Therapy Approach of Neurodegenerative Disorders by Bone Marrow Stem Cells

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Sara Alejandra Litwak

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1. Referent: Prof. Dr. Harald Neumann
2. Referent: Prof. Dr. Michael Hoch

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Abbreviations

Ab: antibody

BBB: blood brain barrier

BDNF: brain derived neurotrophic factor

bFGF: basic fibroblast growth factor

BM: bone marrow

CAM: cell adhesion molecule

CFA: complete Freund's adjuvant

CMV: cytomegalovirus

CNS: central nervous system

DNA: deoxyribonucleic acid

EAE: experimental autoimmune encephalomyelitis

ECM: extracellular matrix

FACS: fluorescence activated cell sorting

FCS: fetal calf serum

FITC: fluoro-isothiocyanate

GA: glatiramer acetate

GAPDH: glyseraldehyde-3-phosphate dehydrogenase

GDNF: glial-cell-line-derived neurotrophic factor

GFP: green fluorescence protein

HSC: hematopoietic stem cells

IFN-γ: interferon-γ

IL-1ß: interleukin-1ß

MCA: middle cerebral artery

MBP: myelin basic protein

MOG: myelin oligodendrocyte glycoprotein

MS: multiple sclerosis

MSC: mesenchymal stem cells

NGF: nerve growth factor

NO: nitric oxide

NSC: neuronal stem cells

NT-3: neurotrophin-3

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PFA: paraformaldehyde

PGK: phosphoglycerate-kinase

PTX: pertussis toxin

RNA: ribonucleic acid

RT: reverse transcription

SEM: standard error of the mean

SCF: stem cell factor

TBM: total bone marrow

TGF-ß: transforming growth factor-ß

TNF-α: tumor necrosis factor-α

TPO: thrombopoietin

VEGF: vascular endothelial growth factor

1. Introduction

1.1. Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the human central nervous system (CNS) affecting a large number of individuals in the Western countries, resulting in a debilitating paralysis and the loss of mental function (Steinman 1996; Hickey 1999; Noseworthy, Lucchinetti et al. 2000).

1.1.1. Clinical aspects

MS often begins in early adulthood with an autoimmune inflammatory strike against components of the myelin sheath. Sensory disturbances, lack of coordination and visual impairment are common features during the progression of the disease (Steinman 2001).

In general terms, the course of the MS can be categorized into two main types as follows (Lublin and Reingold 1996):

1) **Relapsing-remitting.** This is the most common form of MS. It generally occurs in younger people. The disease starts with an attack that lasts from days to weeks, followed by a remission period that can last months to years (when symptoms improve or disappear). Remission periods may be spontaneous or induced by immunosuppressive drugs and mostly alternate between a flare-up of symptoms and periods of deteriorating ability (figure 1.1.A).

2) **Chronic-progressive.** Some MS patients enter in a second chronic phase of the disease characterized by the inability to walk which can lead to wheelchair confinement. About 20% of MS patients (usually those whose first symptoms occur after age 45) present as chronic-progressive without first developing relapsing-remitting MS. Although this chronic-progressive type follows a downhill course, its severity varies widely. Therefore, three variants have been proposed to better describe it:

2a) Secondary-Progressive. About half of the patients with relapsing-remitting MS develop secondary-progressive MS. Clinically it follows a course of progressive nerve and muscle deterioration with occasional acute flare-ups, remissions, and plateaus (figure 1.1.B).

2b) Progressive-Relapsing. In this case, the severity of the disease symptoms increases constantly from the beginning accompanied by acute symptom flare-ups and continued deterioration between relapses. This is a very rare form of MS (figure 1.1.C).

2c) Primary-Progressive. In rare instances, clinical disabilities begin directly with a continuous and gradual deterioration without any remission or flare-up. It occasionally levels off, and minor recovery is even possible. This occurs in about 10% of patients, who tend to be older than average at the time of diagnosis (Hauser and Oksenberg 2006); (www.reutershealth.com) (figure 1.1.D).

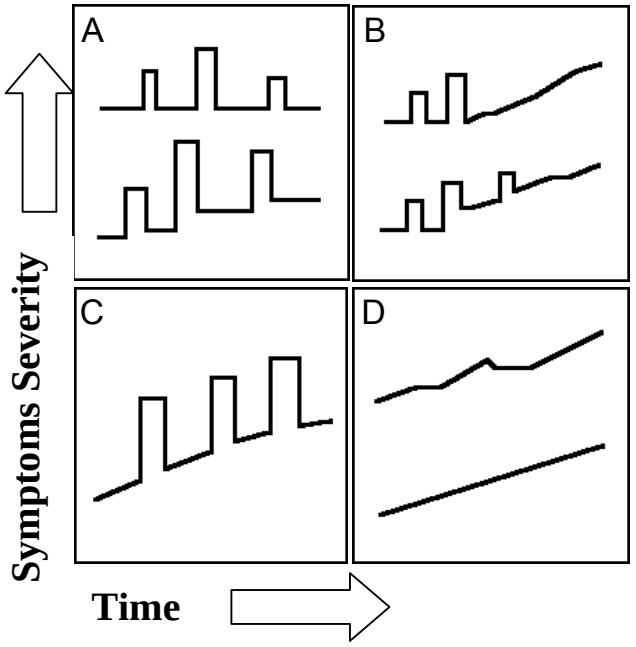


Figure 1.1. Diagrammatic representation of disease severity over time for the different categories of Multiple Sclerosis.A) relapsing-remitting; B) secondary progressive; C) progressive relapsing and D) primary progressive. (Source: www.mult-sclerosis.org)

1.1.2. Disease initiation

The exact events leading to the development of the MS disease are not clearly understood, but there are several molecular and cellular components described that may take part in the destructive cascade leading to myelin damage.

The insult to myelin components is dependent on the activation of an autoimmune response. An ongoing discussion focuses on whether this inflammatory reaction is first initiated within the immune system confines or in response to primary events impacting neuronal cells (Prat and Antel 2005).

Accounting for the first possibility, a long favored hypothesis for MS initiation implicates that auto-reactive T cells that are generated in the systemic compartment gain later on access and persist in the CNS parenchyma, inducing the inflammatory phase of the disease. The main question still present is how exactly these T cells could have been activated for the very first time. It must be considered that a contact between antigen and cells must exist prior to their activation. Moreover, under normal conditions, T cells reacting against a self-antigen, are eliminated by programmed cell death.

To explain how a potential immunogenic antigen can be originated in the CNS and later come in contact with T cells, the main detrimental cell population acting in MS, Walker et al proposed three hypotheses. The first one claims that a certain antigen can be captured in the brain by a dendritic (professional antigen presenting) cell and after the migration of this cell type to the lymph nodes, may initiate an immune response in the periphery. Secondly, they speculate about the idea of the drainage of soluble or particulate antigenic material, or alternatively, the transportation to the lymph nodes of the antigen by other cell types which are not a dendritic type (Walker, Calzascia et al. 2003).

Respect to events primary affecting neuronal cells, there are speculations that acquired acute or persistent infections of these cells could result in tissue antigen release or alternatively that certain viral infections could be connected with the aetiology of MS by molecular mimicry responses. This is the case described for the MS-associated retrovirus (MSRV) (Perron, Garson et al. 1997). MSRV viral particles displayed pro-inflammatory activities both *in vitro*, in human mononuclear cell cultures and *in vivo*, in a severe combined immunodeficient (SCID) humanized mouse model (Nowak,

Januszkiewicz et al. 2003). For instance, a study showed how the surface unit of the MSRV envelope protein specifically induced human monocytes to produce major proinflammatory cytokines. Even more, this surface protein could also trigger the maturation process of dendritic cells able to support a Th1-like type of T helper cell differentiation which are the main cell type involved in proinflammatory diseases as MS (Rolland, Jouvin-Marche et al. 2006).

Factors believed to influence the very first stage of MS range from environmental to genetic background. With respect to the genetic contribution to MS aetiology, it is now believed that the genetic background of a patient might determine the disease susceptibility to a certain extent. The histocompatibility human leukocyte antigen-D (HLA-DRB1) gene is the strongest genetic factor identified so far which influences MS susceptibility (Herrera and Ebers 2003; Dyment, Herrera et al. 2005; Oksenberg and Barcellos 2005). Although until now the exact mechanism by this gene influences susceptibility to MS remain undefined but it is thought to be related with the gene product physiological function in immune responses.
In summary, it is clear that there is not a unique cause of MS initiation. Triggering of MS pathology can be considered as an imbalance between immune and CNS homeostasis.

1.1.3. Molecular pathogenesis

Whereas the mechanisms proposed for the initiation of the immune response in the CNS in MS are still hypothetical, more direct data detailing the consequent effector stage of the disease is available. Current evidence indicates that in this inflammatory neurodegenerative disorder both, adaptive and innate immunity, play a relevant role, not only in initiation, but also in the progression of the disease (Imitola, Chitnis et al. 2006).

Regardless of their location and means of activation, lymphocytes must be specifically recruited to the CNS for development of the MS pathology. Initially, circulating immune cells penetrate the blood brain-barrier (BBB), which normally strongly regulates cell passage, by a process called transmigration, that proceeds according to the multi-step model described elsewhere for cell homing (Mackay 1991; von Andrian

and Mackay 2000). Briefly, in the initial step, "tethering", low affinity bonds between Selectins (on the endothelial cell surface) and oligosaccharides (on the immune cells) reduce the velocity of the T cells to a rolling motion. At the same time, endothelial cells are induced to express Vascular Cell Adhesion Molecule (V-CAM) and class II Major Histocompatibility Complex (MHC) by Interferon (IFN)-γ and Tumor Necrosis Factor (TNF)-α released during the inflammatory response by T cells and macrophages. The final step is called "diapedesis" and consists in the passage of the immune cells into the extracellular matrix (ECM). This last step is mediated by Integrins, in particular α4β1 (Very Late Antigen (VLA)-4), α4β7, lymphocyte function-associated antigen (LFA)-1 on the T cells and Intercellular Cell Adhesion Molecule (I-CAM), V-CAM on the endothelial cells.
Once the activated lymphocytes have extravasated from the blood vessels, they must migrate through an ECM composed mainly of collagen type IV. The α1 integrin subunit plays a relevant role in this binding to the matrix. Also, secretion of several matrix metalloproteases by the lymphocytes allows the access of these cells to the white matter surrounding the axons (Steinman, Martin et al. 2002).
The encephalitogenic T cell response directed against different CNS antigens not only determines the lesional topography of CNS inflammation but also the composition of the inflammatory infiltrates (Schmidt 1999).

In the past few years evidence derived from studies of anti-myelin antibodies (Abs) in CNS lesions of patients with early MS and of MS animal models has led to a renewed interest in the role for B cells, plasma cells and their products in the pathogenesis of MS in addition to that of T cells (Ziemssen and Ziemssen 2005).

At the effector phase, when activated immune cells homed already to the CNS, many molecules are involved in final myelin damage and consequent progression of MS. Abs against myelin components are secreted (Burgoon, Gilden et al. 2004). Complement proteins are activated into a cascade that culminates in membrane attack complexes. Cytokines secreted by activated T cells, including TNF-α and lymphotoxin, have an effect on infiltrating macrophages as well as microglia and astrocytes. Concomitantly, damaged cells produce nitric oxide (NO) and osteopontin. The combined effect of abs, complement, NO and cytokines damages the myelin and

induces the macrophages to phagocyte large pieces of myelin sheath (Steinman, Martin et al. 2002) (figure 1.2).

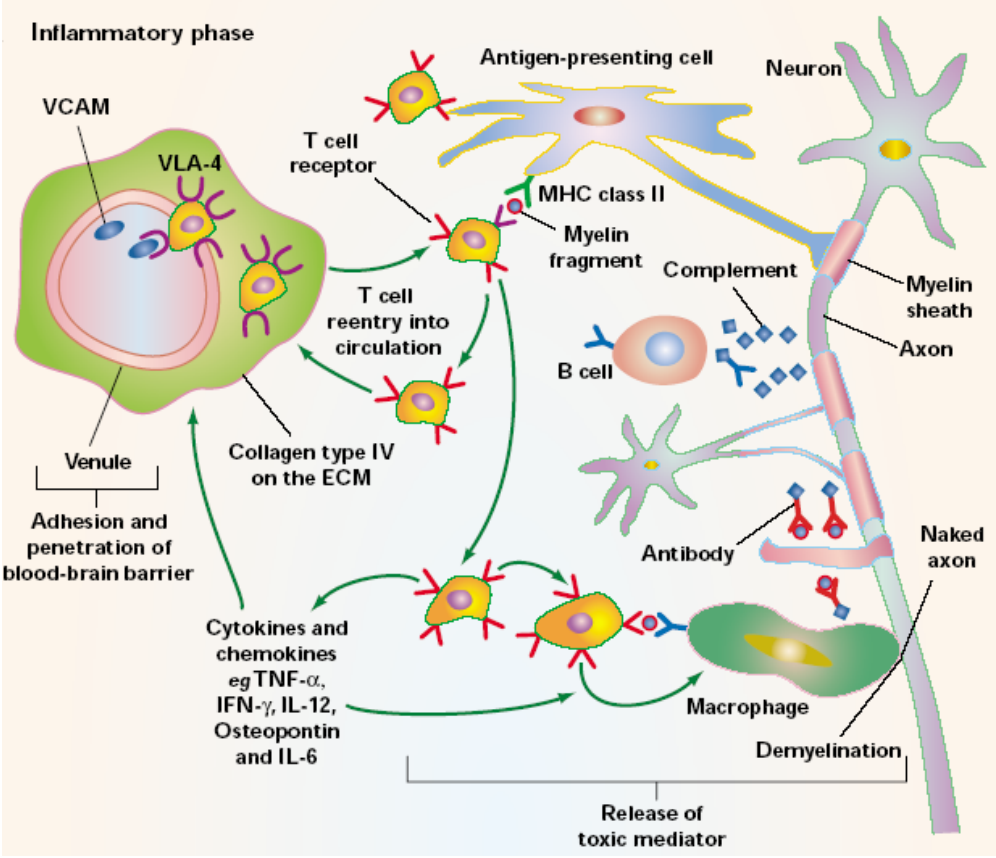


Figure 1.2.General scheme of MS molecular pathogenesis. T and B cells home to the CNS after adhesion molecule up-regulation. Cells encountering an antigen presenting cell release inflammatory cytokines. Demyelination mediated by combined action of Abs, complement and macrophages takes place. (*From Steinman 2001. Nat. Immunol.).*

1.1.4. Current therapy

Current treatments are most effective in the inflammatory phase of the disease and aim to interfere with the many stages of the immune cascade. However, recent evidence has emerged that inflammation may not only be destructive, but may also play a role in tissue repair (Hohlfeld, Kerschensteiner et al. 2006), which makes difficult the task of finding a suitable specific treatment.

Glatiramer acetate (GA; Copaxone, also known as Copolymer 1 or Cop-1), a copolymer of amino acids, has been very effective in the suppression of Experimental Autoimmune Encephalomyelitis (EAE), the animal model for MS. The immunological cross-reaction between the myelin basic protein (MBP) and GA serves as the basis for the suppressive activity of GA, by the induction of antigen-specific suppressor cells. The mode of action of GA consists of strong promiscuous binding to MHC class II molecules and competition with MBP and other myelin proteins (Aharoni, Teitelbaum et al. 1999). Suppressor T cells induced by GA are of the Th2 type. Clinical trials with GA, both, phase II and phase III, were performed in relapsing-remitting MS patients, and demonstrated efficacy in reducing the relapse rate, decreasing magnetic resonance imaging (MRI)-assessed disease activity and burden and slowing progression of disability (Aharoni, Meshorer et al. 2002; Arnon and Sela 2003). After successful clinical trials this GA drug is currently available on the market.

Another commonly used therapeutic agent is the β-IFN in its recombinant form specially to control the relapsing-remitting form of MS. The known effects are its interference with lymphocyte migration and other pleiotropic actions (Steinman 2001). It is pertinent to notice that inhibitors and regulators of many of the above described molecules that take part in the steps of its molecular pathogenesis (e.g. cell adhesion, diapedesis, migration through ECM) are been tested as possible therapies for MS.

Ideally, treatment might be feasible when a combination of more than one specific target could be assessed for each particular MS case. Further increases in the understanding of the pathogenesis of the MS are likely to assist in the identification of new targets for disease-modifying therapies.

1.1.5. Experimental Autoimmune Encephalomyelitis (EAE)

Many animal models of CNS demyelination have been described, reflecting the diversity of clinical manifestations in humans. EAE is the most frequently studied autoimmune model for MS. Although it does not exactly replicate the human counterpart, it shares many similarities and has provided valuable insights into the pathobiology of the disease (Ercolini and Miller 2006).

1.1.5.1. EAE induction

EAE can be induced in a number of species, including rodents and non-human primates through experimental immunization with whole myelin proteins or specific myelin peptides in adjuvant. Alternatively, EAE can also be induced through systemic transfer of primed immune cells (Owens, Wekerle et al. 2001).

In the 1930's, EAE was initially developed to study episodes of paralysis occurring with certain vaccinations. Rivers and collaborators established this model in primates and their work was first published in 1933 (Rivers et al, 1933). More than seventy years had passed and many refinements and variations of the EAE forms have been developed including models for relapsing-remitting (Brown and McFarlin 1981) and progressive forms of MS (Lublin, Maurer et al. 1981).

To induce the disease in mice (and other animals as well), immunization was initially made with whole spinal cord homogenate (Yasuda, Tsumita et al. 1975; Mitsuzawa and Yasuda 1976; Mitsuzawa, Yasuda et al. 1981). Now it is more clear which myelin epitopes are specifically capable of inducing EAE, for example, with respect to the strain of mouse used. Currently, peptides derived from the myelin as proteolipid protein (PLP), MBP or myelin oligodendrocyte glycoprotein (MOG) are used to induce EAE. For the present work, a twenty-aminoacid-immunogenic peptide which is a portion of the MOG molecule (aa 35-55) was chosen.

Additionally, EAE induction depends on the inclusion of complete Freund's adjuvant containing heat-killed mycobacteria (Billiau and Matthys 2001) providing a chronic inflammatory stimuli (Mitsuzawa and Yasuda 1976; Mitsuzawa, Yasuda et al. 1981), and also pertussis toxin (PTX) as adjuvant and disrupter of the BBB. A recent study shows that PTX could use Toll-like receptor 4 signalling to mediate its disease-inducing effect (Kerfoot, Long et al. 2004; Racke, Hu et al. 2005).

1.1.5.2. Molecular and cellular pathogenesis of EAE

In the C57BL/6 (H-2b) mouse strain, EAE induced by MOG_{35-55} is characterized by early (around day 12 post-induction) acute paralysis, followed by a sustained chronic clinical course that gradually stabilizes. Extensive inflammation and demyelination coincide with clinical signs of disease.

As is the case in MS, chemokines and chemokine-receptors are critical in establishing a chemotactic gradient to recruit immune cells to the sites of inflammation in the CNS. Chemokines like IFN-γ-induced protein (IP)-10, Monocyte Chemoattractant Protein (MCP)-1 and RANTES are described to appear with the onset of the EAE symptoms (Elhofy, Kennedy et al. 2002).

Like MS, EAE was classically considered a prototypic T-cell mediated animal model. It is characterized by infiltration of mononuclear cells into the CNS (Fuller, Olson et al. 2004). This disease was viewed in terms of type 1 versus type 2 immunity: the type 1 cytokines IFN-γ and TNF-α promoting disease (Renno, Krakowski et al. 1995), whereas an IL-4-dominated, type 2 response being protective. For instance, it was observed that the adoptive transfer of Th1 clones could induce the disease (Zamvil, Nelson et al. 1985). Although a regulatory role for IFN-γ in EAE has been shown, acting on T cell proliferation and directing chemokine production, with profound implications for the onset and progression of the disease (Tran, Prince et al. 2000), contrasting observations were made in mice lacking Th1 associated molecules as IFN-γ (Ferber, Brocke et al. 1996), IFN-γ receptor and IL-12 receptor as well as IL-12p35 (Becher, Durell et al. 2002) that unexpectedly developed severe EAE as well. Therefore, the initial idea of an exclusively Th1 phenotype accounting for the EAE (and MS) degeneration has been challenged.

In a detailed study using a sensitive single-cell based assay Juedes et al. determined that the MOG immunization resulted in the priming of both Th1 (lymphotoxin, IFN-γ, and TNF-α) as well as Th2 (IL-4) cells in the spleen. Only 7 days after immunization IFN-γ, and TNF-α producing CD4 Th1 cells seemed to be present in the CNS, peaked at day 20, and then waned. At this time points, microglia as well as infiltrating macrophages were additionally responsible for the production of TNF-α (Juedes, Hjelmstrom et al. 2000).

More recent data point to important roles for IL-23 and IL-6 (rather than IL-12 and IFN-γ) in the establishment and persistence of the inflammatory lesion (Langrish, Chen et al. 2005). Moreover, therapeutic approaches with anti-IL-23 or anti-IL6 antibodies could ameliorate EAE (Chen, Langrish et al. 2006), and IL-6 knock out mice showed resistance to EAE (Samoilova, Horton et al. 1998).

On the other hand, IL-10 appears to be the dominant cytokine mediating recovery. The source of IL-10 includes B cells (most probably in the peripheral lymphoid organs). However, the key IL-10-producing cell within the CNS is a CD4+CD25+ T cell population that has regulatory function and is critical for the resolution of the disease (Samoilova, Horton et al. 1998; McGeachy and Anderton 2005).
In the monophasic and relapsing-remitting forms, recovery from the disease is also associated with clearance of inflammatory infiltrates from the CNS.

1.2. Cerebral ischemia

Acute ischemic stroke caused by cerebral artery occlusion leading to infarction of brain tissue and loss of neuronal cells is a serious vascular CNS disorder and it represents a leading cause of death and disability in industrial nations (Haas, Weidner et al. 2005).

1.2.1. Clinical aspects and molecular pathology

Clinical manifestations of transient ischemic attack include: motor dysfunction, sensory alterations, speech or language disturbance and loss of vision.
After an ischemic event an inflammatory response in the brain tissue is elicited (del Zoppo, Ginis et al. 2000). Inflammatory interactions between endothelial cells and leukocytes occur at the BBB level involving cytokines, adhesion molecules, chemokines. These interactions are critical to the pathogenesis of tissue damage in cerebral infarction (Huang, Upadhyay et al. 2006).
At the molecular level, ischemia results in rapid loss of high-energy phosphate compounds and generalized depolarization, which induces release of glutamate and, in selectively vulnerable neurons, opening of both voltage-dependent and glutamate-regulated calcium channels. This allows a large increase in cytosolic $Ca^{(2+)}$ and consequent activation of mu-calpain, calcineurin, and phospholipases with proteolysis of calpain substrates, activation of nitric oxide synthase (NOS), and accumulation of free arachidonic acid, which can induce depletion of $Ca^{(2+)}$ from the intracellular storage compartment.
A kinase that shuts off translation initiation by phosphorylating the initiation factor-2 (eIF)-2α is activated. Early during reperfusion, oxidative metabolism of arachidonate causes a burst of excess oxygen radicals, iron is released from storage proteins, and NO

is generated. Neurons present then altered translation initiation mechanisms that reduce total protein synthesis, and down-regulate survival signal-transduction together with caspase activation leading to programmed cell death (White, Sullivan et al. 2000).

1.2.2. Middle cerebral artery occlusion as a model of cerebral ischemia

Animal models of cerebral infarction are crucial to understand the molecular and cellular mechanisms following ischemic brain injury and to the development of therapeutic interventions for victims of all types of stroke.

Rodents have been used extensively in such research. First established in rats, the middle cerebral artery occlusion (MCAO) as a model for focal cerebral ischemia, has been well accepted as it has shown to be reproducible and to reflect the human clinical settings (Ginsberg and Busto 1989). Since the development of an endovascular method published in 1989, MCAO has been applied commonly in rats and because of technical demands and issues with survival, relatively few laboratories have taken advantage from the MCAO method in the mouse (Duckworth, Butler et al. 2005).

1.3. Neurotrophins

Neurotrophins are a family of structurally related genes whose products have been attributed to have an important role in the regulation and homeostasis of the central and peripheral nervous systems.

The first neurotrophin to be described was the nerve growth factor (NGF), followed by the brain derived neurotrophic factor (BDNF). Structural analysis of these two proteins revealed more than 50% of homology and led to the search for more members of the survival factor family. Thereafter, several members were discovered in a rapid succession: Neurotrophin (NT)-3, NT-4/5 and NT-6 (Shen, Figurov et al. 1997).

In parallel to the discovery of neurotrophic factors, a family of high affinity receptors called TrK (Tyrosine Kinases related) has been characterized. The first one described, TrK A, was isolated from carcinoma cells, and initially was identified as a proto-oncogene. This receptor binds with high affinity to NGF (Lewin 1996; Lewin and Barde 1996).

Later, TrK B, a second member, was found to be functional when BDNF and NT-4/5 bind to it, explaining the similar patterns of biological effects observed for these two neurotrophins (Klein et al., 1991a, 1991b). The last member to be isolated was the TrK C, the receptor for NT-3 (Lamballe, Smeyne et al. 1994). Neurotrophins can also bind to a low affinity receptor, p75 and trigger apoptosis (Ibanez 1994).
It was originally thought that neurotrophin receptors were strictly located along the nervous tissue, but several studies have reported their expression in a wide range of cell types including: muscular (Ochi, Saito et al. 1997), cardiac (Donovan, Hahn et al. 1996), lymphoid (Labouyrie, Parrens et al. 1997), endothelial (Ricci, Greco et al. 2000; Ricci, Greco et al. 2001), bone marrow (BM) tissue (Labouyrie, Dubus et al. 1999) and B cells (Kerschensteiner, Gallmeier et al. 1999). It still remains unclear under which conditions the effects mediated by these receptors are considerable in regard to each particular cell type.

It is therefore important to notice that the biological action of these neurotrophic factors is not tissue restricted but they also exert many pleiotropic effects. Some examples are augmentation of microvascular endothelial cell proliferation and expression of adhesion molecules (Raychaudhuri, Raychaudhuri et al. 2001), inhibition of monocyte migration through the BBB (Flugel, Matsumuro et al. 2001), promotion of neutrophil survival (Kannan, Usami et al. 1992) and *in vitro* migration of muscle cells (Donovan et al., 1995).
Moreover, there is increasing evidence that these factors may play a major role in regulation of immune response. In fact, many neurotrophins and their receptors are produced and act in the immune system and therefore it appears likely that neurotrophins can mediate bidirectional cross-talk between the nervous and immune systems (Kerschensteiner, Stadelmann et al. 2003).

1.3.1. Neuroprotection in neurodegenerative disorders

Treatments of neurodegenerative diseases such as MS should take into account the heterogeneous pathophysiology of the disease. Anti-inflammatory approaches have been proven to be a possible therapy for MS, but a current concept is to declare neuroprotection as an important goal in MS therapy. Neuroprotective strategies in MS

could focus on rescuing degenerating axons, e.g., by reinforcing endogenous neurotrophic action (Kerschensteiner, Stadelmann et al. 2003).

Addressing the neurodegenerative component of the MS disease is an important objective of the treatment, since axonal injury is believed to underlie the accumulation of disability and disease progression. Specific treatment strategies to prevent neurodegeneration need to be developed acting within the CNS. A promising approach is to enhance neuroprotective immunity inside the brain, believed to be mediated, at least in part, by the release of neurotrophic factors within the CNS from infiltrating immune cells (Ziemssen 2005).

Many authors are in favor of the idea of a dual effect of the attacking immune cells, from one hand conferring damage to the neuronal environment, but from the other, mediating protection by production of trophic factors.

Fewer endogenous neurotrophins are present in older, chronic MS plaques (areas of brain tissue scarring and damage) than in the early stages of lesion development. A reasonable strategy for slowing axonal degeneration in this late phase of MS, which is notoriously resistant to other forms of therapy, would be to provide exogenous (therapeutic) neurotrophic support (Hohlfeld, Kerschensteiner et al. 2006).

Regarding the influence that some neurotrophic factors may have during MS, a clinical study performed by Cagguilla et al. observed the correlation between grade of MS and production of several neurotrophic factors, including NT-3 in peripheral blood mononuclear cells. They found increased levels of glial-cell-line-derived neurotrophic factor (GDNF), NGF and NT-3 growth factors in the post-relapse phase in patients with complete remission (Caggiula, Batocchi et al. 2005). In another study, BDNF and its truncated receptor gp145TrKB were found to be present in MS lesions. The number of BDNF reactivity correlated with the lesional demyelinating activity, and the authors suggested a role for some neuronal growth factors not only under normal conditions but also under pathological conditions (Stadelmann, Kerschensteiner et al. 2002).

With respect to the significance of providing trophic support in neurodegenerative cases, Lu et al. have shown functional recovery in a rat model of spinal cord injury upon locally or delivered administration of neurotrophins. When a combinatorial therapeutic approach was used, NT-3 and cAMP contributed to a clear axonal

regeneration beyond spinal cord injury in a strategy designed to enhance spinal cord repair (Lu, Yang et al. 2004).

Neuronal stem cells (NSC) expressing neurotrophic factor genes grafted *in vivo* to cystic dorsal column lesions in the cervical spinal cord of adult rats and supported extensive growth of host axons. Moreover, these NSC were genetically modified to produce NT-3. In this case, effects of NSC on host axons were enhanced. Thus, it has been demonstrated for this particular paradigm of spinal cord injury that NSC can promote host neural repair by secreting intrinsic growth factors, and their regeneration-promoting activities can be modified by neurotrophic gene delivery (Lu, Jones et al. 2003).

Locally injection of fibroblasts also modified to express human NT-3 directly into spinal cord lesions was able to elicit growth of corticospinal axons in chronic stages of injury and improved functional outcome compared to non-growth-factor-treated animals (Tuszynski, Grill et al. 2003). A similar outcome was shown for human BDNF expressing mesenchymal stem cells (MSC) in the same animal model (Lu, Jones et al. 2005). Furthermore, in another study it was shown that the co-graft of NT-3 expressing Schwann cells and NSC enhanced neuronal survival in a rat model of spinal cord injury (Guo, Zeng et al. 2006).

Flugel et al. showed in cell transfer studies that MBP-specific CD4 cells modified to express NGF were unable to mediate clinical EAE alone or in combination with non-transduced MBP specific cells. This was associated with a reduced inflammation in the CNS and they propose an impaired monocyte migration through the BBB as a putative mechanism (Flugel, Matsumuro et al. 2001).

All these data indicate that a combination of cell and trophic support could be considered a suitable approach for recovery of degenerating neuronal tissue. Moreover, effects of neurotrophic factors could be extended beyond nervous tissue when taking into account their influence on the immune system.

1.4. Hematopoietic stem cells

1.4.1. Terminology: stem cells, precursor cells and progenitor cells

By definition, a stem cell is an undifferentiated cell that produces daughter cell which can either remain as a stem cell (self-renewal) or commit to a pathway leading to differentiation. This pathway to differentiation usually involves the daughter becoming a precursor cell which proliferates prior to its differentiation. In general, the terms precursor cell and progenitor cell are used interchangeably.

Mammalian stem cells can be classified according to their developmental potential. They are called either "totipotent", in the case that they can produce all embryonic and extra-embryonic tissues required for development, or "pluripotent" (as in the case of embryonic stem cells, which are derived in culture from epiblast cells), if they can give rise to all cells of the embryo proper.

Adult stem cells present in the organs or tissues of adult animals are considered "multipotent" if they are able to differentiate into more than one cell type. Alternatively, they are called "unipotent" when they are able to contribute only to one mature cell type. Hematopoietic, hepatic, and neuronal stem cells belong to the multipotent category (Raff 2003).

1.4.2. Ontogenesis of hematopoietic stem cells

Hematopoietic stem cells (HSC) are at the foundation of the hematopoietic hierarchy and give rise to all blood lineages in the adult organism. In the early embryonic state of all vertebrates, HSC appear to derive from hemangioblasts, which are common progenitors with the precursors of blood vessels (Dieterlen-Lievre, Pardanaud et al. 2002; Bollerot, Pouget et al. 2005). These hemangioblastic cells condense into aggregates that are called blood islands. The inner cells of these blood islands become HSC, while the outer cells become angioblasts, the precursors of the blood vessels (Choi, Kennedy et al. 1998; Jaffredo, Nottingham et al. 2005) (figure 1.3).

Moreover, many proteins as stem cell leukemia (SCL), a basic helix-loop-helix transcription factor (Chung, Zhang et al. 2002) and Flk-1, a receptor tyrosine kinase, (Schuh, Faloon et al. 1999) are shared by the earliest blood and capillary cells. Also, the transmembrane cell surface glycoprotein CD34 has been described to be expressed

in both, hematopoietic and vascular immature cells (Wood, May et al. 1997; Choi 1998).

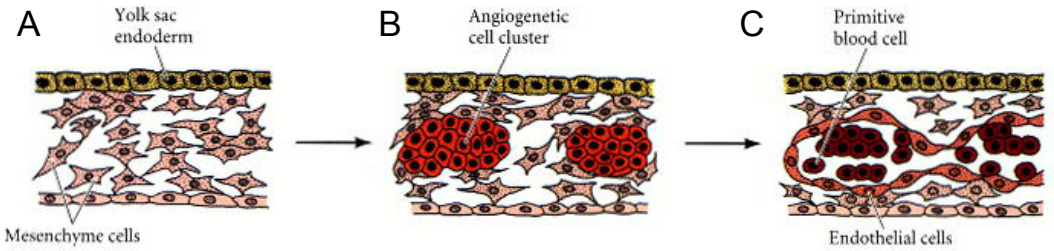


Figure 1.3. Early formation of hematopoietic stem cells and endothelia. Both cell types derive from a common hemangioblast progenitor. A) In the wall of the yolk sac, mesenchymal cells condense. B) Condensed cells form angiogenic clusters. C) Centre of cluster cells form blood cells and outside located cells originate vessel endothelial cells. (Source: Gilbert, S. Developmental Biology. 6th Edition. Sinauer Associates, Inc. 2000).

In murine and human systems, embryonic and fetal development is associated with multiple switching in the sites of hematopoiesis. The phenomenon is initially extra-embryonic, occurring in the blood islands of the yolk sac. There, erythropoiesis takes place and it proceeds synchronously and may be erythropoietin-insensitive, at the same time the yolk sac milieu is not permissive to the development of other cell lines. The final products of hematopoiesis at this stage are nucleated red blood cells. Yolk sac hematopoiesis is considered a primitive (as compared to definitive) form of hematopoiesis (Tavassoli 1991).

HSC later on seem to migrate via the bloodstream to the liver and spleen to seed these tissues, which then carry the burden of hematopoiesis until birth and for some time thereafter. Here some granulopoiesis also occurs, in addition to erythropoiesis. Hematopoiesis is progressively in its definitive form, lacking synchronicity of cell growth with the end red blood cell being enucleated and synthesized hemoglobin of the adult type.

In an adult organism, blood cells originate from the BM and spleen, and are predominantly granulopoietic (Zanjani, Ascensao et al. 1993). In all cases, recognition and binding of HSC to lineage specific stromal cells present in these hematopoietic tissues is called "homing", and initiates the processes of differentiation, proliferation, and maturation of HSC (Konno, Hardy et al. 1990). The homing mechanism is

mediated by a lectin-glycoconjugate interaction. The lectin is on the surface of HSC and progenitor cells with specificity for galactosyl and mannosyl residues on the stromal cells. The binding is subsequently stabilized by membrane-bound proteoglycans, integrin-like receptors, and fibronectin and it is crucial for a proper stem cell maintenance and maturation (Tavassoli 1991; Tavassoli 1994).

1.4.3. Adult bone marrow derived stem cells

BM is the tissue present in the core of almost all long bones of adult mammals and contains an heterogeneous mixture of mature and maturing precursors of endothelial, hematopoietic and mesenchymal cells (Wang, Pierce et al. 2005). The BM derived HSC differentiate into all mature blood cells. The BM derived endothelial progenitors cells will give rise to vascular cells, and the BM derived stromal cells, called also mesenchymal stem cells (MSC), can differentiate into mature cells of multiple tissues including fat, bone and cartilage (Herzog, Chai et al. 2003).

HSC are currently the best characterized multipotent stem cell population and were initially isolated from mouse BM in 1988 (Spangrude, Heimfeld et al. 1988). The ability to recover hematopoietic function by repopulating all lineages comprising the entire blood system of a myeloablated organism functionally defines mammalian HSC (Bhatia 2003). Therefore, distinguishing features of HSC for reconstitution are the requirement of homing to their niche in the BM, extensive self-renewal, and their differentiation into all mature blood cell types (schematically represented in figure 1.4).

Studies emphasize the heterogeneity of the HSC compartment, in terms of proliferation and self-renewal capacities. Currently, there are two classes of mouse HSC that can be distinguished on the basis of cell-surface markers and potential for self-renewal: the long term (LT) repopulating cells can reconstitute the blood system of an irradiated mouse for its lifetime, alternatively the short-term (ST)-HSC can do so only for six weeks.

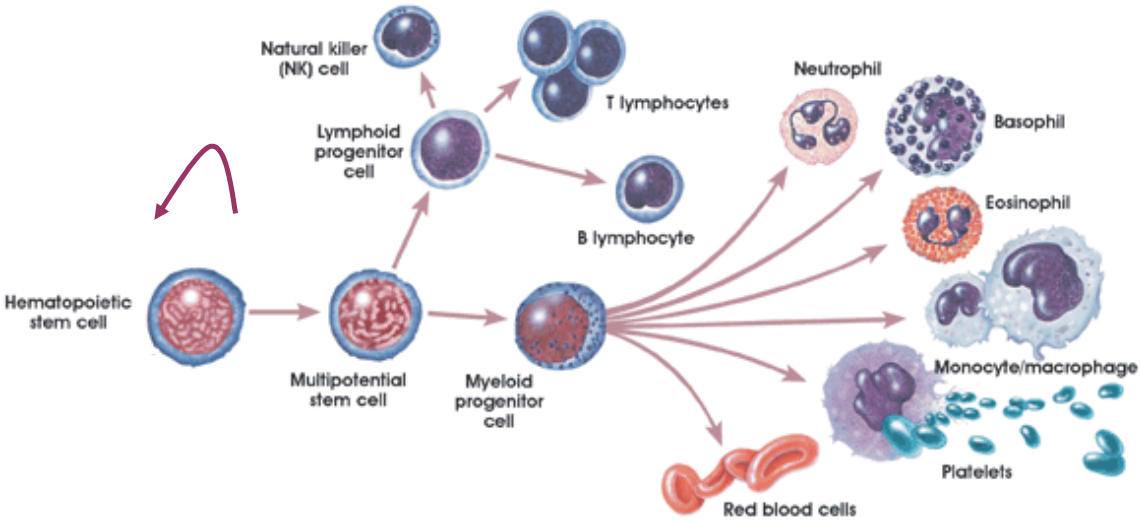


Figure 1.4. Hematopoietic stem cells give rise to all mature blood and immune cells and are able to self-renew. *(Source: stemcells.nih.gov)*

Upon transplantation in a myeloid cell ablated mouse, it was shown that LT repopulating cells remain quiescent in the BM shortly after engraftment, whereas ST-HSC are more rapidly dividing (Neben, Redfearn et al. 1991).
Lanzkron and collaborators observed that transplanted HSC homed normally to spleen and BM. Nevertheless, only cells in this last tissue, were significantly more competent at reconstituting lethally irradiated secondary hosts, suggesting BM to be the niche of the LT-HSC (Lanzkron, Collector et al. 1999a; Lanzkron, Collector et al. 1999b).

A central issue of the HSC biology is to understand the mechanisms behind the regulation of their self-renewal and differentiation as any miss regulation of these processes can be the cause of uncontrolled cell growth and even leukemia.
After isolation from adult mice, it was calculated that around 8% of the LT-HSC entered the cell cycle per day (Cheshier, Morrison et al. 1999). The HSC cell cycle is tightly regulated by external factors such as cytokines and interactions with stromal cells and extra-cellular matrix in the BM microenvironment.
Many groups have implicated the interaction between osteoblasts (bone-forming cells) as the one of the key regulatory components of the HSC microenvironment in the BM. Moreover, contact between Tie2 receptor in HSC and Angiopoietin-1 produced by

osteoblasts was shown to maintain the LT repopulating activity of HSC (Arai, Hirao et al. 2004).

Intrinsic transcription factors as c-Myb, GATA-2, HOX family proteins (Miyake, Brun et al. 2006) and Bmi-1 (Park, Qian et al. 2003) were described to contribute to the maintenance of adult self-renewal of HSC and up to 70% of HSC exhibited detectable levels of telomerase activity associated also with this renewal feature (Morrison, Prowse et al. 1996). On the other hand, cell cycle regulators as p21 and p27 proteins are known to maintain quiescence in these HSC (Ezoe, Matsumura et al. 2004).

In the mouse, LT-HSC make up approximately 0.007% of whole BM and are characterized by their undetectable levels of lineage markers (i.e. B220, CD3, Mac-1, TER-119 and Gr-1), double c-Kit and Sca-1 expression, and low levels of Thy-1 (Morrison, Uchida et al. 1995). The ability to efflux the vital dye Hoescht 33342 is also a feature described for these cells (Jackson, Majka et al. 2002) known as side population, when analyzed by flow cytometry.

There is a current concept of "aging of stem cells" that implies the loss of their self-renewal capacity upon successive transplantation or if they are obtained from an older organism (Morrison, Wandycz et al. 1996; Liang, Van Zant et al. 2005). However, this concept has been challenged by groups showing no stem cell exhaustion after serial transplantations and arguing that instead, extrinsic mechanisms that may affect this aging effects are reversible by administration of exogenous cytokines (Iscove and Nawa 1997; Benveniste, Cantin et al. 2003).

1.4.4. Culture of adult hematopoietic stem cells

The possibility of keeping and expanding HSC *in vitro* would contribute not only to the better understanding of their biology, but also to their genetic manipulation and further use in therapy approaches. Nevertheless, culture and expansion of HSC *in vitro* has been demonstrated not to be an easy challenge. Once they are extracted from their BM niche, HSC are very prone to lose their pluripotency.

The most commonly used stimulating cytokines in HSC culture are thrombopoietin (TPO), Stem Cell Factor (SCF) and IL-6. In general they all promote self-renewal and expansion, and they are used in particular when these cells are genetically modified by

retroviruses. Also the use of the HOXB4 transcription factor has been shown to be highly effective in the HSC expansion, specially of human origin, *in vitro* when used as a protein able to translocate through the HSC membrane (Amsellem, Pflumio et al. 2003; Miyake, Brun et al. 2006).

Expansion *in vitro* of HSC (in particular human type) is a major challenge in cellular therapy, and the development of cell amplification factors is being widely considered.

1.4.5. Migration of bone marrow derived cells into the central nervous system

A classical thought was that HSC derived from BM, after undergoing differentiation, would exclusively give rise to circulating immune and red blood cells, throughout adulthood. Alternatively, it was thought that stromal progenitors from BM would only differentiate into structural cells such as chondrocytes or osteocytes. In the year 2000 this perspective of a limited differentiation capability of these BM cells began to change after Mezey and collaborators (Mezey, Chandross et al. 2000) and Brazelton and collaborators (Brazelton, Rossi et al. 2000) described the migration of BM cells to the CNS, further showing the expression in these BM derived cells of neuronal markers once in the brain region.

Since then, recent publications have mentioned the migration of BM cells into CNS and their establishment as resident microglia (Eglitis and Mezey 1997; Ono, Yoshihara et al. 2003; Asheuer, Pflumio et al. 2004); and possibly neurons (Priller, Persons et al. 2001).

This group of publications have generated enthusiasm in the cell therapy field, in respect to the possibility of using stem cells or their derivatives for therapy of neurodegenerative disorders.

1.4.6. Plasticity versus cell fusion of adult bone marrow derived stem cells

There is indeed controversy about the fate of these BM cells once in the CNS environment. While publications claimed that cells coming from BM after transplantation to maintain their hematopoietic phenotype once they migrated to the brain (Ono, Yoshihara et al. 2003; Massengale, Wagers et al. 2005), others challenged the initial idea of trans-differentiation of BM cells with studies that demonstrated the existence of spontaneous cell fusion events between BM and resident neuronal cell as the cause of fate-switch (Terada, Hamazaki et al. 2002; Alvarez-Dolado, Pardal et al.

2003). Differences in experimental conditions and BM cell population used in each study may account for the cell fate (or fusion event) reported for the brain detected BM cells. At the same time, there are some points to be considered when claiming a cell plasticity case. For instance, which methods were used for final cell phenotype identification (staining and/or functional assays) and if a heterogeneous BM population was selected for the transplantation experiments. All these conditions may bring contradictory interpretations of the fate that the BM cells may have in the host tissue.

However, the reported migration of BM derived cells towards the CNS and passage through the BBB and moreover, their eventual differentiation into resident microglia is a very attractive feature that can be taken into future consideration, in particular as means of transport of therapeutic molecules towards the CNS.

1.4.7. Stem cell therapy for central nervous system disorders

Many attempts have been made in recent years to interfere with degeneration occurring during the progression of CNS diseases. Recent studies have demonstrated the use of stem cells for effective treatment of experimental inflammatory diseases of the CNS. Among them, it has been described that NSC may be engrafted into the injured CNS, migrating to the inflamed areas, and can contribute to functional recovery and tissue repair through their differentiation in neuronal cells and the release of anti-inflammatory factors (Pluchino, Quattrini et al. 2003; Einstein, Grigoriadis et al. 2006).

One of the major concerning points to overcome when using cell based therapy is the ethical issue surrounding stem cells, in particular when using (human) embryonic cells. Adult BM derived stem cells are regarded as promising tools in this respect. Moreover, they have the additional advantages such as avoidance of immune rejection (they can be obtained from the same patient), and reduced chance of tumor formation (which is a usual problem derived from the use of embryonic stem cells).

Accounting for the possible therapeutic use of BM adult stem cells in MS, it was reported the administration of BM derived MSC into EAE induced in mice. It was shown that these cells ameliorated the course of the disease, if applied before disease appearance. A decrease in inflammation and demyelination in the CNS of animals

treated with BM-derived MSC could be observed. *In vitro*, BM cells inhibited T cell proliferation and decreased IFN-γ and TNF-α production. The authors concluded that the cells induce an unresponsive state of autoimmune T-cells (Zappia, Casazza et al. 2005).

In another report, human BM cells were also administered in the same animal model of MS and neurological function recovery was shown. The authors also observed a reduction in the inflammatory infiltrates and in the demyelination. At the same time, levels of BDNF were increased, possibly contributing to oligodendrocyte progenitor survival (Zhang, Li et al. 2005).

The use of adult stem cells has also been reported in regard to other CNS insults as it is the case of cerebral ischemia. Following an ischemic insult, experimental transplantation of BM cells into irradiated recipient animals resulted in engraftment into the ischemic lesions, after stroke. BM derived cells migrated into the ischemic lesion and integrated into the peri-infarct zone to become microglial-like cell type. Moreover, the authors claim that the BM cells took part in a injured tissue repair process (Beck, Voswinckel et al. 2003).

Therefore, much excitement has been generated over the possibility of using stem cells for the treatment of neurodegenerative disorders and many attempts are been made to find a suitable approach. Considering the source of cells and the ethical background adult stem cells present a clear advantage with respect to embryonic stem cells and to study possible applications of this cell type is a major goal to be achieved.

1.5. Lentivirus based vector system

The use of virus-derived vectors for cell genetic modification has opened innumerable perspectives since first developed. They have shown not only to be a powerful tool for the study of relevant molecules by gain/loss of function assays, but also in the field of gene therapy, for potential genetic treatment of many diseases.

1.5.1. Introduction to Lentivirus

The most common viral-derived vector used in preclinical research for gene therapy applications has its origin from the human immunodeficiency virus type-1 (HIV-1).

HIV-1 is a member of the retrovirus (*Retroviridae*) family of viruses. The main feature of this family is that their genome, an RNA molecule, is converted into a double stranded DNA molecule during replication. Within the subfamilies of the retroviruses, the *lentivirinae* is a group associated with slow and progressive diseases affecting the immune system. In fact, the term "Lenti" comes from slow in Latin, referring to its slow and persistent rate of infection.

In general, lentiviruses such as the HIV-1 are considered to have a complex genome. In addition to structural genes like *gag, pol* and *env*, they contain a number of regulatory and accessory genes involved in the modulation of gene expression, assembly of viral particles and structural function alterations in the infected cells (Trono 1995). In total, six accessory genes: *tat, rev, nef, vpr, vpu*, and *vif* are implicated in the viral pathogenesis (Emerman and Malim 1998; Li, Li et al. 2005).

The life cycle of the lentivirus is common to all members of the *Retroviridae* family and can be described by the following steps (schematically represented in figure 1.5):

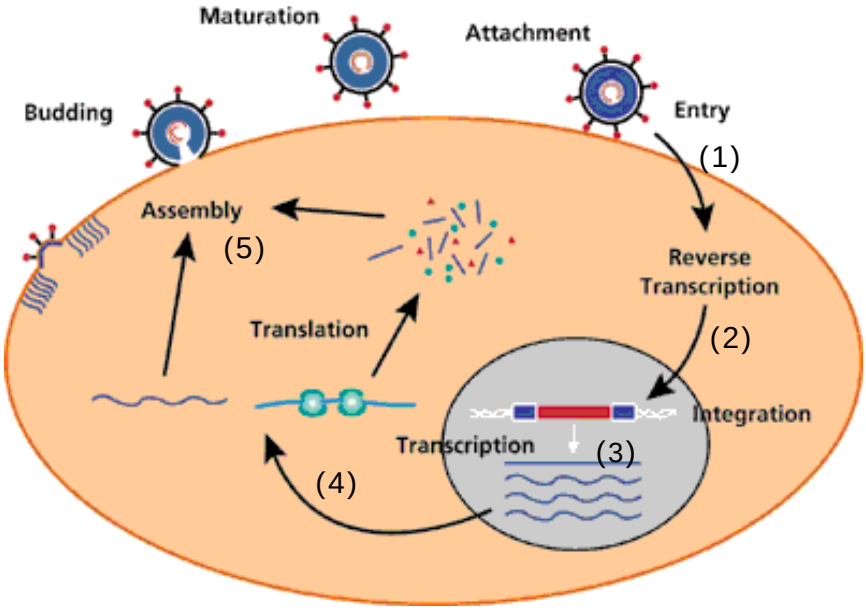
1- Attachment and entry: the interaction between the virus and the target cell occurs via specific receptors (on the cell membrane). Once bound to the surface, the viral and cellular membranes undergo fusion. After this fusion step, the virion nucleoprotein is delivered to the cell cytoplasm where reverse transcription begins.

2- Reverse transcription: synthesis of double stranded DNA from an RNA template is carried out by the reverse transcriptase enzyme (present in the virion nucleoprotein) and uses cellular tRNA as a primer.

3- Integration: once the viral DNA is synthesized, the integrase catalyzes its integration into the host genome and allows the provirus to become a permanent genomic element in the host.

4-Transcription and viral protein synthesis: the first transcription products from the virus code for the REV TAT and NEF. In a late stage, un-spliced and single spliced RNA species are produced.

5-Virion assembly and release: viral genome and structural proteins are packed into the viral particles and released at the plasma membrane.



Firgure 1.5. Schematic representation of a retrovirus life cycle which can be described in 5 steps from attachment of the virus to host cells till the new virion assembly and release.

(Source: www.clontech.com/ expression/retro/images/retro2.gif).

1.5.2. Lentivirus based gene transfer system

The most attractive feature of a system for genetic engineering based on lentivirus is under no doubt the capability of this virus type to infect non-mitotic cells. This is in sharp contrast to their predecessors, the oncoretroviruses, which require cell cycling within hours for transfer of genes into host cells (Roe, Reynolds et al. 1993).

Replication of HIV-1 in non-dividing and slowly proliferating cell populations depends on active import of the viral pre-integration complex (PIC) into the cell nucleus and it is commonly accepted that this complex hijacks the cellular nuclear import machinery to do so. Within the viral proteins known to play a role in the transport of HIV-1 through the nucleopore of the cell are present: “matrix”, “virion protein R” (VPR) (Bukrinsky and Adzhubei 1999) and “integrase” (Bukrinsky, Haggerty et al. 1993; Gallay, Hope et al. 1997).

In general terms, the principle of the lentivirus derived system consists of the construction of replication-defective recombinant chimeric lentiviral particles from three different components: the genomic RNA, the internal structural and enzymatic proteins and the envelope glycoprotein.

In the third generation of the vector system only three genomic RNA sequences from the HIV-1 are remaining (Dull, Zufferey et al. 1998). The viral genetic information contained in the vector is the only transferred to the host cells (not the accessory packaging plasmids) and represents only the 20% of the original viral genomic RNA. Removal from this vector of almost all original viral genes ensures that no potentially immunogenic proteins are expressed in target cells. Some cis-acting flanking sequences were left to ensure the proper packing of the coding cassette. The use of a long terminal repeat (LTR) enables transcription in the absence of *tat.* This LTR also contains a self-inactivating deletion for biosafety and it is detailed later.
The vector system contains three trans-acting elements: *gag* and *pol* which encode for the structural and enzymatic components of the virion and *rev* for the transcriptional regulation necessary for correct *gag* and *pol* expression, *rev* is necessary to prevent splicing of the transfer vector RNA in the producer cell by exporting it out of the nucleus during viral production (Delenda 2004) (figure 1.6).

Although several proteins can pseudotype lentiviral particles (Sandrin, Boson et al. 2002), the G protein of the vesicular stomatitis virus G-(VSV) is the one most commonly used. This envelope protein binds to the phospholipids in the bilayer of the cytoplasmatic membrane in vertebrates and also invertebrates (Burns, Friedmann et al. 1993), conferring a width range of host that can be infected. The G-VSV also allows to achieve high titers with unconcentrated viral particles and it is stable when ultraconcentrated and when frozen particles are required (Reiser 2000).

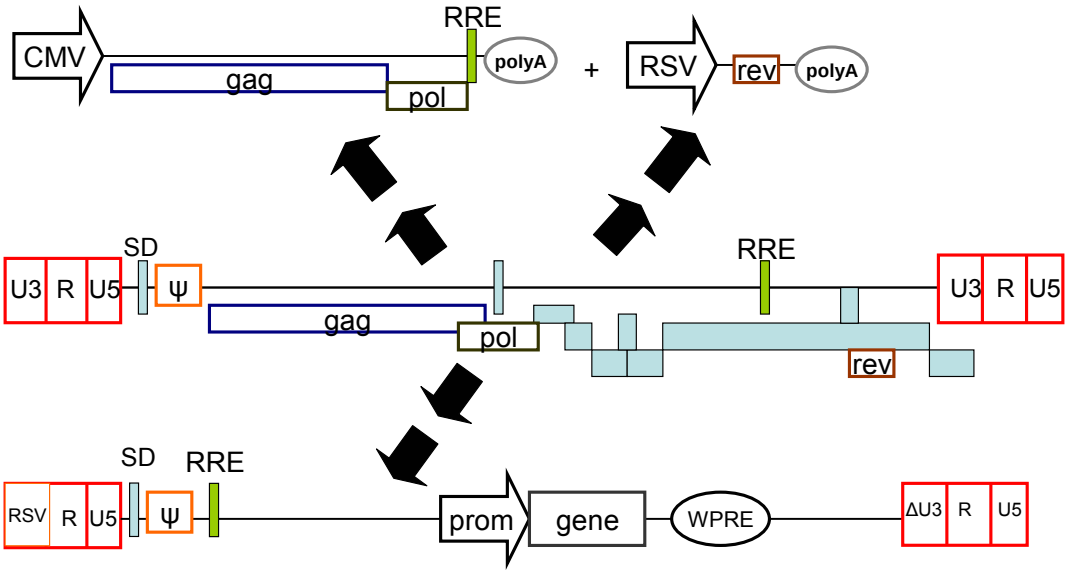


Figure 1.6. Scheme showing the features that were taken from the original HIV-1 genome and elements (middle) for the accessory plasmids (top) and the expression vector (bottom) in the third generation of lentivirus derived system. (Adapted from Salmon T. and Trono D. "Design and production of HIV-derived vectors". Cell biology: a laboratory handbook, 3rd edition. Elsevier, 2005).

CMV: cytomegalovirus promoter
Gag/pol: structural proteins and enzymes for viral replication
RSV: rous sarcoma virus promoter
rev: transcriptional replication protein
polyA: polyadenylation site
U3-R-U5: HIV-1 long terminal repeat
SD: major splice donor
RRE: rev-responsive element
Ψ: HIV-1 packaging signal for encapsidation of genomic RNA
WPRE: Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element
ΔU3: self- inactivating deletion of the U3 part of the LTR : long terminal repeats

1.5.3. Lentivirus production and biosafety

In order to generate replication-incompetent viruses, the vector containing the gene of interest is co-transfected into a packaging cell line together with the accessory plasmids which contain the virion packaging elements.

The cell line of choice for viral particle production is the 293 line, which was derived from human embryonic kidney. Currently for our work we are using the 293 FT, where F stands for fast growing cells and T stands for large T antigen (this is the origin of replication).

As it has its origin in one of the most deadly virus affecting human kind today, many issues concerning biosafety have emerged, but still researchers feel confident with the safety levels reached in lentiviral vectors, especially with the most recent (third) generation that even exceeds the safety standards of oncoretroviral vectors currently used in clinics (Trono 2000).

In general terms, biosafety of a certain vector production system is achieved by distributing the coding sequences of its proteins and regulatory elements in as many independent units as possible. This is the case with the lentiviral system, where the system components were segregated into three helper plasmids. The Gag/pol accessory genes are supplied in trans, namely in a separated vector and are never packed into the viral particles.

In addition, in the expression vector, the 3 prime LTR region has been deleted in the U3 region to remove all transcriptionally active sequences, creating a self-inactivating LTR, this means that once in the target cell the vectors lose the transcriptional capacity of their LTR (Miyoshi, Blomer et al. 1998; Zufferey, Dull et al. 1998).

Finally, sequence overlap between vector and helper sequences has been reduced to less than 10 nucleotides which significant reduces the chance of an homologous recombination event to occur (Trono 2000).

1.5.4. Lentivirus derived vector applications

There are many cell types described to be susceptible to lentivirus-derived vector transduction since the first study by Naldini et al. in 1996 showing stable gene modification of non-dividing cells (Naldini, Blomer et al. 1996). The wide range of cell types shown to be transducible until now includes: epithelial cells (Johnson, Olsen et al. 2000), hepatocytes (VandenDriessche, Thorrez et al. 2002) and retinal cells (Miyoshi, Takahashi et al. 1997). Furthermore, efficient *in vivo* gene delivery and long-term expression in the CNS had been shown successfully (Naldini, Blomer et al. 1996; Zufferey, Nagy et al. 1997).

As mentioned above, the fact that lentiviruses are able to transduce non-dividing or slowly dividing cells has widened the range of hosts where this technology could be applied. Of particular interest for gene therapy are the HSC. In accordance with this, many publications described the efficient transduction of human HSC (CD34+) capable of reconstitute non-obese diabetic/severe combined immunodeficient mice (NOD/SCID) (Uchida, Sutton et al. 1998; Salmon, Kindler et al. 2000; Woods, Fahlman et al. 2000; Trono 2001; Woods, Mikkola et al. 2001). Today, lentiviral vectors are seen as potent tool for genetic engineering and their use in basic research as well as possible clinical applications has generated great expectations.

1.6. Aim of the study

Therapeutic agents currently used for the treatment of neurodegenerative diseases as Multiple Sclerosis and cerebral ischemia are not curative and many problems can be found when attempting to deliver any therapeutic molecule specifically to the lesioned tissue in the CNS. Using adult bone marrow derived lineage negative hematopoietic stem/progenitor cells two different animal models for neurodegeneration and their progress after a cell and molecular therapy approach were examined.
Depending on the mouse model used the following specific goals were chosen:

1) The first biological question of this work was whether hematopoietic stem/progenitor cells derived from bone marrow and genetically modified to express NT-3 would ameliorate the disease course of the animal model for multiple sclerosis.
In that respect, a cell population enriched in progenitor and stem cells was isolated from murine bone marrow and genetically modified to express NT-3. The animal model for multiple sclerosis was induced by immunization with the myelin antigen MOG_{35-55}. Intravenously cell injection was performed and clinical outcome was analyzed.

2) As there is little data on the kinetics and tissue protection of bone marrow hematopoietic stem/progenitor cells entering the ischemic area after intravenous administration, the second biological question was to assess the effects of hematopoietic stem and progenitor cell injection post-infarction in an animal model of transient cerebral ischemia.
For that purpose a transient ischemia animal model was induced by middle cerebral artery occlusion. Bone marrow hematopoietic stem/progenitor cells were injected intravenously 24 hours after ischemia. Brain hemispheres and spleens collected from injected animals were analyzed for cell migration, pathologic phenotype and expression profiles of growth factors and cytokines.

2. Materials and Methods

2.1. Materials

2.1.1. Buffers and solutions

- **(10X) 0.125M Phosphate-Buffered Saline (PBS),** pH 7.3

Components	Concentration	Company
$NaH_2PO_4.H_2O$	0.007M	Roth, Germany
$Na_2HPO_4.7H_2O$	0.034M	Roth, Germany
NaCl	0.6M	Roth, Germany
ddH_2O	up to1 liter	Roth, Germany

- **4% paraformaldehyde (PFA),** pH 7.3

Components	Amount	Company
PFA	20g	Sigma, Germany
NaOH	30ml	Roth, Germany
PBS(10X)	50ml	
ddH_2O	up to 1 liter	Roth, Germany

- **Lysis buffer for erythrocytes**

Components	Concentration	Company
NH_4Cl	0.156M	Roth, Germany
$KHCO_3$	0.01M	Roth, Germany
EDTA	$5x10^{-6}$M	Roth, Germany
ddH_2O		Roth, Germany

- **(10X) TBE Buffer**

Components	Concentration	Company
Tris-Base	1.78M	Roth, Germany
Boric Acid	1.78M	Sigma, Germany
EDTA	0.04M	Roth, Germany
ddH_2O	to 2 liters	Roth, Germany

- **(6X) Loading buffer**

Components	Concentration	Company
EDTA	0,5M	Roth, Germany
Sucrose	60%	Fluke Biochemika, Germany
Bromphenol Blue	0,04%	Sigma, Germany
Xylene Cyanole	0,04%	Sigma, Germany
Ficol-400	2%	Bio-Rad, Germany

- **1% Agarose gel**

Components	Amount	Company
Agarose	0.5g	SeaKem,Cambrex, USA
Etidium Bromide or	1.25ul	Roth, Germany
Gel Star (when gel extraction)	4ul	BioWhittaker Molecular Applications, USA
TBE (1X)	50ml	

- **PCR reaction mix** (50 ul sample)

Components	Amount	Company
dNTP mix (10mM)	1ul	Amersham Bioscience, USA
Taq polymerase(100U/20ul)	0.5ul	Roche, Germany
Forward primer (10pmol/ul)	3ul	MWG, Germany
Reverse primer (10pmol/ul)	3ul	MWG, Germany
Buffer (10X)	5ul	Roche, Germany
dd H_2O	37.5ul	Roth, Germany

- **Reverse transcription (RT) mix** (20 ul sample)

Components	Amount	Company
Total RNA	5ug	
Hexanucleotide Mix (10X)	1uL	Roche, Germany
dNTP mix (10 mM)	1uL	Amersham Bioscience, USA
DTT mix (0.1M)	2uL	Invitrogen, Germany
5XRT 1st Strand Buffer	4uL	Invitrogen, Germany
RT enzyme (200U/ml)	1uL	Invitrogen, Germany
dd H_2O	up to 20ul	Roth, Germany

- **Real time RT-PCR** (25 ul sample)

Components	Amount	Company
SYBR Green Master Mix(2x)	12.5ul	Applied Biosystems, UK
cDNA	1ul	
forward and reverse primer pair mix (10 pmol/ul)	1ul	MWG, Germany
ddH_2O	10.5ul	Roth, Gemany

- **Digestion reaction mix** (20 ul sample)

Components	concentration	Company
Enzyme 1 (10U/ul)	0.5ul	Roche, Germany
Enzyme 2 (10U/ul)	0.5ul	Roche, Germany
Buffer (10X)	2ul	Roche, Germany
insert/plasmid	up to1ug	
ddH_2O	up to 20ul	

- **Ligation reaction mix** (10ul sample)

Components	Concentration	Company
T4 Ligase (1U/ul)	1ul	Roche, Germany
Ligation Buffer (10X)	1ul	Roche, Germany
DNA	8ul	
ddH_2O	10ul	Roth, Germany

3.1.2. Cell culture media and reagents

- **293FT cell line** (based on human embryonic kidney cell line HEK):

Components	Concentration	Company
DMEM		Gibco, Germany
Fetal Calf Serum	10%	PAN, Germany
1% L-Glutamate	1%	Gibco, Germany
1% Penisilin/Streptomycin	1%	Gibco, Germany
1% Glucose	1%	Sigma, Germany
Genetycin (when expanding)	1%	Gibco, Germany

- **Lineage negative cells**

Components	Concentration	Company
Stem Span SFM		Stem Cell Technologies,Vancouver, Canada
rhInterleukin-6	20ng/ml	R&D systems, Germany
rmStem Cell Factor	100ng/ml	R&D systems, Germany
rmThrombopoietin	20ng/mL	Sigma, Germany

- **Other cell culture reagents**

Opti-MEM (reduced serum medium)	Gibco, Germany
Trypsin-EDTA(1X)	Gibco, Germany
Poly-L-Lysine	Sigma, Germany

3.1.3. Antibodies

- **Cell isolation and flow cytometry monoclonal primary antibodies**

Epitope	Specificity	Source	Company
B220/CD45R	B lymphocytes	Rat	BD Pharmingen, USA
CD4	T lymphocytes	Rat	BD Pharmingen, USA
CD8	T lymphocytes	Rat	BD Pharmingen, USA
Gr-1	Granulocytes	Rat	BD Pharmingen, USA
Mac-1(CD11b)	Macrophages	Rat	BD Pharmingen, USA
TER-119	Erythroid cells	Rat	BD Pharmingen, USA
CD45 (LCA)	Pan-hematopoietic (except erythrocytes)	Rat	BD Pharmingen, USA
c-Kit	HSC	Rat	BD Pharmingen, USA
Sca1	HSC	Rat	BD Pharmingen, USA
CXCR4	HSC, B cells	Rat	BD Pharmingen, USA
CXCR3	lymphocytes	Rat	R&D Systems, De
β1-integrin (CD29)	lymphocytes	Rat	BD Pharmingen, USA
α4-integrin (CD49d)	lymphocytes	Rat	BD Pharmingen, USA
Isotype IgG	-	Rat	BD Pharmingen, USA

- **Additional inmunohistochemistry monoclonal primary antibody**

Epitope	Specificity	Source	Company
Myelin Basic Protein	Myelin sheath	rat	Chemicon, Germany

- **Secondary antibodies and other staining reagents**

Fluorophore	Specificity	Source	Company
-	Rat	Goat (magnetic bead conjugated)	Dynal, USA
FITC	Rat	Goat	Dianova, Germany
Cy3	Rat	Goat	Sigma, Germany
FITC	-	Avidin-conjugated	Sigma, Germany
Cy3	-	Avidin-conjugated	Sigma, Germany
-	microglia/ macrophages	Biotinylated IsolectinB4	Sigma, Germany
-	double stranded DNA	DAPI	Sigma, Germany

3.1.4. Primer sequences (all pimers were purchased from MWG, Germany)

- **Table I. Real time RT-PCR primers**

Target	Accession number	Oligo nucleotide	Sequence
IFN-γ	NM_008337	forward	5'-ACTGGCAAAAGGATGGTGAC-3'
		reverse	5'-TGAGCTCATTGAATGCTTGG-3'
IL-1β	NM_008361	forward	5'-ACAACAAAAAAGCCTCGTGCTG-3'
		reverse	5'-CCATTGAGGTGGAGAGCTTTCA-3'
TNF-α	NM_013693	forward	5'-CCGTCAGCCGATTTGCTATCT-3'
		reverse	5'ACGGCAGAGAGGAGGTTGACTT-3'
BDNF	BC034862	forward	5'-GCTGAGCGTGTGTGACAGTA-3'
		reverse	5'-TTGGATACCGGGACTTTCTC-3'
bFGF	NM_008006	forward	5'-GCTGCTGGCTTCTAAGTGTG-3'
		reverse	5'-TACTGCCCAGTTCGTTTCAG-3'
GDNF	NM_010275	forward	5'-CCTCGAAGAGAGAGGAATCG-3'
		reverse	5'-ATAGCCCAAACCCAAGTCAG-3'
NGF	NM_013609	forward	5'-TCTATACTGGCCGCAGTGAG-3'
		reverse	5'-GCTTCAGGGACAGAGTCTCC-3'
NT-3	NM_008742	forward	5'-AAATAGTCACACGGATGCCA-3'
		reverse	5'-GGCAAACTCCTTTGATCCAT-3'
TGF-β	NM_011577	forward	5'-AGGACCTGGGTTGGAAGTGG-3'
		reverse	5'-AGTTGGCATGGTAGCCCTTG-3'
VEGF	BC061468	forward	5'-CGAAGTGGTGAAGTTCATGG-3'
		reverse	5'-AAGATGTCCACCAGGGTCTC-3'
GAPDH	BC083149	forward	5'-ACAACTTTGGCATTGTGGAA-3'
		reverse	5'-GATGCAGGGATGATGTTCTG-3'
CCR1	NM_009912	forward	5'-CTCATGCAGCATAGGAGGCTT-3'
		reverse	5'-ACATGGCATCACCAAAAATCCA-3'
CCR2	NM_009915	forward	5'-ATCCACGGCATACTATCAACATC-3'
		reverse	5'-CAAGGCTCACCATCATCGTAG-3'
CCR5	NM_009917	forward	5'-TTTTCAAGGGTCAGTTCCGAC-3'
		reverse	5'-GGAAGACCATCATGTTACCCAC-3'
CXCR4	NM_009911	forward	5'-GGAGTGGGGTCTGGAGACTAT-3'
		reverse	5'-TTGCCGACTATGCCAGTCAAG-3'
CXCR3	NM_009910	forward	5'-TACCTTGAGGTTAGTGAACGTCA-3'
		reverse	5'-CGCTCTCGTTTTCCCCATAATC-3'
CX3CR1	NM_009910	forward	5'-GAGTATGACGATTCTGCTGAGG-3'
		reverse	5'-CAGACCGAACGTGAAGACGAG-3'

- **Cloning primers**

Target	Oligo	Sequence
PGK prom	Forward	5'-CCATCGATAATTCTACCGGGTAGGGGGAGG-3'
	Reverse	5'-CGCGGATCCGGAGATGAAGGAAGAGGAGAAAC-3'
NT-3	Forward	5'-GGGGATCCAATGTCCATCTTGTTTTATGTGATA-3'
	Reverse	5'-GGCTCGAGTCATGTTCTTCCAATTTTTCTCGAC-3'

- **RT-PCR primers**

Target	Oligo	Sequence
NT-3	Forward	5'-CAATTCCCTCATCATCAAGCTGA-3'
	Reverse	5'-CTCGGTGACTCTTATGTTCTGC-3'
18s	Forward	5'-ATCCATTGGAGGGCAAGTCT-3'
	Reverse	5'-CCGCGGTCCTATTCCATTAT-3'

3.1.5. Consumables

6 and 24-well culture plates	Cellstar, VWR International, Germany
15ml tubes	Cellstar, VWR International, Germany
50ml tubes	Sarstedt, Germany
5ml, 10ml, 25ml pipets	Sarstedt, Germany
Chamber slides	Nunc GmbH, Germany
Cryovials	VWR International, Germany
75cm^2 and 175 cm^2 culture flasks	Sarstedt, Germany
5ml polystyrene round-bottom tubes	BD Falcon, USA
3cm,10cm culture dishes	Sarstedt, Germnay
Bacteria culture 10ml tube	Sarstedt, Germany
500ul, 1000 uL plastic tube	Eppendorf, Germnay
PCR tubes	Biozym Diagnostik, Germany
10ul, 100ul and 1000ul tips	Starlab, Germany
96 well- optical reaction plate	Microamp, Applied Biosystems, Germany
5ml, 10ml syringes	Braun, Omnifix, Labomedic, Germany
Needles	100Sterican, Braun, Germany
Stopcock for infusion	Discofix, Braun, Germany
Glass slices for cryosectioning	Menzel-Glacer, Germany
Bottle top filters (0.25um pore)	Millipore, Germany
Filters (0.45um and 0.2um pore)	Filtropur, Sarstedt, Germany

3.1.6. Equipment and software

Centrifuges	Ultracentrifuge, Sorvall Discovery^{TM} 90SE, Hitachi, Germany Megafuge, 1.OR. Heraeus, Germany Biofuge Fresco, Heraeus, Germany
Cryostat	Micron HM560, Microm Int., Germany
Flow cytometer	FACSCalibur, Becton Dickinson Bioscience, Germany
Electrophoresis gel chambers	Blomed Analitik GmbH, Germany
Power supply	Amersham Bioscience, Germany
Heating block	Stuart Scientific, Germany
Incubators	Heracell240, Heraeus, Germany
Laminar-Air-flow workbench	Herasafe, Heraeus, Germany
Microscopes	Axiovert40CFL, Zeizz, Germany Axiovert200M, Zeizz, Germnay Fluoroview1000 Confocal micr., Olympus, Germany
pH-meter	Hanna Instruments, Germany
Photometer	Biophotometer, Eppendorf, Germany
Real time thermocycler	ABI Prism 5700 Sequence Detection System, Applied Biosystems, UK
Thermocycler	T3, Biometra, Germany
Transiluminator	Dark Reader, Clare Chemical Research, Germany
Vortex	$2X^2$, VelpScientifica, Germany
Scale	Sartorious, Germany
-80°C Freezer	Herafreeze, Heraeus, Germany
Magnetic stirrer	Velp Scientific, Germany

Software	Openlab4.0.1, Improvision, Germany
	CorelDRAW. Graphics Suite 11, Germany
	EndNote v8,Thomson ISI ResearchSoft, USA
	Microsoft Office XP, Microsoft USA, USA
	Olympus FluoView1.4. Olympus, Germany
	SDS 2.2.2, Applied Biosystems, USA
	Cellquest Pro, BD Biosciences, USA

3.1.7. Kits and additional reagents

- **DNA and RNA purification kits**

Kit name	Purpose	Company
Endofree Plasmid Maxiprep	Plasmid extraction (up to 500ug)	
QIAprep Plasmid Miniprep	Plasmid extraction (up to 20ug)	
Min iElute Gel extraction	DNA (70bp-4kb) extraction from agarose gels	All from
Mini Elute Clean up	DNA (70bp-4kb) purification from reactions	Qiagen,
RNeasy Mini	Total RNA (up to 100ug) purification	Germany
RNeasy Mini for lipid tissue	Total RNA (up to 100ug) purification from lipid tissue	

- **Additional reagents**

MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK)	Charité, Berlin
Pertussis toxin	List Biological Laboratories,
Mycobacterium Tuberculosis H37Ra	USA
Incomplete Freund's adjuvant	Difco, USA
Lipofectamine2000 reagent	Difco, USA
Ampicilin	Invitrogen, Germany
LB agar and LB media	Sigma, Germany
DMSO	Fluke Biochemika, Germany
Glycerol	Sigma, Germany
Tissue tek O.C.T. compound	Sigma, Germany
β-Mercaptoethanol	Sakura, NL
Ethanol	Sigma, Germany
Propidium Iodide	Roth, Germany
	Sigma, Germany

2.2. Animals

Green fluorescent protein (GFP) expressing transgenic mice (in these mice, the expression of GFP is under the chicken ß-actin promoter and the cytomegalovirus (CMV) enhancer ensuring the expression of this fluorescent molecule in all tissues with the exception of erythrocytes and hair) were obtained from JAX laboratories and bred in heterozygosis in the animal facility. Normal C57Bl/6 mice were obtained from Charles River (Sulzfeld, Germany). Female animals of 6-8 weeks of age were used for Lin^--HSC isolation and EAE induction. Adult male C57Bl/6N mice weighting 22-27g were used for all stroke experiments.

2.3. Isolation of lineage negative cells (Lin^--HSC) from adult mice BM

To isolate BM cells, mice were sacrificed, skin and muscle were removed from their hind limbs, ends of the bones were cut off and BM from femora and tibiae was flushed out using a 27 gauge needle and 5 ml syringe, and collected in 5 ml of cold phosphate-buffered saline (PBS, 1X). Erythrocytes were lysed using 1ml/mouse of hypotonic solution for 30 sec. Immediately, PBS was added preventing further lysis of white blood cells and the suspension was centrifuged (5 min. 2000 rpm 4°C). After a second washing step, total BM (TBM) cells were collected.

For removing cells positive for blood lineage markers, TBM was incubated for 1 hr in a rotor at 4°C, with a suspension of six types of rat monoclonal antibodies against mice lineage markers as listed bellow:

Surface Marker	*Cell lineage*
B220/CD45R	B lymphocytes
CD4	T lymphocytes
CD8	T lymphocytes
Gr-1	Granulocytes
Mac-1	Macrophages
TER-119	Erythrocytes

(All 1:200, BD Pharmingen, USA)

After a washing step, a population devoid of lineage markers was isolated by incubating the cell and antibodies suspension with sheep anti-rat IgG secondary antibody coupled with immune-magnetic bead (1:10, Dynabeads M-450, Dynal, USA) with rotation during 45 min at 4°C. The bead-bounded cells were depleted from the total population by placing the tube in a magnet (also from Dynal) for 10 min. before

collection of the non-magnetic fraction (Lin$^-$-HSC). Cells were counted before and after selection in a Neubauer chamber of 0.0025 mm^2.

After a washing step, Lin$^-$-HSC were either use for FACS or real time RT-PCR analysis, incubated with viral particles for genetic modification and/or injected into animals for *in-vivo* migration study or therapeutic approach.

2.4. Total Bone Marrow and Lin$^-$-HSC cell analysis

2.4.1. Flow cytometry

TBM cells or Lin$^-$-HSC were incubated with either biotin-conjugated anti-CD45, anti-cKit (1:200, BD Biosciences) or anti-CD11b, anti-TER119, anti-Gr-1, anti-B220, anti-CD8, anti-CD4, anti-Sca1, anti-CD29, anti-CD49d, anti-CXCR4 or anti-CXCR3 rat anti-mouse monoclonal antibodies for 30 min at 4°C (same concentration).

After a washing step, cells were incubated with FITC-conjugated streptavidin in the same concentration (BD Biosciences) or FITC-conjugated goat anti-rat IgG secondary antibody (Dianova, Hamburg, Germany). Isotype matched antibodies (BD Biosciences) were used as negative control. Analysis was done with a FACSCalibur™ flow cytometer (BD Biosciences). Live gating was preformed using propidium iodide (PI, 1ug/ml, Sigma). PI can be excluded from living cells, therefore only cells negative for fluorescence coming from PI are consider for analysis.

2.4.2. RNA extraction, reverse transcription and real-time PCR quantification

RNA was isolated from TBM or Lin$^-$-HSC using RNeasy minikit (Qiagen). The principle of the procedure of this kit is the selective binding of RNA to a silica membrane while the rest of the cell components are washed away.

As starting material, 2 million cells from each population were disrupted by addition of buffer containing ß-mercaptoethanol 1% (Sigma) and homogenized to reduce viscosity of lysates. Afterwards, ethanol 70% was added to provide proper binding conditions to the silica-gel membrane of the columns provided by the kit. Finally, RNA molecules longer than 200 nucleotides are eluted in 30ul of water.

To obtain DNA copy (cDNA), reverse transcription (RT) was performed using approximately 3ug of RNA isolated as described above. SuperScript III reverse transcriptase enzyme (Invitrogen) and hexamer random primers (Roche) were used for this reaction during 1 hour at 50°C. This enzyme is able to synthesize a

complementary DNA strand (and the supplementary copy) from single chain RNA. Thereafter, the product of this synthesis was utilized as template for the relative quantification step. As control of possible genomic DNA contamination, samples without the reverse transcriptase enzyme were prepared.

Relative expression levels of the following cytokines and factors were determined: TNF-α, IFN-γ, IL-1ß, Transforming Growth Factor (TGF)-ß, BDNF, basic Fibroblast Growth Factor (bFGF), Glial-cell-line-derived Neurotrophic factor (GDNF), NGF, NT-3 and Vascular Endothelial Growth Factor (VEGF). List of specific primers for each transcript is detailed in Table I of Materials, primer sequences section.

Real time quantitative Polymerase Chain Reaction (PCR) was performed using an ABI Prism 5700 Sequence Detection System machine (Applied Biosystems), which detects fluorescent signal coming from a reporter during the PCR amplification reaction. SYBR green (emits signal only when intercalates in double stranded DNA) was the fluorescent dye of choice which is included in the PCR master mix from Applied Systems together with the polymerase enzyme (AmpliTaq Gold). The amplification protocol for the GeneAmp 5700 Sequence Detection System Software (v.2.2) was performed. Cycles were as follows: initial denaturalization at 95°C for 10 min, denature at 95°C for 15 sec., annealing and amplification at 60°C for 1 min. repeated 40 times. Non-template control and negative samples from reverse transcription were included. Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous gene expression control. After the reaction, the melting curve program was perfomed to assess the presence of specific products. A peak in the negative first derivative of the fluorescence versus temperature graph indicated a unique specific product in the amplification reaction.

To analyze the values obtained from the real time reaction the comparative Ct (treshold cycle) method was used. Normal brain tissue was used as calibrator sample (gene expression value=1).

2.5. Lentivirus based vector system

2.5.1. Cloning into Lentiviral vectors

Lentiviral vectors of third generation (pLenti6/V5 D-TOPO, Invitrogen) were used for genetic modification of cells. The CMV original promoter was replaced for the phosphoglycerate-kinase (PGK). In hematopoietic derived cells, the PGK promoter allows the ubiquitous and stable in time expression of the desire gene.

The PGK promoter was amplified from the murine stem cell virus plasmid (pMSCV; Clontech) using extended primers for ClaI and BamHI restriction sites (please, refer to Materials, cloning primers).

Downstream the promoter, enhanced GFP (eGFP) reporter gene was inserted into the TOPO cloning sites or for therapeutic approach GFP was cut out using BamHI and XhoI (Roche). The mouse NT-3 gene (NM_008742) of 777 base pairs previously amplified using extended primers was inserted. Accordingly, gene expression was confirmed with transfection of the packaging cell line by fluorescence imaging or RT-PCR (primers detailed in Materials, RT-PCR primers list).

2.5.2. Viral particle production

The 293FT (purchased from Invitrogen and expanded in the laboratory) packaging cell line was kept in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) with 1% penicillin/streptomycin and glucose (Sigma) at 37°C in 10% CO_2.

In order to produce viral particles, 5 million 293FT cells were seeded in 10 cm dishes precoated with poly-L-Lysine (Sigma). After removing the culture medium and washing once with PBS, 5 ml of Opti-MEM medium (Gibco) without antibiotics were added. Cells were transfected using the gene-containing vector (either GFP or NT-3) together with three packaging helper plasmids and Lipofectamine 2000 reagent (Invitrogen).

The principle of this procedure is the entrance of the DNA to the cells after forming lipidic complexes form with the Lipofectamine2000 reagent. Medium was replaced for fresh DMEM 10 hrs post-transfection.

Viral supernatant (10 ml) was collected at 48-72 hrs post-transfection. To increase viral titres, particles were concentrated by ultracentrifugation (25000 rpm for 1 hr. 30min at 4°C) using a Sorvall Discovery^{TM} 90SE ultracentrifuge. Supernatant was

removed, and pellet resuspended by slightly shivering the tubes overnight at 4°C in 500ul of medium. Viral particles were immediately used for Lin$^-$-HSC or control cell line transduction.

2.5.3. Transduction of Lin$^-$-HSC

To genetically manipulate cells to express a fluorescent molecule or alternatively a neurotrophic factor the lentiviral technology (Invitrogen) was preferred.

For transduction Lin$^-$-HSC, cells were normally seeded at a density of $1x10^6$cells/ml in 24-well dish and incubated with 10^8 Transducing Units (TU)/mL of lentiviral particles. First, cells were centrifuge for 90 min. at 2000 rpm and 30°C (spin down protocol) in Stem Span serum free medium (StemCell Technologies Inc., Canada) in the presence of rhIL-6 (20ng/ml), rmSCF (100 ng/ml) and rmTPO (20ng/mL) (all from R&D systems). After centrifugation step, cells were placed in 37°C and 5% CO_2 incubator overnight.

When attempting to improve transduction efficiency, the proteasome inhibitor MG-132 (Calbiochem, Germany) was added together with the cytokines at a concentration 2uM.

Alternatively, the glycoprotein VSV that pseudotypes the lentivirus capside (coded in the plpVSV helper plasmid) was replaced by the SCF, which is the ligand for the cKit receptor. SCF was cloned into the plpVSV packaging vector (G-VSV was replaced by the SCF of 821bp using EcoRI restriction sites). Both vectors (plpVSV and plpSCF) were used to co-transfect 293FT cells when preparing viral particles and transduction followed as before.

The day after Lin$^-$-HSC transduction, medium containing viral particles was removed and fresh medium was added. FACS analysis of eGFP expression was performed between days 3 to 5 post-transduction. Alternatively, injection into EAE animals for therapy approach, took place 24 hrs after cell transduction. To this aim, cells were washed and re-suspended in 300uL of PBS.

2.6. Induction of Experimental Autoimmune Encephalomyelitis

2.6.1. Myelin Oligodendrocyte Glycoprotein (MOG) emulsion

MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK; Charité, Berlin) was emulsified with 1 mg of Mycobacterium Tuberculosis H37Ra (Difco, Detroit, MI) in

incomplete Freund's Adjuvant (Difco). Emulsion was left overnight at 4ºC the day before mice immunization.

2.6.2. Immunization of animals with MOG_{35-55} peptide

For immunization protocol, adult 6-8 weeks old female C57Bl/6 mice were anesthetized and injected subcutaneously in both inguinal lymph node regions with 200 ug of MOG_{35-55} emulsion.

PTX (200 ng, List Biological Laboratories, Campell, CA) was injected on day 0 (intravenously) and 2 (intraperitoneally). Weight and clinical score of animals was checked daily for at least 30 days thereafter unless moribund.

Scoring of clinical symptoms was as follows:

0- Healthy

1- Complete limp tail

2- Weakness of hind limbs

3- Paralysis of hind limbs

4- Weakness of fore limbs

5- Fore and hind limbs paralysis or moribund

Animals with a clinical score less than 1 by day 14 post-immunization were not used for the study. For migration and therapeutic studies, mice were intravenously injected with GFP positive or NT-3 transduced Lin^--HSC 5 days after the onset of disease.

2.7. Detection of GFP+ Lin^--HSC in injected animals.

Animals were transcardially perfused with 20ml of PBS followed by 20ml of cold 4% PFA. Spinal cords and spleens were collected and left overnight in fresh fixative. The day after, tissues were cryoprotected in 2% DMSO (Sigma), 10% glycerol (Sigma) containing PBS for one extra day. Tissues were embedded in Tissue-Tek O.C.T. compound (Sakura, NL), rapidly frozen on dry ice and stored in -80°C till cryosectioning. Slicing was performed in a Micron HM560 cryostat at -20°C. Thickness of slices was between 10 to 20um.

For immunohistochemistry, cryo-sections were re-fixed in PFA 4%, washed and incubated with monoclonal first antibody diluted in 0.1% Bovine Serum Albumin (BSA) and 0.01% Triton-X (1:200, BD Pharmingen) overnight at 4°C. After 2 washing steps, secondary fluorophore conjugated anti-rat IgG was added (1:200, Dianova) for 2 hrs at room temperature. Counterstaining of nuclei was performed using DAPI (1:5000, Sigma).
Sections were analyzed both, by normal flourescence (Zeiss) and confocal microscopy (Olympus)

2.8. Induction of focal cerebral ischemia and Lin$^-$-HSC injection

(In collaboration with the Department of Neurology, University Hospital Göttingen)

Transient focal cerebral ischemia (45 minutes) was induced by occlusion of the middle cerebral artery (MCA) using the intraluminal filament technique (Kilic, Bahr et al. 2001). Briefly, animals were anaesthetized with initially 4% isofluran and then maintaining 1%-1.5% isofluran in 68% N_2O and 30% O_2 using a facial mask. Rectal temperature was maintained between 36.5-37°C employing a feedback-controlled heating system. For assessment of cerebral blood flow (CBF), laser-Doppler flow (LDF) was recorded during all experiments using a flexible 0.5mm fiber-optic probe (Perimed, Sweden) attached to the intact skull overlying the core region of the MCA territory (2mm posterior, 6mm lateral from Bregma). Following a midline neck incision the left common and external carotid artery were isolated and ligated. After placing a microvascular clip (Aesculap, Germany) on the internal carotid artery, an 8-0 silicon resin (Xantopren; Deuker, Germany) coated nylon monofilament (Ethilon, thread diameter 180-200µm; Ethicon, Germany) was introduced through an incision into the distal part of the common carotid artery and, after removing the clip, advanced 9mm distal from the carotid bifurcation for MCA occlusion. Only mice that showed a constant decrease in CBF to at least 30% of baseline values were used for our study. After 45 minutes of ischemia the filament was removed to allow reperfusion of the MCA. LDF recordings were continued for 15 minutes to monitor appropriate reperfusion (>80% of initial CBF).
Twenty-four hours after reperfusion mice were randomly selected to receive either Lin$^-$-HSC or PBS. Firstly, animals were (re-)anaesthetized as described above,

subsequently, the left femoral vein was isolated and a 30 gauge needle attached to a polyethylene micro catheter (PE10) was introduced. Animals were injected with 5 million Lin^--HSC (dissolved in 200µl of PBS) over 20 minutes. Control animals received PBS (200µl) only.

2.9. Brain and spleen tissues real-time RT-PCR analysis

For analysis in brains of cytokine and neurotrophic factor expression following stem cell treatment in cerebral ischemia, Lin^--HSC-treated animals and PBS-control mice were sacrificed 72 hours after intravenously treatment. Brains were carefully removed, and right and left (infarcted) hemispheres were separated. RNA was isolated by the RNeasy Mini Kit for Lipid Tissue (Qiagen). Reverse transcription was performed as described earlier. Levels of the same neurotrophic factors and cytokines mentioned above were determined for the ischemic and healthy hemispheres separately.

To assess peripheral immune activation, spleens were removed 24 hrs post cell or PBS administration. Same procedure as before for RNA isolation RT and real time PCR was followed. Expression of the following transcripts was analyzed: TNF-α, IFN-γ, IL-1β, Chemokine Receptor (CCR)-1, CCR2, CCR-5, CXCR1, CXCR3, CX3CR1.

2.10. Appendix to Materials and Methods

RNA Isolation

Isolation of RNA was performed using the RNeasy Mini Kit (Qiagen, Germany). The protocol used is summarized as follows:

• Take tubes with tissue (e.g. 20-30mg in PBS).

• Add 700µl lysis buffer (inc. 1:100 β-mercaptoethanol), use 350µl if less than 20mg of tissue or less than $5x10^6$ cells (lysis buffer lasts approx. 1 month).

• Homogenize with small syringe (use large first if a lot of tissue). Centrifuge (3min, max).

• Mix 600µl of supernatant with 600µl EtOH (70% in dd H_2O).

• Add 700µl into column and centrifuge (15s, 10000rpm), throw away waste, add rest of mixture and repeat.

• Add 350µl wash buffer RW1 and centrifuge (15s, 1000rpm), throw away waste.

• Mix 10µl DNase1 with 70µl RDD buffer, add to filter and leave for 15min.

• Add 350µl wash buffer RW1 and centrifuge (15s, 10000rpm), throw away waste.

• Add 500µl wash buffer RPE (+EtOH) and centrifuge (15s, 10000rpm), remove waste, repeat and centrifuge (2min, max). Remove waste and centrifuge again to dry (1min, max).

• Place column in tube, add 35µl RNase free H_2O (onto filter), leave for 3 min, centrifuge (1min, 10000), throw away column. RNA is collected in 1.5ml tube.

• Measure concentration (µg/ml) and RNA/protein absorption (260/280) using photometer (Eppendorf), and dilute with ddH_2O accordingly to start with about 3 µg of sample RNA.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed for the amplification of specific cDNAs in order to obtain gene products for insertion into viral expression constructs, as well as for analysis of sequences inserted into such constructs following mini-prep plasmid preparation.

In cases where correct sequences were required a polymerase with high proofreading ability was used, Vent Polymerase (New England Bio Labs), whereas in cases of analysis a standard Taq Polymerase was used (Roche). A master mix was prepared containing all required reagents (see Materials, buffers and solutions) and aliquoted to PCR tubes according to the following protocol:

• Add 47.5µl master mix to each tube.

• Add 2µl of appropriate cDNA (nothing to negative control).

• Place tubes into PCR machine and choose required program (use heated lid!).

Example of PCR program:

Initial Denaturation Step: 94^{o}C 3min

Cycle Step 1 – Denaturation: 94 ^{o}C 1min

Cycle Step 2 – Annealing 55-60 ^{o}C 1min (dependent upon primers used)

Cycle Step 3 - Elongation 74 ^{o}C 1min (3min for Vent Polymerase)

Repeat cycle steps accordingly between 15 to 35 times.

Final Elongation Step: 74 ^{o}C 3min.

PCR products were either stored at 4^{o}C or run on a 1% agarose gel for analysis or extraction of the product.

Agarose Gel Analysis and Extraction

In order to observe PCR products they were run on a 1% agarose gel. For analysis, ethidium bromide was added to the gel to allow visualization under a U.V. lamp. Where extraction of the product was required, "Gelstar" (BioWhittaker Molecular Applications) was used instead of ethidium bromide to allow visualization without the use of an U.V. lamp and thus preventing the possibility of mutation. After setting the gel in the chamber (see Materials, buffers and solutions) proceed as follows:

• Add a mixture of 3µl loading dye and 6µl sample to each lane, using a ladder in one lane to evaluate product size.

• Connect to power supply and set to 120V, 110A for 30min.

• Visualize DNA under a U.V. lamp.

• For extraction, visualize using a transiluminator (Dark Reader), cut out required fragment and extract DNA.

Extraction of DNA from agarose was performed using the QIAquick Gel Extraction Kit

(Qiagen) as follows:

• Excise DNA fragment and weigh.

• Add 3xVol buffer QG to 1xVol gel (max 400mg, normally use 450µl).

• Leave at 50oC for 10 min (vortex every 3min).

• Add 1xVol isopropanol (normally use 150µl), invert several times, place in column, centrifuge 1min, discard flow through. (Qiagen column collects fragments within range of 70bp-10Kb).

• Add 500µl QG buffer, centrifuge 1min, discard flowthrough.

• Add 750µl buffer PE, centrifuge 1min, discard flowthrough, repeat centrifugation.

• Place column in clean tube, add 10µl EB buffer/H2O, leave 1min, centrifuge 1min.

Real-time RT-PCR

Pipet reaction mix accordingly prepared (see Materials, buffers and solutions) into wells. Add 1ul of cDNA. Use a "non-template control" (NTC) well only with master mix. When pipetting, avoid bubbles. Cover the plate, with the plastic lid.

Program on ABI Prism SDS 7000 (Standard Protocol)

Cover T°= 105°C !!

Initial denature: 95°C, 10min

Denature: 95°C, 15sec

Annealing: 60°C, 60sec

Amplification for 40 cycles

Final elongation: 72°C, 10min

To asses if a specific product was obtained, perform dissociation curve analysis. (95°C, 60°C, 95°C ramp rate 2%).

(Alternatively, the PCR outcome can be examined by 3% agarose gel using 5 µl from each reaction)

Analyze the real-time PCR result with the SDS 7000 software. Check to see if there is any bimodal dissociation curve or abnormal amplification plot.

Blunt-end Formation and Dephosphorylation

In cases where "sticky-end" ligation could not be performed blunt-end ligation was used.

This procedure required dephosphorylation of the end terminals of the digested plasmid in order to prevent self-religation.

• Perform restriction digestion in a 20µl mixture as mentioned above.

• Without any manipulation on the reaction mix (e.g. cleaning or changing buffer) add 0.5µl of 10 mM dNTP.

• In order to fill in "sticky-ends" add 1-5 U Klenow Enzyme (Roche) and incubate at 30^{o}C for 15 min.

• Block the reaction by heating at 75^{o}C for 10 min (not necessary if continuing with dephosphorylation).

Proceed with dephosphorylation of the plasmid:

• Extract the necessary DNA fragment into 15µl H2O by gel extraction.

• Add 2µl 10x Buffer and 3U Shrimp Alkaline Phosphatase (Roche), bring to total volume of 20µl.

• Incubate for 60 min at 37oC degrees. Inactivate by heating to 65oC for15 min.

• Purify dephosphorylated plasmid by gel extraction and ligate to blunt-end-insert.

Ligation

Ligation of insert into plasmid was performed using T4 DNA Ligase (Roche). Ligation reactions were normally carried out at 15°C for at least 3 hours. The reaction

mix used was as listed below. Normally a ratio of 1:3 or 1:15 was used for plasmid:insert DNA in a volume of 8µl.

Transformation

Chemically competent bacteria (TOP10 Chemically Competent E.Coli, Invitrogen) were transformed with ligated insert-plasmid DNA and expanded according to the following protocol:

• Defrost chemically competent cells on ice (500µl per tube).
• Aliquot 100µl of cells per transformation and leave on ice for 30min.
• Dilute ligation mix 1:1 and add 0.6µl to 100µl of competent bacteria.
• Heat-shock cells at 42oC in water bath for 1min.
• Return cells to ice for 2min.
• Add 1ml LB medium and incubate in for 45min at 37°C (rotatory shaker, >200rpm).
• Centrifuge for 3min at 7000rpm and remove excess medium.
• Plate onto appropriate selective LB plates and incubate at 37°C overnight.
• Pick colonies and grow in selective LB medium for 10hr at 37°C in shaker.
• Isolate plasmid DNA using mini-prep kit (Qiagen).
• Verify ligation by restriction digest and PCR.
• Prepare high concentrate stock of positive samples using maxi-prep kit (Qiagen).

Transfection

Transfection of cell lines was performed to produce lentiviral particles as well as to confirm the expression of genes cloned into plasmids. Lentiviral particles were produced by co-transfection of the lentiviral plasmid along with plasmids expressing accessory lentiviral genes into a packaging cell line using a lipofectamine-based technique as follows:

• Plate $5x10^6$ 293FT cells in a poly-L-lysine coated 10cm dish one day before transfection to obtain a culture of 80-90% confluence.
• On the day of transfection add antibiotic-free medium to the cells (10ml).
• Prepare DNA-lipofectamine complexes:

9ug packaging mix + 3ug vector in 1.5ml Opti-MEM medium (Gibco).
36ul Lipofectamine 2000 (Invitrogen) in 1.5ml Opti-MEM
leave for 5min at room temperature.
mix gently and leave for 20min at room temperature

• Add transfection mix to cells dropwise, mix gently and leave for 6 hours.

• Add medium containing antibiotics and 2% serum to the transfected cells.

• Remove supernatant 36-72hrs post-transfection and pellet the debris.

As the lentivirus is VSV-G pseudotyped, the supernatant may be stored overnight at 4^{o}C, frozen at -80^{o}C or concentrated by ultracentrifugation (25,000rpm, 4^{o}C, 90min).

Transduction

A number of different transduction protocols can be used depending upon the target cell.

• Supernatant (cell lines)

Cells at a confluency of 30-50% were treated with lentiviral supernatant and kept at 37^{o}C overnight. Fresh medium was added to the cells on the following day.

Expression normally reached its peak 48hr post-transduction.

• Supernatant Spin Infection (cell lines)

Cells at a confluence of 30-50% in 6-well dishes were treated with lentiviral supernatant, centrifuged (2500rpm, 30^{o}C, 90min) and kept at 37^{o}C overnight.

Fresh medium was added to the cells on the following day. Expression normally reached its peak 48hr post-transduction and was generally higher than that observed following regular supernatant treatment.

• Viral Concentrate (primary HSC and cell lines)

Viral particles concentrated by ultracentrifugation were resuspended overnight at 4^{o}C in 500µl serum free medium (e.g. Stem Span). The resuspended viral concentrate was added to the target cells and kept at 37^{o}C overnight. Fresh medium was added to the cells on the following day. Expression normally reached its peak 48hr post-transduction.

• Concentrate Spin Infection (primary HSC and cell lines)

Viral particles concentrated by ultracentrifugation were resuspended overnight at 4^{o}C in 500µl serum free medium. The resuspended viral concentrate was added to the target cells, centrifuged (spin infection: 2500rpm, 30^{o}C, 90min) and kept at 37^{o}C overnight. Fresh medium was added to the cells on the following day. Expression normally reached its peak 48hr post-transduction and was generally higher than that observed following other transduction procedures.

Kill Curve

The pLenti6/V5 plasmid expresses the antibiotic blasticidin to allow selection of transduced cells in culture. The following protocol was used to determine the minimum concentration of blasticidin required to kill non-transduced cells:

• Plate cells in a 6-well plate at a confluency of 25%.
• Add blasticidin at various concentrations, e.g. 0, 2, 4, 6, 8, 10 ug/ml.
• Change medium every 3-4 days.
• Observe the percentage of surviving cells.
• Determine the lowest concentration that kills all cells within 10 days of treatment.

Viral Titre Determination

Titration of the viral titre may be performed using FACS analysis to quantify the number of transduced cells in order to estimate the concentration applied to primary hematopoietic cultures. Cells (can be from a cell line like HeLa) are transduced as follows:

• Plate 5x104 cells per well in a 6 well culture dish.
• Prepare 10-fold serial dilutions of viral stock (10-6, 10-5, 10-4, 10-3, 10-2, 0).
• Add to cells in total volume of 2ml medium containing 6ug/ml polybrene.
• Leave overnight at 37^{o}C.
• Add fresh medium and leave overnight at 37^{o}C.

Transduced cells are allowed to culture for 4 days following transduction and then analyzed by FACS (normally best done using a GFP reporter gene construct). Only dilutions yielding to 1-20% GFP-positive cells should be considered for titer calculations. Below 1%, the FACS may not be accurate enough to give a reliable determination of the number of GFP+ cells. Above 20%, the chance for each GFP+ target cell to be transduced twice significantly increases, resulting in underestimation of the number of transducing particles. The following formula is used to calculate the viral titre:

$$\text{Titer (Hela-transducing units / ml)} = \frac{(5\text{x}104 \text{ Hela cells}) \times (\%\text{ GFP-positive cells}/100)}{\text{volume of supernatant (ml)}}$$

3. Results

3.1. Lin$^-$-HSC isolation from adult mouse bone marrow

There are three main stem cell populations described to be present in mouse BM, the endothelial, the mesenchymal and the HSC, this last consisting of a lesser quantity in the total population (Wang and Spangrude 2003). In an ideal situation, when highly pure HSC need to be obtained, a very accurate sorting method and a large number of mice are required as source of BM, as this stem cell group represents less than 0.007% of the TBM. In addition, many difficulties are found when keeping these pure cells in culture for a long time without losing their stem properties.

To obtain a sufficient cell number for a cell therapy approach, an enriched hematopoietic precursor and stem cell population, here termed Lin$^-$-HSC, was selected for this work. These cells were immuno-magnetically negatively sorted. The protocol consisted of a step of depletion of blood committed cells expressing surface lineage markers. For this purpose, TBM cells were incubated with a mixture of primary abs against six different lineage epitopes (anti-TER-119 for erythrocytes, anti-Mac-1 for macrophages, anti-Gr-1 for granulocytes, anti-CD8 and anti-CD4 for T lymphocytes and anti-B220 for B lymphocytes) and after with magnetic bead conjugated secondary abs.

After a final incubation of the cell suspension in a magnetic column, the non-adherent fraction was used for following experiments.

The non-magnetic fraction obtained after the negative isolation step described above represented an average of 21±5.6 % of the initial TBM population (12 independent isolation times, figure 3.1). This average was reproducible in further isolations.

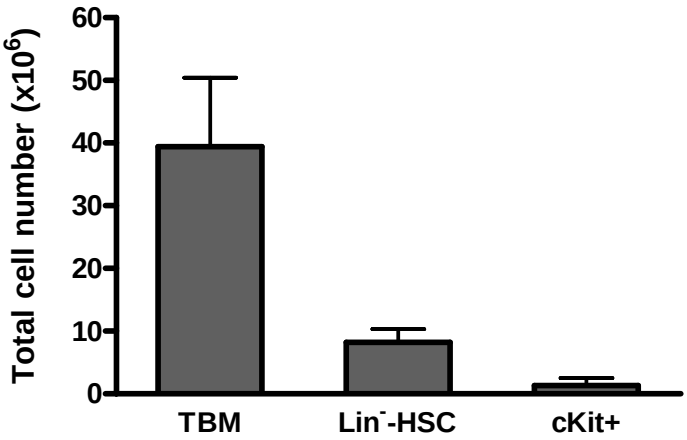


Figure 3.1. Isolation of lineage negative fraction from murine total bone marrow. Bars show the averaged cell number of absolute values per mouse. TBM: total bone marrow cells when freshly isolated from bone. Lin$^-$-HSC: cells after negative selection and number of cKit+ cells in TBM. Data is shown as the mean +/- S.D. (n=12 isolation times).

3.2. Flow cytometry characterization of Lin$^-$-HSC

To assess the reliability of the depletion procedure and describe which molecules are present on the surface of the cells obtained after negative selection, flow cytometry analysis was performed. TBM or selected cells were marked for the six different epitopes used for their isolation, followed by a secondary ab conjugated to a fluorescent molecule.

The Lin$^-$-HSC population obtained after negative selection was devoid of molecular markers for blood differentiated in contrast with TBM cells (figure 3.3A). Therefore, the depletion method selected in this work shown to be a trustworthy protocol to obtain a non-committed enriched cell population.

Further characterization by flow cytometry was performed with respect to surface markers and receptors present in the cells. As they are described markers for stem cells, levels of Sca-1 and c-Kit were assessed. Double positive cells never exceeded 1.2 % of the Lin$^-$-HSC. A representative dot plot for double staining is shown in figure 3.2.

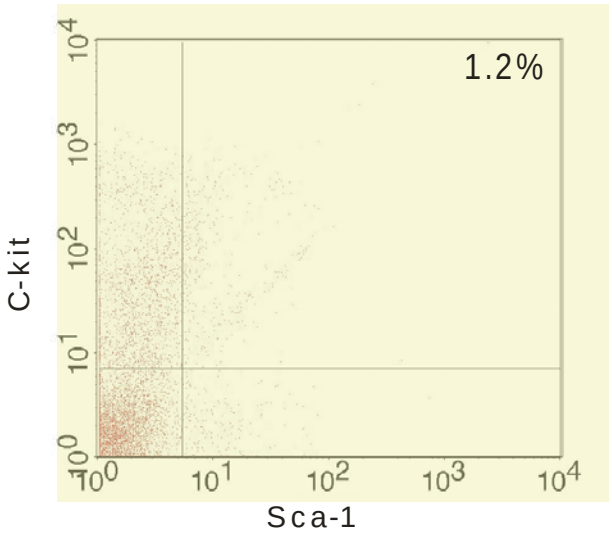


Figure 3.2. Representative flow cytometry dot plot showing percentage of double cKit and Sca1 positive cells in the Lin$^-$-HSC population.

Lin$^-$-HSC were found to be positive for CD45 (up to 58%) and the level of ckit expression shown was 29% (figure 3.3B). Chemokine-chemokine receptor is a system involved in recruitment of cells to sites of inflammation. The expression levels of two of the main chemokine receptors involved in this migration process was checked. No detectable levels of expression of CXCR3 and CXCR4 chemokine receptors in the purified cells were found (figure 3.3B). Once cells are recruited to sites of inflammation firm adhesion between migrating cells and endothelium is mediated by molecules of the Integrin superfamily. These molecules are expressed in the cell surface as heterodimers. Cells were checked for expression of two subunits of these Integrins. The subunit beta-1 was found not to be expressed and only 1% of Lin$^-$-HSC showed alpha-4 subunit signal expression (figure *3*.3B). In Table 3.I, flow cytometry values are summarized for all markers mentioned above.

Therefore, upon isolation, the population of choice for this work was devoid of lineage committed markers while expressing others belonging to a stem/progenitor phenotype. At the same time, they were not having detectable levels of chemokine receptors and of integrins analyzed once freshly obtained. Up to 85% of committed cells were eliminated with this procedure.

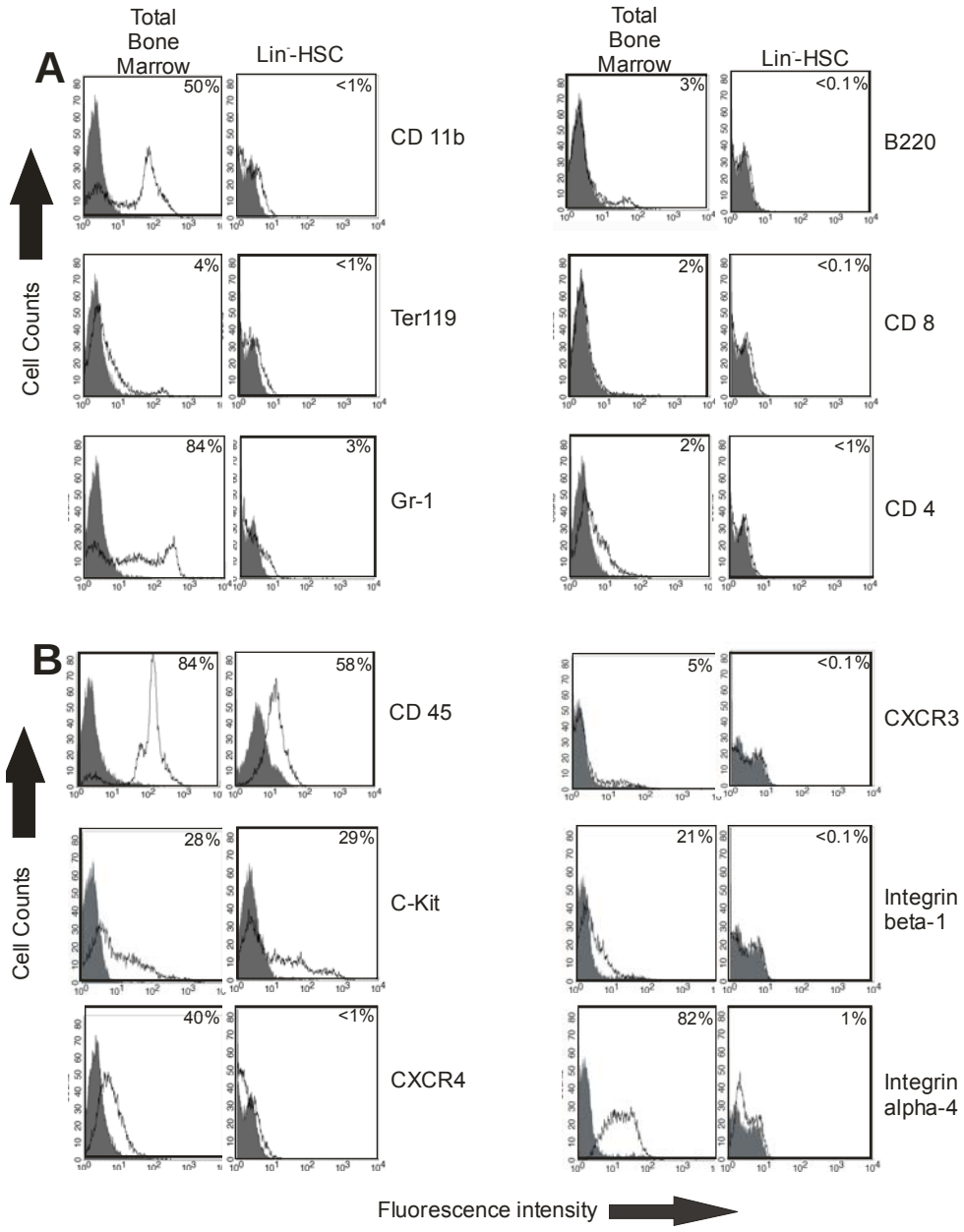


Figure 3.3. Representative flow cytometry analysis histograms of freshly isolated total bone marrow and selected Lin$^-$-HSC. A) After isolation from BM and immuno-magnetic negative selection, Lin$^-$-HSC showed undetectable levels of blood lineage committed cells.

B) Lin$^-$-HSC were positive for CD45 and cKit, while low levels of chemokine receptors and adhesion molecules were detected. Isotype controls are shown in grey (closed histograms). In the upper right corner, representative values of the markers are shown.

Marker	**Total Bone Marrow**	**Lin$^-$-HSC**
CD11b	55.4±3.8	1.7±1.6
TER119	6.0±1. 2	1.5±1.4
Gr-1	34.4±4.6	3.0±2.9
B220	3.4±2.3	0.1±0.1
CD8	1.7±0.8	0.5±0.4
CD4	2.0±1.0	0.5 ±0.1
Ckit	25.9±9.5	18.7±12.2
CD45	84.2±0.1	50.8± 8.7
CXCR3	6.9±2.1	<0.1
CXCR4	44.7±4.7	2.0±1.1
Beta-1	20.84±0.1	<0.1
Alpha-4	72.0±10.0	0.91±0.01

Table 3.I. Percentage of surface markers of Total Bone Marrow and lineage negative cells. Flow cytometry data from 3 independent experiments are shown. Values are presented as the mean ± SD.

3.3. Real time RT-PCR analysis of total bone marrow and Lin$^-$-HSC

As an additional method of characterization of the population used for this work, gene transcription levels were compared between Lin$^-$-HSC and TBM cells and analyzed in relation to their respective counterparts in normal brain tissue for a number of inflammatory cytokines and growth factors.

After isolation, TBM cells, Lin$^-$-HSC and brain tissue were lysed, RNA was extracted and reverse transcription performed. Thereafter, the cDNA obtained from each sample was used as a template for real time RT-PCR. For analysis, the comparative Ct method was used. Expression levels were normalized to brain gene transcripts levels (calibrator expression value = 1). Expression of the following cytokines and growth factors were determined: TNF-α, IFN-γ, IL-1ß, BDNF, bFGF, GDNF, NGF, NT3, TGF-ß, VEGF.

After three independent experiments, no relevant levels of growth factors and trophic factors BDNF, bFGF, GDNF, NGF, NT-3, TGF-ß, VEGF were observed in the Lin$^-$-HSC (figure 3.4A).
At the same time, Lin$^-$-HSC showed significantly decreased gene transcript levels of the pro-inflammatory cytokine TNF-α. Although not significant, also lower levels of IFN-γ, and IL-1ß were seen in the Lin-HSC when compared to TBM cells (figure 3.4B).

Therefore, cells obtained after the immune depletion step are not able *per se* to generate considerable levels of relevant growth factors for neuronal support and survival. In addition, levels of cytokines related to inflammation were reduced in comparison with TBM cells, which are already mature blood cells.

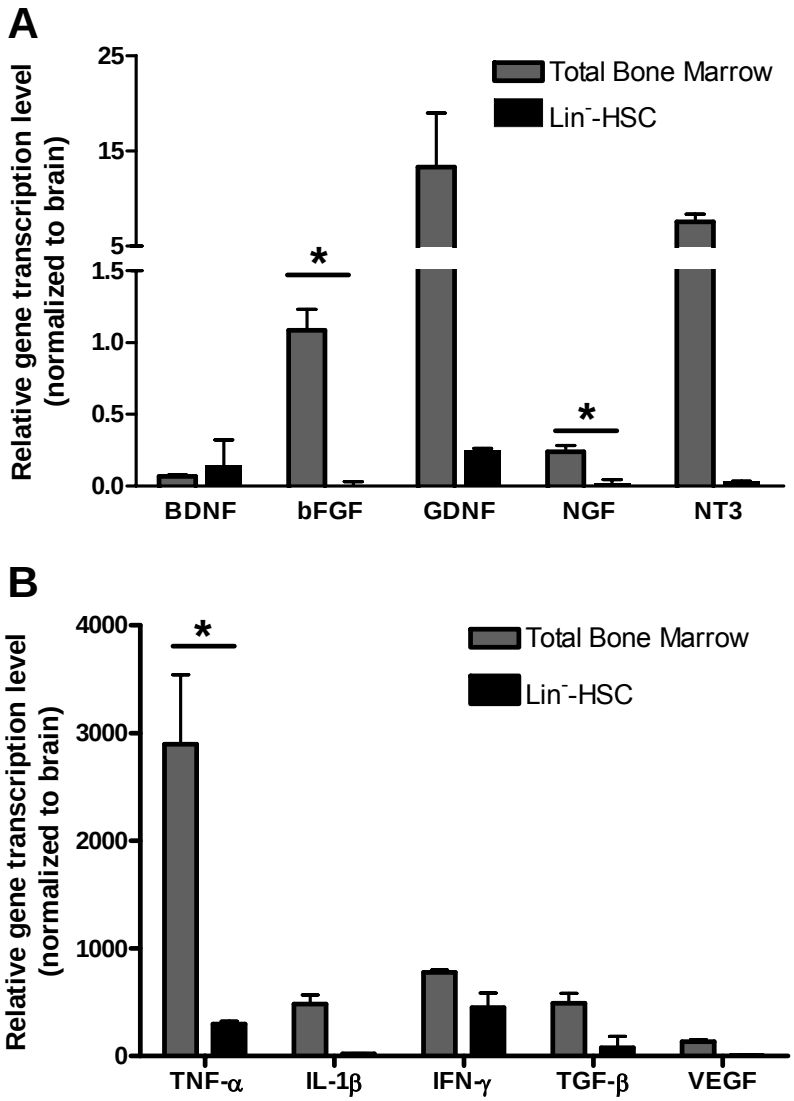


Figure 3.4. Relative gene transcription levels of cytokines and growth factors of Lin$^-$-HSC and total bone marrow cells. The population lacking lineage markers was found not to express considerable levels of growth factors (A) and reduced levels of pro-inflammatory cytokines when compared to total bone marrow (B).

Mean ± S.E.M are shown for the values normalized to expression level in brain (value=1). Data were analyzed using the Mann-Whitney Test (*, $p<0.05$; n=3).

3.4. Induction of Experimental Autoimmune Encephalomyelitis

A mouse model for MS was established. For this purpose, C57Bl/6 female mice were injected with MOG_{35-55} peptide and developed a progressive relapsing disease with a late mild remission. Disease susceptibility observed in this work is detailed in the following table:

Mouse Strain	Incidence	Disease onset	Maximal clinical score
C57Bl/6	64±15%	11±1 days after immunization	3±0.5

Table 3.II. EAE disease susceptibility of C57Bl/6 mice strain used for this work. Incidence (%), mean time of disease onset (days) and mean maximum clinical score ± S.E.M are shown for 9 independent immunization times.

As specified above, the incidence of the disease was between 60% and 70%. Onset of clinical symptoms was observed generally at days 10 to12 after immunization. Maximal clinical score was between 3 and 3.5 and was observed at days 4 to 5 after onset of the symptoms (this was the time point chosen for cell injection in the migration and therapeutic approaches). Only mice that showed onset (score more than 1) of clinical symptoms before day 14 were considered for the following experiments.

A graph showing typical outcome data from four mice in which EAE was induced independently is depicted in figure 3.5. A decrease in the weight can be observed immediately after immunization with a second point of weight loss together with the first clinical manifestations. Minimal weight values coincide with worsening of the clinical symptoms at 4 to 5 days after onset of disease.

Histopathological analysis was performed in order to complement the observations of clinical symptoms of EAE. As expected, large areas of infiltrates which stained positive for IsolectinB4 were observed specially in the peri-vascular regions in spinal cord (figure 3.6A). Staining for CD45 showed also regions of immune cell infiltration (figure 3.6B). MBP immunohistochemistry revealed areas of myelin loss 20 days after EAE induction (figure 3.6B).

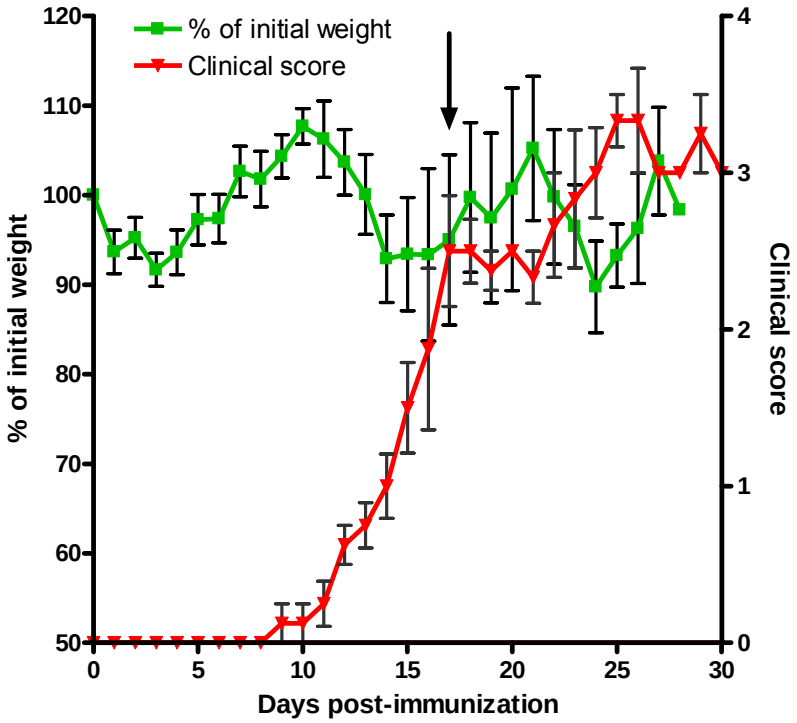


Figure 3.5. Representative diagram of mean values (±S.E.M) of relative (to initial) body weight change and clinical score of 4 independent immunized mice, during course of EAE.

Mice usually lost weight during first 2 days and 4 to 5 days after the first symptoms. This time point coincided with worsening of the clinical score. The arrow represents the cell injection time point chosen for the migration and therapeutic approaches.

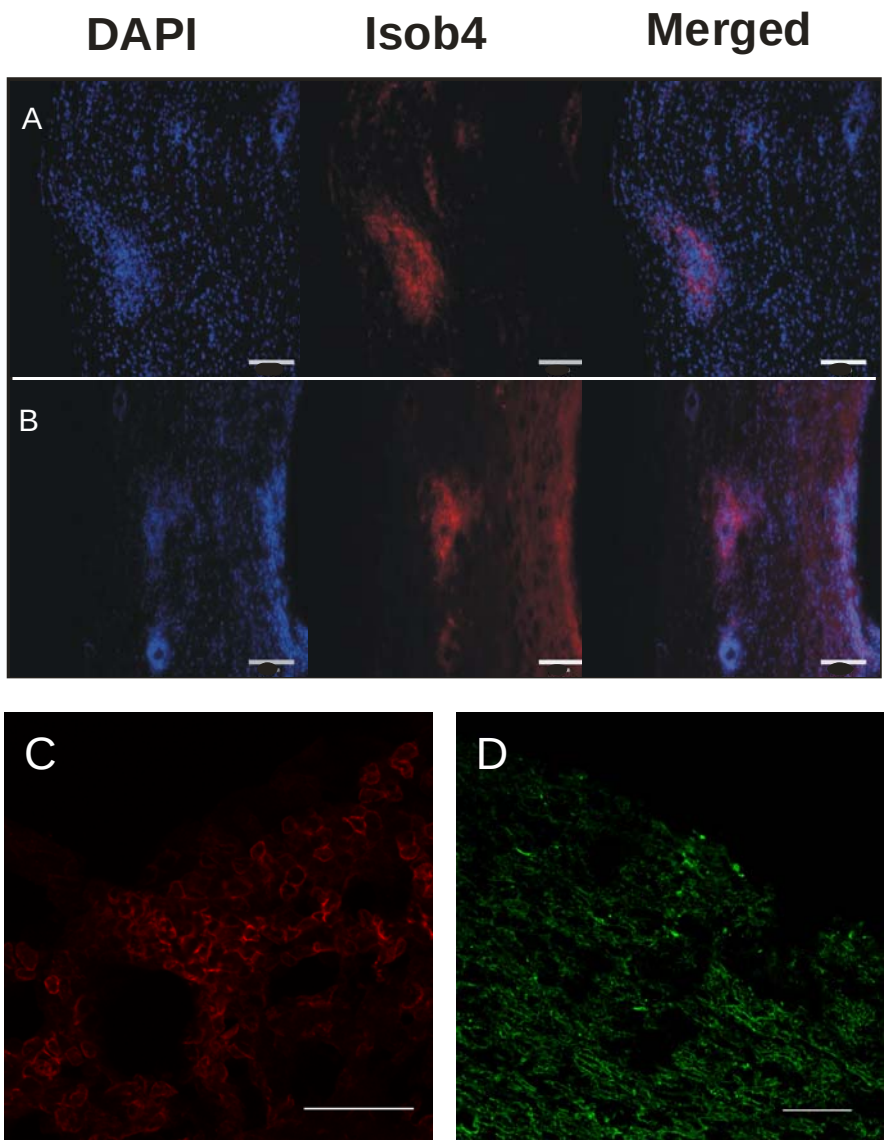


Figure 3.6. Staining of spinal cords from EAE mice at day 20 after disease induction (worsening of clinical symptoms phase). A and B) Fluorescence microscope images. Areas of high number of infiltrates are revealed by the nuclear staining DAPI and stained positive for the macrophage/microglia marker IsolectinB4. C and D) Confocal images. C) Immunostaining for CD45 revealed also areas of infiltration. D) MBP staining allows visualization of damaged myelin regions.
All scale bars: 50 um.

3.5. Lin$^-$-HSC effect and migration after injection into EAE mice

It has already been reported that cells derived from BM can migrate to different tissues including CNS after injection. In contrast to local cell injection protocols, a systemic application was performed while clinical outcome and migratory behaviour of the cells was studied.

Lin$^-$-HSC were isolated from GFP transgenic mice (GFP+ Lin$^-$-HSC). In total, 5 million cells were injected into the tail vein of EAE non-transgenic C57Bl/6 mice. EAE mice were injected with an equal volume of PBS as control. For cell administration, a time point of 5 days after the first clinical symptoms was chosen. Clinical score and weight were followed daily. No changes in disease outcome were observed when comparing GFP+ Lin$^-$-HSC to control PBS injected (figure 3.7).

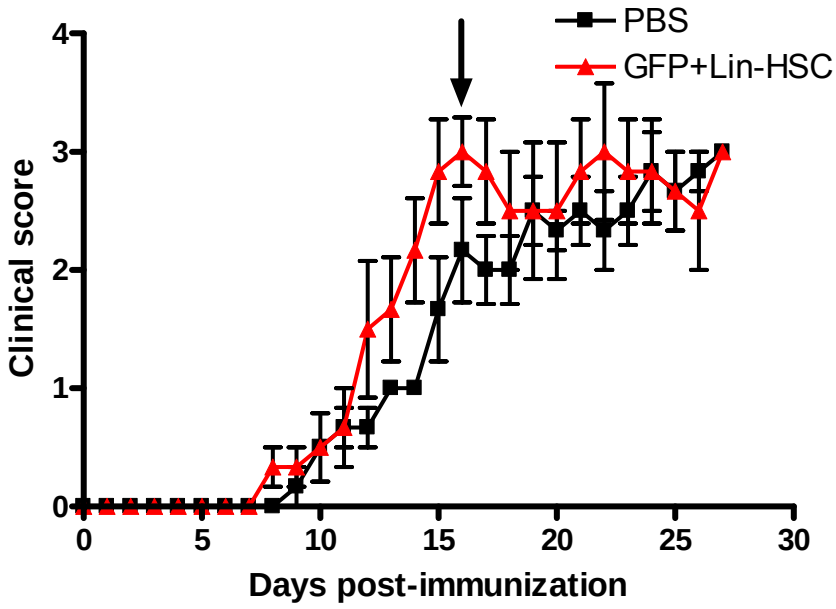


Figure 3.7. Average ± S.E.M of EAE course of animals that were injected with 5 million GFP+Lin$^-$-HSC or PBS 5 days after first symptoms. Mice that received Lin$^-$-HSC did not differ from the PBS injected controls in respect to their clinical score. Arrow: injection time point.

To assess the migratory behaviour, in particular to sites of the CNS where inflammation takes place, histological analysis was performed. As an additional control for migration experiments an equal amount of GFP+ Lin^--HSC were administered to healthy aged matched mice. Animals were sacrificed 5 days after cell administration (21 days after EAE induction) and intra-cardiac perfusion with a fixative was performed. After collecting spinal cords and spleens, tissues were frozen and slices were prepared in a cryostat.

As previously observed under our experimental conditions, GFP+ cells were found in spleen and spinal cords of EAE mice. Quantification of 5 representative spinal cord areas bearing GFP+ cells showed 25.4±14.5 cells/mm^2 (figure 3.8). Healthy controls showed cell migration only towards spleen and no GFP+ cells in the spinal cord could be found.

More detailed morphological analysis of the migrated cells by confocal microscopy was performed. Morphology strongly suggests the GFP+ cells found in spinal cord to present a microglia-like phenotype (figure 3.9).

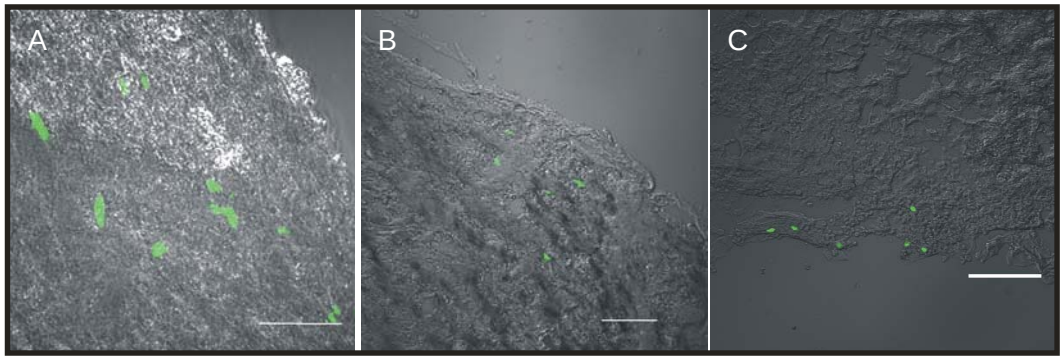


Figure 3.8. Representative confocal images showing GFP+ cells detected within the spinal cord tissue of EAE mice. Cells were intravenously injected 5 days after the onset of symptoms. Mice were analyzed 5 days after cell application. Overlay of green fluorescence and phase contrast is shown. Scale bars A) 50um, B) and C) 100um.

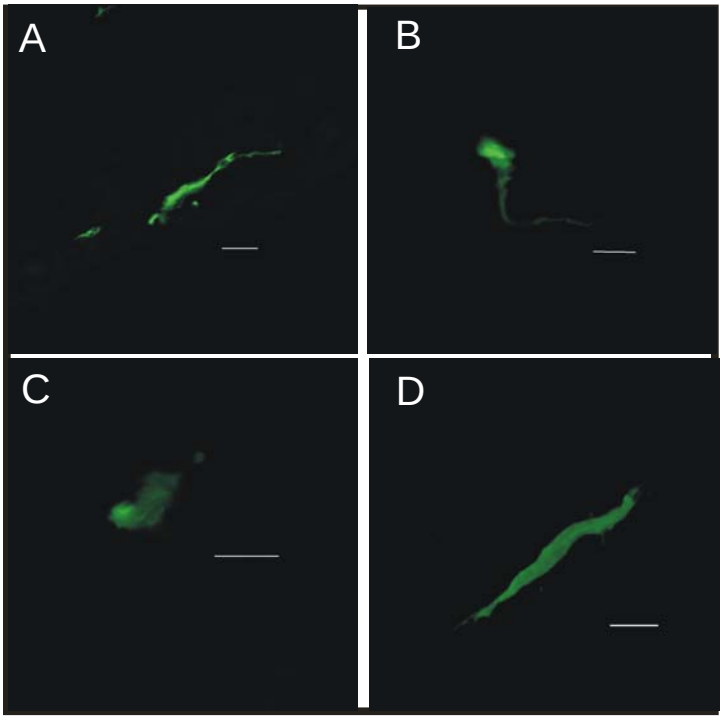


Figure 3.9. A-C) Confocal images and D) 3D reconstruction depicting different morphologies observed in GFP+ Lin$^-$-HSC found in spinal cord 5 days after administration into EAE mice. Scale bars A-D): 10 um.

3.6. Lin$^-$-HSC present undetectable CD45 levels in the EAE spinal cord

To start assessing the phenotype of the Lin$^-$-HSC present once they have migrated to the CNS in EAE mice, immunohistochemistry for CD45 was performed on cryoslices. As mentioned before, CD45 surface molecule expression levels in the freshly isolated Lin$^-$-HSC assessed by flow cytometry was around 58% (figure 3.3 and Table 3.I). Analyzed GFP+ cells found in spinal cord didn't show co-visualization of GFP and CD45 markers (figure 3.10).

Therefore, data may indicate that the phenotype of these injected cells could change to a microglial-like cell type after contact with new cues, in particular within the CNS environment.

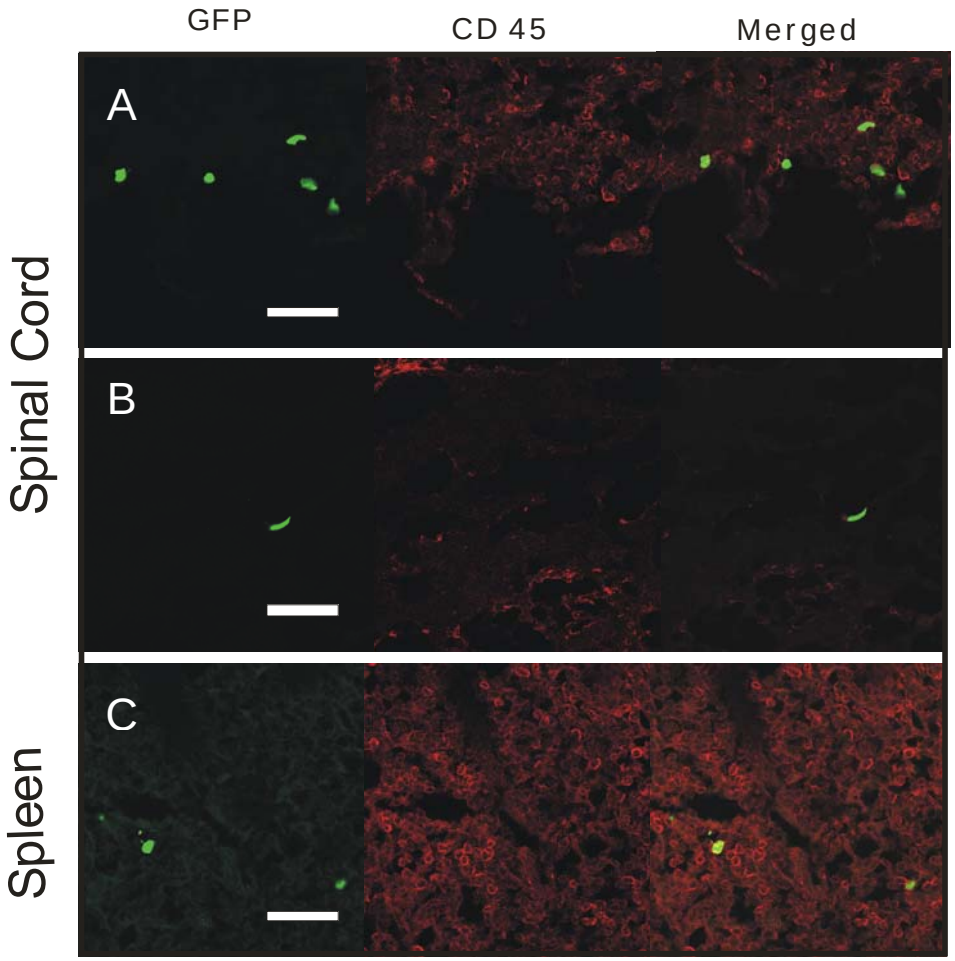


Figure 3.10. Immunohistochemistry analysis of GFP+Lin$^-$-HSC injected into EAE mice and found in spinal cord 5 days after injection. A-B) No colocalization of CD45 and GFP+ signals was observed in spinal cord engrafted cells. C) colocalization of CD45 and GFP found in spleen. Scale bars: 50 um

3.7. Lin$^-$-HSC present a microglia/macrophage-like phenotype in the EAE spinal cord

Further histochemistry analysis was performed to assess which phenotype the Lin$^-$-HSC may present once in the CNS environment. IsolectinB4 labeling revealed that a high number of cells within the spinal cord of EAE mice were positive for this macrophage/microglia marker (figure 3.11). Quantification of GFP+ cells showed that approximately 53% of these cells (n=200) were positive for the microglia/macrophage marker. Therefore a microglia/macrophage surface molecule profile was confirmed for the Lin$^-$-HSC when analyzed in EAE spinal cord 5 days after administration.

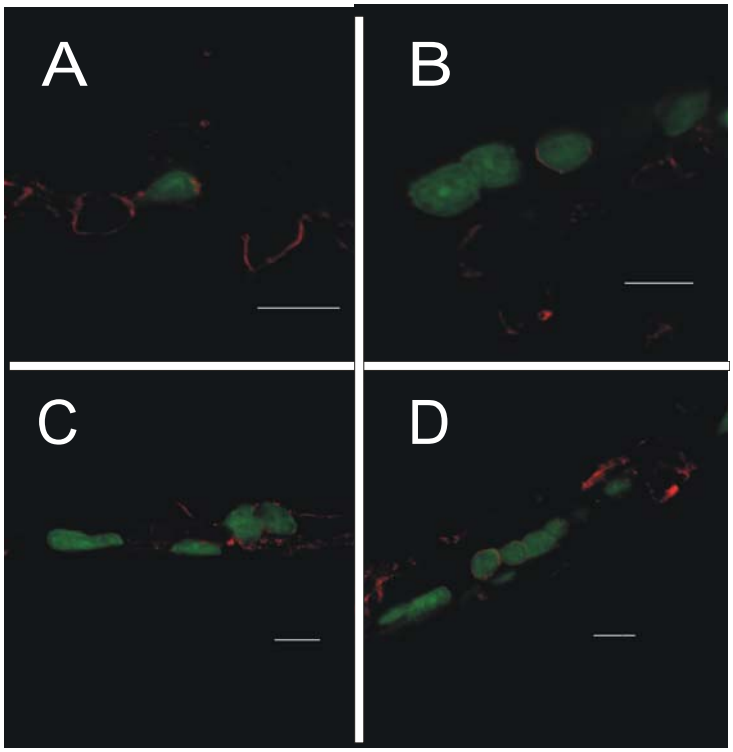


Figure 3.11. A-D) Confocal images showing covisualization of GFP+ cells detected in spinal cord of EAE mice and found to be positive for Isolectin B4 staining (Cy3). Scale bars: 10um

3.8. Lentiviral vectors and Lin$^-$-HSC transduction

To genetically manipulate the cell population chosen in this work, two vectors containing either a fluorescent marker (eGFP) or NT-3 neurotrophic factor were created (please see scheme on figure 3.12).

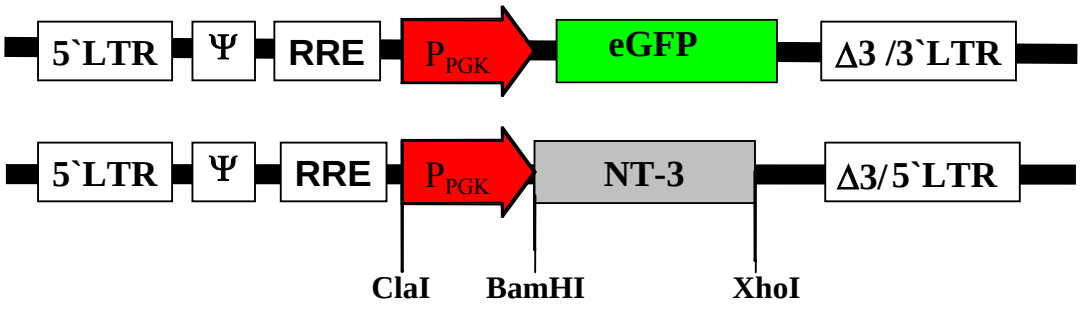


Figure 3.12. Schematic representation of the constructs used to genetically modify the Lin$^-$-HSC. In a Lentiviral vector backbone, the PGK promoter was inserted. Downstream, the eGFP gene was inserted between the TOPO cloning sites. For the therapy approach, the GFP cassette was removed by the use of BamHI and XhoI restriction enzymes and the gene for the mouse NT-3 was inserted by ligation after being amplified with extended primers for the same enzymes

In these vectors the gene of interest is under the control of the PGK promoter. Although intricacies for modifying murine lineage negative cells using lentivirus derived vectors under our experimental conditions were faced before, the system was tested for assessing the percentage of lineage negative cells expressing the reporter gene after transduction.

First, the vector expressing eGFP was used to transfect the packaging cell line 293FT in order to prepare viral particles. Upon transfection almost 100% of the packaging cell line was positive for eGFP (figure 3.13).

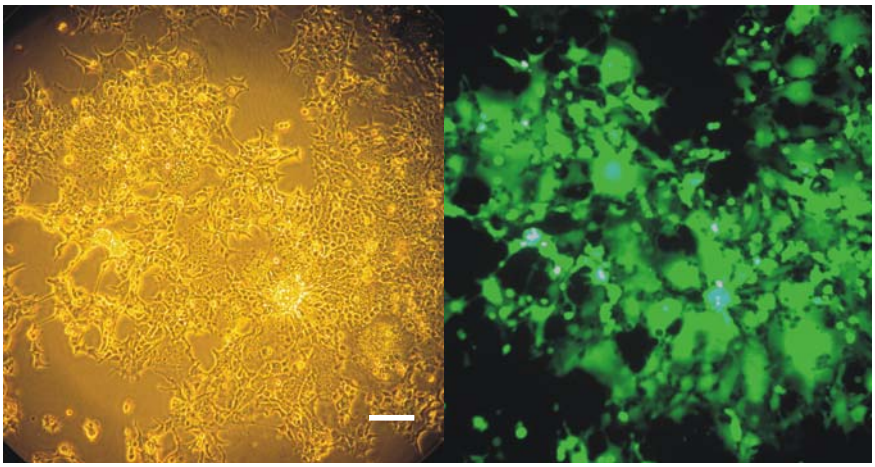


Figure 3.13. Upon transfection using a PGK promoter driven eGFP expression lentiviral construct, the packaging cell line 293FT were 100% positive for eGFP expressing cells Scale bar: 20 um.

After selection, Lin^--HSC were transduced with the eGFP containing viral particles produced as described above in the 293FT cells. When using non-ultracentrifuged freshly collected from the packaging cells line viral supernatant (10^6 TU/ml), no eGFP expression was observed in the Lin^--HSC, when checked after a period of 5 days. Transduced 293FT cell line as procedure control showed 70% of eGFP+ cells.

Thereafter, a protocol to augment viral titer (10^8-10^9 TU/ml) was chosen. Viral fresh supernatants were ultracentrifuged and the pellet containing viral particles were re-suspended in a low volume of media (500ul). Efficiency of lineage negative cell transduction using these concentrated viral particles was analyzed by flow cytometry. Three days after incubation with the concentrated viral particles up to 16% of cells were positive for eGFP (figure 3.14A). Further cell transduction has been in the range of this value.

Alternative strategies were considered during this work to improve transduction efficiency and specificity. The proteasome inhibitor MG-132 was applied during viral incubation with the cells. This pharmacological proteasome inhibition has been reported to increase transduction of human HSC up to 90% (Santoni de Sio, Cascio et al. 2006).

A dose of 2 uM of MG-132 was used. After three independent transduction procedures no significant differences in the percentage of eGFP positive cells was found (figure 3.14B). Higher amounts (4 and 8 uM) of the MG-132 led to massive cell death (assessed by PI staining and flow cytometry analysis), while lower concentrations (0.5 and 1uM) showed even lower levels of GFP+ cells than the non-MG132 treated control (data not shown).

Alternatively, production of a hybrid pseudotyped virus which could be specific for transducing the cKit positive cells was challenged. To that aim, SCF, the ligand for cKit receptor (expressed in the undifferentiated cell population) was cloned into the plpVSV packaging vector. Membrane SCF expression was up to 90% after transfection of the 293FT, analyzed by flow cytometry (data not shown). Virus supernatant was ultra-centrifuged and assessed for the transduction of Lin^--HSC.

In this case of transduction, a ratio of percentage of eGFP positive cells of the relative amount of cKit positive cells was compared between plpVSV and plpVSV+plpSCF pseudotypes. After 3 independent transduction procedures, no significant differences were found between the original G-VSV and the hybrid pseudotyped virus (table 3.III).

Capside pseudotype	***Mean ± SD %eGFP/cKit+ cells (n=3)***
plpVSV	***18.15±9.8***
plpVSV+plpSCF	***27.8±21***

Table 3.III. Averaged percentages of eGFP obtained for the cKit+ fraction of the Lin^--HSC. No significant differences in the eGFP expression levels were found between plpVSV or plpVSV+plpSCF pseudotyped virus particles.

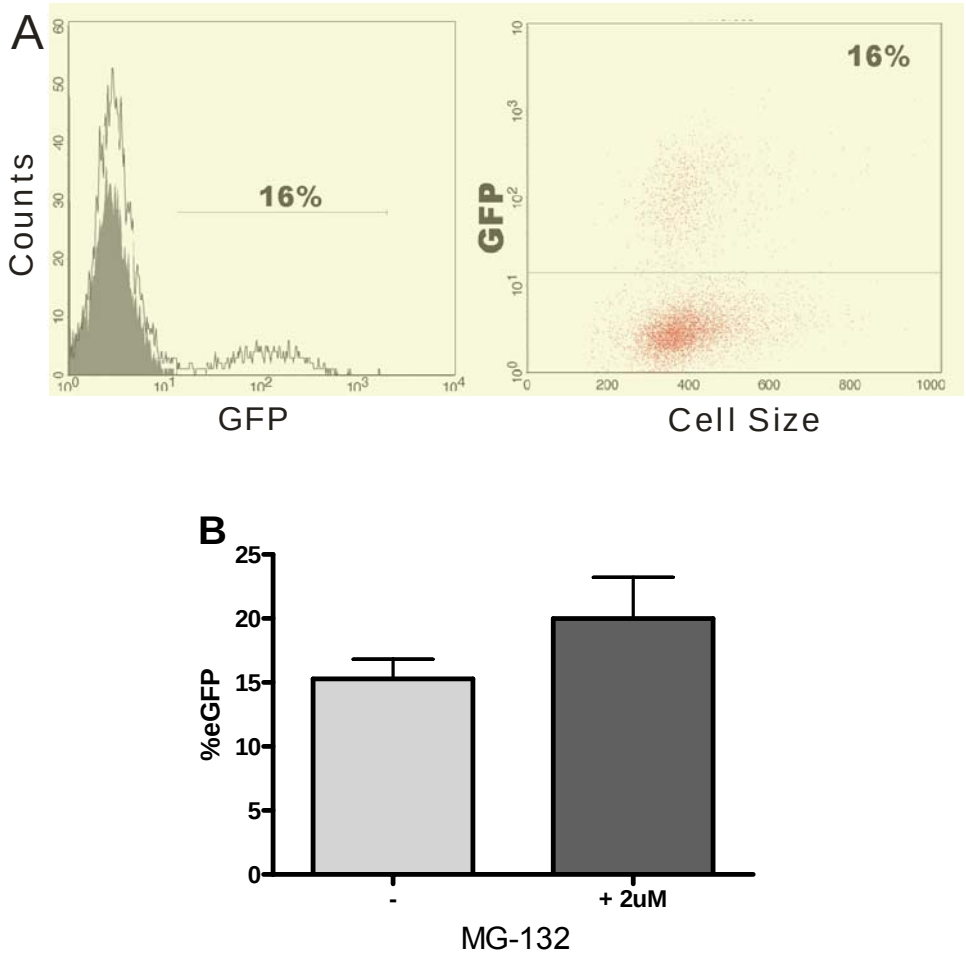


Figure 3.14. Lentiviral gene transfer system was used to genetically manipulate the Lin^--HSC. A) Representative flow cytometry histogram and dot plot showing transduction level achieved assessed by eGFP expression level. Cells were lentivirally transduced using viral titers up to 10^8 TU/ml and analyzed 3 days thereafter. B) A pharmacological inhibitor of the proteasome pathway was used in order to improve transduction levels. A slight but not significant improvement of eGFP levels was observed with a concentration of 2uM (n=3).

3.8.1. Neurotrophin-3 expression

To confirm correct expression of the NT-3 trophic factor selected for the therapeutic approach at the mRNA level, RT-PCR was performed in the 293FT cell line 2 days after transduction. A band for the amplified portion of the NT-3 gene was observed in the agarose gel analysis. No amplification product was detected in the control eGFP transduced cells (figure 3.15).

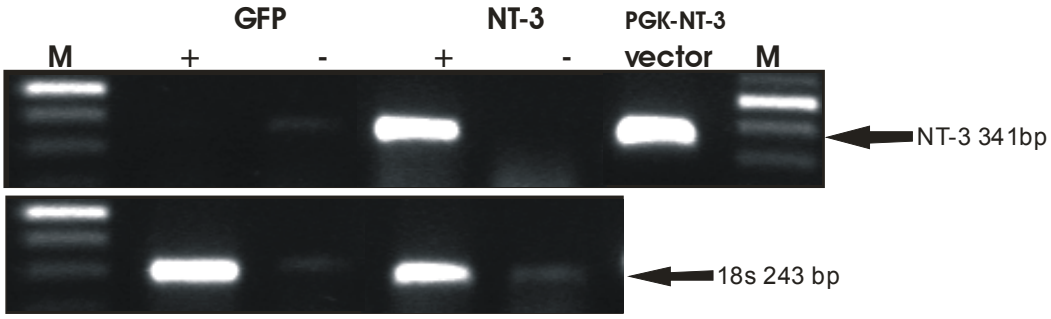


Figure 3.15. Agarose gel analysis of RT-PCR products for NT-3 transcript and 18s after cell transduction. M: size marker (100 base pairs leader). Gene transcripts derived from cells transduced with eGFP or NT-3 were either reverse transcribed (+) or non-transcribed (-) and amplified by PCR. DNA derived from the PGK-NT3 vector was used as a positive control.

3.9. Injection of NT-3 transduced Lin$^-$-HSC into EAE mice

It is clear that several factors are involved in the pathophysiology of MS and its animal model EAE. A successful treatment of such complex diseases would imply a multi-component approach capable of compensating/controlling the neurodegeneration taking place. Nevertheless, it has been shown that a reduced availability of neurotrophic factors may contribute to the neurodegenerative state in these diseases.

A molecular therapy approach was used to asses if the use of cells able to migrate into spinal cord lesion areas (and secondary lymph organs) and eventually provide nervous and immune systems with an over-expression of one of these neurotrophic factors, NT-3 would compensate the symptoms observed during EAE disease progression.
At first, Lin^--HSC were isolated and immediately transduced with lentiviral particles containing the mouse NT-3 gene under the PGK promoter. As control, Lin^--HCS cells were incubated in parallel with PGK-eGFP containing vector.

After transduction, a total of 3-5 million cells expressing either NT-3 or eGFP, were intravenously injected into EAE mice 5 days after disease onset (days 15-16 after MOG_{33-55} immunization). Clinical score and weight of the animals from 4 independent induction procedures were controlled daily. None of them presented any modification of clinical symptoms when compared with eGFP transduced cells as injection controls (figure 3.16).

Spinal cords were taken from animals treated with NT-3 expressing cells, alternatively eGFP expressing cell injected were used as controls. After RNA isolation and reverse transcription, levels of NT-3 were assessed by real-time RT-PCR. On average, spinal cords from mice injected with NT-3 bearing cells, expressed 3 ± 1 fold more NT-3 when compared to spinal cords from eGFP-cell injected mice (data not shown).

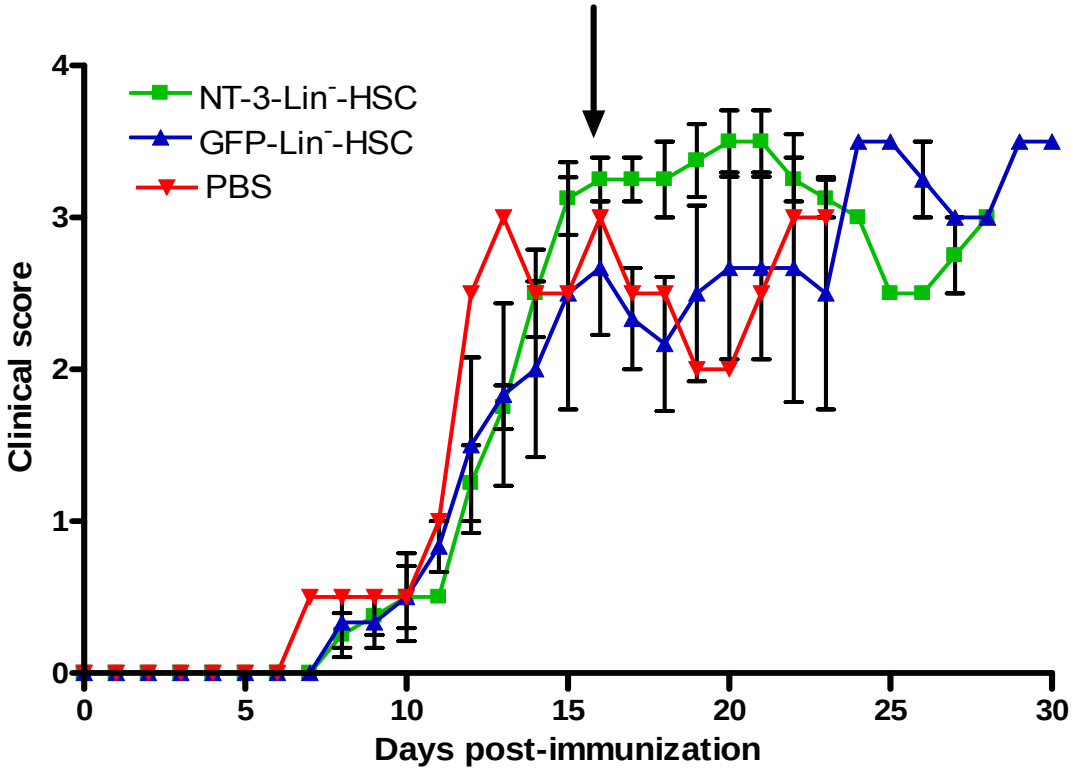


Figure 3.16. Averaged clinical scores observed for EAE mice following injection of Lin⁻-HSC over-expressing NT-3. In total, 3 to 5 million cells were lentivirally transduced and intravenously injected at day 5 after first clinical manifestations (arrow). Same amount of eGFP transduced cells or PBS were used as control. NT-3 expressing cells were not able to modify the clinical course of the disease (n=4).

3.10. Injection of Lin$^-$-HSC into a mouse model of transient cerebral ischemia

To this extent, an interesting cell population coming from BM, the Lin$^-$-HSC were not capable of modifying the course of disease in the animal model for MS. Nevertheless, the cell isolation protocol was reliable and it seemed worthwhile to challenge another model of neurodegeneration for the therapeutic approach of applying this undifferentiated cell population.

In collaboration with the Neurology Department at University Hospital Göttingen, transient focal cerebral ischemia in adult C57Bl/6 mice was induced using the middle cerebral artery occlusion (MCAO) model. A decrease of the brain blood flow to at least 30% of baseline values was controlled by laser Doppler flow (LDF) measurements. After 45 min., reperfusion was allowed and values of blood flow recovered to more than 70% of the initial values. At 24 hours after reperfusion, animals received an intravenous injection of 5 million Lin$^-$- HSC previously isolated from GFP transgenic mice as described before. At 24, 48 and 72 hours after cell administration tissue analysis was performed.

Injected GFP+ Lin$^-$-HSC increased in number over time in the infarcted tissue. The cell number per ischemic hemisphere changed significantly from 146±43 at 48 hours to 798±335 at 72 hours. At 48 hours after cell injection, GFP+ cells were located in the parenchyma (65%) but also in the meninges (13%) and perivascular space (22%). At 72 hours post cell administration, GFP+ cells were mainly (90%) located in the brain parenchyma within the ischemic hemisphere (Schwarting et al. Submitted).

GFP+ cells found within the brain parenchyma 72 hrs after injection displayed a microglial-like phenotype and were positive for Isolectin B4 and CD11b-specific abs (figure 3.17).

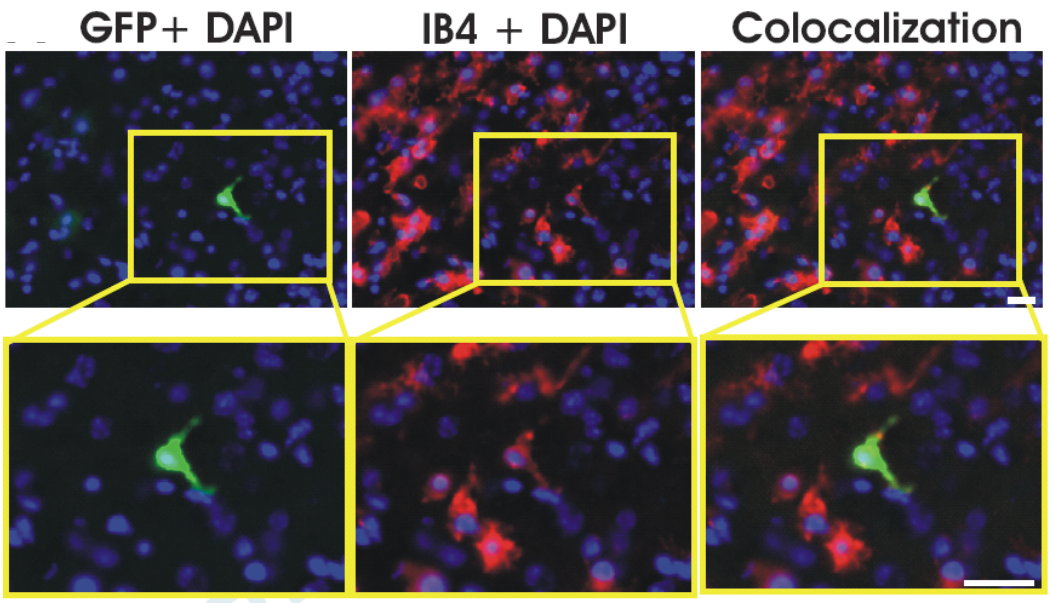


Figure 3.17. Histochemical analysis of GFP+ Lin$^-$-HSC within ischemic hemispheres at 72 hrs post-injection. The microglia/macrophage marker IsolectinB4(IB4-Cy3) was found to colocalize with GFP+ signal, suggesting a microglial fate of the injected cells.. Nuclei was counterstained using DAPI. Scale bars: 20 um.

In addition, infarct size determination was performed on cresyl stained cryo-sections at 72 hours after Lin$^-$-HSC- or PBS-treatment. Lin$^-$-HSC-treated animals (n=6, 18.3±3.9 mm^3) showed significantly smaller infarcts when compared to PBS-treated controls (n=7, 41.3±8.8 mm^3) (Schwarting et al. Submitted). Whereas the cerebral infarcts measured about 50% (49.3±4.9%) of the normal hemisphere in PBS-treated controls, it comprised less than 30% (26.8±4.6) of the contralateral hemisphere in Lin$^-$-HSC-treated mice (Schwarting et al. Submitted).

The number of Isolectin B4 and CD3 cell within ischemic lesions was assessed. Lin$^-$-HSC treatment decreased significantly the number of Isolectin B4 positive cells (from 2887± 194/mm^2 in PBS-injected mice to 2355±275/mm^2) and the same was observed when CD3 cells were quantified (from 1226± 124/mm^2 in PBS injected to 995± 194/mm^2). The application of Lin$^-$-HSC clearly reduced immune infiltration and activation in ischemic hemispheres (Schwarting et al. Submitted).

3.11. Real-time RT-PCR for cytokines and neurotrophic factors in brains derived from Lin$^-$-HSC or PBS-treated animals.

Some reports suggested that stem/progenitor cells could provide trophic support to damaged neuronal tissue. To answer the question of whether exist a difference in the expression levels of a number of cytokines and growth factors between the ischemic hemispheres of Lin$^-$-HSC and PBS injected mice, real time RT-PCR analysis was performed.

RNA was extracted from 9 ischemic brain hemispheres of each group, (PBS or cell-injected mice) as well as from normal mice. As described before, RT was performed and relative quantitative real-time RT-PCR analysis was done. Data was compared to normal brain for the expression of the factors: TNF-α, IFN-γ, TGF-β, BDNF, NT-3, NGF, IL-1ß, bFGF, GDNF or VEGF (figure 3.18).

No significant differences in the growth factors BDNF, bFGF, GDNF, NGF, NT3, TGFβ and VEGF were observed between Lin$^-$-HSC and PBS treated animals. A slight but no significant difference in the gene transcript levels of the pro-inflammatory cytokines TNF-α and IFN-γ was observed.

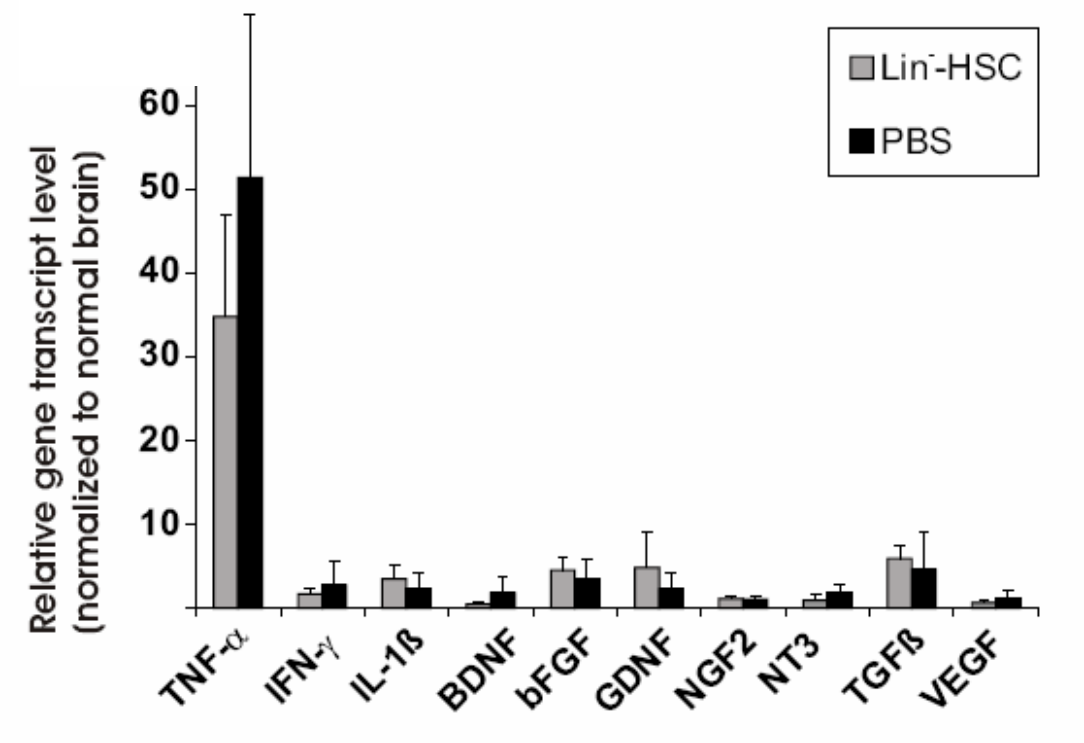


Figure 3.18. Real time RT-PCR analysis of cytokines and growth factors of the ischemic hemispheres from Lin$^-$-HSC injected and PBS mice at 72 hours post-cell administration. No significant differences were found between these two groups. Data was normalized to normal brain tissue (value 1) and is presented as the mean ± S.E.M (n=9 independent experiments).

3.12. Real-time RT-PCR for cytokines and chemokine receptors in spleens derived from Lin$^-$-HSC or PBS-treated animals.

A clear reduction in brain inflammation and together with reduced infract size was found after 72 of Lin$^-$-HSC administration in comparison with PBS treated controls. At the same time, no local trophic support in the brain environment was detected by a number cytokines and trophic factors, it is tempting to speculate about an immune modulation taking place in the periphery of the treated animals

In order to study the grade of immune activation in peripheral immune tissues, spleens derived from normal and ischemic animals (PBS or Lin$^-$-HSC injected) were analyzed at 24 hrs post cell injection.

Real time RT-PCR data showed a tendency to down-regulation of inflammatory cytokines together with chemokine receptors when comparing Lin$^-$-HSC injected animals with PBS controls (figure 3.19).

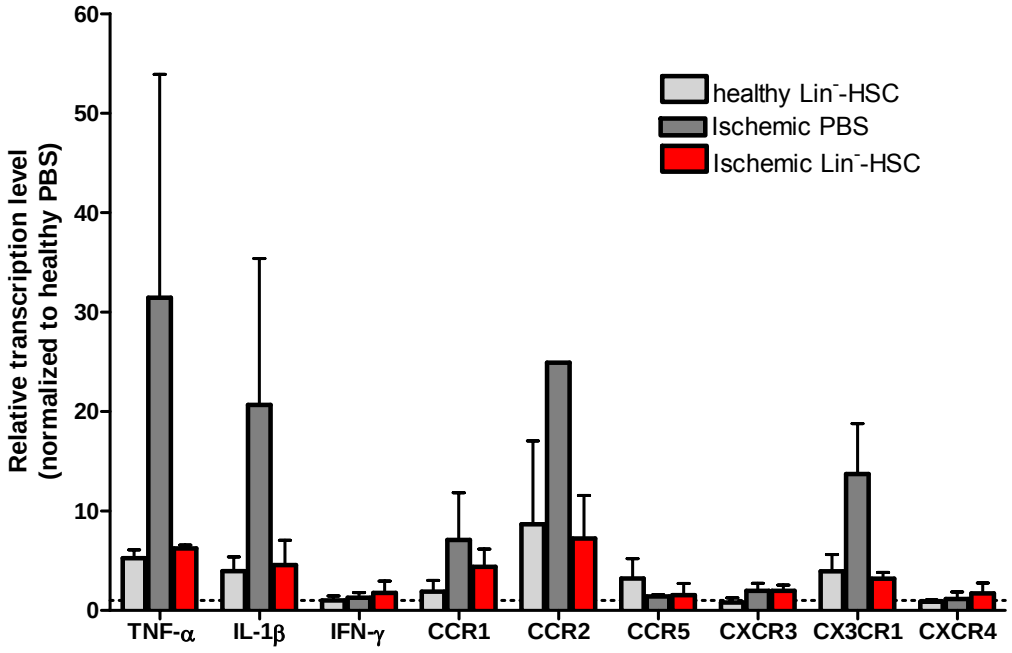


Figure 3.19. Spleen real time PCR for several inflammatory cytokines and chemokine receptors. After 24 hrs of cell administration a tendency of a reduced inflammatory profile and activation was observed. Data from 3 independent experiments are shown (Mean ± S.E.M.).

4. Discussion

In this work a BM population comprising of an enriched stem and progenitor cells was isolated from adult mice BM. Cell surface molecules and relevant gene transcript levels were analyzed confirming the lack of blood committed cell markers and their poor expression of inflammatory and trophic factor molecules when freshly isolated. Moreover, cell migration behavior and potential therapeutic effects were assessed after administering these cells to two different animal models of neurodegeneration. While cells migrated specifically to lesion sites in the spinal cord in the animal model for MS, they were not able to interfere with the course of the disease as a cellular therapy themselves or as a combined cellular and gene therapy approach applying genetically modified cells over-expressing the neuronal growth factor NT-3. On the other hand, cells migrated into damaged hemispheres in an animal model of cerebral ischemia. Moreover, animals that were administered with this enriched stem/progenitor cell population showed a reduction of infarcted tissue and inflammation when compared to controls.

4.1. Isolation and characterization of adult mouse Lin$^-$-HSC

When selecting a population suitable for a prospective cell therapy approach, there are several features to be taken into consideration. Adult stem cells are a promising cell type to take advantage of. They could be relatively easy obtained from the patient, expanded and genetically modified in culture. In addition, ethical issues related with the clinical use of human embryonic derived stem cells could be avoided.

From the many stem cells currently studied for prospective therapeutic use, the adult HSC are one of the best characterized, and they are very promising respect to the range of pathologies for their possible application. This cell type is already used in clinical applications and they are highly effective as for instance, as blood lineage repopulation treatment when High-Dose Chemotherapy is required (e.g. in myeloma cases, HSC are obtained from peripheral blood and re-transplanted into the patient). In addition, the use of the HSC as therapy beyond blood-related diseases, as nervous tissue repair, has started to be explored.

If a high number of extremely pure HSC from the BM is needed for a certain study, then many animals would be required as a cell source and an accurate method to

isolate them would had been necessary, as they represent an extremely small percentage of the whole BM population (<0.007%). Nevertheless, the use of a complete un-selected BM population could introduce artifacts and side effects derived from an uncontrolled immune action of the committed blood cells. In order to obtain a suitable number of cells for a therapy approach, a depletion step of adult BM lineage committed cells was followed for this work. A stem/progenitor cell enriched BM fraction, (lineage negative cells, Lin^--HSC) was obtained through a reproducible negative-selection protocol. The majority of cells showing a mature phenotype were efficiently eliminated.

Since being first described, several markers to clearly define the phenotype of HSC (mouse and human) have been proposed. Although it is now accepted that they may display heterogeneity in their properties (cell cycle status, self renewal, pluripotency) even when isolated as a phenotypically homogeneous population, the described profile of surface markers that currently defines them is: Thy-1.1(low), Lineage(-/lo), Sca-1+ and the exclusion of rhodamine and Hoechst dyes (Uchida, Aguila et al. 1994; Uchida, Jerabek et al. 1996). In this work detected c-Kit tyrosine kinase receptor levels in the Lin^--HSC were acceptable (around 30%). In addition, Lin^--HSC showed a high percentage of the CD45 (a membrane tyrosine phosphatase) surface expression, which is used to distinguish cell of the hematopoietic lineage from the endothelial lineage.
As expected, a relatively high expression level of chemokine receptors (CXCR4 and CXCR3) and integrin subunits (α4 and β1) was observed for TBM preparation as it contains mature immune cells ready to enter into blood circulation. In parallel, negligible surface expression levels of these chemokines and integrins were found in the Lin^--HSC population. This poor migration receptor and adhesion molecule detection under the experimental conditions of this work could be explained in part, by the absence of stimulating factors in the isolation protocol. Supporting this concept, it was shown that human CD34+ cells (mainly HSC), when treated with human recombinant SCF, up-regulated homing related molecules, increasing their *ex vivo* trans-migratory and *in vivo* homing potential (Glimm, Tang et al. 2002; Zheng, Watanabe et al. 2003). It is worth to mention that publications claim the relevance of the CXCR4 receptor in keeping the LT-HSC in an undifferentiated state in the BM niche (Wilson and Trumpp 2006).

In this work, the observed *in vivo* migratory pattern of the Lin$^-$-HSC when applied systemically in two animal models (discuss later) suggests that some chemokine receptors as well as integrins are very likely present in the cell surface once the suitable signal has been trigger *in vivo*. Assessing *in vitro* the response to migratory factors and time points of receptor expression would contribute to understanding the biological response of the population selected for this work.

On the other hand, Lin$^-$-HSC resulted in silent expression of a number of inflammatory cytokine and growth factor transcript levels in contrast with the TBM population. There were reduced levels of all growth factors and cytokines analyzed by real time RT-PCR. This could be in part account for the unsuccessful *per se* EAE symptoms modulation of these Lin$^-$-HSC when applied in the relapsing stage of the disease.

The potential utility of adult stem cells for gene therapy, tissue engineering, and treatment of neurological and other forms of disease is too significant to ignore. And yet knowledge and ability to deliver these forms of therapy in a safe and efficacious manner will require advances in the understanding of the basic biology of stem cells (Spangrude 2003).

Two studies exemplifying the use of HSC for therapeutic purposes are the rescue of retinal degeneration (Otani, Dorrell et al. 2004) and the correction of metachromatic leukodystrophy (Biffi, De Palma et al. 2004).

4.2. Induction of Experimental Autoimmune Encephalomyelitis

An animal model sharing several neurodegenerative features with MS disease was induced following the described immunization protocol. The animals developed a progressive relapsing disease. Incidence of the disease and average clinical scores were acceptable and in agreement with previous reports (Eugster, Frei et al. 1999). Clinical symptoms were consistent with the change in body weight. Immuno-histological analysis revealed a large number of cell-infiltrates in the spinal cord of the animals. Infiltrating cells were IsolectinB4 and CD45 positive and expected to be mainly in the demyelinated regions (Onuki, Ayers et al. 2001).

4.3. Migratory behavior and phenotype of Lin$^-$-HSC injected into EAE mice

Under our experimental conditions, BM derived Lin$^-$-HSC infiltrated spinal cord and spleens after been injected into EAE mice at day 5 after first clinical manifestations. Although GFP+ Lin$^-$-HSC had not effect in the clinical course of EAE, cells showed specific migration towards demyelinating regions and therefore could be a useful tool for specific delivery of potential therapeutic factors into these lesion sites.

Once in the spinal cord region, cells displayed a microglia/macrophage-like morphology in addition to the IsolectinB4 surface expression. At the same time, low levels of the pan-leukocyte marker CD45 was observed as reported before for CNS parenchymal microglia (Zhang, Li et al. 2002). Altogether these data seem to indicate that these cells derived from adult BM could present a microglial/macrophage-like phenotype in the EAE brain environment. A functional characterization (for instance a phagocytocis assay) would help to assess if these BM derived cells are indeed differentiated towards the above claimed phenotype once in the CNS.

Data reporting migration of BM cells towards the CNS is broad, but controversy about the final fate they adopt once in the CNS still exists. As was the case for the morphology and surface marker found in the infiltrating cells during this work, several authors have reported the microglia/macrophage features displayed in cells that migrated into the CNS under BM transplantation or EAE conditions (Corti, Locatelli et al. 2002; Davoust, Vuaillat et al. 2006; Roybon, Ma et al. 2006). In contrast to these reports, Priller et al and Brazelton et al. claimed that these BM cells adopt a neuronal fate once in the brain (Brazelton, Rossi et al. 2000; Priller, Persons et al. 2001). Different experimental models and isolation protocols can be the reason for such dissimilarities.

It is not inexpectable that the majority of cells coming from BM present a microglial phenotype in the CNS as a currently accepted idea is the renewal of microglia population by BM-derived precursor from the circulatory system. Nevertheless, in terms of local repair of damaged tissue it would be interesting to exactly assess which

conditions favour BM cells to adopt a particular phenotype (when certain cell type is desirable to be present in a therapy approach).
In terms of therapeutic delivery of molecules, the migration and establishment of cells in the CNS is an attractive means to take advantage of.

4.4. Lentiviral gene transfer vectors for Lin$^-$-HSC

In order to genetically manipulate the Lin$^-$-HSC looking towards a prospective clinical approach, a third generation lentiviral system was chosen for this work. In terms of bio-safety it is worthy to note that the elements present in the expression and packaging vectors of this gene transfer system are self-inactivating to prevent possible transcription of genes next to the integration site (Miyoshi, Blomer et al. 1998). Transduction levels of Lin$^-$-HSC assessed by reporter gene expression was around 16% using a viral titer of 10^8 TU/ml.
Application of lentiviral system to modify murine HSC is not widely reported, and publications are not consistent in HSC trasduction levels mentioned. Mikkola et al. showed up to 53 percent of GFP+ colonies (assessed by clonogenic progenitor assay from highly pure HSC). Reporter gene expression of the lentiviral vector used in this case was driven by the elongation factor-1 promoter and authors observed genetic mosaicism in the cell progeny. As is the case of the present work, cytokine stimulation was preferred during transduction and correlated with higher transduction efficiency levels (Mikkola, Woods et al. 2000).
In contrast to the levels of transduction reported for lentiviral vectors in HSC from mice, transduction of human HSC (CD34+) has been widely described and high reporter gene expression was repeatedly shown (Akkina, Walton et al. 1996; Case, Price et al. 1999; Douglas, Kelly et al. 1999; Miyoshi, Smith et al. 1999; Kurre, Anandakumar et al. 2004). It is conceivable that an HIV-1 based vector system will be more efficient in human cells than in cells derived from other species, since humans are the natural host of the virus.

Blockade of proteosome activity increased the levels of transduction up to 100 % in human HSC (Santoni de Sio, Cascio et al. 2006) as was not the case for this work where by a slight but not significant increase in the levels of transduction was observed after the same treatment.

In another report, the virus capside has been pseudotyped with early acting cytokines (TPO and SCF) and transduction of human HSC was enhanced (Verhoeyen, Wiznerowicz et al. 2005). In this report, fusion proteins between TPO or SCF and the N terminus of the MLV envelope glycoprotein were used to pseudotype the lentivirus particles. In addition functional display of the SCF and TPO on the viral particles was shown, which was not performed during the present work.

A possible alternative to further obtain a highly transduced population would be the sorting of the cells of interest, but several disadvantages such as low yield, cell contamination and poor cell survival are common problems which are face when exploring this alternative.

4.5. Lack of amelioration of EAE course by NT-3 over-expressing Lin$^-$-HSC

After confirming a specific migratory pattern of the BM derived cells, a therapeutic approach was tested in this work. In order to give local support, a neurotrophic factor was chosen and over-expressed in the Lin$^-$-HSC cells before injecting to the animals at day 5 after first clinical manifestations.

Contrary to the reduction of inflammation observed when applying the cells into the ischemic model (discuss later), the cells were not able *per se* to modify the course of the disease. It can be speculated that the time point in the EAE course of cell application correlates with an advanced stage of inflammation and myelin deterioration. An early cell application time could eventually lead to immune reaction amelioration as was observed in the ischemic model. Supporting a potential immune regulation when injecting bone marrow stem cells during EAE initiation, Zappia et al demonstrated amelioration of the disease through induction of T-cell anergy when bone marrow cells were injected at disease initiation (days 3 and 8 after immunization) (Zappia, Casazza et al. 2005).

With regard to its neuroprotective effect as a therapeutic molecule, NT-3 has been previously shown to exert a positive effect in axonal injury (Guo, Zeng et al. 2006; Narazaki, de Barros Filho et al. 2006), so far data concerning its mode of action at the molecular level have not been published.

In favor of the use of neurotrophic factors as a therapy approach for EAE, CNTF had shown a beneficial effect when administered daily in MOG_{33-55} induced mouse model.

Effects were mediated through a reduction of peri-vascular CNS infiltrates (Linker, Maurer et al. 2002; Kuhlmann, Remington et al. 2006). Similar effects in terms of reduction of inflammation were observed for NGF in a passive transfer EAE model in rats (Flugel, Matsumuro et al. 2001), and in the marmosets EAE model (Villoslada, Hauser et al. 2000).

The therapeutic molecule administration scheme and the animal models used could be one possible difference to the work presented here. An effect for NT-3 when locally delivered has so far not been reported.

Possible reasons for the unsuccessful treatment using NT-3 could be attributed to low availability due to improper local production/secretion of the factor by the Lin$^-$-HSC. Improvement of viral cell transduction titers and therefore gene expression would surely be desirable. Even a local application of NT-3 would eventually account for the efficacy and possible means of action of the molecule in EAE case. Additionaly to the NT-3 production, an *in vitro* functional assay of the neurotrophic molecule (e.g. induction of choline acetil transferase basal forebrain neurons primary culture, or promotion of embryonic dorsal root ganglia neuronal survival) would have shown proper factor activity.

Although a currently concept of CNS therapy is by providing throphic support to damaged CNS environment, it is most likely that a combination of factors rather than a single one could be powerful enough for compensating the detrimental immune attack present in EAE.

4.6. Application of Lin$^-$-HSC into a cerebral ischemia model and real time RT-PCR analysis of brains and spleens

A therapeutic approach in an animal model of cerebral ischemia was tested by administration of Lin$^-$-HSC intravenously, 24 hours post-ischemia. Cells were found in the spleen before they selectively migrated to the lesioned hemisphere at 48-72 hours after administration. In this regard, a time kinetics study may further illustrate the appearance of the cells in the CNS. Several reports have described the migration of BM cells to nervous tissue upon transplantation (Priller, Flugel et al. 2001; Simard and Rivest 2004). Even different time kinetics of cell entry was shown at 24 hours after ischemic insult. In this respect it must be considered that irradiation performed for the BM transplantation might have induced BBB alterations allowing the earliest time point entry of cells in these other report (Priller, Flugel et al. 2001).

Immunohistochemistry using surface markers for microglia/macrophage revealed the differentiation of the applied Lin$^-$-HSC to a microglia-like phenotype as was the case for cells found in the CNS in the EAE model. This part of the work supports the idea that the local environment of injured tissue plays certain role in determining the cell fate of transplanted HSC (Koshizuka, Okada et al. 2004). Hess et al. have reported that a microglial fate was adopted by BM transplanted cells, 3 month after going under MCAO (Hess, Abe et al. 2004).

Despite the low number of Lin$^-$-HSC within the ischemic hemispheres at 72 hours post-ischemia, a decrease in the infarct volume and in the number of post-ischemic apoptotic cells was observed (Schwarting at al. submitted). Also in this case, as observed in EAE approach, the appearance of cells as resident microglia-like may potentially be used as a tool for gene therapy, in terms of delivery of therapeutic molecules directly to the site of injury.

To check if Lin$^-$-HSC could mediate a neuroprotective effect in the ischemic micromilieu real time RT-PCR for gene transcription levels of several neurotrophic factors and cytokines was performed in brain hemispheres. Surprisingly, no significant changes in the neurotrophic factors between HSC and PBS-injected animals were found. A slight but non-significant reduction in the proinflammatory cytokines TNF-α and IFN-γ gene expression levels was detected. In parallel, a decreased in microglia activation in the ischemic hemisphere was observed.
Since the absolute number of Lin$^-$-HSC detected within the ischemic hemisphere at 72 hours after injection is relatively low to mediate a local environment change, one could speculate the systemically applied cells are able to reduce the peripheral immune activation in an earlier time point during cerebral ischemia, which in turn leads to reduced migration of peripheral immune cells into the ischemic lesion.
To that regard, real time RT-PCR assessing cytokine and chemokine receptors expression status was checked in spleens of the injured animals and compared to healthy ones.

It must be mentioned that Lin$^-$-HSC were detected in secondary immune organs such as the spleen at 24 hours after application. Although RT-PCR values presented are

non-significant (due to a low number of samples) it clearly shows a tendency of the Lin$^-$-HSC treatment to counter-regulate the ischemia induced up-regulation of inflammatory cytokines in the spleens and the chemokine receptor expression, which is in accordance with the reduced recruitment of immune cells to the ischemic lesion. A recent study reported the systemically activated immune system within 22 hours after MCAO (Offner, Subramanian et al. 2006).
More spleen samples are needed to complement this observations and additional studies showing interactions between Lin$^-$-HSC and splenocytes would contribute to a better understanding of the observed effects.

Although no clinical effects were observed when applying Lin$^-$-HSC to the animal model of MS, in this second part of the work a novel neuroprotective effect by Lin$^-$-HSC in an experimental model of cerebral ischemia is shown. Future investigations are open for testing further gene and cell therapies in the context of neurodegenerative diseases and prospective clinical applications should eventually be considered after detailed understanding of the molecular and cellular therapeutic basis.

5. Summary

Multiple Sclerosis (MS) and cerebral ischemia are severe neurodegenerative diseases. Treatment in both cases is inadequate as drug delivery to the central nervous system (CNS) represents a difficulty due to the presence of the blood-brain-barrier (BBB) and its control of molecule trafficking. The migration and integration into CNS parenchyma of adult stem cells coming from bone marrow (BM) has been widely reported, suggesting their potential as a cellular therapy in themselves, but also as means of local delivery of therapeutic agents.

In this work, the isolation of a cell population derived from the mouse BM, which is enriched in hematopoietic progenitor/stem cells (here termed Lin$^-$-HSC) and their application in two neurodegenerative paradigms: the animal model for MS, EAE, and the animal model for cerebral ischemia were described. Furthermore, the use of these cells as a delivery system for a potential therapeutic factor, Neurotrophin-3 was studied.

Lin$^-$- HSC were isolated and characterized by flow cytometry and quantitative real time RT-PCR. Lin$^-$-HSC showed no expression of blood lineage differentiated cells neither of chemokine receptors and integrins, but were positive for c-kit (29%) and CD45 (58%).

Relative gene transcript levels of BDNF, bFGF, GDNF, NGF, NT3, TGF-ß, VEGF were not relevant in the Lin$^-$-HSC population when compared to total BM. While the inflammatory cytokines TNF-α, IFN-γ and IL-1ß where reduced in comparison with total BM that contains already matured blood cells.

The EAE model was induced by active immunization with myelin oligodendrocyte glycoprotein. GFP positive Lin$^-$-HSC were intravenously injected at day 5 after first clinical symptoms. Five days after application, cells were found to migrate and remain specifically in the lesion sites of the spinal cord. On average 25.4±14.5 cell/mm^2 were found, they down-regulated their CD45 expression while being positive for the IsolectinB4 marker. Therefore a microglial/macrophage-like phenotype of the migrated cells is suggested. Lin$^-$-HSC were not able to interfere with the EAE symptoms when injected alone, or genetically modified to over-express a neuroprotective factor, Neurotrophin-3. At an advanced state of inflammation, poor local release of the NT-3 factor could explain the luck of success of this approach.

The animal model of cerebral ischemia was established by middle cerebral aorta occlusion. Lin^--HSC were injected 24 hours after ischemia induction. Lin^--HSC migrated in this model into the peri-infarct zone after intravenous application. Cells found in the brain displayed an IsolectinB4 and CD11b marker positive phenotype. Brain hemispheres of Lin^--HSC injected mice showed a reduced infarct size and a decreased recruitment of inflammatory cells to lesion sites when compared with PBS injected controls. Analysis by real time RT-PCR showed that Lin^--HSC were not able to give direct trophic support within ischemic regions but rather influenced by reducing peripheral immune activation as expression levels of a number of cytokines and chemokine receptors were seen to be down-regulated in the spleens derived from cell treated animals.

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8. Erklärung/Declaration

An Eides statt versichere ich, dass ich die Arbeit mit dem Titel „Therapy approach of Neurodegenerative Disorders by Bone Marrow Stem Cells“ selbst und ohne jede Hilfe angefertigt habe, dass diese oder eine ähnliche Arbeit noch keiner anderer Stelle als Dissertation eingereicht wurde. Ich habe früher noch keinen Promotionsversuch unternommen.

This thesis has been written independently and with no other sources and aids than stated.

Bonn, December 2006

Sara Alejandra Litwak

9. Curriculum Vitae

Litwak, Sara Alejandra

Personal Details

Date of Birth: February 5th, 1978.

Place of Birth: Pehuajó, Buenos Aires, Argentina

Nationalities: Argentinan /Italian

Professional Contact Details Institute of Reconstructive Neurobiology,
University Bonn Life and Brain Center,
Sigmund Freud Strasse 25
53127 Bonn-Germany.
Tel: +49-(0)228-688-5542/43
email: slitwak@uni-bonn.de

Personal Contact Details Sebastianstrasse 141
53115, Bonn-Germany
Tel: +49-(0)17624767246
email: saralit2000@hotmail.com

Current position

2003-present **PhD student**. Topic: Therapy approach of neurodegenerative diseases by adult bone marrow stem cells.
Supervisor: Dr. H. Neumann.

(Since 04/05) Neural Regeneration Unit, Institute of Reconstructive Neurobiology at the University Life and Brain Center, Bonn-Germany.

(From 04/2003 to 04/2005) Neuroimmunology Group at the European Neuroscience Institute, Göttingen-Germany.

Education and research experience

1996-2002	Master of Sciences Degree. School of Exact and Natural Sciences, University of Buenos Aires, Buenos Aires, Argentina. Master of Science thesis (07/2002) "Effect of Nerve Growth Factor on Human Vascular Endothelial Cells" Supervisor: Dr. Mirta Schattner.
2000-2002	Lab Assistant. Dept. of Thrombosis and Haemostasis, Hematological Research Institute, National Academy of Medicine, Buenos Aires, Argentina.

Honours and financial support

2005- present	BATII/2 contract, University Hospital Bonn-Germany.
2003-2005	Scholarship, University Hospital Göttingen-Germany.
2001	Travel World Award, International Society of Thrombosis and Haemostasis (ISTH), XVIII Congress Paris, France. "Characterization of endothelial cell infection by Junin virus". R Pozner, R Gómez, M Lazzari, **S Litwak**, N Maugeri, M Schattner.

Submitted publications

"Hematopoietic stem cells reduce postischemic inflammation and act neuroprotective in a mouse model of cerebral ischemia."

S. Schwarting, J. Weise, **SA. Litwak**, W. Hao, M. Bähr and H. Neuman.

Submitted: Brain (2006).

"Generation of a progenitor cell-based packaging cells line for delivery of retroviral pseudotype vectors to gliomas"

Fisher, Y. H., Miletic, H., **Litwak, SA**., Giroglou., T., Stenzel, W., Deckert, M., Neumann, H. and von Laer, D. Submitted:. Journal of Gene Medicine (2006)

"Bystander killing of bone marrow derived tumor infiltrating progenitor cells expressing a suicide gene".

Miletic, H., Fischer, Y., **Litwak, SA**., Giroglou, T., Waerzeggers, Y., Winkeler, S., Li, H., Himmelreich, U., Stenzel, W., Deckert, M., Neumann, H., Jacobs, A.H.,and von Laer, D. Submitted: Molecular Therapy (2006).

Published abstracts and congress contributions

Litwak, SA. ***Oral presentation:*** "CNS therapy by bone marrow derived cells". PhD Seminar Series in Neurobiology, organized by Dr. Steinhäuser. Clinic of Epileptology, University Hospital, Bonn. 2006.

Litwak SA., Ber S., Rochford CD., Neumann H.,
"Targeting Adult Bone Marrow Stem Cells to the Central Nervous System in Experimental Autoimmune Encephalomyelitis" Bonner Forum Biomedizin, Bad Breisig Semester Meeting, Germany. March 2006.

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Additional Information

Languages: Spanish (mother tongue), English (fluent), German (basic)
Computer programs: Word, Excel, End Note, Adobe Photoshop, Corel-Draw, Power Point, Cell Quest Pro, GraphPad Prism, Sequence Detector System 2.2..
Post-degree courses*:* History of Science. CEFIEC. School of Exact and Natural Sciences, University of Buenos Aires.2002.

"No one can really feel at home in the modern world and judge the nature of its problems -and the possible solutions for those problems- unless one has some intelligent notion of what science is up to. Furthermore, initiation into the magnificent world of science brings great esthetic satisfaction, inspiration to youth, fulfillment of the desire to know, and a deeper appreciation of the wonderful potentialities and achievements of the human mind".

Isaac Asimov
Russian-American PhD in biochemistry & writer (1920-1992)