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Generation of Busulfan Chimeric Mice for the Analysis of T Cell Population Dynamics

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[Abstract] This protocol was developed to generate chimeric mice in which T lymphocytes could be stratified by age on the basis of congenic marker expression. The conditioning drug busulfan is used to ablate host haematopoietic stem cells while leaving the peripheral immune system intact. Busulfan treatment is followed by bone marrow transplantation (BMT), with T-cell depleted donor bone marrow bearing a different congenic marker (CD45.2) to that of the host mouse (CD45.1). New cell production post-BMT can thus be tracked by measuring the fraction of CD45.2$^+$ cells over time within a population of interest (Hogan *et al.,* 2015; Gossel *et al.,* 2017).

Keywords: T cells, T cell homeostasis, Bone marrow chimeras, Busulfan, Temporal fate mapping

[Background] Bone marrow chimeras are a valuable tool for studying immune system development and function. Typically, chimeras are generated by irradiation of host mice followed by transplantation with donor bone marrow. Irradiation causes considerable damage to the haematopoietic system, and full immune reconstitution is delayed by weeks to months post-transplant (Fry and Mackall, 2005). The resulting period of lymphopenia drives spontaneous proliferation of naïve T cells and the acquisition of a memory-like phenotype (Goldrath *et al.,* 2004). To avoid the perturbation of immune homeostasis caused by irradiation, we turned our attention to the conditioning drug busulfan. Busulfan is an alkylating agent that is toxic to haematopoietic stem cells (HSC) but does not deplete circulating lymphocytes (Westerhof *et al.,* 2000; Hsieh *et al.,* 2007). Following busulfan conditioning and bone marrow transplantation (BMT), chimeras are indistinguishable from untreated controls: they have normal numbers of naïve and memory CD4 and CD8 T cells, and these cells express normal levels of the proliferation marker Ki67 (Hogan *et al.,* 2015; Gossel *et al.,* 2017). Therefore, busulfan conditioning allows induction of chimerism while preserving peripheral immune homeostasis. By using donor bone marrow bearing a congenic marker, it is possible to distinguish host- or donor-origin cells by flow cytometry. While HSC replacement is never absolute, emergence of donor origin cells can be used as an accurate proxy for *de novo* haematopoetic development since BMT, thereby allowing cells within a population to be stratified by age on the basis of congenic marker expression.

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Materials and Reagents

1. 5 ml syringes (BD, catalog number: 302187)
2. 25 G needles (Terumo, catalog number: GS-351)
3. Insulin syringes (B. Braun Melsungen, catalog number: 9151125)
4. Petri dishes (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 150288)
5. 70 µm cell strainers (Corning, Falcon®, catalog number: 352350)
6. 50 ml tubes (Corning, Falcon®, catalog number: 352070)
7. FACS tubes with lid (Corning, Falcon®, catalog number: 352058)
8. Host mice: B6 CD45.1 adult females (THE JACKSON LABORATORY, catalog number: 002014)
9. Donor mice: C57BL/6 CD45.2 adult females (THE JACKSON LABORATORY, catalog number: 000664)
   Note: One donor typically provides sufficient T-cell depleted bone marrow for two recipients.
10. Busilvex® 6 mg/ml busulfan concentration for infusion (Pierre Fabre)
11. Biotinylated anti-TCRβ (Thermo Fisher Scientific, eBioscience™, catalog number: 13-5961)
12. Biotinylated anti-CD3ε (Thermo Fisher Scientific, eBioscience™, catalog number: 13-0031)
13. Dynabeads M-280 streptavidin (Thermo Fisher Scientific, Invitrogen™, catalog number: 11206D)
14. Fluorescent streptavidin (for example, Streptavidin APC, BioLegend, catalog number: 405207)
15. Fluorescent antibody to CD45.1 (for example, anti-CD45.1 Brilliant Violet 650, BioLegend, catalog number: 110736)
16. Fluorescent antibody to CD45.2 (for example, anti-CD45.2 PE-Dazzle 594, BioLegend, catalog number: 109846)
17. Phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Gibco™, catalog number: 14190)
18. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A7906)
19. PBS/BSA (see Recipes)

Equipment

1. Dissection tools (sharp scissors and forceps)
2. Refrigerated benchtop centrifuge
3. EasySep™ magnet (STEMCELL Technologies, catalog number: 18000)
4. Tube rotator
5. Flow cytometer

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Procedure

Note: The steps in this procedure are summarised in Figure 1. All animal procedures were performed in accordance with UK Home Office regulations.

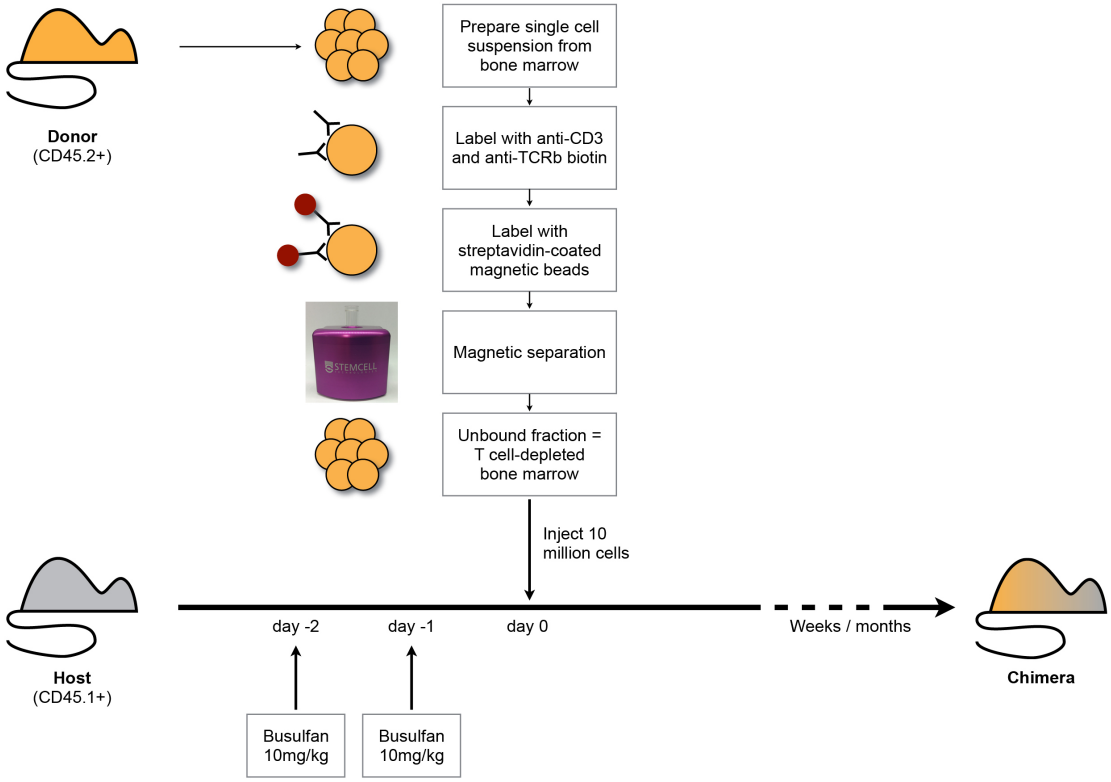

Figure 1. Generation of busulfan chimeric mice. This schematic summarises the principal steps of this protocol for the generation of busulfan chimeric mice. Bone marrow cells from CD45.2$^+$ donor mice are labeled with biotinylated antibodies to CD3 and TCRb, followed by labelling with streptavidin-coated magnetic beads. Labelled cells are immobilised using a magnet, allowing the unbound fraction to be collected. T cell depleted bone marrow cells are then injected into busulfan-conditioned CD45.1$^+$ host mice.

A. Busulfan treatment of host CD45.1 mice (Day -2 and Day -1)
   1. Prepare a fresh working solution of 1 mg/ml busulfan by diluting Busilvex (6 mg/ml busulfan) in sterile PBS.
      Note: This must be prepared fresh each day and cannot be stored.
   2. Ear mark and weigh each mouse individually prior to each injection and calculate the appropriate volume of working solution required to deliver a dose of 10 µg/g busulfan–*i.e.,* 10 µl of 1 mg/ml working solution per gram of body weight.
   3. Day -2: Deliver appropriate dose of busulfan by intraperitoneal injection.
   4. Allow host mice 24 h recovery after injection.

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5. Day -1: Repeat Steps A1-A4 for a second injection of busulfan. The final dose of busulfan in each mouse is thus 20 µg/g, delivered as two doses of 10 µg/g 24 h apart.
   Note: Mice remain healthy and alert after busulfan injection. HSC are depleted within 24 h after busulfan treatment (Westerhof et al., 2000).

B. Preparation of T cell depleted donor CD45.2 bone marrow (Day 0)
   Note: Cell suspensions can be prepared on the bench; a laminar flow hood is not required.
   1. Euthanise donor mice by cervical dislocation and remove the femur and tibia from both hind legs.
   2. Use a 5 ml syringe fitted with a 25 G needle to flush each of the bones with PBS/BSA (see Recipes), collecting the marrow in a Petri dish.
   3. Filter the bone marrow through a 70 µm cell strainer into a 50 ml tube.
   4. Centrifuge at 300 *x g* for 5 min at 4 °C, discard supernatant and resuspend in 1 ml PBS/BSA per donor mouse.
   5. Count cells (expect to recover ~40 million cells per donor mouse) and adjust the volume of cell suspension to a concentration of 20-40 million cells/ml.
   6. Add anti-TCRβ and anti-CD3ε biotinylated antibodies to a final concentration of 1 µg/ml for each, incubate with rotation for 30 min at 4 °C.
   7. Centrifuge at 300 *x g* for 5 min at 4 °C, discard supernatant and resuspend in PBS/BSA at a concentration of at 20-40 million cells/ml.
   8. Transfer cell suspension to 5 ml FACS tubes with lids. Use multiple tubes if necessary, with a volume of 3-4 ml per tube.
   9. Add streptavidin Dynabeads to a bead: cell ratio of approximately 1:1 and incubate with rotation for 30 min at 4 °C.
   10. Insert the tube of beads with cell suspension into the Easysep magnet and incubate for 2 min on ice.
   11. Collect the supernatant containing the unbound fraction of cells into a fresh tube.
   12. Repeat Steps B10-B11 with the collected unbound fraction of cells, to ensure all beads are removed from the cell suspension.
   13. Centrifuge at 300 *x g* for 5 min at 4 °C, discard supernatant and resuspend in 1 ml PBS/BSA per donor mouse.
   14. Count cells, expecting a loss of 10-40% from the original cell count (Step B5) due to depletion and repeated wash steps.
   15. Confirm depletion of T cells by staining samples of pre- and post-depletion cells with fluorescent streptavidin and analysing by flow cytometry. Expect to see ~5% of lymphocytes staining positive in the pre-depletion sample, reduced to < 0.5% of lymphocytes in the post-depletion sample.

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C. Bone marrow transplantation (Day 0)
1. Allow host mice 24 h recovery after the second injection of busulfan before transplantation with T-cell depleted donor bone marrow.
2. Resuspend T-cell depleted bone marrow in PBS to a concentration of at least 50 million cells/ml.
3. Deliver 200 µl (*i.e.,* at least 10 million cells) to host mice by intravenous injection into the tail vein.

Data analysis

1. At the desired timepoint(s) post-BMT, sacrifice chimeras and harvest organs of interest for analysis by flow cytometry. Organs and staining panels vary depending on the cell type of interest, but the inclusion of antibodies to both CD45.1 and CD45.2 is necessary in all staining panels to allow positive identification of cells of host ($CD45.1^+$) or donor ($CD45.2^+$) origin.
2. By 6 weeks post-BMT, we typically see that the fraction of cells in the thymus that are donor-derived has stabilised at approximately 90%, and this is maintained up to one year post-BMT. The extent of stem cell replacement and therefore chimerism in progenitor populations varies between mice, reflecting variability in the ablation of host stem cells in the bone marrow. Thus the output of de novo generated T cells from the thymus is chimeric with respect to donor and host. To account for this, we measure the donor: host ratio within the thymus $CD4^+CD8^+$DP population of all mice as an accurate proxy for stem cell replacement in the bone marrow. With this estimate of stem cell chimerism, it is possible to estimate what fraction of any haematopoietic compartment has been replaced since the time of BMT, so called the fraction of 'new' cells, by the following formula:

$$\frac{\text{Donor fraction in population of interest}}{\text{Donor fraction in thymus DP}} = \text{Fraction of "new" cells in population of interest}$$

Since this calculation in effect normalises data against variability in stem cell chimerism, such estimates of 'fraction new' can be directly compared between individuals regardless of variability of stem cell engraftment following busulfan treatment (Figure 2).
3. Timepoints chosen for analysis of chimeras depend upon the cell population of interest. For naïve CD4 and CD8 T cells, older host-derived cells are gradually replaced by new donor-derived cells until the donor:host ratio stabilises at approximately 30 weeks post-BMT (Figure 2D and Hogan *et al.,* 2015). The replacement kinetics of memory CD4 T cell subsets are similar to naïve cells (Gossel *et al.,* 2017), although the level of saturation varies by population, reflecting differences in underlying homeostatic mechanisms.

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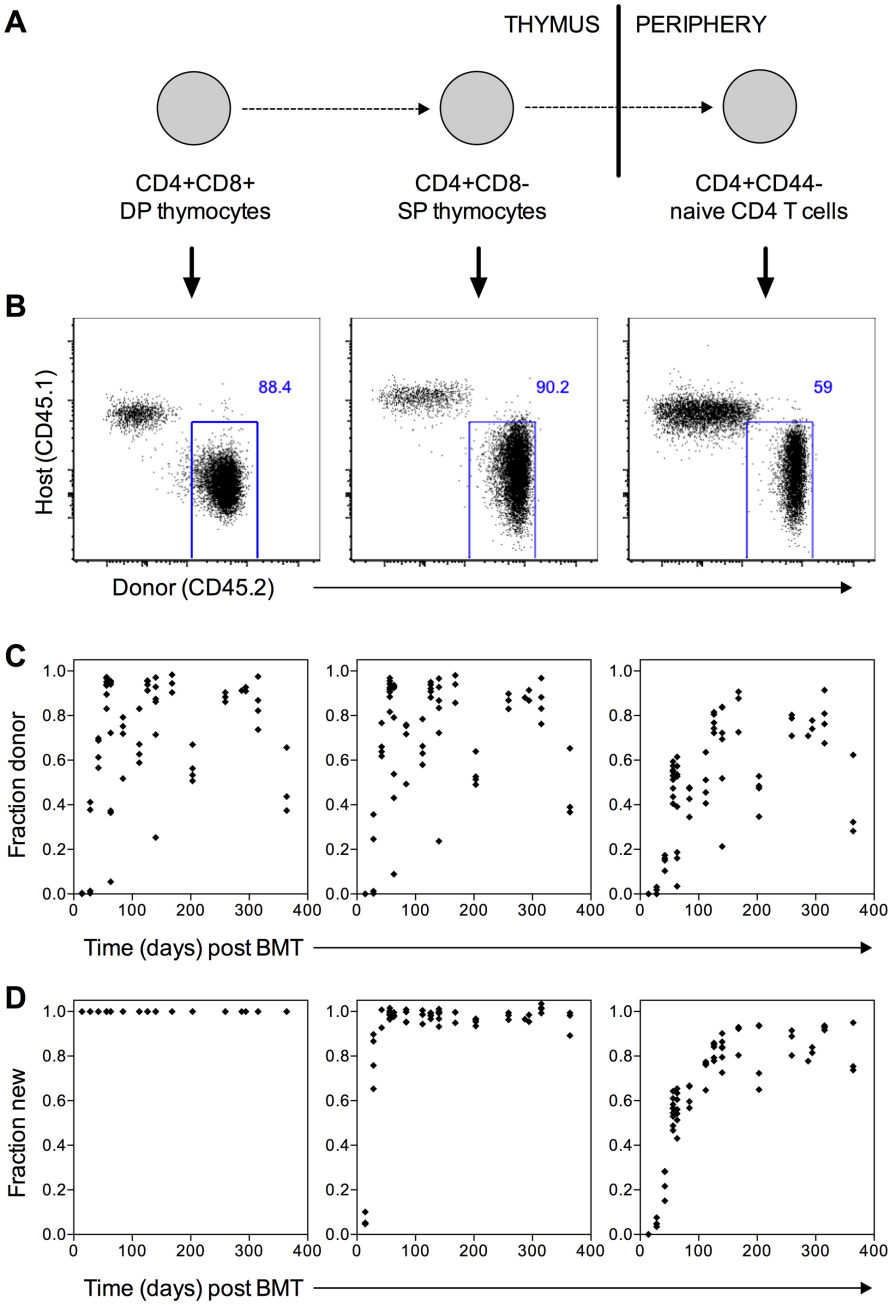

Figure 2. Representative data analysis. A. Schematic of maturation stages for the T cell populations shown in Figures 2B-2D. B. Representative flow cytometry plots showing host ($CD45.1^+$) and donor ($CD45.2^+$) cells in chimeras 8 weeks post-BMT. Left panel shows thymocytes gated on $CD4^+CD8^+$ double positive cells; middle panel shows thymocytes gated on $CD4^+CD8^-$ single positive cells; right panel shows splenocytes gated on naive ($CD4^+CD44^{lo}$) CD4 T cells. B. Fraction of donor cells in DP (left), CD4 SP (middle) and CD4 naive (right) populations at various times post-BMT. Each point represents one mouse. C. Fraction of 'new' cells in DP (left), CD4 SP (middle) and CD4 naive (right) populations at various times post-BMT. Each point represents one mouse. 'Fraction new' is calculated according to the formula (donor fraction in a population of interest)/(donor fraction in thymus DP).

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Notes

1. The busulfan dose used in this protocol (20 µg/g) was specifically tested in B6 CD45.1 mice and may require testing and titration in alternative mouse strains.
2. Delivery of busulfan as two doses of 10 µg/g with 24 h recovery between injections is typically better tolerated by the mice than a single dose of 20 µg/g.
3. The number of bone marrow cells injected can be varied from 10-20 million per mouse. However, doses lower than 10 million cells per mouse may result in a reduced level of chimerism.

Recipes

1. PBS/BSA
   a. Supplement Dulbecco's PBS with 1% BSA
   b. Keep sterile and store at 4 °C for up to 1 month

Acknowledgments

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