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TITLE:

Bone Marrow Transplantation Procedures in Mice to Study Clonal Hematopoiesis

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KEYWORDS:

clonal hematopoiesis, myeloablative pre-conditioning, non-myeloablative conditioning, hematopoietic cell reconstitution, bone marrow transplantation, adoptive transfer

SUMMARY:

We describe three methods of bone marrow transplantation (BMT): BMT with total-body irradiation, BMT with shielded irradiation, and BMT method with no pre-conditioning (adoptive BMT) for the study of clonal hematopoiesis in mouse models.

ABSTRACT:

Clonal hematopoiesis is a prevalent age-associated condition that results from the accumulation of somatic mutations in hematopoietic stem and progenitor cells (HSPCs). Mutations in driver genes, that confer cellular fitness, can lead to the development of expanding HSPC clones that increasingly give rise to progeny leukocytes harboring the somatic mutation. Because clonal hematopoiesis has been associated with heart disease, stroke, and mortality, the development of experimental systems that model these processes is key to understanding the mechanisms that underly this new risk factor. Bone marrow transplantation procedures involving

myeloablative conditioning in mice, such as total-body irradiation (TBI), are commonly employed to study the role of immune cells in cardiovascular diseases. However, simultaneous damage to the bone marrow niche and other sites of interest, such as the heart and brain, is unavoidable with these procedures. Thus, our lab has developed two alternative methods to minimize or avoid possible side effects caused by TBI: 1) bone marrow transplantation with irradiation shielding and 2) adoptive BMT to non-conditioned mice. In shielded organs, the local environment is preserved allowing for the analysis of clonal hematopoiesis while the function of resident immune cells is unperturbed. In contrast, the adoptive BMT to non-conditioned mice has the additional advantage that both the local environments of the organs and the hematopoietic niche are preserved. Here, we compare three different hematopoietic cell reconstitution approaches and discuss their strengths and limitations for studies of clonal hematopoiesis in cardiovascular disease.

INTRODUCTION:

Clonal hematopoiesis (CH) is a condition which is frequently observed in elderly individuals and occurs as a result of an expanded hematopoietic stem and progenitor cell (HSPC) clone carrying a genetic mutation[1]. It has been suggested that by the age of 50, most individuals will have acquired an average of five exonic mutations in each HSPC[2], but most of these mutations will result in little or no phenotypic consequences to the individual. However, if by chance one of these mutations confers a competitive advantage to the HSPC—such as by promoting it's proliferation, self-renewal, survival, or some combination of these—this may lead to the preferential expansion of the mutant clone relative to the other HSPCs. As a result, the mutation will increasingly spread through the hematopoietic system as the mutated HSPC gives rise to mature blood cells, leading to a distinct population of mutated cells within the peripheral blood. While mutations in dozens of different candidate driver genes have been associated with clonal events within the hematopoietic system, among these, mutations in DNA methyltransferase 3 alpha (*DNMT3A*) and ten eleven translocation 2 (*TET2*) are the most prevalent[3]. Several epidemiological studies have found that individuals who carry these genetic mutations have a significantly higher risk of cardiovascular disease (CVD), stroke, and all-causal mortality[3–7]. While these studies have identified that an association exists between CH and increased incidence of CVD and stroke, we do not know whether this relationship is causal or a shared epiphenomenon with the aging process. To gain a better understanding of this association, proper animal models that correctly recapitulate the human condition of CH are required.

Several CH animal models have been established by our group and others using zebrafish, mice, and non-human primates[8–14]. These models often use hematopoietic reconstitution methods by transplantation of genetically modified cells, sometimes using Cre-lox recombination or the CRISPR system. This approach allows for the analysis of a specific gene mutation in hematopoietic cells to assess how it contributes to disease development. In addition, these models often employ congenic or reporter cells to distinguish the effects of mutant cells from normal or wild-type cells. In many cases, a pre-conditioning regimen is required to successfully engraft donor hematopoietic stem cells.

Currently, the transplantation of bone marrow to recipient mice can be divided into two main categories: 1) myeloablative conditioning and 2) non-conditioned transplantation. Myeloablative conditioning can be achieved by one of two methods, namely, total body irradiation (TBI) or chemotherapy[15]. TBI is carried out by subjecting the recipient to a lethal dose of gamma or X-ray irradiation, generating DNA breaks or cross-links within rapidly dividing cells, rendering them irreparable[16]. Busulfan and cyclophosphamide are two commonly used chemotherapy drugs that disrupt the hematopoietic niche and similarly cause DNA damage to rapidly dividing cells. The net result of myeloablative preconditioning is apoptosis of hematopoietic cells, which destroys the recipient's hematopoietic system. This strategy not only allows for the successful engraftment of the donor HSPCs, but can also prevent graft rejection by suppressing the recipient's immune system. However, myeloablative preconditioning has severe side effects such as damage to tissues and organs and their resident immune cells as well as destruction of the native bone marrow niche[17]. Therefore, alternative methods have been proposed to overcome these undesirable side effects, particularly in regard to damage to the organs of interest. These methods include shielded irradiation of recipient mice and the adoptive BMT to non-conditioned mice[9,17]. Shielding the thorax, abdominal cavity, head or other regions from irradiation by the placement of a lead barriers keeps tissues of interest protected from the damaging effects of irradiation and maintains their resident immune cell population. On the other hand, the adoptive BMT of HSPCs to non-conditioned mice has an additional advantage because it preserves the native hematopoietic niche. In this manuscript, we describe the protocols and results of HSPC engraftment after several transplantation regimens in mice, specifically the delivery of HSPC to TBI mice, to mice partially shielded from irradiation, and to non-conditioned mice. The overall goal is to help researchers understand the different physiological effects of each method as well as how they affect experimental outcomes in the setting of CH and cardiovascular disease.

PROTOCOL:

All procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Virginia.

1. Prior to preconditioning

1.1. Place the recipient mice on antibiotic-supplemented water (5 mM sulfamethoxazole, 0.86 mM trimethoprim) ~24 h prior to irradiation. This is necessary to prevent infection, as the immune system will be suppressed following irradiation, and maintained for 2 weeks following irradiation. At this point, supplement mice with a nutritional/hydration gel to encourage feeding and to prevent weight loss and dehydration after irradiation.

2. Preconditioning of recipient mice (optional)

[Place **Figure 1** here]

2.1. Total body irradiation

2.1.1. Place recipient mice into a uniformly sliced pie-cage, or a mouse cage in the reflective

chamber within the calculated radius that receive same irradiation dose; however, a maximum of 8 mice per pie-cage and 5 mice per mouse cage is recommended to ensure uniform irradiation (**Figure 1A,B**).

2.1.2.To achieve complete myeloablation, ensure that recipient mice receive a total radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 4–24 h interval.

NOTE: While optimal engraftment can be obtained by implementing a 4 h interval between fractions, this can be extended to a 24 h interval, which can be helpful when labor and/or the irradiator is unavailable.

2.2. Partially shielded irradiation

2.2.1. Anesthetize the recipient mice by intraperitoneal injection of ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg). The restraint of mouse movement is critical to ensure the uniform irradiation and effective protection of the targeting organs during the shielding process.

2.2.2. For thorax and abdomen shielding, orient the radiation beam of the X-ray irradiator vertically to the mouse (**Figure 1C**).

2.2.2.1. Position the anesthetized mice onto a flat plate, centering the radiation source from above. Place the mice inverted to each other in a supine position with arms and legs fully extended (**Figure 1D,E**).

NOTE: X-ray irradiator facility, for this experiment, allows that two mice at a time can be positioned within the effective radius that allows uniform irradiation. While the effective radius is calculated based on the distance between the radiation source and the tray, the number of animals that can be simultaneously irradiated will depend upon the specific irradiator.

2.2.2.2. Fasten the paws of the mice onto the plate using tape to ensure the mice are immobilized during the irradiation procedure. Place lead shielding so that it covers regions that require protection.

2.2.2.3. For thorax shielding, prepare the lead shield by measuring the length from the mouse's xiphisternum bone to the thymus and calculating the thickness that will provide sufficient protection from the source of irradiation. Place the lead shielding so that the lower end aligns with the xiphisternum bone. The upper end of the lead barrier will fit near the thymus (**Figure 1D**).

2.2.2.4. For abdomen shielding, prepare the lead shield by measuring the length from the mouse's anus to the diaphragm and calculating the thickness that will provide sufficient protection from the source of irradiation. Place the lead shielding so that the lower end aligns with the anus. The upper end of the lead shield will fit below the diaphragm (**Figure 1E**).

NOTE: Localizing the lead shield to be consistent among cohorts may reduce some variation with regard to the size of the mice.

2.2.3. For head shielding, orient the radiation beam of the Cesium irradiator horizontally to the mouse.

2.2.3.1. Carefully tape the forepaws of an anesthetized mouse to the abdomen. This ensures that the arms get a full dose of irradiation and are not covered by the shield.

2.2.3.2. For head shielding, place the mouse in a conical restrainer, which fits inside a lead shield. Once the mouse is inside the conical restrainer, slide the restrainer into the slot within the lead shield (**Figure 1F**). The lead shield should completely cover the mouse's head and ears (~3.2 cm), leaving the rest of the mouse's body exposed for irradiation. The position of the restrainer inside the shield can be adjusted to fit different sized animals by sliding it further inside or outside the shield.

2.2.3.2. Place mice inside the irradiator, perpendicular to the source for irradiation.

2.2.4. Expose mice to two 5.5 Gy fractions of irradiation (total dose of 11 Gy) separated by a 4–24 h interval.

2.2.5. After each irradiation fraction, place the cages with anesthetized mice on heated mats or under red heat lamps to prevent hypothermia and aid in the recovery from anesthesia.

NOTE: Caution must be taken to not overheat the anesthetized mouse when using a lamp since they cannot escape the heat. As described above, the positioning of animals and the thickness of lead shield can differ between studies based upon the specific features of the irradiator (radiation type/direction of beam, etc.). Researchers will need to adjust their experiments accordingly.

3. Bone isolation

NOTE: Ideally, donor mice and recipient mice should be similar in age, and within 8–12 weeks old. Using at least 3 mice as donors (rather than single donor) is preferred to minimize for heterogeneity (even when using mice with the same genotype). Approximately, 40 million unfractionated bone marrow cells can be obtained from six bones (two femurs, two tibias, and two humeri) of a single mouse. Transplantation of 5 million bone marrow cells to each recipient mouse will typically ensure engraftment.

3.1. Euthanize donor mice by cervical dislocation without anesthesia (preferred method to avoid chemical contamination of cells) and place each mouse onto an absorbent pad.

3.2. Disinfect the skin using a 70% ethanol spray.

3.3. Make a small transverse cut in the skin below the rib cage and hold the skin tightly at either side of the incision, tear in opposite directions toward the head and feet. Peel off the skin from all the limbs.

3.4. Cut over the shoulders and the elbow joints, and remove the attached muscles and connective tissues with the aid of a Kimwipe to obtain the humeri.

3.5. Carefully dislocate the hip joints between the femur and hip bones. Use blunt scissors to cut along the femur head and detach the legs. Cut over the knee joint to separate the femur and tibia, and carefully remove the attached muscles and connective tissues with the aid of a Kimwipe to harvest the femur and tibia.

NOTE: Pay special attention to keep the bone epiphysis intact during this step. Discard any broken bones due to loss of sterility. Hip bones and spine bones can be collected in addition to the femur, tibia, and humerus. To collect spine bones, a mortar and a pestle can be used to crush the bones into pieces and harvest the bone marrow cells.

3.6. Place the isolated bones from mice of the same genotype into correspondingly 50 mL conical tube containing 20 mL ice-cold sterile PBS, and keep it on ice until further use. Pay special attention to correctly place the bones into tubes with matched genotypes.

3.7. Repeat the above steps for each donor animal changing gloves in between each mouse. Also, clean scissors and other instruments with 70% ethanol between each mouse.

4. Bone marrow cell isolation

NOTE: Perform the following steps in a biosafety class II cabinet.

4.1. Preparation of tube sets: Make a small hole in the bottom of a sterile 0.5 mL microcentrifuge tube using an 18 G needle and place it into a sterile 1.5 mL microcentrifuge tube, which contains 100 µL of ice-cold sterile PBS at the bottom.

NOTE: As only six bones can fit into the 0.5 mL microcentrifuge tube, it is recommended to prepare sufficient tube sets to process all the bones at the same time.

4.2. Aspirate the PBS and transfer the isolated bones onto a sterile 100 mm cell culture dish. Holding each bone using fine forceps, carefully cut the epiphyses off each end using small scissors that were sterilized in an autoclave. Place the cut bones into the prepared tube sets.

4.3. Centrifuge the tubes at 10,000 x *g* for 35 s at 4 °C.

4.4. After centrifugation, confirm that the bone marrow has been successfully removed from the bones. Bones should appear white and translucent with a relatively large red pellet at the bottom of the 1.5 mL microcentrifuge tube. Discard the 0.5 mL microcentrifuge tube.

NOTE: If the visual inspection fails to detect bone marrow at the bottom of the 1.5 mL tube, cut the bone again and repeat step 4.3.

4.5. Resuspend the bone marrow in 1 mL of ice-cold PBS, then transfer the cell suspension from the same genotype to a matched 50 mL conical tube.

4.6. Dissociate the cells by passing them through an 18 G needle with a 10 mL syringe 10 times.

4.7. Filter single cell suspensions through a 70 µm cell strainer. Add additional ice-cold PBS to a final volume of 10 mL, and resuspend the cells through the gentle use of pipette-aid.

4.8. Centrifuge at 310 x *g* for 10 min at 4 °C.

4.9. Aspirate the supernatant and resuspend the cell pellet with 10 mL of serum-free RPMI media. Spare 30 µL of this material for cell counting.

4.10. Determine cell concentration with a cell counter, and calculate the volume of cell suspension required for the transplantation. For the example of a 100% BMT, 5 x 10^6 bone marrow cells are required for each recipient mouse.

NOTE: For a competitive BMT, prepare a total of 5 x 10^6 bone marrow cells comprising a mixture of donor cells (e.g., CD45.2+) and competitor cells (e.g., CD45.1). Preparing extra bone marrow cells is highly recommended. For example, if there are 10 recipient mice per experimental group, we typically prepare enough cells for 12 recipient mice.

4.13. Transfer the calculated volume of cell suspension into a new 50 mL conical tube. Centrifuge at 310 x *g* for 10 min at 4 °C.

4.14. Aspirate the supernatant and resuspend the cells using the calculated amount of serum-free RPMI medium to achieve the appropriate cell density and volume. Typically, 200 µL is the optimal volume for a retro-orbital injection.

5. Transplantation of bone marrow cells to irradiated mice

5.1. Anesthetize the recipient mice with 5 % isoflurane.

5.2. While mice are anesthetized, slowly inject 200 µL of bone marrow cells into the retro-orbital vein using a 28–30 G needle with an insulin syringe.

5.2.1. Alternatively, perform the delivery of the donor cells by tail vein intravenous injection and femoral intramedullary injection, with a maximum volume of 0.2 mL and 25 µL, respectively.

5.3. Once the cells are injected, place a drop of proparacaine-containing eye-drops onto the

surface of the eye for pain relief. The animal can then be allowed to regain consciousness.

6. Transplantation of bone marrow cells to non-conditioned mice

6.1. Anesthetize the recipient mice by inhalation of 5% isoflurane.

6.2. Inject 5 x 10^6 unfractionated bone marrow cells from either genotype retro-orbitally into non-irradiated recipient mice with 28–30 G insulin syringe.

6.3. Repeat steps 6.1 and 6.2 over 3 consecutive days, such that the recipient mice will be transplanted with a total of 1.5 x 10^7 bone marrow cells.

NOTE: Because the adoptive BMT without pre-conditioning procedure requires bone marrow transplantation for 3 consecutive days, one should attempt to alternate eyes for each injection.

6.4. Post-injection, administer a drop of proparacaine-containing eye drops to the affected eye.

REPRESENTATIVE RESULTS:

To compare the effect of three BMT/pre-conditioning methods on donor cell engraftment, the fractions of donor cells in peripheral blood and heart tissue were analyzed by flow cytometry at 1-month post-BMT. Isolated cells were stained for specific leukocyte markers to identify the different subsets of leukocytes. In these experiments, wild-type (*WT)* C57BL/6 (CD45.2) donor bone marrow cells were delivered to *WT* B6.SJL-*Ptprc*^{a}*Prpc*^{b}/BoyJ (CD45.1) recipient mice to distinguish donor cells from the recipient's cells. The flow cytometry gating strategies that were used are described previously by Wang et al.[9] in **Supplementary Figure1**.

The total body irradiation (TBI) treated group received 5 x 10^6 bone marrow cells following a total lethal radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 4 h interval. In the peripheral blood of recipient mice, monocytes, neutrophils, and B cells were largely ablated and replaced by the progeny of donor bone marrow-derived cells. In addition, the resident cardiac monocyte and neutrophil population in hearts of recipient mice were almost completely replaced by donor-derived cells (**Figure 2A**).

In the partially shielded irradiation group, recipient mice were irradiated with a thorax shield and transplanted with 5 x 10^6 bone marrow cells following a total radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 4 h interval. In contrast to the TBI group, the contribution of donor-derived cells to cardiac immune cells was modest, which probably reflects the combined effects of protecting the local immune cells in the hearts of recipient mice and the physiological repopulation of bone marrow-derived donor cells from the peripheral blood. Recipient mouse bone marrow cells in shielded regions are also likely to have contributed to peripheral blood reconstitution, which reduces the percentage of donor derived cells in peripheral blood compared to the TBI treated group (**Figure 2B**).

In the group without BMT pre-conditioning (adoptive BMT), recipient mice were transplanted

with 5 x 10^6 bone marrow cells over 3 consecutive days. At 4 weeks post-BMT, the portion of donor-derived cells in peripheral blood and heart was detectable. (approximately 5%) **(Figure 2C**). To illustrate how the adoptive BMT model can be applied to the study of the CH model, CD45.2$^+$ donor bone marrow cells (*WT* or *Tet2-/-)* were transplanted into CD45.1$^+$ recipient mice. Recipients without conditioning were transplanted with 5 x 10^6 bone marrow cells each day for 3 consecutive days (for a total of 1.5 x 10^7, n = 5–6 per group). Flow cytometric analysis of peripheral blood was performed 4, 8, 12, and 16 weeks post-transplantation. The *Tet2-deficient* donor cells conferred a competitive advantage and gradually expanded over time; WBCs, monocytes, Ly6C^{hi} monocytes, neutrophils, T cells, and B cells increased significantly over time. Compared to the *Tet2-deficient* donor cells engrafted into recipient mice, recipient mice engrafted with *WT* donor cells showed less significant clonal expansion of donor cells (**Figure 3**). Consistent with the clinical paradigm of clonal hematopoiesis, the expansion of *Tet2-deficient* cells does not impact the absolute numbers of the various blood cell types[9] (data not shown).
[Place **Figure 2** and **Figure 3** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Images showing various preconditioning setups. (**A**) Pie-cage total body irradiation setup using gamma-ray (Cesium-137): The radiation beam comes from the back of the irradiator in the y-axis direction (horizontal radiation). (**B**) Mouse cage total body irradiation setup using X-ray: The mouse cage is placed in the reflective chamber. The radiation beam comes from the top of the irradiator in the shape of a cone (vertical radiation). The distance from the radiation source to the cage is 530 mm. (**C**) Adjustable tray in X-ray irradiator: This setup is used for partially shielded irradiation using X-ray. The radiation beam comes from the top of the irradiator in the shape of a cone (vertical radiation). The distance from the radiation source to the tray is 373 mm, and the radius is 250 mm. (**D**) Thorax-shielding: Anesthetized mice are placed on a tray. The mice are placed inverted to each other in supine positions with arms and legs fully extended. The lower end of the lead-shield is aligned with the xiphisternum bone and the upper end with the thymus. (**E**) Abdominal-shielding: Anesthetized mice are placed as in the thorax-shielding set-up with the lower end of the lead-shield aligned with the anus and the upper end below the diaphragm. (**F**) Head-shielding irradiation setup using gamma-ray (Cesium-137): The anesthetized mouse's forepaws are taped down and the mouse is placed in a conical restrainer. The black lead-shield (marked) covers the mouse's head and ears. The radiation beam comes from the back of the irradiator in the direction of the Y-axis (horizontal radiation).

Figure 2: Flow cytometric analysis of blood and heart using different methods of preconditioning. Flow cytometric analysis of peripheral blood and heart was performed 1 month after bone marrow transplantation. To distinguish donor cells from the recipients' cells, CD45.1$^+$ recipient mice were transplanted with CD45.2$^+$ donor bone marrow cells. (**A**) 5 x 10^6 bone marrow cells were transplanted following two fractions of total body irradiation (2 x 5.5 Gy, 4 h interval, n = 3). (**B**) 5 x 10^6 bone marrow cells were transplanted following two fractions of total body irradiation with thorax-shielding (2 x 5.5 Gy, 4 h interval, n = 10). (**C**) Recipients without conditioning were transplanted with 5 x 10^6 bone marrow cells for 3 consecutive days (for a total of 1.5 x 10^7, n = 4). Data are expressed as mean ± SEM. Total WBCs are defined as CD45$^+$;

Neutrophils as Ly6G$^+$; Ly6C^{hi} monocytes as CD115$^+$, Ly6G$^-$, and Ly6C$^+$; Ly6C^{lo} monocytes as CD115$^+$, Ly6G$^-$,and Ly6C$^-$; B cells as CD45R$^+$; CD4$^+$ T cells as CD3e$^+$ and CD4$^+$; CD8$^+$ T cells as CD3e$^+$ and CD8$^+$; and Macrophages as CD64$^+$, Ly6G$^-$, and Ly6C$^-$. (WBCs: white blood cells, Neut: neutrophils, Mono: monocytes, Mac: macrophages). **Figure 2A,C** have been modified from Wang et al.[9].

Figure 3: Clonal expansion of *Tet2-deficient* cells using adoptive BMT to non-conditioned mice. CD45.2$^+$ donor bone marrow cells (*WT* or *Tet2*-/-) were transplanted into CD45.1$^+$ recipient mice. Recipients without conditioning were transplanted with 5 x 10^6 bone marrow cells each day for 3 consecutive days (for a total of 1.5 x 10^7, n = 5-6 per group). Flow cytometric analysis of peripheral blood was performed after 4, 8, 12, and 16 weeks post-transplantation. The fraction of CD45.2$^+$ donor cells in each population is shown. (**A**) WBCs (**B**) Mono (**C**) Ly6C^{hi} mono (**D**) B cells (**E**) T cells. Data is expressed as mean ± SEM. WBCs are defined as CD45$^+$; Neutrophils as Ly6G$^+$; Ly6C^{hi} monocytes as CD115$^+$, Ly6G$^-$, and Ly6C$^+$; Ly6C^{lo} monocytes as CD115$^+$, Ly6G$^-$,and Ly6C$^-$; B cells as CD45R$^+$; CD4$^+$ T cells as CD3e$^+$ (*WT*: wild-type, WBCs: white blood cells, Mono: monocytes)

Table 1: The efficiency of donor cell engraftment using different dosing intervals. Flow cytometric analysis of peripheral blood was performed 1 month after bone marrow transplantation. *WT* CD45.2$^+$ donor bone marrow cells were transplanted into CD45.1$^+$ recipient mice. 5 x 10^6 bone marrow cells were transplanted following two fractions of total body irradiation (2 x 5.5 Gy) with or without thorax-shielding separated by a 4 h interval or 24 h interval. (n = 3–4 per group).

DISCUSSION:

For studies of clonal hematopoiesis and immune cell function, we described three methods of BMT: BMT with total-body irradiation, BMT with irradiation with partial shielding, and a less commonly used BMT method that involves no pre-conditioning (adoptive BMT). These methods have been used to assess the impact of clonal hematopoiesis on cardiovascular disease. Researchers can modify these methods accordingly to suit the specific purpose of their study.

Clonal hematopoiesis models

Clonal hematopoiesis is the phenomenon in which mutant hematopoietic cells compete with wild-type cells and obtain clonal dominance over time. To create a model of this competition, mice can be administered bone marrow, which consists of a mixture of genetically different cells. Generally, this mixture will include mutant and wild-type cells, which have been labeled with a fluorescent tag or different pan-leukocyte markers (i.e., CD45.1 and CD45.2). For example, when creating models that mimic *Tet2*-mediated CH, we perform a competitive bone marrow transplantation into lethally irradiated recipients that typically involves mixing 90% of cells that originate from CD45.1 *Tet2*$^{+/+}$ donors and 10% of cells that originate from CD45.2 *Tet2*$^{-/-}$, *Tet2*$^{+/-}$ or control *Tet2*$^{+/+}$ donors[8]. Flow cytometry detection of the CD45 variants using specific monoclonal antibodies enables one to distinguish donor cells (CD45.1$^-$/CD45.2$^+$) from competitor cells (CD45.1$^+$/CD45.2$^-$) within the blood, and assess the clonal dynamics of the test cells over time. By doing so, we have been able to observe a gradual increase in donor chimerism of *Tet2-deficient* cells, while the percentage of wild-type cells remain at approximately 10%. This

experimental setting mimics the human scenario of individuals carrying a *TET2* somatic mutation, since these mutations are initially carried by a small number of HSPCs, which will gradually expand over time. Employing this approach in cardiovascular disease models of atherosclerosis and heart failure has led to the documentation of a potential causal link between *Tet2*-mediated CH and CVD[8–10].

When employing these approaches, researchers should take into consideration the possible confounding effects generated by TBI. Although TBI prior to transplantation enables a high degree of HSPC engraftment, this pre-conditioning will lead to several undesirable effects outside of the hematopoietic system. It has been documented that TBI can lead to inflammation, injury, and fibrosis in multiple organ systems including skin, liver, kidneys, lungs, bone marrow, heart, brain, etc.[18–20]. These side effects can negatively impact the cardiovascular organs under study, and they also alter disease pathogenesis[21,22]. A notable example is the effect of irradiation on resident macrophages in the heart. Irradiation of the thorax results in a marked replacement of cardiac-resident macrophages with circulating monocyte-derived macrophages. Studies have shown that cardiac-resident macrophages display distinct characteristics relative to circulating monocyte-derived macrophages that will engraft in the heart following radiation injury. In disease settings, cardiac-resident macrophages are thought to play a cardioprotective role, whereas infiltrating blood-borne macrophages have been reported to promote injury and inflammation[23]. Therefore, it is conceivable that replacing resident cardiac immune cells with blood-borne cells will alter the pathological processes under study, which contribute to cardiovascular disease[24–26]. Similarly, in the brain, TBI results in depletion of resident microglia and replacement by peripherally-derived macrophages[27,28]. While peripherally-derived macrophages can behave like microglial cells, they maintain a unique functional and transcriptional identity compared to monocyte-derived microglia[28]. Therefore, it is possible that the disease sequela may be altered, particularly when studying diseases such as ischemic strokes. In order to avoid these confounding effects, shielding the thorax and head can be recommended. This is advantageous because it provides protection to the heart and brain, respectively; and it also maintains their resident immune cells intact, better recapitulating the human condition of CH. However, as noted previously, shielding results in a lower rate of chimerism compared to TBI pre-conditioning, which essentially eliminates all the host's hematopoietic cells.

Another important impediment in pre-conditioning is its deleterious effect on the bone marrow niche. Although irradiation-induced damage of the BM niche can be restored to a suboptimal extent, it is unclear whether naïve hematopoiesis is recovered in these damaged microenvironments. In addition, transplantation of mixed HSPCs into empty BM initiates a race for proliferation between clones, rather than the simple "competition" for a niche that is occupied by a wild-type HSPC—which is presumably what occurs in CH. Thus, a potentially preferable approach to studying CH may be the adoptive BMT method, whereby BM cells are transferred into recipient mice without pre-conditioning. This adoptive BMT without pre-conditioning method minimally affects the ongoing naïve hematopoiesis, most faithfully recapitulating CH observed in humans[29]. **Figure 2C** shows the level of chimerism at 1-month post-transplantation without pre-conditioning. While the donor chimerism is low at this early timepoint, we find a progressively increasing fraction of *Tet2-deficient* clones over time, as

presented in **Figure 3**. It should be noted that this model is most useful when the mutant cells have a competitive advantage over wild-type cells under homeostatic conditions such as *Tet2-deficient* cells. When *Tet2-deficient* cells are engrafted, there is a marked expansion in various leukocyte populations such as neutrophils, monocytes, NK cells, and B cells. A slower expansion was noted in T cells, presumably due to the longer half-life of this population.

The expansion of *Tet2-deficient* cells has been observed not only in the peripheral blood but also in several other tissues, including bone marrow, liver, and kidney, with different dynamics of hematopoietic cell reconstitution[9]. For example, our lab's previous published paper described the bone marrow cell chimerism of *WT* and *Tet2-deficient* donor cells engrafted into CD45.1 recipient mice 8 months after adoptive BMT[9]. *Tet2-deficient* donor cells transplanted into CD45.1 recipient mice have shown a competitive advantage over immature lineage$^-$Sca1$^+$c-Kit$^+$ (LSK) cells, short-term and long-term HSC cells, and multipotent progenitors (MPPs) compared to that of *WT* donor cells transplanted into the CD45.1 recipient mice. In addition, as *Tet2-deficien*t donor cell engrafted recipient mice, they develop an age-related cardiomyopathy phenotype without exogenous factors causing cardiac dysfunction, thereby recapitulating the effect of clonal hematopoiesis in a manner similar to that of aging humans. This observation was accompanied with increased degree of chimerism in cardiac neutrophils and Ly6C^{hi} monocytes. Collectively, this adoptive BMT regimen can be applied to future studies that could expand our understanding of the association between cardiovascular disease development and clonal hematopoiesis on a more advanced level.

In summary, we described three BMT methods and discussed their application in generating CH models. CH is associated with poorer prognoses in cardiovascular diseases such as atherosclerosis and heart failure. Although considerable progress has been made, the study of the causal links between CH and CVD is still in its infancy, and further investigations are required through the use of optimized animal models. We hope that these protocols allow researchers to select a more physiologically appropriate method of BMT, which minimalizes potential confounding effects on the cardiovascular system, ultimately yielding studies that expand our understanding of how CH contributes to cardiovascular disease.

Design of lead shielding

The thickness of the lead shield will be dictated by the type of irradiation used to induce the myeloablation. X-ray or gamma-ray types of radiation are frequently used for experimental myeloablation but differ in terms of their frequency, wavelength, and photon energy. When it comes to shielding, the photon energy, which describes the energy or speed at which the rays are traveling, is the most important parameter. Typically, radiation source X-rays have an energy of 160 kVp whereas cesium-137 sources, which emit gamma rays, have an energy of 662 KeV. The energy emitted by these radiation sources equates to their penetration power, with higher energies having a greater penetration power. Therefore, a greater thickness of lead shield is required when using cesium source-based irradiators in comparison with using X-ray-based irradiators. X-ray-based irradiation, which we use when we perform thorax and abdominal shielding, requires a 7 mm thick lead shield to provide sufficient protection. However, for cesium 137 sources, which we use when we perform head-shielding, requires lead shields to be at least

1 inch thick to provide sufficient protection.

Lead shields for use in X-ray irradiators can be purchased from commercial vendors. Alternatively, lead sheets can be cut to size to either fit around the animal's body or to fit around a restrainer (see **Figure 1**). When using a cesium-based irradiator, lead bricks, which are considerably thicker should be used and can be custom made by companies that specialize in making these types of shields. For instance, for the headshield, we custom designed a lead brick to hold a 50 mL conical tube restrainer (see **Figure 1F**). The animal is able to fit inside the restrainer, which is then slotted into a hole made in the brick, to provide 1.5 inches of protection from the irradiation. Importantly, all lead shields should be coated either with paint or tape to prevent exposure to lead dust, which can be toxic.

Based on the equipment and its parameters, researchers can design their own lead shields for their sites of interest. Here, we introduced thorax, abdominal, and head shielding; however, other sites such as limb or flank can be considered for shielding as well. In addition, while both radiation sources (Cesium-137 and X-ray) are suitable for bone marrow ablation and successful engraftment, variability in the reconstitution of lymphoid and myeloid cell populations has been observed between Cesium-137 and X-ray irradiation sources[30]. Thus, researchers should take into account the disparate physiological responses to the radiation source for use in studies.

Dosing intervals

Dosage and dosing intervals may affect the efficiency of donor cell engraftment and survival rates. In human patients, high-dose irradiation can cause idiopathic interstitial pneumonia, gastrointestinal injury, and cataract formation. In mouse models, single high-dose irradiation followed by bone marrow transplantation can produce similar results and can also affect survival rates[31]. Therefore, fractionated irradiation is highly recommended for the mouse BMT studies. In addition, the dosing intervals of fractions can impact mouse survival rate and reconstitution rate, leading to different fractions of donor cell chimerism in the hematological organs as well as other tissues[31]. Thus, researchers should be careful in designing a fractionated irradiation dosing schedule for BMT studies.

In the context of survival rate and immune cell reconstitution, our small study showed that a group of mice receiving total body irradiation with a lethal radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 24 h interval group had no significantly different results than the group which received the same TBI dosage at a 4 h interval (see **Table 1**). However, with thorax-shielding BMT, the 24 h interval group appeared to show less efficiency of donor cell chimerism in comparison with the 4 h interval group. A possible explanation for this result is that irradiation with 24 h interval may not have been sufficient to remove the recipient's immunocompetent cells because the prolonged intervals gave recipient mice sufficient time to repair damaged cells. In addition, thorax-shielding protects partial spine bones that also contain the recipient's HSCs. Thus, the remaining and recovered immunocompetent recipient cells may have attacked the donor-derived cells and induced an outcome that showed lower engraftment efficiency.

[Place **Table1** here]

Animal care

Multiple steps involving mice are required for the success of this experiment. Thus, extra attention is required at the following points: First, the delivery of viable donor cells is crucial for successful engraftment. One should be properly trained in the collection of intact BM cells and their injection into recipient mice. Consequences of poor delivery of cells include the failure of donor HSPCs to reconstitute recipient bone marrow leading to mortality. Second, care must be taken after transplantation to avoid infection, particularly following myeloablative therapy. Contact with pathogens can be fatal, as mice become transiently immunodeficient following irradiation. As indicated above, supplementing the drinking water with antibiotics can lower the risk of fatal infection. Moreover, providing recipient animals with nutritional/hydration gel can minimize dehydration and nutritional deficiencies which may occur following irradiation, as irradiation can disrupt the intestinal epithelium leading to diarrhea[32]. Cages should also be replaced frequently to reduce the risk of fecal bacteria contamination, animals should be handled in a proper animal transfer station, and the recipient mice should be monitored carefully for weight loss and any signs of distress or pain.

ACKNOWLEDGMENTS:

This work was supported by US National Institutes of Health grants to K. Walsh (HL131006, HL138014, and HL132564), to S. Sano (HL152174), American Heart Association grant to M. A. Evans (20POST35210098), and a Japan Heart Foundation grant to H. Ogawa.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1: Images showing various preconditioning setups

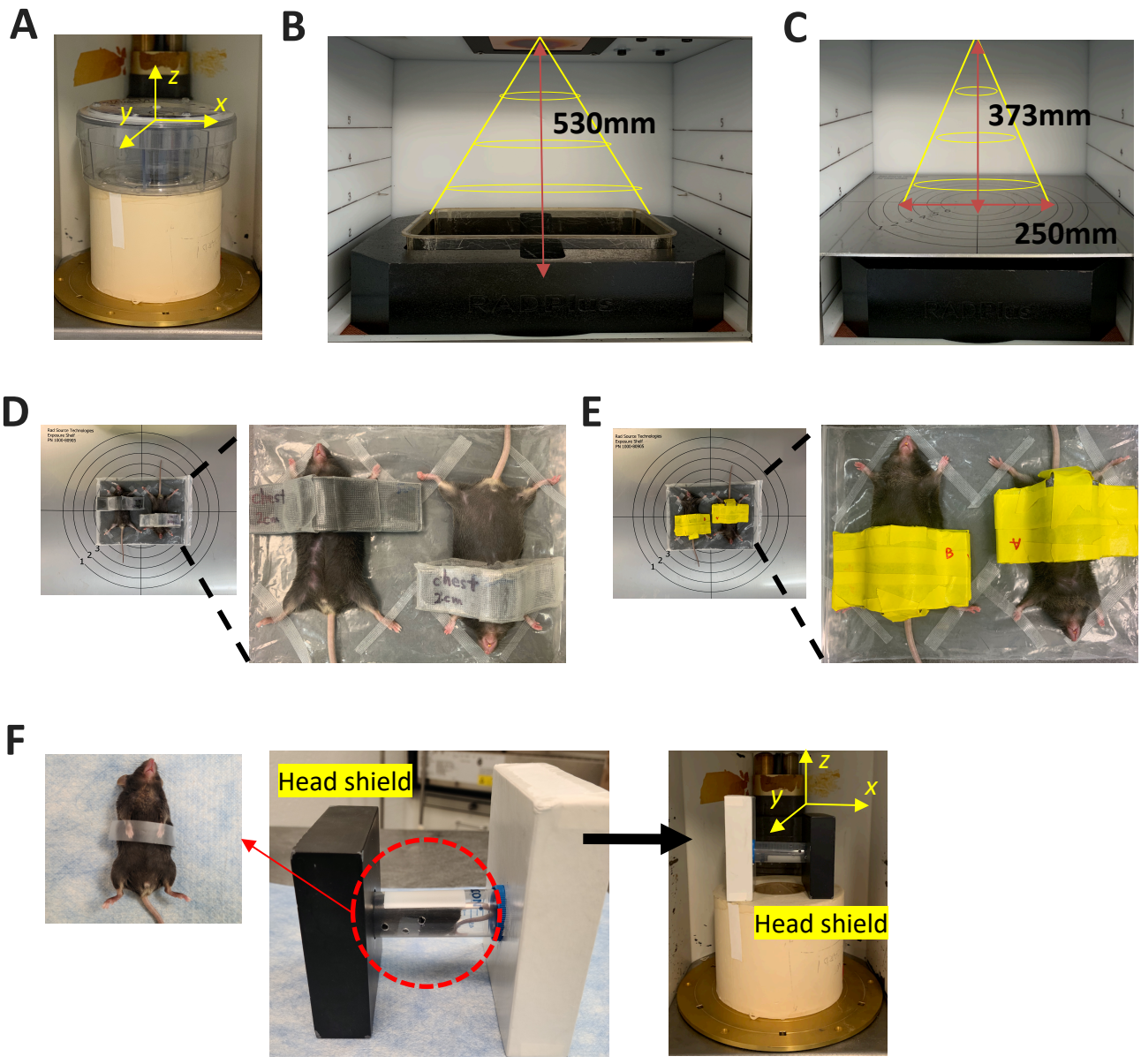


Figure 2: Flow cytometric analysis of blood and heart using different methods of preconditioning.

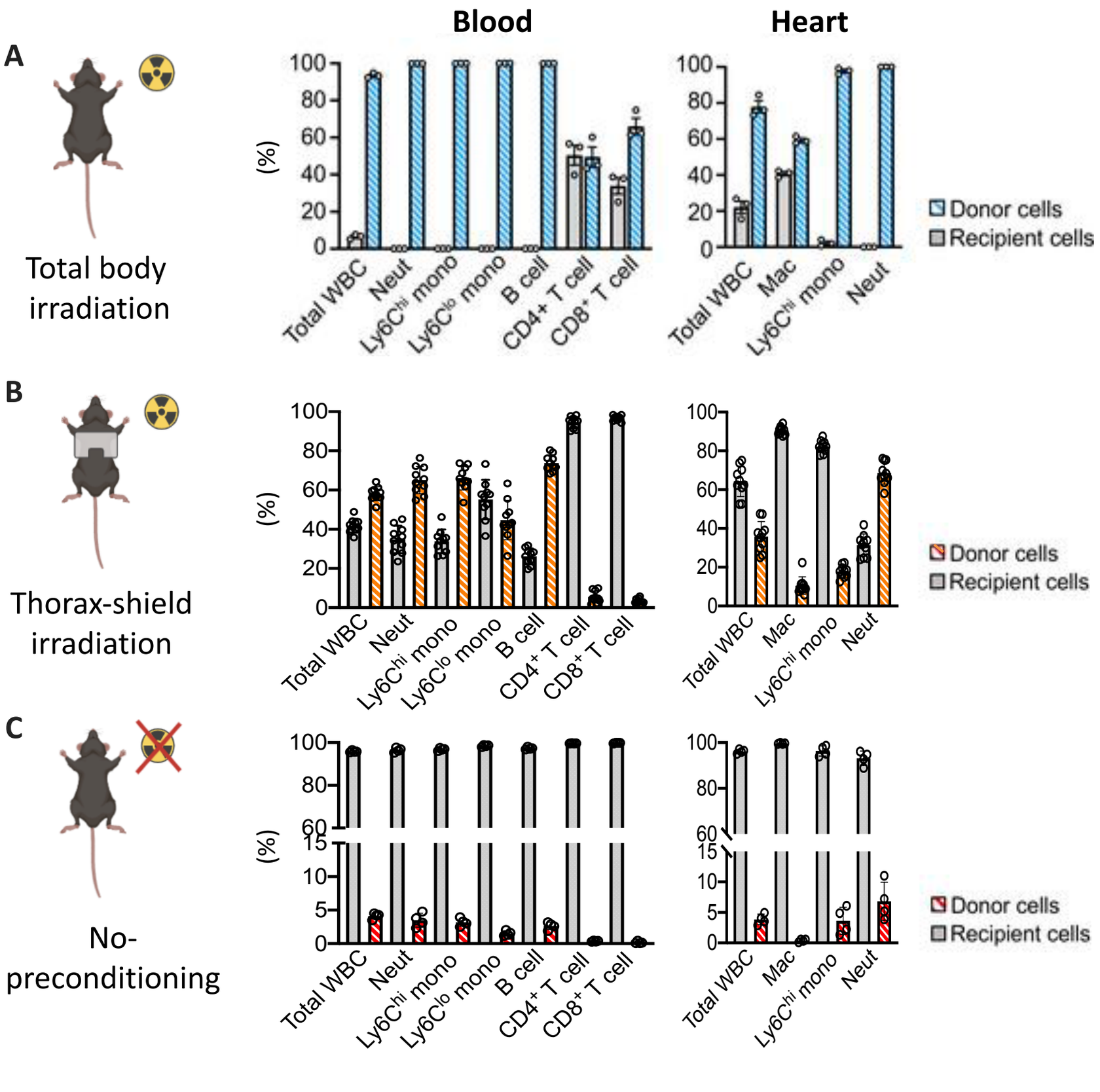


Figure 3. Clonal expansion of *Tet2-deficient* cells using adoptive BMT to non-conditioned mice.

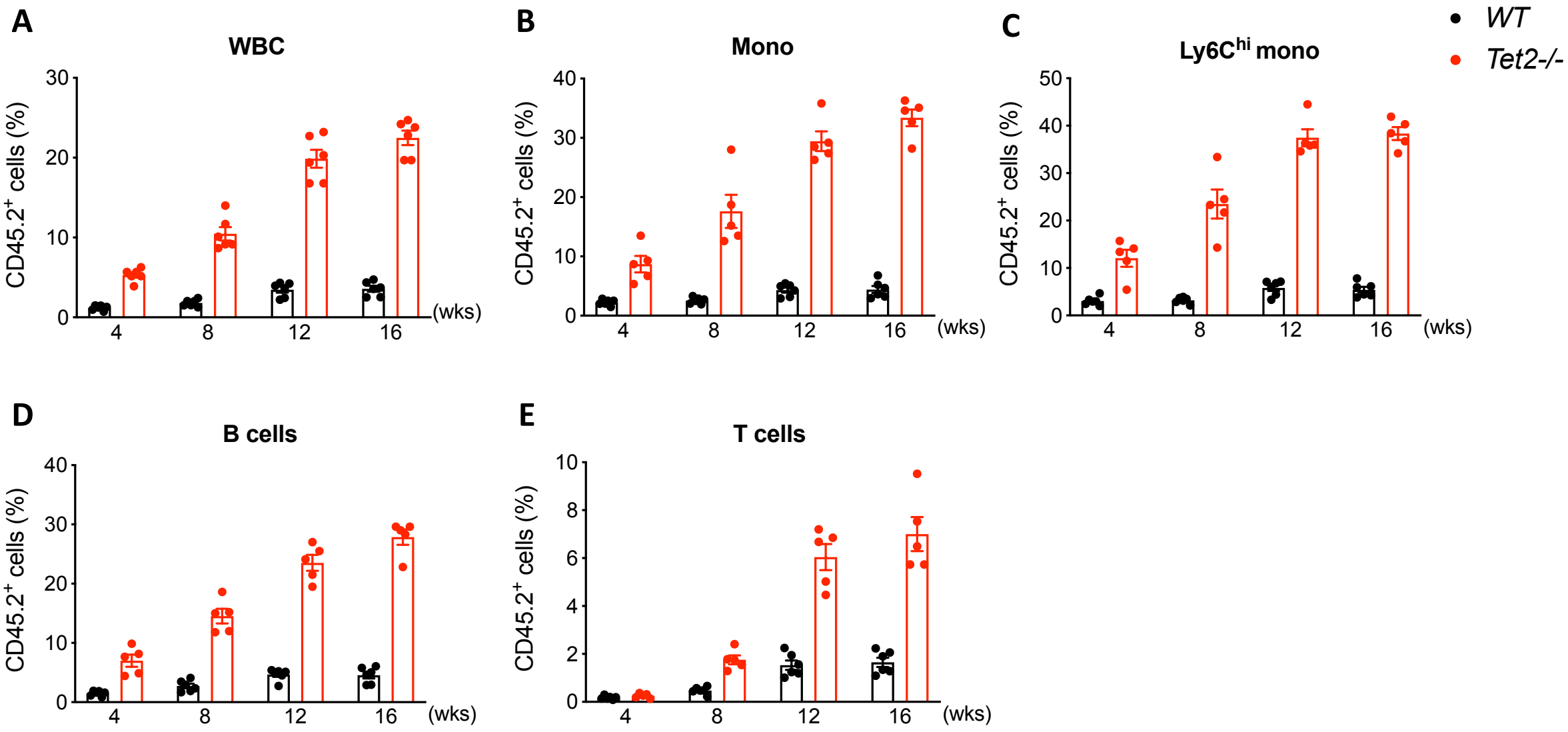


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.5ml microcentrifuge	Fisher Scientific	05-408-121	general supply
1.5ml microcentrifuge	Fisher Scientific	05-408-129	general supply
1/2 cc LO-DOSE INSULIN SYRINGE	EXELINT	26028	general supply
Absolute Ethanol (200 prfof)	Fisher chemical	200559	general supply
BD 1mL Tuberculin Syringes 25G 5/8 Inch Needle	Becton Dickinson	309626	general supply
BD PrecisionGlide Needle 18G (1.22mm X 25mm)	Becton Dickinson	395195	general supply
Cesium-137 Irradiator	J. L. Shepherd	Mark IV	equipment
DietGel 76A	Clear H2O	70-01-5022	general supply
Falcon 100 mm TC-Treated Cell Culture Dish	Life Sciences	353003	general supply
Falcon 50 mL Conical Centrifuge Tubes	Fisher Scientific	352098	general supply
Fisherbrand sterile cell strainers, 70 µm	Fisher Scientific	22363548	general supply
Graefe Forceps	Fine Science Tools	11051-10	general supply
Hardened Fine Scissors	Fine Science Tools	14090-09	general supply
Isothesia (Isoflurane) solution	Henry Schein	29404	Solution
Ketamine	Zoetis	043-304	injection
Kimwipes Delicate Task Wipers	Kimtech Science	KCC34155	general supply
PBS pH7.4 (1X)	Gibco	10010023	Solution
RadDisk – Rodent Irradiator Disk	Braintree Scientific	IRD-P M	general supply
RPMI Medium 1640 (1X)	Gibco	11875-093	Medium
Sulfamethoxazole and Trimethoprim	TEVA	0703-9526-01	injection
Xylazine	Akorn	139-236	injection
X-ray irradiator	Rad source	RS-2000	equipment

JoVE61875
E. Park et al.
"Bone Marrow Transplantation Procedures in Mice to Study Clonal Hematopoiesis and the Role of Immune Cells in Cardiovascular Disease,"

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: Followed.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

Response: Followed.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"

Response: Followed.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Response: Followed.

5. The Protocol should contain only action items that direct the reader to do something.

Response: Followed.

6. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

Response: Followed.

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? For this please include button clicks, knob turns, mechanical actions, etc.

Response: Followed.

8. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: Highlighted.

9. Anesthesia and Euthanasia steps cannot be filmed.

Response: Understood.

10. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e. "This figure has been modified from [citation]."

Response: Followed.

11. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

Response: Followed.

12. Please ensure all figures are referenced in the order. So figure 1 will be cited before Figures 2 or 3.

Response: Followed.

13. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: Followed.

14. Please do not make a separate section for additional comments. This can be integrated in the discussion in the format mentioned above.

Response: Followed.

15. Please remove the figure legends from the uploaded figures.

Response: Followed.

Reviewer #1:

In the submitted manuscript, Park et al. describe bone marrow transplantation methods in mice to study clonal hematopoiesis. The authors compare methodology for bone marrow transplantation into preconditioned (global and localized radiation) and naïve mice. They further provide an example where adoptive transplantation on Tet2 *KO bone marrow into naïve mice results in stable and increasing chimerism over time. The manuscript is clearly written and will be useful to investigators wishing to enter this field. The authors do an excellent job clearly explaining the experimental protocol in sufficient detail and discussing the intricacies of different irradiation sources. I have the following comments and suggestions to improve the manuscript.*

Response: We thank the reviewer for these positive comments.

1. Understanding the difference between 4h and 24h TBI dosing regimens is an important and useful note. It would be helpful to more clearly explain whether any differences are expected between these radiation dosing schedules. Example data would be useful if available. The authors state that optimal engraftment is obtained using the 4h dosing interval and only display results from this protocol.

Response:

We appreciate your detailed comment. We agree that it would be helpful to show the difference between irradiation dosing intervals. Thus, we have added the following to the discussion section. (Line 557-580)

"**Dosing intervals**

Dosage and dosing intervals may affect the efficiency of donor cell engraftment and survival rates. In human patients, high-dose irradiation can cause idiopathic interstitial pneumonia, gastrointestinal injury, and cataract formation in human patients. In mouse models, single high-dose irradiation followed by bone marrow transplantation can produce similar results and can also affect survival rates[31]. Therefore, fractionated irradiation is highly recommended for the mouse BMT studies. In addition, the dosing intervals of fractions can impact mouse survival rate and reconstitution rate, leading to different fractions of donor cell chimerism in the hematological organs as well as other tissues[31]. Thus, researchers should be careful in designing a fractionated irradiation dosing schedule for BMT studies.

In the context of survival rate and immune cell reconstitution, our small study, showed that a group of mice receiving total body irradiation with a lethal radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 24 h interval group had no significantly different results than the group which received the same TBI dosage at a 4 h interval. (see **Table 1**) However, with thorax-shielding BMT, 24 h interval group appeared to show less efficiency of donor cell chimerism by comparison with the 4 h interval group. A possible explanation for this result is that irradiation

with 24 h interval may not have been sufficient to remove the recipient's immunocompetent cells because the prolonged intervals gave recipient mice sufficient time to repair damaged cells. In addition, thorax-shielding protects partial spine bones that also contain recipient's HSCs. Thus, the remaining and recovered immunocompetent recipient cells may have attacked the donor-derived cells and induced an outcome that showed lower engraftment efficiency.

Table1. The efficiency of donor cell engraftment using different dosing intervals.

		WBC (%)	B cell (%)	T cell (%)	Mono (%)	Ly6C^{hi} mono (%)	Ly6C^{lo} mono (%)	Neutrophil (%)
TBI-BMT	4h interval	97.4 ± 1.0	100 ± 0	59.1 ± 18.7	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	24h interval	97.2 ± 2.2	100 ± 0	79.0 ± 8.1	100 ± 0	100 ± 0	100 ± 0	100 ± 0
thorax-shielding BMT	4h interval	56.2 ± 4.0	54.2 ± 6.2	0.5 ± 0.1	66.7 ± 6.1	63.8 ± 6.3	70.8 ± 5.7	82.0 ± 3.8
	24h interval	34.4 ± 3.1	34.8 ± 3.1	2.9 ± 1.7	45.0 ± 3.2	34.2 ± 3.6	56.2 ± 4.9	56 ± 10.0

Flow cytometric analysis of peripheral blood was performed 1 month after bone marrow transplantation. *WT* CD45.2^{+} donor bone marrow cells were transplanted into CD45.1^{+} recipient mice. 5 × 10^{6} bone marrow cells were transplanted following 2 fractions of total body irradiation (2 x 5.5 Gy) with or without thorax-shielding separated by a 4 h interval or 24 h interval. (n=3-4 per each group).

2. In regard to the design of the lead shielding, please provide anatomical landmarks to describe the proper size and position of the shield. This information would be helpful for investigators to properly execute experiments on mice of different sizes.

Response:

We agree that potential readers will be curious about how we designed the size and positioned the shield for different sizes of mice. Based on your comment, we have modified the revised manuscript as follows. (Line 167-169, 171-175, 177-180, 190-195)

2.2.2.3 Place lead shielding so that it covers regions that require protection. Localizing the lead shield to be consistent among cohorts may reduce some variation with regard to the size of the mice.

2.2.2.3.1 For thorax shielding, the lead shield can be prepared by measuring the length from the mouse's xiphisternum bone to the thymus and calculating the thickness that provide sufficient protection from the source of irradiation. Place the lead shielding so that the lower end aligns

with the xiphisternum bone. The upper end of the lead barrier will fit near the thymus. (**Figure 1D**)

2.2.2.3.2 For abdomen shielding, the lead shield can be prepared by measuring the length from the mouse's anus to the diaphragm and calculating the thickness that provide sufficient protection from the source of irradiation. Place the lead shielding so that the lower end aligns with the anus. The upper end of the lead shield will fit below the diaphragm. (**Figure 1E**)

2.2.3.2. For head shielding, the mouse can be placed in a conical restrainer which fits inside a lead shield. Once the mouse is inside the conical restrainer, slide the restrainer into the slot within the lead shield. (**Figure 1F**) The lead shield should completely cover the mouse's head and ears (~3.2 cm), leaving the rest of the mouse's body exposed for irradiation. The position of the restrainer inside the shield can be adjusted to fit different sized animals by sliding it further inside or outside the shield.

3. The authors share clear evidence of immune cell chimerism in the blood and heart. Some investigators may be interested in studying clonal hematopoiesis in hematopoietic organs (bone marrow and spleen). It would be informative to provide information regarding chimerism of WT and Tet2 KO engrafted cells in these hematopoietic compartments. Comparisons between preconditioning with chest shielding and adoptive transplantation into naïve mice would be ideal. This information would be valuable for readers to understand expected changes in hematopoiesis for each of these protocols.

Response:

Thank you for pointing this out. We understand that studies investigating clonal hematopoiesis in hematopoietic organs would be of great worth. Unfortunately, we do not have data comparing the chimerism of *WT* and *Tet2-deficient* cells between thorax-shielding BMT and adoptive BMT to non-conditioned mice with respect to the bone marrow and spleen.

However, our lab's previous published paper described the chimerism of *WT* and *Tet2*-deficient donor cells engrafted into B6.SJL-*Ptprc*^{a}*Prpc*^{b}/BoyJ (CD45.1) recipient mice 8 months after adoptive BMT in bone marrow, heart, liver, and kidney.[9] Thus, we have added the following paragraph to the discussion section. (Line 500-514)

"The expansion of *Tet2-deficient* cells has been observed not only in the peripheral blood but also in several other tissues, including bone marrow, liver, and kidney, with different dynamics of hematopoietic cell reconstitution[9]. For example, our lab's previous published paper described the bone marrow cell chimerism of *WT* and *Tet2-deficient* donor cells engrafted into CD45.1 recipient mice 8 months after adoptive BMT[9]. *Tet2-deficient* donor cells transplanted into CD45.1 recipient mice have shown a competitive advantage over immature lineage^{-}Sca1^{+}c-Kit^{+} (LSK) cells, short-term and long-term HSC cells, and multipotent progenitors (MPPs) compared to that of *WT* donor cells transplanted into the CD45.1 recipient mice. In addition, as *Tet2-deficien*t donor cell engrafted recipient mice, they develop an age-related cardiomyopathy

phenotype without exogenous factors causing cardiac dysfunction, thereby recapitulating the effect of clonal hematopoiesis in a manner similar to that of aging humans. This observation was accompanied with increased degree of chimerism in cardiac neutrophils and Ly6C^{hi} monocytes. Collectively, this adoptive BMT regimen can be applied to future studies that could expand our understanding of the association between cardiovascular disease development and clonal hematopoiesis on a more advanced level."

4. Please discuss in further detail the observation that adoptive transfer of WT bone marrow into naïve WT mice results in 5-10% chimerism that appears to increase over time. This seems high considering that donors should not have a competitive advantage over recipient cells in this experiment.

Response:

We thank the reviewer for their concern. As you mentioned, engrafted *WT* CD45.2^{+} donor cells increase over time, although this increase is much less than the increase produced by *Tet2-deficient* CD45.2^{+} cells. In our understanding, this observation derives from the confounding effects of using congenic mouse. Waterstrat et al., have shown that CD45.2^{+} (C57BL/6) HSPCs have an intrinsic competitive advantage over CD45.1^{+} (B6/SJL-Ptprc^{a} Pepc^{b}/BoyJ). In a competitive BMT assay, where C57BL/6 and B6/SJL-Ptprc^{a} Pepc^{b}/BoyJ donor cells were mixed 50:50 and transplanted into lethally irradiated C57BL/6 recipient mice, CD45.1^{+} HSPCs showed reduced homing efficiency, a decreased number of long-term hematopoietic stem cells (LT-HSCs), and a lower engraftment ratio. Although we are not sure whether this applies the same way in our adoptive BMT, CD45.2^{+} donor cells may possibly present a fitness advantage over CD45.1^{+} (B6/SJL-Ptprc^{a} Pepc^{b}/BoyJ) when a relatively high number of bone marrow cells is injected. To minimize these confounding effects, CD45.1^{STEM} (single targeted exon mutation) recipient mice that have shown functional equivalence to C57BL/6N mice can be considered. (Francois E. Mercier et al.,)

Ref)

1. Waterstrat, A., Liang, Y., Swiderski, C.F., Shelton, B.J., Van Zant, G. Congenic interval of CD45/Ly-5 congenic mice contains multiple genes that may influence hematopoietic stem cell engraftment. *Blood*. **115** (2), 408–417, doi: 10.1182/blood-2008-03-143370 (2010).
2. Mercier, F.E., Sykes, D.B., Scadden, D.T. Single targeted exon mutation creates a true congenic mouse for competitive hematopoietic stem cell transplantation: The C57BL/6-CD45.1STEM mouse. *Stem Cell Reports*. **6** (6), 985–992, doi: 10.1016/j.stemcr.2016.04.010 (2016).

5. Consider including a diagram demonstrating the 0.5ml within 1.5ml microcentrifuge tube setup for isolating bone marrow by centrifugation.

Response:

We thank the reviewer for their suggestion. The setup for the 0.5 mL and 1.5 mL microcentrifuge tube will be shown in the JoVE video to provide better understanding.

6. Please include flow cytometry gating schemes

Response:

We added the following statement to line 341-342.

"The flow cytometry gating strategies that were used are described previously by Wang et al.[9] in Supplementary Figure1."

Minor
*7. Line 324 shielded *region*?*

Response:

We have corrected this error. (Line 354-355) "Recipient mouse bone marrow cells in shielded regions are also likely to have contributed to peripheral blood reconstitution…"

Reviewer #2:

The protocol submitted by Eunbee Park et al. from the laboratory of Kenneth Walsh is of high relevance and has so far not being widely addressed in the field. Total body irradiation used for efficient transplantation and blood cell reconstitution by donor cells hampers accurate systemic studies on the impact of the transplanted cells on multiple organs due to a systemic damage. The authors here describe methods to either protect distinct parts of the recipients´ body by lead shielding or by preventing damaging conditioning regimes at all. They further compared the efficiency of blood cell reconstitution after lethal total body irradiation, partial shielding, and non-conditioning in peripheral blood and heart. The protocol is written in a very concise but easy-to-follow style, and many critical aspects of the protocol have been well discussed. This is a well-written, very useful and applicable description of various transplantation methods which will be of high interest to the field.

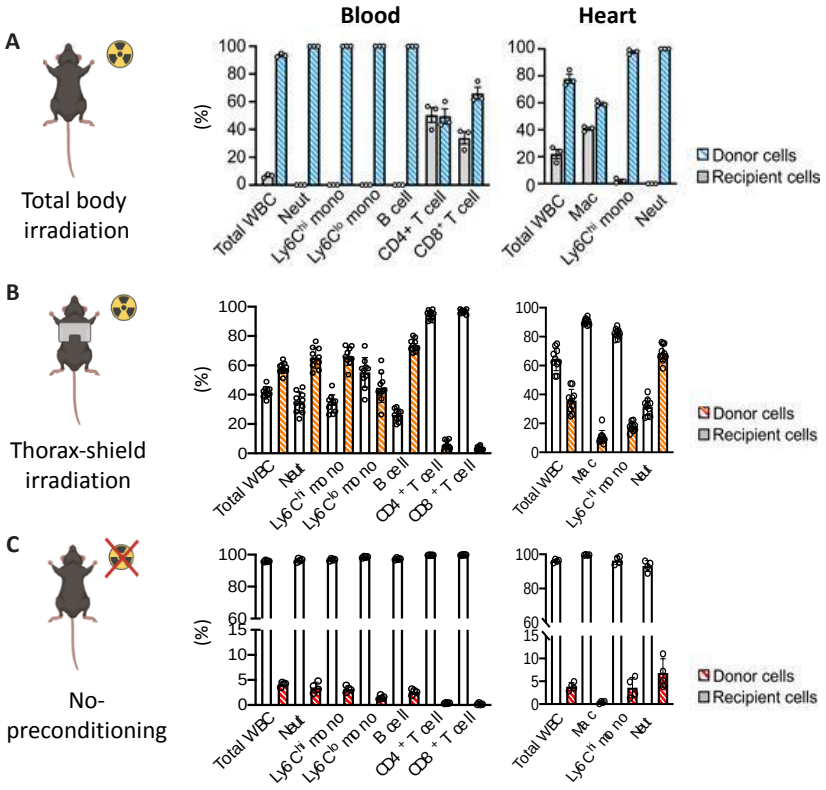
Response: We thank the reviewer for these positive comments.

- How do the authors explain the differences of the donor cell contribution in the heart after thorax shielding and non-conditioning transplantation (Figure 2B and C)? It seems that non-conditioning would allow more donor WBCs (in proportion to the blood cell chimerism) to engraft in the heart than when applying partial shielding with high irradiation. The percentage of donor cell chimerism is hard to see due to the scale of the axis in Fig2C. The authors may split the axis scale for donor and recipient %, to zoom into the donor chimerism.

Response:

In the context of adoptive BMT, the donor cell contributions in the blood and heart are comparable to those of non-conditioned recipient mice. To aid the reader's understanding, we have changed the y-axis scale in Fig2C. In this model, the donor cells' engraftment in both blood and heart is approximately 5% or less in each immune population 1-month post-transplantation.

Figure 2: Flow cytometric analysis of blood and heart using different methods of preconditioning.



- Along the same line, what happens in animals that have been transplanted after thorax shielding at a later time point (3 months after transplantation) concerning the contribution of myeloid cells in the heart? Do the authors have data on later time points as well? Will the resident recipient myeloid cells in the heart be replaced by newly produced donor cells?

Response:

Thank you for this question. Although we do not have data for a later time point post-thorax shielded BMT, we assume that cardiac myeloid cells will be replaced by newly produced donor cells as these recipient mice age. We say this because even in the steady state, without cardiac injury or inflammation, cardiac myeloid cells are substituted by infiltrating monocyte-derived macrophages with age. (Molawi et al and Wang et al)

Ref)

1. Molawi, K. et al. Progressive replacement of embryo-derived cardiac macrophages with age. Journal of Experimental Medicine. 211 (11), 2151–2158, doi: 10.1084/jem.20140639 (2014).
2. Wang, Y. *et al.* Tet2-mediated clonal hematopoiesis in nonconditioned mice accelerates age-associated cardiac dysfunction. *JCI Insight*. **5** (6), doi: 10.1172/jci.insight.135204 (2020).

- Do the authors have data on Tet2-deficient donor cell engraftment in the heart after 3-4 months using the non-conditioned regimen? This would be particularly important for studies on clonal hematopoiesis and cardiovascular insults (e.g. recovery after infarct) to discriminate between the local contributing effects of mutated myeloid cells in the heart vs. a systemic effect.

Response:

We did not include the data for analyzing the heart after 3-4 months *Tet2*-deficient donor cell adoptive BMT to non-conditioned mice in this manuscript. However, Wang et al. (2020) have shown an association between clonal hematopoiesis and cardiac tissue hematopoietic cell chimerism[9]. We have added the following paragraph to the discussion section. (Line 500-514)

"The expansion of *Tet2-deficient* cells has been observed not only in the peripheral blood but also in several other tissues, including bone marrow, liver, and kidney, with different dynamics of hematopoietic cell reconstitution[9]. For example, our lab's previous published paper described the bone marrow cell chimerism of *WT* and *Tet2-deficient* donor cells engrafted into CD45.1 recipient mice 8 months after adoptive BMT[9]. *Tet2-deficient* donor cells transplanted into CD45.1 recipient mice have shown a competitive advantage over immature lineage$^-$Sca1$^+$c-Kit$^+$ (LSK) cells, short-term and long-term HSC cells, and multipotent progenitors (MPPs) compared to that of *WT* donor cells transplanted into the CD45.1 recipient mice. In addition, as *Tet2-deficien*t donor cell engrafted recipient mice, they develop an age-related cardiomyopathy phenotype without exogenous factors causing cardiac dysfunction, thereby recapitulating the effect of clonal hematopoiesis in a manner similar to that of aging humans. This observation was accompanied with increased degree of chimerism in cardiac neutrophils and Ly6C^{hi} monocytes. Collectively, this adoptive BMT regimen can be applied to future studies that could expand our understanding of the association between cardiovascular disease development and clonal hematopoiesis on a more advanced level."

Ref)

1. Wang, Y. *et al.* Tet2-mediated clonal hematopoiesis in nonconditioned mice accelerates age-associated cardiac dysfunction. *JCI Insight*. **5** (6), doi: 10.1172/jci.insight.135204 (2020).

- Do you have confirmed HSC engraftment and self-renewal in non-conditioned adoptive transfer regimens by applying secondary transplantations? These experiments go beyond the scope of the study, but if you have confirmed it experimentally, mention it in the text (not necessarily showing the data).

Response:

This is an Interesting question. A previous study from another group (Moran-Crusio et al., 2011) has shown that *Tet2* deficiency can increase the replating ability in *in vitro* and loss of *Tet2,* leading to increased self-renewal ability using competitive bone marrow transplantation (*Tet2*$^{+/+}$ CD45.2^{+}: *Tet2*$^{-/-}$ CD45.2^{+} = 50: 50) into lethally irradiated recipient mice. Unfortunately, we have not performed experiments confirming the HSC engraftment and self-renewal by doing secondary adoptive BMT with non-conditioned mice. However, it would be interesting to try those experiments in the future and determine whether first transplanted *Tet2-deficient* donor cells retain their self-renewal capability or even enhance their self-renewal capacity in the secondary transplanted mice as shown by *in vitro* experiments.

Ref)

1. Moran-Crusio, K. *et al.* Tet2 Loss Leads to Increased Hematopoietic Stem Cell Self-Renewal and Myeloid Transformation. *Cancer Cell*. **20** (1), 11–24, doi: 10.1016/j.ccr.2011.06.001 (2011).

- Please briefly include the information about the non-conditioned transplantation of Tet2-deficient cells in the main text (page 8, lines 332f) as you have described it in the legend of figure 3.

Response:

To address this concern, we added the following information. (Line 361-371)

"To illustrate how the adoptive BMT model can be applied to the study of the CH model, CD45.2^{+} donor bone marrow cells (*WT* or *Tet2*$^{-/-}$) were transplanted into CD45.1^{+} recipient mice. Recipients without conditioning were transplanted with 5 × 10^{6} bone marrow cells each day for 3 consecutive days (for a total of 1.5 x 10^{7}, n=5-6 per each group). Flow cytometric analysis of peripheral blood was performed 4, 8, 12, and 16 weeks post-transplantation. The *Tet2-deficient* donor cells conferred a competitive advantage and gradually expand over time; WBCs, monocytes, Ly6C^{hi} monocytes, neutrophils, T cells and B cells increased significantly over time. Compared to the Tet2-deficient donor cells engrafted into recipient mice, recipient mice engrafted with WT donor cells showed less significant clonal expansion of donor cells. (Figure 3) Consistent with the clinical paradigm of clonal hematopoiesis, the expansion of Tet2-deficient cells does not impact the absolute numbers of the various blood cell types[9]. (data not shown).

- Page 8, line 335: "..the expansion of *Tet2*-deficient CELLS does… cells is missing.

We apologize for this error. We have now corrected this. (Line 370-371)

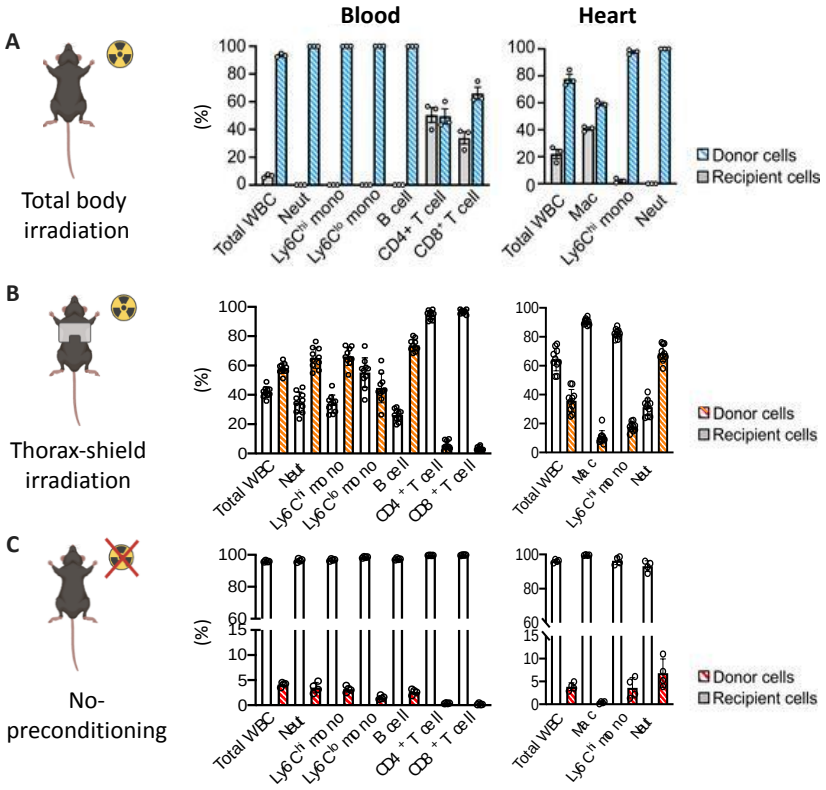
Response:

- The y-axis legend in Figure 2 is missing (%)

Response:

Corrected.

Figure 2: Flow cytometric analysis of blood and heart using different methods of preconditioning.



Reviewer #3:

The manuscript by Park et al., describes three bone marrow transplant methods to investigate how clonal hematopoiesis contributes to cardiovascular disease. The authors provide step by step protocols how to irradiate mice and perform bone marrow transplant and show the strength and the limitation of each procedure. The partially shielded irradiation and non-conditioned adoptive transplant show physiological advantages that are similar to in human cardiovascular disease compared to total body irradiation. Employing this approach in cardiovascular disease models led to successful demonstration of the effect of clonal hematopoiesis in cardiac dysfunction.

Response: We thank the reviewer for these positive comments.

Major Concerns:
-In Protocol section 4, there was not a red blood cell lysis step which is a standard procedure to process bone marrow samples. The authors' prior study included this step in the method section. If red blood cell lysis buffer is not used, the authors should provide their rationales avoiding red blood cell lysis step.

Response:

In our previous study, red blood cell (RBC) lysis was performed on bone marrow cells for the purpose of flow cytometry analysis. However, for transplantation, we do not routinely perform RBC lysis. The steps involved in red blood cell lysing can cause some loss of leukocytes and interfere with the integrity and number of hematopoietic cells. In order to transplant intact bone marrow cells, we do not perform RBC lysis.

-Authors described adoptive transfer of bone marrow to non-conditioned mice would be better suited in studying clonal hematopoiesis in cardiovascular disease but there no data provided in Fig 3 that showed engraftment and clonal expansion of Tet2-deficient cells. Authors should provide figures showing successful engraftment/localization and clonal expansion of Tet2-deficiant cells using adoptive transfer of bone marrow to non-conditioned mice in heart as well since Fig 2 is generated with WT background mice.

Response:

The reader is referred to Figure 3 that documents the time-dependent expansion of mutant cells in various peripheral blood cell types and our paper of Wang et al.[9] that documents the engraftment of donor cells to hematopoietic stem cells, long-term and short-term HSC, multipotent progenitors following adoptive BMT.

In addition, we did not include the data for analyzing the heart at later time points for Tet2-deficient donor cell adoptive BMT to non-conditioned mice in this manuscript. However, Wang et al. (2020) have shown an association between clonal hematopoiesis and cardiac tissue hematopoietic cell chimerism. We have added the following paragraph to the discussion section. (Line 500-514)

"The expansion of *Tet2-deficient* cells has been observed not only in the peripheral blood but also in several other tissues, including bone marrow, liver, and kidney, with different dynamics of hematopoietic cell reconstitution[9]. For example, our lab's previous published paper described the bone marrow cell chimerism of *WT* and *Tet2-deficient* donor cells engrafted into CD45.1 recipient mice 8 months after adoptive BMT[9]. *Tet2-deficient* donor cells transplanted into CD45.1 recipient mice have shown a competitive advantage over immature lineage$^-$Sca1$^+$c-Kit$^+$ (LSK) cells, short-term and long-term HSC cells, and multipotent progenitors (MPPs) compared to that of *WT* donor cells transplanted into the CD45.1 recipient mice. In addition, as *Tet2-deficien*t donor cell engrafted recipient mice, they develop an age-related cardiomyopathy phenotype without exogenous factors causing cardiac dysfunction, thereby recapitulating the effect of clonal hematopoiesis in a manner similar to that of aging humans. This observation was accompanied with increased degree of chimerism in cardiac neutrophils and Ly6C^{hi} monocytes. Collectively, this adoptive BMT regimen can be applied to future studies that could expand our understanding of the association between cardiovascular disease development and clonal hematopoiesis on a more advanced level."

Minor Concerns:
-In Protocol section 3, authors used 2 femurs, 2 tibias, and 2 humeri. It would be helpful to add a note that it is possible to get more bone marrow cells from hip bones (you can use the same procedure as for femur, tibia, and humeri) and spines (using mortar and pestle).

Response:

Added. (line 241-243)

Note: Hip bones and spine bones can be collected in addition to femur, tibia and humerus. To collect spine bones, mortar and pestle can be used to crush bones into pieces and harvest the bone marrow cells.

-In Protocol section 5, it would be helpful to note alternative routes of cell transfer. Tail vain intravenous injection and intrafemural injection can be mentioned.

Response:

Added. (line 313-315)

Note: As an alternative, delivery of the donor cells can be performed by tail vein intravenous injection or femoral intramedullary injection, with a maximum volume of 0.2 mL and 25 µL, respectively.

-In the Representative Results, at the last paragraph, the authors reference that the expansion of Tet2-deficiant does not impact the absolute numbers of the various blood cell types. In fact, there are significant changes in absolute number of cell population in peripheral blood in the referenced manuscript. To clarify this statement, the authors should specify "in cardiac immune cells".

Response:

The absolute numbers of the various immune cell populations in both cardiac and peripheral blood does not display statistically significant differences in these models. While the absolute number of mutant immune cells from the *Tet2-deficient* donor bone marrow cells increases over time, the total absolute number, which is the combined absolute number of recipient and donor cells, does not change. Thus, these models are consistent with the clinical paradigm of clonal hematopoiesis in which the mutant clone expands, but the absolute number of immune cells does not change.

Reviewer #4:

Park et al clearly describe commonly used and less commonly used BMT techniques to assess re-population potential of murine bone marrow from genotypes relevant to studying clonal hematopoiesis (CH), and their potential effects (in terms of immune cell presence) on a few specific organs. As studies of CH emerge that focus on specific organ systems, this protocol provides ideas and data on which method variant may be most beneficial for a specific site or research question. The experimental steps are described in sufficient detail, and the figures provided are practical.

Response: We thank the reviewer for these positive comments.

Major Concerns: None

Minor Concerns:
Lines 124 & 488: "Diet gel" is an incorrect term, as it is a specific product (e.g. "DietGel Recovery, DietGel Boost"). "Nutritional/hydration gel" is a more generic term for this. Please use the specific product name or the general product descriptor name.

Response:

Corrected. In all cases, the term “Diet gel” was changed to “Nutritional/hydration gel”. (Line 123, 593)

135-6: The correct term for a radiation dose to be "split into" smaller doses is "fractionated". Perhaps using "recipient mice receive a total lethal radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 4-24 h interval" will be most accurate and broadly understandable by readers. Similarly on line 138 & 182 etc., a dose would be a "fraction".

Response:

Thank you for your suggestion. We corrected the sentence and changed the term “dose” to “fraction” in the manuscript. (Line 138, 141, 199, 202, 337, 344, 351, 402, 403, 427, 563, 564, 567, 570)

184: Or under red heat lamps (or if not, why not)

Response:

The sentence has been modified as follows: (Line 202-206)

2.2.5. After each irradiation fraction, place the cages with anesthetized mice on heated mats or under red heat lamps to prevent hypothermia and aid in the recovery from anesthesia.

Note: Caution must be taken to not overheat the anesthetized mouse when using a lamp since they cannot escape the heat.

229: Additional note authors should consider adding: Instead of steps 4.1-4.4, bone marrow cell isolation may also be performed by flushing out the marrow by inserting a 23 G needle and syringe at one of the cut ends of the bones, or by crushing bones with a mortar and pestle.

Response:

We added the following statement (line 241-243) according to your suggestion.

"Note: Hip bones and spine bones can be collected in addition to femur, tibia and humerus. To collect spine bones, mortar and pestle can be used to crush bones into pieces and harvest the bone marrow cells."

Section 2.2: Please give specific make and model of the Cesium and X-ray irradiators

Response:

We added to the material section.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.5ml microcentrifuge	Fisher Scientific	05-408-121	general supply
1.5ml microcentrifuge	Fisher Scientific	05-408-129	general supply
1/2 cc LO-DOSE INSULIN SYRINGE	EXELINT	26028	general supply
Absolute Ethanol (200 prfof)	Fisher chemical	200559	general supply
BD 1mL Tuberculin Syringes 25G 5/8 Inch Needle	Becton Dickinson	309626	general supply
BD PrecisionGlide Needle 18G (1.22mm X 25mm)	Becton Dickinson	395195	general supply
Cesium-137 irradiator	J.L. Shepherd	Mark I	equipment
DietGel 76A	Clear H2O	70-01-5022	general supply
Falcon 100 mm TC-Treated Cell Culture Dish	Life Sciences	353003	general supply
Falcon 50 mL Conical Centrifuge Tubes	Fisher Scientific	352098	general supply
Fisherbrand sterile cell strainers, 70 µm	Fisher Scientific	22363548	general supply
Graefe Forceps	Fine Science Tools	11051-10	general supply
Hardened Fine Scissors	Fine Science Tools	14090-09	general supply
Isothesia (Isoflurane) solution	Henry Schein	29404	Solution
Ketamine	Zoetis	043-304	injection
Kimwipes Delicate Task Wipers	Kimtech Science	KCC34155	general supply
PBS pH7.4 (1X)	Gibco	10010023	Solution
RadDisk – Rodent Irradiator Disk	Braintree Scientific	IRD-P M	general supply
RPMI Medium 1640 (1X)	Gibco	11875-093	Medium
Sulfamethoxazole and Trimethoprim	TEVA	0703-9526-01	injection
Xylazine	Akorn	139-236	injection
X-ray irradiator	Rad source	RS-2000	equipment

Section 2.2 would benefit from a diagram (perhaps an addition to Figure 1) for the TBI and partial shielding set-ups. Visualizing the orientation of the mouse bodies, the beam (and the beam's spread/scatter over a specific radius), the flat plane of the irradiator, and the factors that affect the calculations behind the radiation dose (e.g. distance from source, radius) would clarify what is hard to describe in text, and would give context to the statements about limitations on the number of mice that can be irradiated at a time.

Response:

Thank you for your comment. We have modified Figure 1 and its legend as below. (line 377-393)

Figure 1: Images showing various preconditioning set ups

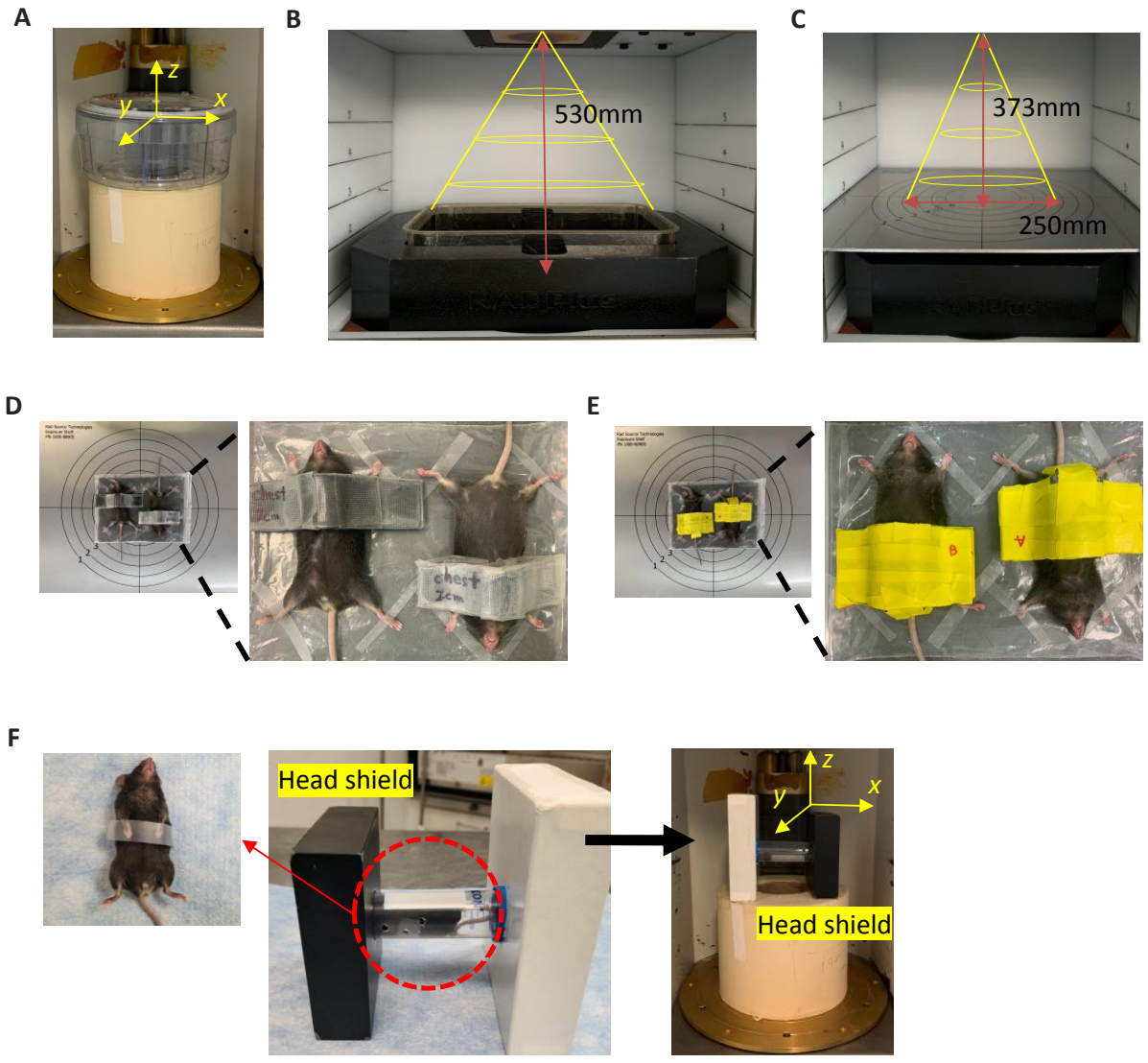


Figure 1: Images showing various preconditioning setups.

(A) Pie-cage total body irradiation setup using gamma-ray (Cesium-137): The radiation beam comes from the back of the irradiator in the y-axis direction (horizontal radiation). **(B)** Mouse cage total body irradiation setup using X-ray: The mouse cage is placed in the reflective chamber. The radiation beam comes from the top of the irradiator in the shape of a cone (vertical radiation). The distance from the radiation source to the cage is 530mm. **(C)** Adjustable tray in X-ray irradiator: This setup is used for partially shielded irradiation using X-ray. The radiation beam comes from the top of the irradiator in the shape of a cone (vertical radiation). The distance from the radiation source to the tray is 373mm, and the radius is 250mm. **(D)** Thorax-shielding: Anesthetized mice are placed on a tray. The mice are placed inverted to each other in supine positions with arms and legs fully extended. The lower end of the lead-shield is aligned with the xiphisternum bone and the upper end with the thymus. **(E)** Abdominal-shielding: Anesthetized mice are placed as in the thorax-shielding set-up with the lower end of the lead-shield aligned with the anus and the upper end below the diaphragm. **(F)** Head-shielding irradiation setup using gamma-ray (Cesium-137): The anesthetized mouse's forepaws are taped down and the mouse is placed in a conical restrainer. The black lead-shield (marked) covers the mouse's head and ears. The radiation beam comes from the back of the irradiator in the y-axis direction (horizontal radiation).

277: Suggest alternative injection sites and volumes

Response:

Added. (line 313-315)

Note: As an alternative, delivery of the donor cells can be performed by tail vein intravenous injection and femoral intramedullary injection, with a maximum volume of 0.2 mL and 25 µL, respectively.

312: 2x 5.5Gy fractions, total dose of 11 Gy

Response:

We have modified the previous sentences as follows: (line 343-344, 349-351)

The total body irradiation (TBI) treated group received 5×10^6 bone marrow cells following a total lethal radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 4 h interval.

In the partially shielded irradiation group, recipient mice were irradiated with a thorax shield and transplanted with 5×10^6 bone marrow cells following a total radiation dose of 11 Gy in two 5.5 Gy fractions separated by a 4 h interval.

336: Include statement "(data not shown)" since it's not part of Fig 3

Response:

Accepted. (line 370-371) Consistent with the clinical paradigm of clonal hematopoiesis, the expansion of *Tet2*-deficient cells does not impact the absolute numbers of the various blood cell types. (Data not shown)

354-5, 365: Clarify that 5 × 10^6 bone marrow cells were administered each day for 3 consecutive days (for a total of 1.5 x 10^7) as mentioned earlier in protocol

Response:

Modified. (line 363) Recipients without conditioning were transplanted with 5×10^6 bone marrow cells each day for 3 consecutive days. (for a total of 1.5×10^7, n=4)

357-358: Cell population characteristics are not clear due to possibly inconsistent use of ";" (this is clear: "Neutrophils as Ly6G+; ", but this is not clear: "Ly6Chigh monocytes as CD115+; Ly6G- and Ly6C+;")

Response:

Based on your comments, we have modified the previous sentence as below: (line 406-409)

WBCs are defined as $CD45^+$; Neutrophils as $Ly6G^+$; $Ly6C^{hi}$ monocytes as $CD115^+$, $Ly6G^-$ and $Ly6C^+$; $Ly6C^{lo}$ monocytes as $CD115^+$, $Ly6G^-$, and $Ly6C^-$; B cells as $CD45R^+$; and T cells as $CD3e^+$.

Discussion:
378: Authors could suggest other sites of interest for shielding

Response:

We are not sure whether it is appropriate to suggest other sites of interest for shielding, specifically at line 378. Line 378 summarizes the overall methods of BMT that we introduced.

We instead added the following sentence in the paragraph under the subtitle “Design of lead shielding”. (line 550-552)

Based on the equipment and its parameters, researchers can design their own lead shields for their sites of interest. Here, we introduced thorax, abdominal, and head shielding; however, other sites such as limb or flank can be considered for shielding as well.

Importantly, the authors comment on which method seems to most faithfully recapitulate what is expected in CH in humans.

Response:

We have modified the original sentence as below: (line 490-492)

This adoptive BMT without pre-conditioning method minimally affects the ongoing naïve hematopoiesis, most faithfully recapitulating CH observed in humans.

Authors should add a statement (in Discussion / Additional comments) that the choice of radiation source (Cesium-137 vs. X-ray) may itself influence the output of the reconstituted bone marrow (see e.g. PMID 26141441)

Response:

We appreciate your detailed comment. We have added the following: (line 552-555)

In addition, while both radiation sources (Cesium-137 and X-ray) are suitable for bone marrow ablation and successful engraftment, variability in the reconstitution of lymphoid and myeloid cell populations has been observed between Cesium-137 and X-ray irradiation sources[30]. Thus, researchers should take into account the disparate physiological responses to the radiation source for use in studies.

492: Mice should be monitored carefully for what? (e.g. Weight loss/cachexia, behaviour change?)

Response:

We appreciate your detailed comment. We have added the following: (line 594-597)

Cages should also be replaced frequently to reduce the risk of fecal bacteria contamination, animals should be handled in a proper animal transfer station, and the recipient mice should be monitored carefully for weight loss and any signs of distress or pain.

Figure 2: Add Y axis labels

Response:

Added.

Given the inclusion and description of brain shielding for BMT (the stroke aspect of cardiovascular disease), the manuscript would be more complete with data from that method as well (rather than data from only the blood and heart in Figure 2). The title does suggest that the

focus of this protocol is the "Role of Immune Cells in Cardiovascular Disease" and the abstract brings up both heart disease and stroke as important associations with CH.

Response:

This is ongoing research, so we are planning to include the brain shield-BMT data in our future full research article.

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		Expected publication date	2020-08-01
		Expected size (number of pages)	12
Author	Eunbee Park et al.	**Standard identifier**	N/A
Publication	Journal of Visualized Experiments		
Publisher	MyJoVE Corporation		

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