

**Targeting Gene Therapy to Neuroinflammatory Lesions in  
Experimental Autoimmune Encephalomyelitis**

**PhD Thesis**

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## **Declaration**

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

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2<sup>nd</sup> December 2004

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## **Abbreviations**

APL – altered peptide ligand

BBB – blood-brain barrier

BDNF – brain derived neurotrophic factor

BM – bone marrow

BMT – bone marrow transplantation

$\beta$ TubIII – Beta-tubulin III

CFA – complete Freund's adjuvant

CMV – cytomegalovirus

CNS – central nervous system

CSF – cerebrospinal fluid

DNA – deoxyribonucleic acid

EAE – experimental autoimmune encephalomyelitis

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

ES cells – embryonic stem cells

FACS – fluorescence activated cell sorting

FCS – fetal calf serum

FITC – fluoro-isothiocyanate

GDNF – glial cell-derived neurotrophic factor

GFAP – glial fibrillary acidic protein

GFP – green fluorescent protein

eGFP – enhanced green fluorescent protein

HIV-1 – human immunodeficiency virus-1  
HSC – hematopoietic stem cell  
HSV – herpes simplex virus  
IFN- $\gamma$  – interferon- $\gamma$   
IL-10 – interleukin-10  
Lin<sup>-</sup>BM – lineage-negative BM  
LTR – long terminal repeat  
LT-HSC – long-term hematopoietic stem cells  
MBP – myelin basic protein  
MHC – multi-histocompatibility complex  
MMP – matrix metalloprotease  
MOG – myelin oligodendrocyte glycoprotein  
MS – multiple sclerosis  
pMSCV – murine stem cell virus  
NGF – nerve growth factor  
NT-3 – neurotrophin-3  
NSCs – neural stem cells  
OPC – oligodendrocyte precursor cell  
PBS – phosphate buffered saline  
PCR – polymerase chain reaction  
PE - phycoerythrin  
PGK – phosphoglycerate kinase  
PFA – paraformaldehyde

PLP – proteolipid protein

PTX – pertussis toxin

RNA – ribonucleic acid

SDF-1 – stromal cell derived factor-1

SIN – self-inactivating

ST-HSC – short-term hematopoietic stem cells

TBE – Tris-Borate EDTA

TCR – T cell receptor

TGF- $\beta$  – transforming growth factor- $\beta$

TNF- $\alpha$  – tumor necrosis factor- $\alpha$

VSV-G – vesicular stomatitis virus G-protein

$\Psi$  – retroviral packaging signal

## Abstract

The blood-brain barrier (BBB) protects the central nervous system (CNS) from harmful substances but at the same time is an obstacle to therapy by blocking the transport of drugs into brain tissue. Studies have reported the migration of hematopoietic progenitor cells past the BBB and their engraftment into the CNS suggesting their potential as a natural vehicle to transport therapeutic agents past this barrier. Hematopoietic progenitor cells (Lin<sup>-</sup>BM) were isolated from the bone marrow of  $\beta$ -actin green fluorescent protein (GFP)-transgenic mice. Transplantation of GFP<sup>+</sup>Lin<sup>-</sup>BM into lethally-irradiated mice showed engraftment of hematopoietic cells into non-hematopoietic tissues, including the brain and spinal cord. Following intravenous administration of these cells to mice with experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (MS), GFP<sup>+</sup> cells were observed to migrate specifically into spinal cord lesions. This migration was correlated with disease severity, with higher engraftment occurring after administration at higher clinical scores. After a period of 5 days, 40 GFP<sup>+</sup> cells/mm<sup>2</sup> were observed in the spinal cord of these EAE mice. Engraftment was only observed in the spleen and bone marrow of healthy mice. Cells engrafting in spinal cord lesions expressed low levels of CD45, of which 68% stained positive for isolectin-B4, suggesting them to have a microglial phenotype. The engrafted cells showed no immunoreactivity for other CNS cell types. Delivery of the anti-inflammatory cytokine interleukin-10 (IL-10) to spinal cord lesions by lentivirally transduced Lin<sup>-</sup>BM showed no improvement in disease course. Thus, it is shown that Lin<sup>-</sup>BM, when administered intravenously in the absence of lethal irradiation, migrate directly to lesions within the damaged spinal cord with little or no engraftment in other tissues and give rise to a microglial population.

## **Introduction**

In order to accommodate its limited power of regeneration, the CNS is protected by the BBB, which has the remarkable ability to keep immunosurveillance of the central nervous system to a minimum and thus prevent any unnecessary immune-mediated tissue damage. However, the BBB often presents difficulty in that it prevents access to the brain parenchyma for many therapeutic agents designed to treat CNS-specific disorders<sup>1, 2</sup>. Many studies indicate that stem cells of the hematopoietic system, under appropriate conditions, may transdifferentiate across lineage boundaries to produce a diverse range of progeny including neurons, hepatocytes, myocardium and skeletal muscle<sup>3-7</sup>. Evidence of overlapping genetic profiles between hematopoietic and neural stem cells even indicates that these cells may share a common genetic programme responsible for maintaining them in such a state<sup>8</sup>. While more recent studies suggest most of these so-called transdifferentiation events to be events of cell fusion as oppose to cellular reprogramming, it has long been accepted that microglia develop during embryogenesis and remain in the central nervous system throughout life. While it is still argued whether these microglia develop from neuroepithelial or hematopoietic precursors, recent data suggest that not only do they develop from hematopoietic precursors but that their formation occurs also in the adult brain<sup>9</sup>. This migration of hematopoietic stem cells across the BBB and their subsequent differentiation provides a specific and natural means for potential therapeutics to enter the CNS and target sites of pathogenesis.

While anti-inflammatory cytokines are the most obvious therapy for neuroinflammatory disorders their systemic administration to patients with MS has been far from successful.

This is due to the fact that cytokines fail to cross the BBB, even when it is inflamed, and because they act locally with a very short half-life<sup>2, 10</sup>. Even if these peptides were to cross the BBB the problem of achieving sufficient diffusion within the brain parenchyma would have to be overcome. Like many peptide therapies, cytokines and neurotrophins have poor pharmacokinetics, are often subject to proteolysis and have pleiotropic effects<sup>11</sup>. In addition, the intravenous administration of cytokines may lead to severe side-effects, one example being the development of nephrotoxicity in multiple sclerosis patients receiving intravenous administration of transforming growth factor- $\beta$  (TGF $\beta$ )<sup>12</sup>. Thus, the successful treatment of MS with anti-inflammatory cytokines requires their specific administration to inflammatory lesions within the CNS. As MS is a chronic disorder its therapy also requires prolonged treatment. Gene therapy offers the possibility of site-directed long-term gene expression and as such is an ideal approach.

The delivery of therapeutic genes to the CNS has been attempted using many approaches. The intracerebral injection of expression constructs leads only to short-term expression of the gene limiting its therapeutic potential in chronic diseases such as multiple sclerosis. The use of first-generation adenoviral vectors is limited by their intrinsic toxicity and immunogenicity preventing clinical application<sup>13</sup>. Advances have been made with the use of herpes simplex virus (HSV) vectors to infect ependymal epithelia of the ventricle walls within the CNS, however, this approach may not be used to target specific sites within the CNS<sup>14</sup>. The short-lived production of cytokines, up to 4 weeks after in-vivo vector mediated delivery, as well as inability to regulate gene transcription, represent important limitations to these approaches.

Bone marrow transplantation (BMT) is an approach which has been in use for many years, e.g. in the treatment of leukaemia, and more recently has been employed to treat CNS-specific lysosomal disorders where it has yielded promising results. BMT has been used to suppress the activation of microglia associated with neurodegeneration in Sandhoff disease<sup>15</sup>, and also to correct the enzymatic defect occurring in the mouse model of globoid cell leukodystrophy<sup>16</sup>. The delivery of therapeutic genes via hematopoietic cells has been used successfully to treat the mouse model of Niemann-Pick disease, another lysosomal disorder<sup>17</sup>, while more recently hematopoietic cells lentivirally transduced to express a transgene have been used to correct the enzymatic defect in metachromatic leukodystrophy<sup>18</sup>. The use of BMT to treat multiple sclerosis, while effective in some cases, is still associated with many risk factors and often gives a variable outcome depending on the state of the disease at the time of transplantation<sup>19</sup>. While BMT has been used successfully in treating CNS disorders and to demonstrate CNS engraftment of hematopoietic cells it is a severe procedure requiring lethal irradiation of subjects. Thus, an optimal approach would bypass this irradiation yet still allow for the CNS engraftment of hematopoietic cells. With this in mind the specific engraftment of lineage-negative hematopoietic cells into inflammatory lesions of the spinal cord following their intravenous administration is demonstrated here.

The cytokine expression profile within inflammatory lesions changes quite dynamically along with the lesions. The ideal therapeutic approach would aim for the prolonged expression of those cytokines present as the immune response is down-regulated. One such cytokine is interleukin-10 (IL-10), an anti-inflammatory mediator secreted by Th1

lymphocytes during immunoregulatory processes. Of the large number of anti-inflammatory cytokines known, IL-10 is well characterized in animal models of MS. Studies in which the IL-10 gene was expressed in the CNS by a viral vector demonstrated that IL-10 ameliorates disease symptoms<sup>20</sup>. Upon lentiviral transduction of the administered lineage-negative hematopoietic cells with the IL-10 gene it was believed that these cells would act as a therapeutic vehicle with the ability to achieve targeted migration to regions of neuroinflammation. While targeted delivery of the therapeutic gene to spinal cord lesions was achieved, no relief of symptoms was observed. The absence of a therapeutic effect may be due to inefficient transduction of the hematopoietic stem/progenitor cell population or due to insufficient production of the therapeutic gene by transduced cells. Both of these obstacles may be overcome with improvements in protocols for gene transfer into hematopoietic stem/progenitor cells. This study demonstrates that hematopoietic stem/progenitor cells can be targeted specifically to lesions of the damaged CNS where they give rise to a mainly microglial cell population. Specific targeting of cells to lesion sites using this approach bypasses the requirement for lethal irradiation when performing bone marrow transplantation and may be used to deliver therapeutic genes in neurodegenerative and neuroinflammatory disorders.

## **EAE: animal model for Multiple Sclerosis**

### **1. Molecular Pathogenesis of EAE**

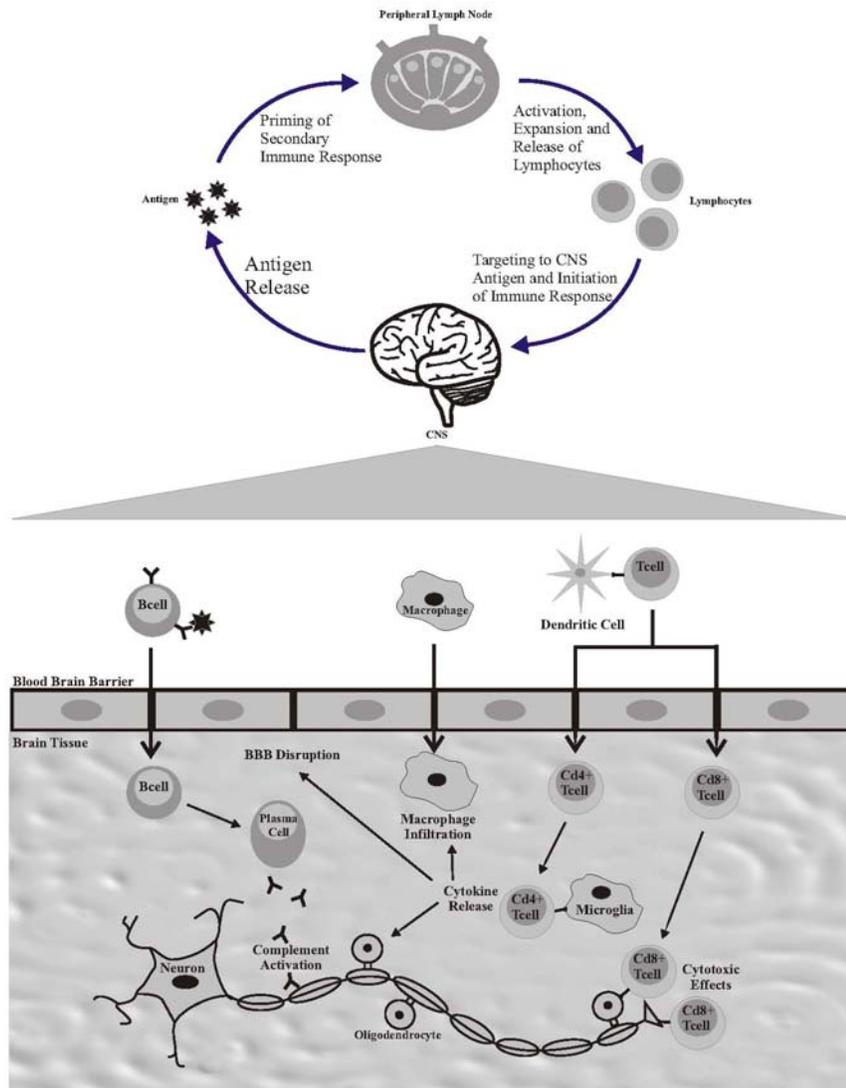
Irrespective of whether the inflammatory-inducing antigen arises from cross-reactive antigens present within the CNS, from brain-resident pathogens, or those released following a degenerative process, antigens released into the periphery reach lymph nodes and the spleen and initiate an acquired immune response. It is likely that T cell antigens are presented by dendritic cells which can load processed antigens onto MHC molecules, allowing for the priming of T lymphocytes. Specific antigen recognition results in the clonal expansion of both T and B lymphocytes and their acquisition of effector functions, followed by their infiltration of the CNS.

Along with an antigen-driven acquired immune response, another requirement for the induction of an immune response in the CNS is a pro-inflammatory milieu leading to the upregulation of MHC molecules, co-stimulatory receptors and inflammatory cytokines. Such a pro-inflammatory milieu also leads to the release of chemokines capable of attracting activated cells via chemokine-chemokine receptor interactions. A specific role for the chemokines IP-10 and RANTES, and their respective receptors, CXCR3 and CCR5, has been suggested by immunohistochemical staining of tissues from MS patients as well as from analysis of cerebrospinal fluid (CSF)<sup>21, 22</sup>. Chemoattraction of activated cells to the CNS requires their migration across the vascular endothelium of the blood brain-barrier (BBB). Adhesion of activated cells to BBB endothelium occurs via adhesion molecules upregulated on endothelia within the pro-inflammatory milieu. Upregulation of

ICAM-I and VCAM-I on the endothelia of MS lesions has been shown, as well as the expression of their respective ligands, LFA-1 and VLA-4, on the infiltrating cells<sup>23-25</sup>. The secretion of matrix metalloproteinases (MMP) at the inflammatory site facilitates infiltration of immune cells across the BBB by disrupting the basement membrane and the extracellular matrix<sup>26-28</sup>. Antigen representation in the CNS is performed mainly by glia and, to a lesser extent, by neurons<sup>29</sup>. Upon reactivation of lymphocytes following antigen recognition, effector functions are initiated, cytokines and antibodies are secreted and other immune cells, such as macrophages, are recruited to the site.

Following reactivation, clonally expanded B cells mature to plasma cells and secrete high levels of immunoglobulin- $\gamma$  (IgG) antibodies which recognize soluble and membrane-bound antigen. IgG1 antibodies bind to antigen on the cell surface, activating the complement cascade leading to direct damage of the antigen expressing cell. Clonally expanded CD8<sup>+</sup> T cells interact with antigen presented by MHC class I expressing glia and neurons<sup>30</sup>. Both oligodendrocytes and neurons can present antigens to CD8<sup>+</sup> T cells via MHC class I and are directly damaged following this interaction. CD8<sup>+</sup> T cells can attack neurites leading to spheroid formation similar to that observed in MS pathology. Reactivated CD4<sup>+</sup> T cells recognize antigen presented by MHC class II on microglia and secrete high amounts of inflammatory cytokines maintaining the pro-inflammatory environment and attracting other immune cells such as macrophages which phagocytose antigen expressing cells and also secrete pro-inflammatory cytokines. Thus, removal of the antigen and antigen-expressing cells results in damage to myelin and axons, after

which the immune cells which have taken part in the response either undergo activation-induced cell-death or exit the lesion site.



**Figure 1:** Scheme depicting events in the pathogenesis of Multiple Sclerosis. Cross-reactive viral antigen or CNS antigen is processed in peripheral lymph nodes and presented to lymphocytes resulting in their clonal expansion, release and migration into the CNS where they are reactivated upon recognition of the myelin-bound antigen. CD8+ T cells interact directly with oligodendrocytes and neurons inducing apoptosis. CD4+ T cells recognize antigen presented by CNS resident microglia and secrete cytokines required for maintenance of the inflammatory environment. These cytokines induce MHC expression on CNS resident cells, disrupt the blood brain barrier to allow further cellular infiltration, and attract peripheral macrophages, further enhancing the immune response.

## 2. Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is an experimental animal model of demyelination and axonal damage which shares many features with MS. The EAE model for MS was first established in 1933 following the discovery that accidental immunization with myelin components led to acute demyelination in humans<sup>31, 32</sup>. EAE is normally induced by active immunization with a specific myelin peptide or by the passive transfer of immunocompetent cells from a primed donor to a non-immune recipient. Disease can be induced in rodents and primates, however, susceptibility, disease course and severity of disease are all dependent on the antigen as well as on the species and strain of the animal used. Earlier EAE models were induced by the injection of whole CNS homogenate, from which it was argued that the myelin peptides myelin basic protein (MBP) and proteolipid protein (PLP) were the major antigens, however, another unknown myelin component found within the CNS, termed M2, was also argued to be the primary antigen<sup>33</sup>. M2 was later described as myelin oligodendrocyte glycoprotein (MOG), studies of which suggested it to be one of the CNS antigens responsible for the initiation of primary autoimmune-mediated demyelination<sup>33-36</sup>. These findings led to the development of MOG-induced models of EAE in rodents and primates<sup>37-40</sup>.

MOG is a CNS-specific type I membrane glycoprotein of the immunoglobulin superfamily expressed mainly on the outermost layer of the myelin sheath, making it an ideal target for antibody mediated demyelination<sup>34, 41-43</sup>. Although accounting for only 0.01-0.05 wt% of the total membrane protein it is highly immunogenic and, unlike other

myelin proteins used to induce EAE, is unique in that it is the only CNS antigen known to induce both an encephalitogenic T-cell response and a demyelinating response in EAE<sup>42</sup>. In rat and marmoset EAE models, demyelination is strictly antibody dependent, while in mice demyelination is mediated by TNF-dependent mechanisms in the absence of a full B-cell response<sup>37, 44-48</sup>. Interestingly, the immunopathological mechanisms in each of these models can be observed in MS<sup>49</sup>.

Active immunization with MOG in mice induces a chronic, non-remitting disease course via activation of a type I immune response directed by autoreactive CD4<sup>+</sup> Th1 cells. The induction of disease requires potentiation of the immune response by complete Freund's adjuvant (CFA) as well as pertussis toxin (PTX). CFA acts to stimulate production of the cytokine interleukin-12 (IL-12) by antigen presenting cells, thus committing the CD4<sup>+</sup> T cell population to a Th1 phenotype<sup>50, 51</sup>. PTX supports antibody and delayed-type hypersensitivity responses and potentiates polyclonal and antigen-specific T cell activation as well as IFN- $\gamma$  production<sup>52-56</sup>. Both CFA and PTX also help to disrupt the BBB facilitating T cell access to the CNS<sup>57, 58</sup>. Demyelination induced in mice following MOG-immunization is mediated by a TNF-dependent pathway and occurs independent of an antibody response<sup>47, 48</sup>. The myelin sheath of mice is particularly sensitive to pro-inflammatory mediators such as TNF and demyelination is the typical response to such an insult in the mouse CNS<sup>46</sup>. Moreover, adoptive transfer of MOG-primed T cells in mice can lead to EAE, and active MOG-EAE can be induced in B cell deficient mice, thus questioning the relevance of a B cell response in disease pathogenesis, however, B cell deficient mice have been shown to be less susceptible to disease<sup>47, 48</sup>.

Regarding the relevance of the MOG-EAE model for MS research, it is now clear that disease pathogenesis in some patients involves a MOG-specific autoimmune response. The MOG-specific T cell response is enhanced in MS patients and MOG-specific antibodies found in serum of MS patients provide direct evidence of MOG being an antigen in MS<sup>59-62</sup>. Although direct evidence for myelin-specific T cells or antibodies in the pathogenesis of MS is still lacking, attempts to modulate the immune response in EAE models have shown promising results and have been used to some success in RR-MS.

### **3. Therapy of EAE**

The complex nature of MS pathogenesis, as well as the heterogeneity observed between patients, makes the development of therapeutics extremely difficult. In addition, as MS is a CNS disorder, if therapeutic agents are to have any effect they must first be able to cross the BBB. Currently used therapies include immunosuppression by glucocorticoids as well as the administration of glatiramer acetate (Copaxone<sup>TM</sup>), a synthetic peptide consisting of four amino acids which simulates myelin basic protein (MBP) and thus competes with various myelin antigens for their presentation to T cells<sup>63</sup>. Other approaches taken include immunomodulatory therapy and bone marrow transplantation, as well as strategies to enhance the process of remyelination. Unfortunately, what is often the outcome in the EAE model is not always observed when transferred to MS patients.

Reports of decreased susceptibility to EAE in IFN- $\gamma$  deficient mice and the demonstration of beneficial effects of IFN- $\gamma$  treatment were very promising and suggested this cytokine to suppress the immune response by inhibiting the proliferation of pathogenic myeloid cell populations<sup>64-67</sup>. However, injection of IFN- $\gamma$  in MS patients exacerbated the disease due to activation of the immune response<sup>68</sup>. The exacerbation of disease course following the neutralization of TNF $\alpha$  in MS patients was also surprising, especially as TNF $\alpha$  was shown to induce demyelination and as the same approach was successful in the treatment of EAE<sup>69, 70</sup>. Shifting the immune response from being Th1 to Th2 mediated has shown very promising results in rodent EAE models, while in monkeys this resulted in severe relapses<sup>71</sup>. Nevertheless, this approach has shown some level of success in a subset of MS patients<sup>72</sup>.

In recent years numerous reports have demonstrated the expression of neural growth factors from cells of the immune system. Expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and glial-cell derived neurotrophic factor (GDNF) have all been reported for cells of the immune system<sup>73-76</sup>. While the exact function of this expression is not well understood it has been suggested that inflammation may in fact have neuroprotective effects. Partial recovery of motor function following spinal cord damage has been reported following the injection of activated macrophages, while autoimmune T cells have been shown to protect neurons from secondary degeneration following damage to the optic nerve<sup>77, 78</sup>. In addition, MBP-specific CD4<sup>+</sup> T cells overexpressing NGF were shown to have beneficial effects on EAE mice by inhibiting the transendothelial migration of monocytes<sup>79</sup>. While many believe

that the effects are neuroprotective, others argue that these factors act to support remyelination by inducing proliferation of oligodendrocytes<sup>80</sup>. Also, it has been demonstrated that BDNF can inhibit MHC class II expression on microglia suggesting these neurotrophic factors to act by regulating the immune response<sup>81</sup>.

Remyelination involves the recruitment of oligodendrocyte progenitor cells (OPCs) from the subventricular zone to the lesion site followed by their differentiation and formation of new myelin sheaths. OPCs isolated from the adult CNS have been shown to remyelinate areas of demyelination following transplantation<sup>82</sup>. Also, successful remyelination by oligodendrocytes produced in-vitro from neural stem cells has been shown to occur in the spinal cord of adult rats<sup>83, 84</sup>. Even more impressive has been the remyelination observed following the intravenous injection of neurospheres<sup>85</sup>. However, the fact that a subset of OPCs express MOG and that some MS patients produce MOG-specific antibodies questions the viability of this approach at the clinical level<sup>86</sup>.

A more recent development in the treatment of immunologically related disorders has been the use of altered peptide ligands (APLs). APLs are analogous to the antigenic peptide with the exception of amino acid substitution(s) in the position required for T cell receptor (TCR) binding. An APL can thus compete with the original antigen for TCR binding and suppress or modulate T cell activation. For example, in the case of MS the ideal APL would bind to uncommitted Th0 cells conferring an anti-inflammatory Th2 phenotype instead of the regular pro-inflammatory Th1 phenotype. The first clinical trials using APLs on MS patients have already taken place using an APL derived from a

fragment of myelin basic protein (MBP), however they have had to be ceased due to exacerbations of disease as well as a high incidence of immediate-type hypersensitivity reactions<sup>87, 88</sup>. It is still not clear if the administered APL induced the exacerbations. It appears that TCR signaling is more complex than previously thought and that if such therapies are to be used then their effect on the T cell should be deciphered as much as possible before their use in clinical trials.

Bone marrow transplantation (BMT) has also been applied to treat EAE. These studies revealed that mice recovered better if treated by syngenic BMT<sup>89, 90</sup>. Given the risk of graft-versus-host disease associated with allogeneic BMT current clinical studies for BMT therapy of autoimmune disorders are using the autologous approach<sup>91</sup>. It is hoped that such an approach may suppress the immune response without compromising remyelination, however, its success is variable and strongly determined by disease stage. The ability of bone marrow cells to cross the blood brain barrier and to reside within the CNS is a unique property and may be taken advantage of in order to deliver therapeutics to lesion sites.

The work performed in this study aims at exploiting this property of bone marrow cells in using them to deliver possibly therapeutic genes across the BBB. The gene of choice in this study is the anti-inflammatory cytokine interleukin-10 (IL-10). IL-10-deficient mice are more susceptible to EAE and develop a more severe disease in comparison to wild-type mice<sup>92</sup>. T cells from such mice show a very strong antigen-specific proliferative response, express very high levels of pro-inflammatory cytokines upon antigen

presentation and induce severe disease when transferred to healthy wild-type mice. Direct injection of adenovirus overexpressing IL-10 into the CNS prevented development of EAE, as did the intracerebral injection of fibroblasts retrovirally transduced to overexpress IL-10<sup>20, 93</sup>. The study employing the use of IL-10-overexpressing fibroblasts did not find any alteration in recruitment of cells to the lesion site, but observed alterations in the phenotype of the cells recruited, mainly an increase in CD8<sup>+</sup> T cells and B cells<sup>93</sup>. No effect was observed following intravenous administration of the IL-10-expressing adenovirus, suggesting the therapeutic effect to be due to effects within the lesion site as oppose to effects on the initiation of the immune response in peripheral lymph nodes<sup>20</sup>. Of particular importance is the disease stage at which treatment is performed as well as the level and duration of IL-10 expression. Injection of various levels of IL-10 before disease onset has no effect, also beneficial effects are not observed following numerous injections of the cytokine but following sustained expression<sup>93</sup>.

# The Hematopoietic System

## 1. The Murine Hematopoietic Stem Cell

A stem cell is commonly referred to as a cell which may self-renew and which has the potential to differentiate into a number of committed cell types. Stem cells are normally categorized according to their differentiation capacity. Totipotent stem cells give rise to all cells of the body as well as extraembryonic cells required for support during development. Pluripotent stem cells are those giving rise to cells of all three germ layers, endodermal, mesodermal and ectodermal, e.g. embryonic stem cells. Restricted, or unipotent, stem cells are those which have the ability to self-renew but may only give rise to a single mature cell type, e.g. primordial germ cells. Adult stem cells belong to the category referred to as multipotent. Multipotent stem cells are defined by their ability to differentiate into more than one cell type, these cells being restricted to a single germ layer, i.e. they are lineage specific. Hematopoietic, neuronal and hepatic stem cells all belong to this category.

The first indication of the existence of HSCs in the adult came from a groundbreaking study published in 1949 demonstrating that irradiated mice could survive if their spleen was protected during whole body irradiation (hematopoiesis occurs in both the spleen and the bone marrow of adult mice)<sup>94</sup>. This work was followed by the first demonstration that injection of spleen or bone marrow cells can rescue lethally irradiated mice<sup>95, 96</sup>. The hypothesis of a hematopoietic stem or progenitor cell was later confirmed by the pioneers of the field of hematopoiesis, Till and McCulloch, in 1961 when they provided the first

example of a quantitative assay for HSCs<sup>97</sup>, followed by the first quantitative description of their self-renewal<sup>98</sup>. The first demonstration of the multipotential differentiation of HSCs was later reported in 1968<sup>99</sup>. The presence of HSCs in-vivo was shown by tracking the progeny of transplanted retrovirally labeled fetal liver cells in mice<sup>100</sup>. Since then an enormous amount of information regarding the hematopoietic stem cell and hematopoiesis has been gathered, making HSCs the best characterized stem cells in the body and leading to their routine use in the clinic for the treatment of diseases such as breast cancer, leukemias and congenital immunodeficiencies.

The physical purification of HSCs was first performed by crude isolation techniques or by centrifugation<sup>101, 102</sup>, however, HSCs can now be isolated by fluorescence-activated cell sorting to give a highly purified population. Based on surface marker expression and self-renewal capability we can now divide HSCs into a number of subpopulations<sup>103, 104</sup>. All mouse HSCs are contained within a lineage negative population ( $\text{Lin}^-$ ) expressing the markers cKit and Sca1, as well as low levels of Thy1.1, on their surface<sup>105</sup>. Within this population are long-term HSCs (LT-HSCs) capable of reconstituting the hematopoietic system of lethally irradiated mice on a long-term basis ( $\geq 3$  months) as well as short-term HSCs (ST-HSCs) capable of rescuing lethally irradiated mice only for a short time period ( $\sim 8$  weeks). These cell populations are distinguished by their levels of the surface marker Flk2. It appears that LT-HSCs express no Flk2 and that this changes as they develop into ST-HSCs which express Flk2 on their surface<sup>106-108</sup>. Another method for the isolation of HSCs is based on their expression of the multidrug efflux pump Abcg2. Upon the staining of bone marrow with Hoechst-33342, HSCs pump out this dye and thus can be

isolated quite easily from the rest of the bone marrow by fluorescence activated cell sorting<sup>109-111</sup>. The population of cells isolated in this fashion is commonly referred to as the side population (SP). The cell population used in this study is the Lin<sup>-</sup> population, a mixed population of bone marrow cells enriched for hematopoietic stem and progenitor cells following the removal of committed cells via magnetically active antibodies specific for cell surface markers expressed on mature cell types (see Materials and Methods).

## **2. Trafficking of Hematopoietic Stem Cells**

It is a well known fact that there is a constant exchange of HSCs between the bone marrow and the peripheral blood in the adult, however, the exact purpose for this migration is not well understood. The presence of HSCs in the peripheral blood suggests that they may be distributed to all organs of the body, but is there a physiological role for them within other organs? A number of theories exist for the explanation of this constant flux of HSCs. It may be that these cells provide for rapid hematopoiesis within the peripheral blood following extreme blood loss. The migration of HSCs may play a role in the decision of the cells to differentiate or to self renew. Or it could be that circulating HSCs are a source of pluripotent cells which may be recruited by various tissues for regeneration in response to an injury, as suggested by many recent reports<sup>112-116</sup>.

The recruitment of migratory HSCs is believed to occur by mechanisms identical to that of inflammatory cells, involving rolling adhesion, via selectins, followed by tight binding, via integrins, then diapedesis and finally chemotaxis along a specific chemokine gradient. HSCs may be classified according to their expression of the chemokine receptor

CXCR4, and they show a high specificity for migration towards stromal derived factor (SDF-1), the endogenous ligand for this receptor<sup>117, 118</sup>. As its name suggests, SDF-1 is expressed by marrow stromal cells within the stem cell niche. Studies show that this chemokine is partly responsible for regulating the flux of HSCs between the bone marrow and peripheral blood as SDF-1 is expressed on bone marrow endothelium and HSCs deficient in CXCR4 show a higher tendency to mobilize from the bone marrow and enter the peripheral blood<sup>119, 120</sup>. Also, inhibition of the membrane bound protease CD26, which cleaves SDF-1, enhances the homing of HSCs to the bone marrow<sup>121</sup>. It is thought that SDF-1 mediated activation of integrins may function in the adhesion of HSCs to the bone marrow endothelium and lead to their extravasation<sup>120</sup>. CXCR4 has been shown to block entry of cells into S-phase of the cell cycle suggesting that this receptor may also play a role in maintaining the quiescent nature of HSCs<sup>122</sup>. Other factors are sure to play a major role in this process as fetal liver HSCs deficient in CXCR4 can successfully rescue lethally irradiated mice<sup>123, 124</sup>. Integrin heterodimers have also been implicated in the homing of HSCs from the blood to the bone marrow<sup>125</sup>. The  $\alpha 1\beta 4$  integrin (very late antigen 4, VLA-4) is expressed on HSCs and binds to fibronectin within the bone marrow thus allowing engraftment<sup>126</sup>. This integrin mediated interaction of HSCs with the extracellular matrix has also been shown to maintain their repopulation potential and quiescence as well as inhibiting apoptosis. The  $\beta 1$ -integrins and their ligands (VCAM-1 and fibronectin) have also been implicated in HSC mobilization into peripheral blood, which is interesting as the mobilization of HSCs is reported to require their proliferation<sup>127, 128</sup>. Cycling HSCs show a higher affinity for fibronectin than their quiescent counterparts. Studies reveal that integrins display non-redundant roles in HSC

homing to bone marrow which are modulated during the cell cycle. Thus HSC trafficking involves a balance between integrin mediated adhesion and migration <sup>129</sup>.

### **3. Bone Marrow Transplantation**

Bone marrow transplantation (BMT) is the therapeutic reconstitution of hematopoiesis following purposeful myeloablative radiation of host bone marrow. The successful recovery after BMT resides in the properties of the cells used for reconstitution. Most transplants are performed using cells from the bone marrow, however, transplants are now being performed using mobilized HSCs from the peripheral blood or cells from umbilical cord blood. The limiting factor is often the number of HSCs which one can obtain and, as already mentioned, the successful ex vivo expansion of HSCs has not yet been achieved. Transplants can be either autologous or allogeneic. Autologous BMT involves the administration of ones own HSCs and is applied when chemotherapeutic doses toxic for endogenous bone marrow are used to achieve tumor kill, however, this approach may often result in remission of the tumor. Allogeneic BMT involves transplantation of HSCs from a HLA-matched donor and is also applied following toxic doses of chemotherapy, however, T cells from the donor enhance the treatment of the cancer as they have a graft-versus-tumor effect. The main complication of allogeneic BMT is the development of graft-versus-host disease in which donor T cells recognize host organs as antigenic, meaning that patients must often receive immunosuppressive therapy in addition. T cell depletion of the donor cells can prevent the development of

graft-versus-host disease, however, for reasons yet unknown, this leads to a dramatic decrease in the efficiency of transplantation<sup>130</sup>.

The interest in treating autoimmune diseases by BMT arose following the apparent recovery of patients suffering from both cancer and an autoimmune disease following treatment of the cancer by allogeneic BMT<sup>131</sup>. Animal studies in BMT have been applied to treat the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). These studies revealed that mice recovered better if treated by syngenic BMT<sup>89, 90</sup>. Given the risk associated with allogeneic BMT current clinical studies for BMT therapy of autoimmune disorders are using the autologous approach<sup>91</sup>.

BMT is also applied in basic research in order to understand the process of hematopoiesis and to elucidate the properties of the hematopoietic stem cell. It must be understood that transplanted HSCs are slightly altered in their properties in comparison to normal physiological conditions. The engraftment of quiescent HSCs was revealed to be more efficient than that of HSCs in the cell cycle, demonstrating that the cell cycle stage of the HSC can dictate successful engraftment. Whether this means that cell cycle determines homing ability, chemotaxis towards the HSC niche or cell-cell interactions remains to be seen, however, it is quite likely that this effect is mediated by effects on chemotaxis as CXCR4 activation, as mentioned above, has been shown to inhibit entry into the cell cycle<sup>122</sup>. An important point to mention here is the fact that HSCs in transplanted mice are more frequently in cell cycle for a period of up to 4 months post-transplantation, and thus are already at a disadvantage when it comes to homing into the bone marrow<sup>132</sup>. BMT studies analyzing the competitive repopulation of the

hematopoietic system between two different HSC sources have also revealed a lot of information on HSC properties. Competitive repopulation studies comparing old and young bone marrow have demonstrated the necessity for the transplantation of a greater number of old HSCs to obtain a reconstitution efficiency equivalent to that of young HSCs, revealing deficiencies in the reconstituting capabilities of HSCs with age<sup>133</sup>. Serial transplantation in mice is limited to 5-7 rounds, suggesting also a limitation to the reconstituting capabilities of the HSC, however, the artifacts introduced by transplantation must again be considered when interpreting this data<sup>134</sup>.

#### **4. Plasticity of Hematopoietic Stem Cells and Cell Fusion**

Plasticity of a cell can be defined as its potential to give rise to more than one defined lineage. Recent studies suggest adding to this definition the ability of a cell to adapt its phenotype in order to accommodate a new environment. Evidence for plasticity between lineages within the hematopoietic system exists<sup>135, 136</sup>, however, the concept of plasticity extending to non-hematopoietic lineages has recently been put forward, challenging the view that tissue specification is determined during embryogenesis. The first studies to suggest the ability of hematopoietic cells to cross their lineage boundary were those revealing the presence of hematopoietic derived cells within non-hematopoietic tissues following bone marrow transplantation or tissue damage<sup>3-5, 9, 137</sup>.

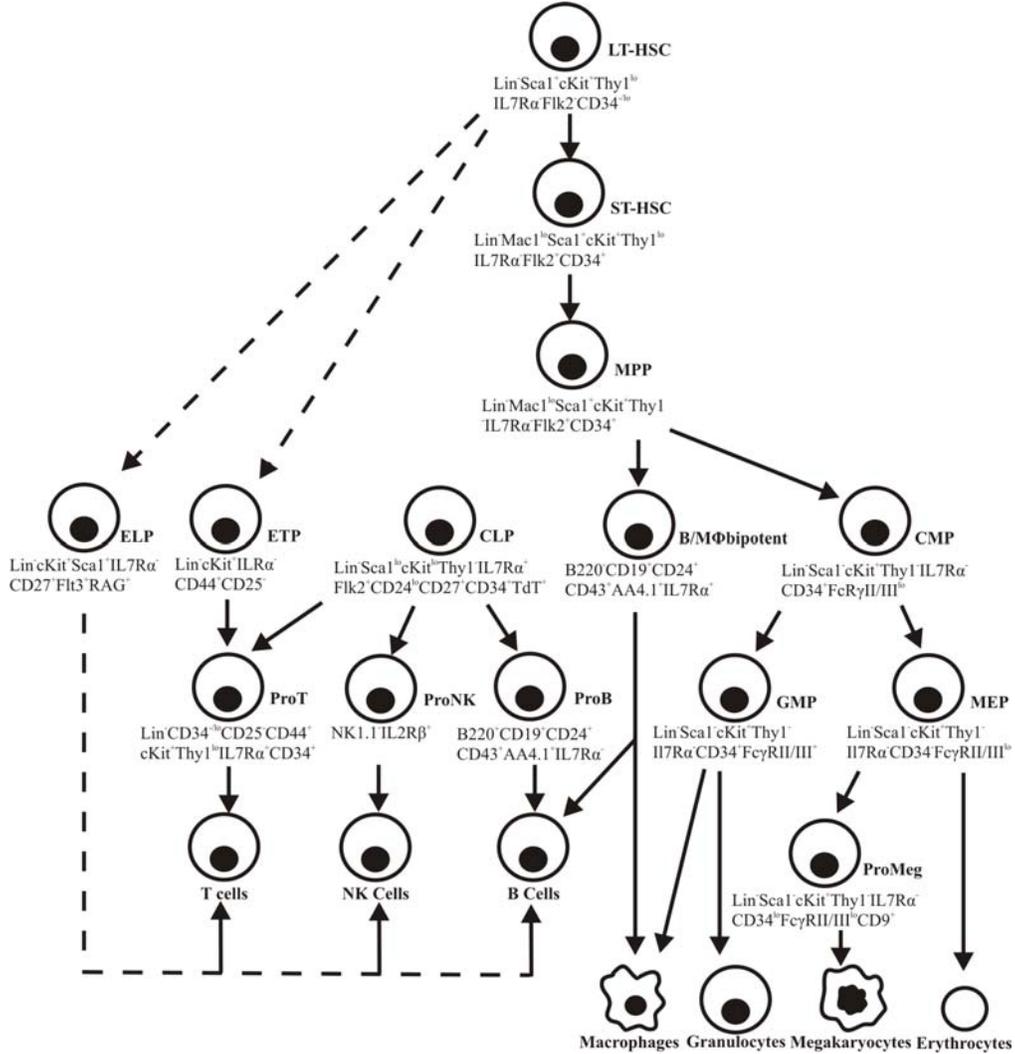
Common weak points coming up in many of these studies are the use of a heterogeneous source of hematopoietic cells for transplantation, as well as the lack of functional data in

order to prove the true phenotype of hematopoietic derived cells in engrafted tissues. The majority of these studies are performed by transplanting total bone marrow, leaving open the question of which cell type may be contributing to this plasticity. In only a few cases have highly purified HSCs been used for transplantation<sup>138-140</sup>. However, even following very stringent purification protocols, a pure population of HSCs is still considered to be somewhat heterogenous<sup>141</sup>. One study in which lethally irradiated mice were rescued following transplantation of a single cell, which contributed to both hematopoietic and non-hematopoietic tissues, argues strongly for plasticity, yet one must still consider the effect of lethal irradiation on the system<sup>142</sup>. One particular model suggests the existence of multiple distinct stem cells within the bone marrow, each of which may give rise to defined lineages, however, while a number of stem cell types have been isolated and shown to have multipotential properties in vitro, unless such cells are shown to exist in vivo this model must remain hypothetical<sup>143</sup>. If we are to truly understand what is happening in these studies we must also understand the full effect of lethal irradiation or other stimuli which have been employed. Regarding definition of the cells observed following transplantation, a common problem is that the phenotype of hematopoietic-derived cells has been determined solely on the basis of immunohistological stainings and morphological analysis with no reference to the acquisition of functional characteristics. To suggest plasticity between lineage boundaries one must first use a well defined, and highly purified, source of input cells followed by evaluation of functional characteristics acquired with their differentiation, otherwise such research can be referred to as nothing more than a glorified form of alchemy.

A foundation for the strong argument against such controversial studies has been the discovery that hematopoietic cells are able to fuse with other cells, raising the possibility that what people have been referring to as plasticity may in fact be the fusion of hematopoietic cells with non-hematopoietic cells. The first report of this phenomenon came about following a study in which an attempt to transdifferentiate neural stem cells (NSCs) into embryonic stem (ES) cells by their coculture resulted in the formation, albeit rarely, of fused NSC-ES cells<sup>144</sup>. This work led to the identification of HSC fusion events in-vivo with particularly sophisticated approaches demonstrating such fusion events to occur only in cell types where fusion is an accepted phenomenon, e.g. Purkinje neurons, hepatocytes and cardiomyocytes<sup>145</sup>. It is now believed by some that these fusion events may contribute to the development or maintenance of particular cell types under normal circumstances, and that fusion, under pathological conditions, may occur as a means of cell survival. Although the concept of HSC plasticity is now losing ground it has revealed the phenomenon of cell fusion which, if understood properly, may be exploited in the future for the design of new cellular therapeutic strategies. In order to clarify a common misuse of terminology, the transdifferentiation of cells, often confused with plasticity, must also be defined here. While plasticity refers to the ability of a stem cell to acquire various mature phenotypes, transdifferentiation is defined by the ability of a mature cell to “de-differentiate” and become a mature cell of another lineage, i.e. the cell reverts along its differential pathway and reprogrammes itself in order to develop along another defined pathway. Examples of in-vivo transdifferentiation have rarely been observed, the only case reported being that of chick retinal epithelium transdifferentiating into neuronal cells<sup>146, 147</sup>. Transdifferentiation is equally as rare in vitro with the only truly convincing

study being the transdifferentiation of mature B cells into macrophages via enforced expression of the transcription factor Pax5<sup>148</sup>.

Previously, the major questions facing researchers within the field of hematopoiesis have been how HSCs may be best isolated, how they may be cultured and expanded in vitro and how their decision to self-renew or differentiate is regulated. Currently we are now left with more questions than anticipated regarding this system. What is the HSC? What is contained within the heterogeneous population of cells now commonly referred to as the HSC population? Are there cells contained within the bone marrow capable of crossing lineage boundaries? Do hematopoietic cells give rise to non-hematopoietic cell types in normal conditions? What are the molecular signals recruiting hematopoietic cells to non-hematopoietic tissues following injury? What is the physiological purpose of cell fusion and how may this occur?



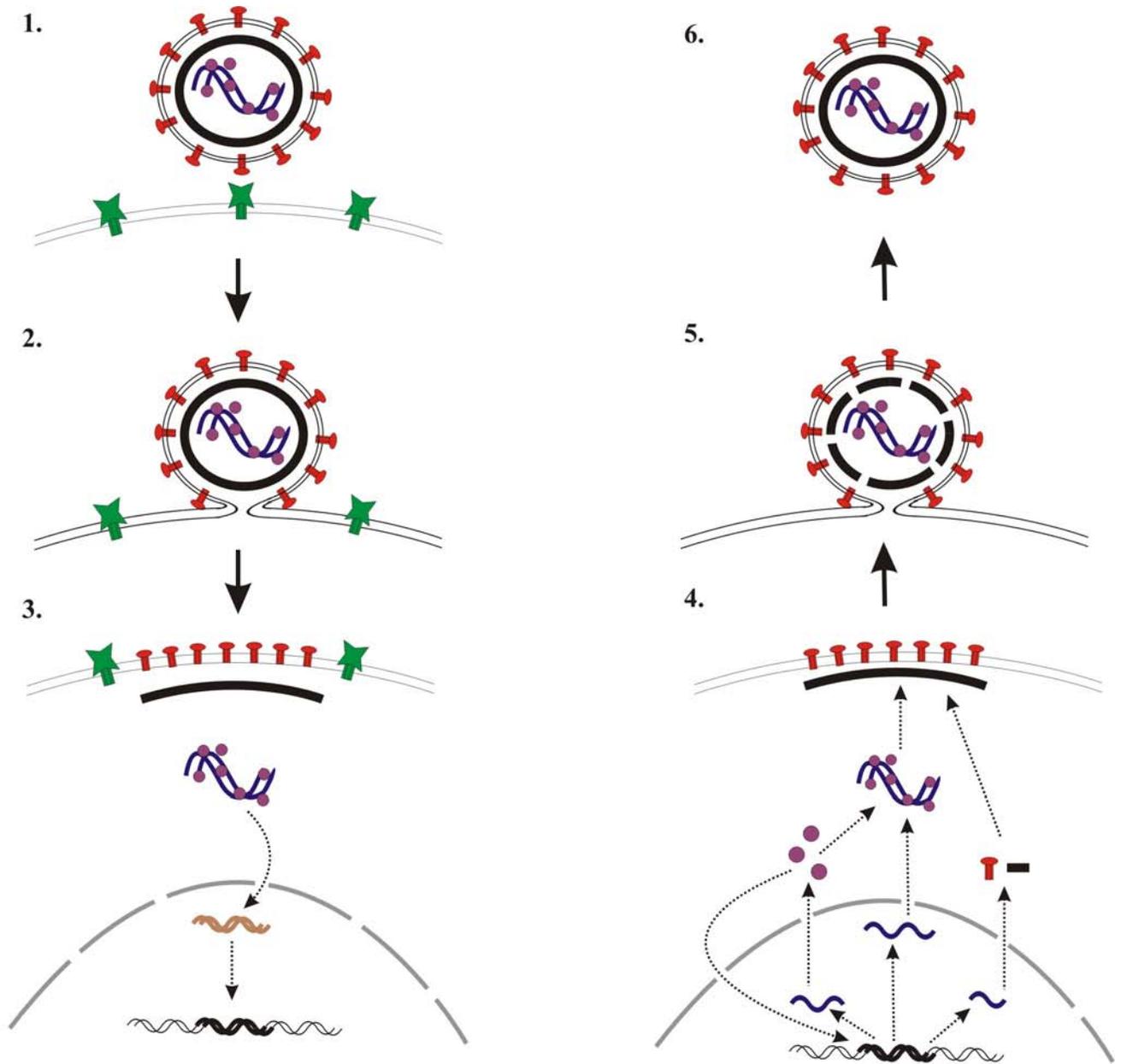
**Figure 2:** Model of hematopoiesis indicating cell surface markers used for isolation of cells. Recent additions include a bipotent progenitor for B lymphocytes and macrophages, the early lymphocyte progenitor (ELP) and the early T lineage progenitor (ETP). Adapted from Kondo et al<sup>149</sup>, Allman et al<sup>150</sup>, Bhandoola et al<sup>151</sup> and Igarashi et al<sup>152</sup>. LT-HSC: Long-TermHSC, ST-HSC: Short-Term HSC, MPP: Multipotent Progenitor, ELP: Early Lymphocyte Progenitor, ETP: Early T Lineage Progenitor, CLP: Common Lymphoid Progenitor, B/MΦ: Bipotent Bcell/Macrophage Progenitor, CMP: Common Myeloid Progenitor, GMP: Granulocyte/Macrophage Progenitor, MEP: Megakaryocyte/Erythrocyte Progenitor.

# Lentiviral Vectors

## 1. An introduction to Lentiviral Vectors

Retroviral vectors have long been considered the ideal gene delivery system. Their ability to integrate into the genome of target cells allows for long term gene expression, they do not induce an immunological response and they have a large cloning capacity (up to 10kb). However, retroviral vectors have a major drawback in that they fail to infect non-mitotic cells<sup>153, 154</sup>. This is a major disadvantage when you consider that many of the targets of gene therapy are cells which are renowned for their quiescent state, e.g. neurons, hepatocytes, myocytes and hematopoietic cells.

The discovery that human immunodeficiency virus-type 1 (HIV-1) can infect both mitotic and non-mitotic cells<sup>155, 156</sup> has led to the development of a new class of retroviral vector to be used for gene therapy. These vectors are termed lentiviral vectors, “lenti” being the latin term for slow, referring to a slow and persistent rate of infection. It is the ability of this newly developed lentiviral system to allow infection of quiescent cells which has brought new hope to the field of gene therapy. While a number of lentiviruses have been described the best understood is HIV-1 and thus most experimental vectors are based on this system.



- Plasma Membrane
- Gag Polyprotein
- Viral RNA
- Viral DNA
- Receptor
- Accessory Protein
- Envelope Glycoprotein
- Integrated Viral DNA
- Cellular DNA
- Nuclear Membrane

**Figure 3: Life Cycle of HIV-1 Based Vectors**

1. Binding of viral particle to surface receptor of target cell via envelope glycoprotein. 2. Fusion of viral envelope with target cell membrane. 3. Uncoating of viral capsid. Viral RNA is reverse transcribed to form double stranded proviral DNA which is translocated into nucleus and integrated into target cell genome. 4. Production of viral transcripts followed by translation of cis-acting regulatory factors. Full length transcripts are packaged with accessory proteins and targeted to cell membrane for assembly of new virions. 5. Assembly of new viral particles and budding from cell membrane of target cell. 6. Mature viral particle capable of infecting other target cells.

## 2. Lentiviral Vectors for Gene Therapy

The first lentiviral vectors to be used experimentally were not intended for use in gene delivery but for the study of HIV-1 pathogenicity. These vectors normally contained the entire viral genome with the exception of the *env* gene. A reporter gene was expressed in place of *env* and the envelope protein was then expressed by another construct<sup>157, 158</sup>. The development of replication incompetent lentiviral vectors designed for the purpose of gene delivery began with the realization that lentiviral vectors may have some benefit over onco-retroviral vectors. The first generation of lentiviral vectors separated structural genes between two constructs. All cis-acting sequences were contained within a third construct expressing the gene of interest<sup>159</sup>. While this system worked it gave low viral titers and was limited to the transduction of the natural target cells of HIV-1. Later vectors expressed structural elements on one construct under the control of the cytomegalovirus (CMV) immediate early promoter. A second vector was used to express the envelope protein, normally the vesicular stomatitis virus G-protein (VSV-G). The VSV-G protein confers pantropic activity on viral particles and allows for greater stability<sup>160, 161</sup>. A third construct contained all required cis-acting sequences, e.g. the 5' and 3' LTRs and  $\psi$ , and the gene of interest under control of the appropriate promoter<sup>162</sup>. Such constructs made expression dependent on the presence of trans-acting proteins expressed from the first construct. While being functional, such vectors raised questions about safety, as homologous regions shared between the different constructs anticipated recombination events which could give rise to replication competent viral particles<sup>163</sup>. The second generation of lentiviral vectors tackled this problem by removing large amounts of the viral genome later discovered unnecessary for gene delivery.

The study described in this thesis employs the use of a third generation lentiviral gene delivery system. The replacement of the U3 region of the 5' LTR with a constitutively active promoter has allowed for the removal of Tat from the system<sup>164</sup>. In addition it was found that Rev can be contributed from a separate vector<sup>165</sup>. Thus, the vector expressing structural and regulatory proteins no longer contains sequences for Tat and Rev. This third generation system is also self-inactivating (SIN) to prevent the possible unwanted expression of genes proximal to the site of integration. Such transcriptional readthrough is due to transcriptional activity of the 5'LTR promoter as well as deficient cleavage and polyadenylation of vector transcripts within the 3'LTR<sup>166</sup>. During reverse transcription, the 5'LTR of the resulting viral DNA is derived from the 3'LTR of the viral RNA. The SIN system was accomplished by deleting the U3 region of the 3' LTR<sup>167</sup>. The modified 3' LTR allows viral packaging but selfinactivates the 5' LTR for biosafety purposes<sup>168</sup>. The element also contains a polyadenylation signal for efficient transcription termination and polyadenylation of mRNA in transduced cells<sup>169, 170</sup>. An added advantage of this modification is that the elimination of transcription from the viral LTR allows the possibility for tissue specific expression upon the use of the appropriate promoter.

### **3. Production of Lentivirus**

The production of functional lentiviral particles is performed by cotransfection of a packaging cell line with four different vectors as described. As Gag and Pol proteins are not assembled accordingly in murine cells, this procedure is performed in a human cell line<sup>171</sup>, the cell line of choice normally being based on 293 cells, a human embryonic kidney cell line. For reasons still unknown, many other common laboratory cell lines, e.g.

HeLa, may be successfully transfected and produce large amounts of viral protein but secrete few viral particles<sup>172</sup>. The cell line used in this study is 293FT. The 293F cell line is a fast growing variant of 293 cells. 293FT cells are a variant of 293F stably expressing the SV40 large T antigen allowing for the replication of plasmids containing the SV40 origin of replication, which is present on the transfer vector used. Viral particles are secreted into the culture medium from which they are collected. Transduction of hematopoietic cells cultured in serum free medium is estimated to require anywhere between  $10^7$  and  $10^8$  transducing units<sup>18, 173</sup>. The pseudotyping of viral particles with the VSV-G protein allows for great stability compared to those with viral glycoproteins. VSV-G pseudotyped particles may be stored at 4°C for 2-3 days, can tolerate a freeze-thaw cycle and may be concentrated 100-fold by ultracentrifugation, all without a significant loss in viral titer<sup>160</sup>. The only disadvantage of using VSV-G pseudotyped virus is its inactivation upon contact with human serum, limiting its experimental use<sup>174</sup>. Secretion of viral particles is maximal 24hr following transfection and decreases two-fold in the second 24 hr period. Low viral titers may be dealt with by altering culture conditions, e.g. decreasing the temperature to 32°C as well as using low serum concentration (2%).

#### **4. Biosafety of Lentiviral Vectors**

The development of the third generation lentiviral gene delivery system has addressed many problems of biosafety regarding the use of lentiviral vectors in the laboratory. The separation of cis and trans acting regions of the viral genome onto separate vectors has enabled the use of this system in scientific and clinical research. Progress in the safety

level of its use has been achieved by decreasing the level of homogeneity between these vectors. However, the presence of some sequences is required on more than one vector, e.g. approximately 300bp of gag is required on both the packaging and transfer vectors, thus the possibility of recombination, while very small, must still be considered. Although, if such an event was to occur, infected cells could not express viral proteins due to the self-inactivating property of the system (SIN), and transport of transcripts to the cytoplasm could not occur in the absence of Rev, which is delivered to packaging cells on its own expression vector. Another concern in clinical research is the possibility of recombination events between engineered virus and natural virus in patients already infected with HIV-1. Studies suggest that were such an event to occur the possibility of recombination between the genome of both viruses is quite likely, resulting in the emergence of a new viral species<sup>175</sup>. The development of SIN lentiviral vectors has decreased the risk of aberrant expression of genes endogenous to the transduced cells, a matter of major concern in clinical gene therapy trials<sup>176</sup>.

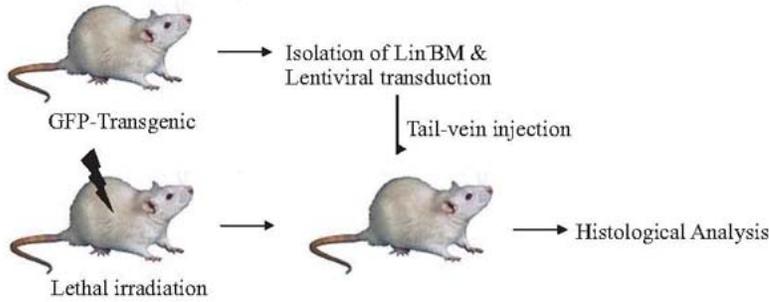
## **Aims of the Study**

Current therapies for the treatment of multiple sclerosis (MS) are problematic in that they cannot be targeted specifically to sites of tissue damage. Using a murine model of MS, Experimental Autoimmune Encephalomyelitis (EAE), the aim of this study was to describe a novel procedure for the delivery of therapeutic genes to the damaged central nervous system (CNS) using hematopoietic stem and progenitor cells (lineage-negative bone marrow cells; Lin<sup>-</sup>BM). The following experiments were performed:

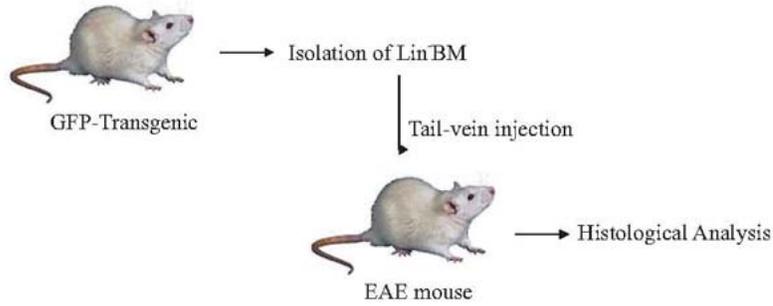
- Transplantation of GFP-labeled Lin<sup>-</sup>BM into lethally-irradiated mice followed by histological analysis to examine the possible engraftment of hematopoietic progenitor cells into non-hematopoietic tissues.
- Analysis of the migration and engraftment of GFP<sup>+</sup>Lin<sup>-</sup>BM following their intravenous administration to EAE mice.
- Phenotypic analysis of hematopoietic-derived cells following their engraftment into the CNS.
- Delivery of the anti-inflammatory cytokine IL-10 to inflammatory lesions in EAE mice using Lin<sup>-</sup>BM, via lentiviral-mediated gene transfer, with the aim of achieving a targeted therapeutic effect.

# Strategy

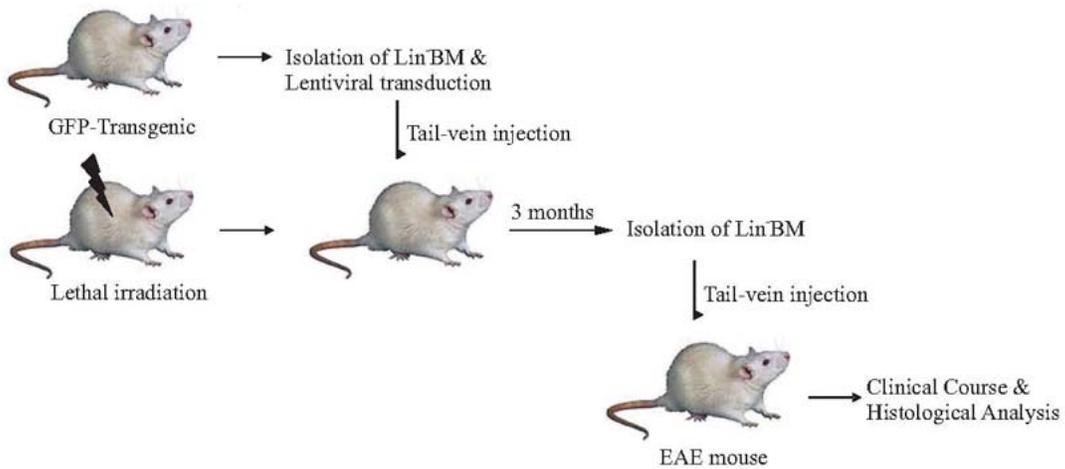
## Bone Marrow Transplantation



## Cell Migration



## EAE Therapy



## Materials and Methods

### 1. Isolation of murine lineage negative bone marrow cells (Lin<sup>-</sup>BM)

6-10 week old C57BL6 mice and  $\beta$ -actin GFP-mice (expressing GFP in all cells under the  $\beta$ -actin promoter) were sacrificed by cervical dislocation following ethyl-ether induced anesthesia. The hind limbs were removed and, using a syringe, the bone marrow was flushed out with phosphate buffered saline (PBS) from the medullary cavities of the tibia and femur bones. Removal of erythrocytes was performed by treatment with lysis buffer (see appendix), cells were suspended in 5ml lysis buffer for 30sec followed by the addition of 5ml PBS to prevent further lysis of white blood cells. Enrichment of c-Kit<sup>+</sup>Sca-1<sup>+</sup> stem cells/progenitor cells was performed by negative selection with the use of a mixture of purified rat anti-mouse monoclonal antibodies specific for the mature cell lineage antigens CDB220 (B lymphocytes), Gr-1 (granulocytes), CD4 (T lymphocytes), CD8 (T lymphocytes), TER119 (erythrocytes) and Mac-1 (macrophages) (all from PharMingen, a concentration of 2 $\mu$ g/ml was used for each antibody). After incubating for 1 hour at 4°C with this primary antibody mixture, cells were washed with PBS and incubated with 1.2x10<sup>7</sup>/ml rat anti-mouse immuno-magnetic beads (Dynal) for 30min at 4°C. Following collection and washing of the non-magnetic fraction the lineage negative cells (Lin<sup>-</sup>) were either analyzed by fluorescence activated cell sorting (FACS) for stem/progenitor cell content, administered to lethally irradiated mice for bone marrow transplantation or directly treated with lentiviral particles and used for in-vivo migration and therapy experiments.

## 2. Construction of replication deficient lentiviral vectors

Lentiviral constructs expressing green fluorescent protein (eGFP) and mouse interleukin-10 (IL-10) were constructed using pLenti6/V5 (Invitrogen). eGFP was cloned into the TOPO-isomerase cloning site whereas IL-10 was cloned between the XhoI and SfuI restriction sites (see Results, figure 2). The cytomegalovirus (CMV) promoter was replaced with the phosphoglycerate-kinase (PGK) promoter. The PGK promoter was obtained from the pMSCV retroviral plasmid (Clontech) by PCR using extended primers allowing the product to be inserted into pLenti6/V5 in place of the CMV promoter using the restriction enzymes ClaI and BamHI. Replacement of eGFP in the pLenti6/V5<sup>CMV</sup>eGFP construct with IL-10 was performed by ligation of a BamHI and XhoI digested fragment produced following PCR amplification of IL-10 with extended primers. Insertion of <sup>PGK</sup>eGFP downstream of <sup>PGK</sup>IL-10 was performed by ligation of a BbrpI and Asp718I digested fragment produced following PCR amplification of the sequence from the pLenti6/V5<sup>PGK</sup>eGFP construct. Confirmation of vector function was confirmed following transfection of packaging cell lines either by fluorescence imaging or ELISA (see Results, figure 2; refer to appendix for transfection and ELISA protocols). ELISA detection of IL-10 was performed using the BD Opt-EIA mouse IL-10 ELISA set (Becton Dickinson). (Refer to appendix for cloning protocols)

Primers used were as follows:

eGFPforward <sup>TOPO</sup>	5'-CACCATGGTGAGCAAGGGCGAGGA-3'
eGFPreverse	5'-TTACTTGTACAGCTCGTCCA-3'
IL-10forward <sup>XhoI</sup>	5'-CCCTCGAGAGAAAAGAGAGCTCCATCATGC-3'
IL-10reverse <sup>SfuI</sup>	5'-GGTTCGAACAGGTGTTTTAGCTTTTCATTTTGA-3'
PGKforward <sup>ClaI</sup>	5'-CCATCGATAATTCTACCGGGTAGGGGAGG-3'
PGKreverse <sup>BamHI</sup>	5'-CGCGGATCCCGGAGATGAGGAAGAGGAGAAC-3'
IL-10forward <sup>BamHI</sup>	5'-GGGGATCCAGAAAAGAGAGCTCCATCATGC-3'
IL10reverse <sup>XhoI</sup>	5'-GGCTCGAGCAGGTGTTTTAGCTTTTCATTTTGA-3'
PGKforward <sup>BbrpI</sup>	5'-GGCACGTGAATTCTACCGGGTAGGGGAGG-3'
eGFPreverse <sup>Asp718I</sup>	5'-GGGGTACCTTACTTGTACAGCTCGTCCA-3'

### **3. Transduction of Lin<sup>-</sup>BM**

For the production of lentiviral particles 293FT cells were cotransfected by means of lipofectamine 2000 (Invitrogen) with the lentiviral construct plus plasmids required for expression of lentiviral genes (pLP1, pLP2, pLP/VSVG). Medium containing viral particles was collected 48hr after transfection and concentrated by ultracentrifugation (25,000rpm, 90min, 4°C).  $1 \times 10^6$  Lin<sup>-</sup>BM were transduced overnight in 500µl Stem Span SFEM expansion medium (Stem Cell Technologies Inc.) containing concentrated viral particles ( $1 \times 10^8$  HeLa transducing units/ml) in 24 well culture dishes (see appendix for detailed transduction protocol).

### **4. Transplantation and Tail-Vein Injection of Lin<sup>-</sup>BM**

For bone marrow transplantation experiments C57Bl/6 mice were lethally irradiated (9Gy) to eradicate endogenous hematopoietic stem/progenitor cells. On the same day  $5 \times 10^6$  Lin<sup>-</sup>BM, transduced overnight with lentiviral particles, were administered under light anesthesia via tail-vein injection. Successful transplantation was confirmed after 4 weeks by FACS analysis of isolated bone marrow. In the study where mice received no irradiation Lin<sup>-</sup>BM were isolated from β-actin GFP-mice (GFP<sup>+</sup>Lin<sup>-</sup>BM) and  $5 \times 10^6$  cells were administered under light anesthesia via tail-vein injection to both EAE and healthy C57Bl/6 mice. In EAE therapy experiments Lin<sup>-</sup>BM was isolated from transplanted mice 3 months after transplantation and administered to EAE mice via the tail vein.

## **5. MOG<sub>35-55</sub> peptide induced EAE**

Immunization of C57/BL6 mice with the myelin oligodendrocyte glycoprotein peptide (MOG<sub>35-55</sub>, SeqLab) was performed under light anesthesia. Complete Freund's adjuvant (CFA) was prepared by suspending 1 ampoule (100mg) of desiccated M.Tuberculosis (Difco Laboratories) in 10ml incomplete Freund's adjuvant (Difco Laboratories). Using CFA, MOG<sub>35-55</sub> (2mg/ml) was diluted 1:1 and emulsified. 50µl was injected into two sites at the base of the tail and 100µl into the right flank. 500µl lyophilised pertussis toxin (PTX, 1ng/µl PBS; List Biological Laboratories Inc.) was injected intraperitoneally to speed up disease progression. A second injection of PTX was given after 24hr, followed by a second injection of 100µl CFA/MOG into the left flank after 7 days. Chronic, non-remitting EAE was induced normally within 10-12 days.

## **6. Detection of GFP<sup>+</sup> cells in recipient animals**

Animals were anesthetized and transcardially perfused with 0.125M PBS followed by 4% paraformaldehyde. After post-fixation in fresh fixative, to prevent the development of post-perfusion artefacts, tissue was cryoprotected at 4°C in 0.125M PBS containing 2% DMSO and 10% glycerol. Tissue was embedded in O.C.T. (Sakura), frozen on dry-ice and stored at -80°C before cryosectioning on a Leica CM1900 cryostat at 20°C. For immunohistochemistry 20µm cryosections were incubated with primary antibodies overnight at 4°C in PBS containing 5% FCS (PAN Biotech) and 0.25% Triton-X (Sigma). Secondary antibody incubations were performed for 1hr at room temperature. Counterstaining of nuclei was performed using DAPI (Sigma). Sections were analyzed by both epifluorescence (Olympus IX70) and confocal (Leica TCS SP2 AOBS) microscopy

for the presence of GFP<sup>+</sup> cells. The antibodies and their optimal concentration for histochemistry are listed in the following table:

#### **Primary Antibodies**

<b>Antigen</b>	<b>Specificity</b>	<b>Source</b>	<b>Class</b>	<b>Company</b>	<b>Concentration</b>
CD45 (Biotinylated)	Leukocytes	Rat	Monoclonal	Pharmingen	1:500
CD3	T lymphocytes	Rat	Monoclonal	Pharmingen	1:1000
GFAP	Astrocytes	Rabbit	Polyclonal	DAKO	1:100
NG2	Oligodendrocytes	Rabbit	Polyclonal	Chemicon	1:100
βTubIII	Neurons	Mouse Ascites	Monoclonal	Sigma	1:400
MBP	Myelin Sheath	Rat	Monoclonal	Chemicon	1:100
VE-Cadherin (CD144)	Endothelia	Rat	Monoclonal	Pharmingen	1:200
APP	Neurons	Rabbit	Monoclonal	Sigma	1:500

#### **Secondary Antibodies**

<b>Fluorophore</b>	<b>Specificity</b>	<b>Source</b>	<b>Company</b>	<b>Concentration</b>
Cy3	Rat	Goat	Sigma	1:200
Cy3	Rabbit	Goat	Sigma	1:200

Microglia were stained using biotinylated-isolectinB4 (3μg/ml), which binds specifically to terminal α-D-galactosyl residues within the plasma membrane of microglia and macrophages. Visualization of biotinylated stainings were performed using streptavidin-conjugated Cy3 (Sigma; 1:200, 1hr, room temperature).

## Appendix to Materials and Methods

### RNA Isolation

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

- Take eppendorf with tissue (e.g. 20-30mg in PBS).
- Add 600µl lysis buffer (inc. 1:100 B-mercaptoethanol), use 300µl if less than 20mg of tissue (lysis buffer lasts approx. 1 month).
- Homogenise with small syringe (use large first if a lot of tissue). Centrifuge (3min, max).
- Mix 600µl of supernatant with 600µl EtOH+DEPC H<sub>2</sub>O.
- 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 DNaseI 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 eppendorf, add 35µl RNase free H<sub>2</sub>O (onto filter), leave for 3min, centrifuge (1min, 10000), throw away column. RNA is collected in eppendorf.

- Measure concentration ( $\mu\text{g/ml}$ ) and RNA/protein absorption (260/280) using photometer (Eppendorf), and dilute with DEPC H<sub>2</sub>O accordingly:

<u>RNA conc. (<math>\mu\text{g/ml}</math>)</u>	<u>DEPC H<sub>2</sub>O</u>
<300	30
300-400	40
400-500	50
>500	60

Reverse transcription (RT)

Reverse transcription of isolated RNA was used to produce cDNA using Superscript RNase H Reverse Transcriptase (Invitrogen). The following reagents were mixed and incubated at 42°C for 1 hour. As a negative control the reaction was performed in the absence of reverse transcriptase (RT). Samples were either stored at -20°C or used immediately for PCR.

	<u>Positive Control</u>	<u>Negative Control</u>
RNA ( $\mu\text{l}$ )	11	11
Hexa-Nucleotide Soln. ( $\mu\text{l}$ )	1	1
5XRT 1 <sup>st</sup> Strand Rxn Buffer ( $\mu\text{l}$ )	4	4
DTT (0.1M) (final: 0.01M) ( $\mu\text{l}$ )	2	2
dNTP (10mM) (final: 0.5mM) ( $\mu\text{l}$ )	1	1
<u>RT (200U/ml) (<math>\mu\text{l}</math>)</u>	<u>1</u>	=
Total ( $\mu\text{l}$ )	20	19

### 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 the 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 and aliquoted to PCR tubes according to the following protocol:

- Master mix:

	<b>1 Tube</b>	<b>2 Tubes</b>	<b>3 Tubes</b>	<b>5 Tubes</b>
<b>H<sub>2</sub>O (μl)</b>	41	82	123	205
<b>dNTPs (μl)</b>	1	2	3	5
<b>10x Buffer</b>	5	10	15	25
<b>(μl)</b>				
<b>Polymerase</b>	0.5	1	1.5	2.5
<b>(μl)</b>				
<b>Total (μl)</b>	47.5	95	142.5	237.5

- Add 48µl master mix to each tube.
- Add 1µl of appropriate cDNA (nothing to –ive control).
- Mix 0.5µl of left and right primers (100pmol/ml) per tube for both sample and control and add 1µl to each tube.

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

Initial Denaturation Step - 94°C 3min

Cycle Step 1 - Denaturation 94°C 1min

Cycle Step 2 – Annealing 55-60°C 1min (dependent upon primers used)

Cycle Step 3 - Elongation 74°C 1min (3min for Vent Polymerase)

Final Elongation Step - 74°C 3min

PCR products were either stored at 4°C or ran 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 a u.v. lamp and thus preventing the possibility of mutation.

- Weigh 0.5g agarose (Sea Kem LE Agarose Cambrex).
- Add to 50ml 1x TBE Buffer and dissolve using microwave at 850 W.

- Add 1.25 $\mu$ l Ethidium Bromide (2.5 $\mu$ l/100ml), or 4 $\mu$ l Gelstar, and set in gel apparatus.
- Add a mixture of 3 $\mu$ l loading dye and 6 $\mu$ l 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 Flu-O-Blu lamp (Biozyme), 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 450 $\mu$ l).
- Leave at 50°C for 10 min (vortex every 3min).
- Add 1xVol isopropanol (normally use 150 $\mu$ l), invert several times, place in column, centrifuge 1min, discard flowthrough. (Qiagen column collects fragments within range of 70bp-10Kb).
- Add 500 $\mu$ l QG buffer, centrifuge 1min, discard flowthrough.
- Add 750 $\mu$ l buffer PE, centrifuge 1min, discard flowthrough, repeat centrifugation.
- Place column in clean tube, add 10 $\mu$ l EB buffer/H<sub>2</sub>O, leave 1min, centrifuge 1min.

### Restriction Digestion

Digestion of DNA with restriction enzymes (Roche) was performed in order to allow ligation of extracted PCR products (inserts) into expression plasmids (e.g. lentivirus), or for analysis of mini prep plasmid preparations. Digestion was performed at 37°C for 1 hour using the reaction mix listed below. Digested insert/plasmid DNA was run on a 1% agarose gel for analysis and extracted when needed for ligation.

0.5µl Enzyme 1

0.5µl Enzyme 2

2µl Buffer

17µl insert/plasmid in H<sub>2</sub>O (up to 1µg)

20µl total reaction volume

### 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 0.5 µl of 10 mM dNTP.

- In order to fill in “sticky-ends” add 1-5 U Klenow Enzyme (Roche) and incubate at 30°C for 15 min.
- Block the reaction by heating at 75°C for 10 min (not necessary if continuing with dephosphorylation).

Proceed with dephosphorylation of the plasmid:

- Extract the necessary DNA fragment into 15µl H<sub>2</sub>O by gel extraction.
- Add 2µl 10x Buffer and 3U Shrimp Alkaline Phosphatase (Roche), bring to total volume of 20µl.
- Incubate for 60 min at 37°C degrees. Inactivate by heating to 65°C for 15 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 1 hour. 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.

1µl T4 Ligase

1µl Ligation Buffer

8µl DNA

10µl total reaction volume

### 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 42°C 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 cotransfection of the lentiviral plasmid along with plasmids expressing accessory

lentiviral genes into a packaging cell line using a lipofectamine-based technique as follows:

- Plate  $5 \times 10^6$  293FT cells in a poly-L-lysine coated 10cm dish one day before transfection to obtain a culture of 80-90% confluency.
- On the day of transfection add antibiotic-free medium to the cells (10ml).
- Prepare DNA-lipofectamine complexes:
  - 9 $\mu$ g packaging mix + 3 $\mu$ g vector in 1.5ml OptiMEMI medium (Gibco).
  - 36 $\mu$ l lipofectamine 2000 (Invitrogen) in 1.5ml OptiMEMI
  - leave for 5min at RT
  - mix gently and leave for 20min at RT
- 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°C, frozen at -80°C or concentrated by ultracentrifugation (25,000rpm, 4°C, 90min).

### Transduction

A number of different transduction protocols were 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°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 confluency of 30-50% in 6-well dishes were treated with lentiviral supernatant, centrifuged (2500rpm, 30°C, 90min) and kept at 37°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 hematopoietic cells and cell lines)

Viral particles concentrated by ultracentrifugation were resuspended overnight at 4°C in 500µl serum free medium (e.g. Stem Span SFEM expansion medium for Lin<sup>+</sup>BM). The resuspended viral concentrate was added to the target cells and kept at 37°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 or spin infection.

- Concentrate Spin Infection (primary hematopoietic cells and cell lines)

Viral particles concentrated by ultracentrifugation were resuspended overnight at 4°C in 500µl serum free medium. The resuspended viral concentrate was added to the target cells, centrifuged (2500rpm, 30°C, 90min) and kept at 37°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 for the 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  $\mu\text{g/ml}$ .
- 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 #1

Titration of viral particle concentration in collected supernatants was performed on HeLa cells in order to estimate the concentration applied to primary hematopoietic cultures.

- Plate  $5 \times 10^4$  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  $6 \mu\text{g/ml}$  polybrene.
- Leave overnight at  $37^\circ\text{C}$ .
- Add fresh medium and leave overnight at  $37^\circ\text{C}$ .
- Add medium containing the appropriate amount of drug for selection (determined by kill curve).
- Change medium every 3-4 days.

- After 10-12 days (when all cells in mock transduction are dead) wash cells twice with PBS.
- Add 1ml crystal violet (1% in 10% EtOH) and leave for 10min at RT.
- Wash cells twice with PBS.
- Count the number of colonies and calculate the titre.

e.g. 46 colonies in  $10^{-5}$  dilution and 5 colonies on  $10^{-6}$  dilution

$$\text{titre} = (46 \times 10^5 + 5 \times 10^6) / 2 = 4.8 \times 10^6 \text{ TU/ml}$$

### Viral Titre Determination #2

Titration of the viral titre may also be performed using FACS analysis to quantify the number of transduced cells. In this protocol cells are transduced as above, however, selection of transduced cells using antibiotics is not performed. Transduced cells are allowed to culture for 4 days following transduction and then analysed 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-positive cells. Above 20%, the chance for each GFP-positive 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:

$$\text{Titer (Hela-transducing units / ml)} = \frac{(5 \times 10^4 \text{ Hela cells}) \times (\% \text{ GFP-positive cells} / 100)}{\text{volume of supernatant (in ml)}}$$

## ELISA

An enzyme-linked immunosorbent assay (ELISA, OptEIA, Becton Dickinson) was used to determine the levels of IL-10 expressed from pLenti6/V5-IL10 constructs. Measurements were made on the 48hr supernatant of cell lines transfected or transduced to produce IL-10. Sample concentrations were calculated from a standard curve prepared from standards measured simultaneously with samples.

- Coat wells of 96-well dish with 100µl of capture antibody diluted in coating buffer (1:125). Seal plate and leave overnight at 4°C.
- Aspirate wells and wash 3 times with wash buffer.
- Block wells with 200µl assay diluent. Leave at RT for 1 hour.
- Aspirate wells and wash 3 times with wash buffer.
- Prepare serial dilution of standard IL-10 preparation (2000, 1000, 500, 250 125, 62.5 31.3 and 0 pg/ml). Add 100µl of standard, sample and control into appropriate wells. Leave at RT for 2 hours.
- Aspirate wells and wash 5 times with wash buffer.
- Prepare working detector by diluting detection antibody (1:250) and avidin-HRP reagent (1:250) in assay diluent. Add 100µl to wells and leave for 1hr at RT.
- Aspirate wells and wash 7 times with wash buffer.
- Add 100µl of substrate solution to each well and incubate for 30min at RT in the dark.
- Add 50µl of stop solution to each well.
- Read absorbance at 450nm within 30min of stopping the reaction.

## Buffers and Solutions

- Lysis Buffer:

0.156 M ammonium chloride (NH<sub>4</sub>Cl)

0.01M potassium hydrogen carbonate (KHCO<sub>3</sub>)

5x10<sup>-6</sup>M EDTA

- 10x concentrated 0.125M Phosphate Buffered Saline (PBS):

0.007M (NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O)

0.034M (Na<sub>2</sub>HPO<sub>4</sub>)

0.6M sodium chloride (NaCl)

Dilute 10.25g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O and 47.7g Na<sub>2</sub>HPO<sub>4</sub> in distilled water and set the pH to 7.3. Add 350.5g NaCl and make solution to 4L with distilled water. Dilute 1:10 with distilled water to use.

- 4% Paraformaldehyde (PFA):

Add 20g PFA to 400ml distilled water and heat to 65°C. Add sodium hydroxide (NaOH) dropwise until the solution becomes transparent. Add 50ml 10x PBS, mix well and allow to cool on ice. Set the pH to 7.3 and bring the volume to 500ml with distilled water. Sterile-filter the solution, aliquot and store at -20°C.

- 10x Concentrated TBE Buffer:

1.78M Tris-Base

1.78M Boric Acid

0.04M EDTA

Dissolve 14.88g EDTA in distilled water and bring the pH to 8 using NaOH.

Dissolve 216g Tris-Base and 110g Boric acid in distilled water and mix with the EDTA solution. Bring the total volume to 2L with distilled water. Dilute 1:10 with distilled water when using.

- 3x Concentrated Loading Buffer (10ml):

2ml 0.5M EDTA

6g Sucrose

0.2ml 2% Bromophenol Blue

0.2ml 2% Xylene Cyanole

0.2g Ficoll-400

3.8ml H<sub>2</sub>O

## Cell Lines and Culture Media

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

DMEM

10% FCS

1% L-Glutamate

1% Antibiotics

(10µl/ml Geneticin (Gibco) when expanding)

- HeLa (human cell line):

DMEM

10% FCS

1% L-Glutamate

1% Antibiotics

- G62 (human glioma cell line):

DMEM

10% FCS

1% L-Glutamate

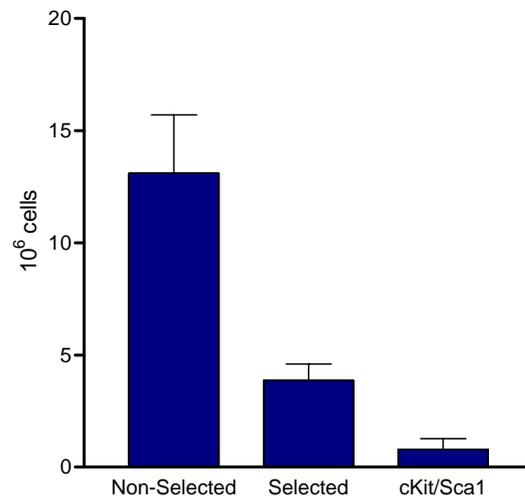
1% Antibiotics

## Results

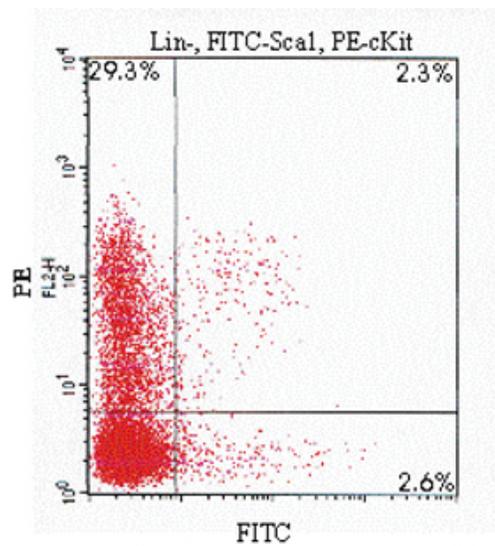
### Isolation of murine lineage negative bone marrow cells ( $Lin^-BM$ ):

Previous studies employing the use of bone marrow cells for therapeutic purposes have taken highly purified stem cells or total bone marrow. Ideally, a pure population of stem cells would be used; however, the isolation of a sufficient number of  $cKit^+Sca1^+$  cells requires their sorting from the bone marrow of many mice. On the other hand, to use a total bone marrow preparation introduces the strong possibility of artifacts arising from the presence of terminally differentiated cells in the preparation. A compromise was taken to use a population devoid of terminally differentiated cells enriched for stem and progenitor cells, the lineage negative population ( $Lin^-BM$ ). A very reliable protocol for the negative selection of differentiated cells from isolated bone marrow has been developed. The average number of cells obtained following both negative selection and c-Kit/Sca-1 analysis was compared (fig.1). The procedure of negative selection can remove up to 85% of all cells to give a population enriched for stem cells.

(A)



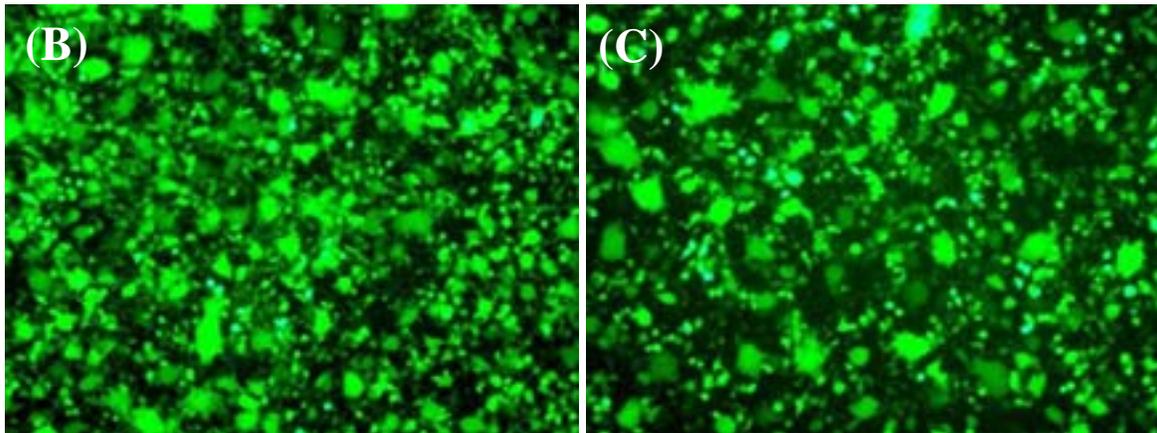
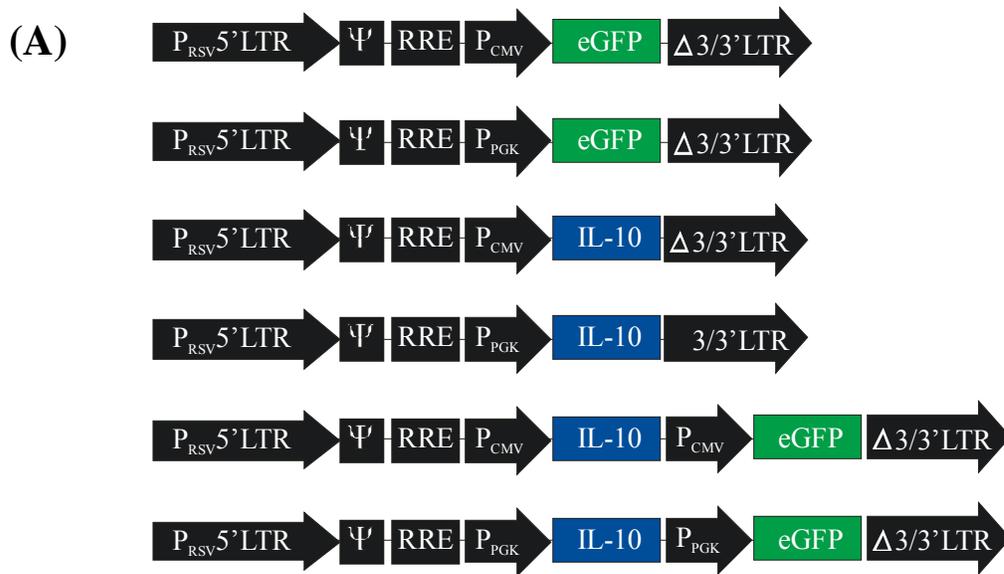
(B)



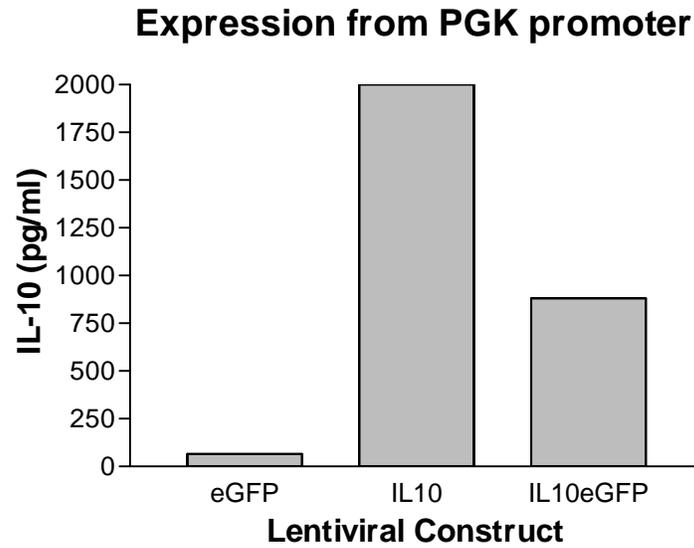
**Figure 1:** Isolation of murine Lin<sup>-</sup>BM. (A) Histogram illustrating the relative number of cells present in untreated bone marrow, lineage negative bone marrow, and the cKit<sup>+</sup>Sca1<sup>+</sup> stem cell population. (B) FACS dot plot illustrating the relative percentage of cKit<sup>+</sup> (PE) and Sca1<sup>+</sup> (FITC) cells in a Lin<sup>-</sup>BM preparation.

Construction of Lentiviral vectors:

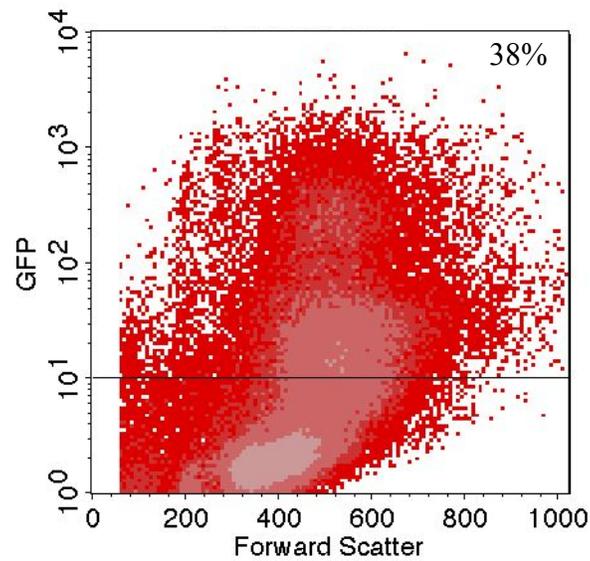
Lentiviral vector constructs were made using pLenti6/V5 (fig.2). While the use of a CMV promoter is the favored choice for transgene expression, its use in hematopoietic cells is often problematic, thus constructs were also cloned using a PGK promoter for comparison. Although expression under the CMV promoter is more efficient, PGK-mediated expression is sustained over longer periods and is more efficient in hematopoietic cell lineages. Successful expression of eGFP from pLenti6/V5-eGFP constructs can be observed where eGFP fluorescence in pLenti6/V5<sup>CMV</sup>eGFP and pLenti6/V5<sup>PGK</sup>eGFP transfected 293FT cells is shown (fig. 2B, 2C). Expression of IL-10 by pLenti6/<sup>V5PGK</sup>IL10 and pLenti6/<sup>V5PGK</sup>IL10<sup>PGK</sup>eGFP constructs was confirmed by ELISA. 48hr-old supernatant from transfected 293FT cells was collected and used to transduce HeLa cells (see Appendix to Materials and Methods). Levels of IL-10 expression from HeLa cells transduced with each lentiviral preparation were compared (fig.3). Highest expression, beyond quantification by ELISA ( $\geq 2000$ pg/ml) was observed when cells were treated with pLenti6/<sup>V5PGK</sup>IL10 viral particles. Intermediate expression was observed following treatment with pLenti6/<sup>V5PGK</sup>IL10<sup>PGK</sup>eGFP particles. IL-10 expression by CMV-promoter constructs was too great for detection by ELISA (sensitivity 30-2000 pg/ml). In order to allow tracking of cells following administration to mice it was decided that pLenti6/<sup>V5PGK</sup>IL10<sup>PGK</sup>eGFP would be used for further experiments, thus its expression in Lin<sup>-</sup>BM was tested by various transduction protocols (see Appendix to Materials and Methods).



**Figure 2:** Construction of lentiviral vectors. (A) Map of various pLenti6/V5 constructs. (B) Transfection of 293FT cells with pLenti6/V5<sup>CMV</sup>eGFP construct (C) Transfection of 293FT cells with pLenti6/V5<sup>PGK</sup>eGFP construct.



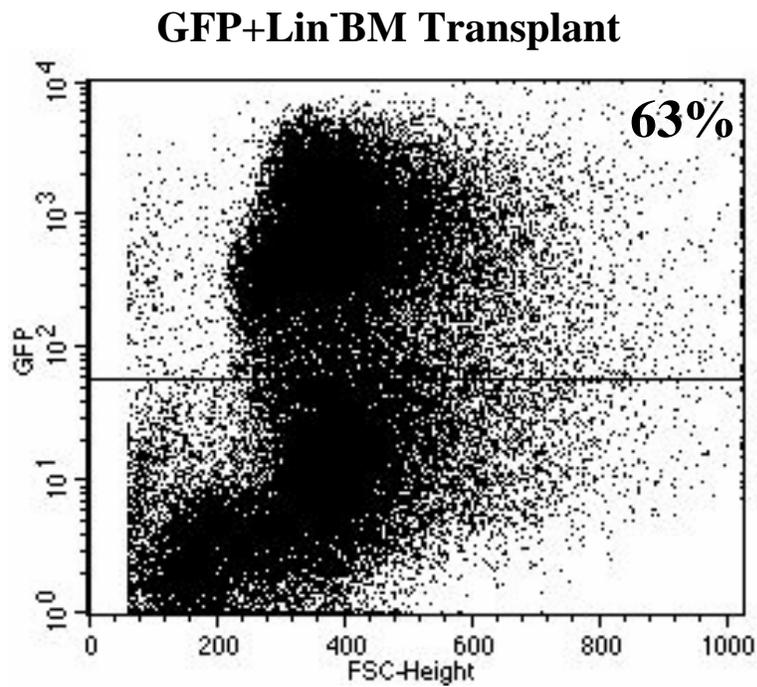
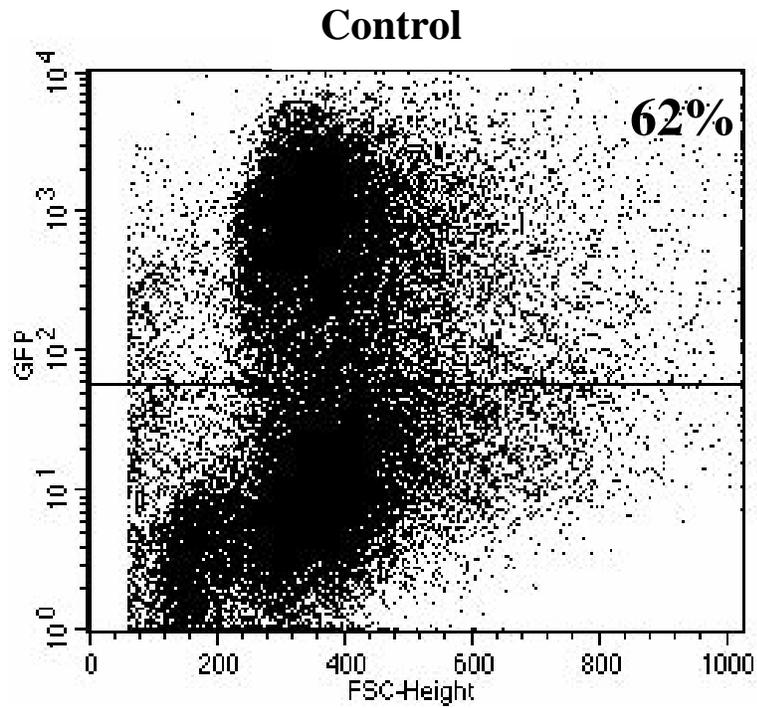
**Figure 3:** ELISA data comparing levels of IL-10 produced by HeLa cells following transduction with different lentivirus preparations (fresh supernatant). Note, expression from the pLenti6/V5<sup>PGK</sup>IL-10 construct is above the detection level of the ELISA assay kit (2000pg/ml).



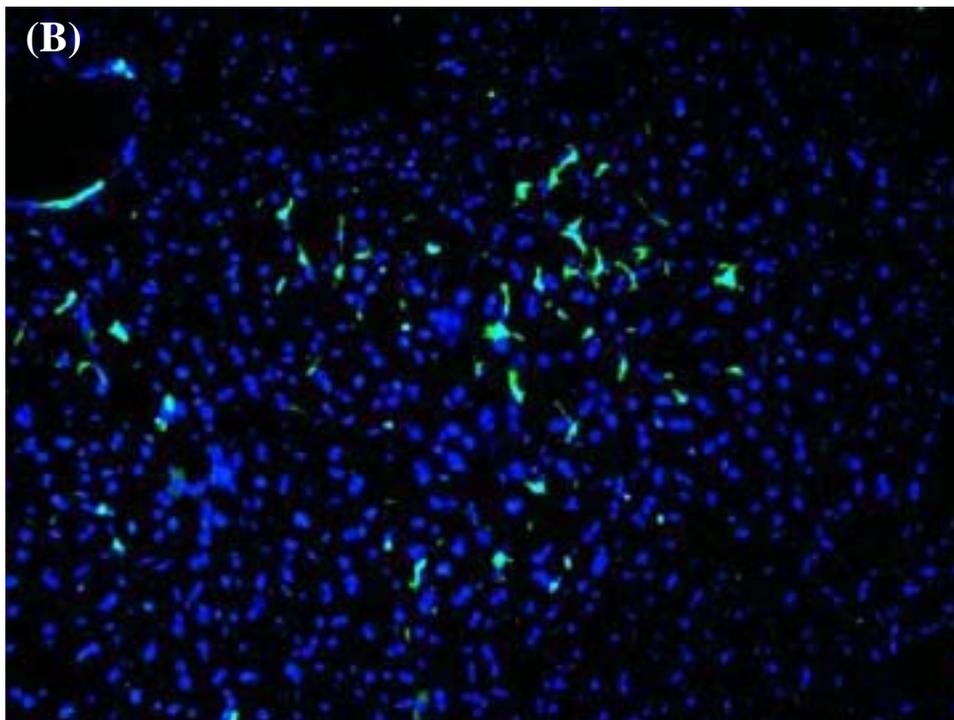
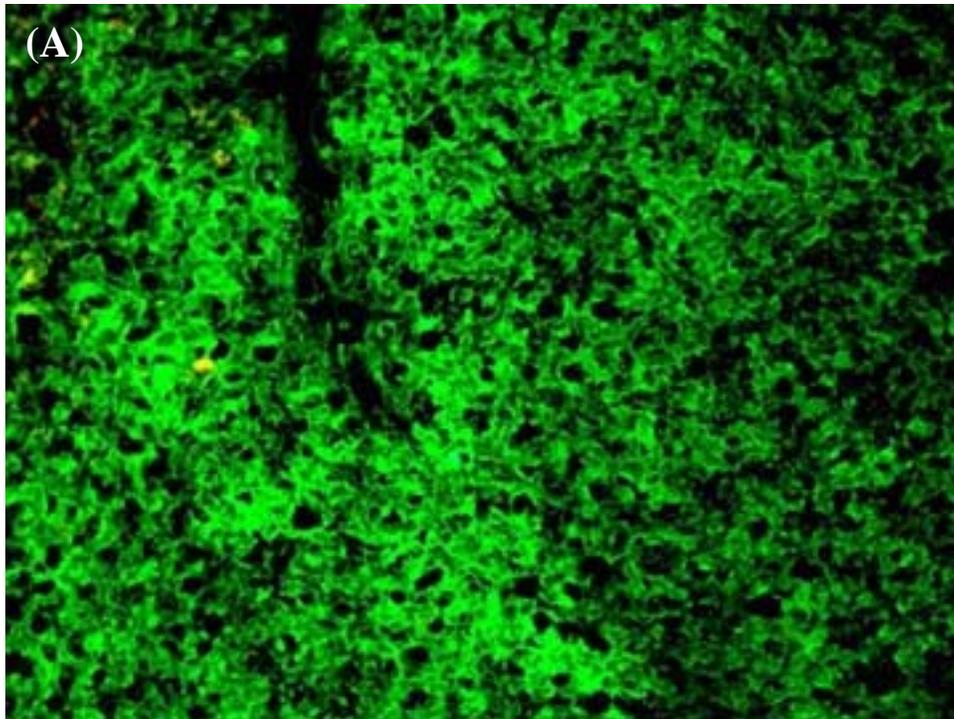
**Figure 4:** pLenti6/V5<sup>PGK</sup>IL-10<sup>PGK</sup>eGFP lentiviral transduction of Lin-BM

Bone marrow transplantation of lethally irradiated C57/Bl6 mice with pLenti6/V5eGFP transduced Lin<sup>-</sup> BM:

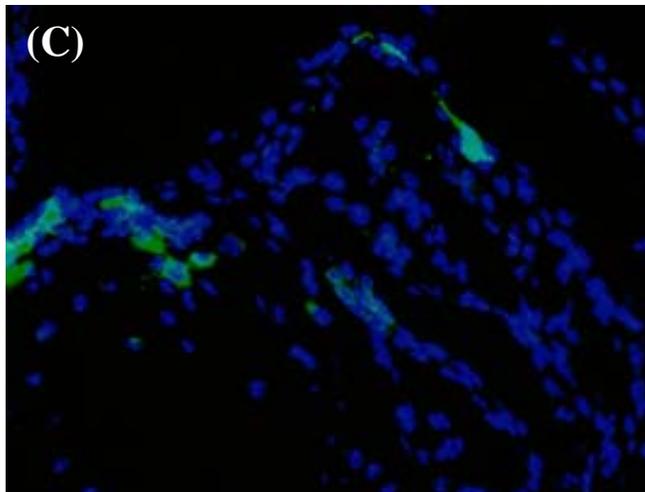
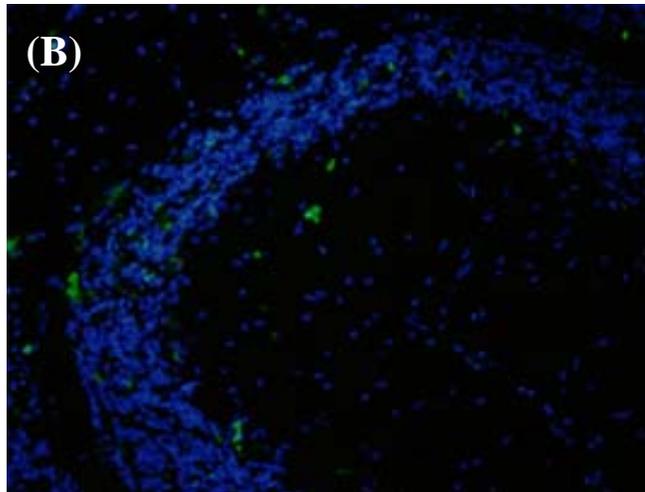
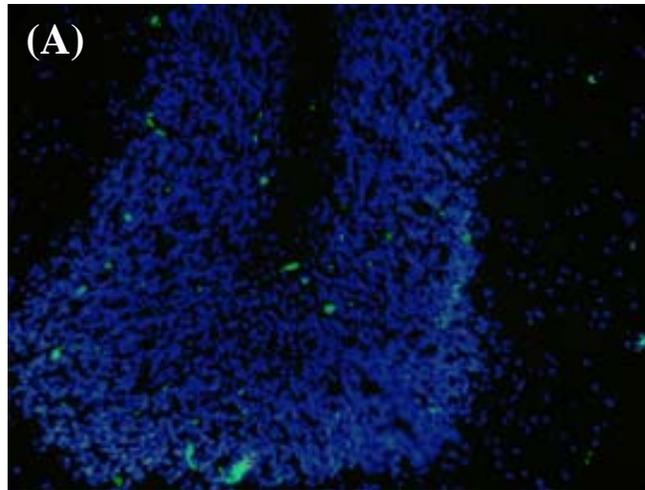
The successful transduction of a Lin<sup>-</sup>BM population with a lentiviral vector was demonstrated (fig.5). Bone marrow from a transplant recipient of pLenti6/V5eGFP-transduced Lin<sup>-</sup>BM is shown by FACS analysis to have an identical percentage of GFP<sup>+</sup> bone marrow cells as in  $\beta$ -actin-GFP transgenic mice. Confirming previous reports, histological analysis showed these GFP<sup>+</sup>Lin<sup>-</sup>BM cells to engraft within many peripheral organs as well as the central nervous system at 4 weeks following transplantation<sup>3, 4</sup> (fig.6-8). As expected almost all cells in the spleen were found to express GFP while a large proportion of GFP<sup>+</sup> cells were observed in the liver (fig.6). In line with previous reports a small number of GFP<sup>+</sup> cells in the cerebellum and choroid plexus of the CNS were also observed (fig, 7). Previously unreported in bone marrow transplantation studies was the engraftment of these cells into the spinal cord. Significant numbers of GFP<sup>+</sup> cells were observed throughout the entire spinal cord (fig.8).



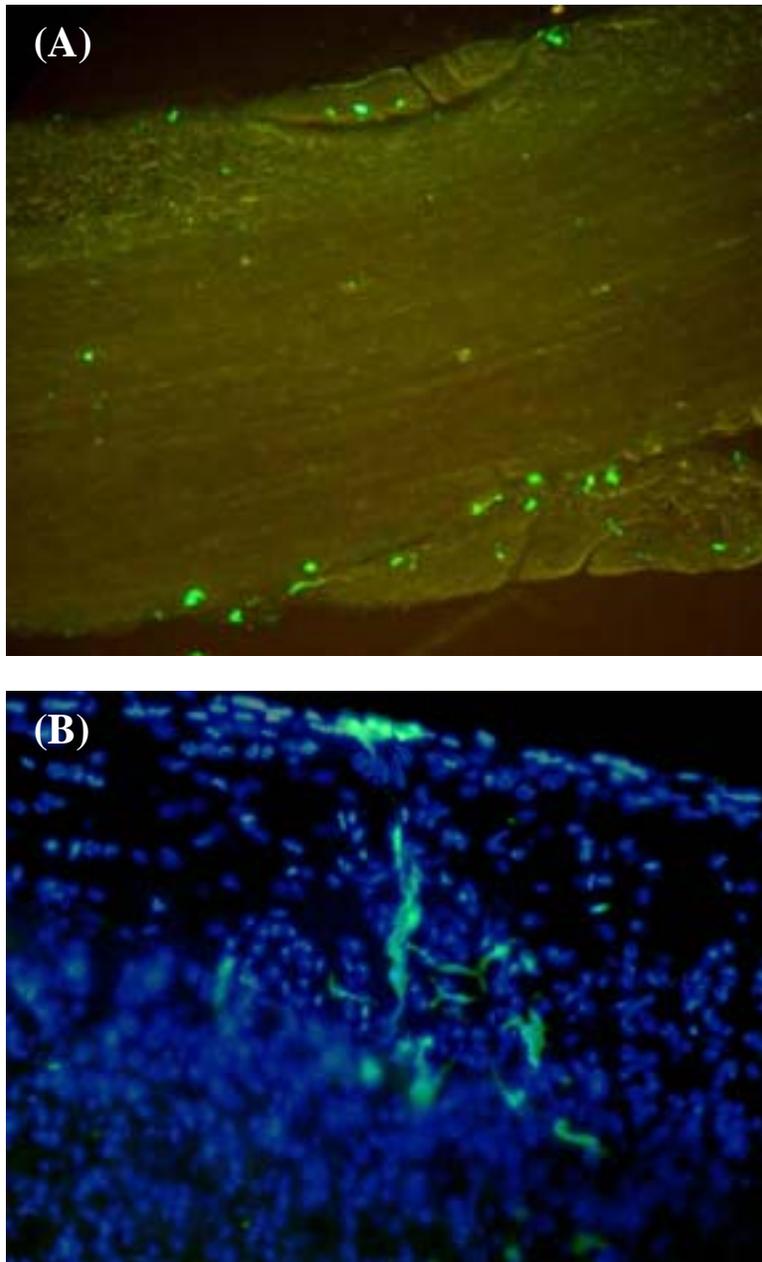
**Figure 5:** Bone marrow transplantation of C57/Bl6 mice with pLenti6/V5-GFP transduced Lin<sup>-</sup> BM. FACS analysis of bone marrow from  $\beta$ -actin-GFP transgenic mouse (control) and from C57/Bl6 mouse 4 weeks after receiving transplant of pLenti6/V5-GFP transduced Lin<sup>-</sup>BM (GFP<sup>+</sup>Lin<sup>-</sup>BM Transplant).



**Figure 6:** Detection of GFP<sup>+</sup> cells in recipient animals. GFP<sup>+</sup> cells found in spleen (A), liver (B) 4 weeks after bone marrow transplantation with pLenti6/V5-GFP transduced Lin<sup>-</sup>BM.



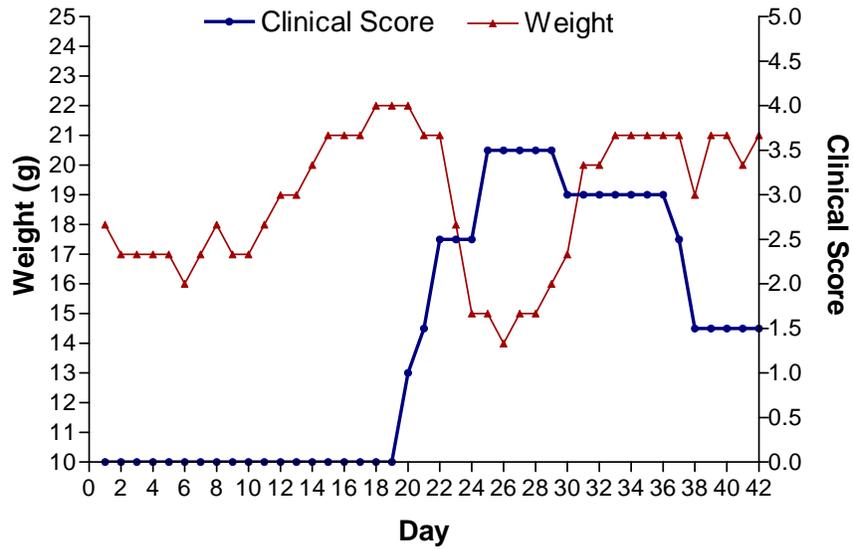
**Figure 7:** Detection of GFP<sup>+</sup> cells in recipient animals. GFP<sup>+</sup> cells found in cerebellum (A, B) and choroids plexus (C) 4 weeks after bone marrow transplantation with pLenti6/V5-GFP transduced Lin<sup>-</sup>BM.



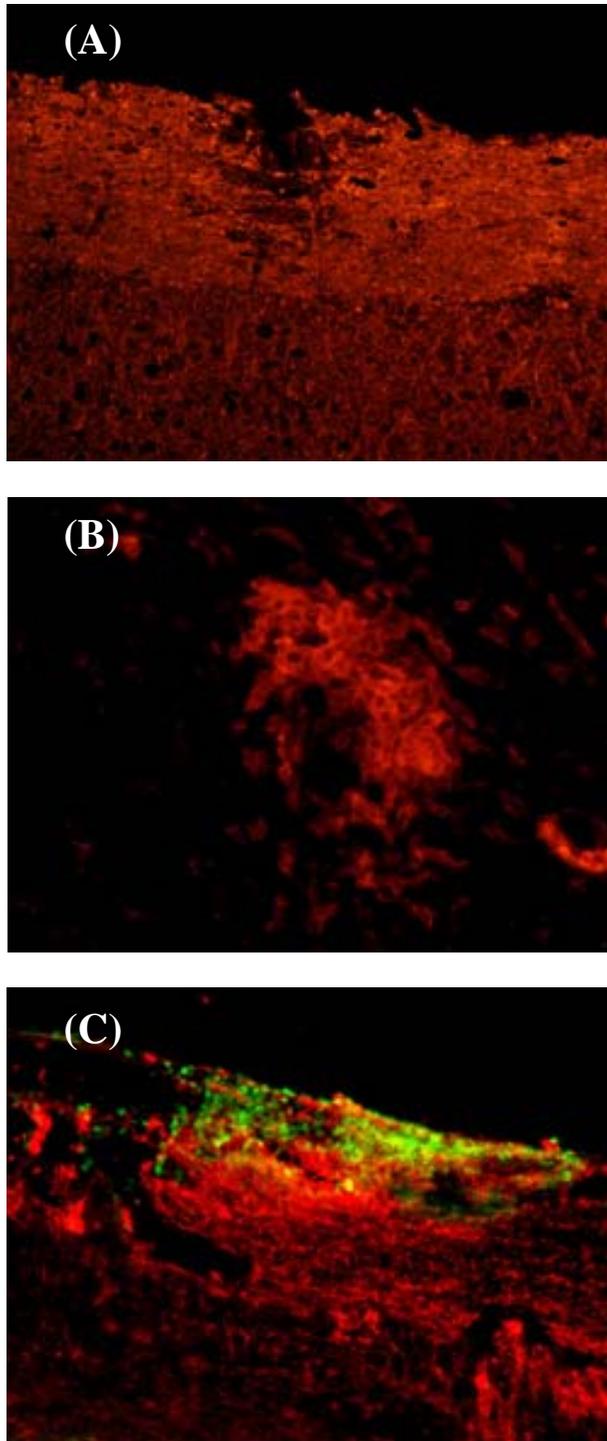
**Figure 8:** Detection of GFP<sup>+</sup> cells in recipient animals. GFP<sup>+</sup> cells found in spinal cord (A, B) 4 weeks after bone marrow transplantation with pLenti6/V5-GFP transduced Lin<sup>-</sup> BM.

*Induction of Experimental Autoimmune Encephalomyelitis (EAE):*

In order to establish a reliable procedure for the induction of EAE in C57/Bl6 mice, the protocol described has been followed (see Materials and Methods). Typical data from a mouse in which the disorder was induced is shown (fig.9). It can be seen quite clearly that there is a correlation between the onset of clinical symptoms and a decrease in the weight of the animal. Supporting the observation of clinical symptoms, histological analysis shows tissue damage consistent with the pathology of MOG<sub>35-55</sub> induced EAE in C57BL/6 mice. Large regions of demyelination were observed as expected following immunostaining for the myelin peptide MBP (fig.10A). Perivascular infiltration of macrophages into these lesions was demonstrated by isolectin-B4 staining (fig.10B). Also shown is the presence of axonal damage, in the form of APP-aggregation, in regions of leukocyte infiltration, as demonstrated by CD45-immunostaining (fig.10C).



**Figure 9:** Induction of MOG<sub>35-55</sub> EAE in C57/Bl6 mice. Representative plot indicating rise in clinical score of a C57/Bl6 mouse paralleled with loss in body weight following MOG<sub>35-55</sub> peptide induced EAE. Clinical score: 0.5, Partial limp tail. 1, Limp tail. 1.5, Limp tail and clumsy gait. 2, Clumsy gait, hind leg paresis. 2.5, Clumsy gait, hind leg paresis (partial dragging). 3, Paralysis of 1 or 2 hind limbs. 3.5, Paralysis of 1 or 2 hind limbs, forelimb weakness. 4, Paralysis of 1 or 2 hind limbs, forelimb paresis. 4.5, Paralysis of 1 or 2 hind limbs, paresis of forelimbs (cannot move or groom). 5, Moribund or dead.

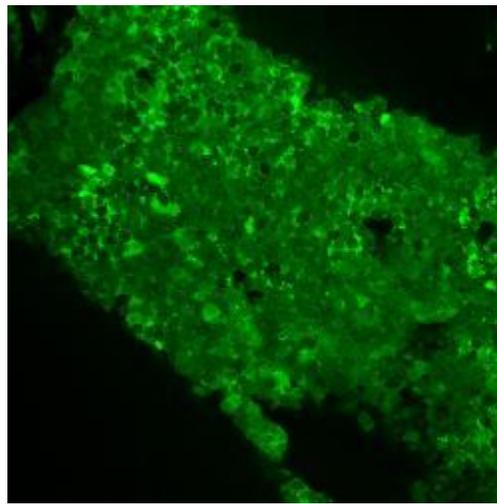


**Figure 10:** Immunofluorescent staining of inflammatory lesions in EAE spinal cord. (A) Cy3-conjugated staining for myelin basic protein (MBP) allows visualization of spinal cord lesions in EAE. (B) Cy3-conjugated isolectin-B4 staining depicting the perivascular infiltration of macrophages into the EAE lesion within the spinal cord. (C) Neuroinflammatory lesions exhibit APP aggregation (Cy3, red) in damaged neuronal axons as well as leukocyte infiltration as demonstrated by CD45 staining (FITC, green).

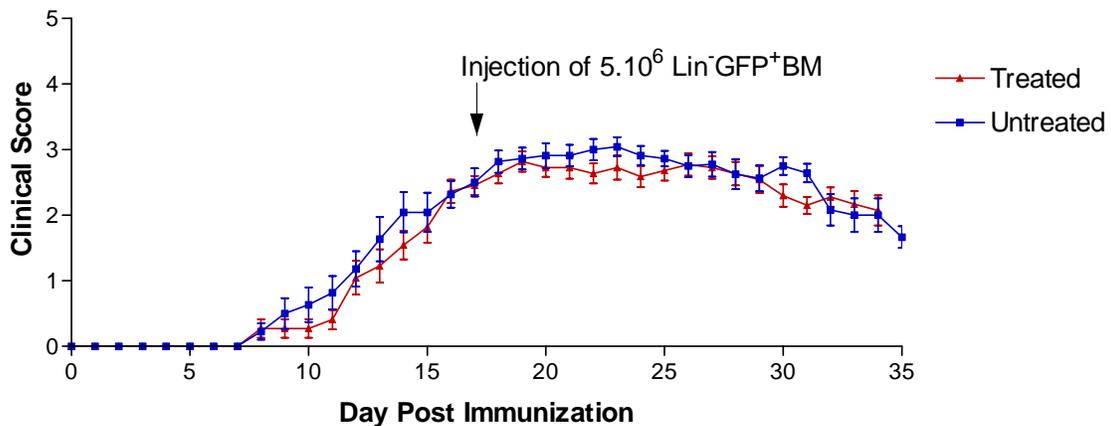
*Infiltration of GFP<sup>+</sup>Lin<sup>-</sup>BM into EAE spinal cord lesions following tail-vein injection:*

GFP<sup>+</sup>Lin<sup>-</sup>BM cells were isolated from  $\beta$ -actin GFP-transgenic mice (fig.11) and administered intravenously to both healthy and EAE mice via tail-vein injection in the absence of lethal irradiation (fig.12). As expected, no therapeutic effect was observed as the administered cells expressed only eGFP as a transgene. However, immediately after injection, GFP<sup>+</sup> cells were observed in inflammatory lesions of the spinal cords of EAE mice (fig.13). An overview of GFP<sup>+</sup>Lin<sup>-</sup>BM cell infiltration in the spinal cord is shown (fig.13A), as well as a typical spinal cord lesion from such a mouse (fig.13B). Quantitative analysis of histological sections shows these cells to be found only in the spleen and bone marrow of healthy mice and, interestingly, in the lesioned spinal cord, spleen and bone marrow of EAE mice (fig.14). Approximately 40 GFP<sup>+</sup> cells/mm<sup>2</sup> were observed in the spinal cords of these mice after 5 days, whereas no such cells were observed in the spinal cords of healthy control mice. Morphological analysis by confocal microscopy suggests these cells to have a microglial phenotype, while others are located perivascularly and appear to be endothelial-like (fig.15). The expression of markers for neurons ( $\beta$ TubIII), astrocytes (GFAP), oligodendrocytes (NG2) and endothelial cells (CD144) was analysed by confocal microscopy, however, these signals were never observed to colocalize with the eGFP signal of the Lin<sup>-</sup>BM cells (fig.16A-D). Labeling with isolectin-B4 demonstrates the majority of the Lin<sup>-</sup>BM within the spinal cord to be microglia (fig. 17). Quantification of cells engrafting into spinal cord lesions shows that migration is optimal when cells are administered during severe stages of disease (clinical score 3) whereas fewer cells are observed to engraft into lesions following their

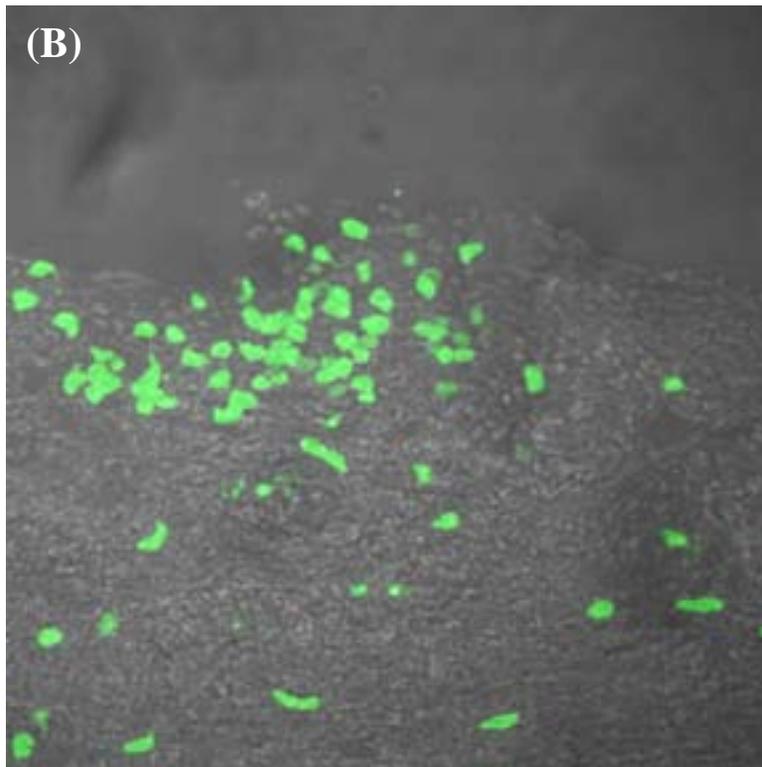
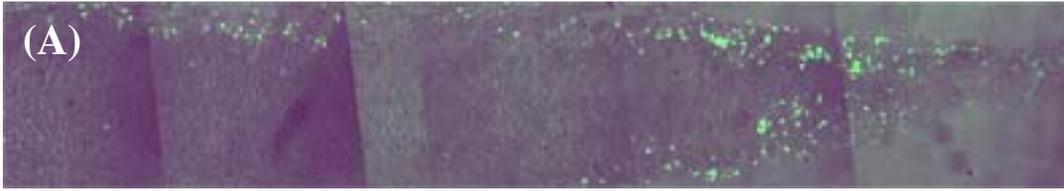
administration at earlier stages (fig.18A). Quantification of isolectin-B4 positive cells within spinal cord lesions tells that approximately 68% of GFP<sup>+</sup> cells within these lesions are of a microglial phenotype (fig.18B). The pan-leukocyte marker CD45 is expressed on the surface of almost the entire Lin<sup>-</sup>BM population, as demonstrated by FACS analysis (fig. 19). Low levels of CD45 expression were observed following confocal microscopy on cells engrafted in spinal cord lesions (fig 20).



**Figure 11:** Confocal image showing a section through the bone marrow of a  $\beta$ -actin-GFP transgenic mouse from which Lin<sup>-</sup>BM cells were isolated for migration studies.

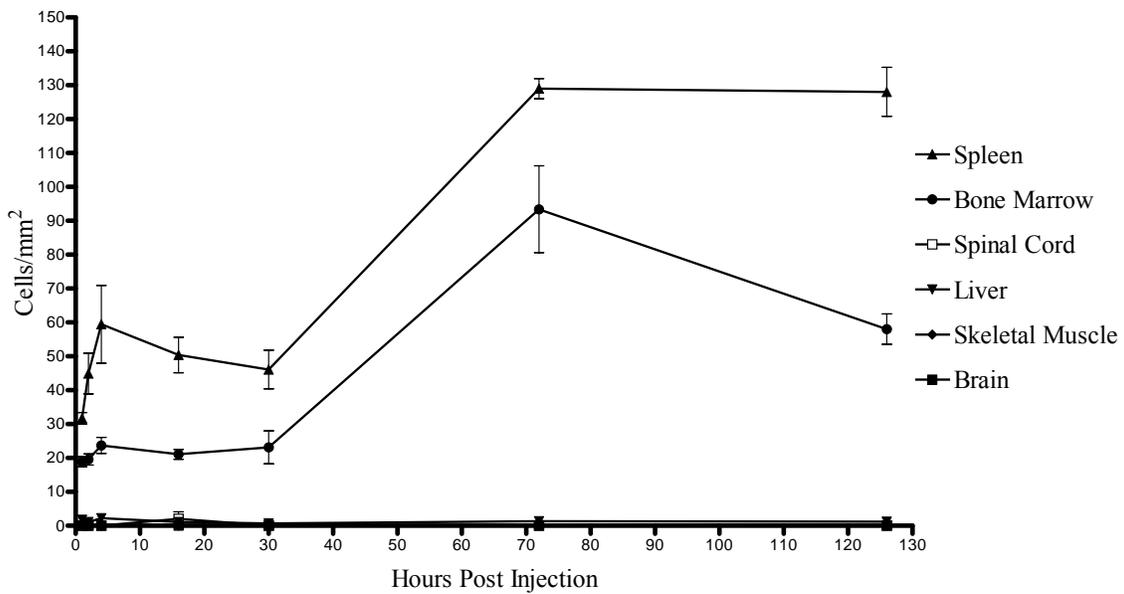


**Figure 12:** Migration of GFP<sup>+</sup>Lin<sup>-</sup>BM to spinal cord lesions in EAE mice. (A) Average clinical history of EAE mice with and without tail-vein GFP<sup>+</sup>Lin<sup>-</sup>BM injection.

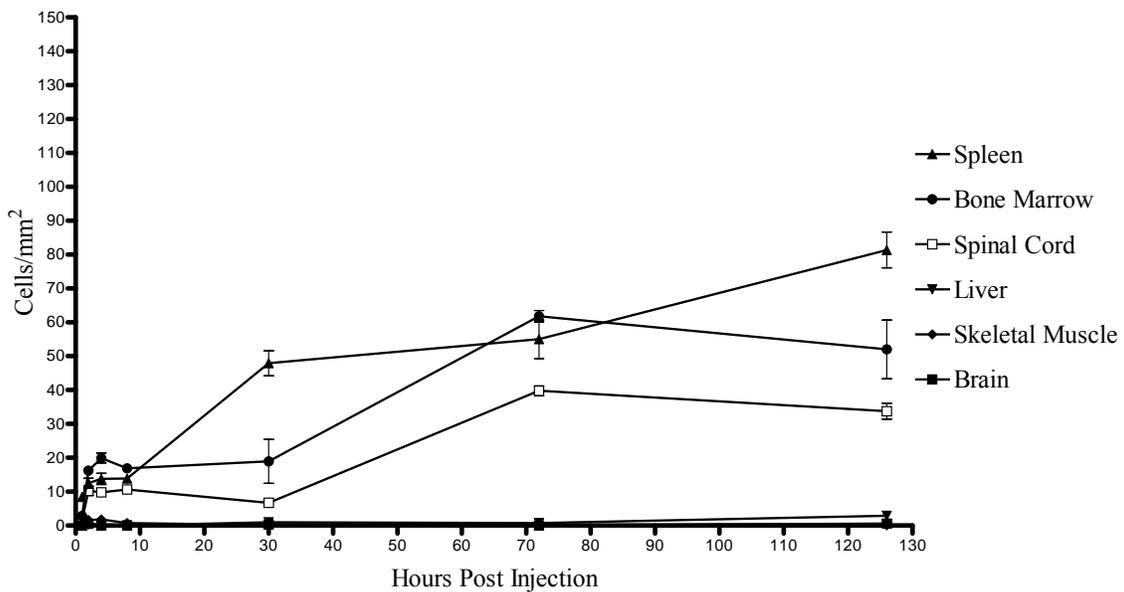


**Figure 13:** Tail-vein injection of GFP<sup>+</sup>Lin<sup>-</sup>BM into EAE mice. GFP<sup>+</sup> cells found in spinal cord lesions of EAE mice 2 weeks after tail-vein injection of GFP<sup>+</sup>Lin<sup>-</sup>BM. GFP<sup>+</sup>Lin<sup>-</sup>BM from  $\beta$ -actin-GFP mice were administered to EAE mice at the peak of disease symptoms via intravenous injection into the tail-vein. (A) Overview of infiltration within posterior region of spinal cord. (B) Detailed view of a single spinal cord lesion.

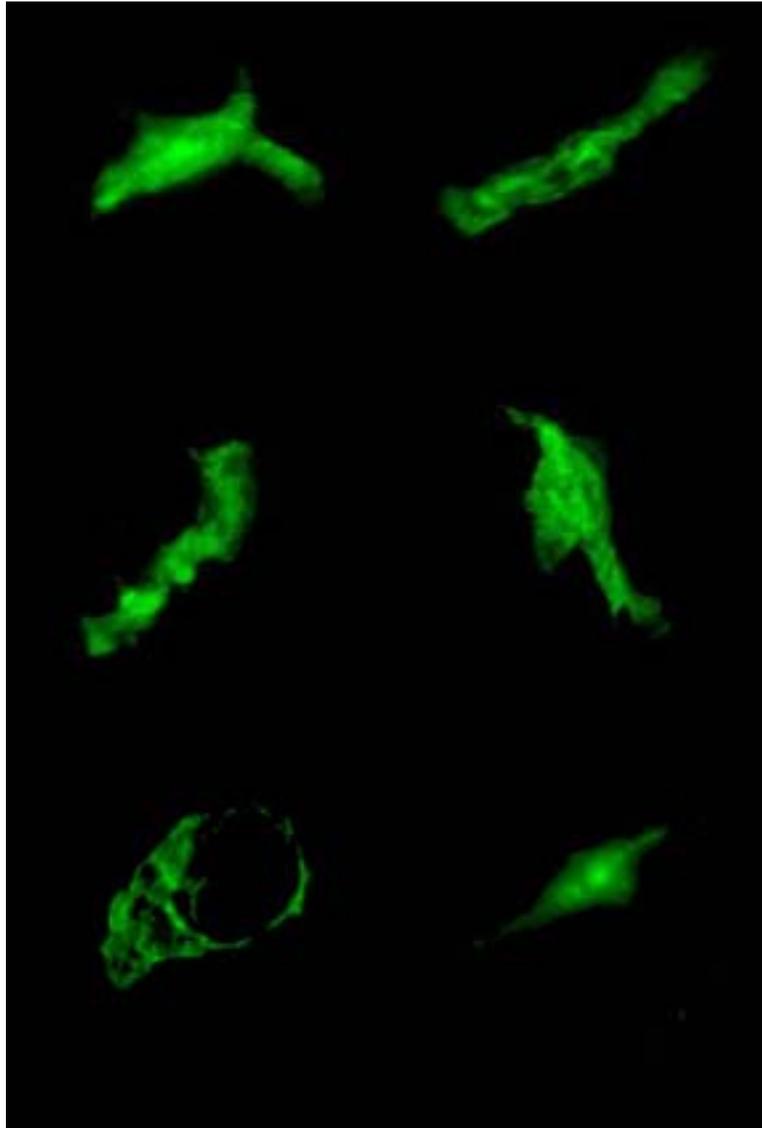
### Migration of hematopoietic precursor cells in healthy mice



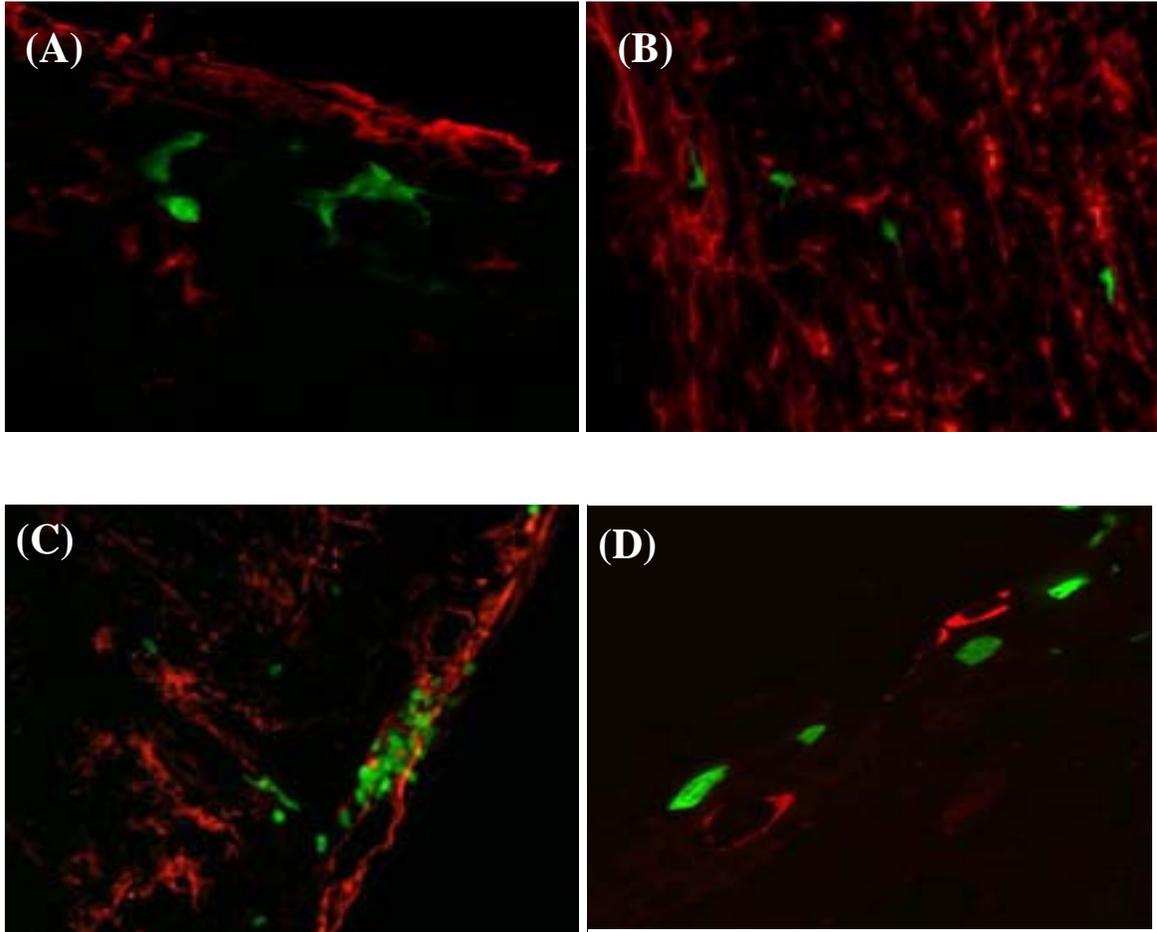
### Migration of hematopoietic precursor cells towards the spinal cord in Experimental Autoimmune Encephalomyelitis



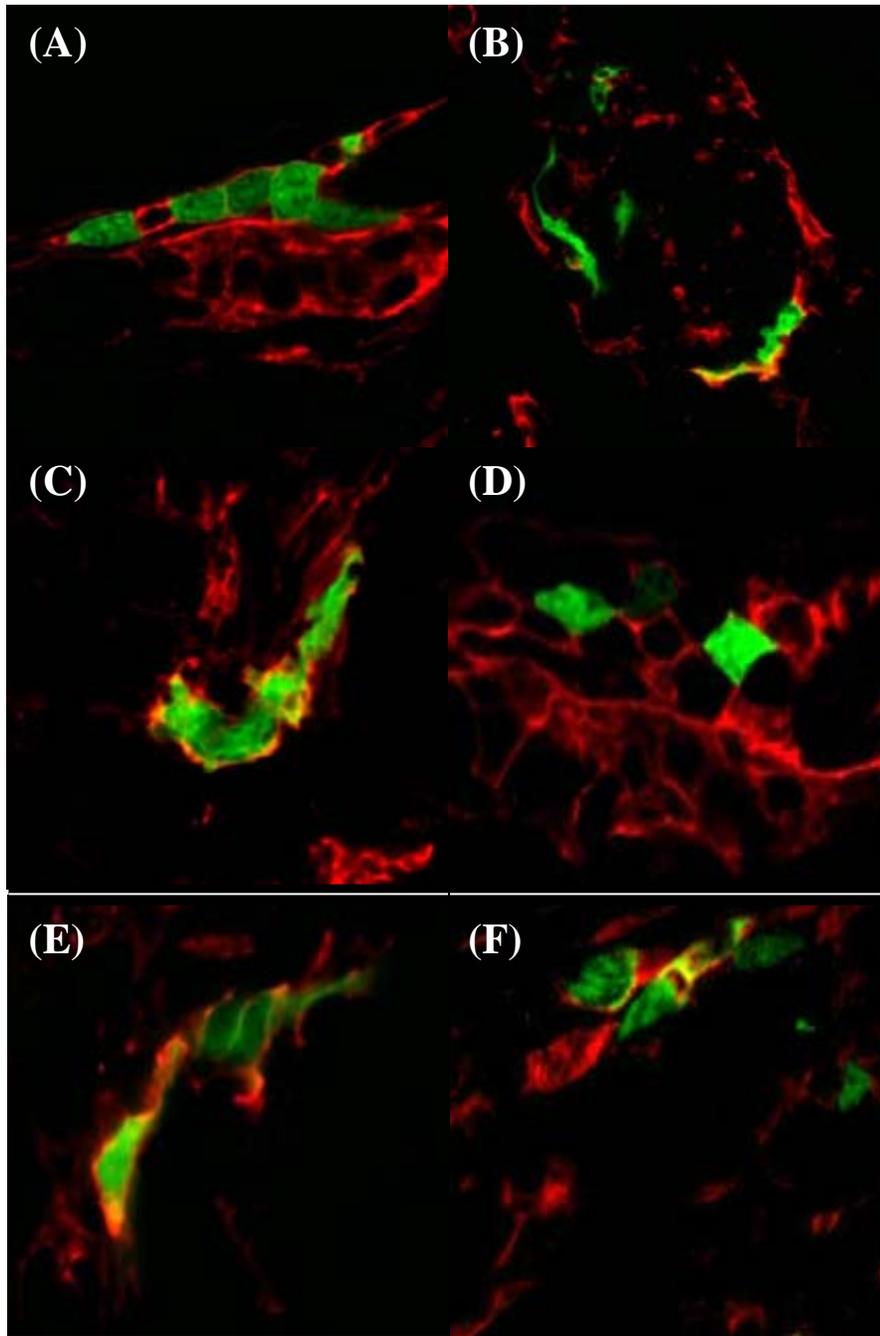
**Figure 14:** Migration of GFP<sup>+</sup>Lin<sup>-</sup>BM to spinal cord lesions in EAE mice. Plots illustrating the number of GFP<sup>+</sup> cells observed in various tissues over 5 days after tail-vein injection of GFP<sup>+</sup>Lin<sup>-</sup>BM in healthy and EAE mice.



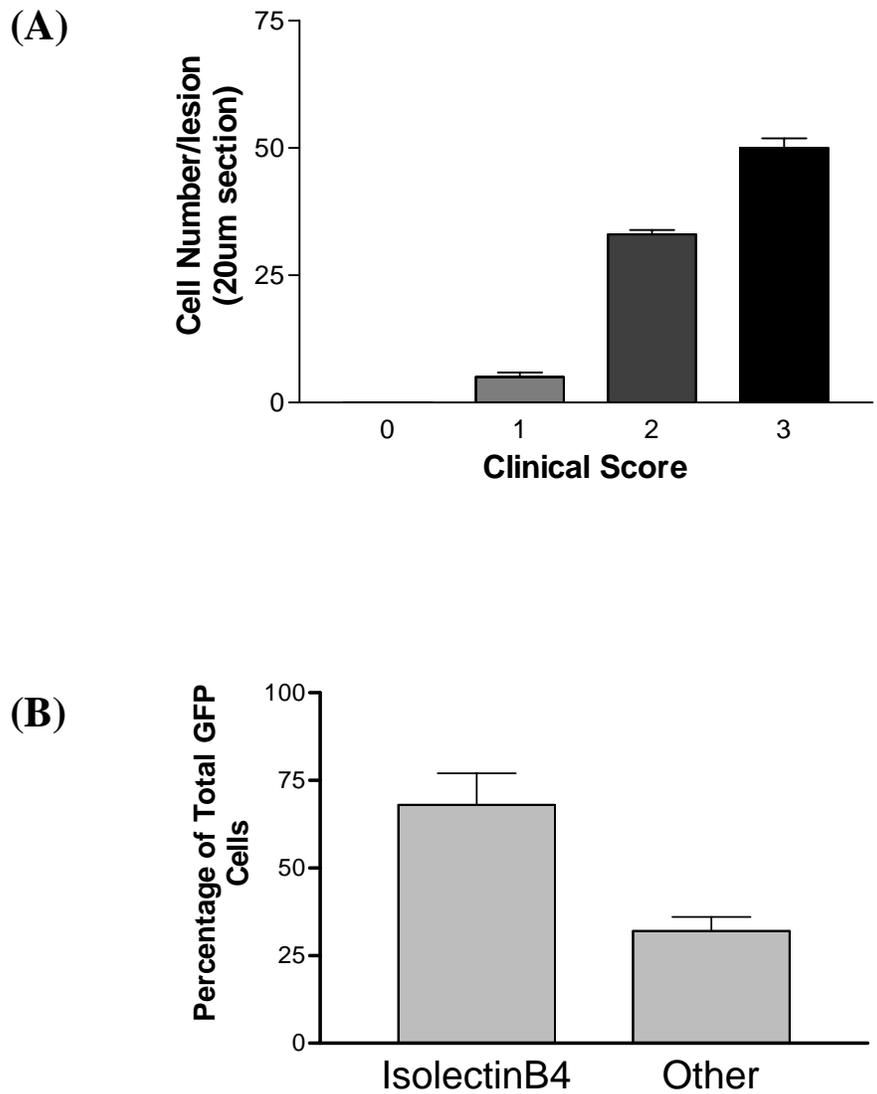
**Figure 15:** Confocal images demonstrating variety of morphologies displayed by GFP<sup>+</sup>Lin<sup>-</sup>BM following engraftment into the damaged spinal cord.



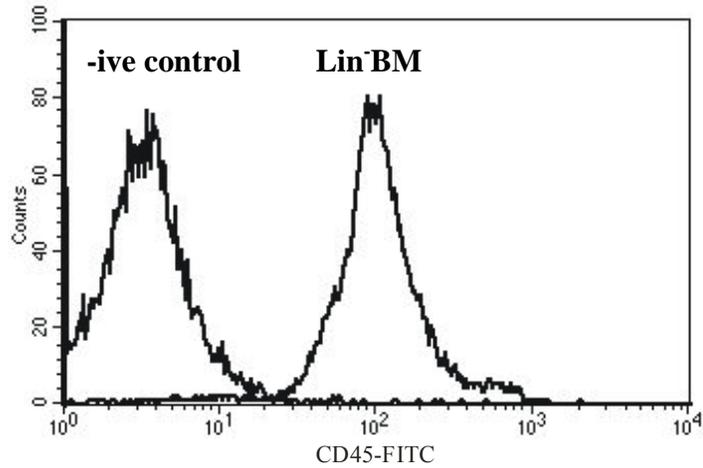
**Figure 16:** Immunohistochemistry analysis of transplanted cells following tail-vein injection of GFP<sup>+</sup>Lin<sup>-</sup>BM into EAE mice. (A) Cy3-conjugated  $\beta$ TubIII staining. (B) Cy3-conjugated GFAP staining. (C) Cy3-conjugated NG2 staining. (D) Cy3-conjugated CD144 staining.



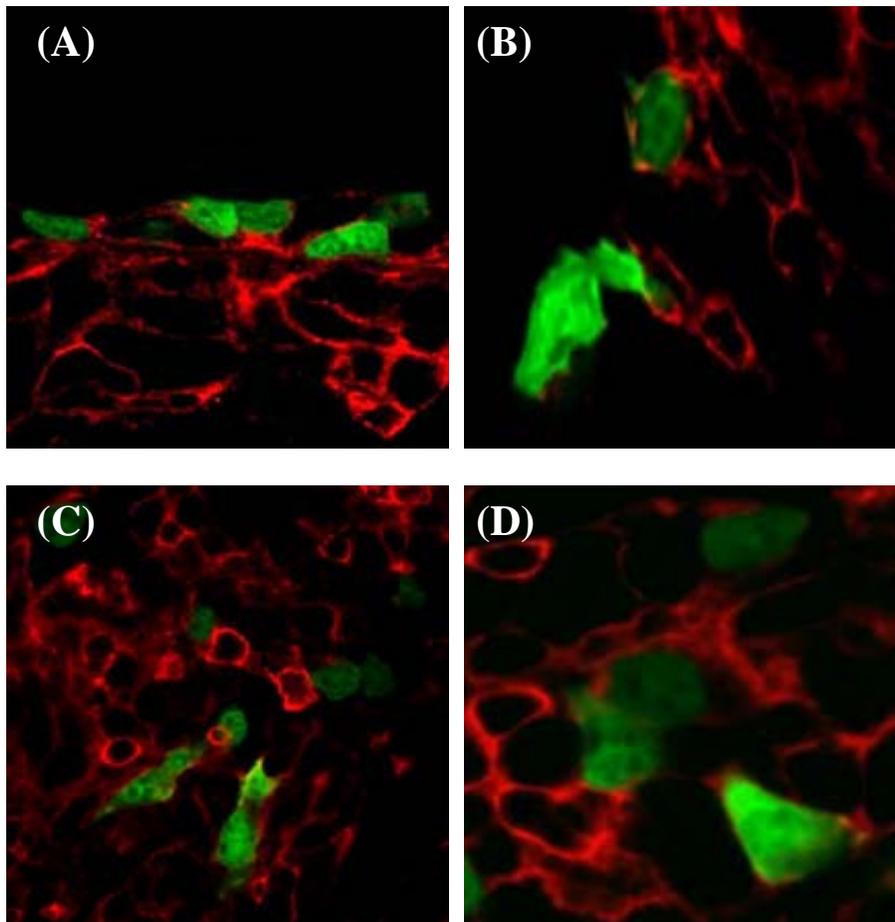
**Figure 17:** (A-F) Isolectin-B4 labeling shows a large subset of GFP<sup>+</sup> cells in the damaged spinal cord to have a microglial phenotype.



**Figure 18:** (A) Quantification of GFP<sup>+</sup>Lin<sup>-</sup>BM cells found in spinal cord lesions of EAE mice following their tail-vein injection at various stages in disease course. (B) Quantification of islectin-B4 positive GFP<sup>+</sup>Lin<sup>-</sup>BM cells in spinal cord lesions of EAE mice (n=300).



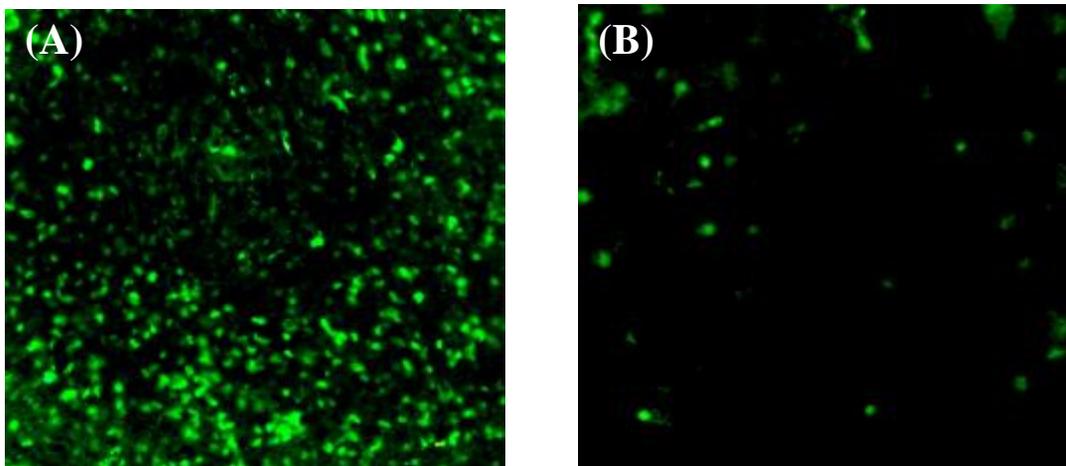
**Figure 19:** Analysis of CD45 expression in Lin<sup>-</sup>BM cells. FACS analysis of freshly prepared Lin<sup>-</sup>BM showing CD45 expression on majority of cells.



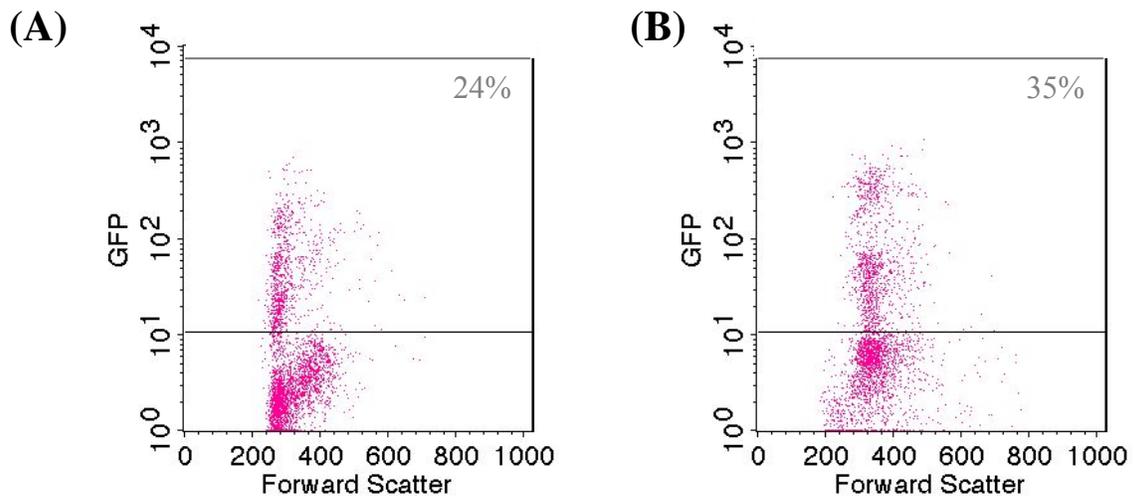
**Figure 20:** Analysis of CD45 expression in Lin<sup>-</sup>BM cells. (A-D) Immunostaining for CD45 on spinal cord sections from EAE mice following tail-vein injection of GFP<sup>+</sup>Lin<sup>-</sup>BM demonstrating loss of CD45 immunoreactivity from Lin<sup>-</sup>BM in-vivo.

Treatment of EAE mice with IL-10 transgenic Lin<sup>-</sup>BM:

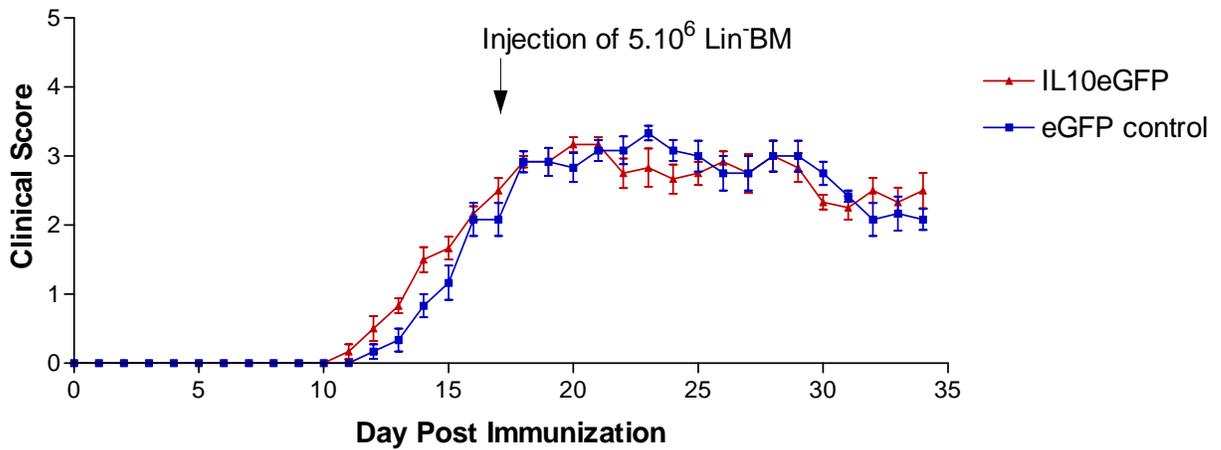
With the discovery that Lin<sup>-</sup>BM target specifically to inflammatory lesions in the spinal cords of EAE mice, the next step was to exploit this finding and attempt the delivery of therapeutic genes to these lesions with the aim of achieving relief in disease symptoms. Due to inefficient transduction of Lin<sup>-</sup>BM in-vitro, an approach was taken whereby Lin<sup>-</sup>BM were transduced overnight with concentrated lentiviral particles (see Materials and Methods) and transplanted into lethally irradiated recipient mice on the following day. Transgenic Lin<sup>-</sup>BM were isolated from transplanted mice after 3 months (see fig.21, 22) and administered to EAE mice via tail vein injection. Lin<sup>-</sup>BM were administered at the peak of disease, when their migration to spinal cord lesions is most efficient (see fig.18A), and mice were then observed for changes in the clinical course of disease over a period of two weeks. Unfortunately, no significant difference between control and treated mice was observed during this period (fig.23).



**Figure 21:** Images of spleen (A) and bone marrow (B) from mice 3 months after transplantation with Lin<sup>-</sup>BM transduced with pLenti6/V5<sup>PGK</sup>IL-10<sup>PGK</sup>eGFP viral particles.



**Figure 22:** FACS analysis of peripheral blood (A) and splenocytes (B) from mice 3 months after transplantation with Lin<sup>-</sup>BM transduced with pLenti6/V5<sup>PGK</sup>IL-10<sup>PGK</sup>eGFP viral particles.



**Figure 23:** Average clinical history of EAE mice following tail vein injection of IL10eGFP<sup>+</sup>Lin<sup>-</sup>BM and eGFP<sup>+</sup>Lin<sup>-</sup>BM (n=6).

## Discussion

### Isolation of murine lineage negative bone marrow cells ( $Lin^-BM$ ):

A very reliable protocol for the negative selection of differentiated cells from isolated bone marrow was developed. The average number of cells obtained following both negative selection and c-Kit/Sca-1 analysis was compared (Results, fig.1). The procedure of negative selection can remove up to 85% of all cells to give a population enriched for stem cells. This enriched population, when examined for cells expressing the hematopoietic stem cell markers c-Kit and Sca-1, has approximately 2.5% of cells positive for both markers. While this 2.5% is also known to consist of some hematopoietic progenitor cells it is still quite a significant yield as the number of stem cells in a pure bone marrow preparation is usually between 0.01% and 0.05%.

A critical parameter in all stem and progenitor cell studies is the purity of the cell population used. In the ideal situation one would use a pure population of stem cells devoid of any differentiated cells, however, the isolation of sufficient numbers of primary stem cells often presents difficulties. With total bone marrow containing very few hematopoietic stem cells, as well as the fact that their in-vitro maintenance and expansion is still not possible, this is clearly a problem. On the other hand, to use a total bone marrow preparation introduces the strong possibility of artifacts arising from the presence of terminally differentiated cells in the preparation. Most studies using hematopoietic cells to treat disease or to deliver therapeutic genes to animal models of disease have

administered cells by bone marrow transplantation<sup>15-18, 177, 178</sup>. While a pure stem cell population is not required to perform bone marrow transplantation, this approach is “dirty” in that hematopoietic cells are delivered to many tissues, leading to possible unwanted side effects due to expression of the therapeutic gene in tissues not affected by the disorder in question. Reconstitution of the hematopoietic system with genetically modified hematopoietic cells may also compromise the normal function of the immune system in recipient animals. In this study a compromise was taken to use a population devoid of terminally differentiated cells which is enriched for stem and progenitor cells, the lineage negative population (Lin<sup>-</sup>BM). Supporting the use of the Lin<sup>-</sup>BM population for therapeutic purposes is a recent study demonstrating the successful treatment of retinal degeneration following their intravitreal injection<sup>179</sup>.

*Construction of Lentiviral vectors:*

This study employs the use of a third generation lentiviral gene delivery system, pLenti6/V5 (Results, fig.2). This third generation system is self-inactivating (SIN) to prevent the possible unwanted expression of genes proximal to the site of integration. The modified 3' LTR allows viral packaging but selfinactivates the 5' LTR for biosafety purposes<sup>168</sup>. The 3' LTR element also contains a polyadenylation signal for efficient transcription termination and polyadenylation of mRNA in transduced cells<sup>169, 170</sup>. An added advantage of this modification is that the elimination of transcription from the viral LTR allows the possibility for tissue specific expression upon the use of the appropriate

promoter. While the use of a CMV promoter is the favored choice for transgene expression, its use in hematopoietic stem cells is often problematic due to strong silencing of transgenes, thus constructs were also cloned using a PGK promoter for comparison. Although normal expression under the CMV promoter is more efficient, PGK-mediated expression is sustained over longer periods and, although still quite low, is more efficient in hematopoietic cell lineages. Successful expression of eGFP from pLenti6/V5-eGFP constructs can be observed where eGFP fluorescence in pLenti6/V5<sup>CMV</sup>eGFP and pLenti6/V5<sup>PGK</sup>eGFP transfected 293FT cells is shown (Results, fig.2B, C).

Supernatant from 293FT cells transfected with pLenti6/V5<sup>PGK</sup>IL10 and pLenti6/V5<sup>PGK</sup>IL10<sup>PGK</sup>eGFP constructs was used to transduce HeLa cells (see Appendix to Materials and Methods). Expression levels of IL-10 were compared to that of a pLenti<sup>PGK</sup>eGFP control in order to determine the optimal vector for transduction of Lin<sup>-</sup>BM (Results, fig.3). Expression levels from pLenti6/V5<sup>PGK</sup>IL10<sup>PGK</sup>eGFP were acceptable, whereas levels of IL-10 expressed from pLenti6/V5<sup>PGK</sup>IL10 were above the detection levels of the ELISA (upper limit 2000pg/ml). The pLenti6/V5<sup>PGK</sup>IL10<sup>PGK</sup>eGFP construct was chosen for use in Lin<sup>-</sup>BM as the expression of eGFP allows for tracking of transduced cells in-vivo. While expression levels from pLenti6/V5<sup>CMV</sup>IL10 and pLenti6/V5<sup>CMV</sup>IL10<sup>CMV</sup>eGFP constructs were beyond quantification by the ELISA assay (upper limit 2000pg/ml) following analysis in HeLa cells, these constructs could not be used for the transduction of Lin<sup>-</sup>BM due to silencing of expression from the CMV promoter in this cell population. Various transduction protocols (see Appendix to

Materials and Methods) using pLenti6/V5<sup>PGK</sup>IL-10<sup>PGK</sup>eGFP were compared to develop a protocol for the optimal transduction of Lin<sup>-</sup>BM (fig.4).

*Bone marrow transplantation of lethally irradiated C57/Bl6 mice with pLenti6/V5eGFP transduced Lin<sup>-</sup> BM:*

Successful transduction of Lin<sup>-</sup>BM with a lentiviral vector was demonstrated in-vivo by bone marrow transplantation (Results, fig.5). FACS analysis of bone marrow from a transplant recipient of pLenti6/V5eGFP-transduced Lin<sup>-</sup>BM is shown to have an identical percentage of GFP<sup>+</sup> bone marrow cells as in  $\beta$ -actin-GFP transgenic mice.

Histological analysis showed these GFP<sup>+</sup>Lin<sup>-</sup>BM cells to engraft within many peripheral organs as well as the central nervous system at 4 weeks following transplantation, thus confirming previous reports that hematopoietic cells may engraft within non-lymphoid tissues <sup>3,4</sup> (Results, fig.6-8). Almost all cells in the spleen were found to express GFP, as expected, while the liver also contained a large proportion of GFP<sup>+</sup> cells (Results, fig.5). With respect to the CNS, as with previous reports a small number of GFP<sup>+</sup> cells were observed in the cerebellum and choroid plexus (Results, fig, 6)<sup>3,4</sup>. Significant numbers of GFP<sup>+</sup> cells were observed throughout the entire spinal cord (Results, fig.7), a finding previously unreported in bone marrow transplantation studies.

In order to interpret these data critically it must be understood that transplanted HSCs are slightly altered in their properties in comparison to normal physiological conditions. HSCs in transplanted mice are more frequently in cell cycle for a period of up to 4 months post-transplantation, and thus are already at a disadvantage when it comes to homing into the bone marrow as the engraftment of quiescent HSCs is known to be more efficient than that of HSCs in the cell cycle<sup>132</sup>. Thus, what is observed following bone marrow transplantation may not be the regular physiological activity of hematopoietic stem cells but a behavior induced under extreme conditions. To truly understand what is happening in these studies one must understand the changes which lethal irradiation confers upon normal physiology and decide if it is this which gives rise to the engraftment of hematopoietic cells in non-lymphoid tissue before concluding that such engraftment may occur under normal physiological conditions. Thus, it cannot be concluded here that Lin<sup>-</sup>BM may give rise to resident cells of the CNS under normal conditions but only under extreme conditions such as those induced by lethal irradiation. What can be concluded from this data, however, is that the entry of hematopoietic cells into the spinal cord may be used for the delivery of therapeutic genes to the pathological inflammatory spinal cord lesions observed in EAE mice.

Induction of Experimental Autoimmune Encephalomyelitis (EAE):

A reliable procedure for the induction of EAE in C57/Bl6 mice was established following the protocol described (see Materials and Methods). A typical disease course from a mouse in which the disorder was induced is shown (Results, fig.9). A correlation between the onset of clinical symptoms and a decrease in body weight of the animal is apparent. Active immunization with MOG<sub>35-55</sub> in C57Bl/6 mice induces a chronic, non-remitting disease course via activation of a type I immune response directed by autoreactive CD4<sup>+</sup> Th1 cells<sup>39</sup>. Consistent with this, such a disease course was observed consistently in immunized mice. Supporting the observation of clinical symptoms, histological analysis shows tissue damage consistent with previous studies on the pathology of MOG<sub>35-55</sub> induced EAE in C57BL/6 mice<sup>39, 180, 181</sup>. Large regions of demyelination were observed as expected following immunostaining for the myelin peptide MBP (Results, fig.10A). Perivascular infiltration of macrophages into these lesions was demonstrated by isolectin-B4 staining (Results, fig.10B). Also shown is the presence of axonal damage, in the form of APP-aggregation, in regions of leukocyte infiltration, as demonstrated by CD45-immunostaining (Results, fig.10C).

Infiltration of GFP<sup>+</sup>Lin<sup>-</sup>BM into EAE spinal cord lesions following tail-vein injection:

With the aim of looking towards a clinical application for the delivery of therapeutic genes to the central nervous system using hematopoietic stem/progenitor cells, an

approach which bypasses the lethal irradiation required for bone marrow transplantation was taken. GFP<sup>+</sup>Lin<sup>-</sup>BM cells were isolated from  $\beta$ -actin GFP-transgenic mice (fig.11) and administered intravenously to both healthy and EAE mice via tail-vein injection without lethal irradiation (Results, fig.12). As the administered cells expressed only eGFP as a transgene, there was no therapeutic effect observed. Immediately after injection, however, GFP<sup>+</sup> cells were observed in inflammatory lesions of the spinal cords of EAE mice (Results, fig.13). Quantification of cell engraftment shows these cells to be found only in the spleen and bone marrow of healthy mice and, interestingly, in the lesioned spinal cord, spleen and bone marrow of EAE mice (Results, fig.14). Approximately 40 GFP<sup>+</sup> cells/mm<sup>2</sup> were observed in the spinal cords of these mice after 5 days, whereas no such cells were observed in the spinal cords of healthy control mice. An overview of the posterior region of the spinal cord of one such treated EAE mouse is shown (fig.13A). Aggregates of GFP<sup>+</sup>Lin<sup>-</sup>BM cells were observed sporadically along the length of the spinal cord at regions corresponding to inflammatory lesions. A typical spinal cord lesion from such a mouse showing infiltration of GFP<sup>+</sup>Lin<sup>-</sup>BM cells is shown (Results, fig.13B).

Confocal microscopy analysis of the morphology of engrafted cells suggests them to have a microglial phenotype, while others are located perivascularly and appear to be endothelial-like (Results, fig.15). Studies analysing the phenotype of CNS-engrafted hematopoietic cells argue for their development of neuronal or glial phenotypes<sup>3, 4, 9, 182, 183</sup>. In order to question such controversial reports, analysis of the Lin<sup>-</sup>BM cells found within EAE spinal cord lesions was performed in order to determine their phenotype. Confocal microscopy was used to analyse the expression of markers for neurons

( $\beta$ TubIII), astrocytes (GFAP), oligodendrocytes (NG2) and endothelial cells (CD144), however, colocalization with the eGFP signal of the Lin<sup>-</sup>BM cells was never observed (Results, fig.16). As the analysis was performed only 2 weeks after administration one may argue that a longer time period may be required if the cells were to transdifferentiate. However, studies have since demonstrated such reports of transdifferentiation to be an artifact of cell fusion events<sup>145</sup>. The fact that the engrafted cells do not express the endothelial marker CD144 suggests that the perivascular cells observed are most likely microglia/pericytes. This is consistent with a recent report claiming that highly purified hematopoietic stem cells give rise to functional endothelial cells in many tissues with the exception of cerebral vasculature<sup>139</sup>. Isolectin-B4 labeling demonstrates the majority of the Lin<sup>-</sup>BM within the spinal cord to have microglial phenotype (Results, fig.17). This finding is in agreement with the theory that microglia are hematopoietic in origin in the adult as well as in the developing brain<sup>184</sup>. Supporting this data are recent reports that hematopoietic cells retain their hematopoietic phenotype upon engraftment into non-hematopoietic tissues<sup>185-187</sup>. In one such study examining the phenotype of hematopoietic derived cells in the CNS following bone marrow transplantation, among the cells that crossed the endothelium of the cerebral cortex, 99.7% were identified as perivascular macrophages while newly formed parenchymal microglia were found in significant numbers only in the cerebellum and at injury sites. This supports strongly the data presented that a subset of the Lin<sup>-</sup>BM found within EAE spinal cord lesions are of a microglial phenotype. Quantification shows that approximately 68% of GFP<sup>+</sup> cells found within spinal cord lesions are isolectin-B4 positive cells and thus are of a microglial phenotype (fig.18B). FACS analysis demonstrates the pan-leukocyte marker CD45 to be

expressed strongly on the surface of almost the entire Lin<sup>-</sup>BM population (fig. 19). Confocal microscopy analysis showed low levels of CD45 expression on cells engrafted in spinal cord lesions (fig 20). This apparent change in CD45 expression may be explained by studies of microglia/macrophages in the CNS of EAE mice suggesting microglia to express low or intermediate levels of CD45 in comparison to infiltrating splenic macrophages which express intermediate or high levels of CD45<sup>188</sup>. Earlier studies have also demonstrated the low level of CD45 expression by microglia in the normal and the inflamed rat CNS<sup>189</sup>.

It has long been accepted that microglia develop during embryogenesis and remain in the central nervous system throughout life, while it has been constantly argued whether they develop from neuroepithelial or hematopoietic precursors. Recent data suggest, however, that not only do they develop from hematopoietic precursors but that their formation occurs also in the healthy adult brain<sup>9</sup>. However, this study was performed by transplanting labeled bone marrow into lethally irradiated mice. Total body irradiation clearly has an effect on all tissues possibly rendering them into a state of shock in which factors signaling for the recruitment of hematopoietic cells are secreted, which would explain the presence of hematopoietic cells within many tissues following bone marrow transplantation. Thus, whether hematopoietic cells contribute to these tissues under normal physiological conditions is strongly debatable. The observed migration of Lin<sup>-</sup>BM to the spleen and bone marrow of healthy mice (Results fig.14) and their significant migration to the spinal cord in EAE mice argues for the migration of Lin<sup>-</sup>BM to non-lymphoid tissues to occur only under conditions of tissue damage. It has also been

suggested that hematopoietic derivatives within the brain may also acquire neuronal phenotypes<sup>3, 114, 184</sup>. Similar studies suggest the ability of hematopoietic cells to give rise to myocardium<sup>115, 187</sup>, hepatocytes<sup>5, 138</sup>, skeletal muscle<sup>116, 190</sup> and endothelium<sup>139</sup>. While it has since been demonstrated that such transdifferentiation is more likely explained by cell fusion events<sup>144, 145</sup>, the general theme observed in these studies is the repopulation of non-hematopoietic tissues with hematopoietic cells following injury or other forms of conditioning, e.g. lethal irradiation. One may thus argue that an injury stimulus is required in order for circulating hematopoietic cells to home into and reside within these tissues.

*Treatment of EAE mice with IL-10 transgenic Lin<sup>-</sup>BM:*

The site specific migration of Lin<sup>-</sup>BM to inflammatory lesions in the spinal cord of EAE mice led to the application of this procedure towards delivering a therapeutic gene to these lesions with the aim of achieving symptomatic and pathological improvements in diseased mice. Inefficient in-vitro transduction of Lin<sup>-</sup>BM with lentiviral particles meant developing an alternative approach for the introduction of a transgene into Lin<sup>-</sup>BM. An approach was taken whereby Lin<sup>-</sup>BM were transduced overnight with concentrated lentiviral particles (see Materials and Methods) and transplanted into lethally irradiated recipient mice on the following day. Following complete reconstitution of bone marrow, 3 months after transplantation, transgenic Lin<sup>-</sup>BM were isolated from recipient mice (see Results, fig.21, 22) and administered to EAE mice via tail vein injection. While this in-vivo preparation of transduced Lin<sup>-</sup>BM turned out to be no more efficient than the in-vitro approach (see Results, fig.4), the in-vivo approach allowed the cells to be kept in

their natural environment and thus avoided the differentiation of Lin<sup>-</sup>BM occurring during in-vitro transduction. Lin<sup>-</sup>BM were administered at the peak of disease, when most efficient migration to spinal cord lesions is observed (see fig.18A). Mice were then observed for changes in the clinical course of disease over a period of two weeks, however, no significant difference between control and treated mice was observed during this period (fig.23).

The absence of a therapeutic effect in EAE mice treated with IL-10 overexpressing Lin<sup>-</sup>BM may be explained by a number of reasons. A most obvious reason would be the inability of IL-10 to down-regulate the immune response. While this cannot be completely ruled out there is convincing evidence suggesting this not to be the case<sup>20, 92, 93</sup>. Direct injection of fibroblasts transduced with IL-10-expressing retroviral vectors into the CNS of EAE mice was demonstrated to ameliorate disease, however, CNS delivery of IL-10-expressing adenovirus failed to do so<sup>93</sup>. Thus, IL-10 actions may depend greatly on the local cytokine microenvironment in areas of IL-10 expression. Other studies of adenoviral-mediated delivery of IL-10 to the CNS reported prevention of disease and blockage of disease progression only when high levels of IL-10 expression were achieved following intracranial injection of virus<sup>20</sup>. The direct migration of Lin<sup>-</sup>BM towards the lesions which was observed, however, should rule out the possibility that IL-10 expression was not targeted to sites of inflammation. While the determination of effective cytokine expression levels is technically very difficult, it is believed that a consistent expression rather than repeated injections of the protein is of more benefit<sup>93</sup>. Induction of EAE was prevented in mice transgenic for human IL-10 with serum levels of 400-700

pg/ml. With ELISA data showing relatively intermediate levels of IL-10 expression from the construct used (approx. 800pg/ml after 48hrs, see Results, fig.3), it may be argued that to achieve a therapeutic dose higher levels must be achieved. Along with the fact that only one third of the hematopoietic cells administered to the diseased mice were expressing the transgene (Results, fig.21), it is thus likely that inefficient levels of IL-10 expression are responsible for the absence of a therapeutical effect.

## Conclusions

A reliable protocol for the isolation of hematopoietic progenitor cells (Lin<sup>-</sup>BM) was developed. Lin<sup>-</sup>BM were isolated from the bone marrow of  $\beta$ -actin GFP-transgenic mice and transplanted into lethally irradiated wild-type recipients in order to analyse the engraftment of hematopoietic progenitors into non-hematopoietic tissues. Engraftment was observed in the brain and spinal cord as well as in other non-hematopoietic tissues. When administered to mice afflicted with experimental autoimmune encephalomyelitis (EAE), a mouse model of the neurodegenerative disorder multiple sclerosis (MS), GFP<sup>+</sup>Lin<sup>-</sup>BM cells were observed to migrate specifically into spinal cord lesions with no engraftment into peripheral tissues or non-inflamed nervous tissue. This targeted migration was not observed in healthy controls and correlated with the severity of disease, optimal migration being observed following the administration of the cells during the peak of the disease course (clinical score 3). The migration of Lin<sup>-</sup>BM to the spleen and bone marrow of healthy mice and their significant migration to the spinal cord in EAE mice argues for the migration of Lin<sup>-</sup>BM to non-lymphoid tissues to occur only under conditions of tissue damage, thus questioning previous claims that microglia in the adult brain are of hematopoietic origin.

An average of 40 GFP<sup>+</sup>cells/mm<sup>2</sup> was observed in the spinal cord of EAE mice, these cells being clustered in regions of demyelination. The observed migration of Lin<sup>-</sup>BM to the spleen and bone marrow of healthy mice (Results fig.14) and their significant migration to the spinal cord in EAE mice argues for the migration of Lin<sup>-</sup>BM to non-lymphoid tissues to occur only under conditions of tissue damage. Immunohistochemical

analysis demonstrated 68% of GFP<sup>+</sup>Lin<sup>-</sup>BM cells engrafted in the spinal cord to be microglia by isolectin-B4 staining and low CD45 immunoreactivity. Transgenic Lin<sup>-</sup>BM, lentivirally transduced to overexpress the anti-inflammatory cytokine IL-10, were administered intravenously to EAE mice at the peak of disease in an attempt to treat disease symptoms by suppressing the local immune response. The use of Lin<sup>-</sup>BM as a targeted gene-therapy delivery system was unsuccessful. The inability to achieve a therapeutic effect was most likely due to insufficient expression of IL-10 within the lesion sites.

Thus, it is shown that lineage-negative hematopoietic progenitor cells, when administered intravenously without prior lethal-irradiation of mice, migrate directly to lesions within the damaged spinal cord with little or no engraftment in other tissues and give rise to a microglial population. With improved gene transfer into hematopoietic progenitors this work may provide a means for the targeted delivery of therapeutic genes to inflammatory lesions of the spinal cord in a site-specific manner.

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Science is facts; just as houses are made of stones, so is science made of facts; but a pile of stones is not a house and a collection of facts is not necessarily science.

**Henri Poincare**

*French mathematician & physicist (1854 - 1912)*