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For Nana, who always had the warmest welcome

Ar dheis Dé go raibh a hanam

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List of abbreviations

APCs	antigen-presenting cells
Arg-1	arginase-1
BDNF	brain-derived neurotrophic factor
BMS	basso mouse scale
Brd-Udr	bromodeoxyuridine
CNS	central nervous system
CXCR4	C-X-C chemokine receptor type 4
CCR5	C-C chemokine receptor type 5
DAPI	4',6-diamidino-2-phenylindole
DCs	dendritic cells
DG	dentate gyrus
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
ERK	extracellular signal-related kinases
ESCs	embryonic stem cells
FIZZ1	found in inflammatory zone 1
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
IBA-1	ionized calcium binding adaptor molecule 1
IL	interleukin

IP	intraperitoneal
Prox1	homeobox protein 1
JAK-STAT	janus-activated kinase-signal transducer, activator of transcription
LIF	leukemia inhibitory factor
LPS	lipopolysaccharides
LFA-1	lymphocyte function-associated antigen 1
LVv	lentiviral vector
MAPK	mitogen-activated protein kinase
MAP-2	microtubule-associated protein 2
MBP	myelin basic protein
MHC-II	major histocompatibility complex
MSC	mesenchymal stem cell
NaCl	sodium chloride
NF	neurofilament
NFATc1	Nuclear Factor of Activated T-Cells 1
NF-κβ	nuclear factor kappa-light-chain-enhancer of activated B cells
NPCs	neural precursor cells
NSCs	neural stem cells
NSPCs	neuronal stem/progenitor cells
nNOS	nitric oxide synthase
Notch1	neurogenic locus notch homolog protein1

NO	nitric oxide
PBS	phosphate**-**buffered saline
Prox1	prospero homeobox protein 1
RFP	red fluorescent protein
ROS	reactive oxygen species
SC1	schwann cell factor 1
SCI	spinal cord injury
STAT	signal transducer and activator of transcription
SVZ	subventricular zone
TBS	tris-buffered saline
Th1	T helper type 1
Th2	T helper type 2
TGF- β	transforming growth factor-beta
TLR	toll-like receptor
TNF	tumour necrosis factor
UCN	urocorticon
WT	wild-type

Chapter I

General Introduction & Aims

Parts of this chapter are based on:

The role of "anti-inflammatory”cytokines in axon regeneration

Vidal PM, Lemmens E, **Dooley D,** and Hendrix S:

Cytokine & Growth Factor Reviews, vol. 24, pp. 1–12, 2013

1.1 Spinal cord injury

Spinal cord injury (SCI) is an insult to the spinal cord resulting in a change, either temporary or permanent, in the cord's normal motor, sensory, or autonomic function and patients with spinal cord injury usually have permanent and often devastating neurologic deficits and disability (1). The global incidence rate of SCI is estimated at 180,000 cases per annum (2). The most common causes of SCI include car accidents (43-50%), falls (18.8-37%) and violence (17.8%). Therefore, the use of seat belts, safety in sports and on construction sites as well as weapon restrictions, all aid in decreasing the number of preventable tragedies that occur annually. The economic burden associated with the decrease in quality of life is a major factor adding to the number of issues that need be addressed when dealing with SCI. The mean cost of treating a patient with traumatic SCI is between US$500,000 and $2 million, depending on elements such as the type of injury suffered or injury location (3).

The two most common types of SCI are contusion and transection injuries, with contusion being the most common in humans (4, 5) and the symptomatology differs depending on the location and severity of the injury. The American Spinal Injury Association (ASIA) have defined a severity classification for impairment as either complete (category A: loss of both sensory and motor function), incomplete (category B, C or D: preservation of either sensory or motor function) or normal (category E). With regard to injury location, about 40% of patients suffer from quadriplegia, due to injuries at the cervical spine level, with the remaining 60% suffering from paraplegia (6). Despite considerable progress in palliative care, there is currently no therapeutic intervention currently available which leads to functional recovery. Throughout this thesis, our SCI model of choice is the T-cut or hemisection injury model (7). We have chosen to work with this model over others (such as contusion), as it enables us to study regenerative processes that can only be investigated when all the axons of a projecting system are lesioned and reduces the amount of spared fibres present. This type of lesion destroys the dorsal, dorsolateral and ventral corticospinal axons (Figure 1.1).

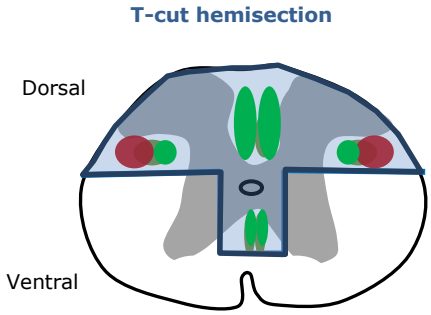

Figure 1.1: Schematic representation of a coronal view of a T-cut hemisection spinal cord injury in rodents. The blue shaded area represents the T-cut lesion which results in complete transection of the corticospinal tract (green) and also part of the rubrospinal tract (red). Image modified from Tuszynski and Steward, 2012).

1.1.2 Pathophysiology

The pathophysiology of SCI can be divided into two phases. Firstly, the primary mechanical injury results in haemorrhage, vascular damage and cell death at the lesion site and this is followed by a secondary inflammatory response. This secondary injury phase is characterised by demyelination, glial cell activation resulting in the production of inhibatory extracellular matrix molecules (e.g. Chondroitin and keratan sulphate proteoglycans, ephrins and semaphorins), glial scar formation as well as immune cell (neutrophils, microglia, macrophages and T cells) migration and infiltration (8-10). In 1928, Ramón y Cajal first described the process in which regenerating axons form so-called dystrophic endbulbs when they encounter the environment of the glial scar (11) and this makes axons regeneration beyond the glial scar, extremely problematic. These events also lead to the secondary loss of neighbouring axons, neurons and oligodendrocytes.

1.1.3 Inflammatory phases & current treatments

Inflammatory responses are a major component of the secondary injury phase and play a key role in regulating the pathogenesis of acute and chronic SCI. On one hand, the inflammatory response is necessary as it leads to clearance of cellular debris, resulting in a more favourable environment for endogenous

repair and axonal sprouting (12, 13), but on the other hand, over-activation of this response can exacerbate damage. Therefore, characterization of these specific phases and maintaining a balance between inflammatory mediators is now being recognized as a vital aspect to consider when designing therapeutic approaches (14)

With the help of well-established rodent models, the four main phases which have been identified after SCI are: acute, sub-acute, early chronic and late chronic. The acute phase which takes place immediately after the injury, lasts for a few hours and leads to an up-regulation of pro-inflammatory cytokines (e.g. interleukin (IL)-1β and tumour necrosis factor (TNF)-α, as well as axonal destruction and demyelination (6, 15). There is an immediate infiltration of neutrophils which reaches its peak one day post injury (16). In the sub-acute phase (days 2-7), the levels of certain pro-inflammatory cytokines start to decrease and there is an influx of microglia, macrophages, T cells and activated astrocytes (15, 17). At this point there is also an increase in certain anti-inflammatory factors such as cytokines, chemokines and neurotrophic mediators (Figure 1.2), which attempt to rebalance the damaged system. The early and late chronic phases can persist for up to weeks or even months and result in a second T cell, macrophage and neutrophil peak in the early chronic phase (16).

Unfortunately to date, there is no treatment available for SCI patients. Current therapies focus on combining immunosuppression (e.g. methylprednisolone) with rehabilitation and palliative care. Although the continual and dual role of the neuroinflammatory response leaves it difficult to decipher upon a single modulatory strategy, the establishment of distinct inflammatory phases is of vital importance when designing therapies. This, in combination with fundamental research strategies have resulted in recent advances in cell-replacement therapy, tissue engineering as well as neural interface systems.

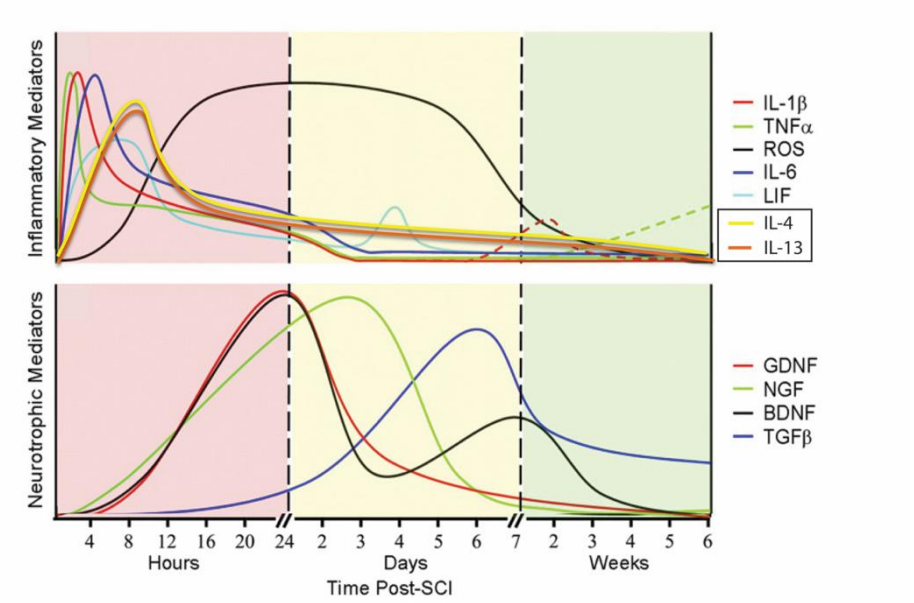

Figure 1.2: Schematic representation of inflammatory and neurotrophic mediator expression following spinal cord injury. The curves represent peaks in pro- and anti-inflammatory mediators as well as neurotrophic factors at various timepoints post injury. Within hours after injury, the chronic phase consists of peaks in pro-inflammatory factors such as IL-1β, TNFα, ROS and IL-6. There is a peak in the anti-inflammatory cytokines IL-4 and IL-13 8 hours post injury. In the later phases spanning from days to weeks, there are peaks in neurotrophic factors such as GDNF, NGF and BDNF which try to rebalance the damaged system after injury (Modified from Popovich & Longbrake 2008).

1.2 Cytokines

Cytokines can be defined as proteins with pleiotropic, redundant, synergetic and/or antagonistic effects which are mediated via several signaling cascades, allowing them to regulate cellular activity (such as proliferation, differentiation and maturation) in a coordinated and interactive manner (18). Cytokines and the cells that secrete them can be classified as either pro-inflammatory (T helper cell type 1 [Th1] or type 1) or anti-inflammatory (T helper type 2 [Th2] or type 2). In most publications, the terms pro-inflammatory, T helper cell type 1 [Th1] or type 1 and anti-inflammatory, T helper cell type 2 [Th2] or type 2 are used interchangeably, which theoretically is incorrect. Generally speaking, type 1 cells

activate macrophages and control infections while type 2 cells activate B cells and help eliminate extracellular parasites (13). Despite this general classification, most cytokines display overlapping functions, exerting both *pro-* and *anti-inflammatory* effects depending on the tissue milieu. This makes it difficult to understand many cytokines' true effects as mediators of the immune response. In this thesis, the focus will be on the so-called "*anti-inflammatory"* cytokines IL-25 and IL-13 and their use as immunomodulators for treatment of SCI.

1.2.1 Interleukin-25

We as well as others have shown that cytokines associated with Th2 cells such as IL-4 (19-21) and IL-10 (22, 23), not only promote neuronal survival and regeneration, but also improve functional outcome after central nervous system (CNS) trauma such as SCI. IL-25 (also known as IL-17E) has been suggested to be a key player in the origin of a type 2 response (24, 25). While research has begun to unravel its importance in immunity in general, conclusive data on the role of IL-25 in the CNS is lacking. Although a limited number of studies are currently available, these tend to point towards a *protective* role of IL-25 in neuroinflammation (reviewed in (26, 27)). For example, IL-25 treatment suppresses Th17 responses and disease symptoms in experimental autoimmune encephalomyelitis (EAE) via IL-13 and is important in maintaining blood-brain barrier function (28, 29). IL-25 expression is downregulated by proinflammatory cytokines such as tumor necrosis factor-α and IL-1β, which increase acutely after trauma. Consistently, IL-25 is reduced in the proinflammatory milieu of CNS lesions (28). These findings, suggest that an increase in IL-25 may possess the therapeutic potential to provide repair after CNS trauma.

1.2.2 IL-25 receptor signaling

IL-25 is a rather unique member of the *pro-inflammatory* IL-17 family and displays the least homology with IL-17(A), which may point towards its potential ability to regulate Th2 responses. IL-25 signals through a heterodimeric receptor complex (IL-25R) which is composed of two subunits, IL-17RA and IL-17RB (30, 31). It has been shown that IL-25 activates NFATc1 and JUNB, which leads to

increased IL-4 expression by Th2 cells (32). Upon ligand binding, IL-25R has been shown to signal through Act1 via interactions with the SEFIR domains (33, 34). IL-25 has also been shown to activate mitogen-activated protein kinases (MAPK)s such as P38 and c-Jun N-terminal kinase (JNK) as well as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (35). Wu et al have identified a novel IL-25 signaling pathway through the activation of Signal transducer and activator of transcription (STAT)5 *(31) and this activation is crucial for T cell development* and Th2 cell differentiation (36). Despite the identification of the IL-25 receptors, few cell populations expressing IL-17RA or IL-17RB have been identified. Antigen-presenting cells (APCs) such as macrophages or dendritic cells (DCs) may be a possible target of IL-25 and IL-17RB has been found to be up-regulated in dendritic cells under Th2-inducing conditions (37). IL-17RA and IL-17RB are also expressed on eosinophils and their expression increases in asthma patients (38).

1.2.3 Interleukin-13

IL-13, a cytokine closely related to IL-4 (39), is a canonical *anti-inflammatory* cytokine, which in some contexts can also be *pro-inflammatory* (40). Functionally, IL-13 is a pleiotropic cytokine, and can display both immunostimulatory or immunosuppressive effects. For example, IL-13 inhibits the expression of pro-inflammatory cytokines (e.g. IL-6, IL-1β, and TNF-α) and other inflammation-associated factors (e.g. nitric oxide (NO), reactive oxygen species (ROS)) both *in vitro* (41) and *in vivo as* IL-13 has also been shown to exert neuroprotective effects in the EAE model of multiple sclerosis, by decreasing inflammatory cell infiltration and axonal loss as well as reducing clinical symptoms (42-44). IL-13 plays an important role in the body's fight against parasites and cancer. On the other hand, it can also contribute to the pathophysiology of allergic diseases such as asthma by promoting immunoglobulin E production (reviewed in (45)). Furthermore, in EAE, IL-13 has been shown to exert neuroprotective effects, decreasing infiltration of inflammatory cells and diminishing axonal loss, and to suppress clinical symptoms (43, 46). Preliminary data from our group has shown that IL-13 promotes neurite growth in primary neurons and organotypic brain slices *in*

vitro. Taken together, these data indicate that IL-13 has the potential to display beneficial immunomodulatory effects following CNS trauma, however, due to it's pleiotropic nature, fine-tuning regarding timing and method of administration is vital.

1.2.4 IL-13 receptor signalling

There are two types of IL-13 receptors, the first consists of a heterodimer containing IL-13Rα1 and IL-4Rα subunits and the latter of which also binds IL-4. The second type is an IL-13 specific receptor and consists of an IL-13Rα2 chain (47). Although IL-13 binds to the IL-13Rα2 chain with a high affinity, it is considered to be primarily a decoy receptor given its short cytoplasmic tail and lacking signalling motif (48). In addition to regulation of IL-13Rα1 and IL-13Rα2 by IL-13 itself *in vivo*, IL-4 and IL-10 are also actively involved. IL-13 first binds to IL-13Rα1 with a low affinity and then IL-4Rα is recruited to the complex which in turn generates a high affinity receptor (49). For this reason, signal transduction via the IL-4Rα is thought to be responsible for the majority of the functional characteristics of IL-4 and IL-13. However, IL-13 also possesses many unique effector functions which differentiates it from IL-4. For example, it has been shown that both IL-4 and IL-13 activate STAT3 and STAT6, while STAT1 is only activated by IL-13 (50). IL-13 is mainly secreted by Th2 cells, but also other T cell subsets, mast cells, dendritic cells, microglia, and macrophages (47, 51). IL-13Rα1 is expressed on many different cell types, including B cells, mast cells, endothelial cells, fibroblasts, monocytes, and macrophages, but is absent on T cells (47). We have also recently identified the expression of the of IL-13R on neurons (data not shown). In the CNS, neurons and microglia work in synergy to reduce brain inflammation via induction of IL-13 and IL-13 has also been shown to directly induce apoptosis in activated microglia (52). It therefore seems plausible that modulating the microglia/macrophage response after SCI by factors such as IL-13, may prove beneficial in down-regulating CNS regenerative-inhibitory factors.

1.3 Microglia & macrophage response in spinal cord injury

Almost all tissues contain several types of microglia and macrophages with specialized functions (53, 54). With regard to the spinal cord, microglia reside in the spinal parenchyma and in their resting state are highly dynamic cells with an ability to continuously scan the surrounding environment for homeostatic irregularities (55, 56). They are responsible for phagocytosis of cellular debris and toxic compounds and are the first cells which respond to infection, inflammation and injury to the CNS (57). Additionally, microglia react to environmental changes by altering their motility, morphology and phagocytic functions by releasing cytokines, chemokines and reactive oxygen species (55). The extent of microglia activation depends on whether they are in a "primed" (sensitised) state upon exposure to a stimulus. Additionally, the type of insult, potency of the stimulus, as well as the microenvironment (proximity of neurons, glial, immune cells etc), all affect the way in which microglia react to CNS injury (58).

Macrophages are derived from monocytes and are recruited to the site of injury sites from the peripheral circulation (59). They are capable of assuming a wide variety of functional states which can influence CNS repair. Similarly to microglia, macrophage phenotypes are determined by the surrounding microenvironment and can change in response to various stimuli (60, 61). This functional adaptability allows macrophage to contribute to repair by promoting/modulating inflammation, removing detrimental triggers, depositing ECM, stimulating cell proliferation, and releasing anti-inflammatory factors (61). However, on the other hand, macrophages also have the potential to hinder different phases of repair and persistent activation can result in chronic inflammation and dysfunctional wound healing (62).

SCI activates microglia and macrophages with different functional phenotypes (63, 64). As outlined above, there is an influx of microglia, macrophages as well as T cells and activated astrocytes in the sub-acute phase (days 2-7) at the lesion site (15, 17). At this stage, blood monocytes migrate to the injury site where they differentiate into macrophages, which, using currently established methodologies, become phenotypically and morphologically indistinguishable

from activated microglia (61). The timing and distribution of monocyte- and microglia-derived macrophage activation after SCI has been previously reviewed (16, 49).

1.3.1 Microglia & macrophage classification

Distinction between brain-resident microglia and blood-borne macrophage is an ongoing issue. A traditional, rather simplistic way to distinguish the varying microglia and macrophage subsets is to divide them into *classically* (M1) or *alternatively activated* (M2) phenotypes (65, 66). M2 microglia/macrophages, differentiate from the classically activated M1 microglia/macrophages and are less inflammatory in nature. They are characterized by a reduced nitric oxide production and secrete lower levels of pro-inflammatory cytokines (67). This subset of M2 microglia/macrophages express markers that differentiate them from *classically activated*, such as Arg-1 and Found in inflammatory zone 1 (FIZZ1) (68). However, a more specific characterization indicates that upregulation of MHC-II (in both M1 and M2 cell subsets) is associated with macrophage activation and joint expression of MHC-II and Arg-1/FIZZ1 is indicative of the neuroprotective and anti-inflammatory, M2a phenotype (69).

1.4 Stem cell therapy for spinal cord injury

Stem cell therapies for SCI have raised a lot of hope amongst patients, doctors and scientists in recent years. Although we are still in the early stages, advances in research have demonstrated the ability of stem cells to provide therapeutic effects in many pre-clinical animal studies (70-72). Despite this progress, a precise mechanism describing the way in which stem cells improve CNS injury is lacking. Potential candidate mechanisms include: direct cell replacement, stimulation of endogenous stem cells and more recently, immunomodulation via transplanted exogenous cells (73-75). This 'by-stander' effect exerted by transplanted cells involves the production of trophic factors which can potentially stimulate the endogenous neuro-protective and/or neuro-repair processes (76, 77). Directing microglia and macrophages towards a more pro-neurogenic, neuroprotective phenotype and the development of an ideal transplantation

environment for stem cells will more than likely involve up- and downregulation of certain inflammatory factors. These factors which display "good'' or "bad'' effects towards stem cells will be discussed in chapter II of this thesis. These data were summarised in a review article which was published in 2014 in the Journal of Pharmacology & Therapeutics.

1.4.1 Neural stem cells

Neural stem cells (NSCs) can be an invaluable tool for CNS repair and low numbers of multipotent stem and precursor cells exist within specialized areas of the adult mammalian brain (78). The stimulation of these endogenous NSCs is one possible strategy to consider in the field of CNS repair. An alternative option may involve the use of previously isolated exogenous NSCs, by means of transplantation to the site of injury. The ability of these exogenous and endogenous NSCs to migrate has been well documented (75, 79, 80) and the differentiative capability of these cells into neurons has been speculated to provide beneficial effects in both degenerative diseases as well as CNS trauma.

1.4.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) possess the ability to exert positive effects in rodent models of spinal cord injury (81, 82). They are a good choice in cell-based therapy as they can modulate oxidative stress and secrete cytokines and growth factors that have immunomodulatory, anti-inflammatory, angiogenic and anti-apoptotic effects (Figure 1.3) (83). Despite the positive effects observed, quite a few hurdles still remain when considering MSC therapy for CNS injury, as the described functions of MSCs have not yet lead to an elusive mode of action.

1.4.3 Which cell type to choose?

Initial studies in our group have investigated the use of both NSC and MSC cell transplantation following SCI. However, for the experimental procedures summarised in this thesis, the focus is on the use of MSCs for the following reasons:

a. When looking towards potential clinical applications, autologous MSC can easily be isolated from bone marrow or other tissues, such as adipose

tissue, dental pulp, placenta and Wharton's jelly (84).

b. MSCs alone possess many strong intrinsic immunomodulatory properties (83).

c. Based on pilot experiments in our lab and previous studies within on group, MSCs display a higher survival rate compared with NSCs, due to new blood vessel formation and appear more stable *in vivo* (85, 86).

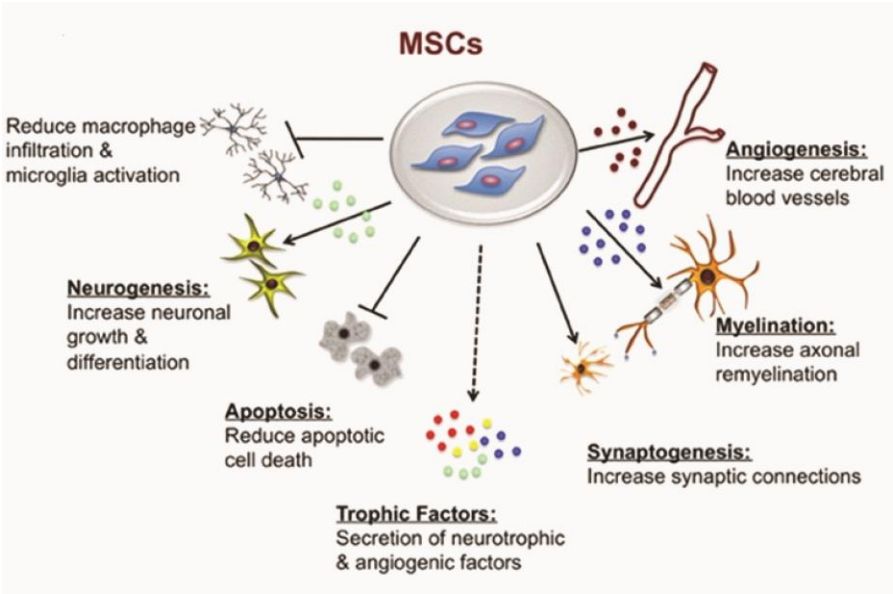

Figure 1.3: Schematic representation of the beneficial characteristics of MSCs. The ways in which MSCs can improve therapeutic outcome following SCI are outlined. E.g. reduction of macrophage infiltration, microglia activation and apoptosis, secretion of trophic factors, increased myelination and angiogenesis may all add to the beneficial effects of MSCs following CNS trauma or disease. Modified from: Castillo-Melendez et al, Frontiers in Neuroscience 2013.

1.5 Aims of this Study

The continual and dual role of the neuroinflammatory response leaves it difficult to decipher upon a single modulatory strategy. Additionally, the role of certain immunomodulatory cytokines which are up- or down-regulated in response to SCI still remains unclear. In this thesis, we focus on the type-2 cytokines: IL-25

and IL-13 and investigate their therapeutic potential for treatment of SCI. Understanding the interaction of these factors with the surrounding microenvironment and exploiting their modulatory effects on cells such as microglia and macrophages, may provide a vital therapeutic tool in developing strategies for treating CNS trauma. Therefore, our primary research question in this thesis is as follows: are IL-25 and IL-13 potential therapeutic immunomodulatory candidates for treatment of SCI?

1.5.1 Therapeutic effects of IL-25 after SCI

We as well as others have shown that cytokines associated with Th2 cells, not only promote neuronal survival and regeneration, but also improve functional outcome after SCI (19-21). Although IL-25 has been suggested to be a key player in the origin of a type 2 response (24, 25), at the beginning of this study, there was a gap in the literature outlining its role in CNS repair. Therefore, the first goal of this study was to investigate whether recombinant murine IL-25, administered either as a single dose locally to the spinal cord or via repeated systemic injections, improves functional recovery after SCI in mice.These results are described in detail in **chapter III** of this thesis and were published in 2016 in the *Journal of Neuroinflammation*.

1.5.2 Therapeutic effects of IL-13 after SCI

In the second part of this study, we focus on investigating the therapeutic potential of the Th2, *anti-inflammatory* cytokine, IL-13. In addition to its immunomodulatory effects, IL-13 is also a well-known inducer of the M2 microglia/macrophage phenotype (87, 88). We recently demonstrated that after SCI in mice, IL-13 levels decrease significantly in the serum and spinal cord within hours after injury (89). Therefore, given the drop in IL-13 levels after injury and it's polarizing capabilities towards a more *neuroprotective* M2 macrophage phenotype, it seems plausible that application of IL-13 in the acute phase after SCI may have therapeutic potential. Therefore the second goal of this study was to investigate the therapeutic effects of recombinant murine IL-13 in a mouse model of SCI. These results are described in detail in **chapter IV** of this thesis.

1.5.3 MSCs as carriers of IL-13 and their therapeutic effects after SCI

Following some inconclusive results upon administration of recombinant IL-13 following SCI, in the final part of this study, we aimed to optimise a continuous local delivery system for IL-13. In order to achieve this, we choose to use MSCs for the reasons outlined above. Therefore, in the final part of this study, we investigated the use of autologous MSCs which were genetically modified to secrete IL-13, as a potential therapeutic strategy to modulate the immune response and improve functional recovery after SCI. We also investigated whether IL-13-secreting MSCs were capable of modulating the complex microglia and macrophage response following SCI and characterised there phenotypes at both the MSC graft and lesion site. Furthermore, we performed detailed histological analysis in $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ mice, allowing us to distinguish between resident microglia and infiltrating macrophages. These results are described in detail in **chapter V** of this thesis and the research manuscript is currently submitted for publication.

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Chapter II

Immunopharmacological intervention for successful neural stem cell therapy: new perspectives in CNS neurogenesis and repair

Based on:

Immunopharmacological intervention for successful neural stem cell therapy: New perspectives in CNS neurogenesis and repair

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2.1 Abstract

The pharmacological support and stimulation of endogenous and transplanted neural stem cells (NSCs) is a major challenge in brain repair. Trauma to the central nervous system (CNS) results in a distinct inflammatory response caused by local and infiltrating immune cells. This makes NSC-supported regeneration difficult due to the presence of inhibitory immune factors which are upregulated around the lesion site. The continual and dual role of the neuroinflammatory response leaves it difficult to decipher upon a single modulatory strategy. Therefore, understanding the influence of cytokines upon regulation of NSC self-renewal, proliferation and differentiation is crucial when designing therapies for CNS repair. There is a plethora of partially conflicting data *in vitro* and *in vivo* on the role of cytokines in modulating the stem cell niche and the milieu around NSC transplants. This is mainly due to the pleiotropic role of many factors. In order for cell-based therapy to thrive, treatment must be phase-specific to the injury and also be personalized for each patient, i.e. taking age, sex, neuroimmune and endocrine status as well as other key parameters into consideration. In this review, we will summarize the most relevant information concerning interleukin (IL)-1, *IL-4, IL-10,* IL-15**,** *IFN-γ,* the neuropoietic cytokine family *and* TNF-α in order to extract promising therapeutic approaches for further research. We will focus on the consequences of neuroinflammation on endogenous brain stem cells and the transplantation environment, the effects of the above cytokines on NSCs, as well as immunopharmacological manipulation of the microenvironment for potential therapeutic use.

2.2 Introduction

Stem cells are a very unique cell type with two fundamental characteristics: multipotency and self-renewal. More specifically, NSCs are an undifferentiated cell type possessing the capacity to generate both glial and neural cell lineages (1). NSCs can be an invaluable tool for CNS repair and low numbers of multipotent stem and precursor cells exist within specialized areas of the adult

mammalian brain (2). The stimulation of these endogenous NSCs is one possible strategy to consider in the field of CNS repair. An alternative option may involve the use of previously isolated exogenous NSCs, by means of transplantation to the site of injury. The ability of these exogenous and endogenous NSCs to migrate has been well documented (3-5) and the differentiative capability of these cells has long since been speculated for reinstating neurons in both degenerative diseases as well as trauma to the CNS. Such capabilities are of vital relevance when considering methods to overcome many of the current challenges associated with application of NSCs in the clinic; namely, low survival, proliferation and differentiation rates. Understanding the interactions between the immune system and NSCs is also crucially important for effective therapies and a method directed at neutralising or downregulating immune-associated mechanisms, represents an affirm approach toward preventing disease progression (6). A recent review by Kokaia et al. has outlined the effects of macrophages and microglia on NSCs (7), however the influence of cytokines on the NSC fate has been neglected and information on their potential use for immunopharmacological intervention is lacking. Varying factors, such as the site of transplantation, method of delivery as well as cytokine concentration, all influence the given immune reaction. Here, we focus on the consequences of neuroinflammation on endogenous brain stem cells and the transplantation environment, as well as the effects of specific groups of cytokines on NSCs. We believe that characterizing the specific phases of selected diseases or injuries such as acute, subacute, early chronic etc, will be necessary before immunopharmacological intervention can be translated from *in vitro* and *in vivo* studies to the clinic. It is of vital importance to understand the implications, be they favorable or deleterious toward NSCs, before one can look toward exploiting cytokines and enhancing the properties of NSCs for therapeutic purposes (8, 9).

2.2.1 CNS injury is associated with 'pro- and anti-inflammatory' processes

Following trauma to the CNS, there is a distinct inflammatory response driven by resident microglial activation, local infiltration of immune cells as well as production of cytokines, chemokines, neurotransmitters and reactive oxygen species (ROS) (10-12). While the primary mechanical injury is restricted to the lesion site, a widely acute inflammatory response including a dramatic influx of neutrophils and later macrophages and T cells, causes rapid death in both neuronal and glial cells. In addition, the secondary pathology is characterized by neuronal and glial apoptosis, increased blood-brain barrier permeability, progressive lesion expansion as well as an elaborate and poorly understood chronic neuroinflammatory response, which may persist for years following the initial trauma (12-15). It is tempting to speculate that this more delayed secondary inflammatory response may be susceptible to certain therapeutic manipulative strategies. One of the main aims to induce CNS regeneration is to promote axonal outgrowth by stimulating the formation of new connections. Anti-inflammatory cytokines as modulators of neurite plasticity and outgrowth, are of pivotal importance in neuroregeneration with many varying effects reported (16).

The continual and dual role of the neuroinflammatory response leaves it difficult to decipher upon a single modulatory strategy. However, despite much debate regarding the detrimental effects of inflammation, many studies have also outlined its significance in tissue repair including a therapeutic potential of activated macrophage/microglial cells in promoting axonal regeneration (11, 17-19). Depending on their requirements, almost all tissues contain macrophages with specialized functions (20, 21). The two varying macrophage subsets were originally represented by the classically (M1) or alternatively activated (M2phenotypes (22). However, M2 macrophages have since been subdivided into M2a, M2b and M2c macrophages, in order to distinguish between phenotypes induced by different stimuli. In short, this new grouping of macrophage populations is based on three different homeostatic activities — host defense, wound healing and immune regulation (23). Additionally,

activation of microglial cells does result in varying response phenotypes and phenotypic diversity leads to functional diversity (24). As outlined above, there is ample evidence indicating that full activation of microglia leads to neurotoxic effects. However, such harmful effects must be based at least in part by the activating conditions involved. To investigate the effects of inflammation on the regenerative capacity of stem cells in the brain, researchers have concentrated on the response of microglial cells following an acute injury and after stimulation with lipopolysaccharides (LPS) (25). However, the use of LPS to outline the toxic potential of microglia *in vitro,* often stimulates defence-oriented reactions and thus, does not offer a broad signal variety.

2.2.3 Inflammation-associated cytokines: Looking past the pro- and anti-inflammatory concept

The dynamic equilibrium that exists between the immune system's pro- and anti-inflammatory response is mediated by pro- and anti-inflammatory factors, including IL-1, IL-4, IL-10, interferon-γ (IFN-*γ*)**,** tumour necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β) (12, 26). However, this concept of defined pro- and anti-inflammatory mediators is that of a simplistic one (16). Cytokines have the capacity to exert varying effects depending on their location and environmental queues. IL-4 for example promotes the proliferation of fibroblasts (27, 28) and endothelial cells (29), while displaying anti-proliferative effects on carcinoma cells (28). The induction of phase-specific effects is also a vital aspect to consider when attempting to classify anti-inflammatory cytokines. Examples of such effects have been described in other disease contexts such as asthma, experimental autoimmune encephalomyelitis (EAE) as well as wound healing and pregnancy. IL-13 is also an example of an anti-inflammatory cytokine which in some contexts can also be pro-inflammatory (30). Studies using anti-IL-13 monoclonal antibodies to treat asthma, have suggested that IL-13 may play a protective role in an acute inflammatory setting, while having a detrimental effect during chronic inflammation (30). Similarly, blocking the IL-4/IL-13 receptor prevents allergic airway sensitisation in asthma, without affecting the established allergy,

suggesting that both cytokines play a predominant role in the acute onset of disease (31).

2.2.4 The stem cell niche

Before looking toward optimal cell-transplantation conditions, one must take into consideration the environment within which endogenous NSCs are located within the adult CNS. These specialized stem cell 'niches' provide support and maintenance to endogenous cells, regulate their proliferative capabilities, and may also be considered to direct downstream differentiation (32). In the case of the NSC niche, physical contact-dependant mechanisms as well as diffusible signals are amongst the factors necessary for cells to thrive within the subventricular zone (SVZ) and the dentate gyrus (DG) (32). Evidence suggests that NSC transplantation may protect the CNS from inflammatory damage via a 'bystander' mechanism rather than by direct cell replacement (9, 33). This neuroprotective ability is mainly exerted by undifferentiated stem cells releasing a milieu of neuroprotective molecules at the site of tissue damage, which is temporally and spatially orchestrated by environmental needs. This milieu contains molecules (such as immunomodulatory substances, neurotrophic growth factors and stem cell regulators), that are constitutively expressed by NSCs for the maintenance of tissue homeostasis, both during development and in adulthood (9). It is important to acknowledge that the inflammatory environment may influence the NSC niche, thereby affecting stem cell survival, self-renewal, migration and neuronal differentiation (9). NSCs appear to reinstate degenerating neurons by regulating the host environment and adapting a chaperone-like role, for example, by possessing the ability to migrate along radial glial cells and in turn contribute to corticogenesis (34). Additionally, exposure to small direct current electric fields, increases the directional migration of NSCs (35). This ability of NSCs to provide cellular scaffolding may affect the mobilization of endogenous stem cells and migration of transplanted NSCs toward the spinal cord injury (SCI) site has also been previously observed (36). Such a result indicates that NSCs may also be used as delivery vehicles for therapeutic proteins due to their migratory capabilities toward the lesion site.

2.3 Regenerative capacity of endogenous CNS stem cells

Endogenous stem cells have been shown to be located within specialised regions of the adult brain - the subgranular zone of the DG of the hippocampus (37) and the lateral ventricle of the SVZ (38). The spinal cord is also home to a specific niche and NSCs have been isolated from the ependymal zone surrounding the central canal (39, 40) In the spinal cord, there is no defined sub-ependymal layer and sustained neurogenesis is not observed (41). Additionally, ependymal cells rather than astrocytes are the primary source of *in vitro* stem cell activity (41). Although there is still much to be learned about the spinal cord ependymal niche, ependymal cells have been seen to be rapidly activated following SCI. They proliferate and differentiate to deliver astrocytes and oligodendrocytes to the injury site. One can therefore conclude that at least a sub-population of spinal cord ependymal cells possess latent NSC properties (41-43).

Although the exact molecular mechanisms affecting NSC proliferation and differentiation have yet to be delineated, several factors are known to regulate neurogenesis. Brain stem cells appear to become 'activated' in response to neuronal trauma and migrate to the site of damage, suggesting that there are factors located at the injury site capable of influencing migration of precursor cells (3, 44). Furthermore, intracerebral administration of stromal-derived factor 1α (SDF-1α) has been seen to enable migration of intravenously injected NSCs into the CNS (45). These findings lead us to believe that a well-coordinated inflammatory reaction is necessary for tissue repair. This should ideally be capable of controlling the secretion of these beneficial factors, whose presence influence cell migration. On the other hand, a prolonged and exacerbated response may lead to a more critical and incessant neuroinflammatory cycle, which may in turn be involved in the acceleration of several neurodegenerative diseases.

2.3.1 Neurogenesis

Neurogenesis and differentiation of endogenous NSCs are also affected by their local environment following disease or injury-induced damage. As activation of astroglia and microglia are common attributes of these pathologies, equilibrium

between protective and toxic mediators controls NSC biology. For example, astrocytes can induce a neuronal phenotype on adult NSCs due to a cell contact mechanism as well as circulating secreted factors (46). In addition, microglia are capable of secreting neurotrophins, cytokines and chemokines which act directly on the NSC niche regulating precursor cell migration, proliferation and differentiation (47). Neurogenesis is inhibited by neuroinflammation by a number of diverse mechanisms, yet little is known about the way in which a pathological environment interacting with reactive microglia, affects precursor cell differentiation. A continual and consistent feature of CNS damage is microglial cell migration toward the injury site, and in turn, consequential activation. There is evidence that newborn neurons generated from stem cells may intermittently replace dead cells following brain injury (3). Thus, to initiate the use of either endogenous brain stem cells or stem cells undergoing transplantation, the establishment of suitable tools to identify the microglial state is necessary. Directing microglia toward a pro-neurogenic phenotype may exemplify a new approach to advocate the regenerative processes within the brain. Other types of immune cells can also induce the survival and proliferation of endogenous NSCs. For example, implantation of dendritic cells (DCs) after SCI, provides trophic support through the release of neurotrophin-3 and modulates the activation of microglia, thereby promoting locomotor repair after SCI (48).

2.4 Cytokines and neural stem cells

The initial idea that stem cell transplants work only via structural cell replacement has been recently challenged, due to regular cellular signaling which takes place between the host and cellular graft (9, 49). The derivation of NSCs from living patients is currently not possible, however, the use of induced pluripotent stem cells may help overcome this limitation (50). Bearing this in mind, there are still many challenges in place when it comes to choosing the most efficient source of NSCs, for example, finding reliable sources of multipotent and pluripotent cells and also controlling their differentiation to generate favourable derivatives (50). Recent observations have shown that a

surprisingly low number of stem cells survive and integrate following transplantation *in vivo* (51). Furthermore, the limited availability and mobilization difficulties associated with endogenous stem cells within the CNS, limit their therapeutic efficiency. These sourcing limitations have lead us to focus more on the bystander effects of stem cells rather than those observed via direct cell grafting (9, 33).

The process of inflammation varies and depending on the environment in place, can either amplify or suppress the activity of endogenous brain stem cells, which in turn will also determine the fate of transplanted NSCs. The distinction between the pro- and anti-neurogenic properties of inflammation may be based upon how macrophages, microglia and/or astrocytes are activated, as well as the duration of the inflammatory process (52). Reactive microgliosis has been shown to contribute to neuronal dysfunction and degeneration via the release of inflammatory factors such as, IL-6, TNF-α, IL-1β, nitric oxide (NO) and ROS. These pro-inflammatory factors can have negative effects on the NSC niche, resulting in reduced neuronal proliferation and differentiation, thereby decreasing neurogenesis (53, 54). However, in contrast, other factors and conditions are involved in the enrichment and stimulation of neurogenesis. It has been previously shown that antigen-specific autoimmune T cells, can increase the ability of microglia enriched cultures to remove glutamate, by tailoring the microglial phenotype (55). This finding suggests that T cells or their cytokines may allow microglia to adopt a particular phenotype that facilitates rather than impairs glutamate clearance, thus regulating at least in part, the onset as well as the shut-down, of the local immune response (55). This may in turn provide a more favorable environment for the survival of stem cell grafts. Additionally, NSCs express receptors which allow them to respond to certain cytokines and trophic factors. Therefore, the inflammatory process trigged in response to injury or disease, as well as that induced by the stem cell transplantation itself, may affect the success of the graft. One may consequently assume that the timing of the transplantation following injury, is of utmost importance when considering the success of the therapy (56). This association between neuroinflammation and neurogenesis and the action of microglia in regulating neurogenesis under pathological conditions, is still under intense

exploration (6, 57, 58). As mentioned previously, the creation of an ideal transplantation environment for NSCs will more than likely involve up- and downregulation of certain inflammatory factors. Although there is still much to be unveiled, particularly due to the dual role of many factors, we will attempt to characterize those which display ''good'' or ''bad'' effects towards NSCs and additionally, summarize those cytokines which should be up- or downregulated within the transplantation environment.

2.4.1 Selected cytokines to be downregulated during pharmacological intervention.

IL-1 family

Members of the IL-1 family exert numerous biological effects acting both directly or indirectly on the CNS (59). The IL-1 family consists of three proteins that are closely related and are products of separate genes. IL-1α and IL-1β are agonists which display identical actions and bind to a single 80kDA cell surface receptor (IL-1R1) (60), which requires an association with a accessory protein in order to carry out signal transduction (61). The third member is IL-1 receptor antagonist (IL-1ra) and as indicated by its name, blocks the actions of IL-1α and IL-1β. It is highly selective and binding to IL-1R1 does not initiate a response between IL-1R1 and AcP (61).

It has been demonstrated that acute LPS stimulation of primary microglia, induces greater levels of mature IL-1α and IL-1β (62). The same group investigated whether neural precursor cells (NPCs) may be a target of IL-1α and studies indicated that IL-1β as well as IL-α, do indeed modulate the properties of NPCs *in vitro* (63, 64). The study established that embryonic cortical NPCs express functional IL-1R1 and IL-1RAcP receptors, which are involved in transducing intracellular signaling via phosphatidylinositol 3-kinases (PI3K) activation, in response to IL-1α stimulation. It has also been shown that IL-1R1 is expressed on adult NPCs derived from the SVZ, indicating that the adult neural cell fate is also influenced by IL-1α. Additionally, recombinant IL-1α

actively enhances NPC differentiation into astrocytes, without altering cell viability and neuronal differentiation (63).

By using techniques such as immunocytochemistry, RT-PCR and ELISA, it has been shown that human multipotent neural progenitor cells express the cytokines IL-1α and IL-1β (65). This, in combination with IL-1R1 and IL-1RAcP receptor expression may suggest the potential for a direct receptor effect via IL-1α or IL-1β (61). IL-1β has also been shown to reduce proliferation and differentiation of NPCs and this appears to be mediated by the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), but not extracellular-signal-regulated kinase (ERK), P38 mitogen-activated protein kinases (P38MAPK) nor nuclear factor-kappa β (NF-κβ) pathways (66). This decrease in cell proliferation was accompanied by a regular pattern of cell differentiation; however, a lower level of glial fibrillary acidic protein (GFAP) protein expression in differentiated NPCs was observed (66). It is also generally accepted that pro-inflammatory cytokines, particularly IL-1β, exert rather detrimental effects following damage to the CNS. Consistently, IL-1 deficient mice display reduced neuronal loss and infarct volumes following ischemic brain damage (67) and in traumatic brain injury, antibodies against IL-1β reduce the loss of hippocampal neurons (68). In contrast, varying *in vitro* models have shown IL-1β to display beneficial effects towards neuronal survival in the CNS (69) and co-administration of IL-1β and NT-3, significantly increases neurite growth in organotypic brain slice cultures when compared to single treatments (70).

Neuropoietic cytokine family

The neuropoietic cytokine family is a group of structurally related cytokines consisting of IL-6, IL-11, IL-27, B cell stimulating factor, cardiotrophin 1 (CT-1), cardiotrophin-like cytokine/cytokine-like factor-1 (CLC/CLF), ciliary neurotrophic factor (CNTF) and leukaemia inhibitory factor (LIF) (71). These cytokines have well established roles in infection, pregnancy as well as muscle, bone and cardiovascular function, however, more recent studies have outlined their signaling involvement in both the developing and adult brain, as well as playing a role in response to CNS injury (71). In mice, LIF, CNTF, CLC/CLF and CT-1 all

contain receptors with identical signal-transducing subunits (LIF receptor (LIFR)-h and gp130) and are therefore called LIF-related cytokines (72). Although IL-6is a member of the neuropoeitic cytokine family, we have decided based on its effects on NSCs and in contrast to the other family members, to categorize it under the *cytokines to be upregulated* sub-heading, thus again verifying the rather pleiotropic nature of this cytokine family. LIF displays many pleiotropic effects within the CNS and therapeutic treatment via systemic application often leads to undesirable side effects (73). The limited potential of LIF to cross the blood brain barrier (74) not only restricts it's therapeutic potential, but also makes it difficult to evaluate the exact role of LIF within CNS disease. However, local production of LIF within the CNS by means of lentiviral vectors, has proven an effective way to overcome this delivery problem (75). Consequently, the effects of LIF and its related cytokines on NSCs are complex and in part contradictory (76). On one hand, early astrocyte formation has been shown *in vitro* following NSC treatment with LIF and CNTF (77). The mechanism of such an effect was shown to be due to activation of the Janus-activated kinase-signal transducer, activator of transcription (JAK-STAT) and mitogen-activated protein kinase (MAPK) signal transduction pathways (77, 78). It is also interesting to note that LIF appears to mediate astrogliogenesis in late embryonic (>E15), but not early (E12-E14) cortical progenitors in mice (77). This may in part be due to the sustained increase in epidermal growth factor receptor (EGFR) expression during development, which in combination with LIF, leads to an increase in astrocyte differentiation through a combined synergistic mechanism (79). On the other hand however, LIF has been shown to support the maintenance and self-renewal of embryonic mouse NSCs *in vitro* (80, 81) via the activation of the JAK–STAT pathway (82).

Signaling via gp130 appears to cause progenitor cells located within the ventricular zone, to re-enter the cell cycle whilst at the same time maintaining the original length of the cell cycle (83). It has also been shown that cells dissociated from LIFR-knockout mice, propagate fewer secondary neurospheres when compared to cells taken from wild-type mice, outlining a decrease in self-renewal capability (84). On the contrary, exogenous LIF appears to promote secondary sphere formation in cells from wild-type animals (85). Neurospheres

secrete endogenous LIF suggesting that *in vitro,* the effect of LIF on the self-renewal of NSCs exists due to an autocrine/paracrine mechanism (77). Although LIF-related cytokines lead to an increase in astrocyte production (78), another important point to outline is the fact that signaling via the LIFR also affects the oligodendrocyte lineage of NSCs. It has been shown that CNTF and LIF are strong modulators of oligodendrocyte-type-2 astrocytes (O2-A) in lineage development (86). These molecules were seen to elevate oligodendrocyte generation in cultures of dividing O-2A progenitors. An increase in myelin basic protein (MBP) also indicated an augmentation in oligodendrocyte maturation and thus, myelination, which may in turn promote oligodendrocyte survival (86).

As mentioned previously, neuropoietic cytokines, primarily LIF and CNTF, play a considerable role in astrocyte differentiation from neural progenitors *in vitro.* Concurrently, studies employing anneurosphere assay derived from embryonic mice, have indicated astrocyte-like cell formation in response to LIF-signaling as shown by an increase in GFAP expression (85, 87). However, given the conflicting observation that LIF and CNTF also appear to maintain NSC survival (84, 85), these findings together denote that some GFAP-positive astrocyte-like cells may generate both neuronal and glial cells, whereas others are in fact differentiated astrocytes. One can therefore conclude that GFAP is not a lineage-specific marker in the context of NSCs (88). Although LIF may be beneficial in maintaining the self-renewal of embryonic mouse NSCs *in vitro,* it's ability to drive NSCs toward an astrocyte lineage and further increase their differentiation, is unfavorable in the context of cell therapy following CNS injury.

IL-15

IL-15 is a pleiotropic, widely expressed pro-inflammatory cytokine that affects a large variety of cell types at varying stages of development and has important functions in both the immune and the nervous system (89). Human cell culture studies have also shown the presence of IL-15 mRNA in microglia, astrocytes and neuronal cell lines (90). IL-15 has been shown to play a role in the development of early inflammatory events in the CNS and is also involved in

glial cell activation as well as neuronal function in response to nerve injury (90, 91). However, because little is known about

the effects of IL-15 on neural cell differentiation, a study using cultured rat NSCs to investigate IL-15 signal transduction and activity subsequently revealed that NSCs and differentiating neurons, but not astrocytes or oligodendrocytes, express the IL-15Rα subunit of the IL-15 receptor (92). The authors also unveiled that IL-15 treatment decreased neurite outgrowth in differentiating neurons but did not alter NSC proliferation. IL-15 may affect neural cell differentiation via a signal transduction pathway involving IL-15Rα and STAT3, as the signal transduction alters MAP-2 protein levels and thus, affects neuronal differentiation from NSCs (92). The involvement of IL-15 in NSC proliferation and/or self-renewal has also been investigated. It's been shown that the cytokine controls NSC self-renewal and enhances neurogenesis with decreased IL-15 levels leading to elevated cell differentiation (93). *In vitro,* IL-15 deficiency results in a defective activation of both the JAK/STAT and the ERK/MAPK pathways in adult NSCs, which are key regulators of NSC proliferation and differentiation (53, 94, 95). Consequently, the effect of IL-15 upon these pathways may be accountable for the maintenance of self-renewal as well as the proliferative capabilities of NSCs within the adult brain, thereby contributing to the governing of neurogenesis during neuropathological states (93). Given the mechanistic effects of IL-15 on NSC proliferation and differentiation, we can conclude that downregulation of this pro-inflammatory cytokine provides a better and more suitable environment for NSCs to thrive.

IFN-γ

IFN-γ is a key inflammatory cytokine, mainly produced by cytotoxic $CD8^+$ T-cells and natural killer cells in the course of neurological diseases such as cerebral trauma (96), stroke (97) and multiple sclerosis (98). One would therefore assume that high levels of IFN-γ would exert detrimental effects upon neurogenesis and stem cells. However, microglia influenced by IL-4 and—surprisingly—by low concentrations of IFN-γ, have been seen to encourage adult neurogenesis and provide neuroprotection *in vitro*. This involves a complex regulation between insulin-like growth factor and TNF-α (99). *IFN-γ* also

increases neuronal (100) and microglial/macrophage (94) differentiation upon direct application to NSCs. However, in sharp contrast, IFN-*γ* hinders the formation of neural colonies in primary SVZ cells, while in neurospheres and NPCs, it reduces proliferation (101, 102). Similarly, the absence of IFN-γ in knockout mice has been shown to result in augmented NSC proliferation and differentiation within the adult DG, which is accompanied by neuroprotection as well as enhanced spatial cognitive performance (103). These data indeed outline the conflicting information which currently exists in the literature and the contradictory role that IFN-γ plays in both in vitro and in vivo with respect to neuroinflammation. However, although IFN-γ is generally thought to beneficially enhance neurogenesis from fetal or adult NSCs, *recent* literature has also provided information to the contrary, outlining dysfunctional development of NSCs under the influence of IFN-γ (104). We therefore hypothesise that downregulating IFN-γ may be beneficial when considering its effects upon endogenous NSCs or those undergoing transplantation.

2.4.2 Selected cytokines to be upregulated during pharmacological intervention.

IL-4 and IL-10

The neuroinflammatory milieu that develops in response to injury is also likely to result in insufficient migration of NSCs upon transplantation. Taking this obstacle into consideration, a previous study has looked at outlining the influence of IL-10 and IL-4 on neurospheres derived from the SVZ of adult mice. The authors found that treatment with IL-4 and IL-10 upregulated the surface adhesion molecule lymphocyte function-associated antigen 1 (LFA-1) and chemokine receptors CXCR4 and CCR5, on NSCs (105). Subsequently, IL-10-treated NSCs displayed significantly higher chemotaxis to the ligands of the above chemokine receptors when compared to untreated cells. In addition, treatment of NSCs with IL-4 also lead to a greater degree of chemotaxis of these cells to RANTES (regulated on activation, normal T cell expressed and secreted) (105). As

mentioned above, microglia modulated by IL-4, encourage adult neurogenesis and provide neuroprotection *in vitro*. (99). Adult NSCs engineered to express IL-10 show a greater ability to induce immune suppression, remyelination, and neuronal repair, thereby possibly outlining a novel approach to improve the efficacy of NSC-based therapy in CNS disease (106). Thus, pre-treatment with anti-inflammatory cytokines such as IL-4 or IL-10 may provide neuroprotection as well as the ability to facilitate migration of NSCs to the site of damage, ultimately resulting in a more favorable environment for the survival and maintenance of transplanted NSCs.

IL-6

IL-6 has been found to stimulate cortical precursor cell differentiation toward astrocyte and oligodendrocyte cell types (78, 107). It also plays a role in adult astrocyte activation as well as functioning as a neuronal neurotrophic and differentiation factor, in both the central and peripheral nervous system (108-110). In contrast, treatment of hippocampal precursor cells with IL-6, decreases neurogenesis *in vitro* (53). This may indicate a cell-type-dependent effect of IL-6, however this needs to be studied in greater depth in order to elucidate such a suggestion.

As mentioned previously, the IL-6 receptor family contains multiple subunit receptors with one common receptor subunit; the gp130 transmembrane protein. However, naturally occurring soluble forms of the integral-membrane receptors also exist (111). It has been shown that the majority of these act as antagonists by competing with the membrane bound receptors for their ligands. The soluble IL-6R (sIL-6R) which is propagated as a result of minimal proteolysis (shedding) then acts as an agonist (111). Hence, the complex of sIL-6R bound to IL-6, is capable of activating target cells expressing gp130 on their cell surface, but lack the membrane-bound IL-6R; a mechanism known as *trans-signaling* (112). Taking that the gp130 protein exists as a common component amongst the IL-6 family, it is interesting to note that every cell in the body expresses gp130. In contrast, only a select few cells express the IL-6R and cells that respond to IL-6 during inflammation, do not express the IL-6R. It may

therefore be of interest to target the IL-6R system in the context of stem cell transplantation for CNS injury, as neutralization of sIL-6R *in vivo* induces mucosal T-cell apoptosis in a model of Crohn's disease (113). Thus, it is tempting to speculate that blockade of IL-6 trans-signaling may therefore decrease T-cell resistance against apoptosis, thereby providing a possible method of controlling inflammation in and surrounding the area of transplantation.

In order to gain insight into the effects of specific cytokines on NSCs, one must take into consideration their effects on stem cell differentiation. A study to further define the functional role of the IL-6R complex during NSC differentiation showed that NSCs do not express a functional IL-6R, nor do they release IL-6. Thus, NSCs do not display a functional response to IL-6 (114). However, in the same study, a highly active fusion protein of IL-6 and sIL-6R, entitled *Hyper-IL-6* (H-IL-6), indicated that H-IL-6 causes NSCs to differentiate specifically into glutamate-responsive neurons, oligodendrocytes as well as phenotypically varying glial cell types. Further analysis revealed that H-IL-6 initiates gliogenesis via activation of STAT-3 and neurogenesis occurs via activation of the MAPK pathway, leading to an augmentation of nuclear phosphorylated CREBB (114). Although there is conflicting information in the literature regarding the effect of neuropoietic cytokines following CNS injury, one interesting approach may be to upregulate IL-6, whilst downregulating LIF, CNTF and CT-1. This may minimise the switch of NSCs toward an astrocytic fate whilst leaving IL-6 in place to promote NSC differentiation into functional neurons.

TNF-α

TNF-α has been documented to possess both positive and negative effects on neurogenesis (115). When it signals through its TNFR1 receptor, it greatly impedes neurogenesis, but conversely, its signaling through TNFR2 supports NSC survival and proliferation (116). TNF-α appears to activate NSC proliferation while inhibiting their differentiation into progenitor cells and an IKK-α/β-dependent proliferation as well as a clear upregulated cyclin D1 expression

following TNF exposure, has been demonstrated (117). This significant increase in proliferation of TNF-treated cells was verified by an increase in neurosphere volume, increased 5-bromodeoxyuridine-labeled (BrdU) incorporation as well as a greater total cell number (117). NSCs constitutively express toll-like receptor (TLR) 2 and TLR4 and following exposure to TNF-α and IFN-γ, this receptor expression is increased. Furthermore, activation of these receptors can lead to production of certain pro-inflammatory cytokines (118) which may lead to an increased inhibitory enviroment. It may therefore be interesting to consider priming endogenous NSCs or even NSCs prior to transplantation, in order to encourage these cells to produce certain cytokines following CNS injury.
Conditioned media from LPS-activated microglia and macrophages has been shown to block neuronal differentiation via the production of TNF-α (119, 120). Consistently, soluble TNF-α receptors as well as pentoxifylline, a TNF-α inhibitor, partially restored neuronal differentiation, establishing that TNF-α is partly responsible for the anti-neurogenic effect of the LPS-conditioned media (119, 120). It has also been demonstrated that NSCs also affect the surrounding environment upon transplantation via a cell contact-dependent mechanism. For example, NSCs co-cultured with macrophages can inhibit *in vitro* macrophage activation, thereby reducing TNF-α level secretion (121). Additionally, co-culture of adult NSCs on a feeder layer of primary astrocytes, results in functional neurogenesis and synapse formation of the stem cell progeny (46). This shows that NSCs derived from adult tissues, similarly to those derived from embryonic tissues; maintain the ability to differentiate into functional neurons whilst possessing fundamental properties of mature CNS neurons.

Although TNF-α is a well-established mediator of inflammation, its signaling mediated by two different receptors appears to generate both positive and negative effects in the case of NSCs. It appears that TNF-α mediates its effect on NSCs via NF-κβ activation, resulting in increased cell proliferation. Such data indicate that upregulation of TNF-α may in fact be favorable towards transplanted NSCs. However, its negative effect on neurogenesis when signaling through the TNFR1 receptor, leaves it yet to be fully elucidated whether this

cytokine is of beneficial or detrimental value to endogenous NSCs or those facing transplantation. To complicate matters even further, the soluble form of TNF- α has an inflammatory function, while transmembrane TNF-α displays anti-inflammatory properties in EAE models (122, 123).

2.5 Conclusions

Cytokines and growth factors are effector molecules of the innate as well as the adaptive immune system and they are involved in the regulation of cellular and humoral responses. Their release may offer plausible hope for potential therapeutic strategies via enhancement and modulation of what is currently a limited repair process. It is therefore of great interest to look at the possibility of exploiting the beneficial effects of cytokines, whilst at the same time, neutralising those with a deleterious impact (Tables 1 and 2). This may, for example, involve the use of factors which induce neurogenesis or inhibit those preventing neurogenesis or ideally, a combination of both. The identification of such a balance is especially important in the context of stem cell therapies where the influence of inflammatory-associated factors is of pivotal importance. Cells are mostly transplanted into a pro-inflammatory milieu and the transplantation mechanism may itself induce a pro-inflammatory response or even result in rejection (88). Despite the initial attempt of the CNS to repair itself in response to injury particularly in the SVZ and the DG (38), it is clear that these repair mechanisms are inadequate in achieving full functional recovery. For these reasons, much research has been directed toward the use of NSCs to replace cells lost though damage, as well as attempting to modulate the immune system and deliver immunomodulatory pro-regenerative factors (124).

One can learn a lot about the exact mechanisms which control *in vivo* development of the CNS from the developing embryo and it is known that similar cues also regulate *in vitro* differentiation of ESCs into neural progenitors and various types of neurons (125). Several studies have taken advantage of these inductive signals identified during embryonic development and have exploited their properties to boost the efficiency of generating neuroectoderm cells and decrease the number of non-neural cell types *in vitro.* For example,

treating mouse ESCs with Wnt and Nodal antagonists (Dkk1 or lefty), to inhibit Wnt and nodal signaling, increases generation of early neuroectoderm based on Sox1 expression (126). Treatment of human NSCs with Noggin has also been seen to encourage their differentiation toward neural progenitors (127). Additionally, it has been shown that the use of a synthetic TGF-β antagonist to inhibit SMAD signaling, leads to greater numbers of neural progenitor production (128). As differentiation of ESCs into NSCs via embryoid body intermediates is closely related to embryonic development of the cortical layers (125), neurogenesis may indeed be something that needs to be mimicked in order for stem cell grafts to succeed therapeutically. CNTF and LIF encourage self-renewal of NSCs as well as astrocyte differentiation (84). This shift to astrocyte differentiation is also seen with the presence of IL-6 in cultured NSCs, thereby decreasing neuronal production (53, 129). LIF's ability to increase the adult NSC population would undoubtedly lead to useful applications in brain injury, whilst curtailing the need for stem cell transplantation. Other promising clinical applications lie in these cytokines' ability to display protective actions towards oligodendrocytes, leading to an increase in the number of myelinated axons. However, some unavoidable hurdles in applying these pleiotropic cytokines still remain. One must establish safe doses to avoid detrimental side effects, but more importantly, a cocktail providing just the right balance of modulatory factors is necessary. This must in turn be phase-specific to the injury and also be personalized for each patient, i.e. taking age, sex, neuroimmune and endocrine status as well as other key parameters into consideration. The re-administration of certain cytokines at specific time-points during for example, SCI, whilst omitting those with no effect, may be one important aspect to consider. This idea may also be implemented quite readily in pre-clinical research given that the neuroinflammatory phases are already well-established in rodent models of SCI.

After investigating both the positive and negative aspects of neuroinflammatory cytokines, one can accept that there is still much more information to be unveiled prior to their use in immune-modulation therapy. Transplantation of NSCs into an environment which possesses a correct balance of positive diffusible signals as well as the ability to neutralise inhibitory effects (Figure 1),

would be an ideal therapeutic situation. This would provide a greater chance for cell migration as well as proliferation, differentiation and formation of functional circuits. But the intrinsic and often pleiopropic nature of these molecules, makes such an approach a difficult one. Indeed, a better comprehension of the mechanisms that control inhibition versus stimulation of neurogenesis during neuroinflammation may be an advantageous approach. Identification of such signals would provide an opportunity to replicate the process of neurogenesis, or even prevent its progression to further characterize the implications, be they detrimental or perhaps even in some cases, beneficial. Furthermore, *in vitro* pre-treatment with cytokines may be an advantageous approach to induce migration of NSCs to the CNS inflammatory core, thus resulting in more substantial and efficacious therapeutic effects. For example, NSCs express TLR2 and TLR4 and because their activation leads to the production of pro-inflammatory cytokines, possible priming of these cells to produce the necessary factors during traumatic conditions, may be an interesting strategy to focus on.

The points raised in this review collectively suggest and indeed support the fact that the inflammatory environment following CNS injury greatly influences the regenerative ability of NSCs. It is clear that understanding the influence of cytokines upon regulation of NSC self-renewal, proliferation and differentiation is a crucial phenomenon to acknowledge when considering pharmacological intervention via cell-based therapy. A model environment capable of maximizing the ameliorative capabilities of NSCs may be all that currently lies between researchers and the exploitation of NSCs as a novel therapeutic tool in the field of neuroregeneration. Viral vectors and gene therapy, even with their known drawbacks, such as potential tumor formation, as well as localized drug delivery by means of implantable pumps, are indeed important options to consider.

2.6 Future therapeutic options

Taking these points into consideration, it may therefore be advantageous to introduce immunopharmacological factors which will downregulate the effects of IL-1, IL-15 and IFN-γ following injury to the CNS. In addition, pre-treatment with ''anti-inflammatory'' cytokines such as IL-4 or IL-10 may provide neuroprotection and facilitate migration of NSCs to the site of damage, thereby

resulting in a more favorable environment for transplanted NSCs to thrive. It is clear that there is conflicting information in the literature regarding the effect of TNF-α and neuropoietic cytokines following CNS injury. One interesting approach may be to upregulate IL-6, whilst downregulating LIF, CNTF and CT-1.
Regarding the future direction of this field, *personalized medicine* will prove an even bigger challenge due to the complex network of the factors outlined above. Thus, identifying the phases of patient disease may be imperative and many of these are already well-established in rodent models. Additionally, targeting these phases specifically in order to increase NSC survival and favor cell differentiation towards oligodendroytes and neurons, is in our eyes the next big hurdle in immunopharmacological research.

Table 2.1: Effects of cytokines on neural stem cell behavior

Cytokine	Effects	Animal model/Region	Ref.
IL-1α	NSCs express IL-1 receptor and so IL-1α increases NSC differentiation into astrocytes, without affecting cell viability and neuronal differentiation	Mouse embryonic NSCs	(63)
IL-1β	IL-1β treatment and IL1-R1 activation of the SAP/JNK pathway inhibit NPC proliferation	Rat embryonic forebrain NPCs	(66)
IL-4	Upregulates surface adhesion molecule LFA-1 and chemokine receptors CXCR4 and CCR5 on NSCs NSCs + IL-4: Progressive disappearance of large tumors	Mouse SVZ aNPCs C57BL6J mice Sprague-Dawley rats	(105) (130)
IL-6	Induces NSC differentiation into both glutamate-responsive neurons & astrocytic cells – NSCs don't express IL-6 receptor	Neurospheres from adult mouse SVZ Mouse ESCs	(71) (131) (132)
IL-10	Increases NSC differentiation into larger numbers of oligodendrocytes and neurons but fewer astrocytes Upregulates surface adhesion molecule LFA-1 and chemokine receptors CXCR4 and CCR5 on NSCs	Mouse SVZ aNPCs	(106) (105)

IL-15	IL-15Rα subunit was expressed in NSCs and neurons, but not oligodendrocytes or astrocytes, IL-15 reduced MAP-2 levels in neurons Modulates proliferation and self-renewal of adult stem cells	Rat NSCs Mouse SVZ NSCs	(92) (93)
CNTF	Induces generation of astrocytes *in vitro*	In late embryonic (>E15), not early (E12-E13) cortical progenitors in mice	(78) (77)
LIF	Promotes growth and proliferation of human NSCs and promotes oligodendrocyte survival	In late embryonic (>E15), not early (E12-E13) cortical progenitors in mice	(77) (131) (133) (134)
TNF-α	Increased TLR2 & TLR4 expression in NSCs upon exposure Increases neurosphere volume, does not cause differentiation into glial cells, activates NF-κβ in NSCs	NSCs from adult rat SVZ Rat derived neurospheres, NSCs from adult rat SVZ	(118) (117)

Table 2.2: Factors mediating therapeutic effects of neural stem cells

Molecule(s)	Therapeutic strategy	Neurogenesis	Species	Model	Effects on NSC differentiation & support	Ref
BDNF	Transplantation of exogenous BDNF-modified NSCs into the ischemic penumbra zone (3 days after tMCAO)	Increased	Rat	tMCAO	Promotion of NSC survival, differentiation and migration in the brain (transplantation reaction favorable)	(135)
	Transplantation of BDNF-modified NSCs to the cortex in the ipsilateral hemisphere	Increased	Rats	Traumatic brain injury	BDNF increased NSCs survival, as well as their differentiation to neurons; increasing regeneration after injury	(136)
GABA	Transplantation of exogenous neural stem/progenitor cells (D6/GFP) 1 week after a brain	Increased	Rat	Thrombotic stroke: photochemical lesion	Differentiation to active neurons capable of forming synapses, no cell migration	(137)

	photochemical lesion One week after *in vitro* differentiation of D6/GFP-derived cells give rise to undifferentiated GABA-ergic neurons	Increased	Mouse	Neural stem/progenitor cells (D6/GFP)	Differentiation to astrocytes and neurons. Transplantation reaction favorable	(137)
GM-CSF	Modulation of endogenous NSCs by local application of GM-CSF at the injury site NSCPCs co-cultured with DCs	Increased	Mouse	Over-hemisection SCI	Induction of differentiation of DCs, leading to proliferation of endogenous NSPCs and later neuronal differentiation in the spinal cord Induction of proliferation and survival of NSPCs in culture	(138) (48)
nNOS/NO	Inhibition of nNos	Increased	Mouse	Cortical	Inhibition of nNOS in	(139)

	by using N5-(1-imino-3-butenyl)-L- ornithine or by nNos gene deletion in NSCs cultures NSCs were co-cultured with nNos-/- neurons for 24 hours Suppression of NO production in the brain by using osmotic minipumps with a NO synthase inhibitor (L-NAME) Analysis of the number of BrdUrd-positive cells in neurogenic brain areas of wildtype	Decreased Decreased	Mouse Rat	NSCs Cortical NSCs Lateral ventricle of an adult brain	embryonic and adult NSCs reduces neurosphere formation and proliferation NO production by neurons inhibits NSCs differentiation and proliferation Pharmacological and genetic deletion of NO production increases proliferation in neurogenic regions (SVZ, RMS and OB) Modulation of endogenous NSCs	(139) (140)

	and NO synthase knockout mice	Decreased	Rat	NO synthase knockout mouse line	(transplantation reaction not studied)	(140)
Prox1/Notch1	NPCs from embryonic mouse spinal cord were transfected with Prox1 Modulation of endogenous NSCs by in ovo electroporation of misexpressed Prox1	Decreased Decreased	Mouse Chicken	Embryonic spinal cord NPCs Neural tube of Prox1 transfected embryos.	Overexpression of Prox1 reduces proliferation and differentiation Reduced proliferation of progenitors by negatively regulating Notch1 expression (transplantation reaction not studied)	(141) (141)
Stat3	Inhibition of Stat3 production using cultured NSC from $Stat3^{flox/flox}$ mouse embryos	Decreased	Mouse	NSCs from $Stat3^{flox/flox}$	Inhibition of Stat3 promotes neurogenesis in NSCs and reduces notch1, notch2, hes5 and hes1 mRNA expression (transplantation	(142)

					reaction not studied)	
SC1/PRMT5 complex	SC1 knock-down in primary NSC by using SC1 specific siRNA oligonucleotides	Increased	Mouse	Cortical primary NSCs	Involved in the switch between proliferation and neurogenesis in NSCs (transplantation reaction not studied)	(143)
TGF-β	NSCs were incubated for 24 hours with different doses of TGF-β	Increased	Rat	Primary adult hippocampal NSCs	Increase in NSC proliferation (Transplantation reaction not studied)	(144)
	Cultured ANPs were treated for 1 week with different doses of TGF-β1	Decreased	Rat	ANPs from the ventricle wall and hippocampus	Decrease in NPC proliferation (transplantation reaction not studied)	(145)
	Intranasal treatment with TGF-β1 after stroke modulates endogenous NSCs	Increased	Mouse	MCAO	Reduction in the number of apoptotic cells, increase in progenitor proliferation in the SVZ (transplantation reaction not studied)	(146)

UCN	NSCs were cultured for 3 days in the presence of different doses of UCN and UCN antagonists	Decreased	Rat	Cortical NSCs and organotypic slices	Inhibition of proliferation (transplantation reaction not studied)	(147)

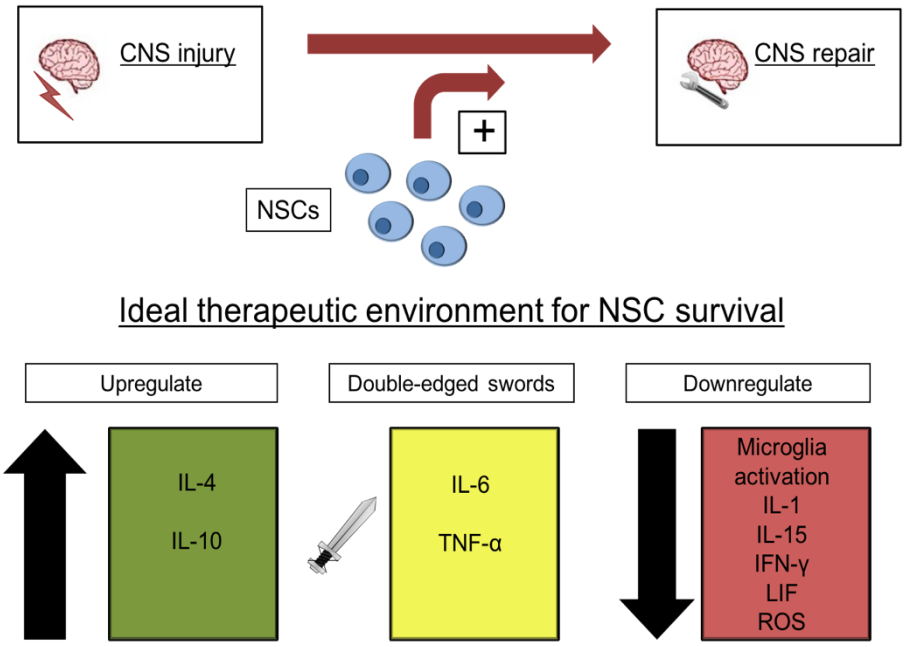

Figure 2.1: Ideal therapeutic environment for NSC survival following CNS injury. This figure illustrates one ideal therapeutic approach for transplantation of NSCs following CNS injury via up- and downregulation of certain environmental factors. Such an approach involves transplantation of NSCs into an environment which possesses a balance between positive diffusible signals and those with inhibitory effects. Upregulation of anti-inflammatory cytokines such as IL-4 and IL-10 (represented in green) and downregulation of microglia activation, ROS and pro-inflammatory cytokines such as IL-1, IL-15, IFN-γ and LIF (represented in red), may prove beneficial. There are also factors which display a ‘’double-edged sword’’ like behaviour. These factors include IL-6 and TNF-α (represented in yellow) and can be either beneficial or detrimental to the NSC environment, depending on the surrounding milieu in place.

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Chapter III

Interleukin-25 is detrimental for recovery after spinal cord injury in mice

Based on:

Interleukin-25 is detrimental for recovery after spinal cord injury in mice

Dooley D, Lemmens E, Ponsaerts P, Hendrix S:

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3.1 Abstract

The cytokine, IL-25, is thought to be critically involved in inducing a type 2 immune response which may contribute to regeneration after CNS trauma. We investigated whether applying recombinant IL-25, locally or systemically, in a mouse model of SCI improves functional and histological recovery. Repeated systemic administration of IL-25 did not influence functional recovery following SCI. In contrast, a single local administration of IL-25 significantly worsened locomotor outcome, which was evident from a decreased BMS score compared with PBS-treated controls. This was accompanied by a significant increase in lesion size, demyelination and T helper cell infiltration. These data show for the first time that IL-25 is either ineffective when applied systemically, or detrimental to spinal cord recovery when applied locally. Our findings question the potential *neuroprotective* role of IL-25 following CNS trauma.

3.2 Introduction

For decades, it has been the general opinion that an imbalanced immune response plays a major role in the pathophysiology of CNS trauma and disease. Inflammation may prove problematic for many repair processes (1), but may also exert beneficial effects when closely regulated. The type 2 response can be characterized by differentiation of $CD4^+$ T helper type 2 (Th2) cells and the production of the type 2 cytokines IL-4, IL-5, IL-9 and IL-13 (2-4). This in turn inhibits phagocytosis (5) and suppresses inflammatory cytokines (6). Therefore, type 2 immune factors can contribute to immune regulation by suppressing excessive pro-inflammatory processes (7, 8).

We as well as others have shown that cytokines associated with Th2 cells such as IL-4 (9-11) and IL-10 (12, 13), not only promote neuronal survival and regeneration, but also improve functional outcome after CNS trauma such as SCI. IL-25 (also known as IL-17E) has been suggested to be a key player in the origin of a type 2 response (2, 14). While research has begun to unravel its importance in immunity in general, conclusive data on the role of IL-25 in the CNS is lacking. Although a limited number of studies are currently available, these tend to point towards a *protective* role of IL-25 in neuroinflammation

(reviewed in (15, 16)). For example, IL-25 treatment suppresses Th17 responses and disease symptoms in EAE and is important in maintaining blood-brain barrier function (17, 18). IL-25 expression is downregulated by proinflammatory cytokines such as tumor necrosis factor-α and IL-1β, which increase acutely after trauma. Consistently, IL-25 is reduced in the proinflammatory milieu of CNS lesions (17). These findings, suggest that an increase in IL-25 may possess the therapeutic potential to provide repair after CNS trauma.

In the present short report, we tested whether recombinant murine IL-25, administered either as a single dose locally to the spinal cord or via repeated systemic injections, improves functional recovery after SCI in mice. While no clinical effect was observed following systemic administration of IL-25, surprisingly, when applied locally, IL-25 lead to a significant decrease in locomotor recovery as well as a substantial increase in lesion size, demyelination and T helper cell infiltration.

3.3 Methods

3.3.1 Spinal cord T-cut hemisection injury

A T-cut hemisection injury was performed as previously described (19-22) in 10-week-old female BALB/c mice (Harlan, The Netherlands). In brief, 10-week-old female BALB/c mice were anesthetized to undergo a partial laminectomy at thoracic level 8. Next, iridectomy scissors were used to transect left and right posterior columns, the dorsal horns and additionally the ventral funiculus. Finally, the muscles were sutured and the back skin closed with wound clips. It is important to mention is that this procedure (i.e. T-cut) results in a complete transection of the corticospinal tract and impairment of several other descending and ascending motor and sensory tracts. All experiments were performed according to the guidelines of EU Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the local ethical committee for animal experimentation at Hasselt University.

3.3.2 Treatment protocol

Mice were treated with recombinant murine IL-25 (500 ng or 1 µg; ImmunoTools, Germany) via two different methods. Mice received either a single, local application of IL-25 (1 µg), by placing a cytokine-saturated gelfoam patch at the lesion site immediately after injury, or systemic administration via repeated intraperitoneal (i.p.) injections (500 ng) once-daily for seven days starting one day before injury. The dose for the local application of IL-25 was chosen based on pilot experiments in our lab, where we observed a non-significant trend towards a decreased functional recovery after SCI following treatment with a lower dose (500 ng/ml; data not shown). The dose for systemic administration was chosen based on a previous study (17). Control animals were treated with vehicle, i.e. phosphate buffered saline (PBS) (n=7-10 mice/group).

3.3.3 Locomotion tests

Starting 1 day after surgery, functional recovery in SCI mice was measured at regular time points for three weeks using the BMS (23) as previosuly described (19, 20, 22). The BMS is a 10-point locomotor rating scale (9 = normal locomotion; 0 = complete hind limb paralysis), in which mice are scored by two investigators blinded to the experimental groups. The given scores are based on hind limb movements made in an open field during a 4-minute interval.

3.3.4 Immunofluorescence protocol

Three weeks after surgery, mice were anesthetized and transcardially perfused with Ringer's solution containing heparin, followed by perfusion with 4% paraformaldehyde. Spinal cord tissue was resected and cryoprotected in sucrose (5% followed by 30%), then stored at -80°C prior to sectioning. Histological analysis was performed on mice receiving a local and systemic application of IL-25 as previously described (20, 22). To analyze lesion size, demyelination, astrogliosis as well as T cell and microglia/macrophage infiltration, cryosections (10 µm) were pretreated with PBS (pH 7.4) containing 10% normal goat serum and 0.05% Triton X-100 for 1h. Sections were then incubated for 2h at room

temperature or overnight at 4°C with the following primary antibodies, diluted in PBS containing 1% normal goat serum and 0.05% Triton X-100: a combination of mouse anti-glial fibrillary acidic protein (GFAP) (1:500; Sigma-Aldrich, Belgium) and rabbit anti-myelin basic protein (MBP) (1:500; Millipore, Belgium), or with rat anti-CD4 (1:500; BD biosciences, Belgium) and rabbit anti-ionized calcium binding adaptor molecule 1 (Iba-1) (1:350; Wako, Germany). Subsequently, sections were washed with PBS, and corresponding secondary antibodies were applied for 1h at room temperature: goat anti-mouse Alexa Fluor 568, goat anti-rabbit Alexa Fluor 488, or goat anti-rat Alexa Fluor 568 (dilution 1:250 in PBS containing 1% normal goat serum and 0.05% Triton X-100; Life Technologies, Belgium). Finally, a DAPI nuclear stain was performed before sections were mounted using anti-fade fluorescent mounting medium (Dako, Germany). Negative controls were prepared by omitting incubation with the primary antibody from the protocol

3.3.5 Image analysis

Image analysis was performed using pictures taken by a Nikon Eclipse 80i microscope (Nikon, Brussels, Belgium), with one series containing a maximum of 8 sections per animal per analysis, as previously described (19, 20), with minor modifications. Lesion size and demyelinated area were defined by delineating the area devoid of GFAP or MBP immunoreactivity, respectively. To quantify the number of infiltrating $CD4^+$ T helper cells, the entire section containing the lesion epicenter as well as the perilesional area was used. CD4-positive but Iba-1 negative cells (to exclude $CD4^+$ microglia/macrophages) were counted manually. For quantification of astrogliosis (GFAP) and microglia/macrophage infiltration (Iba-1), TissueQuest immunofluorescence analysis software (TissueGnostics GmbH, v3.0) was used, as previously described (24). Each slide was analysed at 4X magnification and the number of IBA-1+ and GFAP+ cells at the lesion site and surrounding tissue were quantified based on a DAPI nuclear staining.

3.3.6 Cell cytotoxicity assay

To study the effect of IL-25 on cell survival *in vitro*, we used a human astrocytoma cell line (CCF) (25), a human glial (oligodendrocytic) hybrid cell line (MO3.13) (26), an immortalized murine BV-2 cell line (27) and primary cortical neuronal cells as previously described (3). Primary cortical neuronal cells were isolated from embryonic day 15 (E15) BALB/c mice and were cultured in neuronal media (Neurobasal media, containing 2% B27, 1% glutamine and 1% penicillin/streptomycin). MO3.13, CCF and BV2 cell lines were used to study survival of oligodendrocytes, astrocytes and microglia respectively. All cells were seeded on poly-d-lysine-coated 96-wells at a density of 5 x 10^3 cells/well. Cells were grown under optimal conditions and treated with selected concentrations of IL-25 (5 ng/ml, 50 ng/ml, 500 ng/ml, and 1 µg/ml for 72 hours to measure viability. To measure a potential beneficial effect of IL-25 inhibition on neuronal death, 1 hour after isolation, cells were incubated in B27 deficient media with or without IL-25 for 72 hours. CCF cells were cultured in DMEM/F-12 medium containing 10% FCS and 1% penicillin/streptomycin and BV2 cells were cultured in (DMEM medium, containing 10% FCS and 1% penicillin/streptomycin. MO3.13 cells were cultured in MEM medium with 10% FCS and 1% penicillin/streptomycin. After 70% confluency, MO3.13 cells were differentiated in DMEM medium by removing FCS from the culture medium and adding 100nM 4-α-phorbol 12-myristate 13-acetate (PMA) to the cells for 72 hours (3, 28). Following treatment of all cell types with varying concentrations of IL-25, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (1 mg/ml) was added for 4 h. The cells were lysed in a mixture of dimethyl sulfoxide (DMSO) and glycine (0.1 M), and the absorption was measured at 540nm using a microplate reader (Bio-Rad, Nazareth, Belgium). Cells treated with 10% DMSO were used as positive control.

3.3.7 Statistical analyses

Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., USA). Differences between treatment groups in lesion size, demyelinated area and T cell numbers, were calculated using the Mann-Whitney

U test. Differences in astrogliosis, microglia/macrophage infiltration, as well as in the BMS data were analyzed using the two-way ANOVA for repeated measurements (with Bonferroni post hoc tests). Differences were considered to be significant when $p < 0.05$. Data in graphs are presented as mean ± SEM.

3.4 Results & Discussion

In this short report, we investigated whether increasing levels of IL-25, a potential inducer of a type 2 immune response, can promote functional recovery in a mouse model of SCI. Considering the widespread expression of the receptor A subunit of the IL-17 receptor which forms a complex with the receptor B subunit upon binding with IL-25 (29), we aimed to distinguish between local and systemic effects of treatment. We found that local application of IL-25 led to a significant worsening in motor performance following injury compared with PBS controls (Figure 3.1a; * $p < 0.05$). At the histological level, these results were accompanied by a 30% increase in lesion size (Figure 3.1b, g, h; *** $p < 0.001$) and demyelinated area (Figure 3.1c, i, j; ** $p < 0.01$). Surprisingly, systemic IL-25 treatment did not influence functional recovery (Figure 3.2a). Furthermore, there was no effect of systemic IL-25 treatment on lesion size or demyelinated area (Figure 3.2b, c).

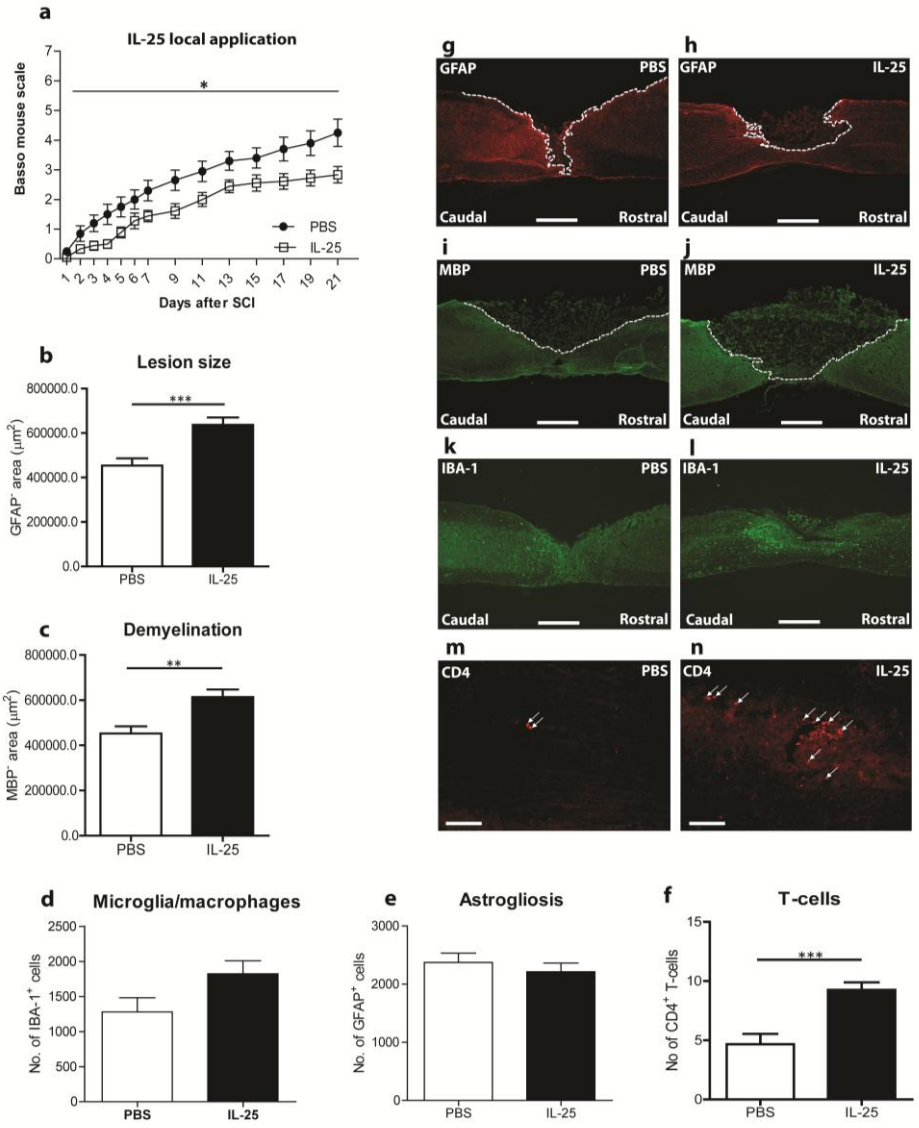
a
IL-25 local application
Basso mouse scale
Days after SCI
PBS
IL-25
b
Lesion size
GFAP⁻ area (µm²)
c
Demyelination
MBP⁻ area (µm²)
d
Microglia/macrophages
No. of IBA-1⁺ cells
e
Astrogliosis
No. of GFAP⁺ cells
f
T-cells
No of CD4⁺ T-cells
g
GFAP PBS
h
GFAP IL-25
i
MBP PBS
j
MBP IL-25
k
IBA-1 PBS
l
IBA-1 IL-25
m
CD4 PBS
n
CD4 IL-25
Caudal
Rostral

Figure 3.1: Local application of IL-25 decreases functional outcome and increases lesion size, demyelination and T-cell infiltration following SCI in mice

(a) Mice receiving local application of IL-25 show a statistically significant decrease in functional outcome when compared to those receiving PBS, as measured by the BMS (*$p < 0.05$), n = 9–10 mice/group. **(b)** Lesion size and **(c)** demyelinated area were quantified by staining for **(g, h)** GFAP and **(i, j)** MBP, respectively, as depicted by the dotted white line. Image analysis revealed a significant increase in **(b)** lesion size and **(c)** demyelinated area in animals treated locally with IL-25, compared with the PBS control group. Quantification of **(d)** Iba-1+ and **(e)** GFAP+ cells after SCI using TissueQuest software revealed no significant difference in **(k, l)** microglia/macrophages numbers or (g, h astrogliosis between animals receiving PBS or IL-25. **(f)** Significantly more CD4+ T cells are present in the spinal cord sections of the **(n)** IL-25-treated mice, compared with **(m)** PBS-treated mice, 3 weeks after SCI. Scale bars of representative photomicrographs: **(g–l)** = 500 µm, m+ n = 50 µm. Data represent mean ± SEM. ***$p < 0.001$, **$p< 0.01$, n = 5–6 mice/group

We also analyzed the presence of microglia/macrophages (Figure 3.1d, k, I & 2d) as well as astrogliosis (Figure 3.1e, g, h & 2e), by quantifying the number of Iba-1+ and GFAP+ cells respectively. However, no significant differences were found between IL-25 treated and control groups, in both local and systemic treatment. Following quantification of perilesional $CD4^+$ T cells, we found a significant increase in the number of cells in tissue sections from mice treated locally with IL-25, compared with PBS controls (Figure 3.1f, m, n; *** $p < 0.001$). There was no effect of systemic IL-25 treatment on the number of $CD4^+$ T cells (Figure 3.2f). The precise role of T cells following CNS injury is still subject to discussion. Although they may display beneficial effects under certain conditions, accumulation of endogenous T cells, may be considered detrimental (7, 20).

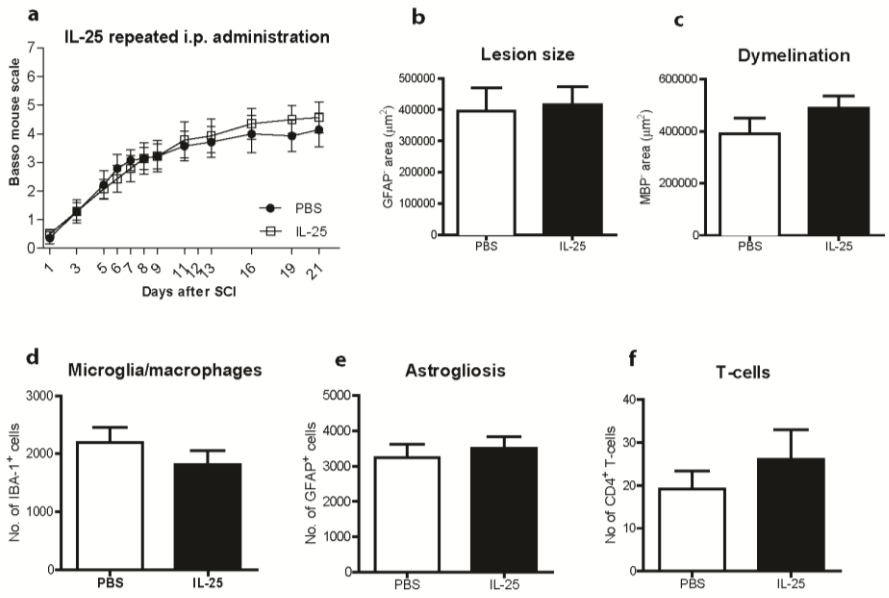

Figure 3.2: Systemic application of IL-25 has no effect on functional or histological outcome following SCI in mice

(a) Repeated i.p. administration of IL-25 has no significant effect on functional outcome when compared to those receiving PBS, as measured by the BMS, n=7 mice/group. Sections were stained for GFAP and MBP to determine the **(b)** lesion size and **(c)** demyelinated area respectively. Image analysis revealed no significant difference in **(b)** lesion size or **(c)** demyelinated area in animals treated systemically with IL-25, compared with the PBS control group. Quantification of **(d)** Iba-1$^+$ and **(e)** GFAP$^+$ cells after SCI using TissueQuest software revealed no significant difference in microglia/macrophages numbers or astrogliosis between animals receiving PBS or IL-25. **(f)** There was no significant difference in the number of CD4$^+$ T cells present in spinal cord sections of IL-25-treated mice, compared with PBS-treated mice. Data represent mean ± SEM, n = 7 mice/group.

We also investigated the effect of IL-25 *in vitro* on cell viability. However, we observed no significant effects of various concentrations of IL-25 on survival of oligodendrocytes, astrocytes, microglia or primary cortical neuronal cells (Figure 3.3a-d). These results may be consistent with the lacking effect of systemic IL-25 treatment *in vivo*. They also indicate that the toxic effect observed locally, is not caused by a direct effect on the above cell types. This suggests that local administration of IL-25 following SCI activates an indirectly mediated cascade of detrimental immune events.

Although a member of the rather *pro-inflammatory* IL-17 family, IL-25 plays a somewhat different role in the context of CNS inflammation. IL-25 mRNA is highly expressed in polarized Th2 cells (14) and IL-25 administration in mice drives the Th2 response, by elevating IL-4 and IL-13 levels (14, 16). Systemic IL-25 regulates the development of autoimmune inflammation mediated by IL-17-producing cells and suppresses EAE symptoms in a relapse-remitting model (14). Additionally, delivery of IL-25 to the CNS in two different models of neuroinflammation, was able to drive microglia and macrophages to a more anti-inflammatory and tissue-protective phenotype (30).

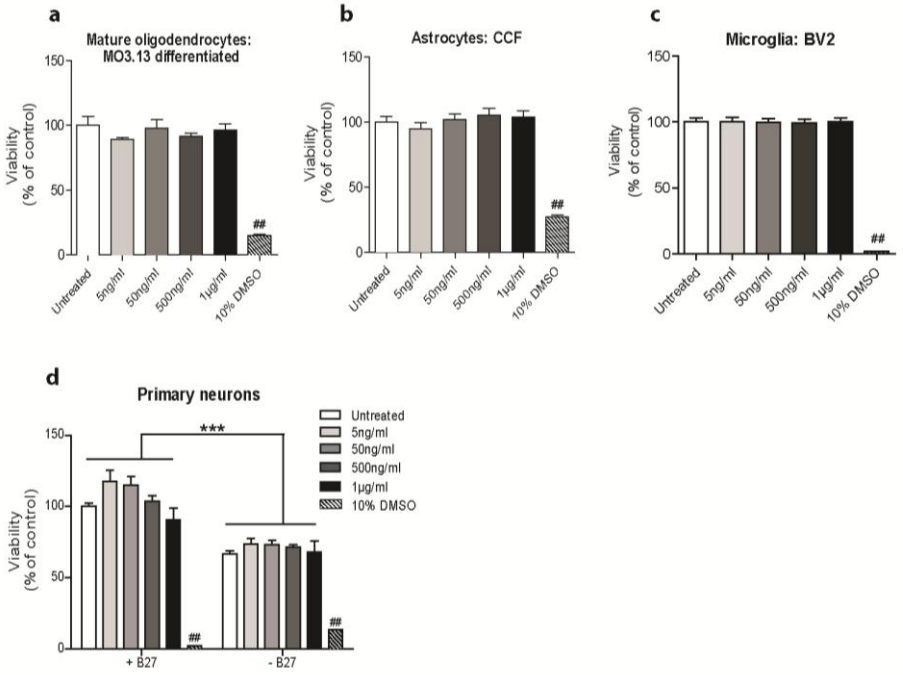

Figure 3.3: IL-25 has no effect on mature oligodendrocyte, astrocyte, microglia or primary neuron cell viability

(a) MO3.13 cells were differentiated to mature oligodendrocytes using PMA for 72 h and were treated for 48 h with selected concentrations of IL-25. **(b, c)** The astrocytic and microglial cell lines (CCF and BV2 respectively) were treated for 48 h with selected concentrations of IL-25. **(d)** Primary neurons were incubated with selected concentrations of IL-25 for 48 h in the presence or absence of B27. B27 deprivation induced a decreased cell viability, but IL-25 treatment had no effect on this. The selected concentrations of IL-25 used for all cell types were: 5 ng/ml, 50 ng/ml, 500 ng/ml and 1 µg/ml. Cell survival was measured using an MTT assay and values are expressed as percentage of the control. **(a-d)** There was no significant effect observed on cell viability in all cell types tested. Data represent mean ± SEM of 1 representative experiment (from 2-3 independent experiments) *** $p < 0.001$.

In contrast to the above positive effects on neuroinflammation, our results indicate that systemic administration of IL-25 after SCI in mice is ineffective in improving functional outcome. This result was surprising given that we as well as others have shown that treatment with cytokines which induce a type-2 response, such as IL-4 and IL-10, are neuroprotective following SCI (11, 12). Differences in systemic versus local administration is a well-known phenomenon (31-33) and our results are consistent with this as local application of IL-25 decreased functional recovery after SCI. Furthermore, we observed that a lower local dose of IL-25 (500 ng/ml) lead to a non-significant trend towards a decrease in functional outcome after SCI (data not shown), indicating that route of administration and dosing are important factors which must be considered prior to use of cytokine therapy.

Interestingly, it was previously demonstrated that intraspinal treatment with IL-10 exacerbated damage and lesion size, while when given systemically; it improved recovery after SCI (12). Taken together, these data reiterate the well-recognized fact that the route of administration is of pivotal importance when determining a therapeutic outcome. Additionally, Mearns et al recently questioned the role of IL-25 in Th2 cell differentiation and the induction of potentially beneficial Th2-cell responses (34). In contrast to previous reports, the authors used reporter mouse technology to show that IL-25 is dispensable during differentiation and development of Th2 cells (34). In our study, IL-25 failed to have an effect systemically and even worsened functional outcome when applied locally. This suggests that the direct involvement of IL-25 in driving a Th2 response remains questionable. Furthermore, based on the current opinion on the role of Th2 cytokines following CNS injury (8), it is safe to suggest that factors which regulate the type 2 immune response, are in turn, key players in CNS pathology.

In this short report, we show for the first time that IL-25 is either ineffective when applied systemically or detrimental to spinal cord recovery when applied locally. These findings indicate that the potential positive effects of IL-25 and its involvement in driving a beneficial type 2 immune response, need to be carefully reconsidered prior to its use therapeutically.

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Chapter IV

Systemic administration of IL-13 improves functional recovery following spinal cord injury in mice

Based on:

Systemic administration of IL-13 improves functional recovery following spinal cord injury in mice

Dooley D*, Lemmens E*, Nelissen S, Vangansewinkel T, Hendrix S:

In preparation.

*equally contributing first author

4.1 Abstract

Increasing evidence suggests that cytokines associated with Th2 cells, contribute to improved therapeutic outcome after trauma to the CNS. We have previously shown that IL-13 potently stimulates neurite outgrowth in primary neurons and organotypic brain slices *in vitro.* Additionally, we have demonstrated that IL-13 levels are significantly decreased in the spinal cord tissue, within hours after after injury in mice. Therefore, in the present study, we investigated whether recombinant murine IL-13, administered either as a single dose locally to the spinal cord or via repeated systemic injections, improves functional outcome after SCI in mice. While no clinical effect was observed following local administration of IL-13, systemic administration of IL-13 lead to a significant increase in locomotor recovery as well as a significant increase in the number of Arg-1^+ *alternatively activated* microglia/macrophages. Taken together, these data indicate that IL-13 may be a promising therapeutic agent for treatment following SCI, however further research is required to optimise the optimal method of administration and determine the mechanistic effects of IL-13 *in vivo*.

4.2 Introduction

To date, regeneration and the recovery of function after as SCI is limited. Trauma induced inflammation combined with the minimal ability of neural tissue to regenerate, are two major factors which hinder recovery. Increasing evidence suggests that cytokines associated with Th2 cells such as IL-4 (1-3) and IL-10 (4, 5), not only promote neuronal survival and regeneration, but also improve functional outcome after CNS trauma such as SCI. IL-13, a cytokine closely related to IL-4 (6), is a canonical *anti-inflammatory* Th2 cytokine, which in some contexts can also be *pro-inflammatory* (7). IL-13 has been shown to exert neuroprotective effects in EAE, by decreasing inflammatory cell infiltration and axonal loss as well as reducing clinical symptoms (8-10). However, at present, there is limited literature available indicating whether IL-13 is capable of promoting repair after CNS trauma. We have previously demonstrated that IL-13 stimulates key processes of regeneration, by modulating neuronal survival

and increasing neurite outgrowth from primary neurons and organotypic brain slices *in vitro* (unpublished observations).

Following CNS trauma, such as SCI, the immune system responds acutely via the induction of a complex pro-inflammatory state, which is accompanied by a lesser, transient anti-inflammatory reaction (11, 12). We have also recently demonstrated that IL-4 levels are significantly decreased in the serum and IL-13 levels are significantly decreased in the spinal cord, within hours after after SCI in mice (13). Therefore, it seems plausible that application of IL-13 in the acute phase after SCI may provide therapeutic potential. In the present study, we tested whether recombinant murine IL-13, administered either as a single dose locally to the spinal cord or via repeated systemic injections, improves functional recovery after SCI in mice. While no clinical effect was observed following a single local administration of IL-13, multiple systemic administrations of IL-13 lead to a significant increase in locomotor recovery as well as a significant increase in the number of Arg-1^+ *alternatively activated* microglia/macrophages.

4.3 Methods

4.3.1 Spinal cord hemisection injury

A T-cut hemisection injury was performed as previously described (13-15) in 10-week-old female BALB/c mice (n=10 per treatment group) (Harlan, The Netherlands). In brief, 10-week-old female BALB/c mice were anesthetized to undergo a partial laminectomy at thoracic level 8. Next, iridectomy scissors were used to transect left and right posterior columns, the dorsal horns and additionally the ventral funiculus. Finally, the muscles were sutured and the back skin closed with wound clips. It is important to mention is that this procedure (i.e. T-cut) results in a complete transection of the corticospinal tract and impairment of several other descending and ascending motor and sensory tracts. All experiments were performed according to the guidelines of EU Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the local ethical committee for animal experimentation at Hasselt University.

4.3.2 Treatment protocol

For systemic recombinant IL-13 treatment, mice were injected once daily i.p. with 500ng of mouse recombinant IL-13 (Peprotech, UK), (the dose of which was chose based on pilot experiments in our lab) in PBS for 7 consecutive days, starting on the day of SCI (i.e. until day 6 post-SCI). For local application of IL-13, mice were treated with 500ng of mouse recombinant IL-13 (Peprotech, UK), by placing a cytokine-saturated gelfoam patch at the lesion site immediately after injury. In both experimental set ups, control animals were treated with PBS.

4.3.3 Locomotion tests

Starting 1 day after surgery, functional recovery in SCI mice was measured at regular time points for 3 or 12 weeks using the BMS (16) as previosuly described (13-15). The BMS is a 10-point locomotor rating scale (9 = normal locomotion; 0 = complete hind limb paralysis), in which mice are scored by two investigators blinded to the experimental groups. The given scores are based on hind limb movements made in an open field during a 4-minute interval.

4.3.4 Immunofluorescence protocol

Three weeks after surgery, mice were anesthetized and transcardially perfused with Ringer's solution containing heparin, followed by perfusion with 4% paraformaldehyde. Spinal cord tissue was resected and cryoprotected in sucrose (5% followed by 30%), then stored at -80°C prior to serial sectioning. Histological analysis was performed on mice receiving a local and systemic application of IL-13 as previously described (15, 17, 18). To analyze lesion size, demyelination, astrogliosis as well as T cell and microglia/macrophage infiltration, serial cryosections (10 µm) were pretreated with PBS (pH 7.4) containing 10% normal goat serum and 0.05% Triton X-100 for 1h. Sections were then incubated for 2h at room temperature or overnight at 4°C with the following primary antibodies, diluted in PBS containing 1% normal goat serum and 0.05% Triton X-100: a combination of mouse GFAP (1:500; Sigma-Aldrich, Belgium) and rabbit MBP (1:500; Millipore, Belgium), or with rat anti-CD4 (1:500; BD biosciences, Belgium) and rabbit Iba-1 (1:350; Wako, Germany). To

identify *alternatively activated* microglia/macrophages, sections were permeabilized using 0.1% Triton X-100 for 30 minutes and treated with 20% serum in Tris-buffered saline (TBS, pH 7.5) for 2 hours. Incubation with primary goat anti-arginase-1 (Arg-1) antibody (Santa Cruz, Germany; sc-18354), diluted 1:50 in TBS containing 10% milk powder (TBS-M), was performed overnight at 4°C. Sections were then washed with PBS, and corresponding secondary antibodies were applied for 1h at room temperature: goat anti-mouse Alexa Fluor 568, goat anti-rabbit Alexa Fluor 488, goat anti-rat Alexa Fluor 568 (dilution 1:250 in PBS containing 1% normal goat serum and 0.05% Triton X-100; Life Technologies, Belgium) or donkey anti-goat DyLight 650 (Abcam, UK). Finally, a DAPI nuclear stain was performed before sections were mounted using anti-fade fluorescent mounting medium (Dako, Germany). Negative controls were prepared by omitting incubation with the primary antibody from the protocol.

4.3.5 Image analysis

Image analysis was performed using pictures taken by a Nikon Eclipse 80i microscope (Nikon, Brussels, Belgium), with one series containing a maximum of 8 sections per animal per analysis, as previously described (15, 17, 19), with minor modifications. Lesion size and demyelinated area were defined by delineating the area devoid of GFAP or MBP immunoreactivity, respectively. To quantify the number of infiltrating $CD4^+$ T helper cells, the entire section containing the lesion epicenter as well as the perilesional area was used. CD4-positive but Iba-1 negative cells (to exclude $CD4^+$ microglia/macrophages) were counted manually. For quantification of *alternatively activated* microglia/macrophages [Arginase (Arg)-1], TissueQuest immunofluorescence analysis software (TissueGnostics GmbH, v3.0) was used, as previously described (15, 20). Each slide was analysed at 10X magnification and the number of Arg-1^+ cells at the lesion site were quantified based on a DAPI nuclear staining.

4.3.6 Statistical analysis

All statistical analyses were performed using Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The BMS locomotion tests as well as histological evaluation of astrogliosis and microglia/macrophage intensities were analysed using a two-way ANOVA for repeated measurements with Bonferroni correction for multiple comparisons. All other differences between two groups were evaluated using the nonparametric Mann-Whitney U-test. Differences were considered statistically significant when $p<0.05$. Data shown represent mean values per experimental group ± SEM.

4.4 Results and Discussion

SCI is accompanied by a complex pro-inflammatory immune response, leading to secondary damage and limited repair. However, increasing evidence suggests that restoring the immune balance may provide a more favourable environment for functional recovery and regeneration. One possible strategy to downregulate this pro-inflammatory state, may be to increase the levels of anti-inflammatory factors. Furthermore, it has been previously demonstrated that anti-inflammatory or Th2/M2 factors such as IL-13 can downregulate the production of pro-inflammatory factors such as TNF-alpha, IL-1beta and IL-6 (21).

To study its potential therapeutic effects, we administered recombinant IL-13, both locally and systemically, in a well-established mouse model SCI. For the local application, mice were treated with recombinant IL-13 (500ng/ml), by placing a cytokine-saturated gelfoam patch at the lesion site immediately after injury and functional recovery was monitored using the BMS. Surprisingly, 3 weeks post injury, IL-13-treated mice showed no functional improvement following treatment, compared with PBS controls (Figure 4.1A).

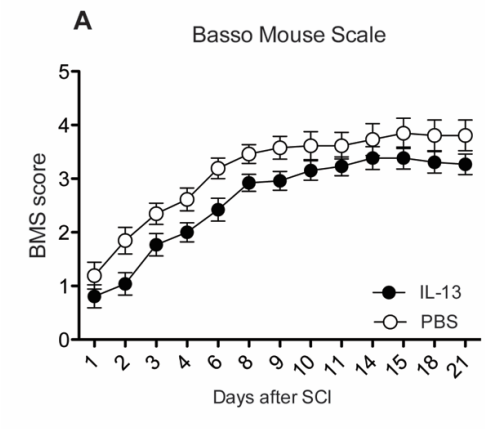

Figure 4.1: Local application of IL-13 has no effect on functional recovery following SCI in mice.
(A) Following SCI, mice were treated with a local application of recombinant IL-13 (500ng/ml), by placing a cytokine-saturated gelfoam patch at the lesion site immediately after injury and functional recovery was monitored using the BMS. IL-13-treated mice showed no functional improvement following treatment, compared with PBS controls, **n=13/group.**

For systemic treatment, mice received recombinant IL-13 at a dose of 500ng/ml, once daily for 7 days, starting on the day of surgery. Similarly to the local application, functional recovery was monitored using the BMS. One week post treatment, mice which received IL-13 displayed a significantly improved functional outcome compared with PBS treated controls, which persisted for 12 weeks post injury (Figure 4.2A). Given this improved clinical effect, we also investigated the effect of systemic IL-13 at the histological level; more specifically on lesion size and demyelination. Surprisingly, we found no significant effect of IL-13 treatment on lesion size and (GFAP) or demyelinated area (MBP) (Figure 4.2B + C respectively).

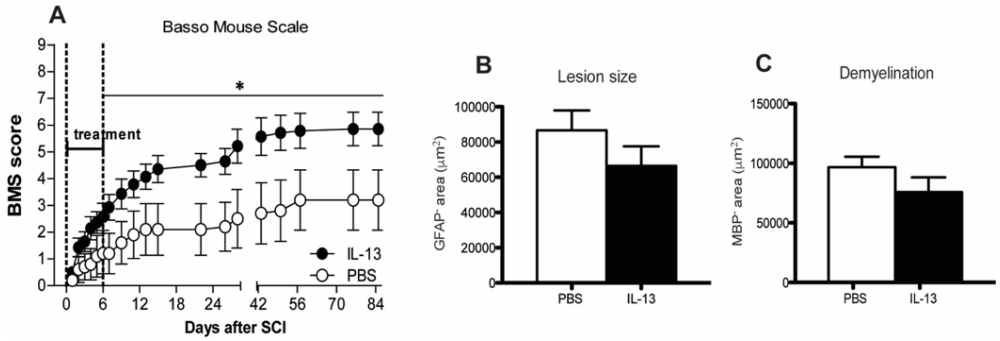

Figure 4.2: Systemic administration of recombinant IL-13 improves functional recovery following SCI in mice.
(A) After subjecting mice to a dorsal T-cut hemisection injury, they received recombinant IL-13 at a dose of 500ng/ml, systemically once daily for 7 days, starting on the day of surgery. Functional recovery was monitored using the Basso mouse scale (BMS). Mice treated with IL-13 displayed a significantly improved functional outcome compared with PBS treated controls. Analysis of **(B)** lesion size and **(C)** demyelinated area revealed no significant differences in IL-13-treated mice compared with PBS controls. *** p < 0.05, n=5-7/group.**

In the case of treatment with IL-10, another anti-inflammatory Th2 cytokine, a decrease in lesion size was observed, however, this was measured two months' post SCI (22). Having ruled out a direct effect of IL-13 on lesion volume and demyelinated area and given that inflammation also plays an important role in defining functional outcome (11), we also quantified the presence of microglia/macrophages and astrogliosis at the lesion site by performing an intensity analysis for Iba-1 (Figure 4.3A). and GFAP respectively (Figure 4.3B). However, for both analyses, no significant differences were found between control and IL- 13 treated mice. Finally, we investigated the presence of *alternatively activated* microglia/macrophages by staining for Arg-1 at the lesion site (Figure 4.3C). Upon quantification, we observed a significant increase in the number of Arg-1^+ microglia/macrophages at the lesion site in IL-13-treated mice compared with PBS controls (Figure 4.3D, E).

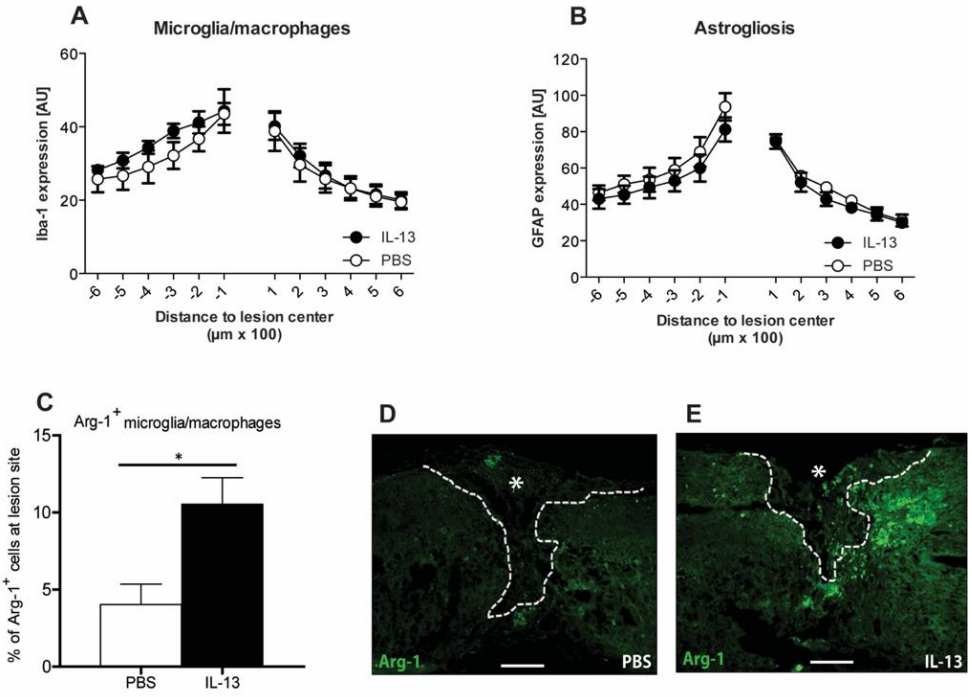

Figure 4.3: Systemic administration of recombinant IL-13 leads to a significant increase in Arg-1$^+$ *alternatively activated* microglia/macrophages.
Immunofluorescence staining for Iba-1 and GFAP revealed no significant differences in **(A)** microglia/macrophage presence or **(B)** astrogliosis. TissueQuest quantification revealed a significant increase in the percentage of **(C)** Arg-1$^+$ cells in **(E)** IL-13-treated compared with **(D)** PBS-treated mice. *** p < 0.05, n=5-7/group.**

IL-13 has been shown to be a key factor, capable of inducing a macrophage phenotype switch from a rather 'detrimental' M1 phenotype to a more 'beneficial' *alternatively activated*, M2 phenotype (6, 23). Therefore, it is tempting to speculate that systemic treatment with IL-13 also leads to a switch *in vivo* towards a more neuroprotective microglia/macrophage phenotype, which may correspond to the improved functional recovery observed in IL-13-treated mice.

Given the limited effect of IL-13 on other inflammatory parameters and lesion remodeling when administered systemically, it is difficult to specifically pin-down the mechanistic effects of IL-13 after SCI. It is also interesting to note the varying results observed in local versus systemic IL-13 treatment, however this is not the first time that we have observed differences based on route of administration using cytokine treatment following SCI (15). Differences in systemic versus local administration is a well-known phenomenon (25-27) and our results are consistent with this as systemic administration of IL-13 improved functional recovery after SCI, while local application had no effect.

4.5 Conclusion

Taken together, our findings indicate that IL-13 has the therapeutic potential to improve locomotion recovery after SCI, most likely via a direct or indirect effect on microglia/macrophages and driving them towards a more alternatively activated, neuroprotective phenotype. However, given the variation observed in route of administration, a more favourable treatment method and further investigation into the mechanistic effects of IL-13, is still needed. These options will be further discussed in **chapter V**.

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Chapter V

Cell-based delivery of interleukin-13 directs alternative activation of macrophages resulting in improved functional and histopathological outcome following spinal cord injury

Based on:

Cell-based delivery of interleukin-13 directs alternative activation of macrophages resulting in improved functional and histopathological outcome following spinal cord injury

Dooley D, Lemmens E, Vangansewinkel T, Le Blon D, Hoornaert C, Ponsaerts P[#] Hendrix S[#]:

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[#] equally contributing senior author

5.1 Abstract

MSC transplantation has been suggested to provide repair after CNS injury, however, the therapeutic effects to date have been rather limited. Therefore, in the present study, rather than focusing on the intrinsic regenerative capacity of MSCs, we aimed to enhance their immunomodulatory properties via genetic engineering, to enable continuous secretion of the *anti-inflammatory* cytokine IL-13. We used MSCs as carriers of IL-13 (MSC/IL-13) and investigated their therapeutic potential, compared with non-engineered MSCs, in a mouse model of SCI. We show for the first time that transplanted MSCs which continuously secrete IL-13, not only significantly improve functional recovery following SCI in mice, but also on the histopathological level, decrease lesion size and demyelinated area by over 40%. Further detailed histological analyses in the $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ transgenic mouse model indicated that transplantation of MSC/IL-13 significantly decreases the number of resident microglia and significantly increases the number of *alternatively activated* macrophages at both the graft and lesion site. Additionally, the number of macrophage-axon contacts in MSC/IL-13-treated mice was decreased by 50%, suggesting a reduction in axonal dieback, and in turn, improved functional outcome. In summary, our data provide first evidence that transplantation of IL-13-secreting MSCs, and the subsequent introduction of M2a macrophages to the injured spinal cord, lead to improved functional and histopathological recovery in a mouse model of SCI.

5.2 Introduction

Stem cell therapies for CNS injury have raised a lot of hope amongst patients, doctors and scientists in recent years. Although we are still in the early stages of developing successful approaches in humans, numerous pre-clinical animal studies support the therapeutic ability of stem cells (1-3). Despite these observations, the dual role of the neuro-inflammatory response following CNS injury, makes stem cell-supported regeneration difficult due to the presence of

inhibitory immune factors which are upregulated in and around the lesion site. Therefore, modulating the inflammatory milieu by upregulating anti-inflammatory cytokines may be crucial when designing therapies for CNS repair (4). With this in mind, using MSCs as an immune-modulating cellular therapy, may exert positive effects in rodent models of SCI (5, 6). Here, we test the hypothesis that using MSCs as carriers for the delivery of the canonical anti-inflammatory cytokine IL-13, may further enhance their therapeutic potential.

Despite much debate regarding the detrimental effects of CNS inflammation, many studies have also outlined its significance in tissue repair, including a therapeutic potential of microglia/macrophages in promoting axonal regeneration (7, 8). Almost all tissues contain several types of phagocytic cell populations, consisting of macrophages and/or microglia, which have specialised functions and distinct phenotypic properties. (9, 10). A rather simplistic but pragmatic way to distinguish the varying microglia/macrophage subsets is to divide them into *classically* (M1) or *alternatively activated* (M2) phenotypes (11, 12). M2 microglia/macrophages differentiate from the *classically activated* M1 microglia/macrophages and are less inflammatory in nature. They are characterised by a reduced nitric oxide production and less secretion of pro-inflammatory cytokines (13). They also express markers such as Arg-1 and *Found in inflammatory zone 1* (FIZZ1) which differentiate them from *classically activated* M1 microglia/macrophages (14). However, a more specific characterisation indicates that upregulation of major histocompatibility complex (MHC)-II (in both M1 and M2 cell subsets) is associated with macrophage activation. It is currently suggested that the joint expression of MHC-II and Arg-1/FIZZ1 is indicative of a neuroprotective and anti-inflammatory, M2a phenotype (15), however, the exact mode-of-action of this polarised cell type has not yet been unravelled. Nevertheless, reducing the pro-inflammatory M1 phenotype upon CNS injury in favour of the beneficial M2a phenotype, is of particular therapeutic interest.

This polarising approach towards an M2a phenotype may be of great therapeutic value, particularly following SCI. After injury, infiltration of axon-attacking

macrophages greatly contributes to axonal retraction and the deleterious phenomenon known as axonal dieback (16, 17). This leads to exacerbation of damage and increased functional deficits. Therefore, in this study, we aimed to target these attacking macrophages and drive them towards a less destructive phenotype, in order to limit axonal dieback and improve functional recovery after SCI. To do this, we chose to use the immunomodulatory cytokine, IL-13, which is a well-known inducer of the M2a microglia/macrophage phenotype (18, 19). IL-13 has also been shown to exert neuroprotective effects in the experimental autoimmune encephalomyelitis model of multiple sclerosis, by decreasing inflammatory cell infiltration and axonal loss as well as reducing clinical symptoms (20-22). We recently demonstrated that after SCI in mice, IL-13 levels decrease significantly in the spinal cord within hours after injury (23). Therefore, given the drop in IL-13 levels after injury and it's polarising capabilities towards a more *neuroprotective* M2 macrophage phenotype, it is plausible that application of IL-13 in the acute phase after SCI may have therapeutic potential.

In order to efficiently deliver IL-13 to the injured spinal cord, we used autologous MSCs genetically engineered to secrete IL-13. We hypothesised that this enhanced cellular therapy is capable of modulating the microglia/macrophage response and improve functional recovery after SCI. In order to support our hypothesis, we investigated the effects of grafting control MSCs and those expressing IL-13 (MSC/IL-13), in a mouse model of SCI. We show for the first time that transplantation of MSCs which continuously secrete IL-13, significantly improve functional recovery and decrease lesion size as well as demyelinated area after SCI. Finally, we propose a mode-of-action in which delivery of IL-13 from MSC grafts to the injured spinal cord, polarises macrophages to a neuroprotective, M2a phenotype, subsequently reducing the number of axon-attacking macrophages and improving functional outcome.

5.3 Materials and methods

5.3.1 Animals

Wild type (WT) BALB/c OlaHsd (strain code 162) and wild type C57BL/6 mice (strain code 027) were obtained from Harlan and Charles River Laboratories respectively. $CX_3CR1^{eGFP/eGFP}$ mice (strain code 005582) and $CCR2^{RFP/RFP}$ mice (strain code 017586) were obtained from Jackson Laboratories. $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ mice were obtained by breeding $CX_3CR1^{eGFP/eGFP}$ mice with $CCR2^{RFP/RFP}$ mice. Resulting double transgenic mice have one allele of the CX_3CR1 gene replaced by eGFP and the other allele of the CCR2 gene replaced by RFP (24). This results in the presence of green fluorescent microglia ($eGFP^+RFP^-$) and red fluorescent infiltrating macrophages/monocytes ($eGFP^-RFP^+$ and $eGFP^+RFP^+$). All animals were housed in a conventional animal facility at Hasselt University or University of Antwerp under regular conditions, i.e. in a temperature-controlled room (20±3°C) on a 12h day-night light cycle and with food and water *ad libitum*. Male mice were used for all experiments, except for those carried out in $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ mice, where equal numbers of males and females were used. All experiments were performed using 8-10-week old mice and were approved by the local ethical committees and were performed according to the guidelines described on the protection of animals used for scientific purposes at Hasselt University (EU Directives 2010/63) and University of Antwerp (2011/13 and 2012/39).

5.3.2 Isolation, genetic engineering and culturing of MSCs

In this study, we used two previously established and characterised bone marrow-derived MSC lines originally derived from BALB/c and C57BL/6 mice (25, 26). Both the parental BALB/c and C57BL/6 MSC lines, as well as derivatives thereof genetically engineered to express IL-13, were used for transplantation experiments. For generation of BALB/c and C57BL/6 MSC/IL-13, the pCHMWS-mIL-13-IRES-Pac lentiviral (LV) vector was used for MSC transduction according to previously optimised procedures (27, 28). Following LV transduction, Pac-expressing MSCs were selected with puromycin (10 µg/mL; InvivoGen).

Expression of IL-13 was confirmed by murine IL-13 ELISA (eBioscience). For routine expansion, all MSC lines were cultured in standard cell culture plasticware (well plates and/or culture flasks) in complete expansion medium as previously described (27, 28). Culture medium of MSC/IL-13 was further supplemented with 5µg/mL puromycin (InvivoGen), in both C57BL/6 and BALB/c-derived lines. All MSC cultures were kept at 37°C and 5% CO_2 and were passaged 1:5 following 0.05% trypsin-EDTA (Invitrogen) treatment every 5 to 7 days. BALB/c-derived MSCs were used in experiments carried out in WT BALB/c mice and C57BL/6 MSCs were used for transplantation experiments in WT C57BL/6 and transgenic C57BL/6 $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ mice.

5.3.3 Spinal cord hemisection injury

A T-cut spinal cord hemisection injury was performed as previously described (n=10 per treatment group). (23, 29-33). Briefly, 8-10-week old anesthetized mice underwent a partial laminectomy at thoracic level T8. Iridectomy scissors were used to transect left and right dorsal funiculi, the dorsal horns and additionally the ventral funiculus (34). The back muscles were sutured and the skin was closed with wound clips. Bladders were manually voided daily until animals were able to urinate independently.

5.3.4 Cell transplantation

For transplantation experiments, MSC and MSC/IL-13 cell populations were harvested via trypsin-EDTA treatment. Cells were then washed twice with NaCl, resuspended in NaCl and kept on ice until spinal cord transplantation. The animals were divided into three groups: those receiving an injection of MSCs, MSC/IL-13 or NaCl (control). A motorised stereotaxic injector pump (Stoelting, Ireland) with a 34-gauge needle attached to a 10µl Hamilton Syringe was positioned 3mm rostral to the lesion site. The needle was stereotactically inserted into the spinal cord at a depth of 1mm and $5x10^4$ cells in 1.5µl NaCl were injected over a four minute time period. The needle was subsequently kept in place for an additional four minutes to allow pressure equilibration and

prevent backflow of the injected cell suspension. For transplantation experiments carried out in $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ mice, no NaCl control group used, given that the research objectives in question concerned potential differences between IL-13-secreting MSCs and control MSCs. Furthermore, $1.5x10^5$ cells were grafted to allow for more detailed histological quantification.

5.3.5 Locomotion tests

Starting 1 day after surgery, functional recovery in SCI mice was measured for 4 weeks using the BMS (35). The BMS is a 10-point locomotor rating scale (9 = normal locomotion; 0 = complete hind limb paralysis), in which mice are scored by two investigators blinded to the experimental groups. The given scores are based on hind limb movements made in an open field during a 4-minute interval.

5.3.6 Immunofluorescence analysis

Spinal cord cryosections (10µm) cut serially, were obtained from animals transcardially perfused 4 weeks post injury with ringer solution containing heparin, followed by 4% paraformaldehyde in 0.1M PBS. To determine lesion size, demyelination, gliosis, and inflammatory infiltrate, cryosections were pre-incubated with 10% serum in PBS containing 0.5% Triton X-100 for 30 minutes at RT. The following primary antibodies were then incubated overnight at 4°C: mouse GFAP (Sigma-Aldrich, Belgium; G3893), rabbit MBP (Merck Millipore, Belgium, AB980), rabbit Iba-1 (Wako, Germany; 016-20001), goat anti-Iba-1 (Abcam, UK; ab107159), rat anti-CD4 (BD Biosciences, Belgium; 553043), rabbit anti-neurofilament (NF) (ThermoFisher Scientific, Belgium; T.400.5) and rat anti-MHC-II (Santa Cruz; sc-59322). To identify *alternatively activated* microglia/macrophages, sections were permeabilized using 0.1% Triton X-100 for 30 minutes and treated with 20% serum in Tris-buffered saline (TBS, pH 7.5) for 2 hours. Incubation with primary goat anti-Arg-1 antibody (Santa Cruz, Germany; sc-18354), diluted 1:50 in TBS containing 10% milk powder (TBS-M), was performed overnight at 4°C. Following repeated washing steps, secondary

antibodies were applied for 1 hour at RT. These consisted of: donkey anti-goat Alexa fluor 555 (Thermo Fisher Scientific, Belgium; A21432), donkey anti-goat DyLight 650 (Abcam, UK; ab96934), goat anti-rat Alexa fluor 568 (Thermo Fisher Scientific, Belgium; A11077), goat anti-rat DyLight 650 (Abcam, UK; ab6565), goat anti-mouse Alexa fluor 568 (Thermo Fisher Scientific, Belgium; A11004), goat anti-rabbit Alexa fluor 488 (Thermo Fisher Scientific, Belgium; A11008) and goat anti-rat Alexa fluor 488 (Thermo Fisher Scientific, Belgium; A11006). DAPI (1:1000; Sigma-Aldrich, Belgium) counterstaining was performed for 10 minutes and sections were mounted using fluorescence mounting medium (DAKO, Belgium). Immunofluorescence analysis was performed using a Nikon Eclipse 80i fluorescence microscope and NIS-Elements Viewer 4.0 software was used for image processing.

5.3.7 Histological quantification in WT BALB/c and WT C57BL/6 mice

For measurement of lesion size and demyelinated area, 5 to 7 serial sections per animal (WT BALB/c: 6-8 animals per group, WT C57BL/6: 9-10 animals per group) containing the lesion centre as well as consecutive rostral and caudal areas were analysed, as previously described (23, 29, 30, 32, 33). Briefly, lesion size was evaluated using anti-GFAP immunofluorescence, while the demyelinated area was evaluated using anti-MBP immunofluorescence, by delineating the area devoid of staining. For measurement of astrogliosis and inflammatory infiltrate in WT BALB/c mice, 5 to 7 sections per animal (7-9 animals per group) were analysed. Quantification of astrogliosis (GFAP expression) and microglial activation (Iba-1 expression) was performed using an intensity analysis with ImageJ open source software (NIH), within square areas measuring 100µm X 100µm extending 600µm rostral to 600µm caudal from the lesion epicentre. Lesion area and graft area were identified as outlined in Figure 5.3A. To quantify *classically activated* and *alternatively activated* microglia/macrophages at the lesion or graft site, sections were stained for MHC-II and Arg-1 respectively. Intensity analysis was performed within square areas measuring 350µm X 350µm, placed at the site with the greatest fluorescence staining intensity. T-helper cells were identified by double staining

against CD4 and Iba-1 in order to exclude CD4+ microglial cells and quantified by counting the number of CD4+ T cells throughout the entire spinal cord section as previously described (23, 29, 30, 32, 33). Quantification of microglia/macrophage and axon interactions was performed by counting the number of contacts between neurofilament$^+$ dystrophic axon bulbs and Iba-1$^+$ microglia/macrophages. Dystrophic axonal bulbs were identified based on their globular and bulbus morphology extending from an axon fibre as previosly described (36, 37). A contact was determined when a cell-cell interaction was observed between a dystrophic axonal bulb and an Iba-1+ cell which contained a DAPI+ nucleus. Analysis was performed in two standardised areas rostral and caudal from the lesion epicentre and the mean number of contacts in these two areas was calculated per animal (16, 17).

5.3.8 Histological quantification in $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ mice

For quantitative phenotypic analyses of macrophage and/or microglia responses at both the lesion and graft site, 5 to 7 sections per animal (graft: 6-11 animals per group, lesion: 9-14 animals per group) were analysed using TissueQuest immunofluorescence analysis software 14 days post injury (TissueGnostics GmbH, v3.0), as previously described (27, 36, 37). For each of region of interest (graft/lesion site), an entire picture taken at 10X magnification was used for quantification and the surface area in the XY plane was determined. According to previously established procedures, the following parameters were quantified at the lesion and graft site: the cellular density of eGFP$^-$RFP$^+$ macrophages ($CCR2^{RFP/+}$), eGFP$^+$RFP$^+$ double positive microglia/macrophages ($CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$) as well as eGFP$^+$RFP$^-$ microglia ($CX_3CR1^{eGFP/+}$) in both MSC and MSC/IL-13-treated groups. Although the presence of a double positive $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ population is prominent, we hypothesise that these cells are blood derived and of peripheral origin given that it is a known limitation that $CCR2^{RFP/+}$ monocytes can down-regulate their reporter over time and show phenotypic evolution (38). Therefore, we consider this double population throughout the rest of the manuscript as one which falls under the macrophage

classification. Based on the above cell density calculations, the proportion of microglia or macrophages at the graft and lesion site expressing MHC-II or Arg-1 were calculated as follows: (i) $eGFP^{-}RFP^{+}MHC\text{-}II^{+}/Arg\text{-}1^{+}$ cells, (ii) $eGFP^{+}RFP^{+}MHC\text{-}II^{+}/Arg\text{-}1^{+}$ cells, (iii) $eGFP^{+}RFP^{-}MHC\text{-}II^{+}/Arg\text{-}1^{+}$ cells, (iv) $eGFP^{-}RFP^{+}MHC\text{-}II^{-}/Arg\text{-}1^{-}$ cells, (v) $eGFP^{+}RFP^{+}MHC\text{-}II^{-}/Arg\text{-}1^{-}$ cells, (vi) $eGFP^{+}RFP^{-}MHC\text{-}II^{-}/Arg\text{-}1^{-}$ cells. Put simply, we identify the number of *classically* or *alternatively* activated $CCR2^{RFP/+}$ macrophages and $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ macrophages or $CX_3CR1^{eGFP/+}$ microglia, at both the graft and lesion site.

5.3.9 Statistical analysis

All statistical analyses were performed using Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The BMS locomotion tests as well as histological evaluation of astrogliosis and microglia/macrophage intensities were analysed using a two-way ANOVA for repeated measurements with Bonferroni correction for multiple comparisons. All other differences between two groups were evaluated using the nonparametric Mann-Whitney U-test. Differences were considered statistically significant when $p<0.05$. Data shown represent mean values per experimental group ± SEM.

5.4 Results

5.4.1 Transplantation of MSC/IL-13 improves functional recovery and reduces lesion size and demyelinated area.

In the first part of this study, we investigated whether MSCs which were genetically engineered to express IL-13 could improve functional recovery following SCI in BALB/c and C57BL/6 mice. Mice were treated with either vehicle (NaCl), control MSCs (MSC) or IL-13-secreting MSCs (MSC/IL-13) immediately after SCI and functional recovery was measured 4 weeks post injury using the BMS. In BALB/c mice, both MSC and MSC/IL-13-treated animals displayed a significantly improved functional recovery, compared with NaCl controls (Figure

5.1A). Lesion size quantification revealed a significant decrease in mice receiving transplantation of MSC/IL-13 compared with control MSCs and NaCl (Figure 5.1B). Similarly, demyelinated area was significantly decreased in MSC/IL-13-treated mice compared with control MSC and NaCl (Figure 5.1C). In contrast to Balb/C mice, treatment with MSC/IL-13 in C57BL/6 mice, significantly improved functional recovery compared with control MSCs or NaCl (Figure 5.1D). Additionally, there was a corresponding significant decrease in both lesion size (Figure 5.1E) and demyelinated area (Figure 5.1F) in MSC/IL-13 compared with control MSCs or NaCl. In both mouse backgrounds, immunofluorescence stainings for GFAP (Figure 5.1G-I) and MBP (Figure 5.1J-L) were used to analyse lesion size and demyelinated area respectively. Taken together, these data demonstrate that on a functional level, BALB/c mice can benefit from both MSC and MSC/IL-13 grafts, while C57BL/6 mice require MSC/IL-13 grafts for improved outcome. However, on the histopathological level, both BALB/c and C57BL/6 mice benefit from MSC/IL-13 grafts to reduce lesion size and demyelination.

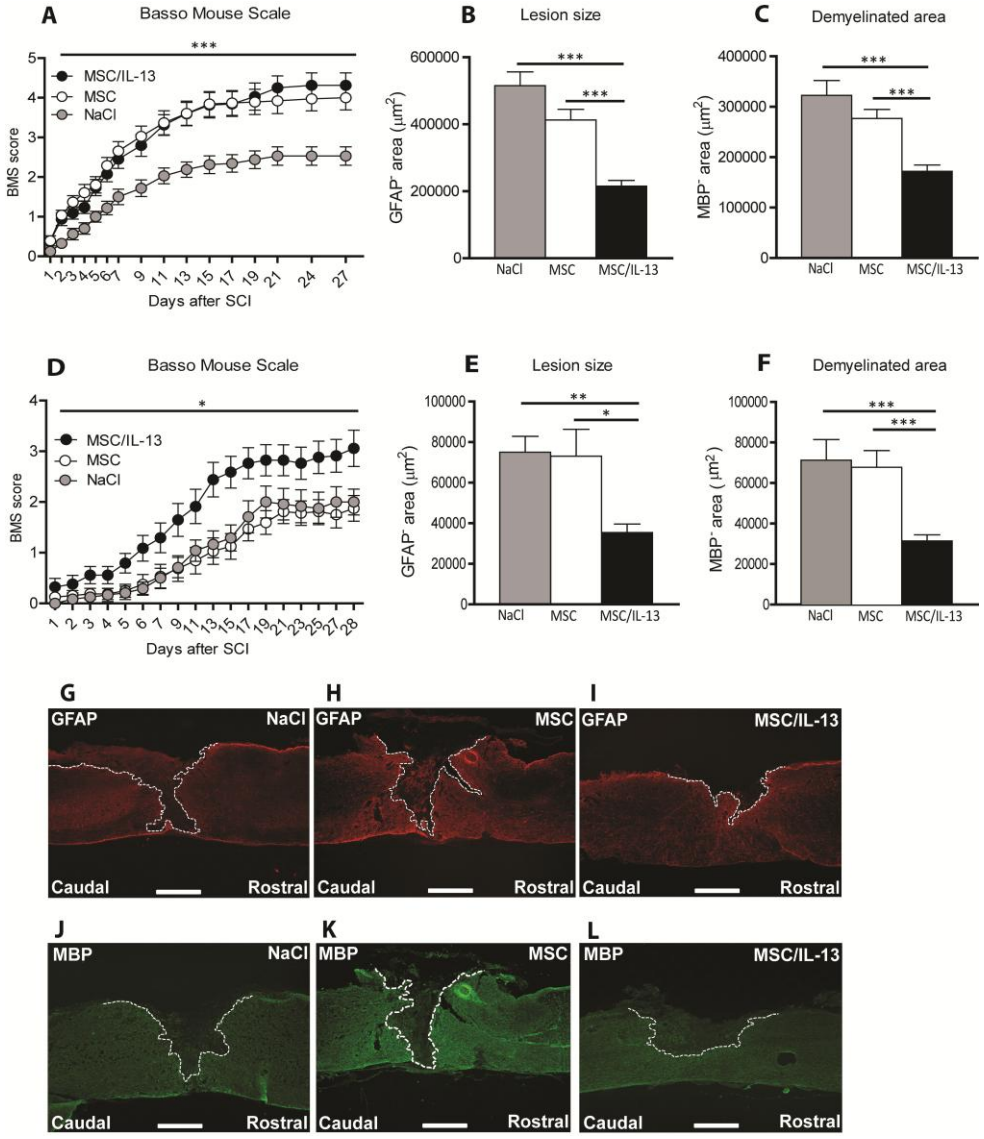
A
Basso Mouse Scale
***
MSC/IL-13
MSC
NaCl
BMS score
Days after SCI
B
Lesion size
***
***
GFAP- area (µm2)
NaCl
MSC
MSC/IL-13
C
Demyelinated area
***
***
MBP- area (µm2)
NaCl
MSC
MSC/IL-13
D
Basso Mouse Scale
*
MSC/IL-13
MSC
NaCl
BMS score
Days after SCI
E
Lesion size
**
*
GFAP- area (µm2)
NaCl
MSC
MSC/IL-13
F
Demyelinated area
***
***
MBP- area (µm2)
NaCl
MSC
MSC/IL-13
G
GFAP
NaCl
Caudal
Rostral
H
GFAP
MSC
Caudal
Rostral
I
GFAP
MSC/IL-13
Caudal
Rostral
J
MBP
NaCl
Caudal
Rostral
K
MBP
MSC
Caudal
Rostral
L
MBP
MSC/IL-13
Caudal
Rostral

Figure 5.1: Transplantation of MSC/IL-13 improves functional recovery and reduces lesion size and demyelinated area in mice following SCI.
(A) BALB/c mice receiving transplantation of MSCs or MSC/IL-13 show a significantly increased BMS score following SCI. ***** p < 0.0001 and * p < 0.05, n = 16-19/group.** Image analysis revealed a significant decrease in **(B)** lesion size and **(C)** demyelinated area in the MSC/IL-13 treated animals, compared with MSC and NaCl groups ***** p < 0.0001, n = 6-8/group. (D)** C57BL/6 mice receiving transplantation of MSC/IL-13 show a significantly increased BMS score following SCI, compared with MSC or NaCl controls. *** p < 0.05, n=12-17/group**. Image analysis for GFAP and MBP staining also revealed a significant decrease in **(E)** lesion size and **(F)** demyelinated area respectively in MSC/IL-13-treated animals, compared with MSC and NaCl controls ***** p < 0.0001, ** p < 0.01, * p < 0.05; n = 8-10/group.** Representative photomicrographs are shown of BALB/c spinal cord sections including the injury epicentre of **(G, J)**, NaCl-treated **(H, K)** MSC and **(I, L)** MCS/IL-13-treated mice. Sections were stained for **(G-I)** GFAP and **(J-L)** MBP to determine the lesion size and demyelinated area as depicted by the dotted white line. Scale bars G-L = 500μm.

5.4.2 MSC/IL-13 transplantation has no significant effect on the presence of microglia/macrophages at the lesion site in both BALB/c and C57BL/6 mice, but leads to a significant reduction in astrogliosis in C57BL/6 mice

In a first attempt to understand why MSC/IL-13, but not MSC grafts, reduce lesion size and demyelination, we investigated the degree of microglia/macrophage (Figure 5.2A, I) and astroglial (Figure 5.2B, J) responses in both BALB/c and C57BL/6 mice respectively, 4 weeks post injury. For analysis, quantification of Iba-1 (Figure 5.2C-E, K-M) and GFAP (Figure 5.2F-H, N-P) intensity was performed 600μm caudal and 600μm rostral from the lesion site, in squares measuring 100μm x 100μm in BALB/c and C57BL/6 mice respectively. There was no significant difference observed in the presence of microglia/macrophages in mice treated with MSC or MSC/IL-13 compared with NaCl controls in both BALB/c and C57BL/6 mouse backgrounds (Figure 5.2A, I respectively). Additionally, there was no significant difference in astrogliosis in MSC or MSC/IL-13 treated mice compared with NaCl controls in BALB/c mice

(Figure 5.2B). However, there was a significant reduction in astrogliosis in C57BL/6 mice treated with MSC/IL-13, compared with MSC on NaCl controls (Figure 5.2J).

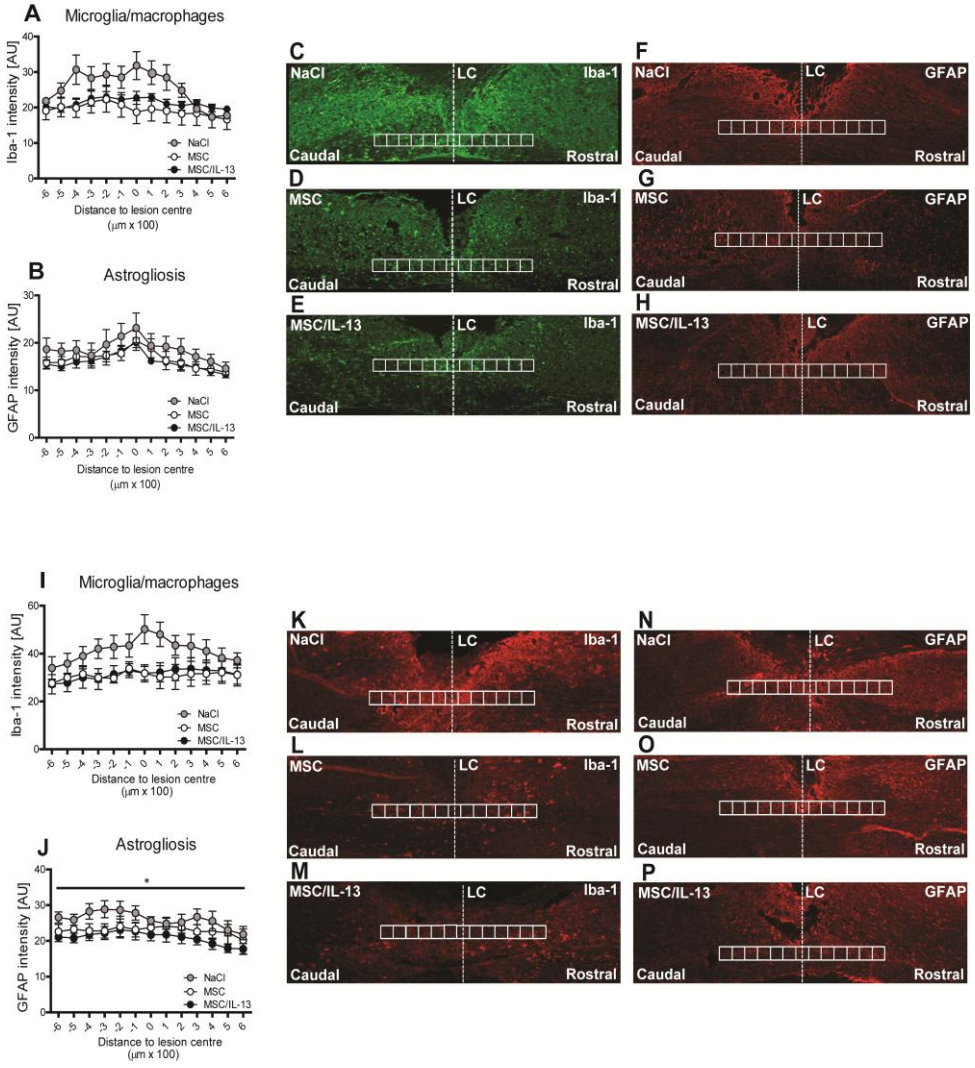

Figure 5.2: MSC/IL-13 transplantation has no significant effect on the presence of microglia/macrophages at the lesion site in both BALB/c and C57BL/6 mice, but leads to a significant reduction in astrogliosis in C57BL/6 mice

In BALB/c mice, there was no significant difference observed in the presence of **(A)** microglia/macrophages or **(B)** astrogliosis in **(C,F)** NaCl **(D,G)** MSC or **(E,H)** MSC/IL-13 treated animals as measured via intensity analysis for Iba-1 and GFAP respectively. In C57BL/6 mice, there was no significant difference observed in the presence of **(I)** microglia/macrophages in **(K)** NaCl **(L)** MSC or **(M)** MSC/IL-13 treated animals. However, quantification of GFAP in C57BL/6 mice revealed a significant decrease in **(J)** astrogliosis in mice treated with **(P)** MSC/IL-13 compared with **(O)** MSC or **(N)** NaCl treated mice. **(C-H, K-P)** All analyses were quantified within square areas of 100μm X 100μm just below the lesion site, extending 600μm rostral to 600μm caudal from the lesion epicentre. Data represents mean ± SEM, *** p < 0.05; n = 7-11/group.**

5.4.3 Transplantation of MSC/IL-13 increases the number of neuroprotective, *alternatively activated* macrophages at the graft site and increases the number of CD4+ T-cells throughout the spinal cord

To further investigate the effects of MSC/IL-13 grafting, we characterised the *classically activated* and *alternatively activated* microglia/macrophage phenotypes at the level of the graft site (Figure 3A: (ii)) by performing an intensity analysis for MHC-II and Arg-1 respectively. While there was no difference in MHC-II intensity between the MSC and MSC/IL-13-graft (Figure 5.3B, D + F), there was a significant increase in Arg-1 intensity within the MSC/IL-13 graft region compared with that of the MSC graft (Figure 5.3G, I + K). The graft site regions of interest (ROI) analysed using MHC-II and Arg-1 intensity analysis are shown in Figure 5.3C+E (white box) and Figure 5.3H+J respectively. The ROIs in Figure 5.3C+E are shown at a higher magnification in Figure 3D+F and the ROIs in Figure 5.3H+J are shown in Figure 5.3I+K. These data strongly suggest that the secretion of IL-13 from MSC grafts, significantly increase the presence of an *alternatively activated* microglia/macrophage phenotype at the graft site. Quantification of CD4+ T cells throughout the spinal cord revealed a significant increase in the number of CD4+ T cells in MSC/IL-13

treated mice, compared with MSC and NaCl treated controls both BALB/c (Figure 5.3L-O) and C57BL/6 Figure 5.3P-S) mouse backgrounds.

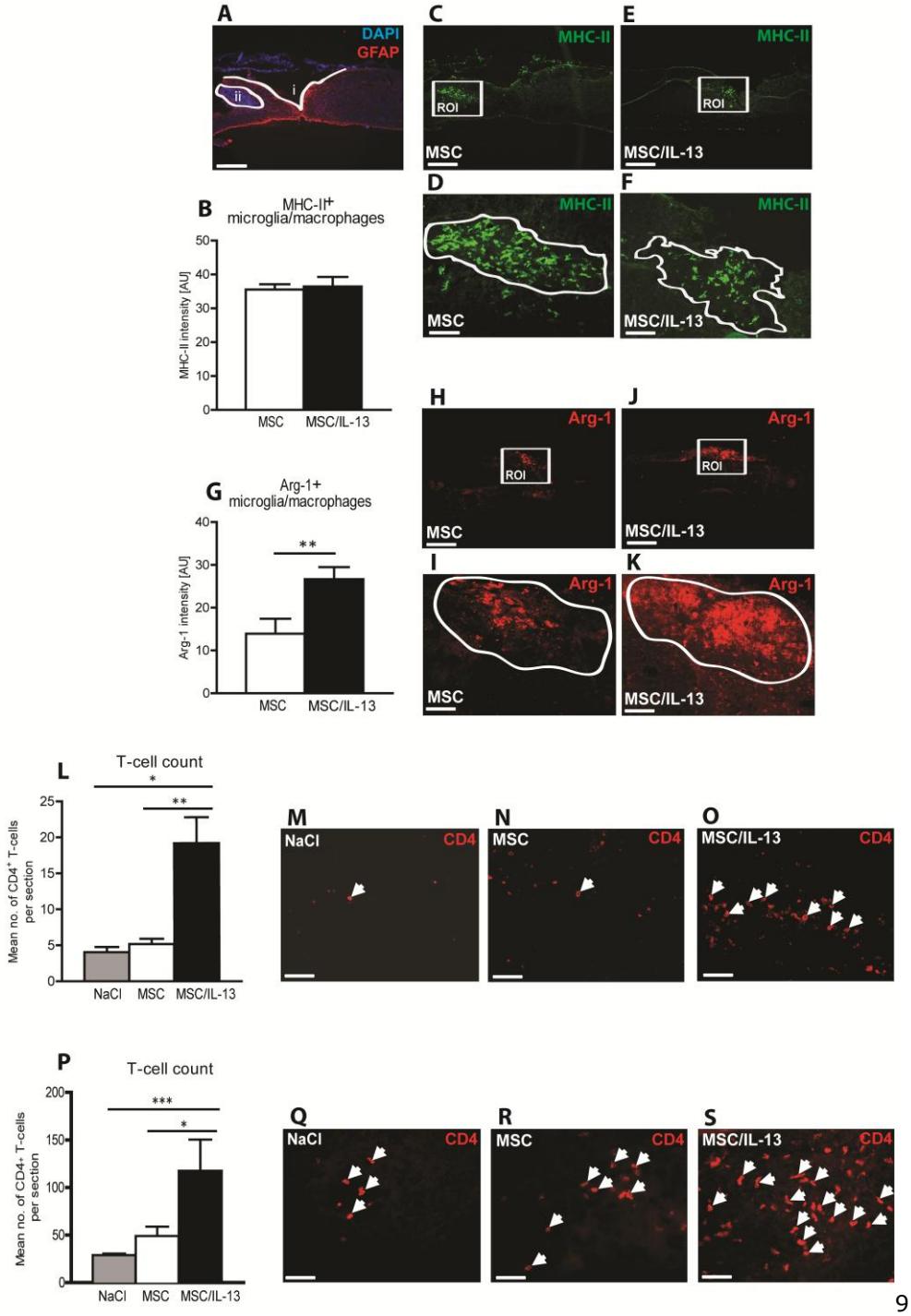
A
DAPI
GFAP
i
ii
C
MHC-II
ROI
MSC
E
MHC-II
ROI
MSC/IL-13
D
MHC-II
MSC
F
MHC-II
MSC/IL-13
B
MHC-II+ microglia/macrophages
MHC-II intensity [AU]
50
40
30
20
10
0
MSC
MSC/IL-13
H
Arg-1
ROI
MSC
J
Arg-1
ROI
MSC/IL-13
G
Arg-1+ microglia/macrophages
Arg-1 intensity [AU]
40
30
20
10
0
**
MSC
MSC/IL-13
I
Arg-1
MSC
K
Arg-1
MSC/IL-13
L
T-cell count
*
**
Mean no. of CD4+ T-cells per section
25
20
15
10
5
0
NaCl
MSC
MSC/IL-13
M
NaCl
CD4
N
MSC
CD4
O
MSC/IL-13
CD4
P
T-cell count
***
*
Mean no. of CD4+ T-cells per section
200
150
100
50
0
NaCl
MSC
MSC/IL-13
Q
NaCl
CD4
R
MSC
CD4
S
MSC/IL-13
CD4

Figure 5.3: Transplantation of MSC/IL-13 increases the number of neuroprotective, *alternatively activated* macrophages at the graft site and increases the number of CD4+ T-cells throughout the spinal cord
(A) To identify the lesion and graft site regions of interest, we have included an overview of a SCI section stained with GFAP+DAPI containing the (i) V-shaped lesion site and (ii) DAPI+ intense MSC graft site encapsulated by GFAP+ astrocytes. To determine the presence of **(B)** *classically activated* and **(G)** *alternatively activated* microglia/macrophages, sections were stained for **(C-F)** MHC-II or **(H-K)** Arg-1 respectively. **(B)** There was no significant difference in MHC-II levels between MSC and MSC/IL-13 graft regions. **(G)** There was a significant increase in Arg-1 levels within the MSC/IL-13 graft region compared to the MSC graft. Representative photomicrographs indicating the graft locations in **(C+E)** MHC-II and **(H+J)** Arg-1 stained sections (white boxes) within the corresponding regions of interest (ROI) are shown. Areas shown in **D+F** are higher magnifications of the ROIs shown in **C+E** and areas shown in **I+K** are higher magnifications of the ROIs shown in **H+J** (white boxes). Scale bars: A,C,E, H+J = 500μm and D+F, I+K = 100μm. Data represent mean ± SEM. **** p < 0.01; n = 6-8/group.** CD4 staining in spinal cord sections revealed a significant increase in the number of $CD4^+$ T cells in both **(L)** BALB/c and **(P)** C57BL/6 mice treated with MSC/IL-13, compared with MSC or NaCl-treated mice 4 weeks after SCI. Representative photomicrographs of spinal cord sections treated with **(M+Q)** NaCl, **(N+R)** MSC or **(O+S)** MSC/IL-13 from BALB/c and C57BL/6 mice respectively are shown. White arrow heads in **M-O** and **Q-S** indicate $CD4^+$ T-cells. Scale bars: M-O and Q-S = 50μm. Data represents mean ± SEM, ***** p < 0.0001, ** p < 0.01, * p < 0.05; n = 7-11/group.**

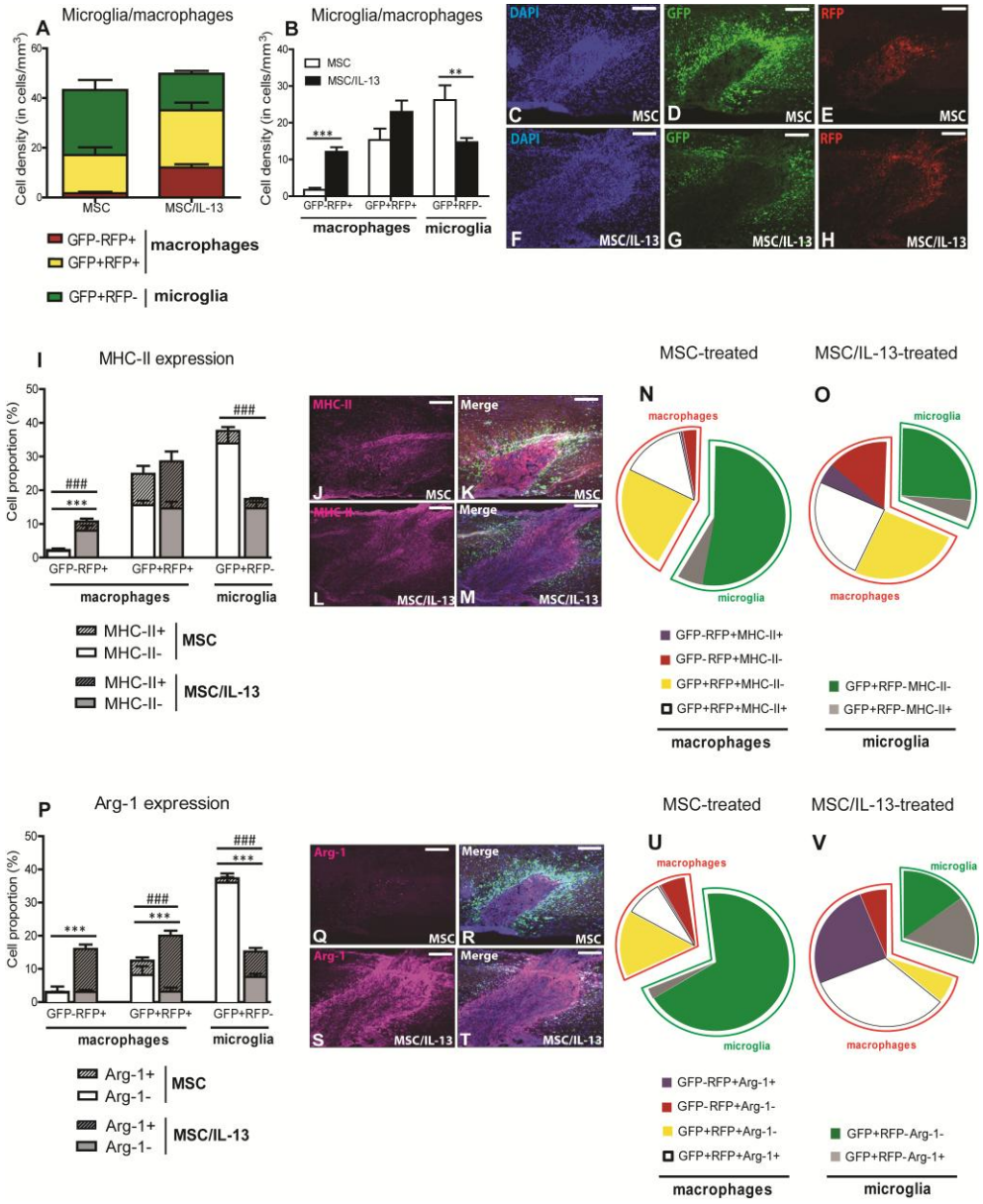
A
Microglia/macrophages
Cell density (in cells/mm3)
MSC
MSC/IL-13
GFP-RFP+
GFP+RFP+
macrophages
GFP+RFP-
microglia
B
Microglia/macrophages
MSC
MSC/IL-13
***
**
GFP-RFP+
GFP+RFP+
GFP+RFP-
macrophages
microglia
C DAPI MSC
D GFP MSC
E RFP MSC
F DAPI MSC/IL-13
G GFP MSC/IL-13
H RFP MSC/IL-13
I
MHC-II expression
Cell proportion (%)
###
***
GFP-RFP+
GFP+RFP+
GFP+RFP-
macrophages
microglia
MHC-II+
MHC-II-
MSC
MHC-II+
MHC-II-
MSC/IL-13
J MHC-II MSC
K Merge MSC
L MHC-II MSC/IL-13
M Merge MSC/IL-13
MSC-treated
MSC/IL-13-treated
N
macrophages
microglia
O
microglia
macrophages
GFP-RFP+MHC-II+
GFP-RFP+MHC-II-
GFP+RFP+MHC-II-
GFP+RFP+MHC-II+
macrophages
GFP+RFP-MHC-II-
GFP+RFP-MHC-II+
microglia
P
Arg-1 expression
Cell proportion (%)
***
###
GFP-RFP+
GFP+RFP+
GFP+RFP-
macrophages
microglia
Arg-1+
Arg-1-
MSC
Arg-1+
Arg-1-
MSC/IL-13
Q Arg-1 MSC
R Merge MSC
S Arg-1 MSC/IL-13
T Merge MSC/IL-13
MSC-treated
MSC/IL-13-treated
U
macrophages
microglia
V
microglia
macrophages
GFP-RFP+Arg-1+
GFP-RFP+Arg-1-
GFP+RFP+Arg-1-
GFP+RFP+Arg-1+
macrophages
GFP+RFP-Arg-1-
GFP+RFP-Arg-1+
microglia

Figure 5.4: Transplantation of MSC/IL-13 increases the number of neuroprotective, *alternatively activated* macrophages and decreases the number of microglia at the graft site

(A) Cell density quantification at the graft site revealed no significant differences in the total number of microglia/macrophages in MSC or MSC/IL-13-treated mice. **(B)** Further characterisation of the microglia/macrophage populations revealed a significant increase in the number of GFP^-RFP^+ macrophages and a significant decrease in GFP^+RFP^- microglia in MSC/IL-13-treated mice compared with MSC controls. **(C+F)** $DAPI^+$ cells localises the position of the graft. Representative photomicrographs of **(D+G)** $CX_3CR1^{eGFP/+}$ microglia and **(E+H)** $CCR2^{RFP/+}$ macrophages are shown from MSC and MSC-IL-13-treated mice respectively. **(I)** Detailed phenotypic analysis of microglia/macrophage populations expressing MHC-II revealed a significant increase in the number of $GFP^-RFP^+MHC\text{-}II^+$ and $GFP^-RFP^+MHC\text{-}II^-$ macrophages as well as a significant decrease in $GFP^+RFP^-MHC\text{-}II^-$ microglia in **(L)** MSC/IL-13-treated animals compared with **(J)** MSC controls. MHC-II activation is shown in the top shaded bar stacks and **###** represents significant differences in $MHC\text{-}II^-$ cells and ******* in $MHC\text{-}II^+$ cells**.** A representative photomicrograph outlining the distribution of $DAPI^+/GFP^+/RFP^+/MHC\text{-}II^+$ cells at the graft site in **(K)** MSC and **(M)** MSC/IL-treated animals is shown. The corresponding relative distribution of MHC-II-expressing microglia and macrophages at **(N)** control MSC and **(O)** MSC/IL-13 graft sites is shown. **(P)** Analysis of microglia/macrophage populations expressing Arg-1 revealed a significant increase in the number of $GFP^-RFP^+Arg\text{-}1^+$ and $GFP^+RFP^+Arg\text{-}1^+$ macrophages, $GFP^+RFP^-Arg\text{-}1^+$ microglia, as well as a significant decrease in the number of $GFP^+RFP^+Arg\text{-}1^-$ macrophages and $GFP^+RFP^-Arg\text{-}1^-$ microglia in **(S)** MSC/IL-13-treated animals compared with **(Q)** MSC controls. Arg-1 activation is shown in the top shaded bar stacks and **###** represents significant differences in $Arg\text{-}1^-$ cells and ******* in $Arg\text{-}1^+$ cells. A representative photomicrograph outlining the distribution of $DAPI^+/GFP^+/RFP^+/Arg\text{-}1^+$ cells at the graft site in **(R)** MSC and **(T)** MSC/IL-treated animals is shown. The corresponding relative distribution of Arg-1-expressing microglia and/or macrophages at **(U)** control MSC and **(V)** MSC/IL-13 graft sites is shown. Microglia and macrophages are encircled in red and green respectively. Immunofluorescence colours: BLUE = DAPI, GREEN = microglia, RED = macrophages, MAGENTA = $Arg1^+$ or $MHC\text{-}II^+$ microglia/macrophages. Scale bars C-H, J-M and Q-T = 200μm. Data represent mean ± SEM. ***** & ### p < 0.0001, ** p < 0.01, * p < 0.05; n = 6-11/group.**

5.4.4 Transplantation of MSC/IL-13 increases the number of neuroprotective, *alternatively activated* macrophages and decreases the number of microglia at the graft site

The data provided above demonstrate that MSC/IL-13 grafts significantly increase the presence of *alternatively activated* microglia/macrophage phenotypes at the level of the graft site. However, in order to investigate which of the two cell types (microglia or macrophages) was responsible for the increase in Arg-1 expression, we took advantage of the C57BL/6 $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ mouse model. This model allows us to distinguish between $eGFP^+RFP^-$ microglia (green) as well as $eGFP^-RFP^+$ macrophages (red) and $eGFP^+RFP^+$ macrophages (yellow) as outlined in the materials and methods section. Following SCI, we grafted MSC or MSC/IL-13 and investigated the graft-immune response by performing immunofluorescence stainings for MHC-II and Arg-1. We then calculated the cell density of microglia and macrophages at the graft site. Although there was no significant difference observed in the total microglia/macrophage cell density when comparing MSC and MSC/IL-13 grafts (Figure 5.4A), there was a clear difference in the origin and phenotype of infiltrating immune cells in MSC/IL-13 grafts as compared to those in MSC grafts. Further quantification revealed a significant increase in the number of $eGFP^-RFP^+$ macrophages and significant decrease in the number of $eGFP^+RFP^-$ microglia in MSC/IL-13-treated, compared with control MSCs. There was no significant difference in the number of $eGFP^+RFP^+$ graft-infiltrating macrophages between MSC and MSC/IL-13 grafts (Figure 5.4B, C-H). Based on these cell density calculations, the proportion of MHC-II^+ or Arg-1^+ expressing cells within the $eGFP^-RFP^+$ and $eGFP^+RFP^+$ macrophage population, as well as within the $eGFP^+RFP^-$ microglia population, was calculated as outlined in the materials and methods (Figure 5.4I + P respectively). For MHC-II expression (Figure 5.4I-M), there was a significant increase in the number of MHC-II^- and MHC-II^+ $eGFP^-RFP^+$ macrophages at the graft site in MSC/IL-13-treated mice compared with control MSCs. The proportion of MHC-II^- and MHC-II^+ $eGFP^+RFP^+$ macrophages at the graft site was unaltered between MSC and MSC/IL-13 grafts. In contrast, we noted a significant reduction in MHC-II^- $eGFP^+RFP^-$ microglia at the MSC/IL-

13 graft site as compared to the MSC graft, while the low number of MHC-II$^+$ eGFP$^+$RFP$^-$ microglia remained unaltered. This relative distribution of MHC-II expression within microglia and macrophage populations in MSC and MSC/IL-13 grafts is also represented in the corresponding pie charts (Figure 5.4N+O). When comparing Arg-1 expression between MSC and MSC/IL-13 grafts (Figure 5.4P-T), there was a significant increase in the Arg-1$^+$ eGFP$^-$RFP$^+$ and eGFP$^+$RFP$^+$ macrophage populations, as well as the eGFP$^+$RFP$^-$ microglia population, in the MSC/IL-13-treated mice. Subsequently, there was a significant decrease in the Arg-1$^-$ eGFP$^+$RFP$^+$ macrophage population and Arg-1$^-$ eGFP$^+$RFP$^-$ microglia population, while the low amount of Arg-1$^-$ eGFP$^-$RFP$^+$ macrophages remained unchanged. The distribution of Arg-1 expression within microglia and macrophage populations in MSC and MSC/IL-13 grafts is also represented in the corresponding pie charts (Figure 5.4U+V). In summary, these results indicate that the number of macrophages is significantly higher and the number of microglia is significantly lower at the MSC/IL-13 graft site compared to control MSC grafts. Of the total macrophage populations present in MSC/IL-13 grafts, 30% express MHC-II (Figure 5.4O) while 53% express Arg-1 (Figure 5.4V) [purple + white segments]. Of the microglia present in MSC/IL-13 grafts, 5% express MHC-II (Figure 5.4O), while 15% express Arg-1 (Figure 5.4V) [grey segments]. Taken together, we demonstrate that there is a 10% increase in the number of MHC-II$^+$ immune cells and a 50% increase in the number of Arg-1$^+$ immune cells at MSC/IL-13 graft site compared to control MSC grafts. These results indicate that the secretion of IL-13 from MSC grafts induces a broad spectrum of *alternatively activated* infiltrating macrophages at the graft site.

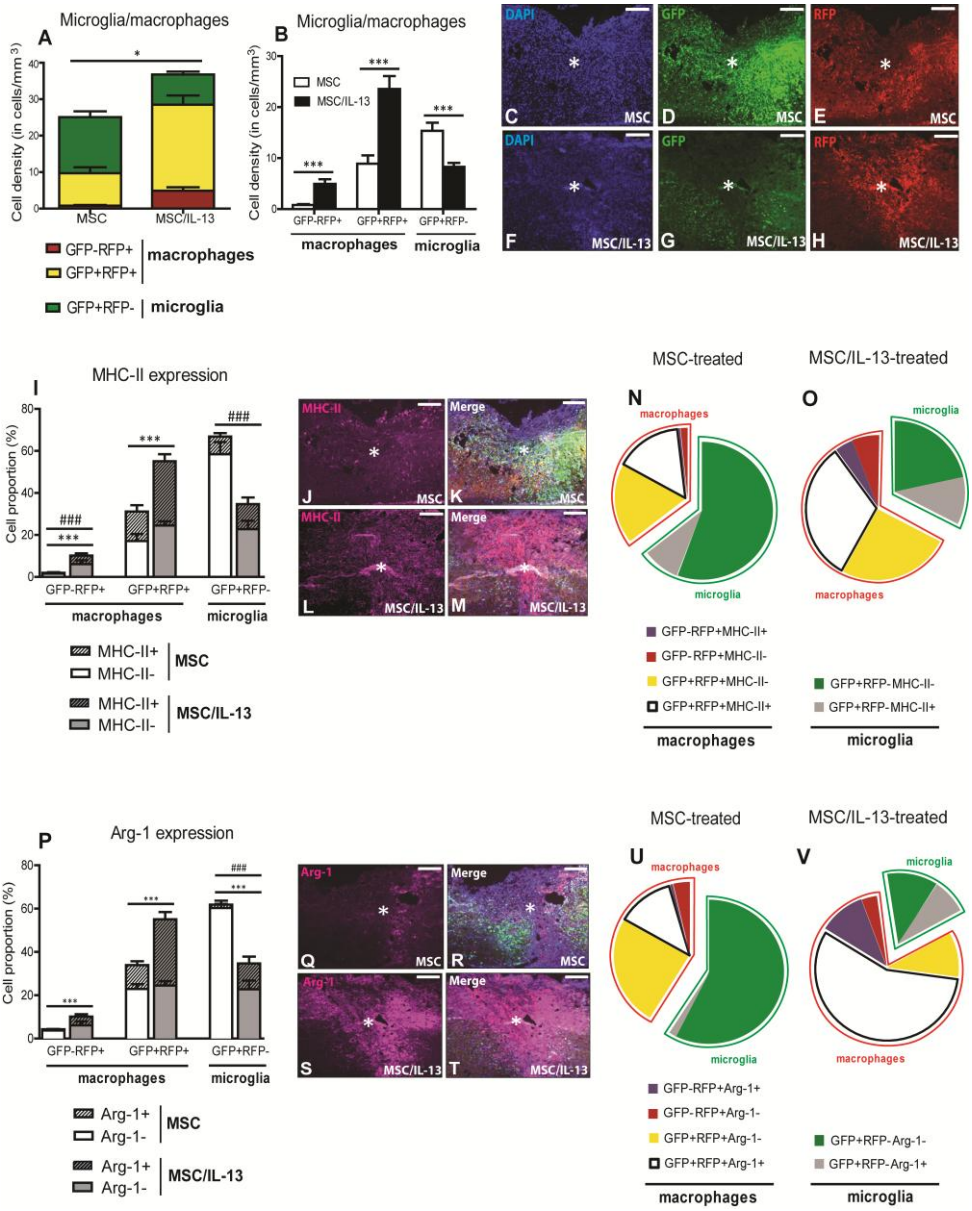
A
Microglia/macrophages
Cell density (in cells/mm 3)
MSC
MSC/IL-13
GFP-RFP+
GFP+RFP+
macrophages
GFP+RFP-
microglia
B
Microglia/macrophages
Cell density (in cells/mm-3)
MSC
MSC/IL-13
GFP-RFP+
GFP+RFP+
GFP+RFP-
macrophages
microglia
C DAPI MSC
D GFP MSC
E RFP MSC
F DAPI MSC/IL-13
G GFP MSC/IL-13
H RFP MSC/IL-13
I
MHC-II expression
Cell proportion (%)
GFP-RFP+
GFP+RFP+
GFP+RFP-
macrophages
microglia
MHC-II+
MHC-II-
MSC
MHC-II+
MHC-II-
MSC/IL-13
J MHC-II MSC
K Merge MSC
L MHC-II MSC/IL-13
M Merge MSC/IL-13
MSC-treated
MSC/IL-13-treated
N
macrophages
microglia
O
microglia
macrophages
GFP-RFP+MHC-II+
GFP-RFP+MHC-II-
GFP+RFP+MHC-II-
GFP+RFP+MHC-II+
macrophages
GFP+RFP-MHC-II-
GFP+RFP-MHC-II+
microglia
P
Arg-1 expression
Cell proportion (%)
GFP-RFP+
GFP+RFP+
GFP+RFP-
macrophages
microglia
Arg-1+
Arg-1-
MSC
Arg-1+
Arg-1-
MSC/IL-13
Q Arg-1 MSC
R Merge MSC
S Arg-1 MSC/IL-13
T Merge MSC/IL-13
MSC-treated
MSC/IL-13-treated
U
macrophages
microglia
V
microglia
macrophages
GFP-RFP+Arg-1+
GFP-RFP+Arg-1-
GFP+RFP+Arg-1-
GFP+RFP+Arg-1+
macrophages
GFP+RFP-Arg-1-
GFP+RFP-Arg-1+
microglia

Figure 5.5: Transplantation of MSC/IL-13 increases the number of infiltrating neuroprotective, *alternatively activated* macrophages and decreases the number of microglia at the lesion site

(A) Cell density quantification at the graft site revealed a significant increase in the number of microglia/macrophages present between MSC and MSC/IL-13 treated animals. **(B)** Further characterisation of the microglia/macrophage populations revealed a significant increase in the number of GFP^-RFP^+ and GFP^+RFP^+ macrophages as well as a significant decrease in GFP^+RFP^- microglia in MSC/IL-13-treated mice compared with MSC controls. **(C+F)** $DAPI^+$ cells localises the lesion site and **(*)** denotes the lesion epicentre. Representative photomicrographs of **(D+G)** $CX_3CR1^{eGFP/+}$ microglia and **(E+H)** $CCR2^{RFP/+}$ macrophages at the lesion site are shown from MSC and MSC-IL-13-treated mice respectively. **(I)** Detailed phenotypic analysis of microglia/macrophage populations expressing MHC-II revealed a significant increase in the number of $GFP^-RFP^+MHC\text{-}II^+$ and $GFP^-RFP^+MHC\text{-}II^-$ macrophages as well as a significant decrease in $GFP^+RFP^-MHC\text{-}II^-$ microglia in **(L)** MSC/IL-13-treated animals compared with **(J)** MSC controls. MHC-II activation is shown in the top shaded bar stacks and **###** represents significant differences in $MHC\text{-}II^-$ cells and ******* in $MHC\text{-}II^+$ cells. A representative photomicrograph outlining the distribution of $DAPI^+/GFP^+/RFP^+/MHC\text{-}II^+$ cells at the lesion site in **(K)** MSC and **(M)** MSC/IL-treated animals is shown. The corresponding relative distribution of MHC-II-expressing microglia and macrophages at **(N)** control MSC and **(O)** MSC/IL-13 lesion sites is shown. **(P)** Analysis of microglia/macrophage populations expressing Arg-1 revealed a significant increase in the number of $GFP^-RFP^+Arg\text{-}1^+$, $GFP^+RFP^+Arg\text{-}1^+$ macrophages and $GFP^+RFP^-Arg\text{-}1^+$ microglia, as well as a significant decrease in the number of $GFP^+RFP^+Arg\text{-}1^-$ macrophages and $GFP^+RFP^-Arg\text{-}1^-$ microglia in **(S)** MSC/IL-13-treated animals compared with **(Q)** MSC controls. Arg-1 activation is shown in the top shaded bar stacks and **###** represents significant differences in $Arg\text{-}1^-$ cells and ******* in $Arg\text{-}1^+$ cells. A representative photomicrograph outlining the distribution of $DAPI^+/GFP^+/RFP^+/Arg\text{-}1^+$ cells at the lesion site in **(R)** MSC and **(T)** MSC/IL-treated animals is shown. The corresponding relative distribution of Arg-1-expressing microglia and macrophages at **(U)** control MSC and **(V)** MSC/IL-13 graft sites is shown. Microglia and macrophages are encircled in red and green respectively. Immunofluorescence colours: BLUE = DAPI, GREEN = microglia, RED = macrophages, MAGENTA = $Arg1^+$ or $MHC\text{-}II^+$ microglia/macrophages). Scale bars C-H, J-M and Q-T = 200μm. Data represent mean ± SEM. ***** & ### p < 0.0001, * p < 0.05; n = 9-14/group.**

5.4.5 Transplantation of MSC/IL-13 increases the number of infiltrating neuroprotective, *alternatively activated* macrophages and decreases the number of microglia at the lesion site

Although analysis on the effects of IL-13 at the MSC graft site showed an increase in the number of neuroprotective, *alternatively activated* macrophages present, we hypothesised that a shift in the microglia/macrophage response at the spinal cord lesion site may also be possible, given the strong clinical benefit observed in MSC/IL-13-treated mice. Therefore, we also investigated the parameters described above at the lesion site in $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ mice receiving MSC or MSC/IL-13 following SCI. Cell density quantification at the lesion site revealed a significant increase in the total number of microglia/macrophages in mice treated with MSC/IL-13 compared with control MSCs (Figure 5.5A). More specifically, there was a significant increase in the number of $eGFP^-/RFP^+$ macrophages (red) and $eGFP^+/RFP^+$ macrophages (yellow), while the number of $eGFP^+/RFP^-$ microglia (green) was significantly decreased, in MSC/IL-13-treated compared with control MSC-treated mice (Figure 5.5B, C-H). Similarly to the method described above at the graft site, we also calculated the proportion of MHC-II^+ or Arg-1^+ expressing microglia and macrophages at the lesion site. For MHC-II expression (Figure 5.5I-M), there was a significant increase in the numbers of MHC-II^- and MHC-II^+ $eGFP^-RFP^+$ macrophages at the lesion site in MSC/IL-13-treated mice compared with control MSCs. The proportion of MHC-II^- $eGFP^+RFP^+$ macrophages at the lesion site was unaltered between MSC and MSC/IL-13 grafts, while the number of MHC-II^+ $eGFP^+RFP^+$ macrophages at the lesion site was significantly increased following MSC/IL-13 grafting. In contrast, we noted a significant reduction in MHC-II^- $eGFP^+RFP^-$ microglia at the lesion site following MSC/IL-13 grafting, compared with control MSCs, while the number of MHC-II^+ $eGFP^+RFP^-$ microglia remained unchanged. This relative distribution of MHC-II expression within microglia and macrophage populations at the lesion site following MSC and MSC/IL-13 grafting, is also represented in the corresponding pie charts (Figure 5.5N+O). When comparing Arg-1 expression at the lesion site following MSC and MSC/IL-13 grafting (Figure 5.5P-T), there was a significant increase in Arg-1^+ $eGFP^-RFP^+$

and $eGFP^+RFP^+$ macrophage populations, as well as the $eGFP^+RFP^-$ microglia population, in MSC/IL-13-treated mice. Subsequently, there was a significant decrease in the Arg-1^- $eGFP^+RFP^-$ microglia population, while Arg-1^- $eGFP^-RFP^+$ and Arg-1^- $eGFP^+RFP^+$ macrophage populations remained unaltered. This relative distribution of Arg-1 expression within microglia and macrophage populations at the lesion site following MSC and MSC/IL-13 grafting, is also represented in the corresponding pie charts (Figure 5.5U+V). These results indicate that similarly to the graft site, the number of macrophages is significantly higher and the number of microglia is significantly lower at the lesion site following MSC/IL-13 grafting as compared with control MSCs. Of the macrophage populations present at the lesion site following MSC/IL-13 grafting, 36% express MHC-II (Figure 5.5O), while 67% express Arg-1 (Figure 5.5V) [purple + white segments]. Of the microglia present at the lesion site following MSC/IL-13 grafting, 11% express MHC-II (Figure 5.5O), while 8% express Arg-1 (Figure 5.5V) [grey segments]. This demonstrates that there is a 24% increase in the number of MHC-II^+ immune cells and a 60% increase in the number of Arg-1^+ immune cells at the lesion site following MSC/IL-13 grafting as compared to control MSCs. Taken together, these results indicate that the secretion of IL-13 from MSC grafts (and/or the presence of *alternatively activated* microglia/macrophages at the MSC/IL-13 graft site), leads to an increased infiltration of peripheral macrophages visible at the lesion site. These macrophages in turn appear to undergo *alternative activation*, thereby providing neuroprotection and improved therapeutic outcome following SCI.

5.4.6 Transplantation of MSC/IL-13 decreases the number of macrophage-axon interactions

Finally, we investigated how the presence of *alternatively activated* microglia and macrophages at the lesion site following MSC/IL-13 grafting, may have influenced the corresponding SCI pathology in both BALB/c (Figure 5.6A) and C57BL/6 (Figure 5.6H) mice. For this, we quantified the number of microglia/macrophage-axon interactions using Iba-1 and neurofilament staining.

In both mouse backgrounds, we analysed two areas rostral and caudal from the lesion epicentre (Figure 5.6B-D, I-K: white boxes) and counted the number of microglia/macrophage-axon contacts (Figure 5.6E-G, L-N). In BALB/c mice, we observed a significant decrease in the number of microglia/macrophage-axon contacts in both MSC (Figure 5.6F) and MSC/IL-13 (Figure 5.6G) treated mice compared with NaCl control mice (Figure 5.6E). In C57BL/6 mice, we obsreved a significant decrease in the number of microglia/macrophage-axon contacts in MSC/IL-13 treated mice (Figure 5.6N) compared with MSC-treated (Figure 5.6M) and NaCl control mice (Figure 5.6L). These results indicate that both MSC and MSC/IL-13 (in a BALB/c background) and MSC/IL-13 (in a C57BL/6 background), may be driving *activated* macrophages located at the lesion site to a more alternatively *actived,* neuroprotective phenotype. This in turn leads to a reduction in the number of destructive macrophage-axon contacts.

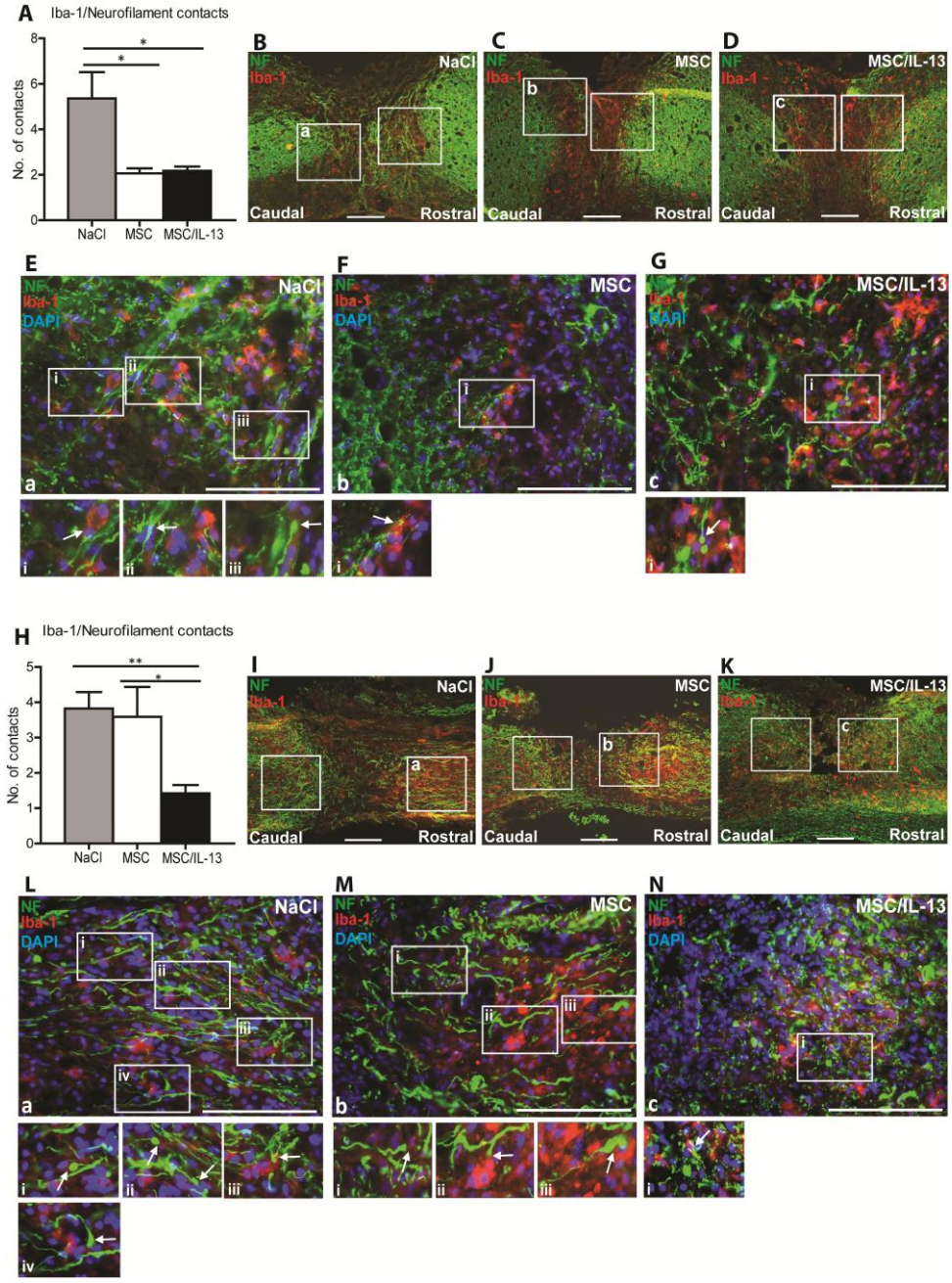
A
Iba-1/Neurofilament contacts
No. of contacts
NaCl
MSC
MSC/IL-13
B
C
D
NF
Iba-1
DAPI
Caudal
Rostral
E
F
G
H
Iba-1/Neurofilament contacts
I
J
K
L
M
N

Figure 5.6: Transplantation of MSC/IL-13 decreases the number of microglia/macrophage-axon contacts
(A) Quantification of microglia/macrophage and axon contacts in BALB/c mice following staining for Iba-1 and neurofilament respectively, revealed a significant decrease in the number of contacts in both MSC and MSC/IL-13 treated mice compared with NaCl controls. Representative photomicrographs from **(B)** NaCl, **(C)** MSC and **(D)** MSC/IL-13 treated BALB/c mice indicates the areas (two white boxed regions) where microglia/macrophage and axon contacts were quantified rostral and caudal from the lesion epicentre. A larger magnification of the white boxes labelled **(a, b, c)** are shown in **(E, F, G)** respectively. The white boxed regions **(i-iii)** in photomicrographs **(E-G)** are shown at a higher magnification to indicate examples of microglia/macrophage and axon contacts. **(H**) Quantification of microglia/macrophage and axon contacts in C57BL/6 mice, revealed a significant decrease in the number of contacts in MSC/IL-13 treated C57BL/6 mice compared with MSC and NaCl controls. Representative photomicrographs of the areas quantified (two white boxed regions) are shown from **(I)** NaCl, **(J)** MSC and **(K)** MSC/IL-13 treated mice. A larger magnification of the white boxes labelled **(a, b, c)** are shown in **(L, M, N)** respectively. The white boxed regions **(i-iv)** in photomicrographs **(L-M)** are shown at a higher magnification to indicate examples of microglia/macrophage and axon contacts. Scale bars B-D, I-K = 200μm, E-G, L-N = 50μm. ***** p < 0.0001, * p < 0.05; n = 4-10/group.**

5.5 Discussion

The goal of this study was to compare the potentially beneficial properties and unravel key immune response changes following engraftment of control MSCs or MSCs overexpressing IL-13 in a well-established mouse SCI model. Although treatment with MSCs has been previously shown to exert positive effects in rodent models of SCI (5, 6), their pro-longed therapeutic effects and success in human clinical trials have been limited (41, 42). Genetic modification of MSCs, for example by overexpression of neurotrophic or growth factors, can further enhance their well-known beneficial effects and improve therapeutic outcome following CNS trauma (reviewed in (43)). In this study, we show that transplanted MSCs, which continuously secrete IL-13, significantly improve histopathological and functional recovery compared with NaCl controls following

SCI in BALB/c and C57BL/6 mice. The correlation between histopathological (i.e. decreased lesion size, demyelinated area and astrogliosis) and functional recovery was highly evident in C57BL/6 mice following grafting of IL-13 producing MSCs. Interestingly, in BALB/c mice, IL-13 contributed to histopathological recovery (decreased lesion size and demyelinated area), but did not further promote an additional functional improvement induced by control MSCs. This variation may be attributed to the well-recognised immunological phenomenon that BALB/c mice are more Th2-orientated whilst C57BL/6 are more Th1-orientated (44, 45). Additionally, it has been reported that BALB/c mice display a higher recovery following SCI compared with C57BL/6 mice as measured using the BMS (35). This may explain the one point difference in the final BMS score between BALB/c and C57BL/6 mice (score of ~4 versus ~3 respectively). Furthermore, these data suggest that treatment with IL-13 may be unable to further enhance what is already a rather *Th2-primed* microenvironment (Hendrix and Nitsch, 2007) resulting in a ceiling effect in BALB/c mice.

It has been well described that IL-13 is capable of polarising microglia and macrophages towards an *alternatively activated* M2a phenotype (25, 46). Therefore, we first investigated whether transplantation of MSCs secreting IL-13 could influence the microglia/macrophage phenotype *in vivo* within the MSC graft site. Our data confirm that MSC graft-associated microglia/macrophages can be efficiently driven towards an Arg-1 expressing state of *alternative activation in vivo*, in both BALB/c and C57BL/6 mice. Given the lacking effect on microglia/macrophage cell number following treatment with MSC or MSC/IL-13, we consider cell phenotype (classically or alternatively activated), to be the most critical factor of interest when determining effects on functional outcome. Further discrimination between MSC-associated microglia and macrophages following grafting of IL-13 producing MSCs in $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ C57BL/6 mice revealed that the majority of Arg-1 expressing cells are of peripheral monocyte/macrophage origin. The peripheral origin of Arg-1 expressing cells is however not surprising as control MSC grafts already attract, in addition to

microglia, high numbers of peripheral monocytes/macrophages. Subsequently, IL-13 is able to further modulate the MSC graft site by increasing the number of Arg-1 expressing macrophages and microglia and decreasing the overall number of microglia. Although subject to debate and further investigation, we may argue that Arg-1 expressing microglia and macrophages are induced *in situ* upon contact with IL-13-secreting MSCs, rather than being specifically attracted.

T cell analysis within the spinal cord showed a significantly increased number of CD4+ T cells in animals treated with MSC/IL-13. An additional double staining for CD4 and FOXP3 (data not shown), ruled out the presence of $FOXP3^+$ regulatory T cells. The specific subtype of the T cells is unclear and previous studies from our group revealed that specific T cell immunophenotyping after CNS injury can be challenging and prone to artifacts, due to the low number of T cells present in the CNS (47). The high number of T cells in MSC/IL-13 treated mice may be due to the restriction of T cell chemotaxis by IL-13 (48), thereby leading to an accumulation of T cells within an area where they are highly activated. Since transplantation of MSC/IL-13 exerts beneficial effects on the injured spinal cord, it is tempting to speculate that the attracted T cells are also those with beneficial properties (Hendrix and Nitsch, 2007).

As the introduction of *alternatively activated* microglia and macrophages is established by the presence of IL-13 at the graft site, we hypothesised that a phenotypic shift in the microglia/macrophage response may also be possible at the spinal cord lesion site. Similarly to our observations at the MSC/IL-13 graft site, we observed an increase in Arg-1 expressing macrophages and microglia and an overall decrease in microglia, at the lesion site in mice receiving MSC/IL-13 grafts. An interesting question consequently arises as to how Arg-1 expressing microglia/macrophages are induced at the lesion site. We can speculate, that the induction of *alternatively activated* microglia/macrophages at the lesion site may be due to passive diffusion of IL-13 from the MSC/IL-13 graft site. Upon contact with lesion-associated microglia or infiltrating macrophages, this may in turn result in a shift towards a more neuroprotective cell phenotype. Although this hypothesis seems highly plausible, one cannot exclude the

possibility that other factors (aside from IL-13), may be secreted from the MSC graft or *alternatively activated* microglia/macrophages at the MSC/IL-13 graft site. These derived factors, either alone or in combination, may also influence the phenotypic properties of lesion-associated microglia/macrophages. Furthermore, as discussed above, specific recruitment of *alternatively activated* monocytes/macrophages directly from peripheral blood may not be evident, however, their recruitment via the MSC/IL-13 graft site cannot be excluded. An important observation which should be taken into consideration for future experiments, is the presence of the double positive $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ cell population. We as well as others hypothesise that these double positive cells are blood derived macrophages given the known limitation that $CCR2^{RFP/+}$ monocytes can down-regulate their reporter over time and show phenotypic evolution (40). It has also been shown that resident microglia consist primarily of $CX_3CR1^{eGFP/+}$ cells, while blood-derived macrophages are made up of both $CX_3CR1^{eGFP/+}$ as well as $CCR2^{RFP/+}$ macrophage populations (Evans et al., 2014). In this study, we therefore consider these double positive cells to be part of the macrophage population. The arrival of promising microglia-specific markers (49, 50) may be useful in future studies to clarify whether the double-positive cells are primarily infiltrating macrophages. It is clear that evaluation of the inflammatory infiltrate is that of a complex one and variation occurs not only in the type of disease/trauma model but also in the time-point under investigation.

Although the variation in immunological background between BALB/c and C57BL/6 **i**s a well know phenomenon (44, 45), we have not observed any immunological differences in our transplantation systems in either mouse strain. As shown in this study, transplantation of MSC/IL-13 leads to a significant increase in Arg1+ cells at the graft sites in both BALB/c and C57BL/6 mice (Figures 3G + 4P respectively). Additionally, our group has performed an in depth analysis of graft site remodelling upon MSC transplantation in the CNS of BALB/c, FBV or C57BL/6 mouse backgrounds (25, 26, 39). Upon MSC transplantation in all of the above mouse strains, cells undergo hypoxic stress within the first 24 hours and the core of the graft becomes apoptotic and

necrotic. This is followed by early infiltration of neutrophils on day 1. From days 3-7, the graft becomes infiltrated by macrophages and surrounded by microglia. At this point, astrocytes begin forming a barrier which encapsulates the graft (51). Based on these studies and our current results, we conclude that there is no obvious difference in the graft site response between BALB/c and C57BL/6 mice.

Finally, we suggest a potential mode-of-action for the observed neuroprotective effects following grafting of IL-13 producing MSCs. Based on our data, and in agreement with current literature, we can put forward two mechanistic explanations. Firstly, it has been shown that a reduction in CX_3CR1 signaling on microglia/macrophages reduces their pro-inflammatory nature and leads to improved outcome following spinal cord injury (52). Therefore, the decrease in $eGFP^+RFP^-$ microglia, which we observed at the lesion site following grafting of MSC/IL-13, may correlate with the improved functional outcome shown in MSC/IL-13-treated mice following SCI. The way in which IL-13 actually reduces the number of microglia in this model remains speculative, however it may be explained by a previously described ability of IL-13 to directly induce apoptosis in activated microglia (53). Secondly, it has recently been demonstrated that CNS axons undergo lengthy retraction from the site of damage following SCI and that activated macrophages play a direct role in this retraction via destructive physical interactions with the injured axons (16, 17, 37). Therefore, the observed conversion of lesion-infiltrating macrophages into an Arg-1 expressing *alternatively activated* phenotype upon MSC/IL-13 grafting, may have rendered these cells less neuro-destructive. The latter suggests a correlation with the significant decrease in macrophage-axon contacts observed at the lesion site in BALB/c mice following treatment with MSC or MSC/IL-13 and in C57BL/6 mice following treatment with MSC/IL-13. It is tempting to speculate that a corresponding reduction in axonal dieback, may lead to the significantly improved histopathological and functional outcome observed following SCI. In BALB/c mice, the reduction in the number of macrophage-axon contacts in MSC and MSC/IL-13 treated animals correlates with the increased functional outcome

observed in MSC and MSC/IL-13 treated mice, compared with NaCl controls. On the other hand, in C57BL/6 mice, only animals treated with MSC/IL-13 displayed an improved functional recovery and the decrease in the number of macrophage-axon contacts was also observed exclusively in MSC/IL-13 treated mice. We hypothesise that an increase in *alternatively activated* macrophages and microglia may promote wound healing, regeneration and functional recovery via the secretion of pro-regenerative factors such as IL-10, insulin-like growth factor-1 (IGF-1) and vascular growth factor-A (VEGF-A) (54). Taken together, these data provide evidence that MSCs can be successfully used as carriers for the local delivery of a beneficial cytokine such as IL-13 and lead to improved functional and histopathological recovery in a mouse model of SCI (Figure 5.7).

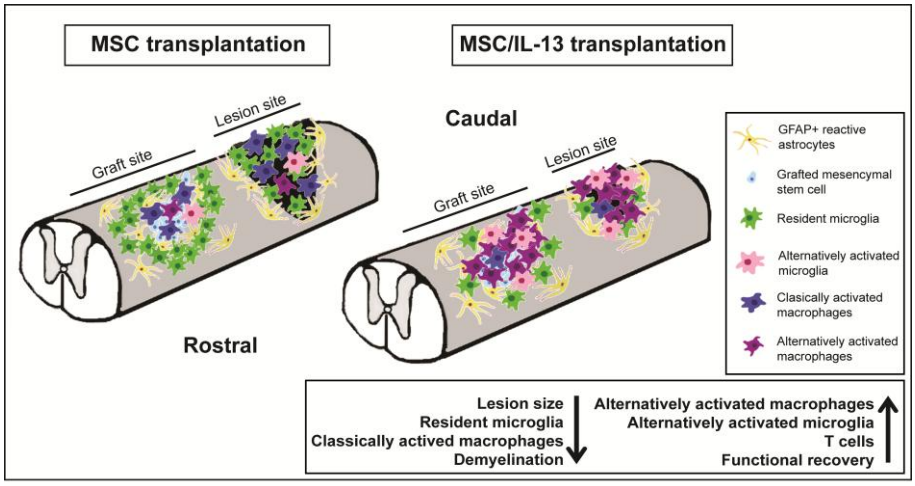

Figure 5.7: Schematic representation of the lesion and graft site in MSC compared with MSC/IL-13 treated mice following SCI.

In MSC treated mice, the lesion and graft site contain classically activated macrophages as well as a low number of alternatively activated macrophages and microglia. Both regions are encapsulated by GFAP+ astrocytes. The lesion area consists of resident microglia within and around the lesion site, whilst the graft site contains resident microglia at the graft border. Following treatment

with MSC/IL-13, the histological appearance and cell distribution differs substantially within the spinal cord. There is a decrease in lesion size as well as classically activated macrophages and resident microglia at both the lesion and graft site. There is also a dramatic increase in the number of alternatively activated macrophages and microglia at both the lesion and graft site. Both the lesion and graft sites remain encapsulated by GFAP+ astrocytes. Treatment with MSC/IL-13 alters the immune cell distribution and phenotype leading to improved functional recovery in a mouse model of SCI.

5.6 Conclusion

In conclusion, this study demonstrates that transplantation of IL-13-secreting MSCs significantly improves functional recovery following SCI in mice. Grafted MSC/IL-13 decrease lesion size and demyelinated area by over 40%, presumably by locally decreasing the number of resident microglia and increasing the number of *alternatively activated* macrophages, at both the graft and lesion site. These changes to the immune environment point towards a switch to a less neuro-destructive macrophage phenotype, as suggested by the significant reduction in macrophage-axon contacts at the lesion site. These data provide evidence that MSCs can be successfully used as carriers for the local delivery of a beneficial cytokine such as IL-13 and lead to improved functional and histopathological recovery in a mouse model of SCI.

5.7 References

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Chapter VI

Conclusions & Future Perspectives

In order to put the work described in this thesis into perspective, referring to the original research question is a good starting point: Is immunomodulation a potential therapy for spinal cord injury repair? As outlined in chapters III, IV and V, the answer to this question very much depends on a number of varying factors, i.e. immunomodulatory factor in question, method of administration, dosing etc. In order to answer this question, the described research has led to a number of scientific observations and conclusions. These are outlined and discussed below.

6.1 IL-25: an unattractive therapeutic target

We as well as others have shown that cytokines associated with Th2 cells such as IL-4 (1-3) and IL-10 (4, 5), are capable of promoting neuronal survival and regeneration, and also improve functional outcome after SCI. IL-25, a member of the IL-17 family, has been suggested to be a key player in the origin of a type 2 response (6, 7). Given that the limited studies available at the beginning of this project suggested a *protective* role of IL-25 in neuroinflammation (reviewed in (8, 9)), we aimed to investigate it's therapeutic potential after SCI. As outline in **chapter III**, we investigated whether increasing levels of IL-25, can promote functional recovery in a mouse model of SCI. Furthermore, we aimed to distinguish between local and systemic effects of treatment. To our surprise, we found that local application of IL-25 led to a significant worsening in motor performance after SCI, compared with PBS controls. At the histological level, these results were accompanied by a 30% increase in lesion size and demyelinated area. On the other hand, systemic IL-25 treatment did not influence functional recovery and there was no effect of systemic IL-25 treatment on lesion size or demyelinated area. These results indicate that both dosing and route of administration play a major role in determining the therapeutic effect of IL-25.

Furthermore, our results were rather unexpected as systemic IL-25 has been previously shown to suppresses EAE symptoms in a relapse-remitting model (7). Additionally, delivery of IL-25 to the CNS in two different models of neuroinflammation, was able to drive microglia and macrophages to a more

anti-inflammatory and tissue-protective phenotype (10). Upon investigation of the way in which IL-25 may be exerting its detrimental effects, we observed no significant effects of various concentrations of IL-25 on survival of oligodendrocytes, astrocytes, microglia or primary cortical neuronal cells *in vitro.* Therefore, the mechanistic action of IL-25 remains unclear. However, given that it is not caused by a direct effect on the above cell types, it is probable that administration of IL-25 following SCI activates an indirectly mediated cascade of detrimental immune events.

Additionally, Mearns et al recently questioned the role of IL-25 in the induction of a potentially beneficial Th2-cell response and demonstrated that IL-25 is dispensable during the differentiation and development of Th2 cells (11). We demonstrated that IL-25 failed to have an effect systemically and even worsened functional outcome when applied locally. This suggests that the direct involvement of IL-25 in driving a Th2 response remains questionable. Therefore, one can conclude that IL-25 is not a promising candidate for immunomodulatory therapy after SCI.

6.2 IL-13: a potential therapeutic target

In continuation with our hypothesis that cytokines associated with inducing a Th2 response may have therapeutic potential for treatment of SCI, our next factor of interest was IL-13. IL-13 has been shown to be a key factor, capable of inducing a macrophage phenotype switch from a rather 'detrimental' M1 phenotype to a more 'beneficial' *alternatively activated*, M2 phenotype (12, 13). Therefore, we hypothesised that treatment with IL-13 may also lead to a switch *in vivo* towards a more neuroprotective microglia/macrophage phenotype, resulting in improved functional recovery and therapeutic outcome.

6.2.1 Treatment with recombinant IL-13: prospects vs. limitations

IL-13, a cytokine closely related to IL-4 (13), is a canonical *anti-inflammatory* Th2 cytokine, which in some contexts can also be *pro-inflammatory* (14). IL-13 has been shown to exert neuroprotective effects in EAE, by decreasing inflammatory cell infiltration and axonal loss as well as reducing clinical

symptoms (15-17). Similarly to the IL-25 experimental set-up, we tested whether recombinant murine IL-13, administered either as a single dose locally to the spinal cord or via repeated systemic injections, improves functional recovery after SCI in mice, as outlined in **chapter IV**. While no clinical effect was observed following local administration of IL-13, systemic administration of IL-13 lead to a significant increase in functional outcome as well as a significant increase in the number of Arg-1^+ *alternatively activated* microglia/macrophages. Following these results, we have once again demonstrated that route of administration is a crucial aspect to consider when developing cytokine therapy for CNS trauma. Systemic administration of IL-13 resulted in a significantly improved functional outcome compared with PBS treated controls, which persisted for 12 weeks post injury. Surprisingly, we found no significant effect of IL-13 treatment on lesion size, demyelination or astrogliosis, ruling out any direct effect of IL-13 on lesion remodelling. However, there was a significant increase in the number of Arg-1^+ microglia/macrophages at the lesion site in IL-13-treated mice, suggesting a potential link between *neuroprotective* microglia/macrophages and improved functional outcome. Taken together, these results make it difficult to pin-down the exact mechanistic effect of IL-13 treatment. Systemic administration proves promising, however further research at this stage is still required to fine-tune the method of administration and identify the link between polarization of microglia/macrophages and functional recovery. Therefore, one can conclude that IL-13 is a promising candidate for immunomodulatory therapy after SCI, but further investigation is necessary to fine-tune its therapeutic potential.

6.3 MSCs as carriers of IL-13: an attractive therapeutic target

To build upon the use of recombinant IL-13 in the previous section, here we put forward our method of choice to continuously deliver local IL-13 to the injured spinal cord. As described in **chapter V**, to do this, we made use of MSCs genetically modified to secrete IL-13. We transplanted MSC/IL-13 immediately following SCI and investigated their therapeutic effects compared with control

MSCs. Our results indicated that transplanted MSCs which continuously secrete IL-13, not only significantly improve functional recovery following SCI in mice, but also decrease lesion size and demyelinated area by over 40%. Detailed histological analyses in $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ transgenic mice indicated that transplantation of MSC/IL-13 decreases the number of resident microglia and increases the number of *alternatively activated* macrophages at both the graft and lesion site. Additionally, we observed a reduced number of macrophage-axon contacts in the MSC/IL-13 group, potentially linked to a reduction in axonal dieback and improved functional outcome.

6.3.1 MSC/IL-13 improve functional recovery and histopathological outcome

Although treatment with MSCs has been previously shown to exert positive effects in rodent models of SCI (18, 19), their pro-longed therapeutic effects and success in human clinical trials have been limited (20, 21). Genetic modification of MSCs, for example by overexpression of neurotrophic or growth factors, can further enhance their well-known beneficial effects and improve therapeutic outcome following CNS trauma (reviewed in (22)). Here, we have shown for the first time that transplanted MSCs, which continuously secrete IL-13, significantly improve histopathological and functional recovery compared with NaCl controls following SCI in both BALB/c and C57BL/6 mice.

While the correlation between histopathological and functional recovery was highly evident in C57BL/6 mice following grafting of IL-13 producing MSCs, this was less clear in BALB/c mice where IL-13 contributed solely to histopathological, but not functional recovery. This variation may be attributed to the well-recognised immunological phenomenon that BALB/c mice are more Th2-orientated whilst C57BL/6 are more Th1-orientated (23, 24). This suggests that IL-13 may be unable to further enhance what is already a rather *Th2-primed* microenvironment in BALB/c mice. Nevertheless, we have demonstrated that in order to achieve substantial tissue protection and/or remodelling at the SCI lesion site, the presence of IL-13 in both C57BL/6 as well as BALB/c mice, is required.

6.3.2 The $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ transgenic mouse model: an invaluable tool for cell phenotyping and quantification following SCI

In order to differentiate between resident microglia and infiltrating macrophages/monocytes, we took advantage of the $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ transgenic mouse model. These mice were cross bred in house at Antwerp University. Resulting double transgenic mice have one allele of the CX_3CR1 gene replaced by eGFP and the other allele of the CCR2 gene replaced by RFP (25). This results in the presence of green fluorescent microglia and red fluorescent infiltrating macrophages/monocytes. This model, combined with the use of TissueQuest software, allowed us to quantify in detail, microglia and macrophage cell densities and phenotypes at both the graft and lesion site. We have demonstrated that the use of TissueQuest is highly quantitative and also more accurate than a standard intensity analysis, using for example Image J. Additionally, this transgenic mouse model and quantification method has also been successfully implemented in various experimental setups by our collaborators at Antwerp University (25-27). We therefore recommend that this be used as the gold-standard method for detailed graft-host cell quantification upon stem cell transplantation.

6.3.3 Successful induction of M2a, *alternatively activated* microglia and macrophages *in vivo* following treatment with MSC/IL-13

Numerous studies have demonstrated that IL-13 is a key factor in inducing a macrophage phenotype switch from a rather *pro-inflammatory*, 'detrimental' M1 phenotype to a more 'beneficial' *alternatively activated*, M2 phenotype (12, 13). However, these studies have demonstrated this phenomenon *in vitro*, which does not always translate well to the *in vivo* situation, given the complex microenvironment that exists. Following systemic administration of IL-13 after SCI, we observed improved functional outcome and an increase in the number of Arg-1^+ *alternatively activated* microglia/macrophages at the lesion site. In our studies using MSC/IL-13, our method of delivery was more localised. Using the $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ transgenic mouse model, we demonstrated an upregulation of both MHC-II and Arg-1, primarily on peripheral macrophages invading the graft and lesion site. Given that the joint expression of MHC-II and Arg-1 is indicative of a neuroprotective, anti-inflammatory, M2a phenotype (28),

this suggests that the secretion of IL-13 leads to a successful induction of M2a macrophages at both the MSC transplantation site and spinal cord lesion area. Additionally, a collaborative research project running in parallel at Antwerp University also demonstrated efficient *in vivo* induction of M2 *alternatively activated* microglia and macrophages following transplantation of MSC/IL-13 in the cuprizone model of neuroinflammation (Le Blon et al, *under revision*).

6.3.4 Decrease in the number of axon-macrophage contacts following treatment with MSC/IL-13: the missing mechanistic link?

In order to piece together the link between *alternatively activated* microglia and macrophages and an improved functional and histopathological outcome following SCI, we took to the literature in an attempt to understand how IL-13 may be exerting its beneficial effects. It is a well-known phenomenon that CNS axons undergo lengthy retraction from the site of damage following SCI, but we learned that activated macrophages play a direct role in this retraction via cell-cell interactions with the injured axons (29, 30). Therefore, the observed increase in Arg-1 expressing, *alternatively activated* lesion-infiltrating macrophages upon MSC/IL-13 grafting, may have rendered these cells less neuro-destructive. We observed a potentially correlative significant decrease in the number of macrophage-axon contacts at the lesion site in MSC/IL-13-treated mice compared with control MSC- or NaCl-treated mice. This in turn may have reduced the level of axonal dieback, resulting in an improved histopathological and functional outcome. Taken together, one can conclude that MSCs can be successfully used as carriers for the local delivery of IL-13 and that this delivery system is a highly attractive candidate for immunomodulatory therapy after SCI.

6.4 Future perspectives

Previous research within the Department of Morphology at Hasselt University has greatly contributed to our understanding of cytokine therapy following SCI (3, 31, 32). Additionally, research at the Laboratory of Experimental Hematology at Antwerp University has greatly contributed to our understanding of the neuro-immune response following MSC transplantation in the CNS (26, 33, 34). In this

thesis, we have combined these areas of expertise by investigating the therapeutic effects of immunomodulatory cytokine treatment as well as MSC transplantation following SCI. We also focused on discriminating between microglia and macrophages in order to underline the phenotypic responses following MSC and MSC/IL-13 transplantation. Therefore, our findings build upon the research at both Hasselt and Antwerp Universities and open the door to a variety of new research questions, which are outlined below.

6.4.1 What are the alternative methods for local delivery of IL-13 following SCI?

Although the data discussed in this thesis are of huge fundamental importance, we must look forward towards translating this to a clinical setting. In parallel with this study, our groups are currently working on assessing these alternatives. One ongoing possibility, is the use of allogeneic ('off-the shelf') instead of autologous, IL-13-secreting MSCs. We have already shown that IL-13-secretion from allogeneic MSC grafts can prolong their immunological survival compared with control allogeneic MSCs (33), however their therapeutic efficacy following SCI, still needs to be established. Despite this progress, there are still several safety concerns (e.g. immunogenicity and tumorogenicity), which are inevitably associated with stem cell transplantation and limit its applicability clinically. Therefore, we are also currently investigating the effects of local or intravenous administration of IL-13-producing macrophages following SCI. The choice of genetically-engineered macrophages for delivery of IL-13 is clinically relevant given that they can be generated in a relatively short period of time (35) compared with autologous MSCs. Additionally, for intravenous cell administration, we have previously shown that macrophage/dendritic cell populations, can, unlike MSC populations, safely pass the lung circulation (36). Furthermore, we have recently shown that *alternative activation* of microglia/macrophages *in vivo* requires sustained stimulation with IL-13 (33), which makes macrophages genetically engineered to continuously secrete IL-13, a logical choice.

6.4.2 What is the mechanism behind the beneficial effects of IL-13 following SCI?

In Chapter V, the results obtained following transplantation of MSC or MSC/IL-13 in a mouse model of SCI were discussed in detail. In short, mice treated with MSC/IL-13 showed a significant improvement in both clinical and histopathological outcome following SCI. However, the question regarding how IL-13 secreted from MSCs is exerting these beneficial effects, still remains unanswered. The most plausible mechanism as discussed, may be a direct effect of IL-13 on microglia apoptosis. Alternatively, IL-13 may act via an indirect induction of on *M2a-activated* macrophages. However, it is most likely a combination of both. In order to further investigate this mechanism of action, future experiments will include transplantation of IL-13-producing MSC in IL-4 receptor type II knockout (IL4RII-/-) mice, given this receptor binds both IL-4 and IL-13 (13, 37). Transplantation of MSC/IL-13 in this knockout model, will enable us to investigate the effects of IL-13 signaling in depth. This, in combination with transplantation experiments using IL-13-secreting macrophages, will help reveal whether IL-13 receptor signalling in either microglia or macrophages, is more relevant in mediating IL-13-induced neuroprotection. Unravelling the true mechanism of action of IL-13 is currently one of our top priorities. Given that this research question is currently being tackled, we hope to determine whether IL-13 has a therapeutic future in human clinical trials in the near future.

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Summary

SCI is a devastating pathology which has a significant impact on life expectancy and quality, and also bears considerable economic burden. Despite considerable progress in palliative care, there is currently no therapeutic intervention available which leads to functional recovery. Therefore, there is an urgent need to develop new strategies and therapies. Inflammatory responses are a major component of the secondary injury phase and play a key role in regulating the pathogenesis of acute and chronic SCI. However, the continual and dual role of the neuroinflammatory response leaves it difficult to decide upon a single modulatory strategy. Additionally, the role of certain immunomodulatory cytokines which are up- or down-regulated in response to SCI still remains unclear. In this thesis, we focus on the type-2 cytokines: IL-25 and IL-13 and investigate their therapeutic potential for treatment of SCI. Understanding the interaction of these factors with the surrounding microenvironment and exploiting their modulatory effects on cells such as microglia and macrophages, may provide a vital therapeutic tool in developing strategies for treating CNS trauma.

Firstly, we examined the effects of local or systemic application of IL-25 after SCI **(Chapter III).** The data presented in this thesis demonstrated that IL-25 failed to have an effect systemically and even worsened functional outcome when applied locally. Therefore, one can conclude that IL-25 is not a promising candidate for immunomodulatory therapy after SCI.

In the second part of this study, we focused on investigating the therapeutic potential of the Th2, *anti-inflammatory* cytokine, IL-13. In addition to its immunomodulatory effects, IL-13 is also a well-known inducer of the M2 microglia/macrophage phenotype. We demonstrated that local application of IL-13 had no effect on functional recovery. However, systemic application significantly improved recovery after SCI and also lead to a significant increase in the number of Arg-1^{+} *alternatively activated* microglia/macrophages. **(Chapter IV).** These results make it difficult however, to pin-down the exact mechanistic effect of IL-13 treatment. Systemic administration proves promising, but further research at this stage is still required to identify the link between polarisation of microglia/macrophages and functional recovery. Therefore, one can conclude that IL-13 is a promising candidate for

immunomodulatory therapy after SCI, but further investigation is necessary to fine-tune its therapeutic potential.

In the final part of this thesis, we aimed to optimise a continuous local delivery system for IL-13. In order to achieve this, we choose to use MSCs as carriers, given that these stem cells already possess the ability to exert positive effects in rodent models of SCI. We investigated the use of autologous MSCs which were genetically modified to secrete IL-13, as a potential therapeutic strategy to modulate the immune response and improve functional recovery after SCI. Our results indicated that transplanted MSCs which continuously secrete IL-13, not only significantly improve functional recovery following SCI in mice, but also decrease lesion size and demyelinated area by over 40%. Detailed histological analyses in the $CX_3CR1^{eGFP/+}$ $CCR2^{RFP/+}$ transgenic mouse model indicated that transplantation of MSC/IL-13 decreases the number of resident microglia and increases the number of *alternatively activated* macrophages at both the graft and lesion site. Additionally, we observed a reduced number of macrophage-axon contacts in the MSC/IL-13 group, potentially linked to a reduction in axonal dieback and improved functional outcome **(Chapter V).** These data indicate that MSCs can be successfully used as carriers for the local delivery of IL-13 and that this delivery system is a highly attractive candidate for immunomodulatory therapy after SCI.

Taken together, we have demonstrated in this thesis that immunomodulatory therapy using IL-13, provides great therapeutic potential for treatment of SCI. However, further research is still required to identify the exact mechanisms behind these beneficial effects.

Nederlandstalige samenvatting

Een ruggenmergletsel is een ernstige aandoening met een significante impact op de levensverwachting en –kwaliteit. Daarenboven heeft het ook een belangrijke economische impact. Ondanks de grote vooruitgang in de palliatieve zorg is er tot op heden geen therapeutische interventie beschikbaar die kan leiden tot functioneel herstel. Daarom is het van belang om nieuwe strategieën en therapieën te ontwikkelen. De secundaire schade na een ruggenmergletsel is grotendeels het gevolg van inflammatoire reacties. Deze spelen een belangrijke rol in de pathogenese van zowel acute als chronische ruggenmergschade. De voortdurende en tweedelige rol van de neuro-inflammatoire respons maakt het moeilijk om één strategie te vinden om deze te moduleren. Bovendien is de functie van bepaalde immuun-modulerende cytokinen, die na ruggenmergschade hoger of lager tot expressie komen, nog onbekend. In deze thesis ligt de focus op type-2 cytokinen: IL-25 en IL-13 en het onderzoek naar hun therapeutisch potentieel als behandeling voor ruggenmergletsels. De interactie van deze factoren met hun omgeving en het gebruik van hun modulerende effecten op bepaalde celtypes zoals microglia en macrofagen, kunnen een belangrijke therapeutische tool vormen in het ontwikkelen van strategieën voor de behandeling van CZS trauma.

Ten eerste hebben we de effecten van lokale of systemische toediening van IL-25 na ruggenmergschade onderzocht (**Hoofdstuk III**). De resultaten in deze thesis geven aan dat IL-25 geen effect heeft na systemische administratie. Daarenboven verslechterde het functioneel herstel na lokale toediening. Hieruit kan men besluiten dat IL-25 niet veelbelovend is als een immuun-modulerende therapie na een ruggenmergletsel. In het tweede deel van deze studie ligt de nadruk op het therapeutisch potentieel van IL-13; een Th2 en anti-inflammatoir cytokine. Naast de immuun-modulerende effecten is IL-13 ook gekend voor de inductie van het M2 microglia/macrofaag fenotype. Wij tonen aan dat lokale toediening van IL-13 geen effecten op functioneel herstel heeft. Daarentegen verbeterde het functioneel herstel significant na systemische toediening. Daarnaast deed IL-13 ook het aantal Arg-1^{+} alternatief geactiveerde microglia/macrofagen significant stijgen. (**Hoofdstuk IV**). Deze resultaten maken het moeilijk om het exacte mechanisme van de effecten van IL-13 aan te duiden. Systemische toediening is veelbelovend maar verder onderzoek is

vereist om de link tussen microglia/macrofaag polarisatie en functioneel herstel te identificeren. Hieruit kan men besluiten dat IL-13 een goede kandidaat is voor immuun-modulerende therapie na ruggenmergschade, maar verder onderzoek is nodig om het therapeutische potentieel te finetunen. Het laatste deel van deze thesis had tot doel de continue lokale toediening van IL-13 te optimaliseren. Om dit te bereiken hebben we mesenchymale stamcellen (MSC) als dragers van IL-13 gekozen, aangezien deze stamcellen reeds positieve effecten bereikten in knaagdiermodellen voor een ruggenmergletsel. Wij hebben het gebruik van autologe MSCs, genetisch gemanipuleerd om IL-13 uit te scheiden, onderzocht als mogelijke therapeutische strategie om het immuunsysteem te beïnvloeden en functioneel herstel te verbeteren na ruggenmergschade. Onze resultaten tonen aan dat getransplanteerde MSC, die continu IL-13 uitscheiden, niet alleen functioneel herstel na een ruggenmergletsel significant bevorderen, maar ook de laesie-grootte en het gedemyeliniseerde gebied verkleinen met 40%. Een gedetailleerde histologische analyse bij het CX3CR1$^{eGFP/+}$ CCR2$^{RFP/+}$ transgeen muismodel toonde aan dat transplantatie van MSC/IL-13 het aantal endogene microglia doet dalen en het aantal alternatief geactiveerde macrofagen doet stijgen in het getransplanteerde gebied en het laesie-gebied. Daarnaast zien we ook een daling in het aantal macrofaag-axon contacten in de MSC/IL-13 groep, mogelijk gelinkt aan een daling in axonretractie en verbeterd functioneel herstel (**Hoofdstuk V**). Deze resultaten tonen aan dat MSC succesvol gebruikt kunnen worden als dragers voor de lokale toediening van IL-13 en dat dit toedieningssysteem zeer geschikt is als immuun-modulerende therapie voor ruggenmergschade.

Samengevat, met deze thesis hebben wij aangetoond dat immuun-modulerende therapie door middel van IL-13 een belangrijk therapeutisch potentieel bevat voor de behandeling van ruggenmergschade. Verder onderzoek is noodzakelijk om de juiste mechanismen achter deze veelbelovende effecten te identificeren.

Curriculum Vitae

Personal Details

Name: Dearbhaile Dooley
Nationality: Irish
Date of birth: 05/08/1989

Education

2011-present: Joint PhD Candidate in Biomedical Science/Anatomy Teaching Assistant
Hasselt University, Belgium – Supervisor: Prof. Sven Hendrix
University of Antwerp, Belgium – Supervisor: Prof. Peter Ponsaerts

2006-2010: BSc (Honours) – Anatomy major, Pharmacology minor
National University of Ireland, Galway

Publications

Dooley D, Lemmens E, Vangansewinkel T, Le Blon D, Hoornaert C, Ponsaerts P, Hendrix S:
IL-13-secreting MSCs promote infiltration of M2a macrophages and improve functional recovery following spinal cord injury
Stem Cell Reports, Under Revision; **IF = 7.023**

Dooley D, Lemmens E, Ponsaerts P, Hendrix S:
Interleukin-25 is detrimental for recovery after spinal cord injury in mice
Journal of Neuroinflammation, vol. 13, 2016: **IF = 5.408**

Dooley D, Vidal PM, and Hendrix S:
Immunopharmacological intervention for successful neural stem cell therapy: New perspectives in CNS neurogenesis and repair
Pharmacology & Therapeutics, vol. 141, pp. 21–31, 2014; **IF = 7.745**

Hoornaert C, Luyckx E, Reekmans K, Dhainaut M, Guglielmetti C, Le Blon D, **Dooley D,** Fransen E, Daans J, Verbeeck L, Quarta A, De Vocht N, Lemmens E, Van der Linden A, Roobrouck V, Verfaillie C, Hendrix S, Berneman Z, Ponsaerts P:
In vivo IL13-primed macrophages contribute to reduced alloantigen-specific T cell activation and prolong immunological survival of allogeneic mesenchymal stem cell implants
Stem Cells, in press, 2016; **IF = 6.523**

Schönfeld LM, Jahanshahi A, Lemmens E, Schipper S, **Dooley D**, Joosten E, Temel Y, Hendrix S:
Long-term motor deficits after controlled cortical impact in rats can be detected by fine motor skill tests but not by automated gait analysis
Journal of Neurotrauma, in press, 2016; **IF = 4.377**

Vangansewinkel T, Geurts N, Quanten K, Nelissen S, Lemmens S, Geboes L, **Dooley D**, Vidal PM, Pejler G and Hendrix S:
Mast cells promote scar remodeling and functional recovery after spinal cord injury via mouse mast cell protease 6
FASEB Journal, vol. 30, pp. 2040-2057, 2016; **IF = 5.034**

Lemmens S, Brone B, **Dooley D**, Hendrix S, and Geurts N:
Alpha-Adrenoceptor Modulation in Central Nervous System Trauma: Pain, Spasms, and Paralysis An Unlucky Triad
Medicinal Research Reviews, 2014, vol. 4 pp. 653-77, 2015; **IF = 8.226**

Nelissen S, Vangansewinkel T, Geurts N, Geboes L, Lemmens E, Vidal PM, Lemmens S, Willems L, Boato F, **Dooley D**, Pehl D, Pejler G, Maurer M, Metz M, and Hendrix S:
Mast cells protect from post-traumatic spinal cord damage in mice by degrading inflammation-associated cytokines via mouse mast cell protease 4
Neurobiology of Disease, vol. 62, pp. 260–272, 2014; **IF = 5.624**

Vidal PM, Lemmens E, **Dooley D**, and Hendrix S:
The role of "anti-inflammatory"cytokines in axon regeneration
Cytokine & Growth Factor Reviews, vol. 24, pp. 1–12, 2013; **IF = 6.537**

Invited talks

2016: Knowledge for Growth, Gent, Belgium
Title: *IL-13-secreting MSCs promote infiltration of M2a macrophages and improve functional recovery following spinal cord injury*

2015: EMBO meeting: Cell therapy today: achievements, hopes and hypes Manchester, United Kingdom
Title: *Immuomodulatory therapy for spinal cord injury via IL-13-secreting stem cells*

Dutch anatomical society (NAV) annual meeting, Lunteren, The Netherlands
Title: *Immuomodulatory therapy for spinal cord injury*

2014: EMBO workshop: Development & regeneration of the spinal cord, Sitges, Spain
Title: *Mesenchymal stem cells overexpressing IL-13 decrease lesion size and demyelination after spinal cord injury*

Selected Poster Presentations

2016: BSCDB Spring meeting: Stem Cells as Disease Model and for Gene Therapy, Brussels, Belgium
Dooley D., Lemmens E., Vangansewinkel T., Le Blon D., Hoornaert C., Ponsaerts P., Hendrix S: *IL-13-secreting MSCs promote infiltration of M2a macrophages and improve functional recovery following spinal cord injury*

Knowledge for Growth, Gent, Belgium

Dooley D., Lemmens E., Vangansewinkel T., Le Blon D., Hoornaert C., Ponsaerts P., Hendrix S: *IL-13-secreting MSCs promote infiltration of M2a macrophages and improve functional recovery following spinal cord injury*

2015: The International Spinal Cord Repair Meeting (ISCORE 15), Barcelona, Spain

Dooley D., Lemmens E., Vangansewinkel T., Le Blon D., Hoornaert C., Ponsaerts P., Hendrix S: *Mesenchymal stem cells overexpressing IL-13 decrease lesion size and demyelination after spinal cord injury*

15th ESNI course Prague, Czech Republic

Dooley D., Lemmens E., Vangansewinkel T., Le Blon D., Hoornaert C., Ponsaerts P., Hendrix S: *Mesenchymal stem cells overexpressing IL-13 decrease lesion size and demyelination after spinal cord injury*

Interuniversity Stem Cell Symposium, Leuven, Belgium

Dooley D., Lemmens E., Vangansewinkel T., Lemmens S., De Vocht N., Le Blon D., Ponsaerts P., Hendrix S: *Mesenchymal stem cells overexpressing IL-13 decrease lesion size and demyelination after spinal cord injury*

2014: 9th FENS Forum of Neuroscience, Milan, Italy

Dooley D., Lemmens E., Vangansewinkel T., Lemmens S., De Vocht N., Le Blon D., Ponsaerts P., Hendrix S: *Mesenchymal stem cells overexpressing IL-13 decrease lesion size and demyelination after spinal cord injury*

Belgian Society for Stem Cell Research, Liège, Belgium

Dooley D., Lemmens E., Vangansewinkel T., Lemmens S., De Vocht N., Le Blon D., Ponsaerts P., Hendrix S: *Mesenchymal*

stem cells overexpressing IL-13 decrease lesion size and demyelination after spinal cord injury

2013: EPFL SV-Life Science Symposium, *Lausanne*, Switzerland
Dooley D., Lemmens E., Vangansewinkel T., Lemmens S., De Vocht N., Ponsaerts P., Hendrix S: *Immunomodulatory therapy for spinal cord injury via IL-13 –secreting stem cells*

WOG symposium, Antwerp, Belgium
Dooley D., Lemmens E., Vangansewinkel T., Lemmens S., De Vocht N., Ponsaerts P., Hendrix S: *Immunomodulatory therapy for spinal cord injury via IL-13 –secreting stem cells*

2012: Summer School on Neural Stem Cells in Development and Disease, Levico Terme, Trento, Italy
Dooley D., Lemmens E., Lemmens S., Hendrix S: *Immunomodulatory therapy for spinal cord injury via IL-13 – secreting stem cells*

Awards

2010: Poster presentation prize
Neuroscience Ireland annual meeting, University College Dublin, Ireland
Title: *Response of astrocytes to Cyclosporin-A and neural stem cell treatment in contused rat spinal cord*

Grants

2015: Travel Grant awarded by Belgian Society for Cell and Developmental Biology
For attending: *EMBO meeting: Cell Therapy today: achievements, hopes & hypes, Manchester, United Kingdom*

Travel Grant awarded by Flemish Research Foundation (FWO)
International Mobility Grant awarded by Hasselt University
For attending: *15th ESNI course Prague, Czech Republic*

2014: Travel Grant awarded by Flemish Research Foundation (FWO)
For attending: *9th FENS FORUM of Neuroscience, Milan, Italy*

2012: International Mobility Grant awarded by Hasselt University
For attending: *Neural Stem Cell Summer School, Trento, Italy*

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Dank u - Merci beaucoup - Go raibh míle maith agaibh - Thank you

Dearbhaile, August 2016

"Do not wait to strike till the iron is hot; but make it hot by striking"
W.B. Yeats

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"Dá fhada an lá tagann an tráthnóna"

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"Níl aon tinteán mar do thinteán féin"