery of mice after spinal cord hemisection. This is most likely due to the low numbers of mice, 4 or 7 per group, that were used to perform this experiment. Significance between some groups will probably be reached if this experiment is repeated. For both BMS and Rotarod performance, there is a trend that IL-13 treatment reduced functional recovery of the mice after SCI. This is in line with preliminary results from our own research group, in which spinal cord hemisection was performed followed by the addition of an IL-13 or PBS containing gelfoam in a large number of animals. The IL-13 treated group had a significant reduced clinical outcome compared to the PBS treated control group, as measured by the BMS test. Moreover, our data demonstrated that

the detrimental effect of IL-13 on the Rotarod performance disappeared after 21 days. This is probably due to the fact that IL-13 only has an effect in the acute phase after SCI, which was also shown by our research group. In this experiment, IL-13 was given 3 days after SCI and had no effect on the clinical outcome as compared to control mice. Until now, not much is known about the role of IL-13 in the CNS. Whereas a number of studies have reported beneficial effects of IL-13, such as a reduced duration, severity and incidence of the EAE disease course, harmful effects of IL-13 have also been documented. These are especially in the context of allergy, for instance, allergic airway inflammation is inhibited by treatment with an anti-IL-13 monoclonal antibody in a mouse asthma model [58, 59]. Moreover, a recent study has indicated that IL-13 induced microglial NADPH oxidase-derived oxidative stress, which leaded to the degeneration of hippocampal neurons *in vivo* [60]. In this study, thrombin was injected into the hippocampus leading to significant loss of hippocampal neurons. This neurotoxicity was accompanied by activation of microglial cells, as well as activation of NADPH oxidase, generation of reactive oxygen species and oxidative damage in the hippocampal area. In addition, intrahippocampal injection of thrombin was associated with increased levels of IL-13 and immunoreactivity against IL-13 was solely detected in activated microglial cells. IL-13-neutralizing antibodies rescued hippocampal neurons from thrombin toxicity, in addition to inhibiting NADPH oxidase activation, reactive oxygen production and oxidative damage. These data indicate that IL-13 plays a major role in hippocampal neurotoxicity [60]. In order to confirm these and our results, as well as to elucidate the involved mechanisms, further research has to focus on the possible detrimental effects of IL-13 in the CNS.

We have also revealed in this experiment that NSC transplantation had no effect on the functional recovery of the mice after SCI, as measured by both BMS and Rotarod performance. To confirm these results this experiment has to be repeated, as a low number of mice was used. These data further suggest that the improvement of functional recovery in the previous experiment was the result of rejection of the transplanted cells, as an allogeneic transplantation was performed.

Combining the immunomodulatory and regenerative properties of NSCs and IL-13 had no effect on the BMS score, however, a positive trend was seen in the Rotarod test. Again, this has to be repeated as only 4 mice per group were used. Finally, IL-13 had no effect on the outcome after NSC grafting in the spinal cord lesion.

Since the previous experiment and preliminary data of the laboratory of experimental hematology of the university of Antwerp indicated that the NSCs did not survive three weeks following transplantation in the CNS, a mouse was perfused on day 3, 7 and 28 post transplantation in the second *in vivo* experiment. The morphology of the NSCs suggested that they survived for 3 and 7 days in the spinal cord lesion. However, after 28 days, their appearance indicated that they were probably phagocytosed by resident microglia and/or infiltrating macrophages. This is concluded since they had a more round morphology, and the eGFP expression was not homogenous throughout the

cells and had a reduced intensity compared to the eGFP-positive NSCs 3 and 7 days post transplantation. Moreover, in the previous experiment the same morphology was observed 3 weeks after transplantation in the damaged spinal cord and staining for IBA-1 expression revealed that a number of NSCs were phagocytosed by microglia/macrophages. Whether these NSCs were phagocytosed by microglia/macrophages 28 days post grafting, has to be further investigated by immunohistochemical analysis with macrophage/microglia markers (IBA-1, MAC-3, F4/80, CD11b). Moreover, further research has to focus on the cause of phagocytosis, as well as on improving survival of the NSCs after transplantation in the CNS.

5. Conclusion

In conclusion, allogeneic transplantation of NSCs in the lesion after spinal cord hemisection resulted in an improved functional recovery of the treated mice. The immunosuppressive properties of the NSCs could account for this improvement, as a reduction, however not significant, in lesion size and demyelinated area was measured. It cannot be excluded that the improvement of clinical outcome was the result of an immune response against the transplant. This was further supported by the second *in vivo* experiment, since no difference in functional recovery was found between the control and NSC grafted group after SCI. However, as only 4 mice per group were included in this experiment, this has to be repeated in order to make a definitive conclusion. Three weeks after transplantation no differentiation of NSCs into neurons was observed, indicating that cell replacement is not the reason for the enhanced functional outcome of the NSC transplanted mice. Differentiation of the NSCs into oligodendrocytes and astrocytes could not be excluded in this experiment. Moreover, three weeks after grafting a number of NSCs was phagocytosed by resident microglial cells and/or infiltrating macrophages.

In the second *in vivo* experiment, the combined immunomodulatory and regenerative effects of NSCs and IL-13 were investigated. As mentioned previously, transplantation of NSCs in the spinal cord lesion had no effect on the clinical outcome of the grafted mice. Combined treatment of NSCs and IL-13 had also no effect on the functional recovery of the mice. In contrast, there was a trend indicating that IL-13 alone impaired clinical recovery of mice after SCI. As low numbers of mice were used in this experiment, 4 or 7 mice per group, this has to be repeated. Furthermore, in this experiment survival was analyzed at day 3, 7 and 28, since in the first experiment not all NSCs survived 3 weeks after transplantation. The morphology of the NSCs suggested that they survived 3 and 7 days post grafting, however, after 28 days they were probably phagocytosed.

This study revealed that further research has to focus on improving the survival of the NSCs after transplantation, as well as on the mechanisms by which NSCs could improve the clinical outcome after grafting in the damaged spinal cord. Furthermore, IL-13 is probably not the ideal factor to combine with NSCs as treatment for SCI, as it impairs functional recovery of the treated mice. NSCs could be used as carriers of other anti-inflammatory cytokines, for example IL-4, as preliminary results from our research group have indicated that IL-4 does improve clinical outcome and promotes axonal regeneration.

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