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TITLE:

Bone Marrow Transplantation Platform to Investigate the Role of Dendritic Cells in Graft-versus-Host Disease

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SUMMARY:

Graft-versus-host disease is a major complication after allogeneic bone marrow transplantation. Dendritic cells play a critical role in the pathogenesis of graft-versus-host disease. The current article describes a novel bone marrow transplantation platform to investigate the role of dendritic cells in the development of graft-versus-host disease and the graft-versus-leukemia effect.

ABSTRACT:

Allogeneic bone marrow transplantation (BMT) is an effective therapy for hematological malignancies due to the graft-versus-leukemia (GVL) effect to eradicate tumors. However, its application is limited by the development of graft-versus-host disease (GVHD), a major complication of BMT. GVHD is evoked when T-cells in the donor grafts recognize alloantigen expressed by recipient cells and mount unwanted immunological attacks against recipient

healthy tissues. Thus, traditional therapies are designed to suppress donor T-cell alloreactivity. However, these approaches substantially impair the GVL effect so that the recipient's survival is not improved. Understanding the effects of therapeutic approaches on BMT, GVL, and GVHD, is thus essential. Due to the antigen-presenting and cytokine-secreting capacities to stimulate donor T-cells, recipient dendritic cells (DCs) play a significant role in the induction of GVHD. Therefore, targeting recipient DCs becomes a potential approach for controlling GVHD. This work provides a description of a novel BMT platform to investigate how host DCs regulate GVH and GVL responses after transplantation. Also presented is an effective BMT model to study the biology of GVHD and GVL after transplantation.

INTRODUCTION:

Allogeneic hematopoietic stem cell transplantation (BMT) is an effective therapy to treat hematological malignancies[1,2] through the graft-versus-leukemia (GVL) effect[3]. However, donor lymphocytes always mount unwanted immunological attacks against recipient tissues, a process called graft-versus-host disease (GVHD)[4].

Murine models of GVHD are an effective tool to study the biology of GVHD and the GVL response[5]. Mice are a cost-effective research animal model. They are small and efficiently dosed with molecules and biologics at early phases of development[6]. Mice are ideal research animals for genetic manipulation studies because they are genetically well defined, which is ideal for studying biological pathways and mechanisms[6]. Several mouse major histocompatibility complex (MHC) MHC-mismatched models of GVHD have been well established, such as C57BL/6 ($H2^b$) to BALB/c ($H2^d$) and FVB ($H2^q$)→C57BL/6 ($H2^b$)[5,7]. These are particularly valuable models to determine the role of individual cell types, genes, and factors that affect GVHD. Transplantation from C57/BL/6 ($H2^b$) parental donors to recipients with mutations in MHC I (B6.C-$H2^{bm1}$) and/or MHC II (B6.C-$H2b^{m12}$) revealed that a mismatch in both MHC class I and class II is an important requirement for the development of acute GVHD. This suggests that both $CD4^+$ and $CD8^+$ T-cells are required for disease development[7,8]. GVHD is also involved in an inflammatory cascade known as the 'pro-inflammatory cytokine storm'[9]. The most common conditioning method in murine models is total body irradiation (TBI) by X-ray or ^{137}Cs. This leads to the recipient's bone marrow ablation, thereby allowing donor stem cell engraftment and preventing rejection of the graft. This is done by limiting the proliferation of recipient T-cells in response to donor cells. Additionally, genetic disparities play an important role in disease induction, which also depends on minor MHC-mismatch[10]. Therefore, myeloablative irradiation dose varies in different mouse strains (e.g., BALB/c→ C57BL/6).

Activation of donor T-cells by host antigen presenting cells (APCs) is essential for GVHD development. Among the APCs, dendritic cells (DCs) are the most potent. They are inheritably capable of inducing GVHD due to their superior antigen uptake, expression of T-cell co-stimulatory molecules, and production of pro-inflammatory cytokines that polarize T-cells into pathogenic subsets. Recipient DCs are critical for facilitating T-cell priming and GVHD induction after transplantation[11,12]. Accordingly, DCs have become interesting targets in the treatment of GVHD[12].

TBI is required to enhance the donor cell engraftment. Due to the TBI effect, recipient DCs are activated and survive for a short time after the transplantation[12]. Despite major advancements in the usage of bioluminescence or fluorescence, establishing an effective model to study the role of recipient DCs in GVHD is still challenging.

Because donor T-cells are the driving force for GVL activity, treatment strategies using immunosuppressive drugs such as steroids to suppress T-cell alloreactivity often cause tumor relapse or infection[13]. Therefore, targeting recipient DCs may provide an alternative approach to treat GVHD while preserving the GVL effect and avoiding infection.

In brief, the current study provides a platform to understand how different types of signaling in recipient DCs regulates GVHD development and the GVL effect after BMT.

PROTOCOL:

The experimental procedures were approved by the Institutional Animal Care and Use Committee of University of Central Florida.

1. GVHD induction

NOTE: Allogeneic bone marrow (BM) cell transplantation (step 1.2) is performed within 24 h after irradiation. All procedures described below are performed in a sterile environment. Perform the procedure in a tissue culture hood and use filtered reagents.

1.1. Day 0: Prepare the recipient mice.

1.1.1. Use female wild type (WT) mice on a BALB/c background ($CD45.2^{+}H2k^{d+}$), 10–12 weeks old as recipients.

1.1.2. Ear-tag and weigh the recipients before TBI. Then, place up to 11 mice in the irradiation chamber and keep it in the irradiator. Irradiate at a single dose of 700 cGy for 10–15 min.

1.1.3. Return irradiated animals to the cages and house them in pathogen-free facilities before the transplant.

NOTE: The minimal body weight of the recipients should be around 20 g. Use this weight as the reference to calculate the body weight loss during the experimental period.

1.2. Day 1: Prepare T-cell depleted bone marrow (TCD-BM) from the donor mice.

1.2.1. Euthanize CD45.1/Ly5.1 C57BL/6 mice by CO_2 asphyxiation and wait for 2–3 min until they are unconscious. Perform cervical dislocation as a secondary euthanasia if necessary.

1.2.2. Put each mouse on a clean working board. Sanitize the fur and the skin with 70%

isopropanol.

1.2.3. Collect the tibia and femur from both legs using forceps and scissors. Put them in ice-cold RPMI containing 1% FCS and 100 U/mL penicillin/streptomycin and 2mM L-glutamine (1% RPMI) in 50 mL conical tubes. Clean the femur and the tibia bones thoroughly by removing all the muscle tissues using forceps and scissors. Transfer the femur and the tibia from the 50 mL conical tubes to a 92 mm diameter Petri dish containing 1% RPMI.

1.2.4. Using scissors, cut the ends of the femur or tibia. Fill a 50 mL tube with 25 mL of 1% RPMI 1640 medium. Use this to fill a 3 mL syringe attached to a 26 G needle with 3 mL of 1% RPMI. Insert this syringe into the bone and push the plunger to flush the bone marrow (BM) out of the cavity into the collection tube with 1% RPMI (~3 mL/bone).

1.2.5. Repeat step 1.2.4 for all the remaining tibia and femur bones from other donor mice. Keep all the collection tubes on ice.

1.2.6. Make the single cell suspension by flushing the bone marrow pieces through a 75 µm mesh cell strainer. Collect the single cell suspension in a 50 mL tube.

1.2.7. Centrifuge tubes at 800 x *g* for 5 min at 4 ˚C. Aspirate and discard the supernatant. Resuspend the pellet in PBS buffer containing 0.5% bovine serum albumin (BSA) at 20 x 10^6 cells/mL. Save an aliquot of 2 x 10^6 cells for purity staining.

1.2.8. Add Thy 1 antibody at 0.05 µg/10^6 cells[14] and incubate for 30 min at 4 ˚C. Wash once with 25 mL ice-cold PBS. Resuspend at 20 x 10^6/mL in 0.5% BSA/10% young rabbit complement/2% DNase (10,000 U/mL in sterile H_2O). Incubate for 45 min at 37 °C and wash 2x as before.

NOTE: T-cells are depleted using a mAb specific for Thy1, a protein expressed by all T-cells, but not other leukocytes.

1.2.9. Resuspend the cells in 20 mL of PBS buffer containing 0.5% BSA. Count the bone marrow cells in 1% acetic acid using a hemocytometer. Keep an aliquot of 2 x 10^6 cells for a purity check by flow cytometry after cell purification.

NOTE: One donor mouse normally generates about 25 x 10^6 TCD-BM.

1.2.10. Use flow cytometry to confirm a successful T-cell depletion. Stain 1 x 10^6 cells, preserved from steps 1.2.7 (before T-cell depletion) and 1.2.9 (TCD-BM after T-cell depletion) with the following antibodies: α-CD3 (17A2), α-CD4 (GK1.5), and α-CD8α (53-6.7).

1.3. Day 1: Prepare T-cells from the donor mice

1.3.1. Use CD45.2$^+$H2k^{b+}C57BL/6 wild type (WT) mice as donors for T-cells.

1.3.2. Euthanize C57BL/6 mice by carbon dioxide (CO_2) asphyxiation as described in 1.2.1.

1.3.3. Clean the fur and skin of the mouse thoroughly with 70% ethanol. Excise spleens and lymph nodes and separate them into a single cell suspension of splenocytes using a syringe plunger and 40 µm mesh strainers. Wash the strainer and syringe plunger with 1% RPMI (1% FBS containing RPMI media) to collect all splenocytes.

1.3.4. Centrifuge the cell suspension at 800 x *g* for 5 min at 4 °C. Add 5 mL of ACK lysing buffer (1 mM Na_2EDTA, 10 mM $KHCO_3$, 144 mM NH_4Cl, pH 7.2) after discarding the supernatant. Incubate the cell suspension for 5 min at room temperature.

NOTE: ACK lysis buffer is used for lysing red blood cells.

1.3.5. Add 5 mL of 1% RPMI to stop lysis. Centrifuge at 800 x *g* for 5 min at 4 °C. Discard the supernatant.

1.3.6. Prepare ice-cold magnetic-activated cell sorting (MACS) buffer (0.5% BSA, 2 mM EDTA in PBS, pH 7.2). Degas the buffer before use. Resuspend the cell pellets in 5 mL of MACS buffer.

1.3.7. Count the splenocytes and check for the live and dead cells using a hemocytometer and 1% trypan blue. Save an aliquot of 2 x 10^6 cells to evaluate the purification yield with flow cytometry analysis.

1.3.8. Resuspend the splenocytes at the concentration of 200 x 10^6/mL in MACS buffer. Add 0.03 µL of biotin-anti-mouse-Ter-119, 0.03 µL of biotin anti-mouse-CD11b, 0.03 µL of biotin anti-mouse -CD45R, and 0.03 µL of biotin anti-mouse -DX5 per 10^6 cells. Incubate for 15 min at 4 °C.

NOTE: Biotin-anti-mouse-Ter-119, biotin anti-mouse-CD11b, biotin anti-mouse -CD45R, and biotin anti-mouse -DX5 were used to react with erythroid, granulocytes, B-cells, and NK cells respectively. Therefore, these cell subsets are depleted in following step[15].

1.3.9. Add 10 mL of ice-cold MACS buffer to the cell suspension. Centrifuge at 800 x *g* for 5 min at 4 °C. Discard the supernatant.

1.3.10. Resuspend the cell pellets in the MACS buffer at a concentration of 100 x 10^6/mL. Add anti-biotin microbeads (0.22 µL/10^6 cells) to the splenocyte suspension. Mix well and incubate for an additional 15 min at 4 °C. Wash the cell suspension once with 10 mL of ice-cold MACS buffer. Centrifuge at 800 x *g* for 5 min at 4 °C and discard the supernatant.

1.3.11. Put a magnetic separating column in the magnetic field. Rinse the column with 3 mL of MACS buffer. Drop the cell suspension onto the column. Collect the flow-through consisting of unbound, enriched T-cells, in a new 15 mL conical tube. Wash the MS column with 3 mL of ice-cold MACS buffer.

NOTE: Ensure that the column is empty prior to performing the washing steps.

1.3.12. Centrifuge the cell suspension at 800 x *g* for 5 min at 4 °C. Resuspend the cell pellet in 5 mL of MACS buffer.

1.3.13. Count the cells in 1% trypan blue using a hemocytometer. Save an aliquot of 2 x 10^6 cells for a purity check by flow cytometry.

NOTE: The average yield of splenic T-cells isolated by this method is ~20−25 x 10^6 cells per mouse.

1.3.14. Confirm the yield of T-cell enrichment by flow cytometry. Stain 1 x 10^6 cells, preserved from steps 1.3.7 (before T-cell depletion) and 1.3.13 (TCD-BM after T-cell depletion), with the following antibodies: α-CD3 (17A2), α-CD4 (GK1.5), and α-CD8α (53-6.7).

1.4. Day 1: Inject irradiated mice with donor T-cells and TCD-BM.

1.4.1. Wash the TCD-BM and T-cells 2x with PBS (800 x *g* for 5 min at 4 °C). Resuspend the cells in ice-cold PBS for injection. Adjust the cell concentration to 20 x 10^6/mL for TCD BM and 4 x 10^6/mL for T-cells.

1.4.2. Heat the animal using a heating lamp to increase the visibility of the bilateral tail veins. If necessary, place the mouse in a restrainer.

1.4.3. Clean the surface of the tail using 70% isopropanol. Inject TCD-BM (5 x 10^6 cells/mouse) with or without T-cells (0.75 x 10^6 cells/mouse). Be careful not to introduce any air into the syringe.

1.4.4. Remove the needle and apply an antiseptic swab directly to the injection site 5−10 s to stop any bleeding.

1.5. Assess GVHD on days 2−80.

1.5.1. Keep track of the animal survival. Monitor the clinical signs of GVHD adapted from the scoring system established previously by Cook et al.[16] and the body weight of the recipient mice 2x per week. Use the body weight determined prior to the TBI to calculate the body weight loss.

1.5.2. Weigh each mouse individually. Score the weight loss as follows: grade 0 = less than 10%; grade 1 = 10%−20%; grade 2 = more than 20%.

1.5.3. Score the posture sign of the recipients: grade 0 = no hunch; grade 0.5 = slight hunch but straightens when walking; grade 1 = animal stays hunched when walking; grade 1.5 = animal does not straighten out; grade 2.0 = animal stand on rear toes.

1.5.4. Score the mobility sign of the recipients: grade 0 = very active; grade 0.5 = slower than

naive mice; grade 1.0 = moves only when poked; grade 1.5 = moves slightly when poked; grade 2.0 = does not move when poked.

1.5.5. Score the skin of the recipients: grade 0 = no abrasions, lesions, or scaling; grade 0.5 = redness in one specific area; grade 1 = abrasion in one area or mild abrasion in two areas; grade 1.5 = serious abrasions in two or more areas; grade 2.0 = severe abrasions, cracking skin, dried blood.

1.5.6. Score the fur of the recipients: grade 0 = no abnormal signs; grade 0.5 = ridging on the side of belly or nape of neck, grade 1.0 = ridging across or the side of belly and neck; grade 1.5 = unkempt matted and ruffed fur; grade 2.0 = badly matted fur on belly and back.

1.5.7. Score the diarrhea of the recipients: grade 0 = no diarrhea; grade 0.5 = slight and soft stool; grade 1.0 = mild (yellow stool); grade 1.5 = moderate (yellow stool with a little blood); grade 2 = severe (light yellow and bloody stool, "dried cake stools" appear at the anal area).

2. Cotransplantation model

2.1. Generate bone marrow-derived dendritic cells (BM-DCs).

2.1.1. Isolate bone marrow from the femurs and tibias of WT or factor B (fB) $^{-/-}$ mice on the B6 background as described in steps 1.2.3–1.2.4. Spin the cells at 800 x *g* for 5 min.

2.1.2. Resuspend the pellet in 5 mL of ACK lysing buffer for red blood cell lysis. Incubate the cell suspension for 5 min on ice. Add 10 mL of 1% RPMI to the cell suspension to stop the lysis and centrifuge at 800 x *g* for 5 min at 4 ˚C. Discard the supernatant.

2.1.3. Resuspend the cell pellets in 10 mL of culture media (RPMI 1460 containing 10% FBS, 100 U/mL of penicillin/streptomycin, 2mM l-glutamine, and 50 mM β-mercaptoethanol) and adjust the volume to meet the final concentration of 2 x 10^6 cells/mL.

2.1.4. Add 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) to the cell suspension. Culture the bone marrow cells in 100 x 15 mm Petri dishes at 37 ˚C in 5% CO_2 for 6 days.

2.1.5. Replace half of the ongoing culture media (about 5 mL) with fresh media containing 40 ng/mL GM-CSF on day 3.

2.1.6. Prewarm the culture media in a water bath at 37 ˚C. Collect about 5 mL of the media from the bone marrow culture dishes. Centrifuge at 800 x *g* for 5 min at 4 ˚C. Discard the supernatant. Resuspend the cell pellet in 5 mL culture media containing 40 ng/mL GM-CSF.

2.1.7. Add 25 µg/mL lipopolysaccharide (LPS) to the media on day 6 of the culture to mature the BM-DCs. Save an aliquot of 2 x 10^6 cells to determine the DC differentiation efficacy by flow

cytometry.

2.1.8. Collect matured BM-DCs: Use cell lifters to softly scrape DCs from the Petri dishes. Collect all the cell suspensions in 50 mL conical tubes. Centrifuge at 800 x *g*, for 5 min at 4 ˚C. Discard the supernatant. Wash the cell pellets 3x with 50 mL of ice-cold PBS. Save about 2 x 10^6 BM-DCs for immunological phenotype analysis by flow cytometry.

2.2. Perform dendritic cell co-transplantation of BMT (DC-cotransplanting BMT).

NOTE: Use FVB ($H2k^q$) mice as donors for T-cells and BM cells. Irradiate B6 Ly5.1 recipient mice at a dose of 1,100 cGy (2 doses, 3 h interval). See details in step 1.1.

2.2.1. Isolate BM from femurs and tibias of the FVB donor mice. See details in steps 1.2.3−1.2.10.

NOTE: Use total isolated bone marrow instead of TCD-BM in this model.

2.2.2. Purify T-cells from the spleens and lymph nodes of the FVB donor mice. See details in steps 1.3.1-1.3.13.

2.2.3. Inject BM (5 x 10^6/mouse), T-cells (0.5 x 10^6/mouse) with BM-DCs (2 x 10^6/mouse) on day 0 of the experimental course.

2.2.4. On day 3 after the transplantation, examine the donor DC reconstitution by flow cytometry.

2.2.4.1. Collect blood from the eyes of the recipients.

2.2.4.2. Anesthetize the CD45.1/Ly5.1 B6 mice by 3% isoflurane inhalation. Check for the depth of anesthesia by the lack of response to a toe pinch.

2.2.4.3. Place a sterile glass pipette tube in the medial canthus of the eye directed caudally at a 30−45° angle from the plane of the nose. Apply pressure while gently rotating the tube.

2.2.4.4. Drop the blood into a 10 µL heparin-containing sterile 1.5 mL microcentrifuge tubes. Transfer 50 µL of blood into 5 mL glass flow tubes.

2.2.4.5. Add 2 mL of ACK lysing buffer. Incubate at 37 ˚C in water bath for 45 min. Add 2 mL of FACS staining buffer. Centrifuge at 800 x *g* for 5 min at 4 ˚C. Discard the supernatant.

2.2.4.6. Resuspend the cell pellets with 200 µL of FACS buffer containing appropriate flow staining antibodies (live/death yellow, α-H-$2K^b$, α-CD45.1, α-CD45.2, α-CD11c, and α-MHCII). Incubate for 15 min at 4 ˚C in the dark. Wash 2x with 1 mL FACS buffer. Resuspend the cell pellets in 200 µL of FACS buffer and perform the analysis by flow cytometry.

3. GVHD/GVL models of BMT

3.1. Perform GVHD/GVL induction.

3.1.1. Culture the luciferase transduced A20 B cell lymphoma in RPMI culture media.

3.1.2. Irradiate BALB/c background WT or Ly5.1 recipient at 700 cGy (single dose). See details in step 1.1.

3.1.3. Isolate TCD-BM from the femurs and tibias of B6. Ly5.1 donor mice. See details in steps 1.2.1−1.2.10.

3.1.4. Purify T-cells from spleens and lymph nodes of the C57BL/6 donor mice. See details in steps 1.3.1−1.3.15.

3.1.5. Wash the A20 lymphoma 2x with 25 mL of PBS. Resuspend the cell pellets in 10 mL of ice-cold PBS. Take a cell suspension aliquot (10 µL) and count the cells using 1% trypan blue and a hemocytometer. Adjust the cell concentration to 20,000 cells/mL.

3.1.6. Inject TCD-BM (5 x 10^6/mouse) with or without T-cells (0.75 x 10^6/mouse) and A20 lymphoma (5,000 cells/mouse).

3.1.7. Follow up on the recipient survival, GVHD clinical signs, and body weight loss during the experimental course. See details in step 1.5.

3.2. Perform bioluminescent imaging.

3.2.1. Monitor the tumor growth in the transplanted recipient by injecting the recipient mice with 4 mg of D-luciferin. Incubate for 5 min to ensure luciferin reacts with luciferase.

3.2.2. Anesthetize the mouse using 3% isoflurane in the chamber of a bioluminescence imager and image the recipients for 5 min in field D and the exposure time of 1 min.

3.2.3. Analyze data using image-analyzing software. Change the scale of the pseudo color images for best results.

NOTE: All the pictures must be at the same scale across experiments.

3.2.4. Use the image-analyzing software to determine the regions of interest and quantify the signal density by calculating the flux (photons/s) being emitted from each region of interest.

REPRESENTATIVE RESULTS:

The major MHC-mismatched B6 ($H2k^b$)-BALB/C ($H2k^d$) model closely corresponded to GVHD development after the transplantation (**Figure 2**). All six GVHD clinical signs established

previously by Cooke et al.[16] occurred in the recipients transplanted with WT-B6 T-cells but not in the recipients transplanted with BM alone (step 1.5), which represented the GVHD-negative group. There are two phases in GVHD development in this model. First, the peak of severity is approximately 11 days after the transplantation, followed by a reduction in the clinical scores and body weight recovery up to 16 days. In this phase, several mechanisms such as irradiation-induced inflammation and engraftment syndrome drives the disease pathogenicity and GVHD. The recipients uniformly succumb to GVHD about 30−40 days post-transplant.

At least 85% of the BM differentiated into DCs (**Figure 3A**). Interestingly, transplantation with $fB^{-/-}$DCs improved the recipient survival and GVHD clinical score **(Figure 3B**,**C**). Given that $fB^{-/-}$DCs have less antigen presenting capacity demonstrated by lower MHCII expression and reduced co-stimulatory receptor expression[17], the co-transplantation protocol may be sufficient for examining various signaling or targets in recipient DCs in GVHD development after BMT.

The T-cell purity was 90% after enrichment (**Figure 4A**). Luciferase-transduced A20 B cell lymphoma allows monitoring tumor growth in live animals (**Figure 4B**). In this model, if the recipients died without any signal and a high GVHD clinical score, it was concluded that they died of GVHD. All the WT BALB/c recipients that received BM alone plus A20 died of tumor relapse (**Figure 3B**). By contrast, if the animal died of higher signal density, it was concluded that they died of tumor relapse. As demonstrated in **Figure 3B**, WT BALB/c recipients transplanted with BM and T-cells from $ACC1^{fl/fl}$B6 donor ($ACC1^{+/+}$ T-cells) died of GVHD. If animals died with signals of disease, it was concluded that they died of GVHD and tumor relapse. The animals that received BM and T-cells from the $ACC1^{fl/fl}$ x CD4 cre B6 donor ($ACC1^{-/-}$ T-cells) died of both GVHD and tumor relapse (**Figure 3B**). Animals can be placed back in the cage to be imaged at a later time point or euthanized for ex vivo imaging. Using the software, the tumor mass in the animal can also analyzed individually (**Figure 3B**).

FIGURE AND TABLE LEGENDS

Figure 1: Schematic representation of the BMT procedure. (**A**) Scheme for MHC-mismatched B6→BALB/c BMT model. (**B**) Scheme for DC co-transplanting FVB→B6 model. (**C**) Scheme for B6→BALB/c GVHD/GVL model.

Figure 2: Major MHC-mismatched B6→BALB/c GVHD model. BALB/c mice were lethally irradiated and transplanted with 5 x 10^6 BM alone or with 0.75 x 10^6 T-cells. (**A**) Survival data, (**B**) body weight loss, and (**C**) clinical score data of the recipients of BM alone or with T-cells.

Figure 3: **DC co-transplanting HCT model**. BM was isolated from WT and $fb^{-/-}$B6 mice and differentiated into DCs by culturing with GM-CSF. (**A**) The purity of DCs was examined by flow cytometry by staining with CD11c and MHCII. Lethally irradiated B6 recipients were transplanted with BM (3 × 10^6/mouse) plus purified T-cells (1 × 10^6/mouse) from the FVB donors. The recipients also received 2 x 10^6 WT or $fB^{-/-}$B6 BM-DCs cells on the day of transplantation. The survival (**B**) and clinical score (**C**) are shown.

Figure 4: Major-MHC mismatched B6→BALB/c GVHD/GVL model. WT BALB/c recipients were

transplanted with TCD-BM (5×10^6/mouse) alone or with ACC1 $^{+/+}$ T-cells or ACC1 $^{+/+}$ T-cells (1×10^6/mouse) isolated from B6 background donor mice. In addition, recipients received 2×10^3 A20-luc at the time of transplant. T-cell purity was examined by flow cytometry through staining with live/death yellow, CD3, CD4, and CD8 flow antibodies (**A**). The recipients were monitored for tumor growth determined by whole-body bioluminescence imaging (BLI) (**B**).

DISCUSSION:

The use of stem cells to suit a particular individual is an effective approach to treat advanced and resistant cancers[18]. Small molecule pharmaceuticals, however, have long remained a primary focus of personalized cancer therapy. On the other hand, in cellular therapy a multitude of interactions between donor and host can decisively influence the treatment outcomes, such as the development of GVHD after BMT[1].

Major MHC-mismatched mouse models of BMT are a valuable tool in understanding the biology of GVHD and testing the efficacy of drugs in its treatment. Among of them, C57BL/6 ($H2^b$) to BALB/c ($H2^d$) and FVB ($H2^q$)→C57BL/6 ($H2^b$) are well-established models[5,7]. These models incorporate either myeloablative radiation conditioning as a single dose (BALB/c) or a fractionated dose (C57BL/6) in which 3–8 hour intervals are required to decrease gut toxicity[5]. Both models are dependent on both $CD8^+$ and $CD4^+$ T-cells. In these models, GVHD severity and survival are the main outcomes measured, and the transplanted recipient has consistent rapid kinetics and 100% penetrance. In order to monitor T-cell migration and expansion, T-cells from luciferase-transduced donor mice should be used for in vivo bioluminescence imaging[19]. However, while 90% of the BMT performed are MHC-matched, the B6-BALB/c model does not perfectly resemble the clinical situation. The discovery of the MHC and minor histocompatibility antigens (miHAs) has significantly contributed to advancing the field of BMT[10]. Minor MHC-mismatched GVHD mouse models more closely mimic patient GVHD[20]. Conditioning intensity to induce donor cell engraftment causes tissue damage and can affect the GVHD outcome[21]. Conditioning regimens in murine models often involves TBI in contrast to chemotherapy in clinical settings[22,23]. Therefore, an immunosuppressive chemotherapy model has been used to mimic a reduced conditional intensity in clinics. The mouse model was C57BL/6 ($H2^b$) to BALB/c ($H2^d$) and transplanted recipients developed clinical and histological symptoms associated with GVHD[24].

The potential advantage of the co-transplanted protocol is to test the role of recipient DCs without depending specifically on CD11c depleted mice. Because the BM-DC generation was performed ex vivo, this protocol can also be applied to test the role of other cell types such as macrophages or neutrophils in GVHD simply by modifying the culture conditions. Using $CD45.1^+$ B6 mice as recipients allows researchers to distinguish BM-DCs ($CD45.2^+$ $CD11c^+$) from recipient DCs ($CD45.1^+$ $CD11c^+$) by flow analysis. The flexible number of cells generated ex vivo and adopted into the transplanted recipients is another benefit of the co-transplantation protocol. Furthermore, ex vivo culture allows us to screen the potential drugs to control GVHD.

The ability to track tumor patterns in vivo is a powerful tool that has the potential to test whether a drug can affect GVL activity. Using this GVHD/GVL model, tumor progression and metastasis

can be monitored in live animals[16]. Moreover, this setting can be used for testing the GVL effect in multiple cancers.

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DISCLOSURES:

The authors have no conflicts of interest.

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Figure 1.

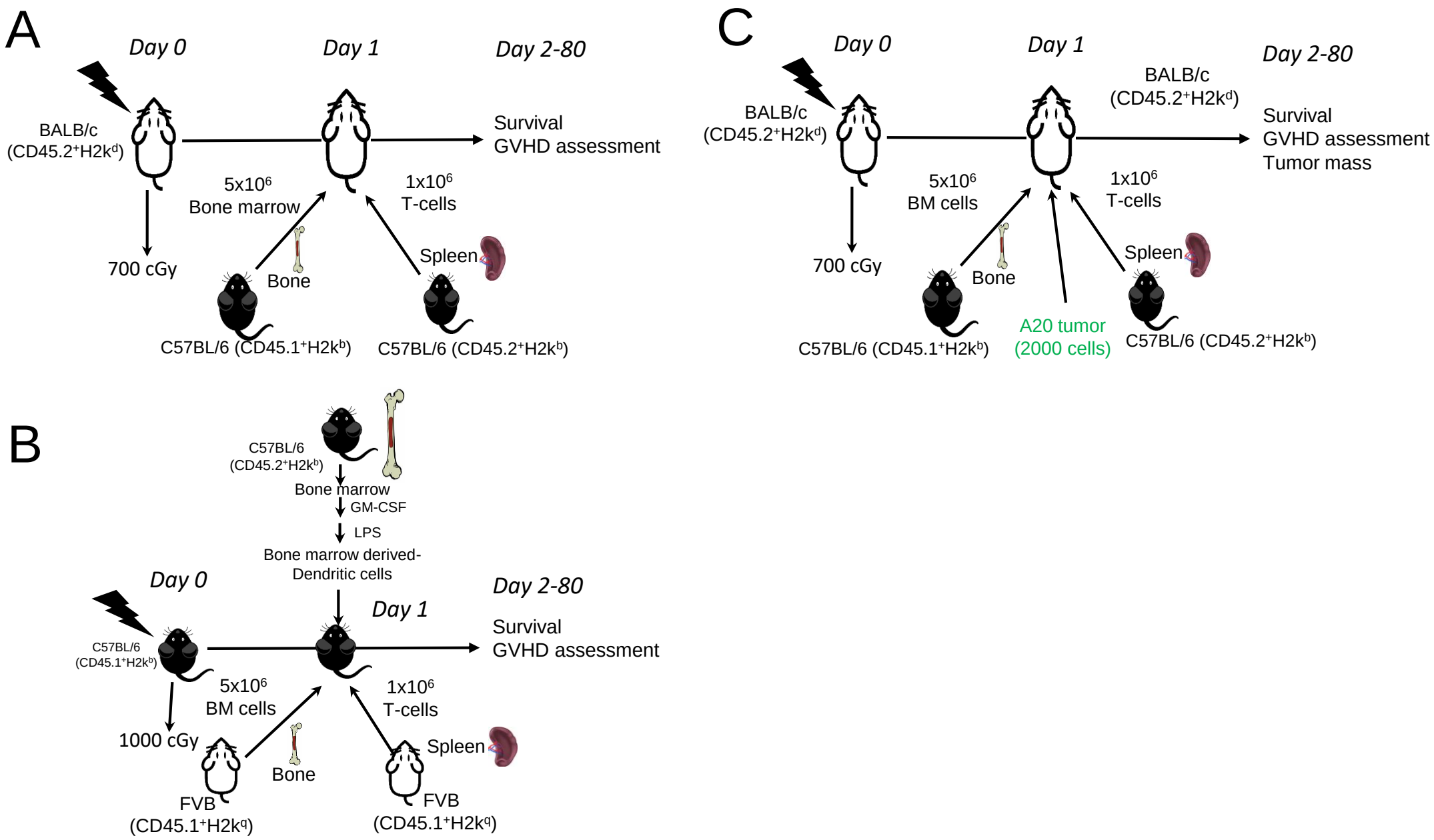


Figure 2.

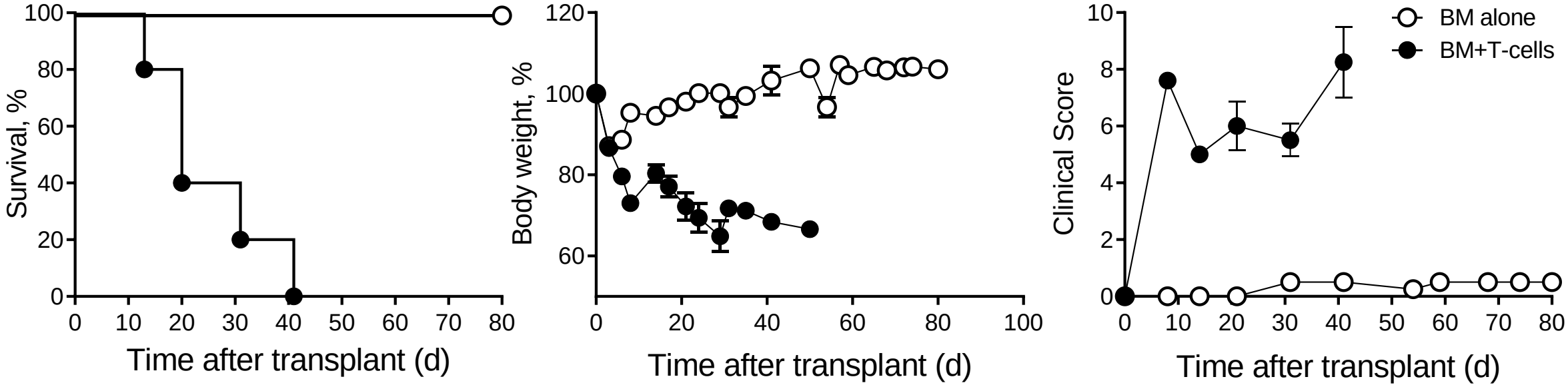


Figure 3.

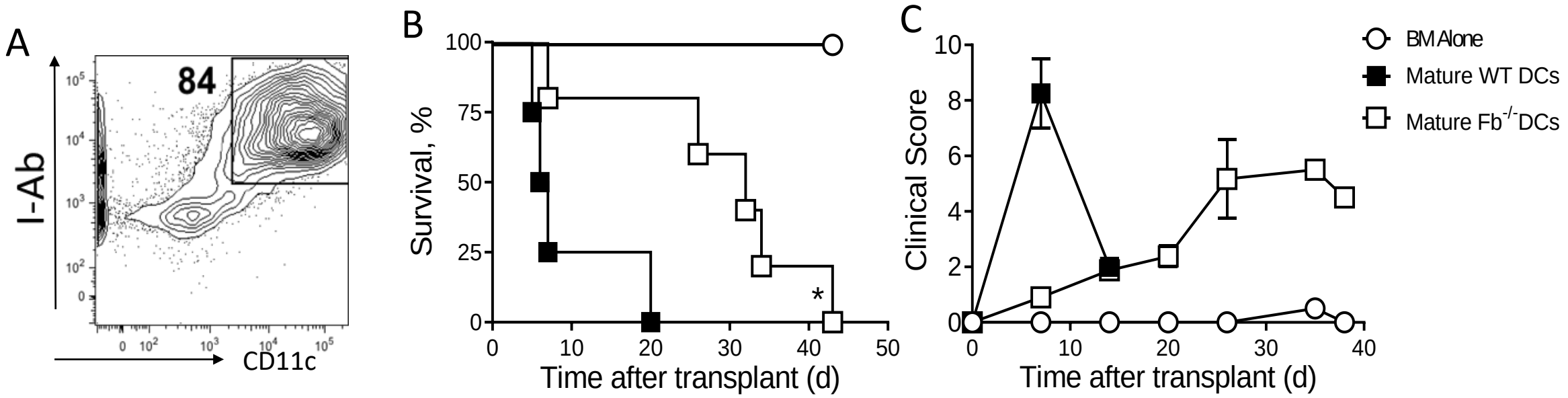
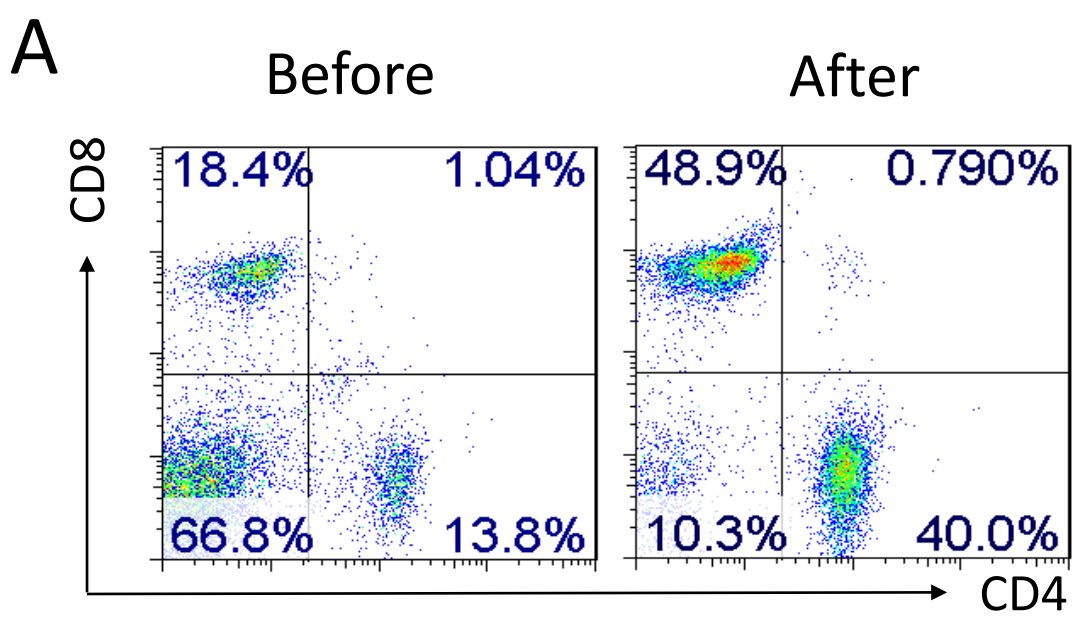
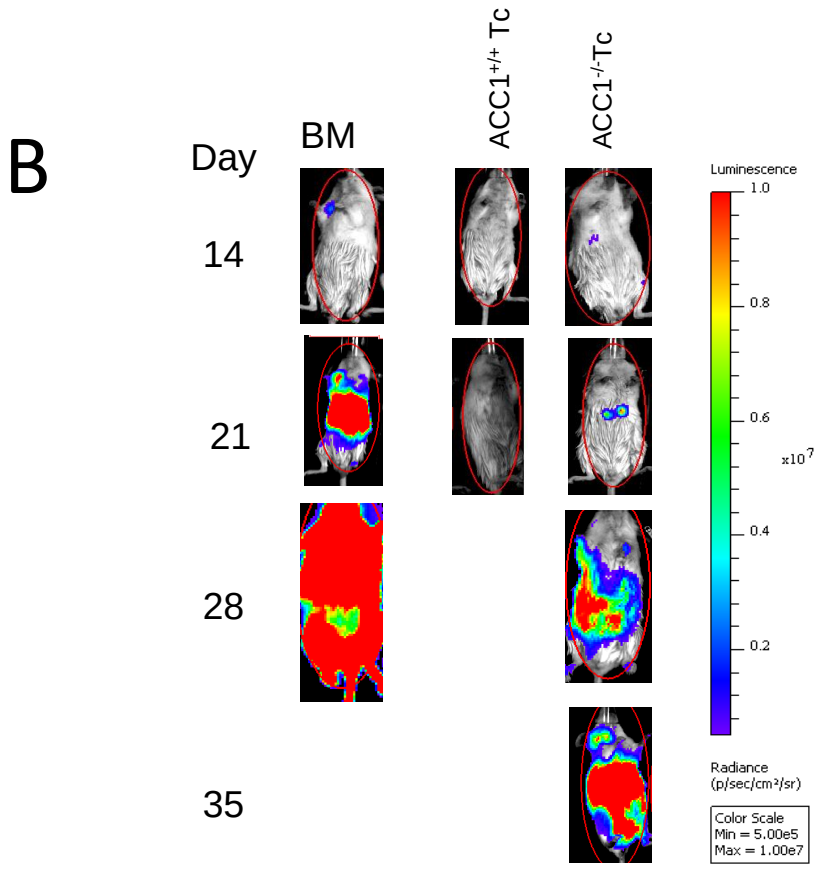


Figure 4.





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Name of Material/Equipment	Company	Catalogue Number
0.5 M EDTA pH 8.0 100ML	Fisher Scientific	BP2482100
10X PBS	Fisher Scientific	BP3994
A20 B-cell lymphoma	University of Central Florida	In house
ACC1 fl/fl	Jackson Lab	30954
ACC1 fl/fl CD4cre	University of Central Florida	
Anti-Biotin MicroBeads	Miltenyi Biotec	130-090-485
Anti-Human/Mouse CD45R (B220)	Thermo Fisher Scientific	13-0452-85
Anti-mouse B220 FITC	Thermo Fisher Scientific	10452-85
Anti-mouse CD11c- AF700	Thermo Fisher Scientific	117319
Anti-Mouse CD25 PE	Thermo Fisher Scientific	12-0251-82
Anti-Mouse CD4 Biotin	Thermo Fisher Scientific	13-0041-86
Anti-Mouse CD4 eFluor® 450 (Pacific Blue® replacement	Thermo Fisher Scientific	48-0042-82
Anti-mouse CD45.1 PE	Thermo Fisher Scientific	12-0900-83
Anti-Mouse CD8a APC	Thermo Fisher Scientific	17-0081-83
Anti-mouse H-2Kb PerCP-Fluor 710	Thermo Fisher Scientific	46-5958-82
Anti-mouse MHC Class II-antibody APC	Thermo Fisher Scientific	17-5320-82
Anti-Mouse TER-119 Biotin	Thermo Fisher Scientific	13-5921-85
Anti-Thy1.2	Bio Excel	BE0066
B6 fB $^{-/-}$ mice	University of Central Florida	In house
B6.Ly5.1 (CD45.1^{+}) mice	Charles River	564
BALB/c mice	Charles River	028
C57BL/6 mice	Charles River	027
CD11b	Thermo Fisher Scientific	13-0112-85
CD25-biotin	Thermo Fisher Scientific	13-0251-82
CD45R	Thermo Fisher Scientific	13-0452-82
CD49b Monoclonal Antibody (DX5)-biotin	Thermo Fisher Scientific	13-5971-82

Cell strainer 40 uM	Thermo Fisher Scientific	22363547
Cell strainer 70 uM	Thermo Fisher Scientific	22363548
D-Luciferin	Goldbio	LUCK-1G
Fetal Bovine Serum (FBS)	Atlanta Bilogicals R&D system	D17051
Flow cytometry tubes	Fisher Scientific	352008
FVB/NCrl	Charles River	207
Lipopolysacharide (LPS)	Millipore Sigma	L4391-1MG
LS column	Mitenyi Biotec	130-042-401
MidiMACS	Miltenyi Biotec	130-042-302
New Brunswick Galaxy 170R incubator	Eppendorf	Galaxy 170 R
units penicillin / 10,000 mg/ml strep)	GIBCO	15140
RPMI 1640	Thermo Fisher Scienctific	11875-093
TER119	Thermo Fisher Scientific	13-5921-82
Xenogen IVIS-200	Perkin Elmer	Xenogen IVIS-200
X-RAD 320 Biological Irradiator	Precision X-RAY	X-RAD 320

Comments/Description
MACS buffer
MACS buffer
GVL experiment
GVL experiment
GVL experiment
T-cell enrichment
T-cell enrichment
Flow cytometry analysis
Flow cytometry analysis
Flow staining
T-cell enrichment
Flow staining
Flow cytometry analysis
Flow cytometry analysis
Flow cytometry analysis
Flow cytometry analysis
T-cell enrichment
BM generation
Recipients
Donors
Transplant recipients
Donors/Recipients
T-cell enrichment
T-cell enrichment
T-cell enrichment
T-cell enrichment

Cell preparation
Cell preparation
Live animal imaging
Cell Culture
Flow cytometry analysis
Donors
DC mature
Cell preparation
T-cell enrichment
Cell Culture
Media
Media
T-cell enrichment
Live animal imaging
Total Body Irradiation

Rebuttal Letter

Dear Editor,

Thank you so much for your insightful comments. We have carefully revised our manuscript. Please find these parts in the track-changed and yellow highlighted parts.

Sincerely yours

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