Published in final edited form as:

Exp Mol Pathol. 2013 August ; 95(1): 7–17. doi:10.1016/j.yexmp.2013.03.010.

Perivascular, but not Parenchymal, Cerebral Engraftment of Donor Cells after Non-Myeloablative Bone Marrow Transplantation

Yue Yang, Nikolas L. Jorstad, Christine Shiao, Makenzie K. Cherne, Shawn B. Khademi, Kathleen S. Montine, Thomas J. Montine, and C. Dirk Keene*

Department of Pathology, University of Washington, Seattle, WA

Abstract

Myeloablative (MyA) bone marrow transplantation (BMT) results in robust engraftment of BMT-derived cells in the central nervous system (CNS) and is neuroprotective in diverse experimental models of neurodegenerative diseases of brain and retina. However, MyA irradiation is associated with significant morbidity and mortality and does not represent a viable therapeutic option for the elderly. Non-myeloablative (NMyA) BMT is less toxic, but it is not known if the therapeutic efficacy observed with MyA BMT is preserved. As a first step to address this important gap in knowledge, we evaluated and compared engraftment characteristics of BMT-derived monocytes/microglia using several clinically relevant NMyA pretransplant conditioning regimens in C57BL/6 mice. These included chemotherapy (fludarabine and cyclophosphamide) with or without 2 Gy irradiation, and 5.5 Gy irradiation alone. Each regimen was followed by transplantation of whole bone marrow from green fluorescent protein-expressing wild type (wt) mice. While stable hematopoietic engraftment occurred, to varying degrees, in all NMyA regimens, only 5.5 Gy irradiation resulted in significant engraftment of BMT-derived cells in brain, where these cells were exclusively localized to perivascular, leptomeningeal, and related anatomic regions. Engraftment in retina under 5.5 Gy NMyA conditions was significantly reduced compared to MyA, but robust engraftment was identified in optic nerve. Advancing the therapeutic applications of BMT to neurodegenerative diseases will require identification of the barrier mechanisms MyA, but not NMyA, is able to overcome.

Introduction

Myeloablative (MyA) pretransplant conditioning followed by bone marrow transplantation (BMT) is neuroprotective in a variety of animal models of neurodegenerative disease, including Alzheimer's disease (Keene et al., 2010; Malm et al., 2008; Naert and Rivest, 2012; Simard et al., 2006), amyotrophic lateral sclerosis (Corti et al., 2004; Ohnishi et al., 2009) Huntington's disease (Kwan et al., 2012), and glaucoma (Anderson et al., 2005). The anatomic distribution, phenotype, and turnover of monocytes/microglia within the central nervous system (CNS) appear to be crucial for the modulation of neurological disease (Djukic et al., 2006; Malm et al., 2005; Mildner et al., 2007; Priller et al., 2006). Successful MyA BMT achieves engraftment of circulating donor monocytes within the CNS as

© 2013 Elsevier Inc. All rights reserved.

*Address correspondence to: C. Dirk Keene, M.D., Ph.D., Harborview Medical Center, Box 359791, 325 Ninth Ave, Seattle, WA 98104, Ph. 206 897-4934, Fax: 206 897-5249, cdkeene@uw.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

perivascular and parenchymal monocytes/microglia (Priller et al., 2001; Simard and Rivest, 2004), resulting in a chimeric CNS monocyte-microglia population that can modulate disease-related innate immune response to mediate a reduction in neurotoxicity (Cobbold et al., 1986; Hanisch and Kettenmann, 2007; Pollack et al., 2009; Prinz et al., 2011; Ransohoff and Cardona, 2010; Rivest, 2009; Sharabi and Sachs, 1989; Shie et al., 2009).

Clinically, however, MyA BMT is associated with significant morbidity and mortality and is used almost exclusively to treat life-threatening malignant cancers of the blood, including leukemias and lymphomas. MyA BMT is poorly tolerated in elderly patients and is therefore not likely to be used to treat age-related neurodegenerative diseases even if BMT-mediated neuroprotection in rodents could be recapitulated in human disease. By contrast, non-myeloablative (NMyA) BMT regimens have been developed specifically to treat patients with hematologic malignancies, such as the elderly, who are too frail or sick to tolerate conventional MyA BMT. In addition, NMyA BMT applications are currently under intense clinical investigation for multiple sclerosis (Burt et al., 2009), lupus (Burt et al., 2006), diabetes (Voltarelli et al., 2007), and other non-malignant conditions (Annaloro et al., 2009; Tyndall and Saccardi, 2005). Thus, NMyA preconditioning could provide a more appropriate risk/benefit ratio to elderly patients in the early stages of neurodegenerative diseases. While several studies have established that recruitment of donor cells to the CNS parenchyma after BMT requires some level of preconditioning irradiation (Grathwohl et al., 2009; Malm et al., 2005; Mildner et al., 2007; Simard et al., 2006; Stalder et al., 2005), the level is not yet known.

NMyA preconditioning regimens consist of low dose irradiation (Shelburne and Bevans, 2009) and/or low dose chemotherapy (Cartier et al., 2009) delivered prior to the BMT. The sublethal irradiation dose used in NMyA preconditioning has been proposed to enhance long-term donor marrow chimerism by inducing proliferative signals after the initial phase of homing (Andrade et al., 2011). However, in order to be a useful therapy for neurodegenerative disease, NMyA preconditioning would probably also have to extend to CNS engraftment of BMT-derived cells. We sought to address this critical gap in knowledge by characterizing CNS engraftment of BMT-derived cells under clinically relevant NMyA preconditioning regimens that result in stable hematopoietic engraftment in the host.

Materials and Methods

Mice

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). BMT was performed in 2-month-old female recipient mice using 6-week-old male mice homozygous for green fluorescent protein (GFP) as donors. GFP expression is under control of the β -actin promoter and cytomegalovirus enhancer. The mice were kept under standard conditions with food and water ad libitum. All protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

Pre-transplant conditioning regimens

We chose three BMT pretransplant conditioning regimens that result in stable hematopoietic engraftment in rodents. Regimen I is a chemotherapeutic approach used in a mouse model of diabetes mellitus to induce robust tolerance to allogeneic islet cell transplants (Guo et al., 2003). We administered fludarabine (400 mg/kg) and cyclophosphamide (200 mg/kg) intraperitoneally (IP) in volumes of 10 μ L/10 g body weight on day -1 before BMT (n=6). Regimen II combines sublethal dose irradiation and chemotherapy to achieve stable hematopoietic engraftment (Luznik et al., 2001). We administered fludarabine (200 mg/kg) and cyclophosphamide (100 mg/kg) intraperitoneally on day -1 followed by 2 Gy irradiation

on day 0 before BMT the following day (n=7). Regimen III uses irradiation only and is based on experiments by Down et al (Down et al., 1991) that demonstrated 50% hematopoietic engraftment between 4 and 6 Gy whole body irradiation. We exposed mice to 5.5 Gy whole body irradiation on day 0 followed by BMT the next day (n=8). Regimen IV is the positive control group who received MyA BMT consisting of 10.5 Gy lethal dose irradiation on day 0 followed by BMT one day later (n=9). In all cases, irradiation was performed with single dose irradiation to the whole body at 2 Gy per minute from a Cesium-137 source (JL Shepherd, Model 81-14, San Fernando, CA). Mice were monitored for 4 months and CNS engraftment was then determined. Hematopoietic engraftment was determined 1, 2 and 4 months after BMT. Additional groups received MyA BMT one (n=5) and two (n=5) months post transplantation.

Generation of chimeric mice

BMT was performed as previously described (Keene et al., 2010). Whole bone marrow was isolated from 6-week-old male C57BL/6;GFP transgenic mice by flushing the femurs and tibias with RPMI media with 10% fetal bovine serum. The samples were combined, passed through a 25-gauge needle filtered through a 70 μ m nylon mesh, and centrifuged. Erythrocytes were lysed in ammonium chloride potassium (ACK) buffer (Invitrogen, Carlsbad, CA) and the remaining leukocytes were resuspended in sterile PBS at a concentration of $\sim 2.5 \times 10^4$ viable nucleated cells per μ l. All the mice received $\sim 5 \times 10^6$ cells via retroorbital venous plexus injections one day after preconditioning with different regimens. Mice were sacrificed for analysis of engraftment 1, 2 or 4 months after BMT.

Tissue collection and processing

Tissue dissection—Mice were anesthetized with Avertin 4 months post transplantation. Blood was drawn via cardiac puncture before the mice were transcardially perfused with ice-cold phosphate buffered saline (PBS). Brains were rapidly removed from the skulls and divided by mid-sagittal section. One hemibrain was dissected on ice and cortex, striatum and hippocampus were immediately placed in cold Hank's Balanced Salt Solution (HBSS) and processed for flow cytometric analysis of BMT-derived cell engraftment. The other hemibrain was postfixed for 2 d in 4% paraformaldehyde, pH 7.6 and then placed in PBS solution containing 30% (w/v) sucrose for 2 d at 4°C. Eyes were rapidly oriented and then enucleated and the cornea and lens were removed under a dissecting microscope. Eye cups were then post-fixed in 4% PFA for 24 hours at 4°C and then cryoprotected in 5%, 10% and 20% sucrose for 30 min each. Eye cups were oriented, embedded in Optimal Cutting Temperature Compound (Tissue Tek, Torrance, CA) and stored at -80°C .

Tissue sectioning—Frozen brains were mounted on a microtome and cut into 40 μ m coronal sections on a Leica CM3050 cryostat (Leica Microsystems GmbH, Wetzlar, Germany). Sections were collected in cold cryoprotectant solution (0.05 mol/L sodium phosphate buffer, pH 7.3, 30% ethylene glycol and 20% glycerol) and stored at -20°C . The frozen eyes were serially sectioned on a cryostat at 40 μ m thickness and mounted on a repeating series of slides in order to ensure an unbiased sample. Sections were stored at -20°C .

Isolation of monocytes/microglia from brain

Mononuclear cells were isolated from the brain homogenates as described previously (Cardona et al., 2006). Briefly, cortex, hippocampus and striatum were dissociated into a cell suspension by gentle homogenization in Hank's Balanced Salt Solution (HBSS). The cell suspension was passed through a 70 μ m nylon cell strainer and centrifuged at 300g for 7 min. Supernatants were removed and cell pellets were resuspended in 70% isotonic Percoll

(GE-healthcare, Uppsala, Sweden). A discontinuous Percoll density gradient was set up as follows: 70%, 35%, and 0% isotonic Percoll. The gradient was centrifuged for 30 min at 1200 g. Microglia were collected from the interphase between the 70% and 35% Percoll layers (Anglen et al., 2003; Frank et al., 2006). Cells were washed and then resuspended in HBSS for staining.

Flow cytometry

Hematopoietic engraftment of donor (GFP⁺) cells was assessed by flow cytometry. Fresh blood samples were exposed to red blood cell lysing buffer (Sigma-Aldrich, St. Louis, MO), and cells were then washed several times in buffer (HBSS containing 2% fetal bovine serum) followed by incubation with primary antibodies on ice for 30 min. Cells were fixed with 1% paraformaldehyde and then analyzed on a flow cytometer (LSR II flow cytometer, BD Biosciences, Franklin Lakes, NJ). Peripheral engraftment was determined as percentage of GFP⁺ cells divided by total cell number. Multilineage differentiation of BMT-derived cells was determined by staining with eFluor 450-conjugated CD3 (T cells), PerCP-Cy5.5-conjugated CD19 (B cells), allophycocyanin-conjugated Gr-1 (neutrophils) and PE-conjugated CD11b (monocytes/macrophages) antibodies (eBioscience, San Diego, CA).

For CNS engraftment, flow cytometry was performed on isolated mononuclear cells from cortex, hippocampus and striatum. The cells were washed and stained with PE-Cy7-conjugated CD11b and Alexa Fluorescence 700-conjugated CD45 antibodies for 60 min. The cell suspension was analyzed to identify the population of CD11b⁺CD45⁺ cells. Engraftment of BMT-derived cells was determined by dividing the CD11b⁺CD45⁺GFP⁺ cell population by total CD11b⁺CD45⁺ monocytes/microglia.

Immunofluorescence and unbiased stereological analysis

Every sixth section of brain and every tenth section of eye were used in each immunostaining paradigm. Immunofluorescence staining was performed according to previously published protocols (Keene et al., 2010). Primary antibodies included anti-Iba-1 (Wako, Richmond, VA; 1:500), anti-CD11b (AbD Serotec, Raleigh, NC; 1:500), anti-CD31 (BD Pharmingen, San Diego, CA, 1:200) and anti-synaptophysin (Millipore, Billerica, MA; 1:200); species-appropriate secondary antibodies were conjugated to Cy3 (1:400; Jackson ImmunoResearch, West Grove, PA) and Cy5 (1:400; Jackson ImmunoResearch, West Grove, PA). Prolong-gold anti-fade with 4', 6-diaminido-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA) was used for coverslipping and nuclear counterstain. All images were captured using an FV1000 confocal microscope (Olympus, Center Valley, PA). Quantification of Iba-1⁺ or CD11b⁺ microglia and BM-derived (GFP⁺) monocytes/microglia in brain was performed using systematic random sampling. Every sixth brain section (240 μ m apart) was analyzed at 400 $\times$ magnification using a Nikon inverted fluorescence microscope (Mellville, NY) and StereoInvestigator software (MicroBrightField, Williston, VT). A fractionator was used with a counting frame measuring 100 μ m $\times$ 100 μ m applied every 500 μ m. Cells in cerebral cortex, hippocampus, and striatum were assessed as Iba-1⁺, GFP⁺ or double-labeled in regions with synaptophysin or without synaptophysin (cortex only).

Because BMT-derived cell engraftment in brains of chimeras pretreated with 5.5 Gy irradiation was localized to perivascular spaces only, regions in cortex with GFP⁺ cells but without synaptophysin were further analyzed for quantification of perivascular BMT-derived cell engraftment in mice conditioned with 5.5 Gy or 10.5 Gy irradiation. For this analysis six regions were randomly selected for each brain section. Cells were assessed as Iba-1⁺, GFP⁺ or double labeled.

Quantification of retinal CD11b⁺ microglia and BM-derived (GFP⁺) mononuclear cells was performed in every tenth retinal section from one eye of each mouse at 200× magnification using a Nikon Eclipse 80i upright fluorescent microscope (Melville, NY). Every CD11b⁺ or GFP⁺ cell in the retina ganglion cell layer (GCL), inner plexiform layer (IPL) and outer plexiform layer (OPL) was recorded as CD11b⁺, GFP⁺ or double- labeled. Cell counts were normalized to the length of retina from each section. For all stereology assessments in brain and retina experimenters were blinded to treatment conditions.

Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed by unpaired Student *t*-test, one- or two-way analysis of variance (ANOVA), followed by Bonferroni post hoc test using GraphPad Prism software (San Diego, CA). Statistical significance was defined as *P* < 0.05.

Results

CNS engraftment kinetics of donor monocytes/microglia cells after MyA BMT

We first characterized CNS engraftment kinetics following MyA (10.5 Gy) preconditioning and BMT at 1, 2 and 4 months. Percent engraftment was determined by flow cytometric assessment of tissue from cortex, hippocampus, and striatum. Monocytic cells were identified as those immunopositive for both CD11b and CD45. We confirmed time-dependent increases in engraftment in all three regions (Fig 1A). At 1 month post-BMT, engraftment in cortex ($10.4 \pm 1.5\%$), hippocampus ($6.9 \pm 0.9\%$) or striatum ($8.1 \pm 1.2\%$) was low, but approximately doubled at 2 months and again at four months in cortex ($36.3 \pm 3.6\%$), hippocampus ($34.5 \pm 3.2\%$) and striatum ($42.4 \pm 2.5\%$). Immunofluorescence histology of contralateral hemisphere confirmed increasing engraftment in all three brain regions over time.

In order to correlate histomorphologic endpoints with flow cytometric analysis of CNS BMT-engraftment we quantified monocyte/microglia engraftment in contralateral cortex, hippocampus, and striatum from 4 month old MyA BMT mice using unbiased stereological methods. Adjacent sections were stained with Iba-1 and CD11b with similar patterns of immunoreactivity, but Iba-1 antibodies decorated the cells more uniformly (Fig 1B) and were thus used for quantification. Confocal images revealed abundant ramified CD11b⁺ microglia in cortex (Fig 1B) and corresponding ramified Iba-1⁺ cells in the hippocampus and striatum (Fig 2A). A subset of Iba-1⁺ and CD11b⁺ cells in each brain region was GFP⁺, and GFP⁺ cells negative for Iba-1 or CD11b were extremely rare. BMT-derived neurons were never seen. Unbiased stereological analysis revealed that $35.7 \pm 3.5\%$ of Iba-1⁺ microglia in cortex, $39.2 \pm 1.5\%$ in hippocampus, and $38.3 \pm 2.7\%$ in striatum were BMT-derived (Fig 2B, black bars). The results are consistent with the 4 month engraftment percentages determined by flow cytometry of contralateral cortex, hippocampus, and striatum (Fig 2B, white bars). This represented sufficient MyA engraftment to enable detection of lower levels of engraftment using NMyA treatments. Based on these results, we chose 4 months BMT-sacrifice latency to test the effects of NMyA pretransplant conditioning on CNS BM engraftment.

Hematopoietic and CNS engraftment efficiency with NMyA BMT

We tested three NMyA pretransplant conditioning regimens (diagrammed in Fig 3) based on established protocols (Down et al., 1991; Guo et al., 2003; Luo et al., 2005) that had been shown to induce stable mixed hematopoietic chimerism in mice: low dose chemotherapy alone (Regimen I), low dose chemotherapy plus low dose irradiation (2 Gy) (Regimen II), and low dose (5.5 Gy) irradiation alone (Regimen III). MyA (10.5 Gy) irradiation was

performed as the positive control. BMT followed after 1 day for all regimens. Flow cytometric assessment of hematopoietic engraftment was assessed on blood drawn at 1, 2, and 4 months post-transplantation (Fig 4A). As expected, by one month MyA controls exhibited a high level of hematopoietic engraftment ($95.1 \pm 1.03\%$ at 1 month post BMT). Regimen III mice also achieved high level of engraftment ($91.5 \pm 1.19\%$) that was not significantly different from MyA BMT recipient mice. Peripheral engraftment was lowest in Regimen I mice ($18.7 \pm 3.5\%$ at 1 month post BMT), while Regimen II mice exhibited intermediate levels of peripheral engraftment ($45.1 \pm 2.6\%$ at 1 month) that were significantly increased compared with Regimen I mice ($***P < 0.001$, two-way ANOVA with Bonferroni post hoc test). Regimens I and II mice were significantly lower than Regimen III mice ($###P < 0.001$, two-way ANOVA with Bonferroni post hoc test). Hematopoietic engraftment for all four regimens was stable over four months (Fig 4A), and multilineage hematopoietic differentiation was not different between groups (Fig 4B). Thus, of the NMyA regimens tested, Regimen III resulted in robust and stable hematopoietic engraftment that was similar to MyA positive controls.

Because correlation between hematopoietic and CNS BMT-derived cell engraftment efficiencies is not well-established, we used flow cytometry to quantify CNS engraftment after each of the NMyA BMT regimens described above. As previously shown in Fig 1A, MyA BMT resulted in greater than 30% chimerism of monocyte-lineage cells in the cortex, hippocampus, and striatum 4 months post-transplant (Fig 4C). Despite the high level of hematopoietic engraftment observed in Regimen III mice, CNS engraftment 4 months post-transplant was low in cortex ($6.7 \pm 0.4\%$), hippocampus ($13.9 \pm 1.9\%$) and striatum ($10.8 \pm 2.4\%$) (Fig 4C, $***P < 0.001$; two-way ANOVA with Bonferroni post hoc test). CNS engraftment in Regimen I or II mice was almost undetectable by flow cytometry in each of the tested brain regions (Fig 4C). Fluorescence histology revealed expected numbers of GFP⁺ cells in MyA BMT mice, while GFP⁺ cells were scarce in brain sections from Regimen I or II mice (Fig 4D). GFP⁺ cells identified in brain sections from Regimen III mice appeared to be localized to vessels and the leptomeninges (Fig 4D).

Histomorphology and microscopic distribution of BMT-derived monocytes/microglia in Regimen III mice

Examination of Iba-1 immunostained brain sections from Regimen III mice revealed that GFP⁺ cells were selectively localized to the leptomeninges (Fig 5A–C), choroid plexus (Fig 5D–F), circumventricular organs (Fig 5G–I), or around blood vessels (Fig 5J–L). Unlike the typical diffuse parenchymal engraftment seen repeatedly with MyA pretransplant conditioning, Regimen III mice showed no definite parenchymal engraftment. Moreover, BMT-derived CNS cells were less ramified with blunted processes and had reduced immunoreactivity for Iba-1 (Fig 4), which is typically strong in MyA BMT-derived CNS cells (Fig 2A). On the basis of their morphology, these GFP⁺ cells were classified into distinct categories including elongate, amoeboid, and rod-shaped. Elongate cells were the most abundant GFP⁺ cells (Fig 6A–D). Also common were amoeboid cells, which were ovoid to elongate with abundant cytoplasm and occasional small projections (Fig 6E–L). Rod cells were closely associated with blood vessels (Fig 6M–P). CD31 immunostaining was negative in GFP⁺ cells but demonstrated close association with the vascular wall by BMT-derived cells, which resided predominantly on the parenchymal side of the vessel and never entirely within the lumen (Fig 6Q–T).

Brain sections from MyA and Regimen III mice were immunostained with synaptophysin and Iba-1 to further evaluate for parenchymal vs. perivascular engraftment in cerebral cortex. Synaptophysin staining was positive in parenchyma around neuronal soma but spared perivascular spaces. As expected, confocal microscopic analysis revealed GFP⁺ cells within synaptophysin immunopositive parenchyma in sections from MyA BMT-recipient

mice, however, GFP⁺ cells in Regimen III brains were confined to synaptophysin-immunonegative regions (Fig 7A). We found no significant differences in perivascular engraftment percentage between MyA and Regimen III mice (Fig 7B).

Histomorphology and microscopic distribution of BMT-derived monocytes/microglia in retina after NMyA pretransplant conditioning

Engraftment of MyA BMT-derived cells is more efficient in retina compared with the brain (Boettcher et al., 2008; Xu et al., 2007), so we evaluated retina and optic nerves of NMyA BMT mice with MyA BMT controls. Retinal microglia/macrophages were identified by CD11b (Fig 8A) and Iba-1 immunostaining. Under MyA conditions most of the CD11b⁺ retina microglia were BMT-derived, were predominantly localized to the retina ganglion cell, inner, and outer plexiform layers, and exhibited classical ramified morphology. As in the brain, retinas from Regimen III mice had fewer engrafted BMT-derived cells and they had a less ramified cytomorphology (Fig 8A). Quantification of GFP⁺ and CD11b⁺ cells in retina demonstrated 85–95% engraftment of BMT-derived monocytes/microglia in MyA BMT recipients, whereas only 5–10% BMT-derived cell engraftment was observed in Regimen III mouse retinas (Fig 8B). BMT-derived cells were scarce in retinas from Regimen I and II mice (data not shown).

Examination of non-retinal ocular tissues revealed engraftment of BMT-derived cells in ciliary body, choroid, optic nerve, and extraocular soft tissues in MyA and Regimen III BMT recipients, while eyes from Regimen I and II mice had only rare periocular and choroidal donor cells and no engraftment in the optic nerve (Fig 9A). There were similar engraftment patterns and cytomorphology of donor cells in Regimen III mice and MyA BMT mice optic nerves, where BMT-derived cells exhibited a mostly ramified morphology but were only weakly stained for Iba-1 (Fig 9B). Similar to our findings in brain, these data suggest that 5.5 Gy pretransplant irradiation is sufficient for engraftment of BMT-derived cells in the perivascular and arachnoidal tissues, but does not result in significant engraftment in the retina proper. However, Regimen III, but not Regimens I or II, does appear to be sufficient for engraftment of transplant-derived microglia-like cells within the optic nerve.

Discussion

We report progressive CNS engraftment of BMT-derived cells under MyA pretransplant conditioning and confirm stable hematopoietic chimerism with three different NMyA BMT regimens. However, while Regimen III (5.5 Gy preconditioning) was sufficient to mediate engraftment of BMT-derived monocytes/microglia within vascular and perivascular compartments of the CNS, none of the NMyA regimens resulted in significant parenchymal engraftment of the brain or retina. This evidence suggests that parenchymal engraftment of BMT-derived cells requires high dose irradiation and challenges the therapeutic potential of NMyA BMT for neurodegenerative diseases that require engraftment of donor cells into the neural parenchymal compartment.

Clinical NMyA BMT regimens employ less toxic preconditioning therapies such as low dose irradiation (Shelburne and Bevens, 2009), T lymphocyte modulation, low dose chemotherapy, or a combination of these and achieve variable hematopoietic chimerism (Micol et al., 2007). In this study we focused on three regimens that had demonstrated efficacy in establishing stable hematopoietic chimerism, but we cannot exclude the possibility that other, as-yet untested NMyA preconditioning regimens more effectively stimulate CNS engraftment.

In the present study, mice pretreated with Regimens I and II achieved peripheral engraftment of approximately 20% and 40%, respectively, far lower than 5.5 or 10.5 Gy BMT. It has been suggested that even low dose irradiation improves stem cell engraftment through effects on the marrow microenvironment in a dose-dependent manner (Tomita et al., 1994), and the major effect of sublethal irradiation is to enhance long-term donor chimerism by inducing proliferative signals after the initial phase of homing (Andrade et al., 2011). In this study high-level hematopoietic engraftment (90%) was achieved with 5.5 Gy irradiation (Regimen III), and this extended to perivascular CNS engraftment that was not different from MyA controls. However, even with these high levels of hematopoietic engraftment, CNS parenchymal engraftment was not achieved. Unfortunately, this points to an irradiation threshold for CNS parenchymal engraftment that likely requires lethal or near lethal doses of irradiation, since mortality from irradiation without BMT begins to be seen at 6 Gy in mice (Down et al., 1991).

The perivascular BMT-derived cells in Regimen III mice appear to be best characterized as perivascular monocytes/macrophages. It is well-established that perivascular macrophages and microglial cells are replaced by BMT-derived monocytic cells in the CNS following lethal dose irradiation (Eglitis and Mezey, 1997; Priller et al., 2001; Simard and Rivest, 2004). This is achieved through circulating monocytes exiting the vascular lumen through the endothelial cell-glia limitans into the perivascular (Virchow-Robin) space as monocytes/macrophages and then entering the neural parenchyma as microglia. Our findings support the concept that most of BMT-derived cells within the CNS in Regimen III mice were perivascular monocytes/macrophages based on morphology and immunohistology (Vallieres and Sawchenko, 2003). Further support for this concept comes from lower levels of Iba-1 expression than would be expected for microglia, underscoring the fact that these cells are functionally and phenotypically distinct (Streit et al., 1999).

The recruitment of BM-derived circulating endothelial precursors has been observed in peripheral organs (Kalka et al., 2000; Kocher et al., 2001; Shintani et al., 2001; Takahashi et al., 1999) and in the choroid plexus under basal conditions and after ischemia (Zhang et al., 2002). However, we did not observe a single GFP⁺, CD31⁺ cell, consistent with previous observations by others that none of the BM-derived cells found throughout the brain displayed endothelial characteristics (Vallieres and Sawchenko, 2003).

MyA BMT results in near-complete replacement of retinal microglia with BMT-derived cells, an effect far greater than that seen in the brain. While Regimens I and II were not sufficient for BMT-derived cell engraftment in retina or optic nerve, Regimen III resulted in rare donor-derived cells in retina with increased CD11b immunofluorescence. Moreover, optic nerve engraftment was nearly indistinguishable from MyA controls. These data suggest that barriers to engraftment in brain that require MyA irradiation may be more easily breached in retina and may even be absent in optic nerve. Similar to cerebral non-parenchymal choroid plexus, leptomeninges, and perivascular space, non-neural regions of the eye (including the choroid) appear to have less formidable barriers to engraftment.

Although these data appear to suggest a physical barrier to engraftment, there are multiple possible mechanisms. Several studies have been performed in transplantation or parabiont paradigms in which the CNS is shielded from irradiation. In each case, uniformly low to negative engraftment has been observed (Ajami et al., 2007; Kaneko et al., 2008; Mildner et al., 2007). The relatively high dose (10.5 Gy) of radiation used in MyA preconditioning appears to alter the blood brain barrier (BBB), thus permitting the physical transit of cells into the CNS (Diserbo et al., 2002; Yuan et al., 2003). Consistent with this, we observed that 5.5 Gy (Regimen III), but not 2 Gy (Regimen II), resulted in engraftment of cells across the BBB into the perivascular space; however, 5.5 Gy was insufficient to mediate parenchymal

engraftment. Taken together, this suggests that multiple levels of injury are required to achieve complete CNS engraftment. Another possibility is that measurable CNS engraftment only occurs following complete myeloablation, through some undetermined mechanism. This seems unlikely considering the high levels of peripheral engraftment achieved with 5.5 Gy.

Recruitment of microglia into the CNS is dependent upon chemokine and cytokine signaling (El Khoury and Luster, 2008). MyA irradiation initiates the release of these factors including vascular endothelial growth factor (VEGF), TNF α , and CCL2 (Lee et al., 2010; Nordal et al., 2004). Retinal para-inflammation, characterized by the chemokine CCL2-CCR2 pathway, is crucial for the recruitment of BM-derived myeloid cells (Chen et al., 2012). Furthermore, control of monocyte migration into the CNS in conditions like amyotrophic lateral sclerosis, experimental autoimmune encephalitis, stroke, traumatic brain injury, and even Alzheimer's disease is known to depend on chemokine and cytokine signaling mechanisms (Malm et al., 2005). Additional studies of modulation of expression of chemokines, cytokines, and complement components by MyA and NMyA pretransplant conditioning regimens is needed to determine the role these paracrine signaling pathways play in CNS engraftment.

This study provides the first comprehensive profile of BMT-derived hematopoietic cells and CNS engraftment under diverse NMyA pretransplant conditions. Although NMyA preconditioning failed to effectively promote CNS engraftment of BM cells, studies are underway in our laboratory to determine whether CNS perivascular engraftment might be the source of at least some of the neuroprotective effects of BMT in experimental neurodegenerative disease. Identification of specific barrier mechanisms to CNS engraftment and how MyA, but not NMyA, BMT barriers may allow future therapies to be designed to replicate the effects of MyA irradiation on CNS BM engraftment and neuroprotection whilst sparing patients serious radiation-associated side effects.

Acknowledgments

Supported by NIH grants P50AG05136 and the Nancy and Buster Alvord Endowment. We thank Samantha Rice, Meilany Wijaya, Kelsey Hinton, Jing Huang, Dan Possin, Dr. Carole Wilson, and Dr. "Aru" Arumuganathan (Benaroya Research Institute) for expert technical assistance, and Aimee Schantz and Amy Look for administrative support.

Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
CNS	central nervous system
MyA	myeloablative
NMyA	non-myeloablative
BMT	bone marrow transplant
GCL	ganglion cell layer
GFP	green fluorescent protein
Gy	Gray
HBSS	Hank's balanced salt solution

IPL	inner plexiform layer
OPL	outer plexiform layer
PBS	phosphate buffered saline
wt	wild type

References

- Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci.* 2007; 10:1538–43. [PubMed: 18026097]
- Anderson MG, Libby RT, Gould DB, Smith RS, John SW. High-dose radiation with bone marrow transfer prevents neurodegeneration in an inherited glaucoma. *Proc Natl Acad Sci U S A.* 2005; 102:4566–71. [PubMed: 15758074]
- Andrade J, Ge S, Symbatyan G, Rosol MS, Olch AJ, Crooks GM. Effects of sublethal irradiation on patterns of engraftment after murine bone marrow transplantation. *Biol Blood Marrow Transplant.* 2011; 17:608–19. [PubMed: 21176787]
- Anglen CS, Truckenmiller ME, Schell TD, Bonneau RH. The dual role of CD8+ T lymphocytes in the development of stress-induced herpes simplex encephalitis. *J Neuroimmunol.* 2003; 140:13–27. [PubMed: 12864968]
- Annaloro C, Onida F, Lambertenghi Delilieri G. Autologous hematopoietic stem cell transplantation in autoimmune diseases. *Expert Rev Hematol.* 2009; 2:699–715. [PubMed: 21082959]
- Boettcher C, Ulbricht E, Helmlinger D, Mack AF, Reichenbach A, Wiedemann P, Wagner HJ, Seeliger MW, Bringmann A, Priller J. Long-term engraftment of systemically transplanted, gene-modified bone marrow-derived cells in the adult mouse retina. *Br J Ophthalmol.* 2008; 92:272–5. [PubMed: 18227206]
- Burt RK, Loh Y, Cohen B, Stefoski D, Balabanov R, Katsamakis G, Oyama Y, Russell EJ, Stern J, Muraro P, Rose J, Testori A, Bucha J, Jovanovic B, Milanetti F, Storek J, Voltarelli JC, Burns WH. Autologous non-myeloablative haemopoietic stem cell transplantation in relapsing-remitting multiple sclerosis: a phase I/II study. *Lancet Neurol.* 2009; 8:244–53. [PubMed: 19186105]
- Burt RK, Traynor A, Statkute L, Barr WG, Rosa R, Schroeder J, Verda L, Krosnjak N, Quigley K, Yaung K, Villa Bs M, Takahashi M, Jovanovic B, Oyama Y. Nonmyeloablative hematopoietic stem cell transplantation for systemic lupus erythematosus. *Jama.* 2006; 295:527–35. [PubMed: 16449618]
- Cardona AE, Huang D, Sasse ME, Ransohoff RM. Isolation of murine microglial cells for RNA analysis or flow cytometry. *Nat Protoc.* 2006; 1:1947–51. [PubMed: 17487181]
- Cartier N, Hacein-Bey-Abina S, Bartholomae CC, Veres G, Schmidt M, Kutschera I, Vidaud M, Abel U, Dal-Cortivo L, Caccavelli L, Mahlaoui N, Kiermer V, Mittelstaedt D, Bellesme C, Lahlou N, Lefrere F, Blanche S, Audit M, Payen E, Leboulch P, l'Homme B, Bougneres P, Von Kalle C, Fischer A, Cavazzana-Calvo M, Aubourg P. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science.* 2009; 326:818–23. [PubMed: 19892975]
- Chen M, Zhao J, Luo C, Pandi SP, Penalva RG, Fitzgerald DC, Xu H. Para-inflammation-mediated retinal recruitment of bone marrow-derived myeloid cells following whole-body irradiation is CCL2 dependent. *Glia.* 2012; 60:833–42. [PubMed: 22362506]
- Cobbold SP, Martin G, Qin S, Waldmann H. Monoclonal antibodies to promote marrow engraftment and tissue graft tolerance. *Nature.* 1986; 323:164–6. [PubMed: 3528866]
- Corti S, Locatelli F, Donadoni C, Guglieri M, Papadimitriou D, Strazzer S, Del Bo R, Comi GP. Wild-type bone marrow cells ameliorate the phenotype of SOD1-G93A ALS mice and contribute to CNS, heart and skeletal muscle tissues. *Brain.* 2004; 127:2518–32. [PubMed: 15469951]
- Diserbo M, Agin A, Lamproglou I, Mauris J, Staali F, Multon E, Amourette C. Blood-brain barrier permeability after gamma whole-body irradiation: an in vivo microdialysis study. *Can J Physiol Pharmacol.* 2002; 80:670–8. [PubMed: 12182325]

- Djukic M, Mildner A, Schmidt H, Czesnik D, Bruck W, Priller J, Nau R, Prinz M. Circulating monocytes engraft in the brain, differentiate into microglia and contribute to the pathology following meningitis in mice. *Brain*. 2006; 129:2394–403. [PubMed: 16891321]
- Down JD, Tarbell NJ, Thames HD, Mauch PM. Syngeneic and allogeneic bone marrow engraftment after total body irradiation: dependence on dose, dose rate, and fractionation. *Blood*. 1991; 77:661–9. [PubMed: 1991176]
- Eglitis MA, Mezey E. Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc Natl Acad Sci U S A*. 1997; 94:4080–5. [PubMed: 9108108]
- El Khoury J, Luster AD. Mechanisms of microglia accumulation in Alzheimer's disease: therapeutic implications. *Trends Pharmacol Sci*. 2008; 29:626–32. [PubMed: 18835047]
- Frank MG, Wieseler-Frank JL, Watkins LR, Maier SF. Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: immunophenotypic and functional characteristics. *J Neurosci Methods*. 2006; 151:121–30. [PubMed: 16125247]
- Grathwohl SA, Kalin RE, Bolmont T, Prokop S, Winkelmann G, Kaeser SA, Odenthal J, Radde R, Eldh T, Gandy S, Aguzzi A, Staufenbiel M, Mathews PM, Wolburg H, Heppner FL, Jucker M. Formation and maintenance of Alzheimer's disease beta-amyloid plaques in the absence of microglia. *Nat Neurosci*. 2009; 12:1361–3. [PubMed: 19838177]
- Guo Z, Wu T, Sozen H, Pan Y, Heuss N, Kalscheuer H, Sutherland DE, Blazar BR, Hering BJ. A substantial level of donor hematopoietic chimerism is required to protect donor-specific islet grafts in diabetic NOD mice. *Transplantation*. 2003; 75:909–15. [PubMed: 12698073]
- Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci*. 2007; 10:1387–94. [PubMed: 17965659]
- Kalka C, Asahara T, Krone W, Isner JM. Angiogenesis and vasculogenesis. Therapeutic strategies for stimulation of postnatal neovascularization. *Herz*. 2000; 25:611–22. [PubMed: 11076319]
- Kaneko H, Nishiguchi KM, Nakamura M, Kachi S, Terasaki H. Characteristics of bone marrow-derived microglia in the normal and injured retina. *Invest Ophthalmol Vis Sci*. 2008; 49:4162–8. [PubMed: 18487364]
- Keene CD, Chang RC, Lopez-Yglesias AH, Shalloway BR, Sokal I, Li X, Reed PJ, Keene LM, Montine KS, Breyer RM, Rockhill JK, Montine TJ. Suppressed accumulation of cerebral amyloid {beta} peptides in aged transgenic Alzheimer's disease mice by transplantation with wild-type or prostaglandin E2 receptor subtype 2-null bone marrow. *Am J Pathol*. 2010; 177:346–54. [PubMed: 20522650]
- Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med*. 2001; 7:430–6. [PubMed: 11283669]
- Kwan W, Magnusson A, Chou A, Adame A, Carson MJ, Kohsaka S, Masliah E, Moller T, Ransohoff R, Tabrizi SJ, Bjorkqvist M, Muchowski PJ. Bone marrow transplantation confers modest benefits in mouse models of Huntington's disease. *J Neurosci*. 2012; 32:133–42. [PubMed: 22219276]
- Lee WH, Sonntag WE, Mitschelen M, Yan H, Lee YW. Irradiation induces regionally specific alterations in pro-inflammatory environments in rat brain. *Int J Radiat Biol*. 2010; 86:132–44. [PubMed: 20148699]
- Luo B, Nanji SA, Schur CD, Pawlick RL, Anderson CC, Shapiro AM. Robust tolerance to fully allogeneic islet transplants achieved by chimerism with minimal conditioning. *Transplantation*. 2005; 80:370–7. [PubMed: 16082333]
- Luznik L, Jalla S, Engstrom LW, Iannone R, Fuchs EJ. Durable engraftment of major histocompatibility complex-incompatible cells after nonmyeloablative conditioning with fludarabine, low-dose total body irradiation, and posttransplantation cyclophosphamide. *Blood*. 2001; 98:3456–64. [PubMed: 11719388]
- Malm TM, Koistinaho M, Parepalo M, Vatanen T, Ooka A, Karlsson S, Koistinaho J. Bone-marrow-derived cells contribute to the recruitment of microglial cells in response to beta-amyloid deposition in APP/PS1 double transgenic Alzheimer mice. *Neurobiol Dis*. 2005; 18:134–42. [PubMed: 15649704]

- Malm TM, Magga J, Kuh GF, Vatanen T, Koistinaho M, Koistinaho J. Minocycline reduces engraftment and activation of bone marrow-derived cells but sustains their phagocytic activity in a mouse model of Alzheimer's disease. *Glia*. 2008; 56:1767–79. [PubMed: 18649403]
- Micol JB, Berthon C, Tricot S, Terriou L, Darre S, Cracco P, Boulanger F, Bauters F, Jouet JP, Yakoub-Agha I. Allogeneic stem-cell transplantation with fludarabine and 2-Gy TBI-based conditioning regimen for chronic hematological malignancy: a study of 25 consecutive patients and a literature review. *Leuk Lymphoma*. 2007; 48:321–9. [PubMed: 17325892]
- Mildner A, Schmidt H, Nitsche M, Merkler D, Hanisch UK, Mack M, Heikenwalder M, Bruck W, Priller J, Prinz M. Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. *Nat Neurosci*. 2007; 10:1544–53. [PubMed: 18026096]
- Naert G, Rivest S. Hematopoietic CC-chemokine receptor 2 (CCR2) competent cells are protective for the cognitive impairments and amyloid pathology in a transgenic mouse model of Alzheimer's disease. *Mol Med*. 2012; 18:297–313. [PubMed: 22160221]
- Nordal RA, Nagy A, Pintilie M, Wong CS. Hypoxia and hypoxia-inducible factor-1 target genes in central nervous system radiation injury: a role for vascular endothelial growth factor. *Clin Cancer Res*. 2004; 10:3342–53. [PubMed: 15161688]
- Ohnishi S, Ito H, Suzuki Y, Adachi Y, Wate R, Zhang J, Nakano S, Kusaka H, Ikehara S. Intra-bone marrow-bone marrow transplantation slows disease progression and prolongs survival in G93A mutant SOD1 transgenic mice, an animal model mouse for amyotrophic lateral sclerosis. *Brain Res*. 2009; 1296:216–24. [PubMed: 19686706]
- Pollack SM, O'Connor TP Jr, Hashash J, Tabbara IA. Nonmyeloablative and reduced-intensity conditioning for allogeneic hematopoietic stem cell transplantation: a clinical review. *Am J Clin Oncol*. 2009; 32:618–28. [PubMed: 19593085]
- Priller J, Flugel A, Wehner T, Boentert M, Haas CA, Prinz M, Fernandez-Klett F, Prass K, Bechmann I, de Boer BA, Frotscher M, Kreutzberg GW, Persons DA, Dirnagl U. Targeting gene-modified hematopoietic cells to the central nervous system: use of green fluorescent protein uncovers microglial engraftment. *Nat Med*. 2001; 7:1356–61. [PubMed: 11726978]
- Priller J, Prinz M, Heikenwalder M, Zeller N, Schwarz P, Heppner FL, Aguzzi A. Early and rapid engraftment of bone marrow-derived microglia in scrapie. *J Neurosci*. 2006; 26:11753–62. [PubMed: 17093096]
- Prinz M, Priller J, Sisodia SS, Ransohoff RM. Heterogeneity of CNS myeloid cells and their roles in neurodegeneration. *Nat Neurosci*. 2011; 14:1227–35. [PubMed: 21952260]
- Ransohoff RM, Cardona AE. The myeloid cells of the central nervous system parenchyma. *Nature*. 2010; 468:253–62. [PubMed: 21068834]
- Rivest S. Regulation of innate immune responses in the brain. *Nat Rev Immunol*. 2009; 9:429–39. [PubMed: 19461673]
- Sharabi Y, Sachs DH. Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. *J Exp Med*. 1989; 169:493–502. [PubMed: 2562984]
- Shelburne N, Bevans M. Non-myeloablative allogeneic hematopoietic stem cell transplantation. *Semin Oncol Nurs*. 2009; 25:120–8. [PubMed: 19411015]
- Shie FS, Nivison M, Hsu PC, Montine TJ. Modulation of microglial innate immunity in Alzheimer's disease by activation of peroxisome proliferator-activated receptor gamma. *Curr Med Chem*. 2009; 16:643–51. [PubMed: 19199928]
- Shintani S, Murohara T, Ikeda H, Ueno T, Sasaki K, Duan J, Imaizumi T. Augmentation of postnatal neovascularization with autologous bone marrow transplantation. *Circulation*. 2001; 103:897–903. [PubMed: 11171801]
- Simard AR, Rivest S. Bone marrow stem cells have the ability to populate the entire central nervous system into fully differentiated parenchymal microglia. *Faseb J*. 2004; 18:998–1000. [PubMed: 15084516]
- Simard AR, Soulet D, Gowing G, Julien JP, Rivest S. Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. *Neuron*. 2006; 49:489–502. [PubMed: 16476660]

- Stalder M, Birsan T, Hausen B, Borie DC, Morris RE. Immunosuppressive effects of surgery assessed by flow cytometry in nonhuman primates after nephrectomy. *Transpl Int*. 2005; 18:1158–65. [PubMed: 16162103]
- Streit WJ, Walter SA, Pennell NA. Reactive microgliosis. *Prog Neurobiol*. 1999; 57:563–81. [PubMed: 10221782]
- Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med*. 1999; 5:434–8. [PubMed: 10202935]
- Tomita Y, Sachs DH, Sykes M. Myelosuppressive conditioning is required to achieve engraftment of pluripotent stem cells contained in moderate doses of syngeneic bone marrow. *Blood*. 1994; 83:939–48. [PubMed: 7906567]
- Tyndall A, Saccardi R. Haematopoietic stem cell transplantation in the treatment of severe autoimmune disease: results from phase I/II studies, prospective randomized trials and future directions. *Clin Exp Immunol*. 2005; 141:1–9. [PubMed: 15958063]
- Vallieres L, Sawchenko PE. Bone marrow-derived cells that populate the adult mouse brain preserve their hematopoietic identity. *J Neurosci*. 2003; 23:5197–207. [PubMed: 12832544]
- Voltarelli JC, Couri CE, Stracieri AB, Oliveira MC, Moraes DA, Pieroni F, Coutinho M, Malmegrim KC, Foss-Freitas MC, Simoes BP, Foss MC, Squiers E, Burt RK. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. *Jama*. 2007; 297:1568–76. [PubMed: 17426276]
- Xu H, Chen M, Mayer EJ, Forrester JV, Dick AD. Turnover of resident retinal microglia in the normal adult mouse. *Glia*. 2007; 55:1189–98. [PubMed: 17600341]
- Yuan H, Gaber MW, McColgan T, Naimark MD, Kiani MF, Merchant TE. Radiation-induced permeability and leukocyte adhesion in the rat blood-brain barrier: modulation with anti-ICAM-1 antibodies. *Brain Res*. 2003; 969:59–69. [PubMed: 12676365]
- Zhang ZG, Zhang L, Jiang Q, Chopp M. Bone marrow-derived endothelial progenitor cells participate in cerebral neovascularization after focal cerebral ischemia in the adult mouse. *Circ Res*. 2002; 90:284–8. [PubMed: 11861416]

Fig. 1. Kinetics of BM-derived mononuclear cells engraftment in brain with MyA BMT. **A.** Monocytic cells isolated from cortex, hippocampus and striatum of chimeras preconditioned with 10.5 Gy were analyzed using flow cytometry. Monocytes/microglia were identified by CD11b⁺CD45⁺ staining. Quantitation of the percentage of GFP⁺ cells in CD11b⁺CD45⁺ cells in cortex, hippocampus and striatum of mice that received myeloablative BMT at different time points. Data are mean $\pm$ SEM, n=6–9. (*P < 0.05, **P < 0.01, two-way ANOVA analysis using Bonferroni post hoc test). **B.** Representative immunofluorescence photomicrographs of cerebral cortex from MyA BMT recipient mouse at 4 months post transplant. Microglia are identified with anti CD11b stains (red) and BMT-derived cells are GFP⁺ (green). Merged images demonstrate that all GFP⁺ cells are also CD11b⁺. Scale bar, 25 μ m.

Fig. 2. Unbiased stereological analysis of BMT-derived microglia engraftment in brain of MyA BMT recipients validates flow cytometric engraftment analysis. **A.** Confocal images show GFP⁺ cells (green) that were co-immunopositive for Iba-1 (red) in hippocampus (left) and striatum (right) from a representative mouse preconditioned with 10.5 Gy. GFP⁺ cells with arrows are shown in greater detail on the lower panel as individual and merged color channels and illustrate the overlap between Iba-1 and GFP that is the case for nearly all GFP⁺ cells. Scale bar, 30 μ m. **B.** Quantification of engraftment of BM-derived mononuclear cells in cortex, hippocampus, and striatum is compared between unbiased stereological analysis (black bars) and flow cytometry (white bars). No statistically significant differences in engraftment were identified between the two methods.

Fig. 3. NMyA and MyA pre-transplant conditioning regimens and transplantation protocols. Experiments were conducted with three disparate NMyA preconditioning regimens, including fludarabine and cyclophosphamide i.p. injection without irradiation (Regimen I), fludarabine and cyclophosphamide i.p. injection combined with 2 Gy whole body irradiation (Regimen II), and 5.5 Gy whole body irradiation (Regimen III). MyA (10.5 Gy whole body irradiation) served as a positive control (IV). BMT was performed 24 hours after irradiation and were sacrificed for engraftment analysis at the indicated time points.

Fig. 4.

Hematopoietic and central engraftment of BMT-derived cells under NMyA and MyA BMT regimens. **A.** Flow cytometric analysis of engraftment of GFP⁺ donor cells in peripheral blood in chimeras that received MyA or NMyA BMT at 1, 2 and 4 months after transplantation. Increased engraftment was seen in mice that received regimen II compared with regimen I NMyA pretreatment (n=6–7, ****P* < 0.001, two-way ANOVA with Bonferroni post hoc test). Less efficient engraftment of GFP⁺ donor cells was observed in mice that received regimen I or II NMyA pretreatment (n=6–9, ****P* < 0.001 versus regimen I. ###*P* < 0.001 versus regimen III, two-way ANOVA with Bonferroni post hoc test). **B.** Flow cytometric analysis of peripheral blood for hematopoietic lineage differentiation of GFP⁺ cells in mice 4 months after BMT. GFP fluorescence was measured in T cells, B cells, neutrophils and monocytes/macrophages. Peripheral blood were stained with the T cell marker CD3, the B cell marker CD19, the neutrophil marker Gr-1 and CD11b to stain the monocytes and macrophages. No significant differences in hematopoietic lineage differentiation were identified between conditioning regimens. **C.** Quantitation of the percent of total GFP⁺CD11b⁺CD45⁺ mononuclear cells in cortex, hippocampus and striatum at 4 months post transplantation using flow cytometry revealed reduced engraftment in all NMyA regimens compared with MyA controls (n=6–9, ****P* < 0.001 versus MyA, two-way ANOVA analysis using Bonferroni post hoc test). **D.** Microscopic analysis of engraftment and distribution of BMT-derived cells in cortex of chimeras preconditioned with I, II, III or IV regimens. Confocal images show representative examples of GFP⁺ cells (green) counterstained with DAPI (blue). Classically ramified cells were observed in mice that received MyA BMT. Elongated or amoeboid cells were found in mice preconditioned with 5.5 Gy and were limited to perivascular spaces. BM-derived cells were extremely rare in cortex of chimeras preconditioned with regimens I and II. All results (A–C) are expressed as the mean ± SEM. Scale bar, 100 μm.

Fig. 5. Neuroanatomic distribution of BMT-derived cells in brains of chimeric mice 4 months after BMT following 5.5 Gy NMyA irradiation. Immunofluorescence microscopy revealed monocytic cells in the leptomeninges (**A–C**), choroid plexus (**D–F**) and subfornical organ (**G–I**). GFP⁺ cells were also found in regions associated with blood vessels (**J–L**). Sections were stained for Iba-1 (red). GFP was detected as green. Lep, leptomeninges; SFO, subfornical organ; ChP, choroid plexus; BV, blood vessel. Scale bar, 100 μ m.

Fig. 6.

Cytomorphology of BMT-derived cells found in brains of mice that received 5.5 Gy NMyA BMT. Confocal images show GFP⁺ cells (green) with elongated (A-D), ameboid (E-L), or rod (M-P) shaped morphology. Iba-1 immunofluorescence (red) is weakly positive in BMT-derived cells. Scale bar: 50 μ m. Q-T Confocal images reveal that GFP⁺ (green) cells counterstained with DAPI (blue) are negative for CD31 (red), and are intimately associated with blood vessels. Scale bar: 50 μ m.

Fig. 7. Perivascular, but not parenchymal, brain engraftment of BMT-derived cells in NMyA 5.5 Gy BMT recipient mice. **A.** Brain sections from Regimen III or MyA controls stained with Iba-1 (red) and synaptophysin (purple) antibodies and counterstained with DAPI (blue). Confocal images showed ramified GFP⁺ cells (green) engrafted in synaptophysin⁺ cortex parenchyma that were positive for Iba-1 and GFP in MyA BMT recipients. In contrast, elongate and amoeboid GFP⁺ cells with low Iba-1 expression engrafted in perivascular patterns in synaptophysin negative areas of cortex in 5.5 Gy BMT mice. Scale bar: 100 μ m. **B.** Quantitation of perivascular BMT-derived engraftment between MyA and 5.5 Gy NMyA BMT recipients revealed similar perivascular engraftment efficiencies between Regimen III mice and MyA controls.. All results are expressed as the mean $\pm$ SEM, n = 6–9.

Fig. 8. Limited NMyA BMT engraftment in retina. **A.** Histologic retinal sections from mice 4 months after BMT, stained with CD11b antibody (red) were analyzed with confocal microscopy revealing mostly ramified CD11b⁺GFP⁺ cells in GCL, IPL and OPL in MyA BMT recipients while GFP⁺ cells (green) in 5.5 Gy NMyA mice exhibited a less ramified morphology and variable CD11b immunopositivity. **B.** Quantification of the percent engraftment of total CD11b⁺ cells that were GFP⁺ in GCL, IPL and OPL of the retina of chimeras preconditioned with 10.5 Gy or 5.5 Gy irradiation revealed significantly reduced levels of BMT-derived cells under 5.5 Gy NMyA conditions. No donor cells were identified in NMyA regimens I and II (data not shown). All results are expressed as the mean $\pm$ SEM, $n = 6-9$. Scale bar: 100 μ m (inset 10 μ m).

Fig. 9. Robust NMyA BMT engraftment in optic nerve. **A.** Abundance of BMT-derived (GFP⁺) cells in optic nerve (white circles) of mice transplanted with BM following MyA and 5.5 Gy NMyA preconditioning but only rare cells in NMyA regimens I and II. **B.** Confocal images show numerous GFP⁺ cells engrafted in optic nerve of chimeric mice pretreated with 10.5 Gy or 5.5 Gy irradiation. Scale bar: 50 μm. Cells in boxes are shown in greater detail on the lower panel revealed robust optic nerve engraftment but weak immunostaining of GFP⁺ cells with Iba-1.