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Modeling chronic graft versus host disease in mice using allogeneic bone marrow and splenocyte transfer

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Abstract

This unit describes a protocol for allogeneic bone marrow and splenocyte transfer for the modeling of chronic graft versus host disease (cGVHD) in mice. Preclinical models provide clinically relevant platforms for mechanistic and therapeutic studies that may inform the treatment of patients suffering from cGVHD, a common and potentially severe complication of allogeneic hematopoietic stem cell transplantation (alloHSCT). Most murine models of cGVHD depend on the transfer of major histocompatibility complex (MHC)-mismatched bone marrow and whole splenocytes (or purified T cells) into an irradiated recipient. The bone marrow contains hematopoietic stem and progenitor cells necessary to reconstitute the irradiated host hematopoietic system, while splenocytes contain T cells that mediate cGVHD. Of note, specific mouse strains, splenocyte dose, bone marrow quantity, and irradiation doses vary widely across different cGVHD models. Here, we describe donor bone marrow and splenocyte preparation, recipient irradiation and intravenous injection of donor cells, and clinical monitoring for disease emergence and progression.

Keywords

Graft versus host disease; allogeneic hematopoietic stem cell transplantation; splenocyte transfer; disease modeling

Introduction

Allogeneic transplantation and modeling of chronic graft versus host disease in mice

This unit describes a protocol for allogeneic hematopoietic stem cell transplantation via whole bone marrow transfer and adoptive splenocyte transfer for the modeling of chronic graft versus host disease (cGVHD) in mice. Preclinical models provide invaluable tools for mechanistic and therapeutic studies that may inform the treatment of patients suffering from cGVHD (Boyiadzis et al., 2015). cGVHD arises in up to 50% of patients following allogeneic hematopoietic stem cell transplantation (alloHSCT), a life-saving therapy for refractory leukemia, lymphoma, marrow failure states, and non-malignant conditions such as

thalassemia and sickle cell anemia (Palmer et al., 2016). cGVHD causes inflammation and fibrosis in multiple organs including the skin, eyes, liver, intestines, and lungs. Although steroids and other immunosuppressants are mainstay therapies, only ibrutinib carries FDA approval for cGVHD treatment (Miklos et al., 2017), and all currently used therapies have variable efficacy. Overall, despite great advances in our understanding of transplant immunology, GVHD is a major obstacle to post-transplant quality of life and overall survival.

Most murine models of cGVHD depend on the transfer of bone marrow and splenocytes partially or fully-mismatched at major histocompatibility complexes (MHC) into an irradiated recipient. Bone marrow cells contain hematopoietic stem and progenitor cells necessary for hematopoietic reconstitution following irradiation, while splenocytes contain T cells and other immune cells that mediate GVHD. Broadly, spleen-derived donor T cells expressing a specific T-cell receptor interact with APCs expressing host MHCs or minor histocompatibility antigens, resulting in donor T cell recognition of host tissue as foreign, initiating immune attack, activation of B lymphocytes and autoantibody production, stimulation of macrophages, and production of pro-fibrotic elements (MacDonald et al., 2017; Zeiser and Blazar, 2017). Some protocols call for the further purification of donor bone marrow (e.g. T cell depletion) and splenocytes (e.g. T cell enrichment), topics beyond the scope of this protocol (see, for example, (Wu et al., 2013)). Furthermore, specific mouse strains, splenocyte dose, bone marrow quantity, and irradiation doses vary widely across different cGVHD models (Schroeder and DiPersio, 2011). This protocol does not intend to cover all strain-dose combinations, and a detailed review can be found in Schroeder et al (Schroeder and DiPersio, 2011).

This unit includes a protocol for donor bone marrow and splenocyte preparation (Basic Protocol 1), and recipient irradiation, intravenous donor cell inoculum injection, and clinical monitoring for disease emergence and progression (Basic Protocol 2).

Basic Protocol 1

Preparation of donor cells for allogeneic transplantation

This protocol describes donor mouse dissection, splenocyte isolation, and bone marrow isolation for adoptive bone marrow and splenocyte transfer into irradiated recipient mice. Whole bone marrow isolated from the femur serves as source for hematopoietic stem cells. Whole spleen cells isolated from the spleen serve as the source for T cells and other immune cells. In this protocol, further purification of donor bone marrow or splenocytes is not described although some cGVHD protocols may require this (for example, (Wu et al., 2013)).

Materials—Donor mice, age- and sex-matched, of the same strain, typically 6–10 weeks old, either bred in-house or obtained from a commercial vendor (e.g. The Jackson Laboratory; Taconic). Our laboratory typically uses ~8 week old male B10.D2 mice (The Jackson Laboratory #000461) as donors and ~8 week old male BALB/cJ mice (The Jackson Laboratory #000651) as recipients. However, donor and recipient strains vary by specific cGVHD model.

Surgical scissors and forceps

70 μ m pre-separation sterile filters (e.g. Miltenyi 130–095-823)

3 mL syringes

1 mL syringes

23 gauge 1” needles

50 mL conical tubes

Sterile petri dishes

Hemocytometer or comparable cell enumeration device

Dissection buffer (see recipe in ‘Reagents and Solutions’)

ACK RBC lysis buffer (see recipe in “Reagents and Solutions”)

PBS

Isolate femurs and spleens

1. Euthanize donor mouse according to institutionally approved animal protocols, per Washington University School of Medicine in St. Louis IUCAC approval.

Note: Mouse dissection is performed with sterile surgical instruments on a clean lab bench. Once organs (i.e. spleens and femurs) are isolated in sterile dishes, further purification should be performed in sterile conditions under a laminar flow hood.

2. Arrange supinated (i.e. on back) mouse on clean surgical drape and spray abdominal skin with 70% ethanol.
3. Gently lift abdominal skin with forceps and make ~1 cm incision with surgical scissors.
4. Gripping with the 1st and 2nd fingers of both hands, retract abdominal skin cranially far enough to expose peritoneum and caudally to expose the tibia (Figure 1A).
5. Using surgical scissors, snip peritoneal lining near spleen on left side of abdomen and gently retract spleen with forceps (Figure 1B). Place spleen (Figure 1C) in a covered petri dish with a few mL of PBS at room temperature or on ice if dissection of all spleens is expected to take more than one hour. Repeat for all donor spleens. A single petri dish with pooled donor spleens of the same strain and/or treatment condition can be used.

Note: Clean dissection tools with 70% ethanol and a task wipe between dissections. Take care to avoid contamination from puncturing the gastrointestinal tract. Shearing or tearing the spleen may reduce splenocyte yield – this can be avoided by gentle retraction.

6. Using surgical scissors, snip femur free from the tibia and the iliac (Figure 1A and 1D).
7. Remove muscle and proximal and distal joint tissue from femur using either scissors or task wipes (Figure 1E). Place femur in a petri dish with a few mL of PBS at room temperature or on ice if dissection of all femurs is expected to take more than one hour. Repeat for all donor femurs. A single petri dish with pooled donor femurs of the same strain and/or treatment condition can be used.

Note: Clean dissection tools with 70% ethanol and a task wipe between dissections. Breaking the femur mid-shaft may reduce bone marrow yield and incomplete removal of muscle and connective tissue may contaminate final specimen.

Splenocyte isolation from spleens (Perform under sterile conditions in laminar flow hood)

8. Under laminar flow hood, carefully aspirate excess PBS from the petri dish, leaving ~1 mL PBS and spleens. Gently crush spleens with the plunger end of a sterile 3 mL syringe, disrupting the splenic capsule and freeing white and red blood cells into the residual PBS.
9. Add 0.5–1 mL of ACK RBC lysis buffer per spleen to petri dish and mix with plunger end of sterile syringe used in step 1. Incubate at room temperature for 2–5 minutes.
10. Using a 10ml sterile pipette and pipette aid, transfer cell suspension from petri dish through a 70 μ m sterile filter nested in a sterile 50 mL conical tube. Take care to avoid transferring the splenic capsule and connective tissue, as this may clog the filter.
11. Wash petri dish with ~10 mL sterile PBS and transfer remaining suspension through the filter into the 50 mL tube. Fill the tube to 50 mL final volume with PBS.
12. Centrifuge cell suspension at 500g for 5 minutes. Discard supernatant.
13. Resuspend cell pellet in 10 mL of sterile dissection buffer by aspirating and expelling the solution numerous times using a 10 mL pipette.
14. Fill the tube to 50 mL final volume with dissection buffer and mix gently by repeated inversion.
15. Remove ~15–20 μ L for enumeration by hemacytometer or comparable device. A typical adult spleen yields 50–100 $\times$ 10⁶ splenocytes and a single femur from an adult mouse yields 15–20 $\times$ 10⁶ bone marrow mononuclear cells.
16. Centrifuge cell suspension at 500g for 5 minutes. Discard supernatant.
17. Resuspend in dissection buffer at a concentration sufficient to inject desired quantity of splenocytes in 100 μ L of dissection buffer. For example, if plan calls for transfer of 10 $\times$ 10⁶ splenocytes into each recipient mouse, resuspend the

splenocytes at 100×10^6 per mL of dissection buffer. Keep on ice until Basic Protocol 2.

Bone marrow isolation from femurs (perform under sterile conditions in laminar flow hood)

18. Under a laminar flow hood and sterile conditions aspirate PBS for petri dish containing femurs. Grasp femur with forceps with femoral head up and cut femoral head off with scissors (Figure 2). Invert the femur, and position vertically over sterile 50 mL conical.
19. Insert a 23 g needle, affixed to a 3 mL syringe containing 3 mL of sterile dissection buffer into the femoral cortex, down the longitudinal axis of the femur, through the intercondylar fossa (Figure 2).
20. Flush femoral cortex with dissection buffer into 50 mL conical. Repeat flush twice using 3 mL of buffer aspirated from the 50 mL conical; this ensures maximum yield of bone marrow from the femoral cortex. Post-flushing, bone should be pale yellow to white in color. Repeat for all donor femurs.
21. Fill 50 mL conical tube containing the bone marrow to volume with dissection buffer.
22. Centrifuge the collected bone marrow fluid at 500g for 5 minutes at room temperature. Discard supernatant.
23. Resuspend cell pellet in 5 mL sterile ACK RBC lysis buffer. Incubate at room temperature for 2–5 minutes.
24. Filter the suspension through a sterile 70 μ m filter into a new sterile 50 mL conical tube. Wash original 50 mL conical tube with ~10 mL sterile PBS and filter that solution into new 50 mL tube.
25. Fill a new 50 mL conical tube to a final volume of 50 mL with dissection buffer and mix gently by inversion.
26. Remove ~15–20 μ L for enumeration by hemacytometer or comparable device for resuspension at standard concentration as described in step 28.
27. Centrifuge cell suspension at 500g for 5 minutes at room temperature. Discard supernatant.
28. Resuspend cell pellet in dissection buffer at a concentration sufficient to inject desired quantity of whole bone marrow in 100 μ L of dissection buffer. Keep on ice until used in Basic Protocol 2.

Preparation of donor cell inoculum

29. Mix bone marrow suspension and splenocyte suspension at a ratio of 1:1 and place on ice until ready to inject recipients. Mice should be injected same day as donor inoculum preparation, preferably within 2 hours after isolation of the inoculum.

30. Each recipient will receive 200 μ L of the donor cell inoculum
31. For the control group (bone marrow only recipients), mix bone marrow suspension 1:1 with dissection buffer.

Basic Protocol 2

Irradiation of recipient mice, intravenous transplantation of allogeneic cells, and monitoring for clinical signs of cGVHD emergence and progression

Recipient mice must be irradiated or otherwise subjected to myeloablation in preparation for hematopoietic stem cell transplant and splenocyte adoptive transfer. Bone marrow cells containing hematopoietic stem cells and splenocytes are injected intravenously into the lateral tail vein in a single injection. Clinical signs of GVHD, including weight loss, diarrhea, fur loss (alopecia), and scaling, (Tables 1 and 2) are monitored over the following weeks. In typical cGVHD models, chronic-type symptoms (e.g. alopecia, scaling) arise in 30–60 days (Figure 3A-B).

Materials list

Recipient mice (example): 8-week old male BALB/cJ mice (The Jackson Laboratory #000651)

Irradiator

[*Please ask author to include relevant vendor information for irradiator]

27 gauge 0.5" needles

1 mL syringes

Heat lamp (optional) can be used to warm mice and increase tail vein blood flow

Small rodent restrainer (optional)

Irradiation

1. Weigh recipient mice just prior to irradiation. This information will be used when monitoring the mice for disease progression.
2. Perform irradiation of recipient mice 8–24 hours prior to injection of donor cell inoculum. Irradiation should be performed according to institutional and machine-specific guidelines.

Note: time of radiation exposure varies widely by irradiator and cGVHD model. The essential protocol involves exposing mice to a specific radiation dose (e.g. 750 cGy) using an institutionally approved and maintained irradiator, according to that machine's dosage table, which varies based on isotope decay.

IV injection

3. Optional: place a heat lamp near animals to increase body temperature, which dilates and increases visualization of the tail vein.
4. Optional: place the recipient mouse in a small rodent restrainer to help immobilize the animal.
5. Aspirate 1 mL of donor inoculum into a 1 mL syringe affixed with a 27 gauge 0.5" needle.
6. Using the non-dominant hand, pull the recipient's tail taut and identify the lateral tail vein (either left or right vein).
7. Carefully insert the 27 gauge needle, bevel side up, ~2 mm into the lateral tail vein, with the needle angled ~15° from parallel to the tail vein.
8. Gently inject 200 µL of donor cell inoculum.

Note: A successful intravenous injection will have minimal back pressure – do not force, as this indicates injection into the subcutaneous tissue. If successful, blood will visibly clear from the tail vein. Inject distally on the tail vein and work proximally if unsuccessful on the first attempt.

Monitoring the progression of cGVHD in mice

9. Score and weigh mice twice weekly, starting 3–4 days after donor cell injection. Perform scoring and weighing in a blind fashion.

Table 1 describes overall GVHD scoring (Cooke et al., 1996) and Table 2 describes skin-specific chronic GVHD scoring (Young et al., 2012). Both scoring systems should be used when assessing cG

VHD, especially as aGVHD may precede cGVHD.

Reagents and Solutions

Dissection buffer—Phosphate buffered saline containing the following:

Na₂EDTA 2 mM

Bovine serum albumin 0.1%

Sterile filter and store at 4C for up to 1 year

ACK (ammonium chloride potassium) red blood cell (RBC) lysis buffer—

Double-distilled water containing the following:

NH₄Cl 150 mM

KHCO₃ 10 mM

Na₂EDTA 0.1 mM

Sterile filter and store at room temperature for up to 1 year

Commentary

Background

Allogeneic hematopoietic stem cell transplantation (AlloHSCT) is a potentially curative procedure for relapsed and refractory blood cancer. However, acute and chronic GVHD are major sources of non-relapse morbidity and mortality following alloHSCT, and few effective therapies for GVHD exist (Arai et al., 2015; Boyiadzis et al., 2015; Schroeder et al., 2017). In mice, acute GVHD arises in days to a few weeks after transplant, with diarrhea and weight loss the predominant features. Chronic GVHD typically arises >30 days, with alopecia, scaling, and organ fibrosis the predominant features. Mouse models of GVHD provide experimental platforms for the preclinical testing of novel therapies and mechanistic discoveries (Chu and Gress, 2008; Schroeder and DiPersio, 2011; Zeiser and Blazar, 2016).

Several models of murine chronic GVHD have been described, and no single murine model to date fully recapitulates the scope of human disease (Schroeder and DiPersio, 2011). Most cGVHD mouse models depend on transfer of bone marrow and splenocytes into irradiated recipients that have partial MHC mismatches (e.g. B10.D2 donor into BALB/cJ recipient). In part, this MHC mismatch causes donor T cells to recognize and attack host (recipient) tissue as foreign, contributing to the inflammatory environment that potentiates autoantibody production and fibrosis crucial to cGVHD pathogenesis (Zeiser and Blazar, 2017).

A full discussion of specific cGVHD models is beyond the scope of this protocol, and a comprehensive review can be found in Schroeder et al (Schroeder and DiPersio, 2011). Table 3 presents specific details on three different commonly used cGVHD models. Importantly, no current mouse model recapitulates all aspects of human disease, and investigators commonly rely on multiple mouse models to perform cGVHD mechanistic and therapeutic studies.

Preclinical models provide a testing platform for novel therapies for cGVHD. For example, the only FDA-approved therapy for cGVHD is ibrutinib, a Bruton's tyrosine kinase (BTK) and interleukin-2-inducible T-cell kinase (ITK) inhibitor. Foundational evidence for its efficacy and mechanism of action in cGVHD derived from prior studies in two different mouse cGVHD models (Dubovsky et al., 2014; Miklos et al., 2017; Schutt et al., 2015). Doubtlessly, murine models of cGVHD will continue to serve as important tools for unraveling mechanisms and discovering new therapies in this disease.

Critical parameters and troubleshooting

Protocols may result in variable phenotypes depending on laboratory and facility factors (e.g. local microbiome) (Schroeder and DiPersio, 2011)(Zeiser and Blazar, 2016).

Of note, specific models must be tested and validated in individual laboratories, as environmental factors (e.g. microbiome, diet, antibiotics) and procedural variables (e.g. irradiation dose, scoring methodology) can create large phenotypic variation.

Mouse strain, donor splenocyte quantity, and whole bone marrow quantity vary by specific cGVHD model. Even within identical established cGVHD models (a full review of established models can be found in (Schroeder and DiPersio, 2011) and (Zeiser and Blazar, 2016)) investigators at different institutions (or even different laboratories) may need to alter donor cell inoculum quantity, recipient irradiation dosage, and post-transplant prophylaxis (e.g. antibiotics) to recapitulate published phenotypes. Thus, before the investigator undertakes larger-scale experiments, we suggest performing a straightforward splenocyte dose-response experiment using experimental mice (irradiated mice receiving splenocytes and bone marrow) compared against irradiated mice receiving only whole bone marrow, the latter of which develop very mild or no clinically apparent GVHD (Schroeder and DiPersio, 2011; Zeiser and Blazar, 2016). An example experiment testing three different splenocyte doses (e.g. 10×10^6 , 20×10^6 , 40×10^6 cells) could use $n=5$ recipient mice per splenocyte dose and whole bone marrow only groups, thus requiring 20 recipient mice and ~5 donor mice (assuming a splenocyte yield of $50\text{--}100 \times 10^6$ per donor). In this way, the investigator can reproduce established cGVHD phenotypes using a sufficient splenocyte dose administered to the recipient mice and subsequently test new therapies or mechanisms for cGVHD.

Statistical analysis

Statistical analysis of weights and GVHD scores can be performed by unpaired student's *t* test between experimental (splenocyte recipients) and control (bone marrow only recipients) mice.

Understanding the Results—Adult spleens yield $50\text{--}100 \times 10^6$ viable cells, and adult femurs yield $15\text{--}20 \times 10^6$ viable cells, although these yields can vary substantially based on technique, mouse age, and mouse gender. Gender of donor and recipient should be kept consistent throughout experiments, as this will affect phenotype. Most protocols require far more splenocytes than bone marrow cells per recipient, and thus splenocyte dose typically dictates the quantity of donors to purchase or breed.

In the early post-transplant period (~1 week), most mice will demonstrate mild weight loss (<10%), mild hunching, and mild fur ruffling. However, with skilled injection of the donor cell inoculum – which includes whole bone marrow and, thus, hematopoietic stem cells critical to host bone marrow reconstitution – all recipient mice should survive post-irradiation. Although post-irradiation antibiotics may reduce mortality related to infection, these can affect the microbiome and may unpredictably influence the course and severity of cGVHD. Therefore, consistency across experiments is key. Clinical signs of cGVHD emerge in 30–60 days, with high variability in timing and clinical signs depending on the specific model used (Representative data from the B10.D2 BALB/c model shown in Figure 3A-B).

Time considerations—Cell isolation and tail vein injection can require a full day of labor, depending on operator experience and size of experiment. Irradiation of animals should be performed the night before or the morning of transplantation. If larger doses of radiation (typically >1000 cGy) are required, a fractionated schedule (2 doses separated by 6

– 8 hours) may be used to decrease toxicity. Chronic GVHD phenotype development typically takes 30 or more days but depends considerably on model selected.

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Literature cited

- Arai S, Arora M, Wang T, Spellman SR, He W, Couriel DR, Urbano-Ispizua A, Cutler CS, Bacigalupo AA, Battiwalla M, et al. (2015). Increasing incidence of chronic graft-versus-host disease in allogeneic transplantation: a report from the Center for International Blood and Marrow Transplant Research. *Biol Blood Marrow Transplant* 21, 266–274. [PubMed: 25445023]
- Boyiadzis M, Arora M, Klein JP, Hassebroek A, Hemmer M, Urbano-Ispizua A, Antin JH, Bolwell BJ, Cahn JY, Cairo MS, et al. (2015). Impact of Chronic Graft-versus-Host Disease on Late Relapse and Survival on 7,489 Patients after Myeloablative Allogeneic Hematopoietic Cell Transplantation for Leukemia. *Clin Cancer Res* 21, 2020–2028. [PubMed: 25348512]
- Chu YW, and Gress RE (2008). Murine models of chronic graft-versus-host disease: insights and unresolved issues. *Biol Blood Marrow Transplant* 14, 365–378. [PubMed: 18342778]
- Cooke KR, Kobzik L, Martin TR, Brewer J, Delmonte J, Jr., Crawford JM, and Ferrara JL (1996). An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. *Blood* 88, 3230–3239. [PubMed: 8963063]
- Du J, Paz K, Flynn R, Vulic A, Robinson TM, Lineburg KE, Alexander KA, Meng J, Roy S, Panoskaltsis-Mortari A, et al. (2017). Pirfenidone ameliorates murine chronic GVHD through inhibition of macrophage infiltration and TGF-beta production. *Blood* 129, 2570–2580. [PubMed: 28254742]
- Dubovsky JA, Flynn R, Du J, Harrington BK, Zhong Y, Kaffenberger B, Yang C, Towns WH, Lehman A, Johnson AJ, et al. (2014). Ibrutinib treatment ameliorates murine chronic graft-versus-host disease. *J Clin Invest* 124, 4867–4876. [PubMed: 25271622]
- MacDonald KPA, Hill GR, and Blazar BR (2017). Chronic graft-versus-host disease: biological insights from preclinical and clinical studies. *Blood* 129, 13–21. [PubMed: 27821504]
- Miklos D, Cutler CS, Arora M, Waller EK, Jagasia M, Pusic I, Flowers ME, Logan AC, Nakamura R, Blazar BR, et al. (2017). Ibrutinib for chronic graft-versus-host disease after failure of prior therapy. *Blood* 130, 2243–2250. [PubMed: 28924018]
- Palmer J, Chai X, Pidala J, Inamoto Y, Martin PJ, Storer B, Pusic I, Flowers ME, Arora M, Pavletic SZ, and Lee SJ (2016). Predictors of survival, nonrelapse mortality, and failure-free survival in patients treated for chronic graft-versus-host disease. *Blood* 127, 160–166. [PubMed: 26527676]
- Schroeder MA, Choi J, Staser K, and DiPersio JF (2017). The Role of Janus Kinase Signaling in Graft-Versus-Host Disease and Graft Versus Leukemia. *Biol Blood Marrow Transplant*
- Schroeder MA, and DiPersio JF (2011). Mouse models of graft-versus-host disease: advances and limitations. *Dis Model Mech* 4, 318–333. [PubMed: 21558065]
- Schutt SD, Fu J, Nguyen H, Bastian D, Heinrichs J, Wu Y, Liu C, McDonald DG, Pidala J, and Yu XZ (2015). Inhibition of BTK and ITK with Ibrutinib Is Effective in the Prevention of Chronic Graft-versus-Host Disease in Mice. *PLoS One* 10, e0137641. [PubMed: 26348529]
- Wu T, Young JS, Johnston H, Ni X, Deng R, Racine J, Wang M, Wang A, Todorov I, Wang J, and Zeng D (2013). Thymic damage, impaired negative selection, and development of chronic graft-versus-host disease caused by donor CD4+ and CD8+ T cells. *J Immunol* 191, 488–499. [PubMed: 23709681]

- Young JS, Wu T, Chen Y, Zhao D, Liu H, Yi T, Johnston H, Racine J, Li X, Wang A, et al. (2012). Donor B cells in transplants augment clonal expansion and survival of pathogenic CD4+ T cells that mediate autoimmune-like chronic graft-versus-host disease. *J Immunol* 189, 222–233. [PubMed: 22649197]
- Zeiser R, and Blazar BR (2016). Preclinical models of acute and chronic graft-versus-host disease: how predictive are they for a successful clinical translation? *Blood* 127, 3117–3126. [PubMed: 26994149]
- Zeiser R, and Blazar BR (2017). Pathophysiology of Chronic Graft-versus-Host Disease and Therapeutic Targets. *N Engl J Med* 377, 2565–2579. [PubMed: 29281578]

Significance statement

Chronic graft versus host disease (cGVHD) is a major cause of non-relapse morbidity and mortality following allogeneic hematopoietic stem cell transplantation. Few effective therapies for cGVHD exist. Preclinical animal models of cGVHD provide clinically relevant platforms for mechanistic studies and the testing of novel therapies. Several models have been developed, each recapitulating some, but not all, pathological aspects of human disease. Here, we describe a detailed protocol for allogeneic bone marrow and splenocyte transfer to generate cGVHD in mice, with a particular emphasis on donor cell isolation, recipient injection technique, and clinical monitoring for disease signs and progression.

Figure 1. Overview of spleen and femur dissection.

Abdominal skin is retracted cranially far enough to expose the peritoneum and caudally to expose the tibia (A; boundaries of the iliac and distal tibia denoted by dashed lines). The peritoneal lining is snipped on the left side of the abdomen and the spleen gently retracted with forceps (B-C). Next, the leg from the iliac to the distal tibia is removed by snipping with scissors (A and D). Using a task wipe, the tibia and iliac are gently separated from the femur and excess muscle and connective tissue removed (E).

Figure 2. Cleaned mouse femur – major anatomical landmarks.

Example of an intact mouse femur completely cleaned of connective tissue and muscle. Using either scissors or gentle pressure, the femoral head and knee joint at the intercondylar fossa are removed to allow easy passage of fluid. The needle tip used for flushing the femoral cortex is inserted into the intercondylar fossa.

Figure 3. Chronic GVHD development in a scleroderma model.

Irradiated BALB/c mice receiving B10.D2 whole bone marrow cells and $100\text{--}120 \times 10^6$ whole splenocytes develop alopecia, scaling, and skin thickening as compared to whole bone marrow recipients only (A). Only mice receiving splenocytes developed cGVHD, as quantitatively demonstrated using the cGVHD scoring system from Table 2 (B).

Table 1:

Acute GVHD scoring (maximum score of 10); modified from (Cooke et al., 1996)

	Grade 0	Grade 1	Grade 2
Weight loss	<10%	>10% to <25%	>25%
Posture	No hunching	Hunched at rest	Impaired movement
Activity	Active	Sluggish	Stationary
Fur	No ruffling, groomed	Mild ruffling	Severe ruffling
Skin	Hair and skin intact	Scaling of paws and tails	Alopecia areas

Table 2:

Chronic GVHD scoring; modified from (Young et al., 2012)

Grade	
0.5	No alopecia, erosions/ulcers present
1	Alopecia <1 cm ²
2	Alopecia 1–3 cm ²
3	Alopecia >15% BSA*
4	Alopecia >30% BSA

BSA: body surface area

Table 3:

Select mouse models of chronic GVHD

Model	Donor Strain	Recipient Strain	Conditioning	Cells and dose	Outcome
Scleroderma model	B10.D2	BALB/c	600–900 cGy	TCD BM + $1-12 \times 10^7$ whole splenocytes or $\sim 1 \times 10^6$ purified T cells	Scleroderma (scaling, alopecia, fibrosis)
Scleroderma model	B6	B6D2F1	1110 cGy (2 fractions)	TCD BM + 1×10^6 purified T cells	Scleroderma (scaling, alopecia, fibrosis)
BO model	B6	B10.BR	Cytosan + 830 cGy	TCD BM + 75,000 purified T cells	Pulmonary fibrosis

BO = bronchiolitis obliterans; TCD BM = T-cell deplete bone marrow; cGy = centigray; B6 = C57/Bl6; B6D2F1 = (C57/Bl6 x DBA/2)F1; Cytosan dose: 120 mg/kg/day days -3, -2

Modified from Schroeder et al.(Schroeder and DiPersio, 2011) with additions from Du et al (Du et al., 2017)