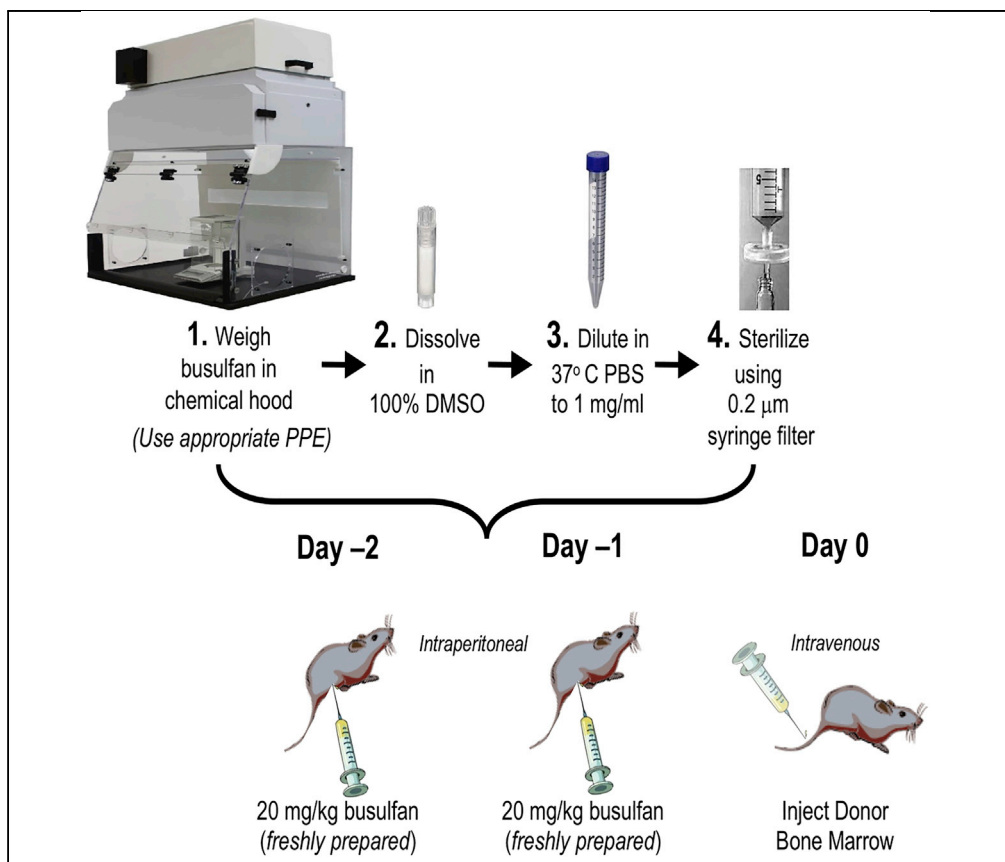


## Protocol

# Use of Busulfan to Condition Mice for Bone Marrow Transplantation



Myeloablative gamma irradiation has traditionally been used to condition mice prior to bone marrow transplantation. However, irradiation induces high levels of inflammation that may alter patterns of reconstitution. In addition, gamma irradiators are being removed from many facilities for security reasons. Alternative conditioning regimens are thus needed. Here, we describe a protocol for the use of busulfan to condition mice for bone marrow transplantation and several of the variables to consider for effective implementation.

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### HIGHLIGHTS

Use of busulfan as an alternative to gamma irradiation for conditioning mice

Examples of how busulfan affects hematopoietic stem and progenitor cells

Procedures for determining busulfan dose and administration schedule

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## Protocol

## Use of Busulfan to Condition Mice for Bone Marrow Transplantation

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## SUMMARY

Myeloablative gamma irradiation has traditionally been used to condition mice prior to bone marrow transplantation. However, irradiation induces high levels of inflammation that may alter patterns of reconstitution. In addition, gamma irradiators are being removed from many facilities for security reasons. Alternative conditioning regimens are thus needed. Here, we describe a protocol for the use of busulfan to condition mice for bone marrow transplantation and several of the variables to consider for effective implementation.

For complete details on the use and execution of this protocol, please refer to Montecino-Rodriguez et al. (2019).

## BEFORE YOU BEGIN

Successful bone marrow transplantation requires that recipients be conditioned to deplete endogenous stem and progenitor cells and facilitate donor stem cell engraftment. Lethal gamma irradiation is the most widely used conditioning regimen, but the conditions in irradiated mice may be distinct from those in animals under steady state conditions (Busch and Rodewald, 2016) and bias the differentiation of transplanted hematopoietic stem cells (HSCs) toward myelopoiesis (Lu et al., 2019). As we recently discussed (Dorshkind et al., 2020), the high levels of inflammation induced by irradiation may account for this effect. Another issue is that many institutions in the United States are removing gamma irradiators due to security concerns. Thus, alternative conditioning regimens are needed.

Here, we describe the use of the alkylating drug busulfan to condition mice prior to bone marrow transplantation. Efficient conditioning of mice with busulfan is dependent on optimizing the dosage, administration schedule, and timing of when to transplant. While these parameters have been discussed in part in earlier studies (Xun et al., 1994; Hsieh et al., 2007; Jopling and Rosendaal, 2001; Westerhof et al., 2000; Yeager et al., 1991), no single report has done so comprehensively. We synthesize lessons learned from our recent use of busulfan (Montecino-Rodriguez et al., 2019) with these earlier studies that have examined some of the variables involved in the use of this drug.

## ⌚ Timing: days to weeks

1. Calculate the total amount of 1,4-Butanediol dimethanesulfonate (busulfan) that will be needed to treat the number of mice to be conditioned with the chosen dose. The example described herein is based on treatment of 10 mice with a total dose of 40 mg/kg, administered in two separate doses of 20 mg/kg of busulfan per mouse at 24 h intervals on two consecutive days. This dose was chosen based on preliminary experiments in which the potential of varying doses of busulfan



to deplete stem and progenitor cells in the bone marrow of recipients was tested. Details of how this was done are provided in the [Expected Outcomes](#) section below.

Assuming that an 8 - 10 week old, young adult mouse typically weighs 20 grams, administration of 0.8 mg of busulfan per mouse will equal a 40 mg/kg dose. If 10 mice are injected, a total of 8 mg of busulfan will be needed.

2. Order a sufficient stock of busulfan. The drug is supplied in crystalline form and should be stored at  $-20^{\circ}\text{C}$  according to the supplier recommendations.

The busulfan working solution, prepared as described below, will need to be sterilized by filtration, and this will result in some loss. This should be taken into account when calculating how much busulfan to order. For example, while 8 mg of busulfan in solution is needed to condition 10 mice, 10 mg of busulfan stock solution is prepared.

⌚ **Timing: weeks**

3. Busulfan treated mice may become immunocompromised. As a precaution, animals should be placed in sterile cages with sterile bedding, food, and water 1 week prior to initiation of busulfan treatment.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Purified CD16/32 (Clone: 93)	Thermo Fisher Scientific	Cat#: 14-0161-86
CD45.1 eFluor 780 (Clone A20)	Thermo Fisher Scientific	Cat#: 47-0453-82
CD45.2 FITC (Clone 104)	BD Pharmingen	Cat#: 553772
CD150 PE (Clone: TC15-12F12.2)	BioLegend	Cat#: 115904
Sca-1 PerCP/Cy5.5 (Clone: D7)	Thermo Fisher Scientific	Cat#: 45-5981-82
CD48 FITC (Clone: HM48-1)	BioLegend	Cat#: 103404
CD117(c-Kit)-APC (Clone: ACK2)	Thermo Fisher Scientific	Cat#: 17-1172-83
CD135 Pacific Blue (Clone: A2F10)	Biolegend	Cat#: 135314
CD127 PE Cy7 (Clone: eBio5B1/199)	Thermo Fisher Scientific	Cat#: 25-127382
CD34 Pacific Blue (Clone: SA376A4)	Biolegend	Cat#: 152208
CD16/32 PE (Clone: 93)	Thermo Fisher Scientific	Cat#: 12-0161-83
Streptavidin APCeF780	Thermo Fisher Scientific	Cat#: 47-4317-82
CD11b Biotin (Clone: M1/70)	Thermo Fisher Scientific	Cat#: 13-0112-85
CD3ε Biotin (Clone: 145-2C11)	Thermo Fisher Scientific	Cat#: 13-0031-85
TCRβ Biotin (Clone: H57-597)	Thermo Fisher Scientific	Cat#: 13-5961-85
TCRγδ Biotin (Clone: UC7-13D5)	Thermo Fisher Scientific	Cat#: 13-5811-85
NK1.1 Biotin (Clone: PK136)	Thermo Fisher Scientific	Cat#: 13-5941-85
Gr-1 Biotin (Clone: RB6-8C5)	Thermo Fisher Scientific	Cat#: 11-5931-85
IgM Biotin (Clone: 11/41)	Thermo Fisher Scientific	Cat#: 13-5790-85
CD8α FITC (Clone: 53-6.7)	Thermo Fisher Scientific	Cat#: 13-0081-85
Ter-119 FITC (Clone: TER-119)	Thermo Fisher Scientific	Cat#: 13-5921-85

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Polyclonal Goat anti-mouse IgM PE	Southern Biotech	Cat#: 1020-09
CD45R(B220) PerCP Cy5.5 (Clone: RA3-6B2)	Thermo Fisher Scientific	Cat#: 35-0452-82
CD43 APC (Clone: S7)	BD Pharmingen	Cat#: 560663
CD24(HSA) PE Cy7 (Clone: M1/69)	BD Pharmingen	Cat#: 560536
Ly-51(BP-1) Biotin (Clone: 6C3)	BD Pharmingen	Cat#: 553159
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
1,4-Butanediol dimethanesulfonate (Busulfan)	Cayman Chemicals	Cat#: 14843
Dulbecco's Phosphate Buffered Saline (Powder)	Gibco	Cat#: 21600-010
Dimethyl Sulfoxide (DMSO)	Corning	Cat#: 25-950-CQC
<b>Experimental Models: Organisms/Strains</b>		
CD45.2 C57BL/6J Mice	The Jackson Laboratory	JAX: 000664
CD45.1 C57BL/6J Mice (B6.SJL-Ptprc <sup>a</sup> Pepc <sup>b</sup> /BoyJ)	The Jackson Laboratory	JAX: 002014
<b>Software and Algorithms</b>		
FlowJo (v10)	FlowJo	<a href="https://www.flowjo.com/">https://www.flowjo.com/</a>
Graphpad Prism (v8)	Graphpad	<a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a>
<b>Other</b>		
Analytical Balance (Readability 0.1 mg)	Fisher Scientific	n/a
Balance Draft Shield	Fisher Scientific	Cat#: 50-855-986
Compact Scale (Readability 0.1 g)	Fisher Scientific	n/a
Nalgene General Long-Term Storage Cryogenic Tubes (1 mL)	Thermo Fisher Scientific	Cat#: 03-337-7C
Falcon™ 15 mL Conical Centrifuge Tubes	Fisher Scientific	Cat#: 14-959-53A
Corning™ RC Syringe Filters (0.2 μm)	Fisher Scientific	Cat#: 09-754-23
Exel International Insulin Syringes (0.5 mL)	Fisher Scientific	Cat#: 14-841-32
Cesium-137 Mark 1 Irradiator	JLShepherd	Model 68A

## MATERIALS AND EQUIPMENT

We used ≥98% pure busulfan from Cayman Chemicals. We have not tested busulfan from other suppliers. However, drug obtained from other vendors should be equally effective as long as similarly high purity material is used.

Clinical grade busulfan, supplied as a colorless, sterile solution, can be obtained from most medical center pharmacies. Its use would avoid the necessity of preparing stock solutions and meet any institutional requirements that only clinical grade material be used in experimental animals. However, it is packaged as 8 single use vials that, depending on where it is purchased, may cost ~\$4,000. This cost may be prohibitive for many laboratories. In contrast, 50 grams of busulfan crystals costs approximately \$75.

## STEP-BY-STEP METHOD DETAILS

### Conditioning Mice with Busulfan

⌚ Timing: Begin 2 days before bone marrow transplantation

This example describes the conditioning of 10 mice with 40 mg/kg of busulfan (administered in 2 doses of 20 mg/kg) per mouse administered over the course of two days. We used 8 - 12 week old female C57BL/6 mice obtained from the Jackson Laboratory in this example. These mice typically weigh between 18 and 22 grams.

Fresh working solutions are prepared each day.

1. In order to achieve a total dose of 40 mg/kg of busulfan, 0.8 mg of busulfan will need to be administered to a 20 gram mouse. In the example we describe, mice were treated with 0.4 mg of drug on days -2 and -1 prior to bone marrow transplantation (day 0). However, as discussed in the “Expected Outcomes” section, there is some, albeit limited, flexibility as to when hematopoietic cells are transplanted after the last busulfan injection.
2. Two days before bone marrow transplantation (day -2), weigh 5 mg of busulfan in a fume hood using a microbalance shielded from drafts. Resuspend the busulfan crystals in 0.5 mL of 20°C–25°C DMSO in a 1 mL sterile tube with screwcap (we use cryogenic vials). We do not recommend using diluted solutions of DMSO, as we encountered difficulty in dissolving drug when this was done. Cap the tube tightly and shake vigorously to dissolve the drug. Even though 4 mg of busulfan will be needed for the 10 mice to be conditioned, excess stock solution is made to account for loss of material during sterilization by filtration. Also, some mice may weigh more than 20 grams, necessitating additional working solution.
3. Once all the busulfan crystals have dissolved, add the mixture to 4.5 mL of Ca<sup>++</sup> and Mg<sup>++</sup> free PBS pre-warmed to 37°C in a 15 mL polypropylene screw cap tube. Vortex this solution as needed to dissolve any precipitates that may have formed. This working solution now contains 1 mg/mL of busulfan and a final DMSO concentration of 10%. Ensure that DMSO levels do not exceed 10%, as higher levels of this solvent may not be tolerated by mice.
4. Sterilize the 1 mg/mL busulfan working solution by filtration through a 0.2 µm syringe filter. Note that the busulfan/DMSO stock solution described in step 2 cannot be sterilized this way as the high DMSO concentration will damage the filter.

⚠ **CRITICAL:** Busulfan is toxic and only personnel with the appropriate training should work with this drug. All procedures, including weighing of drug, should be performed in a chemical fume hood by experienced laboratory personnel wearing appropriate personal protective equipment. The institution’s office of environmental health and safety should be consulted if laboratory personnel require advice on the use of busulfan. Needles, syringes, plastics, and unused busulfan stock solution should be considered as hazardous waste and should be disposed of according to institutional regulations.

5. Weigh each mouse on a portable balance to determine the volume of the busulfan working solution to be injected. For example, the goal is to treat mice with 0.4 mg of busulfan, which would equal a 20 mg/kg dose. Therefore, 0.4 mL of the 1 mg/mL working solution should be loaded into a 0.5 mL insulin syringe fitted with a 29-gauge needle. These specific syringes, described in the [Key Resources Table](#), are used because they allow delivery of a precise amount of solution.

⚠ **CRITICAL:** Busulfan has an established instability in aqueous preparations and exhibits sensitivity to hydrolysis that is influenced by phosphate buffer components ([Hassan and Ehrsson, 1986](#); [Houot et al., 2013](#)). That is why we prepare the material immediately before injection and do not store these solutions for any length of time. We also avoid cooling the material to 4°C as this will result in precipitation of the busulfan crystals and inaccurate dose delivery. We also do not freeze busulfan solutions in order to avoid potential problems with loss of activity.

6. The calculated volume of drug is delivered via an intraperitoneal injection.
7. Repeat steps 3–7 24 h later on day -1 before transplantation.

8. Twenty-four hours later, on day 0, transplant donor bone marrow cells into the busulfan-conditioned mice.

The precise composition of the donor cells is variable. In some cases, total bone marrow is transplanted, but purified HSCs may also be used. When purified HSCs are transplanted into lethally irradiated recipients, they are usually mixed with a small number of total bone marrow cells. This “carrier marrow” is thought to ensure survival of the myeloablated recipients until the donor HSCs generate enough mature blood cells to do so. Our experience is that carrier marrow is not needed when purified HSCs are transplanted into Busulfan-conditioned recipients (Montecino-Rodriguez et al., 2019). The reason for this is discussed in more detail below.

9. Busulfan treated mice may be temporarily immunocompromised and should be placed in sterile cages with sterilized bedding, food, and water for 3 weeks following drug treatment and transplantation. We never treat mice with antibiotics because of their potential to inhibit hematopoiesis (Josefsdottir et al., 2017).

### EXPECTED OUTCOMES

Preliminary experiments in small groups of mice should be conducted to define the dose of busulfan to be used and the administration schedule. Since the goal of conditioning is to deplete endogenous hematopoietic stem and progenitor cells, it is important to confirm that the protocol to be used targets these populations. We recommend that the frequency and total number of lineage negative (Lin<sup>-</sup>) CD117(c-Kit)<sup>+</sup> Sca-1<sup>+</sup> CD150<sup>+</sup> CD135<sup>-</sup> CD48<sup>-</sup> HSCs and Lin<sup>-</sup> CD117(c-Kit)<sup>+</sup> Sca-1<sup>+</sup> CD150<sup>-</sup> multipotential progenitors (MPPs) be quantified following conditioning to do so.

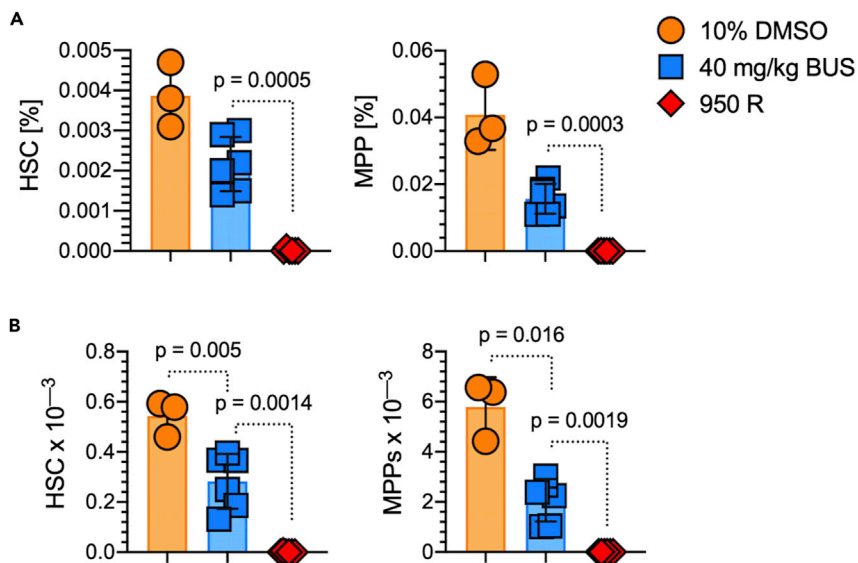
We analyzed these populations in 8 - 12-week-old female C57BL/6 mice conditioned with 40 mg/kg (2× doses of 20 mg/kg) of busulfan. An additional cohort of mice was conditioned with 950R of irradiation, delivered as a split 475 R dose from a cesium-137 irradiator the day prior to sacrifice of the mice. The inclusion of these mice allowed a side by side comparison of the effects of busulfan versus gamma irradiation conditioning on hematopoietic progenitors. All animal procedures were approved by the UCLA Institutional Animal Care and Use Committee. The antibodies used for the analysis of HSCs and MPPs are listed in the [Key Resources Table](#). However, this protocol does not describe how to acquire and analyze FACS data.

Twenty-four hours following the last dose of busulfan, HSCs and MPPs were present in the bone marrow of treated mice but their number was significantly reduced compared to control mice treated with a 10% DMSO solution. This reduction was not as severe as observed in the irradiated animals where few HSCs and MPPs were detected ([Figure 1](#)).

However, when mice were analyzed 72 h following the last busulfan dose, few HSCs and progenitor cells, which are included in the Lin<sup>-</sup> CD117(c-Kit)<sup>+</sup> Sca-1<sup>+</sup> gate, were detected ([Figure 2](#)). This indicates that even though HSCs and MPPs were still present in mice 24 h after busulfan conditioning, most of these cells were likely damaged and destined to die. Thus, while busulfan conditioning effectively depletes stem and progenitor cells, it does not do so as rapidly as gamma irradiation.

No difference in total bone marrow cell number was observed between busulfan and 10% DMSO treated control mice, while there was a significant depletion of hematopoietic cells in the 950R irradiated mice ([Figure 3](#)). This observation may explain why carrier marrow is not needed when purified HSCs are transplanted into busulfan-conditioned recipients.

The data in [Figures 1 through 3](#) provide examples of the assays that should be performed to identify the busulfan dose and treatment schedule to be used. The underlying assumption is that if a given



**Figure 1. Analysis of HSCs and MPPs in Mice Conditioned with Busulfan or Gamma Irradiation**

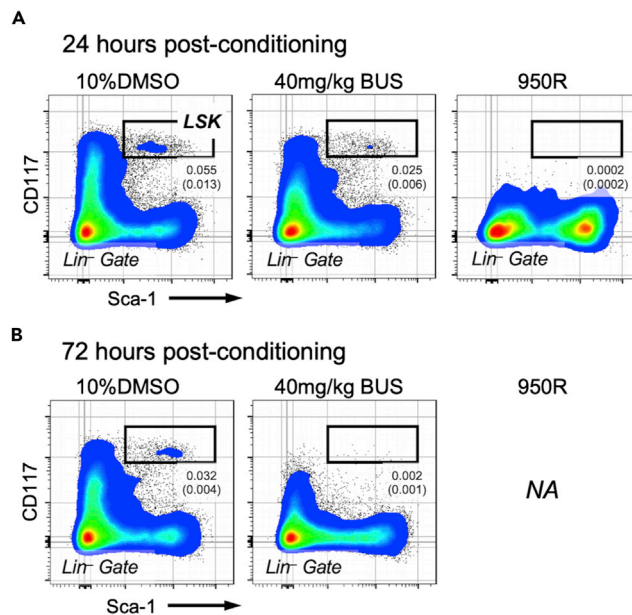
Frequency (A) and number (B) of Lin<sup>-</sup> CD117(c-Kit)<sup>+</sup> Sca-1<sup>+</sup> CD150<sup>+</sup> CD135<sup>-</sup> CD48<sup>-</sup> HSCs and Lin<sup>-</sup> CD117(c-Kit)<sup>+</sup> Sca-1<sup>+</sup> CD150<sup>-</sup> multipotential progenitors (MPPs) in mice treated with 10% DMSO in PBS, 40 mg/kg Busulfan, or 950R of gamma irradiation. Mice received two intraperitoneal injections on days -2 and -1 with either 10% DMSO in PBS (n = 3) or 20 mg/kg busulfan in PBS (n = 6) solutions. Bone marrow was harvested 24 h following the second injection. Gamma irradiation conditioned mice (n = 6) received a dose of 950R administered as two split doses of 475R delivered 8 h apart using a cesium-137 irradiator. Bone marrow was harvested from mice 24 h later. Data are expressed as means  $\pm$  SD. Indicated p values for the differences of mean between groups was determined using a two-tailed, unpaired Student t test ( $p = 0.05$ ).

dose(s) effectively depletes stem and progenitor cells, it is likely donor HSCs will efficiently engraft in the conditioned mice. This has proven to be the case.

A focus of our research is on how aging affects the lymphoid potential of hematopoietic stem cells. As we recently reviewed, a widely accepted view is that hematopoietic system aging results from stable, irreversible defects that accumulate in HSCs (Dorshkind et al., 2020). This conclusion is based on the transplantation of old total, as well as lymphoid biased (Ly-HSCs) or myeloid biased (My-HSCs) stem cells (Muller-Sieburg et al., 2004) into mice conditioned with lethal irradiation (Beerman et al., 2010), which as noted induces high levels of inflammation. However, we found that levels of donor chimerism 5 weeks post transplantation were equivalent when 200 Ly-HSCs from young or old C57BL/6 CD45.2 donor mice were transplanted into C57BL/6 CD45.1 recipient mice that were conditioned with 40 mg/kg of busulfan (2  $\times$  20 mg/kg on days -2 and -1) (Figure 4A). In addition, there was no difference in the number of B cell progenitors in recipients of young or old lymphoid biased stem cells (Figure 4B) (Montecino-Rodriguez et al., 2019). Despite the acquisition of a myeloid gene signature by the old Ly-HSCs (Montecino-Rodriguez et al., 2019), they did not generate elevated numbers of common myeloid progenitors, granulocyte-macrophage progenitors, or megakaryocyte-erythroid progenitors in recipient mice.

## QUANTIFICATION AND STATISTICAL ANALYSIS

We routinely include 6 - 8 recipients in bone marrow transplantation experiments. When the procedures outlined above are followed, all mice survive conditioning and transplantation. Our experience is that large recipient group sizes are critical for adequate statistical analysis, particularly when the donor cells are experimentally manipulated in any way. The data in the figures are expressed as



**Figure 2. Analysis of Hematopoietic Progenitors in Mice One and Three Days following Busulfan Conditioning**

(A) Representative FACS plots showing the frequency of Lin<sup>-</sup> CD117(c-Kit)<sup>+</sup> Sca-1<sup>+</sup> cells in mice 24 h following treatment with 10% DMSO/PBS (n = 3), 40 mg/kg busulfan (n = 6), or 950R of gamma irradiation as described in the Figure 1 legend. These mice are the same as in Figure 1.

(B) Representative FACS plots showing the frequency of Lin<sup>-</sup> CD117(c-Kit)<sup>+</sup> Sca-1<sup>+</sup> cells 72 h following treatment with 10% DMSO/PBS (n = 3) or 40 mg/kg busulfan (n = 6). Treatment of the animals is described in the Figure 1 legend. Mean and (SD) frequencies of LSK cells are indicated. NA, not available.

means ± SD. Statistical significance for the differences between groups was determined by a two-tailed, unpaired Student t test ( $\alpha = 0.05$ ).

## LIMITATIONS

The above sections have discussed a number of variables that must be optimized to achieve high levels of donor cell engraftment, and ultimately high levels of donor cell chimerism, in busulfan conditioned recipients. It is strongly recommended that preliminary experiments be conducted to establish them for the age and strain of mice used. The following parameters should be considered:

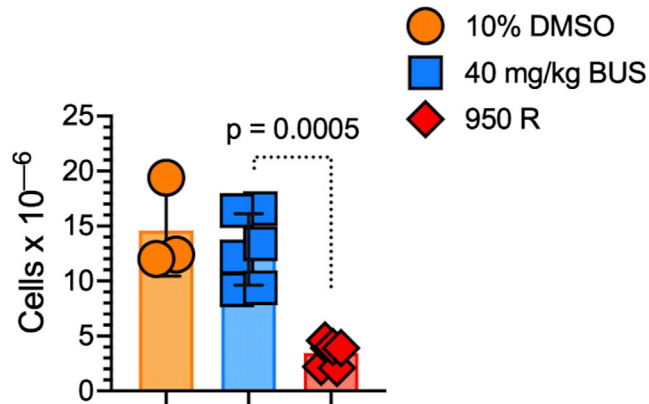
### Age of Recipients

Busulfan may be metabolized more rapidly in young children thus decreasing the effective dose (Nguyen et al., 2004). Therefore, recipient age should be taken into consideration if the experimental question requires the use of very young mice, as this may necessitate the use of higher doses of drug in order to condition them effectively.

### Busulfan Dose

Previous studies have demonstrated that mice will tolerate a wide range of busulfan doses, and in some studies up to 150 mg/kg (Jopling and Rosendaal, 2001) has been used. Reconstitution of donor bone marrow cells in peripheral blood, lymph nodes, and peripheral blood of C57/BL6 mice conditioned with 10, 20, 35, 50, 80, and 100 mg/kg of busulfan has been compared. Similar, high levels of donor lymphoid chimerism, ranging up to 100%, were observed in mice treated with doses of 20 mg/kg and higher. However, donor chimerism in mice treated with 10 mg/kg of busulfan was at least 50% lower (Yeager et al., 1991). This trend was confirmed in a subsequent study (Hsieh et al., 2007). As we discuss below, preliminary experiments





**Figure 3. Number of Bone Marrow Cells in Mice Conditioned with Busulfan or Gamma Irradiation**

Mice were analyzed 24 h after busulfan or irradiation conditioning. The number of animals in each group and the conditioning regimens used are described in the [Figure 1](#) legend.

must be conducted to establish the optimal dose of busulfan needed for the particular strains of mice being used.

#### Administration Schedule

It has been proposed that busulfan delivered in split doses administered over several days is more effective than a single injection ([Jopling and Rosendaal, 2001](#)). This may be particularly critical if high doses of drug are being used, as mice may not survive treatment with single large doses.

#### Transplantation Timing

One study reported that bone marrow cells can be transplanted up to 20 days after the last busulfan injection and that efficient donor chimerism is still achieved ([Hsieh et al., 2007](#)). This result suggests that there is minimal expansion of surviving endogenous stem and progenitor cells for several weeks following conditioning. However, we observed high variability in levels of donor cell chimerism when mice received a bone marrow transplant one week following the last busulfan injection. This could result from recovery of host hematopoiesis in some animals, thereby impeding engraftment of donor cells. Because of this, we recommend that donor bone marrow be transplanted 24 h following the last busulfan injection.

#### Syngeneic versus Allogeneic Transplantation

We have used busulfan to condition recipient mice prior to transplantation with syngeneic bone marrow. However, there may be instances, particularly in some immunologic studies, where there are histocompatibility differences between donors and recipients. There is an established literature in which busulfan combined with cyclophosphamide is used to condition donors prior to allogeneic bone marrow transplantation ([Tutschka and Santos, 1975](#); [Westerhof et al., 2000](#); [Xun et al., 1994](#)). Cyclophosphamide has immune suppressive properties, which is thought to increase donor cell chimerism and decrease graft versus host disease ([Tutschka and Santos, 1975](#)). Thus, combined conditioning regimens should be considered when allogeneic transplantation models are being used.

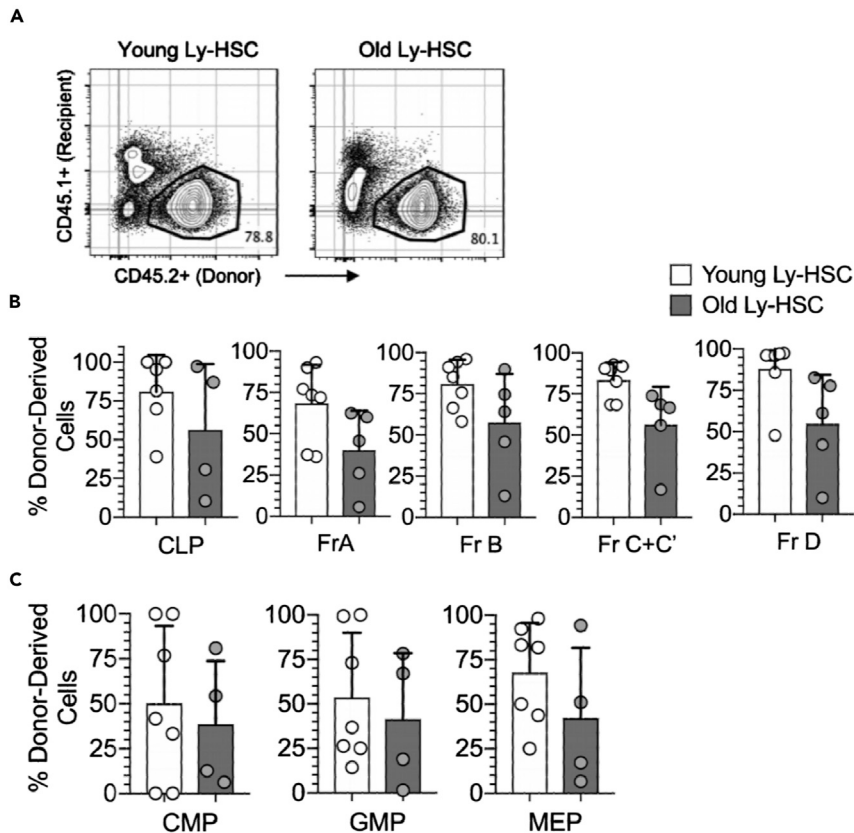
## TROUBLESHOOTING

### Problem

Mice become ill and die in days or weeks following busulfan treatment.

### Potential Solutions

There are several issues to consider in this case:



**Figure 4. Young and Old Ly-HSCs Exhibit Similar Levels of Reconstitution in Busulfan Treated Recipients**

200 Ly-HSCs isolated from young (8–12 weeks) or old (17–18 months) CD45.2<sup>+</sup> C57BL/6 donors were transplanted into CD45.1<sup>+</sup> C57BL/6 recipients. Recipients were conditioned with 40 mg/kg of busulfan (2 × 20 mg/kg on days –2 and –1) before transplantation of donor HSCs on day 0.

(A) representative FACS plots showing resolution of CD45.1<sup>+</sup> recipient and CD45.2<sup>+</sup> donor hematopoietic cells in the bone marrow of busulfan-conditioned CD45.1<sup>+</sup> C57BL/6 mice 5 weeks after transplantation.

(B) Relative frequency of donor derived lymphoid populations defined as: common lymphoid progenitors (CLP, Lin<sup>–</sup> CD117(c-Kit)<sup>+lo</sup> Sca-1<sup>+lo</sup> CD135<sup>+</sup> CD127<sup>+</sup>); pre-pro-B (FrA, surface (s) IgM<sup>–</sup> CD45R(B220)<sup>+</sup> CD43<sup>+</sup> CD24(HSA)<sup>–</sup> Ly-51<sup>–</sup>); early pro-B (FrB, sIgM<sup>–</sup> CD45R(B220)<sup>+</sup> CD43<sup>+</sup> CD24(HSA)<sup>+</sup> Ly-51<sup>–</sup>); late pro-B (FrC+C', sIgM<sup>–</sup> CD45R(B220)<sup>+</sup> CD43<sup>+</sup> CD24(HSA)<sup>+</sup> Ly-51<sup>+</sup>) and pre-B cells (FrD, sIgM<sup>–</sup> CD45R(B220)<sup>+</sup> CD43<sup>–</sup>) in recipients of 200 young and 200 old Ly-HSCs at 5 weeks post reconstitution.

(C) Relative frequency of donor derived myeloid lineage cells defined as: common myeloid progenitors (CMP, Lin<sup>–</sup> CD117(c-Kit)<sup>+</sup> Sca-1<sup>–</sup> CD32/16<sup>–</sup> CD34<sup>+</sup>); granulocyte-macrophage progenitors (GMP, Lin<sup>–</sup> CD117(c-Kit)<sup>+</sup> Sca-1<sup>–</sup> CD32/16<sup>+</sup> CD34<sup>+</sup>); and megakaryocyte-erythroid progenitors (MEP, Lin<sup>–</sup> CD117(c-Kit)<sup>+</sup> Sca-1<sup>–</sup> CD32/16<sup>–</sup> CD34<sup>–</sup>) in recipients of 200 young and 200 old Ly-HSCs at 5 weeks post reconstitution. These figures are from Montecino-Rodriguez et al. (2019).

- If the final concentration of DMSO used to dissolve busulfan is too high, it may not be tolerated by mice. Thus, particular attention should be paid to the initial preparation of stocks and to limit the final concentration of DMSO to no more than 10%.
- It is essential that mice be weighed to determine the exact amount of drug to be injected. Do not assume that all mice, even animals of the same age and sex, will be the same weight. Use precisely graduated syringes to ensure delivery of the precise dose of busulfan.
- Perform preliminary experiments to establish the appropriate dose of busulfan for the mouse strain to be used. Some strains could exhibit increased drug sensitivity, so it may be necessary to lower the dose.

- It may be necessary to decrease the busulfan dose injected daily while increasing the number of days the drug is administered. This may allow mice to more effectively metabolize drug over a longer period of time.
- Any myeloablated animal may be temporarily immunocompromised. Therefore, ensure that mice are housed in sterilized micro-isolator cages with sterile bedding, food, and water 1 week prior to and 3 weeks after transplantation.

### Problem

Low donor cell chimerism in busulfan treated mice

### Potential Solutions

If it is necessary to achieve very high levels of donor chimerism, the concentration of busulfan used for conditioning may need to be increased. Pilot experiments should be conducted in which HSC frequency and number are quantified in mice conditioned with a range of concentrations to identify the drug dose that most effectively depletes HSCs.

Figure 1 shows that HSCs can be detected in the bone marrow 1 day after the last busulfan treatment. However, by 3 days few HSCs can be detected (Figure 2). Thus, higher levels of donor chimerism might be obtained by delaying injection of donor bone marrow by up to 3 days after the completion of busulfan conditioning.

However, it is also important to consider that low donor chimerism may be due to the properties of the HSCs that are transplanted. As discussed above, the HSC compartment is heterogeneous and includes lymphoid and myeloid biased HSCs (Muller-Sieburg et al., 2004). We observed high levels of donor chimerism 5 weeks after transplantation of mice with lymphoid biased HSCs. However, donor chimerism was significantly lower in cohorts of mice analyzed 16 weeks post reconstitution (Montecino-Rodriguez et al., 2019). This outcome likely reflects the fact that Ly-HSCs have more limited self-renewal potential compared to other HSCs and may likely exhaust long term.

## RESOURCE AVAILABILITY

### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Encarnacion Montecino-Rodriguez ([emontecino@mednet.ucla.edu](mailto:emontecino@mednet.ucla.edu))

### Materials Availability

This study did not generate new unique reagents.

### Data and Code Availability

No large data sets were generated or analyzed in this study.

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## AUTHOR CONTRIBUTIONS

E.M.-R. performed all of the experimental work and data analysis described in this study. E.M.-R. and K.D. were involved in designing the experiments and writing this manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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