Video Article

Busulfan as a Myelosuppressive Agent for Generating Stable High-level Bone Marrow Chimerism in Mice

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Abstract

Bone marrow transplantation (BMT) is often used to replace the bone marrow (BM) compartment of recipient mice with BM cells expressing a distinct biomarker isolated from donor mice. This technique allows for identification of donor-derived hematopoietic cells within the recipient mice, and can be used to isolate and characterize donor cells using various biochemical techniques. BMT typically relies on myeloablative conditioning with total body irradiation to generate niche space within the BM compartment of recipient mice for donor cell engraftment. The protocol we describe here uses myelosuppressive conditioning with the chemotherapeutic agent busulfan. Unlike irradiation, which requires the use of specialized facilities, busulfan conditioning is performed using intraperitoneal injections of 20 mg/kg busulfan until a total dose of 60-100 mg/kg has been administered. Moreover, myeloablative irradiation can have toxic side effects and requires successful engraftment of donor cells for survival of recipient mice. In contrast, busulfan conditioning using these doses is generally well tolerated and mice survive without donor cell support. Donor BM cells are isolated from the femurs and tibiae of mice ubiquitously expressing green fluorescent protein (GFP), and injected into the lateral tail vein of conditioned recipient mice. BM chimerism is estimated by quantifying the number of GFP+ cells within the peripheral blood following BMT. Levels of chimerism >80% are typically observed in the peripheral blood 3-4 weeks post-transplant and remain established for at least 1 year. As with irradiation, conditioning with busulfan and BMT allows for the accumulation of donor BM-derived cells within the central nervous system (CNS), particularly in mouse models of neurodegeneration. This busulfan-mediated CNS accumulation may be more physiological than total body irradiation, as the busulfan treatment is less toxic and CNS inflammation appears to be less extensive. We hypothesize that these cells can be genetically engineered to deliver therapeutics to the CNS.

Video Link

The video component of this article can be found at https://www.jove.com/video/52553/

Introduction

Recently, there has been considerable interest in the roles of microglia and bone marrow (BM)-derived cells (BMDCs) in the central nervous system (CNS), both during disease as well as with normal aging. Microglia, the resident immune cells of the CNS, are now known to develop in the CNS following the entry of primitive myeloid progenitors during embryogenesis[1]. Microglia retain aspects of their myelomonocytic lineage well into adult life. While evidence suggests that the contribution of BMDCs to the microglial pool is minimal in healthy adult animals[1], the role BMDCs play in the progression of various neurodegenerative diseases remains unclear. This uncertainty is compounded by the fact that it is difficult to distinguish BMDCs that accumulate within the CNS from endogenous resident microglia, as no universally accepted discriminating immunohistochemical marker has been identified. In order to monitor BMDCs *in vivo*, we employ a BM transplantation (BMT) protocol whereby endogenous BM cells of a recipient mouse are replaced with those of a donor mouse ubiquitously expressing green fluorescent protein (GFP) under control of the β-actin promoter. This protocol permits the determination of both the localization and morphology of GFP+ BMDCs within the recipient mouse using immunohistochemistry, and facilitates further characterization of these cells using fluorescence-activated cell sorting (FACS) followed by subsequent biochemical assays.

For a successful BMT, recipient BM cells need to be ablated (termed myeloablation) in order to generate niche space within the recipient BM to allow for donor cell engraftment. Often, myeloablation is achieved using total body gamma irradiation which induces double strand breaks in DNA that leads to cell death, particularly in actively dividing cells such as hematopoietic progenitor cells[2,3]. The irradiation protocols are done to induce sufficient BM cell death such that animal lethality results if the recipient animal does not achieve adequate donor cell engraftment from BMT (so called 'lethal irradiation'). However, irradiation requires a specialized facility and equipment, along with veterinary and animal husbandry resources not available to researchers at all institutions. Furthermore, myeloablative irradiation can cause potentially lethal damage to other tissues, and due to immunosuppresion, irradiated animals are more susceptible to secondary infections[3]. As such, reduced intensity conditioning

(so called 'RIC' protocols) regimens have been developed that are intended to minimize potentially toxic side effects of the conditioning protocol in patients, particularly for use in at risk populations such as children and the elderly[4].

Some RIC protocols rely upon chemotherapeutics such as busulfan in order to condition the BM compartment. Busulfan is a bifunctional DNA alkylating agent often used clinically as an alternative to irradiation[5,6]. Notably, busulfan can be safely administered to mice by intraperitoneal (IP) injection and does not require the specialized facilities and equipment necessary to irradiate mice. Busulfan conditioning has been used extensively in our lab[7], as well as in several other recent publications[8–11]. When doses of 60-100 mg/kg are employed, high degrees of stable chimerism (>80% GFP+ cells) can be established in the peripheral blood and BM[7]. Importantly, at these doses myeloablation is not complete and as such mice are able to survive without receiving support BM cells (K. Peake, J. Manning, C. Lewis, and C. Krieger, unpublished observations). Moreover, with myelosuppressive busulfan conditioning there is rapid reconstitution of peripheral blood myelomonocytic cells by donor cells. However, the replacement of peripheral blood lymphocytes by donor cells is slower, highlighting the lack of immunosuppression that occurs with 60-100 mg/kg doses of busulfan compared to total body irradiation[7].

We have successfully used busulfan-induced chimerism to investigate BMDCs in wild-type mice, as well as in mouse models of the neurodegenerative disorders amyotrophic lateral sclerosis (ALS)[7] and Alzheimer disease. It has been observed that under certain conditions significant numbers of GFP+ BMDCs accumulate within the CNS[7,12]. Importantly, like myeloablative conditioning with irradiation, busulfan conditioning followed by BMT is sufficient to allow for GFP+ BMDC accumulation within the CNS in both wild-type mice, and mice with neurological disorders[7–11]. As a major obstacle in treating neurodegenerative disorders is the ability to get therapeutic molecules from the circulation into the CNS where they can exert beneficial effects, this raises the possibility that BMDCs could be engineered to express therapeutic molecules, and subsequently used as a vehicle to deliver therapeutics to the CNS of busulfan conditioned recipients, a mechanism we are actively investigating. Although we have found that myeloablative conditioning with irradiation leads to greater accumulation of GFP+ BMDCs in the CNS compared to busulfan conditioning[7], irradiation may have considerable toxicity in patients with neurodegenerative disorders and in murine models of CNS disease.

While we, and many others, have used BMT models to study BMDCs in neurodegenerative disorders, the ability to largely replace the BM compartment of a recipient mouse with a distinctly identifiable population of BM cells is an invaluable tool for studying various aspects of the hematopoietic system. This includes a wide array of research topics such as hematopoietic lineage development, leukemia, organ transplantation, graft-versus-host disease, and immunobiology.

Protocol

NOTE: Ethics Statement: This protocol has been reviewed and approved by the Animal Care Committee of Simon Fraser University (UACC; permit numbers 1037K-12 and 1060K-03) and is in compliance with the Canadian Council on Animal Care, the NIH Guide for the Care and Use of Laboratory Animals, and the EEC Council Directive.

1. Special Considerations

NOTE: Busulfan is cytotoxic. Handle and dispose according to the material safety data sheet and institutional guidelines.

1. Perform all techniques aseptically in a laminar flow hood.
2. Sterilize instruments prior to use by wrapping in an appropriate packaging material, such as a peel package, and autoclaving.
3. While the risk for infection is lower with busulfan conditioning compared to irradiation, handle animals only in a laminar flow hood for the first 2 weeks following transplantation.

2. Conditioning of Recipient Mice

1. Dilute busulfan to 3 mg/ml with sterile phosphate buffered saline (PBS).
   NOTE: It is important to make a fresh dilution of busulfan just prior to use each day of injection.
2. Administer 20 mg/kg of diluted busulfan to recipient mice via IP injection daily.
3. Repeat daily IP injections of 20 mg/kg busulfan until a total dose of 60-100 mg/kg has been delivered (*i.e.,* for an 80 mg/kg total dose, administer 20 mg/kg of busulfan for 4 consecutive days).

3. Isolation of Donor Bone Marrow Cells

NOTE: This protocol has been successfully used for isolating and preparing BM cells from up to 5 donor mice. Typically the cell yield per mouse is approximately 30-40 million BMDCs, which is sufficient to transplant 12-16 recipient mice. If more donor mice are needed the protocol may need to be adjusted accordingly.

1. Following the last day of busulfan conditioning euthanize a GFP donor mouse (one to six months old) using CO_2 (or by other euthanasia procedure accepted at institution). To avoid graft complications use syngeneic donors that are the same sex as the recipients.
2. Spray mouse with 70% ethanol. Lift skin at the abdomen and using surgical scissors make an incision through the skin from the abdominal cavity up the leg towards the ankle. Holding the foot, firmly pull the skin from the ankle towards the hip exposing the leg tissue.
3. Trim away muscle and fat tissue from the femur to expose the hip joint.
4. While gently pulling on the foot to extend the leg, press the scissors against the hip joint. Cut just above the head of the femur taking care not to cut the femur itself.
5. To help maintain sterility, hold the leg by the foot and clean any remaining tissue from the bones by rubbing the bone surface with autoclaved tissues.
6. Separate the femur and tibia by cutting through the knee joint and place the femur in a culture dish containing sterile PBS. Incubate on ice.

7. Remove and discard the fibula by cutting at the points where the fibula connects to the tibia. Place the tibia in the culture dish with the femur and incubate on ice.
8. Repeat steps 3.2-3.7 for the other leg, and if necessary, additional donor mice. Following removal of the bones, sterilize the surgical tools with a hot bead sterilizer or use a new set of sterile tools for the subsequent steps.
9. For the femurs, hold the femur with forceps and using surgical scissors carefully 'shave' the distal ends off the bone. Remove as little of the bone as necessary to expose the BM cavity.
10. Fill a syringe with 3 ml of sterile PBS and attach a 23 G needle. Carefully bore the needle into the BM cavity and flush the BM into a sterile culture dish. Be sure to scrape the medullary cavity with the needle point to ensure removal of all desired cells. Following extraction, ensure that the red BM is no longer visible and the bone now appears white.
11. Repeat steps 3.9-3.10 for subsequent femurs, pooling all of the BM in the same culture dish.
12. For the tibiae, hold the tibia with forceps and carefully 'shave' the end where the tibia was attached to the knee to expose the BM cavity. Make a second cut along the bone where the visible red BM ends.
13. Fill a syringe with 3 ml of sterile PBS and attach a 25 G needle. Gently insert the needle into the BM cavity (from the end that was attached to the knee) and flush the BM into the culture dish containing the BM from the femurs. Scrape the walls of the BM cavity with the needle to remove all cells. Following extraction, ensure that the red BM is no longer visible and the bone now appears white.
14. Repeat steps 3.12-3.13 for subsequent tibiae, pooling all of the BM in the same culture dish.
15. Gently triturate the BM with a 1 ml pipette tip to dissociate the cells.
16. Pass the cell suspension through a 40 µM basket filter into a sterile 50 mL centrifuge tube. Rinse the dish with PBS to get any remaining cells and transfer this through the filter into the centrifuge tube.
17. Centrifuge for 5 min at 450 x g at 4 °C. Remove and discard the supernatant.
18. Re-suspend the pellet in 3 ml of erythrocyte lysing buffer. Incubate on ice for 8.5 min.
19. Add ~30 ml of sterile PBS to quench the lysing buffer.
20. Centrifuge for 5 min at 450 x g at 4 °C. Remove and discard the supernatant.
21. Re-suspend the pellet in 1 ml of sterile PBS. Keep on ice.
22. Take a small aliquot of the cell homogenate and dilute with PBS (1:100 for 1 mouse; 1:200 for 2 mice, etc.) to quantify the cell number using a haemocytometer.
23. Dilute the cell suspension to 8 x 10^6 cells/ml and incubate on ice until injection.

4. Bone Marrow Transplantation

1. Fill a 0.5 ml syringe (with a 28 G insulin needle) with 300 µl of diluted cell suspension for each mouse to be injected. Be sure to remove any air bubbles present in the syringe.
2. Place the mouse cage containing the conditioned recipient mice on a heating pad and allow the tail veins to dilate.
3. Take a recipient mouse and place in the restraining device. Wipe the tail with surgical gauze soaked with 70% ethanol.
4. Firmly hold the tail and with the bevel up, gently insert the needle into one of the lateral tail veins and inject the cell suspension.
5. Hold surgical gauze on the injection site until bleeding stops prior to introducing the mouse into a new clean cage.

5. Estimating the Extent of BM Chimerism

NOTE: Chimerism levels are typically variable in the peripheral blood at 1 week post-BMT, and as such chimerism levels are normally estimated at least 2 weeks post-BMT. Levels of chimerism should reach >80% GFP+ cells in the peripheral blood within 3-4 weeks post-BMT.

1. Prepare FACS buffer (2 mM EDTA + 2% fetal bovine serum in PBS).
   NOTE: FACS buffer can be stored at 4 °C for several weeks.
2. Place the mouse in a restraining tube and shave the fur on the posterior leg to expose the lateral saphenous vein.
3. Thinly coat the leg with petroleum jelly and pierce the saphenous vein with a 25 G needle.
4. Collect approximately 50 µl of blood with a heparin coated capillary tube. Add the blood to a micro-centrifuge tube containing 1 ml of FACS buffer. Mix the tube by inversion and store on ice.
5. Hold surgical gauze on the injection site until bleeding stops before returning mouse back to the cage.
6. Centrifuge the blood samples for 5 min at 900 x g. Remove and discard the supernatant.
7. Re-suspend the pellet in 500 µl of erythrocyte lysing buffer. Incubate on ice for 8.5 min.
8. Add 1 ml of FACS buffer to quench the lysing buffer.
9. Centrifuge the samples for 5 min at 900 x g. Remove and discard the supernatant.
10. Perform a second lysis step in 500 µl of erythrocyte lysing buffer. Incubate on ice for 8.5 min.
11. Add 1 ml of FACS buffer to quench the lysing buffer.
12. Centrifuge the samples for 5 min at 900 x g. Remove and discard the supernatant. Check that the pellet is now white.
    NOTE: If the pellet still appears red, an additional lysis step may be needed before proceeding to the next step.
13. Re-suspend the pellet in 1 ml of FACS buffer. Centrifuge the samples for 5 min at 900 x g. Remove and discard the supernatant.
14. Re-suspend the pellet in 300 µl of FACS buffer and quantify GFP+ cells using flow cytometry (NOTE: At this point any desired immunostaining of the cells can be performed, using standard techniques, prior to flow cytometric analysis).

Representative Results

The administration of 60-100 mg/kg of busulfan is generally well tolerated by mice. However, animals typically lose weight during the conditioning phase (~5-10%) and thus the diet may need to be supplemented with treats such as irradiated sunflower seeds to encourage eating. Levels of chimerism of >80% GFP+ cells in the peripheral blood and BM should be consistently achievable using 60-100 mg/kg busulfan[7]. Our laboratory has successfully used this protocol to generate over 100 chimeric mice with >80% GFP+ cells in the peripheral blood and BM, and virtually all of our BMTs are successful when using syngeneic donors and recipients. **Figure 1** shows the levels of chimerism quantified weekly in the

peripheral blood of mice conditioned with 100 mg/kg busulfan receiving a successful syngeneic BMT (n = 3) and mice conditioned with 80 mg/kg busulfan receiving an unsuccessful allogeneic BMT (n = 3). Chimerism levels >80% are typically established by 3-4 weeks post-BMT. **Figure 2** is representative FACS data of peripheral blood showing a successful and unsuccessful BMT. **Figure 3** shows that this chimerism remains established for at least one year in the BM (n = 3), and that conditioning with vehicle is not sufficient to induce BM chimerism (n = 3). While there are no significant differences in the levels of BM chimerism achieved using doses of 60-100 mg/kg busulfan, GFP+ cells accumulate within the CNS in a dose-dependent manner. Moreover, this accumulation is dramatically increased in neurodegenerative disorders such as ALS[7]. **Figure 4** illustrates GFP+ cell accumulation within the lumbar region of the spinal cord in a wild-type (n >20) and ALS mouse model (n >20).

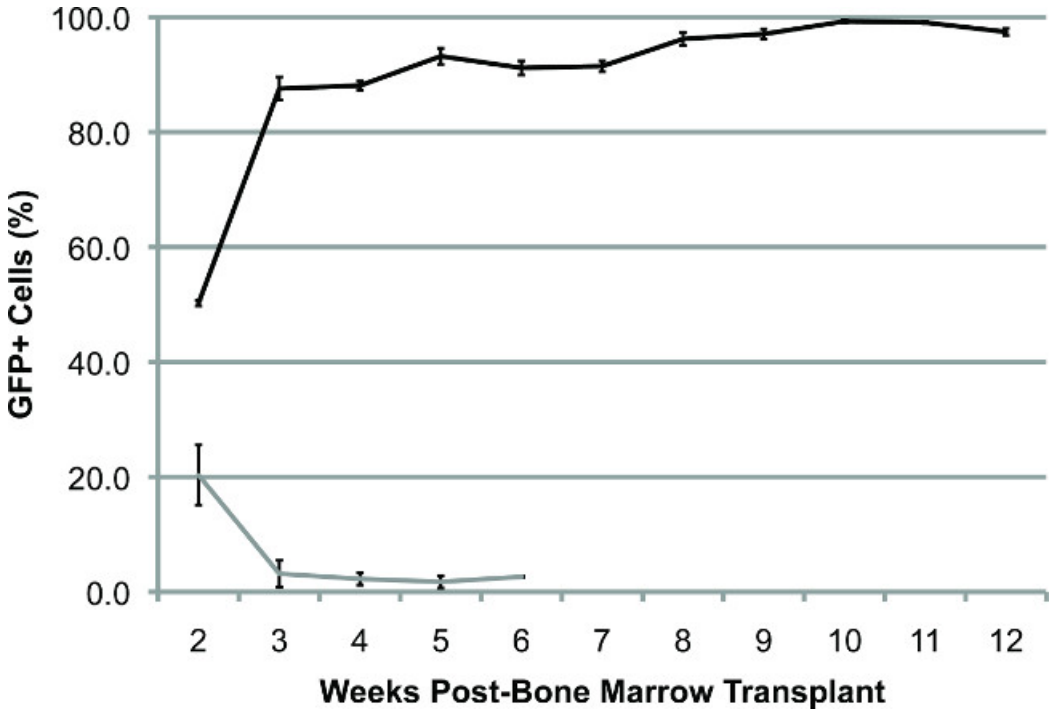

Figure 1. Recipient mice conditioned with busulfan and transplanted with GFP+ bone marrow cells from a syngeneic donor achieve a high level of sustained chimerism in the blood. Weekly flow cytometric data showing GFP+ chimerism in the peripheral blood of successfully transplanted mice conditioned with 100 mg/kg of busulfan followed by syngeneic bone marrow transplantation (black) and unsuccessfully transplanted mice conditioned with 80 mg/kg of busulfan followed by allogeneic bone marrow transplantation (grey). Results are means from n = 3 mice/group, error bars represent SEM.

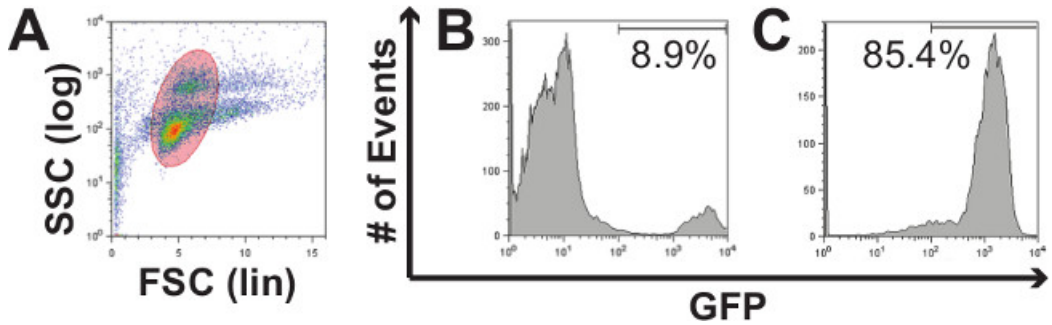

Figure 2. Representative flow cytometric data showing GFP+ cells in the peripheral blood 3 weeks after bone marrow transplantation in mice conditioned with 100 mg/kg of busulfan. (A) Scatterplot showing gating strategy, **(B)** an unsuccessful allogeneic bone marrow transplantation, and **(C)** a successful syngeneic bone marrow transplantation. Results are representative from n > 3 mice. Please click here to view a larger version of this figure.

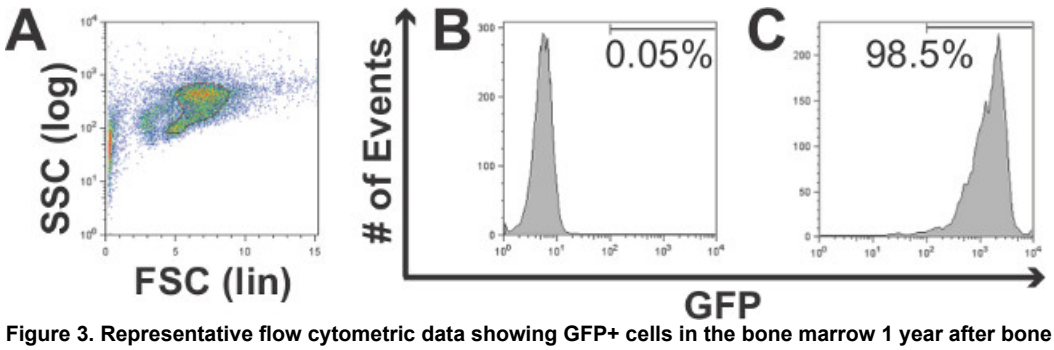

Figure 3. Representative flow cytometric data showing GFP+ cells in the bone marrow 1 year after bone marrow transplantation. (A) Scatterplot showing gating strategy, **(B)** vehicle conditioned mice, and **(C)** 100 mg/kg busulfan conditioned mice. Results are representative from n = 3 mice. Please click here to view a larger version of this figure.

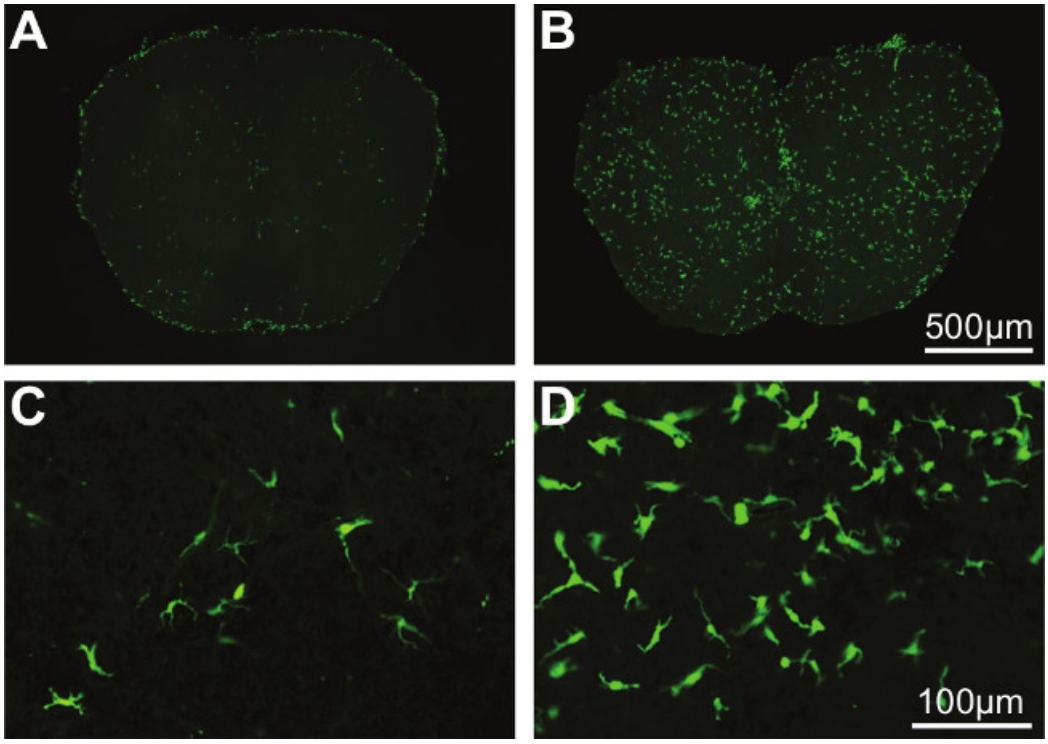

Figure 4. Bone marrow derived cells accumulate in the CNS of busulfan conditioned mice given a bone marrow transplant. Immunohistochemical analysis of sections of the lumbar region of the spinal cord isolated from **(A**, **C)** wild type mice and **(B**, **D)** late disease stage ALS mice. GFP+ cells are shown in green. Results are representative images from n >20 mice for each model. Please click here to view a larger version of this figure.

Discussion

Busulfan conditioning allows for the generation of high-level BM chimerism in mice with hematopoietic cells that are readily detectable. This conditioning can be performed without the need for irradiation facilities. Moreover, myelosuppression with the doses of busulfan outlined in this protocol is well tolerated, minimizing toxic side effects caused by myeloablative doses of irradiation, and thus may be a more suitable technique to generate BM chimerism in young, old, or diseased mice that are more susceptible to myeloablative irradiation. Some RIC protocols use low, non-myeloablative doses of total body irradiation to minimize the adverse side effects, but these protocols still require irradiation facilities and, depending on the dose of irradiation, may result in chimerism levels that are lower than those achievable with busulfan conditioning[13]. Recently, treosulfan has been used for BM conditioning[8,14]. Like busulfan, treosulfan conditioning and BMT is capable of generating mice with a high degree of chimerism in the BM[14]. However, treosulfan is not readily attainable in North America, and the dose of treosulfan required to achieve comparable levels of chimerism to busulfan conditioning is significantly higher than busulfan (~5-10 fold)[14]. Moreover, at these effective doses treosulfan appears to have more toxic side effects in mice compared to busulfan (K. Peake, J. Manning, and C. Krieger, unpublished observations). Peripheral blood chimerism can also be established through generation of parabiotic mice, which involves surgically linking the circulation of a recipient mouse to a donor mouse. Unlike whole BM transplants, parabiosis allows for peripheral blood chimerism to be established in a more physiological manner without introducing BM-restricted progenitor cells into the circulation, although parabiosis is a technically challenging procedure that only results in ~50% donor cells in the peripheral blood of recipient mice[15].

The protocol outlined here relies upon using recipient and donor mice that are the same sex, as well as using syngeneic recipient and donor mouse strains, in order to minimize the possibility of transplant rejection. Although many suitable donor and recipient strains are available

commercially, establishing chimerism in mismatched allogeneic mice (*i.e.,* differ significantly in MHC haplotype) can be problematic. In such cases where allogeneic mice are the only suitable mice available, a preliminary study should first be carried out on a small group of mice to determine if busulfan conditioning is capable of leading to a high degree of stable chimerism. We have had some success transplanting donor BMDCs isolated from C57BL/6-GFP mice into C57BL/6;SJL recipients, though not all transplanted mice established sustained chimerism (~30%). The addition of cyclophosphamide, a strong immunosuppressive agent often used in conjunction with busulfan clinically[16], may be used to improve the success rate of the allogeneic transplants. However, when C57BL/6-GFP cells were transplanted into a triple transgenic mouse model of Alzheimer disease on a mixed C57BL/6;129 genetic background, busulfan and cyclophosphamide conditioning were insufficient for generating stable BM chimeras (**Figure 2B**), whereas stable engraftment was possible following lethal irradiation. Thus, in situations where allogeneic transplants are required, irradiation may be a more appropriate conditioning regimen due to the complete myeloablation and severe immunosuppression that occurs.

Several additional measures should be taken in order to maximize the chimerism and consistency of this protocol. It is crucial that fresh dilutions of busulfan are prepared for injection, ideally just prior to busulfan administration, as the potency of busulfan appears to be reduced with long-term storage. Additionally, when triturating the BM to dissociate the cells, it is important to do so gently so as not to damage the BM cells. While this may result in some clumps of tissue that are not completely dissociated, and consequently a slightly lower yield, the overall health and quality of the dissociated cells will be better. Likewise, any steps that require re-suspension of the pellet should also be done gently.

High levels of chimerism can consistently be attained with 60, 80 and 100 mg/kg doses of busulfan[7] (**Figures 1**-**3**). Moreover, there is a dose-dependent increase in the accumulation of GFP+ cells in the CNS of wild-type[8,9] and ALS mice[7]. We and others have also observed an increase in the number of accumulating GFP+ cells within the CNS of mouse models of neurodegeneration compared to wild-type mice[7,8,10]. Lethal irradiation results in an even greater number of GFP+ BMDCs compared to 60-100 mg/kg doses of busulfan[7] although one recent study suggests that a myeloablative dose of busulfan (125 mg/kg) results in a higher number of GFP+ cells in the brain compared to total body irradiation[9]. Evidence suggests that BMDC entry into the CNS of lethally irradiated mice may be due, at least in part, to disruption of the blood-brain barrier (BBB). While busulfan does not appear to disrupt the BBB based upon the absence of serum proteins (albumin) within the CNS[11], busulfan is able to readily cross the BBB[17,18] and thus it has been suggested that busulfan conditioning may open up niche space within the CNS that is subsequently filled by BMDCs[8]. Interestingly, the findings of Wilkinson *et al.* suggest that the GFP+ cells accumulate in the CNS of busulfan conditioned mice due to a chemokine recruitment mechanism while GFP+ cell accumulation in lethally irradiated mice appears to be due to inflammatory mechanisms generated by the irradiation itself[9]. Treosulfan does not appear to condition the CNS and consequently few GFP+ cells were found within the CNS of treosulfan conditioned mice[8]. It remains to be determined the effect different doses of busulfan may have on tissues other than the CNS, although busulfan is known to target the liver, lungs, and kidneys[6]. That busulfan may condition other tissues should be taken into consideration when choosing a dose of busulfan for new experiments.

While this busulfan conditioning protocol is relatively simple to perform, the generation of mice with chimeric BM is a powerful tool that can be used to investigate hematopoietic cells. The wide range of transgenic mice commercially available, along with the ability to genetically engineer mice and BMDCs, dramatically increases the potential of this protocol. For instance, various mouse models exist where GFP expression is restricted to specific cell types and lineages, such as CX3CR1-GFP mice that express GFP predominantly in myelomonocytic lineage cells[19]. These mice can be used as donors allowing for the investigation of specific hematopoietic cell populations *in vivo*. A number of different fluorophores also exist in addition to GFP, which can be used in order to transplant and monitor BMDCs from two (or more) distinct donors, a technique that can be employed for competition assays[20]. Moreover, a number of different transgenic mouse models of disease exist that can be used as recipients to investigate the role of BMDCs in a variety of disorders. Following transplantation, donor cells can be analyzed using immunohistochemistry, or isolated by FACS and investigated using a range of biochemical techniques. Furthermore, advanced imaging technologies such as two-photon microscopy can be implemented for live *in vivo* detection of fluorophores such as GFP[21]. Ultimately, it is envisioned that BMDCs could be genetically engineered and used to deliver therapeutic molecules to target tissues such as the CNS in busulfan-conditioned recipients.

Disclosures

The authors have nothing to disclose.

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References

1. Ginhoux, F., *et al.* Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science.* **330**, (6005), 841-845 (2010).
2. Hickey, W. F., Kimura, H. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science (New York, N. Y.).* **239**, (4837), 290-292 (1988).
3. Duran-Struuck, R., Dysko, R. C. Principles of bone marrow transplantation (BMT): providing optimal veterinary and husbandry care to irradiated mice in BMT studies. *Journal of the American Association for Laboratory Animal Science: JAALAS.* **48**, (1), 11-22 (2009).
4. Jimenez, M., Ercilla, G., Martinez, C. Immune reconstitution after allogeneic stem cell transplantation with reduced-intensity conditioning regimens. *Leukemia.* **21**, (8), 1628-1637 (2007).

5. Ellen Nath, C., John Shaw, P. Busulphan in blood and marrow transplantation: dose, route, frequency and role of therapeutic drug monitoring. *Current clinical pharmacology.* **2**, (1), 75-91 (2007).
6. Galaup, A., Paci, A. Pharmacology of dimethanesulfonate alkylating agents: busulfan and treosulfan. *Expert Opinion on Drug Metabolism & Toxicology.* **9**, (3), 333-347 (2013).
7. Lewis, C. -A. B., *et al.* Myelosuppressive Conditioning Using Busulfan Enables Bone Marrow Cell Accumulation in the Spinal Cord of a Mouse Model of Amyotrophic Lateral Sclerosis. *PLoS ONE.* **8**, (4), e60661 (2013).
8. Capotondo, A., *et al.* Brain conditioning is instrumental for successful microglia reconstitution following hematopoietic stem cell transplantation. *Proceedings of the National Academy of Sciences.* **109**, (37), 15018-15023 (2012).
9. Wilkinson, F. L., Sergijenko, A., Langford-Smith, K. J., Malinowska, M., Wynn, R. F., Bigger, B. W. Busulfan Conditioning Enhances Engraftment of Hematopoietic Donor-derived Cells in the Brain Compared With Irradiation. *Molecular Therapy.* **21**, (4), 868-876 (2013).
10. Lampron, A., Pimentel-Coelho, P. M., Rivest, S. Migration of bone marrow-derived cells into the CNS in models of neurodegeneration: Naturally Occurring Migration of BMDC into the CNS. *Journal of Comparative Neurology.* 3863-3876 (2013).
11. Kierdorf, K., Katzmarski, N., Haas, C. A., Prinz, M. Bone Marrow Cell Recruitment to the Brain in the Absence of Irradiation or Parabiosis Bias. *PLoS ONE*. **8**, (3), e58544 (2013).
12. Ajami, B., Bennett, J. L., Krieger, C., McNagny, K. M., Rossi, F. M. V. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nature Neuroscience.* **14**, (9), 1142-1149 (2011).
13. Down, J. D., Tarbell, N. J., Thames, H. D., Mauch, P. M. Syngeneic and allogeneic bone marrow engraftment after total body irradiation: dependence on dose, dose rate, and fractionation. *Blood.* **77**, (3), 661-669 (1991).
14. Van Pel, M., van Breugel, D. W. J. G., Vos, W., Ploemacher, R. E., Boog, C. J. P. Towards a myeloablative regimen with clinical potential: I. Treosulfan conditioning and bone marrow transplantation allow induction of donor-specific tolerance for skin grafts across full MHC barriers. *Bone Marrow Transplantation.* **32**, (1), 15-22 (2003).
15. Ajami, B., Bennett, J. L., Krieger, C., Tetzlaff, W., Rossi, F. M. V. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nature Neuroscience.* **10**, (12), 1538-1543 (2007).
16. Andersson, B. S., *et al.* Conditioning therapy with intravenous busulfan and cyclophosphamide (IV BuCy2) for hematologic malignancies prior to allogeneic stem cell transplantation: a phase II study. *Biology of Blood and Marrow Transplantation.* **8**, (3), 145-154 (2002).
17. Hassan, M., *et al.* Cerebrospinal fluid and plasma concentrations of busulfan during high-dose therapy. *Bone Marrow Transplantation.* **4**, (1), 113-114 (1989).
18. Hassan, M., *et al.* In vivo distribution of [11C]-busulfan in cynomolgus monkey and in the brain of a human patient. *Cancer Chemotherapy and Pharmacology.* **30**, (2), 81-85 (1992).
19. Jung, S., *et al.* Analysis of Fractalkine Receptor CX3CR1 Function by Targeted Deletion and Green Fluorescent Protein Reporter Gene Insertion. *Molecular and Cellular Biology.* **20**, (11), 4106-4114 (2000).
20. Harrison, D. E. Competitive repopulation: a new assay for long-term stem cell functional capacity. *Blood.* **55**, (1), 77-81 (1980).
21. Evans, T. A., *et al.* High-resolution intravital imaging reveals that blood-derived macrophages but not resident microglia facilitate secondary axonal dieback in traumatic spinal cord injury. *Experimental Neurology.* **254**, 109-120 (2014).

