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TREATING GM1 GANGLIOSIDOSIS WITH EX VIVO HEMATOPOIETIC STEM CELL GENE THERAPY WITHOUT USING TOTAL BODY IRRADIATION

A Masters Thesis Presented

By

Michael Whalen

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

AUGUST 31 2011

BIOLOGY

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August 31 2011

Dedication

This manuscript is dedicated to Linette L. Letendre for her love, support and refusal to let me quit.

Acknowledgements

Thank you, all members of the Esteves' Lab for all the help you provided me over the last two years and for being a wonderful lab family. Thank you, Lord Davide Gianni, Sourav Choudury, Dwijit Guhasarker, Stanley Leroy and Cara Weismann for teaching me many different lab protocols and providing constant technical assistance. Thank you Lorelei Stoica for your critical review of my qualifying examination presentation and your constant support. Thank you Diane Golebiowski for reminding me of all the 80's toys I have long forgotten and Stacy Maitland for your endless supply of humorous cat YouTube videos. I will never be able to get hall of the mountain kings out of my head. Thank you, Dr. Guangping Gao, Dr. Claudio Punzo, Dr. Chris Mueller and Dr. Thomas Fazzio for being on my Qualifying Exam Committee. Thank you Dr. Guangping Gao, Dr. Claudio Punzo, and Dr. Miguel Sena-Esteves for being on my Master's Thesis Committee. And finally thank you, Dr. Miguel Sena-Esteves for accepting me into your lab and being a wonderful, thought-provoking and attentive mentor throughout my time at Umass.

Abstract

GM1 gangliosidosis is an autosomal recessive lysosomal storage disease, caused by a deficiency in the enzyme β-galactosidase. The disease affects the CNS, liver, kidney, heart and skeletal system, leading to severe neurodegeneration and death. We propose to treat this disorder using ex vivo hematopoietic stem cell therapy. The effectiveness of this therapy requires the recruitment of transduced donor cells to the CNS. This is only found to occur after mice are conditioned with total body irradiation, due to the increase in CNS cytokine production and blood brain barrier permeability that occurs. As the use of total body irradiation in pediatric patients has been linked to future developmental problems, this myeloablation approach is often avoided in younger patients in favor of a conditioning regimen using the chemotherapy drugs, busulfan and cyclophosphamide. Whether donor cells can enter the CNS when a busulfan and cyclophosphamide conditioning regimen is used has not been determined. In this study we plan to quantify the cytokine and blood-brain barrier permeability increases necessary for donor cells to be recruited to the CNS after total body irradiation. We will then investigate whether busulfan and cyclophosphamide conditioning and/or the chronic neuroinflammation present in GM1 mice can produce similar conditions and facilitate the recruitment of donor hematopoietic stem cells to the CNS. Finally we will assess whether ex vivo hematopoietic stem cell gene therapy is still an effective therapy when busulfan and cyclophosphamide are used for myeloablative conditioning.

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List of Abbreviations and Nomenclature

GM1 = Monosialotetrahexosylganglioside, also short-hand for the disease GM1 gangliosidosis

β-Gal = β-galacosidase

CNS = Central Nervous System

BBB = Blood Brain Barrier

AAV = Adeno-Associated Virus

LV = Lentivirus

HSC = Hematopoietic Stem Cells

BMT = Bone Marrow Transplant

ALD = X-linked Adrenoleukodystrophy

WT = Wild-type

TBI = Total Body Irradiation

Bu = Busulfan

Cy = Cyclophosphamide

TNF-α = Tumor Necrosis Factor-α

IL-1β = Interleukin-1β

CCL2 = Chemokine CC Motif Ligand 2

CCL5 = Chemokine CC Motif Ligand 5

CCR2 = Chemokine CC Motif Receptor 2

CCR5 = Chemokine CC Motif Receptor 5

AD = Alzheimer's Disease

MS = Multiple Sclerosis

WNV = West Nile Virus

TNF-R1 = Tumor Necrosis Factor Receptor 1

EAE = Experimental Autoimmune Encephalomyelitis

Gy = Gray

Sham-TBI = Treatment where mice are put in the apparatus used to deliver radiation but no radiation is administered

GFP = Green Fluorescent Protein

BuCy2 = Commonly used myeloablative condition regimen where 16 mg/kg of busulfan is given over 4 days followed by 120 mg/kg of cyclophosphamide over 2 days, with two days occurring between the last dose and the injection of donor cells

IRES = Internal Ribosome Entry Site

X-gal = bromo-chloro-indolyl-galactopyranoside, which is a compound that can be cleaved by β-galactosidase to produce a blue insoluble product

Background and Significance:

GM1 Gangliosidosis

GM1 gangliosidosis is an autosomal recessive lysosomal storage disease caused by a deficiency in the enzyme β-galactosidase (β-gal) (Suzuki et al., 2001). This enzyme is responsible for hydrolyzing the terminal β-galactosyl residue from GM1 gangliosides, glycoproteins and glycosaminoglycans. When enzyme activity is low or absent due to a mutation in the β-gal gene the ganglioside GM1 and its asialo derivative GA1 accumulate in the lysosomes of various tissues, particularly those of the central nervous system (CNS) leading to severe neurodegeneration (Brunetti-Pierri and Scaglia, 2008; Suzuki et al., 2001).

GM1 gangliosidosis shows phenotypic variance, even amongst members of the same family, with severity being correlated to how strongly enzyme activity has been reduced (Suzuki et al, 1978). The disease is classified into 3 types: (1) infantile, (2) juvenile and (3) adult, based on the onset of symptoms (Brunetti-Pierri and Scaglia, 2008). In the infantile form, which is the most prevalent form, symptoms generally present themselves between birth and 6 months of age. The disease progresses very rapidly, with severe neurodegeneration and death often occurring a few years after onset. While the neurological symptoms are generally the most pronounced, skeletal dysplasia as well as hepatosplenomegaly are often observed in GM1 patients (Suzuki et al., 2001),

with cardiomyopathy appearing in around a third of patients (Brunetti-Pierri and Scaglia, 2008)).

The only treatment currently available to patients with GM1 gangliosidosis is to provide supportive care and address their symptoms (Brunetti-Pierri and Scaglia, 2008). As this disease's major pathology is found in the CNS and β-gal cannot cross the blood brain barrier (BBB), enzyme replacement therapy is not a viable option (Samoylova et al, 2008). Being a recessive, single gene disorder, however, GM1 gangliosidosis is an ideal candidate for gene therapy.

Gene therapy can only be considered for GM1, however, due to a critical property of β-gal. Like most lysosomal enzymes, β-gal can be secrete by one cell and taken up by another, a process known as cross correction. As lysosomal enzymes are produced they are first glycosylated in the endoplasmic reticulum and then directed to the Golgi apparatus. There they receive a phosphate group on the 6 position of their terminal mannose residue. This modification enables the enzyme to bind to the mannose-6-phosphate/IGF II receptor. Binding to this receptor allows the enzyme to be transported through the endocytotic pathway to its destination, the mature lysosomes. Some of the enzyme, however, never makes it to the lysosome and is instead redirected to the plasma membrane where it is secreted from the cell. As the mannose-6-phosphate/IGF II receptor is found on the surface of all cells, the enzyme can then be taken up by other cells and directed to their lysosomes (Broekman et al 2008; Sands and Davidson 2006). This phenomenon of cross correction is critically important for the application of gene therapy to GM1 as it allows many cells to receive the necessary enzyme when

significantly less transduced cells are present. Without this process gene therapy would be a far less attractive treatment option for lysosomal storage diseases, including GM1.

Researchers have taken advantage of this fact, as well as the ability of the brain to distribute the enzyme by diffusion, axonal transport and cerebrospinal fluid flow (Broekman et al., 2008), and shown great success treating the neurological aspects of the disease in the GM1 mouse model with strategically placed intracranial injections of AAV vectors capable of delivering the transgene to neurons at high efficiency (Broekman et al, 2007; Baek et al 2010).

While this research has shown promise for treating the neurological aspects of the disease, it provides no therapeutic benefit to the visceral or skeletal pathologies. This can be of particular importance as patients with the infantile form of the disease have died of cardiac failure (Suzuki et al., 2001). To address this issue we plan to investigate a gene therapy strategy that provides systemic treatment of the disease.

Ex vivo Hematopoietic Stem Cell Gene Therapy

Ex vivo hematopoietic stem cell (HSC) gene therapy is a strategy where a patient's own HSCs are collected and then transduced with a lentiviral (LV) vector in vitro. The modified HSCs are then returned to the patient as a bone marrow transplant (BMT). Since a LV vector will be used, the transgene will be integrated into the bone marrow cell's DNA and therefore be present in every cell derived from that progenitor. The transduced HSCs will reconstitute the hematopoietic system and secrete the

necessary transgene, thereby providing systemic correction. Recent studies in mouse models have shown ex vivo gene therapy using LV vectors to be a highly successful systemic treatment for multiple lysosomal storage diseases with neurological components (Visigalli et al., 2010; Gentner et al, 2010; van Til et al. 2010) as well the neurodegenerative disorder metachromatic leukodystrophy (Biffi et al 2004; Biffi et al 2006). Notably, two patients with X-Linked Adrenoloeukodystrophy (ALD), for whom a matched donor could not be found, were successfully treated with autologous ex vivo HSC gene therapy using LV vectors (Cartier et al. 2009).

Ex vivo HSC gene therapy has previously been used to treat GM1 gangliosidosis in the mouse model, although a gammaretrovirus vector was used to transduce the HSCs (Sano et al 2005). Vectors derived from gammaretroviruses used in previous clinical trials led to cases of leukemia in 5 of the patients treated, due to insertional mutagenesis (Hacein-Bey-Abina et al 2003; Hacein-Bey-Abina et al 2008; Howe et al 2008). The increased risk of oncogenesis with gammaretroviral vectors is believed to be a result of their propensity to integrate near the transcription start site of actively transcribing genes and the presences of strong enhancers in their long terminal repeats (Cattoglio et al 2007; Diechmann et al 2007; Wu et al 2003). LV vectors, however, lack the integration preferences observed with gammaretroviral vectors and display low genotoxicity (Cattoglio et al 2007; Montini et al 2006). The use of self-inactivating LV vectors, which lack intrinsic enhancer activity, also obviates the later problem (Matrai et al 2010). A long term study in rhesus macaques found LV vectors to result in a much more favorable integration profile with no cases of oligoclonal expansion or oncogenesis 4 years after

being transplanted (Kim et al 2009). LV vectors were also found to transduce HSC far more efficiently than gammaretroviral vectors (Naldini 2009). These features make LV vectors the superior choice for ex vivo gene therapy. With the successful use of this treatment in other disease models and the proof of principle study done in GM1 mice, we plan to investigate the therapeutic potential of ex vivo gene therapy for GM1 gangliosidosis using a more clinically applicable LV vector.

Effects of Total Body Irradiation

As β-gal cannot cross the BBB, the success of the proposed therapy depends on modified donor HSCs being recruited to the CNS, where they would cross the BBB and permanently establish themselves in the brain parenchyma as microglia. These cells could then overexpress and secrete the necessary transgene, which would be taken up by the surrounding cells. Unfortunately, studies in wildtype (WT) mice have suggested that donor HSCs are only able to establish themselves in the CNS when it is pre-conditioned with radiation (Mildner et al 2007; Ajami et al 2007). As the use of total body irradiation (TBI) in pediatric patients has been linked to future developmental problems (Hopewell 1998), this myeloablation approach is often avoided in younger patients in favor of a conditioning regimen using the chemotherapy drugs, busulfan (Bu) and cyclophosphamide (Cy) (Miano et al 2001; Prasad and Kurtzberg 2010; Shi-Xia et al, 2010). All the above mentioned successful mouse studies used TBI as a myeloablative conditioning regimen. Whether the success observed in these studies can be replicated

when a Bu and Cy conditioning regimen is used has not been determined and is of critical importance to our proposed therapy.

The successful human clinical trial of ex vivo gene therapy in ALD patients mentioned above did, however, utilize a Bu and Cy conditioning regimen. While they could not directly detect donor cells in the CNS, the researchers assumed that donor cells did establish themselves throughout the CNS based on the phenotypic success observed. Lysosomal storage diseases with neurological involvement have also been treated with allogeneic BMT using Bu and Cy for myeloablative conditioning with mixed results (Begley et al 2008; Krivit et al 1999; Ehlert et al 2007; Prasad and Kurtzberg 2010). Partial correction of the neurological phenotype occurs in certain diseases, suggesting that under the right conditions donor cells may be recruited to the CNS without radiation. These potentially important conditions and the processes that create them remain unknown.

The successful ALD trial and the trials of allogenic BMT in different lysosomal storage diseases, where donor cells entered the CNS without radiation, differ from the Mildner et al study demonstrating that donor cells could not infiltrate the CNS unless radiation was used in two major ways: 1) Instead of sham-irradiation, a Bu and Cy conditioning regimen was used. 2) Instead of performing a bone marrow transplant in wild-type mice, the trials were performed on diseased patients. While the interspecies differences could also account for the success of the trials that did not use radiation, this is very unlikely as evidence suggests peripheral myeloid cells are normally excluded from the CNS by the blood brain barrier in non-diseased humans (Cardona et al 2008;

Rezai-Zadeh et al 2009), much like they are in mice. This suggests that either the Bu and Cy conditioning regimen or the effects of the diseases are creating conditions in the CNS similar to those observed post-TBI that facilitate the recruitment of donor cells to the CNS. In this study we intend to determine the conditions post-TBI that facilitate donor cell recruitment to the CNS and then determine whether the Bu and Cy conditioning regimen and/or the effects of GM1 gangliosidosis create similar conditions and facilitate the recruitment of donor cells to the CNS.

Changes caused by Total Body Irradiation

TBI causes certain physiological changes in the CNS that promote the recruitment of donor cells. Primarily, TBI causes tissue damage leading to cytokine/chemokine induction in the CNS and an increase in BBB permeability (Diserbo et al 2002; Li et al 2003).

Cytokines are small cell-signaling molecules that are secreted by cells of the immune system, and microglia and astrocytes in the CNS. Chemokines are a family of cytokines named for their ability to induce chemotaxis. Cells with matching chemokine receptors generally move along a chemokine gradient to areas of higher concentration. Chemokines play a prominent role in the recruitment and activation of tissue infiltrating leukocytes (Conductier et al 2010, Yadav et al 2010). The cytokines tumor necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β), and chemokines CCL2 (Lee et al 2010) and CCL5 (Mildner et al 2007) are all upregulated in the CNS post TBI. All of these

cytokines have also been implicated in leukocyte recruitment to the CNS in multiple disease models as detailed in a later section.

CCL2 was the first CC motif chemokine to be discovered and is one of the most widely studied chemokines (Conductier et al 2010). When mice that were myeloablated with TBI received a transplant of HSCs from a CCR2 knockout mouse, CNS recruitment of donor cells was very significantly reduced, suggesting its ligand CCL2 plays a critical role (Mildner et al 2007). In order for leukocytes to leave the bloodstream and enter a tissue, they must first firmly adhere to the surface of the vascular endothelial cells that make up the capillary's walls. CCL2 is involved in the firm adhesion process (Kuziel et al 1997). Specifically, CCL2 activates β1 and β2 integrins on the cell surface of rolling leukocytes thereby increasing their avidity to cellular adhesion molecules on the surface of the vascular endothelial cells that make up the blood brain barrier (Laudanna et al 2002; Weber et al 1996). In addition it has been suggested that CCL2 may also play a role in changing the shape of the infiltrating cell to allow extravasation across the BBB (Weber et al 1999). Finally, along with the role it plays in recruiting leukocytes to sites of neuroinflammation, CCL2 also appears to alter the distribution of the tight junction proteins that make up the BBB and thereby cause an increase in BBB permeability (Dimitrijelkovic et al 2006; Stamatovic et al 2005).

Much like CCL2, CCL5 is also involved in the chemotaxis and firm adhesion of leukocytes to the surface of vascular endothelial cells (dos Santos et al 2005). CCL5 activates β1 integrins on the surface of leukocytes that express the CCR5 receptor

(Ubogu et al 2006; Weber et al 1996). The cells that are still recruited to the CNS post-TBI in CCR2-/- mice most likely do so in a CCL5/CCR5 dependent manner.

TNF-α and IL-1β are pro-inflammatory cytokines that play a central role in the innate immune response (Montgomery et al 2011; Shaftel et al 2007). Both cytokines are found to upregulate the expression of CCL2 and key cellular adhesion molecules. TNF-α has also been found to upregulate CCL5 expression in human brain endothelial cells (Subilea et al 2009). They both play an important role in the recruitment of leukocytes to the CNS in multiple disease models (Konsman et al 2007; Larochelle et al 2011; Montogomery et al 2011; Shaftel et al 2007). Leukocyte infiltration of the hippocampus was even observed in transgenic mice that chronically overexpress IL-1β in the hippocampus (Shaftel et al 2007). In addition to their role as chemokine regulators, both TNF-α and IL-1β appear to increase blood brain barrier permeability (Larochelle et al 2011; Shaftel et al 2007).

While cytokine induction clearly plays a key role in the recruitment of donor cells to the CNS post-TBI, a study using cuprizone treatment to induce a similar localized increase of cytokine production in the CNS, without overtly damaging the BBB, demonstrated that cytokine increases alone are not sufficient (Mildner et al 2007). In this study cuprizone treatment caused the production of TNF- α, CCL2 and CCL5 at levels comparable to those seen post-TBI, yet donor cells were not recruited to the site of inflammation without precondition the CNS with TBI. This study provides strong evidence that an increase in BBB permeability, beyond that created by cytokines alone, is also required. The cause behind the increase in BBB permeability seen after irradiation is

not definitively known but most likely the result of vascular endothelial cell apoptosis (Li et al 2003; Li et al 2004), and disruption of the endothelial cell tight junction proteins that maintain the BBB (Kaya et al 2004). As increased BBB permeability has been associated with increased leukocyte infiltration into the CNS (Alvarez and Teale 2006), it will most likely be a good indicator of when donor cell will be recruited to the CNS.

Busulfan and Cyclophosphamide

Whether Bu and Cy conditioning causes an induction in CNS cytokines and increase in BBB permeability similar to that observed post-TBI and facilitates the recruitment of donor cells to the CNS has not been investigated. Bu and Cy are myeloablative pre-conditioning drugs that cross-link DNA, prevent cell division and cause apoptosis. Both have been shown to cross the BBB (Hassan, Ehrsson et al. 1996; Yule, Price et al. 1997), cause damage to vascular endothelial cells (Ohtani, Nakamura et al. 2006; Vassord, Lapouméroulie et al. 2008; Zeng, Yan et al. 2008; Zeng, Jia et al. 2010) and lead to cases of neurotoxicity (Morgia, Mondini et al. 2004). While cytokine induction in the CNS has not been assessed to my knowledge, studies have shown the conditioning regimen to cause cytokine increases in the serum, although these increases occur later and are less drastic than those caused by TBI (Xun et al 1994). In total these studies suggest that Bu and Cy conditioning may cause increased cytokine production in the CNS and increased BBB permeability. In this study we plan to investigate this and

determine whether Bu and Cy conditioning can facilitate donor cell recruitment to the CNS.

Leukocyte Recruitment to the CNS in Neuropathologies

While once considered immunologically isolated, it is now generally accepted that leukocytes will enter the CNS (without the need for pre-conditioning with TBI) as a response to certain neuropathologies such as Alzheimer's Disease (AD) (Town et al 2008), multiple sclerosis (MS) (Lim 2011) and West Nile Virus (WNV) encephalitis (Getts et al 2008), where neuroinflammation and BBB disruption occur as the disease progresses. The same four cytokines involved in the recruitment of myeloid cells to the CNS post-TBI (CCL2, CCL5, TNF-a and IL-B) have also been implicated in these diseases and appear to be required for myeloid cell entry to the CNS regardless of the inflammatory stimuli.

CCL2 (Sokolova et al 2009), CCL5 (Tripathy et al 2010), TNF-α and IL-1β (Veerhuis et al 1999) are all upregulated in the brains of AD patients. The recruitment of leukocytes to the brain was found to be dependent on CCR2 expression, in a mouse model of AD (El Koury 2007). Additionally, increased numbers of leukocytes are seen in the brain of AD mice that either constitutively express IL-1 (Shaftel et al 2007) or lack transforming growth factor β-SMAD2/3 signaling (Town et al 2008). Transforming growth factor β is known to be an immunosuppressive cytokine that can inhibit TNF-α

and IL-1β production (Martiney et al 1998). Finally BBB integrity is compromised in AD (Bower et al 2007; Lim 2011;Ujile et al 2003).

Chemokines also play a prominent role in the recruitment of leukocytes to the CNS during WNV encephalitis. CCL5 expression is significantly upregulated in the CNS of mice infected with the WNV. Furthermore, mice lacking the CCR5 receptor have significantly less CNS infiltration of leukocytes and reduced survival as a result (Glass et al 2005). In accordance with this research, humans with a detrimental mutation in the CCR5 gene were found to have a greater risk of symptomatic WNV encephalitis (Glass et al 2006). TNF-R1-/- mice also showed decreased survival compared to wild-type mice when infected with WNV, suggesting TNF-α may also play a role in leukocyte recruitment to the CNS in this disease model (Shrestha et al 2008). Finally, CCL2 is also strongly upregulated during WNV infection in mice but whether it plays a role in the recruitment peripheral myeloid cells to the CNS (Getts 2008) or instead simply stimulates proliferation of leukocytes in the bloodstream (Lim et al 2010), thereby indirectly increasing the number of infiltrating leukocytes found in the brain, is still a matter of debate.

MS is an inflammatory disease characterized by a demyelination and axonal degeneration. Leukocyte infiltration of the CNS is a cardinal sign of pathogenesis and chemokines appear to play a key role in the process. While researchers have found MS patients to have lower levels of CCL2 in their cerebral-spinal-fluid (Malmestrom et al 2006), CCL2 is found at increased levels in active and chronic lesions (Mahad et al 2003). CCL2 was found to have a major role in the disease progression of experimental

autoimmune encephalomyelitis (EAE) in mice, as CCL2-/- mice only receive a mild case of the disease with drastically reduced leukocyte recruitment to the CNS (Huang et al 2001). The chemokine CCL5 is also found at increased levels in lesions of MS patients (Sorensen 1999) and in the brains of EAE mice (dos Santos 2005), although CCR5-/- mice are still susceptible to EAE (Tran et al 2000). Leukocyte adhesion was significantly reduced in the EAE mice model if either CCL2 or CCL5 were reduced using antibodies (dos Santos 2005). BBB integrity is also compromised in MS (Lim 2011)

The same conditions that allow peripheral myeloid cells to enter the CNS in the neuropathologies mentioned above may also occur in GM1 gangliosidosis. Neuroinflammation occurs in GM1 mice as early as two months, which is four months before symptoms appear. TNF-α and IL-1β were found to increase as the disease progresses and symptoms become more severe (Jeyakumar et al 2003). Increases in CCL2 and CCL5 (along with CCR5's two other ligands CCL3 and CCL4) were also observed in mice at three and four months of age (Sano et al 2005). Finally, increases in BBB permeability have been observed in GM1 mice at six and eight months, although whether BBB permeability increases occur earlier has not been examined (Jeyakumar et al 2003). In this study we plan to investigate whether the increased cytokine production and BBB permeability observed in GM1 mice will facilitate the recruitment of donor cells to the CNS and allow GM1 gangliosidosis to be treated using ex vivo gene therapy without the use of TBI.

Goals of Study

In this study I plan to quantify the cytokine and BBB permeability increases necessary for donor HSC cells to be recruited to the CNS post-TBI. I will then investigate whether Bu and Cy conditioning and/or the chronic neuroinflammation present in GM1 mice can produce similar conditions and facilitate the recruitment of donor HSC cells to the CNS. Finally I will assess whether ex vivo HSC gene therapy is still an effective therapy when Bu and Cy are used for myeloablative conditioning, given the levels of neuroinflammation and BBB disruption present.

This study will clearly define the potential of ex vivo HSC gene therapy as a treatment for GM1 gangliosidosis. It will also determine the myeloablative conditioning strategy needed for successful treatment of the disease and provide a model for researchers treating other neuropathologies with BMT. Establishing how CNS cytokine levels and BBB permeability effect donor cell recruitment to the CNS will provide researchers with parameters to use to determine whether ex vivo HSC gene therapy can serve as an effective therapy for their disease. Physicians may also utilize these parameters to determine the appropriate myeloablation strategy (Bu and Cy or TBI) depending on the patient's neuroinflammatory status. For example if a patients CNS cytokine levels, as measured in their cerebral spinal fluid, are high enough to facilitate the recruitment of donor cells to the CNS then a Bu and Cy conditioning regimen may be used. If the cytokine levels are not high enough then the negative effects of TBI must be

weighed against the benefits of treatment to determine whether treatment, using TBI for a myeloablative condition regimen, should be attempted.

General Hypothesis: The CNS cytokine production and blood-brain barrier disruption that occur as a result of the disease will facilitate donor cell recruitment to the CNS and allow GM1 gangliosidosis to be treated with ex vivo hematopoietic stem cell gene therapy without the use of total body irradiation.

Specific Aims:

Aim 1: Determine whether Bu and Cy conditioning creates conditions in the CNS similar to TBI and results in the recruitment of donor cells to the CNS

Aim 1.1: Determine the changes in CNS cytokine production and BBB permeability that occur post-TBI, and how they correlate to donor cell recruitment to the CNS

Hypothesis: Donor cell recruitment to the CNS will be increased when donor cells are injected at times of increased CNS cytokine production and BBB permeability

Rationale: The increased production of certain cytokines (TNF-α, IL-1β, CCL2, CCL5, CCL4 and CCL3) and the increase in BBB permeability that occur post-TBI both play important roles in the recruitment of donor cells to the CNS. Understanding when these changes occur post-TBI and how they correlate to donor cell recruitment will allow us to gauge whether other conditioning regimes may facilitate the recruitment of donor cells and when the optimum time post-conditioning is to inject the donor cells. As HSCs are cleared from the bloodstream within a few hours after injection (Lapidot et al 2005),

injecting the cells at the optimum time after myeloablation will be critical for achieving maximal recruitment to the CNS. The importance of this point is further emphasized by the finding that progenitor cells that are not normally found in the blood stream are the only cells capable of permanently establishing themselves in the CNS as microglia (Ajami et al 2007). Finally, if TBI is found to be required, the results of these experiments will show the optimum time to inject the donor cells post-TBI for maximal CNS recruitment.

Experimental Design: CNS cytokine production and BBB permeability will be measured along a time course in the brain and spinal cord of both GM1 (Hahn et al 1997) and WT (C57BL/6) mice that receive a treatment of either 8.5 Gy of TBI (Sano et al 2005) or sham-TBI (Table 1). The first measurement will be 4 hours after TBI, which is when cells would normally be injected for the BMT (Han et al 2006; Hickey et al 1992), and will be referred to as time point 0 (Figure 1a). Subsequent measurements will be made at 4, 12, 24, 48, and 72 hours after time point 0, as well as 7 days after. As a control cytokine levels, BBB permeability and donor cell recruitment will also be assessed in three mice that receive no myeloablative treatment. The cytokines TNF-α, IL-1β, CCL2, CCL5, and CCR5's other two ligands CCL4 and CCL3 will be measured using ELISA assays. BBB permeability will be measured using sodium fluorescein dye (376 Da) and Evans Blue dye (67 kDa) simultaneously (Hawkins and Egleton 2006; Liu et al 2009).

Table 1

Aim 1.1: CNS cytokine levels, BBB permeability and donor cell recruitment to the CNS post TBI

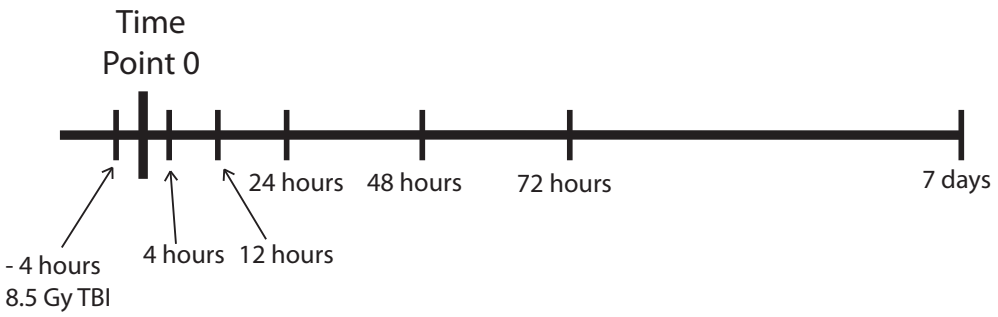
*Time points are as follows: 0, 4, 12, 24, 48, 72, hours and 7 days (Figure 1)

*As a control cytokine levels, BBB permeability and donor cells recruitment to the CNS will be measured in three WT and three GM1 mice that receive no myeloablative treatment

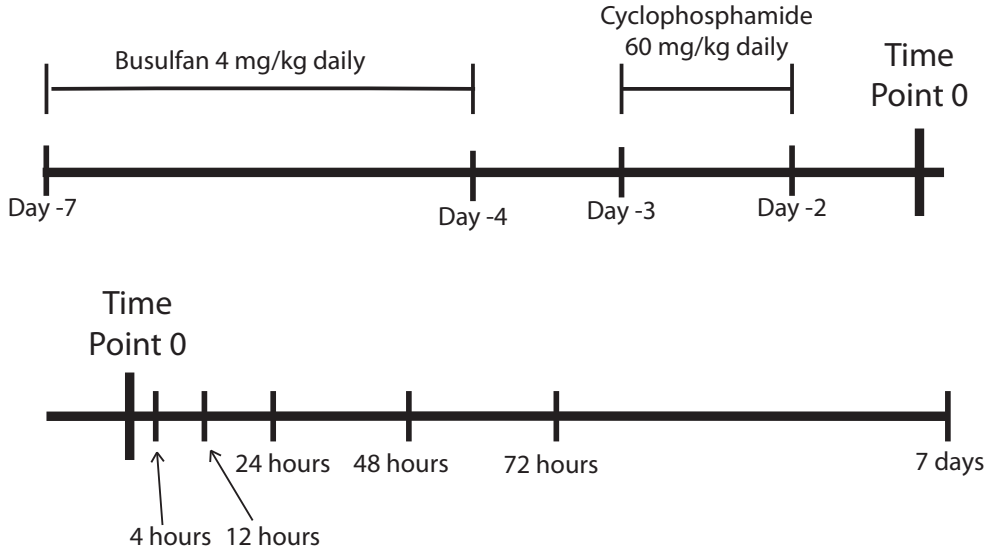
Readout	Geno type	Myelo-ablation	Cells Injected (10^6/ mouse)	Time Point Injected	Read-out Time Points	Mice per Time Point	Total mice per exp
BBB permeability	β-gal -/-	TBI	None	n/a	All Time Points	3	21
BBB permeability	β-gal +/+	TBI	None	n/a	All Time Points	3	21
BBB permeability	β-gal -/-	Sham	None	n/a	All Time Points	3	21
BBB permeability	β-gal +/+	Sham	None	n/a	All Time Points	3	21
Cytokine levels	β-gal -/-	TBI	None	n/a	All Time Points	3	21
Cytokine levels	β-gal +/+	TBI	None	n/a	All Time Points	3	21
Cytokine levels	β-gal -/-	Sham	None	n/a	All Time Points	3	21
Cytokine levels	β-gal +/+	Sham	None	n/a	All Time Points	3	21
Donor cells in CNS	β-gal -/-	TBI	ACTβ-eGFP HSCs	All except 0	12 hours after injection	3	18
Donor cells in CNS	β-gal +/+	TBI	ACTβ-eGFP HSCs	All except 0	12 hours after injection	3	18
Donor cells in CNS	β-gal -/-	Sham	ACTβ-eGFP HSCs	All except 0	12 hours after injection	3	18
Donor cells in CNS	β-gal +/+	Sham	ACTβ-eGFP HSCs	All except 0	12 hours after injection	3	18

Figure 1: (a) Time point for total body irradiation (TBI) and measurements post-TBI (b) BuCy2 myeloablation treatment schedule and measurement time points

A.



B.



To test the hypothesis that increased CNS cytokine levels and BBB permeability will lead to increased donor cell recruitment to the CNS, 10^6 HSCs isolated from a male ACTβ-eGFP mice, which constitutively expresses GFP in all myeloid cells, will be injected into one month old female GM1 and WT mice at all the different time points after TBI or sham-TBI along the time course. Twelve hours after the cells are injected, when all cells that are going to enter the CNS should have already been recruited there, the mice will be perfused with 1xPBS and sacrificed. The left hemisphere will be embedded in OTC and used to assess donor cell presence and distribution using immunohistochemistry. This hemisphere will be cut into 20 μm sections and stained with a GFP primary antibody to identify donor cells. The right hemisphere will be divided into the cerebrum, cerebellum and brain stem and used to quantify donor cell presence in these regions by flow cytometry with a 488 nm filter. Donor cell presence in the spinal cord will be quantified by flow cytometry as well.

Prior to completing these experiments, a preliminary experiment must be performed to confirm that almost all the cells that are going to enter the CNS will do so within twelve hours after being injected. While HSCs are known to leave the bloodstream and enter tissues a few hours after being injected and the injected donor cells are assumed to enter the CNS at this time, this has never been proven. HSCs are known to occasionally leave tissues and reenter the bloodstream briefly after their initial exit (Lapidot et al 2005) but not in number sufficient to account for the amount seen in the

brain. While few, if any, other options exist, we still must prove that almost all the cells that will establish themselves in the CNS will do so within the first 12 hours after injection. This point is critical for the timing of the experiment detailed above but modifications can be made if unexpected results are found.

To determine when donor cells enter the brain after being injected into the bloodstream, 10^6 HSCs isolated from ACTβ-eGFP mice will be injected into both GM1 and wild-type C57Bl/6 mice 4 hours after they receive a does of 8.5 Gy of TBI or sham-TBI at time point 0 (Table 2). Three mice from each of the four groups will then be sacrificed at 4, 12, and 24 hours after time point 0. Serum samples will be taken before sacrifice to quantify the number of GFP+ donor cells present in the blood. Donor cell presence and distribution will be assessed as described above.

Expected Results: Although they will most likely peak at different times, I expect to see a strong increase in cytokine production for at least the first 24 hours after TBI (Lee et al 2010). I expect BBB permeability to be increased at time point 0, peak at 24 hours and then decline (Yuan et al 2003; Diserbo et al 2002). As HSCs leave the blood stream within a few hours, I expect my preliminary experiment to show that almost all the cells that will enter the CNS will do so within 12 hours from injection.

I expect that the greatest number of donor cells will be recruited to the CNS when cells are injected at the time point when BBB permeability peaks, provided strong cytokine production also occurs. In general, I expect to see that increased cytokine levels and BBB permeability will correlate with increased donor cell recruitment to the CNS,

Table 2

Mice groups used in preliminary experiment to determine when donor cells enter the CNS

Readout	Geno type	Myelo-ablation	Cells Injected (10^6/ mouse)	Time Point Injected	Read-out Time Points	Mice per Time Point	Total mice per exp.
Donor cells in CNS	β-gal -/-	None	ACTβ-eGFP HSCs	0	4, 12, 24 hours	3	9
Donor cells in CNS	β-gal +/+	None	ACTβ-eGFP HSCs	0	4, 12, 24 hours	3	9

confirming that at least one of these readouts is a good predictive parameter for determining when donor cells will enter the CNS.

Potential Problems and Alternative Approaches: If all the donor cells that are going to establish themselves in the CNS have not done so by the twelve hour time point, then donor cell recruitment to the CNS will have to be assessed at a later time point when all the cells have entered the CNS such as 24 hours. If cells are found to continual enter the CNS past 24 hours then donor cell presence will have to be assessed at the 12 hour time point as a ratio of donor cells in the CNS to donor cells in the bloodstream.

If cytokine levels and BBB permeability are found not to correlate with donor cell recruitment to the CNS, then other potential chemokines and cellular adhesion molecules will be assessed. If no cytokines/chemokines can be found that correlate with donor cell recruitment to the CNS, cytokine levels and BBB permeability will not be measured in future experiments and donor cell entry into the CNS will be assessed empirically by testing all time points. This outcome is highly unlikely though.

Aim 1.2: Determine whether myeloablation using Bu and Cy causes changes in cytokine levels and BBB permeability similar to those observed with TBI, and if these changes can facilitate donor cell recruitment to the CNS.

Hypothesis: Bu and Cy conditioning will cause a slight increase CNS cytokine levels and BBB permeability and facilitate a slight increase of donor cell recruitment to the CNS but not to the levels observed post-TBI

Rationale: As mentioned previously, Bu and Cy cause damage to vascular endothelial cells, which may increase BBB permeability. Both drugs also cross the BBB and occasionally cause neurotoxicity, suggesting they may also cause an increase in CNS cytokine levels. Determining whether Bu and Cy conditioning causes these changes and facilitates the recruitment of donor cells to the CNS is critical for the application of our proposed therapy. If it does result in the CNS recruitment of donor cells, these experiments should also identify the optimum time for injecting the cells after conditioning.

Experimental Design: The effect of Bu and Cy conditioning on BBB permeability, cytokine induction and donor cell recruitment to the CNS will be assessed in 1 month old GM1 and WT mice (Table 3). A standard Bu and Cy conditioning regimen called BuCy2 will be used (Mengarelli, Iori et al. 2002; Gupta, Lazarus et al. 2003). This conditioning regime consists of 4 mg/kg of Bu given daily for four days (days -7 to -4) followed by 60 mg/kg of Cy given daily for two days (days -3 and -2) (Figure 1b). For a BMT, donor cells would normally be injected 48 hours after the last dose of Cy and this will be denoted as time point 0. Measurements of CNS cytokine levels and BBB permeability will be performed as described in aim 1.1 along the same time course for both GM1 and WT mice receiving either BuCy2 conditioning or 1xPBS injections.

To assess donor cell recruitment to the CNS after conditioning, 10^6 HSCs isolated from a male ACTβ-eGFP mice will be injected at time point 0 into both GM1 and WT mice receiving either BuCy2 conditioning or 1xPBS injections. If an increase in cytokine induction and/or BBB permeability is found at any of the other time points after time

Table 3

Aim 1.2: CNS cytokine levels, BBB permeability and donor cell recruitment to the CNS post BuCy2

*time points are as follows: 0, 4, 12, 24, 48, 72, hours and 7 days (Figure 1b)

Readout	Geno type	Myelo-ablation	Cells Injected (10^6 / mouse)	Time Point Injected	Read-out Time Points	Mice per Time Point	Total mice per exp.
BBB permeability	β-gal -/-	BuCy2	None	n/a	All Time Points	3	21
BBB permeability	β-gal +/+	BuCy2	None	n/a	All Time Points	3	21
BBB permeability	β-gal -/-	None 1xPBS	None	n/a	All Time Points	3	21
BBB permeability	β-gal +/+	None 1xPBS	None	n/a	All Time Points	3	21
Cytokine levels	β-gal -/-	BuCy2	None	n/a	All Time Points	3	21
Cytokine levels	β-gal +/+	BuCy2	None	n/a	All Time Points	3	21
Cytokine levels	β-gal -/-	None 1xPBS	None	n/a	All Time Points	3	21
Cytokine levels	β-gal +/+	None 1xPBS	None	n/a	All Time Points	3	21
Donor cells in CNS	β-gal -/-	BuCy2	ACTβ-eGFP HSCs	0 and as indicated	12 hours after injection	3	3-21
Donor cells in CNS	β-gal +/+	BuCy2	ACTβ-eGFP HSCs	0 and as indicated	12 hours after injection	3	3-21
Donor cells in CNS	β-gal -/-	None 1xPBS	ACTβ-eGFP HSCs	0 and as indicated	12 hours after injection	3	3-21
Donor cells in CNS	β-gal +/+	None 1xPBS	ACTβ-eGFP HSCs	0 and as indicated	12 hours after injection	3	3-21

point 0, donor cells will also be injected at those time points, with cell recruitment to the CNS being measured as described in aim 1.1 twelve hours after the injection. As a control cytokine levels, BBB permeability and donor cell recruitment will also be assessed in three mice that receive no myeloablative treatment.

Expected Results: I expect BuCy2 conditioning to cause increases in CNS cytokine levels at the 48-hour, 72-hour and 7-day time points based on the observed increase in the serum at these times (Xun et al 1994). These increases will not be to the degree observed post-TBI though. BBB permeability may increase at the 24-hour, 48-hour, and 72-hour time points, coinciding with damage to vascular endothelial cells observed at these times (Zeng et al 2010). These increases may be sufficient to facilitate the recruitment of donor cells to the CNS but probably not to the level seen post-TBI. If increased infiltration is observed, it should occur when cytokine levels and BBB permeability are both increased.

Potential Problems and Alternative Approaches: If the data suggests that either cytokine induction or BBB permeability may peak at a point between two time points, further time points may be assessed before determining when to inject the donor cells.

Aim 2: Determine whether donor cells are recruited to the CNS of GM1 mice as cytokine production and BBB permeability change with disease progression

Hypothesis: Donor cells will be recruited to the CNS of GM1 mice when cytokine production and BBB permeability increase with disease progression.

Rationale: Myeloid cell infiltration of the CNS, without pre-conditioning the CNS with TBI, has been observed in certain neurodegenerative diseases such as AD (Town et al

2008), MS (Lim 2011) and WNV encephalitis (Getts et al 2008), where chronic neuroinflammation and increased BBB permeability occur. Given that chronic neuroinflammation and increased BBB permeability have both been found to occur in the GM1 mouse model (Jeyakumar et al 2003), myeloid cell recruitment to the CNS may occur to varying degrees as the disease progresses. Determining if, when, and to what degree donors cells get recruited to the CNS at different time points in disease progression will enable us to determine when, during the course of the disease, the proposed therapy may be effective without TBI.

Experimental Design: Cytokine levels in the CNS, BBB permeability and donor cell recruitment to the CNS will be assessed in 1, 2, 3, 4, and 6-month-old GM1 and WT mice receiving either BuCy2 treatment or 1xPBS injections (Table 4). Cytokine levels and BBB permeability will be measured at the time point post-BuCy2 conditioning that was shown to facilitate the greatest recruitment of donor cells to the CNS from the results of aim 1.2. If no time point was shown to facilitate donor cell recruitment to the CNS, then time point 0 will be used. Cytokine levels and BBB permeability will be measured as described in aim 1.

To assess donor cell recruitment to the CNS as the disease progresses, mice will receive a tail vein injection of 10^6 cells isolate from a ACTβ-eGFP mouse at the same time point after BuCy2 treatment that cytokine levels and BBB permeability were

Table 4

Aim 2: CNS cytokine levels, BBB permeability and donor cell recruitment to the CNS during GM1 disease progression

*For all mice receiving BuCy2 conditioning, assays will be performed at the time point post BuCy2 that is found in aim 1.2 to results in maximal donor cell recruitment to the CNS. Cells will also be injected at this time point with assays being performed one week after injection.

Experiment	Geno type	Myelo-ablation	Age of Mice (months)	Cells Injected (10^6/mouse)	Mice per Age Group	Total mice per exp.
BBB permeability	β-gal -/-	BuCy2	1, 2, 3, 4, and 6	None	3	18
BBB permeability	β-gal +/+	BuCy2	1, 2, 3, 4, and 6	None	3	18
BBB permeability	β-gal -/-	None	1, 2, 3, 4, and 6	None	3	18
BBB permeability	β-gal +/+	None	1, 2, 3, 4, and 6	None	3	18
Cytokine levels	β-gal -/-	BuCy2	1, 2, 3, 4, and 6	None	3	18
Cytokine levels	β-gal +/+	BuCy2	1, 2, 3, 4, and 6	None	3	18
Cytokine levels	β-gal -/-	None	1, 2, 3, 4, and 6	None	3	18
Cytokine levels	β-gal +/+	None	1, 2, 3, 4, and 6	None	3	18
Donor cells in CNS	β-gal -/-	BuCy2	1 and as indicated	GFP LV HSCs	3	3-18
Donor cells in CNS	β-gal +/+	BuCy2	1 and as indicated	GFP LV HSCs	3	3-18
Donor cells in CNS	β-gal -/-	None	1 and as indicated	GFP LV HSCs	3	3-18
Donor cells in CNS	β-gal +/+	None	1 and as indicated	GFP LV HSCs	3	3-18

measured at. Donor cell presence and distribution in the CNS will be measured as described in aim 1.1 twelve hours after cells are injected.

Expected Results: Without BuCy2 conditioning, I expect CNS cytokine production to begin around 1 or 2 months of age increase up to the 6-month age group. I expect an increase in BBB permeability to begin at 3 or 4 months and to steadily increase as the disease progresses. If BuCy2 condition is found in aim 1.2 not to effect donor cell recruitment to the CNS, I expect donor cell recruitment to the CNS to start to occur around 3 or 4 months of age, when BBB permeability begins to increase and cytokines are strongly induced. If BuCy2 is found to effect donor cell recruitment to the CNS, the effects of myeloablative conditioning may further exacerbate the neuroinflammation that occurs as the disease progresses allowing for even greater recruitment of donor cells in the CNS at 3 and 4 months and potentially earlier recruitment as well. The degree of donor cell recruitment should reach levels similar to that observed post-TBI when cytokine and BBB permeability levels approach the levels established in aim 1.1.

Aim 3: Determine whether ex vivo HSC gene therapy using BuCy2 as a myeloablative conditioning regimen can provide therapeutic benefits to GM1 mice equivalent to when TBI is used.

Hypothesis: If donor cell recruitment to the CNS is found to reach levels observed with TBI conditioning when BuCy2 conditioning is used in pre-symptomatic mice, then the therapeutic effects should be similar.

Rationale: While the data gathered from aims 1 and 2 will give a better picture of how effective the proposed therapy may be when TBI is not used, experiments still must be done to directly compare the actual therapeutic benefits. The data gathered from aims 1 and 2 should allow for optimization of both conditioning regimens so a direct comparison of the therapeutic benefits can be made. If donor cell recruitment to the CNS is not found to occur after BuCy2 conditioning or at any point during the disease progression of GM1 gangliosidosis, I will compare therapeutic benefits in 1 month old mice only to insure that similar benefits cannot occur without donor cell recruitment to the CNS.

Experimental Design: To compare the efficiency of the therapy when BuCy2 conditioning is used as opposed to TBI, the therapeutic benefits of ex vivo HSC gene therapy will be assessed two and six months after one month old GM1 and WT mice receive a BMT of modified HSCs using either TBI or BuCy2 treatment as a myeloablative conditioning regimen. If the results of Aim 2 suggest that donor cell recruitment to the CNS without TBI would be increased in older GM1 mice, the efficiency of the therapy using either TBI or BuCy2 treatment will also be compared in those age group(s). Instead of receiving donor cells from ACTβ-eGFP mice, experimental groups will receive HSCs isolated from C57Bl/6 mice that have been transduced with LV vectors (Biffi et al 2004) to deliver either 1) the β-gal transgene along with a GFP transgene under control of an internal ribosome entry site (IRES) or 2) the mCherry transgene with the GFP transgene under control of an IRES as a control. Therapeutic benefits will be assessed in the treatment groups listed in Table 5, with n = 5 for each treatment group.

Table 5

Aim 3: Comparison of therapeutic benefits of BMT with modified HSCs when BuCy2 is used as a myeloablative conditioning regimen vs. TBI

Experiment	Genotype	Myeloablation	Cells injected (10^6/mouse)	Mice per group
Group 1	β-gal -/-	BuCy2	β-gal + GFP HSCs	5
Group 2	β-gal -/-	TBI	β-gal + GFP HSCs	5
Group 3	β-gal -/-	BuCy2	GFP HSCs	5
Group 4	β-gal -/-	TBI	GFP HSCs	5
Group 5	β-gal -/-	BuCy2	Unmodified HSCs	5
Group 6	β-gal -/-	TBI	Unmodified HSCs	5
Group 7	β-gal -/-	None	None	5
Group 8	β-gal +/+	None	None	5

Therapeutic benefit will be assessed by the following readouts:

1) *Donor Chimerism:* Donor chimerism will be assessed using fluorescence activity cell sorting analysis of peripheral blood cells.

2) *Donor cells in the CNS:* Donor cell presence in the CNS will be assessed as described in aim 1.1.

3) *β-gal production and distribution in CNS:* B-gal production and distribution in the CNS will be assessed by performing X-gal staining on 20 µm sections (Broekman et al 2007). A fluorometric assay using 4-methylumbelliferyl_β_D-galactopyranoside as a fluorogenic substrate for β-gal (Baek, R.C. et al 2010) will be used to quantify β-gal production in the brain, spinal cord, liver, spleen, heart, lungs, kidneys and serum with this assay.

4) *Lysosomal storage:* Lysosomal storage will be assessed histologically using Filipin staining (Broekman, M.L.D. et al 2007). Individual gangliosides will be purified, separated and then quantified by HPTLC (Broekman, M.L.D. et al 2007)

5) *Phenotypic Correction:* Neuromotor abilities will be assessed monthly. Rotarod testing will be used to assess coordination and balance (Ingram et al 1981). Open field testing will be used to measure activity (Holland et al 1968). Finally, gait disturbances will be assessed by painting the mice's feet and placing them in a corridor lined with white paper (Tilson e al 1978).

6) *Survival Analysis:* A separate set of mice (n =5) for each group will also be used for a survival assay. Weights will be measured weekly and behavioral analyses will be done

monthly. Mice will be sacrificed when they have lost 20% of their maximum body weight.

Expected Results: I expect that GM1 mice that receive treatment with BuCy2 conditioning at 1 month will not fair as well as those that receive treatment with TBI conditioning due to limited donor cell recruitment to the CNS. Mice receiving treatment with BuCy2 conditioning at 3or 4 months, when donor cell recruitment to the CNS should be much higher, should fair just as well those receiving treatment with TBI. I expect treatment of 6 month old mice to halt disease progression, with treatment using BuCy2 conditioning possibly being even more effective, as it would do less injury to the mice's already damaged brain. If the BuCy2 conditioning is found to lead to the same amount of donor cell recruitment to the CNS as TBI in aim 1.2, then the therapeutic effects of each myeloablation treatment should be the same regardless of the mouse's age.

Potential Problems and Alternative Approaches: While the therapy is unlikely to reverse the neurological phenotype observed in 6 month old mice, if the results of aim 2 suggest that donor cells are recruited to the CNS at a high level at this age, a study of the therapeutic benefits of BMT with modified HSCs will still be done with this age group as the disease will most likely not be diagnosed in patients until symptoms appear and the ability of this therapy to halt disease progression should be addressed.

Concluding Remarks: The results of this study should determine the therapeutic potential of ex vivo HSC gene therapy for treating GM1 gangliosidosis. It will also

address whether conditioning regimens may affect the outcome of the therapy for GM1 gangliosidosis, as well as the many other neuropathologies for which this approach is being investigated (Biffi et al 2006; Gentner et al 2010; Visigalli et al 2010). Understanding how conditioning regimens effect donor cell recruitment to the CNS will help physicians make appropriate decisions when treating neuropathologies with BMT. While this study does not address mechanistically how CNS cytokine induction and increased BBB permeability effects donor cell recruitment to the CNS, by correlating these readouts to donor cell recruitment in three separate processes (TBI conditioning, BuCy2 conditioning and neuropathology), this study should firmly establish their link to donor cell recruitment to the CNS. Establishing parameters for the degree of CNS cytokine production and BBB permeability increase needed for the recruitment of donor cells to the CNS will enable researchers to predict whether ex vivo HSC gene therapy will be effective for treating their disease. Physicians may also use these parameters as a guideline for determining the appropriate myeloablative condition regimen to use on a patient-to-patient basis. Finally the results of this study may suggest means of improving donor cell recruitment to the CNS, such as transiently increasing BBB permeability, and thus open new avenues of potential research.

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