Journal of Visualized Experiments

Induction and scoring of graft-versus-host disease in a xenogeneic murine model and quantification of human T cells in mouse tissues using digital PCR

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59107R2
Full Title:	Induction and scoring of graft-versus-host disease in a xenogeneic murine model and quantification of human T cells in mouse tissues using digital PCR
Keywords:	human; immunosuppression; t cell; graft-versus-host disease; xenogeneic; xenogenicGVHD; digital PCR; CD3
Corresponding Author:	Mary A. Markiewicz University of Kansas Medical Center Kansas City, KS UNITED STATES
Corresponding Author's Institution:	University of Kansas Medical Center
Corresponding Author E-Mail:	mmarkiewicz@kumc.edu
Order of Authors:	Amara Seng
	Mary A. Markiewicz
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Kansas City, KS USA

JoVE submission JoVE59107

Department of Microbiology, Molecular Genetics & Immunology

February 19, 2019
Alisha DSouza, Ph.D.
Senior Review Editor
JoVE
617.674.1888

Subject: RE: Revisions required for your JoVE submission JoVE59107R1 - [EMID:e9e4d9c1e4b80050]

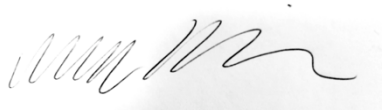
Dear Dr. DSouza,

Thank you for your e-mail dated 11-Feb-2019. The feedback from the reviewers is greatly appreciated. We have reviewed the reviewers' comments and revised the manuscript accordingly.

Please find attached a point-by-point response to the editorial and reviewers' concerns.

We hope the revised version is now suitable for publication and look forward to hearing from you in due course.

Best regards,



Mary A. Markiewicz, Ph.D.
Assistant Professor
Microbiology, Molecular Genetics & Immunology
Scientific Director Flow Core
University of Kansas Medical Center

TITLE:

Induction and Scoring of Graft-Versus-Host Disease in a Xenogeneic Murine Model and Quantification of Human T Cells in Mouse Tissues using Digital PCR

AUTHORS AND AFFILIATIONS:

Amara Seng[1] and Mary A. Markiewicz[1]

[1]Department of Microbiology, Molecular Genetics, and Immunology, University of Kansas Medical Center, Kansas City, KS 66160

Corresponding author:

Mary A. Markiewicz (mmarkiewicz@kumc.edu)

Email Addresses of Co-authors:

Amara Seng (aseng@kumc.edu)

KEYWORDS:

human, immunosuppression, T cell, graft-versus-host disease, xenogeneic GVHD, digital PCR, CD3

SUMMARY:

Here, we present a protocol to induce and score disease in a xenogeneic graft-versus-host disease (xenoGVHD) model. xenoGVHD provides an in vivo model to study immunosuppression of human T cells. Additionally, we describe how to detect human T cells in tissues with digital PCR as a tool to quantify immunosuppression.

ABSTRACT:

Acute graft-versus-host disease (GVHD) is a significant limitation for patients receiving hematopoietic stem cell transplant as therapy for hematological deficiencies and malignancies. Acute GVHD occurs when donor T cells recognize host tissues as a foreign antigen and mount an immune response to the host. Current treatments involve toxic immunosuppressive drugs that render patients susceptible to infection and recurrence. Thus, there is ongoing research to provide an acute GVHD therapy that can effectively target donor T cells and reduce side effects. Much of this pre-clinical work uses the xenogenic GVHD (xenoGVHD) murine model that allows for testing of immunosuppressive therapies on human cells rather than murine cells in an in vivo system. This protocol outlines how to induce xenoGVHD and how to blind and standardize clinical scoring to ensure consistent results. Additionally, this protocol describes how to use digital PCR to detect human T cells in mouse tissues, which can subsequently be used to quantify efficacy of tested therapies. The xenoGVHD model not only provides a model to test GVHD therapies but any therapy that can suppress human T cells, which could then be applied to many inflammatory diseases.

INTRODUCTION:

Allogeneic hematopoietic stem cell transplant (HSCT) has become routine treatment for patients suffering from hematological malignancies such as leukemia with poor prognosis. A significant

complication of HSCT is acute graft-versus-host disease (GVHD). A 2012 study reported that acute GVHD developed in 39% of HSCT patients receiving transplants from sibling donors and 59% of patients receiving transplants from unrelated donors[1]. Acute GVHD occurs when donor-derived T cells attack recipient's organs. The only successful therapy for GVHD is treatment with highly immunosuppressive drugs[2], which are highly toxic and increase the risk of infection and tumor recurrence. Thus, despite improvements that have been made in acute GVHD survival in recent years[3-5], there is still a critical need for improved GVHD therapies with minimal toxicity that promote long-term remission.

The overall goal of the following methods is to induce and score xenogeneic GVHD (xenoGVHD). The xenoGVHD model was developed as a tool to induce acute GVHD with human cells rather than murine cells allowing for more direct translation of pre-clinical GVHD research to clinical trials[6]. This model involves intravenously injecting human peripheral blood mononuclear cells (PBMC) into NOD-SCID IL-2Rγnull (NSG) mice that are sublethally irradiated. Injected human T cells are activated by human antigen presenting cells (APCs) presenting murine antigen and the activated T cells migrate to distant tissues resulting in systemic inflammation and ultimately death[6-10]. Disease pathology and progression in the xenoGVHD model closely mimic human acute GVHD. Specifically, the pathogenic human T cells are reactive to murine major histocompatibility complex (MHC) proteins, which is similar to the T cell alloreactivity in human GVHD[6,9]. The primary advantage of the xenoGVHD model over the mouse MHC-mismatch model, the other widely used GVHD model, is it allows for testing of therapies on human cells rather than murine cells. This allows for testing of products that can directly be translated to the clinic without any modifications because they are made to target human cells. Recently, this model has been used to test a human anti-IL-2 antibody[11], human thymic regulatory T cells (Tregs)[12] and human mesenchymal stem cells[13] as potential treatments for acute GVHD. In a wider context, this model can be used as an in vivo suppression assay for any drug or cell type that can suppress human T cell activity. For example, Stockis et al.[14] used the xenoGVHD model to study the effect of blocking integrin αVβ8 on Treg suppressive activity in vivo. Thus, the xenoGVHD model can provide insight into the mechanism of any therapy targeting T cells in an in vivo setting.

An additional method described in this protocol is how to detect human T cells in mouse tissues using digital polymerase chain reaction (dPCR). The goal of this method is to offer a tool to quantify migration and proliferation of T cells in target tissues, which measure efficacy of immunosuppressive therapies being tested in this model. dPCR is a relatively novel method for quantification of nucleic acids[15]. Briefly, the PCR reaction mixture is divided into partitions that contain small numbers of the target sequence or no target at all. The target sequence is then amplified and detected using DNA intercalating dyes or fluorescent target-specific probes. dPCR quantifies the number of copies of target sequence based on the fraction of positive partitions and Poisson's statistics[15,16]. Detecting T cells with dPCR requires much less tissue compared with other alternative methods, including flow cytometry and histology, and can be performed on frozen or fixed tissue. dPCR does not require a standard curve to determine copy numbers, nor are technical replicates required. This reduces the amount of reagent and template DNA needed for dPCR compared to traditional quantitative PCR (qPCR)[16]. Partitioning the PCR reaction into sub-reactions in dPCR effectively concentrates targets[17]. Thus, dPCR is primarily a tool for

detection of rare targets in a large amount of non-target DNA. For example, dPCR is being used to detect bacterial contamination in milk[18], identify rare mutations in the estrogen receptor gene[19], and detect circulating tumor DNA in the blood of patients[20]. In this protocol, dPCR serves as an efficient tool for detecting and quantifying human T cells in tissues of mice with xenoGVHD.

PROTOCOL:

All mouse experiments were performed in compliance, and with approval from, the University of Kansas Medical Center Institutional Animal Care and Use Committee. All healthy human blood samples were obtained under informed consent and with approval from the Institutional Review Board at the University of Kansas Medical Center.

1. Irradiation of NSG Mice

1.1. One day prior to PBMC injection, irradiate 8–12-week old NSG mice (either sex can be used). In a sterile biosafety cabinet, place mice in a sterilized pie cage or microisolator. Irradiate mice in a Cs^{137} source or small animal irradiator (e.g., RS 2000) with a total dose of 150 cGy with slow rotation to ensure even irradiation.

1.2. Place mice into clean cages in a sterile biosafety cabinet.

2. Preparation of human PBMC for injection

2.1. Collect enough healthy human blood to isolate 1.1 x 10^7 PBMC per mouse. Dilute the heparinized blood in an equal volume of 2% fetal bovine serum (FBS) in phosphate buffered saline (PBS).

NOTE: With this protocol, the PMBC yield is generally 0.5–1 x 10^7 per 10 mL of whole blood. Each mouse will receive 10^7 PBMC in 100 µL of PBS. The extra 1 x 10^6 in 10 µL per mouse ensures that each mouse receives the full dosage of PBMC, should there be any issues with filling syringes.

2.2. Add 15 mL lymphocyte separation density gradient medium (e.g., Ficoll) to a 50 mL conical tube and then carefully overlay up to 25 mL of diluted blood on top of the density gradient. Centrifuge the tube containing density gradient and diluted blood at 400 x *g* for 40 min at room temperature without brake.

2.3. Harvest the PBMC interface into 10 mL of PBS in a 50 mL conical tube. Centrifuge the cells at 400 x *g* for 10 min. Remove the supernatant.

2.4. Loosen pellet by flicking tube and resuspend in 10 mL PBS to wash the PBMC. Centrifuge the cells at 400 x *g* for 5 min.

2.5. (Optional) Discard the supernatant and lyse red blood cells (RBCs) in the cell pellet by adding a volume of Ammonium-Chloride-Potassium (ACK) lysis buffer that is equal to the pellet volume. Gently resuspend the pellet and swirl the tube for 30–60 s. Fill tube with serum-free RPMI media

and centrifuge the tube at 400 x *g* for 5 min.

2.6. Remove the supernatant and re-suspend the cell pellet in 5 mL of PBS. Count the cells using trypan blue exclusion with a hemocytometer and a microscope.

2.7. Centrifuge the cells at 400 x *g* for 5 min. Remove the supernatant and resuspend cells at 1 x 10^8 cells/mL in PBS.

3. Retro-orbital injection of human PBMC into mice[21]

3.1. Place an anesthesia chamber in a laminar flow hood to maintain sterility. Pre-charge the anesthesia chamber with 5% isoflurane and 1 L/min oxygen flow rate for 5 min.

3.2. Reduce isoflurane to 2% and put mice from the same cage into the anesthesia chamber. Once mice lose righting, anesthetize mice for 5 min in chamber. During this time, pre-fill syringe with 100 µL (1 x 10^7 cells) of cell suspension.

3.3. Place mouse on a heating pad with its nose in a nose cone to maintain anesthesia. Check for loss of consciousness by pinching a foot pad and checking for lack of reflex.

3.4. Restrain mouse with the thumb and middle finger. Insert the 28 G 1/2 in needle with the bevel down lateral to the medial canthus, through the conjunctival membrane until the back of the eye is reached. Slightly retract the needle and slowly inject 100 µL of cells (1 x 10^7 cells).

3.5. Fully retract the needle and properly dispose of the syringe and needle. Close the eyelid and apply mild pressure to the injection site with a gauze sponge.

3.6. Examine the injection site for swelling or other visible trauma. Allow the mouse to regain consciousness in a sterile cage lined with paper towels to prevent aspiration of bedding before moving to home cage.

NOTE: Tail vein injection is an alternative method of intravenous injection of PBMC for this protocol as described by Macholz et al.[22].

4. Clinical Scoring of acute GVHD in Mice (Figure 1)[23]

4.1. Measure GVHD score every other day until mice reach a score of a 2 and then every day until day of sacrifice.

4.1.1. Place the cage in a laminar flow hood, remove the food and water and put the lid back on. Score **activity** by observing mice for 5 min and assign scores as follows: 0 = mouse starts walking within a couple minutes and keeps walking around cage, 1 = mouse takes a more than a couple minutes to get up and walks slowly around cage, 2 = mouse doesn't get up in 5 min and only walks when touched.

4.2. Weigh each mouse in a glass beaker and give a **weight loss** score: 0 = <10% change, 1 = 10–25% change and 2 = >25% change.

4.3. While the mouse is still in the beaker, inspect for **posture**: 0 = normal, 1 = hunching at rest, 2 = hunching impairs movement; **fur texture**: 0 = normal, 1 = mild to moderate ruffling, 2 = severe ruffling, and **skin integrity** : 0 = normal, 1 = scaling of paws/tail, 2 = obvious areas of denuded skin (look at ears, tail and paws for scaling).

4.4. With five categories and a score of 0–2 for each category, a mouse can reach a maximum score of 10. When a mouse reaches a score of 7 or greater or reaches 42 days post-injection, euthanize mouse via CO_2 euthanasia or other method approved by the local Institutional Animal Care and Use Committee.

NOTE: To ensure accuracy of results, the scoring is performed by a researcher who is blinded to the treatment groups[24]. Additionally, although >20% of body weight loss is the recommended humane endpoint for many IACUC protocols, with additional justification IACUC approval of this protocol can be obtained.

5. Harvesting tissues for genomic DNA from euthanized mice and isolating genomic DNA

5.1. Dissect mice using sterile surgical tools. Cut a small piece of tissue about 3 mm x 0.5 mm in size from an organ of interest, e.g., lung, liver, or spleen. Weigh the tissue sample and place the tissue piece in a sterile 1.5 mL tube. If collecting multiple tissues, wash tools with ethanol between cutting each organ.

5.2. Freeze tissues by submerging the tubes containing the tissue in liquid nitrogen until it stops bubbling and store in -80 °C overnight. Thaw the tissue samples and lyse them according to a genomic DNA (gDNA) isolation kit.

5.3. Isolate genomic DNA according to manufacturer's instructions as described in the kit. Be sure to note the amount of tissue that was processed per microliter of eluted gDNA (mg/µL).

NOTE: Frozen tissues can be stored at -80 °C for processing at a later time.

6. Quantification of human T cells using digital PCR (Figure 2)

6.1. Prepare digital PCR reactions according to the protocol for DNA binding dyes for the digital PCR machine being used. Use the following primers specific for human CD3 epsilon genomic DNA (NCBI Reference Sequence: NG_007383.1); Forward Primer: AGGCTGCCTTAACTCCCAAG, Reverse Primer: GCCCTACCAGCTGTGGAAAC. These primers will yield a single band of 105 bp.

NOTE: Add 0.5 µL of a restriction enzyme, such as HindIII, to each reaction to digest the genomic DNA. Be sure to test out different gDNA dilutions to optimize separation of positive and negative

droplets. Additionally, a subset of infiltrating T cells may be non-pathogenic regulatory T cells. These cells can be quantified using additional primers for regulatory T cell markers.

6.2. Carry out the digital PCR reaction under the following conditions: Lid at 105 °C, 95 °C for 10 min (1 cycle); 95 °C for 30 s, ramp 2 °C/s and 55 °C for 1 min, ramp 2 °C/s (40 cycles); 72 °C for 10 min, 12 °C hold.

NOTE: These parameters may need to be adjusted depending on digital PCR equipment.

6.3. Acquire digital PCR data with analysis software. Report data as numbers of copies per mg of tissue using the following equation: (number of copies/µL) x (µL of gDNA in reaction) x (dilution factor)/(mg of tissue/µL of total gDNA)

REPRESENTATIVE RESULTS:

Sublethally irradiated 8–12-week old NSG mice of both sexes that received human PBMC started displaying clinical signs of GVHD around day 10 post injection compared to negative control mice that received PBS only (**Figure 1A**). XenoGVHD mice had a median survival of 23.5 days (**Figure 1B**). With digital PCR, CD3 epsilon positive human T cells could be detected in the lung and liver samples of mice that received human PBMC. Tissue samples from mice injected with PBS were used as controls (**Figure 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: **GVHD disease progression.** Sublethally irradiated 8–12-week-old male and female NSG mice were retro-orbitally injected with 1 x 10^7 human PBMC (n = 6) or PBS (n = 4) as a negative control. Data shown are the combined results of three independent experiments. (**A**) GVHD score was measured every other day until mice reached a score of a 2 and then every day until day of sacrifice. Data reported at each time point for GVHD score is the mean ± SEM score of the live mice combined with the last scores of any deceased mice in each group. * $p < 0.05$ as determined by Mann Whitney U test. (**B**) Kaplan-Meier curve of survival. Death was marked when GVHD score was ≥7. * $p < 0.05$ as determined by log-rank test.

Figure 2: Detection of human T cells using digital PCR. Lung and liver samples were collected from mice that were retro-orbitally injected with PBS (n = 3) or 1 x 10^7 human PBMC (n = 3) when mice reached a GVHD score of ≥7 or 42 days post injection. Data were collected from 3 independent experiments. gDNA was isolated and digital PCR was used to determine the copies of human CD3 epsilon per milligram of tissue. (**A**) Representative digital PCR plot of lung and liver samples from a mouse injected with PBS or PBMC. (**B**) Quantification of copies of human CD3 epsilon per millgram of tissue from mice injected with PBS or PBMC. * $p \leq 0.05$ as determined by Mann Whitney U test.

DISCUSSION:

Disease progression is generally consistent in the xenoGVHD model, even with injection of PBMC from different donors, so multiple experiments can be combined. The key steps required to

maintain this consistency are proper i.v. injection technique, blinding and consistent scoring. A study by Nervi et al.[25] demonstrated that compared to intravenous tail vein injection, retro-orbital injections of PBMC resulted in more consistent engraftment and more severe GVHD. Leon-Rico et al.[26] also demonstrated that retro-orbital injections resulted in more consistent hematopoietic stem cell engraftment in mice compared to tail vein injection. However, if necessary, tail vein injections can be used as an alternative method in the xenoGVHD model[27-29].

The problem of variability of outcomes associated with tail vein injections can be reduced by increasing the number of subjects. Additionally, it is important that the person scoring the mice is blinded to mouse treatment to avoid bias in scoring of more subjective criteria such as activity or fur texture. The importance of blinded GVHD scoring has also been demonstrated in the clinic[24]. The person injecting the mice should not be the same person scoring the mice. If this is not possible, control and treatment tubes can be randomized and labeled with new ID's by another person (i.e., control is A, treatment is B) so injections can be blinded. Mouse scoring should be performed around the same time every day and on the same days post-injection between different experiments.

One potential obstacle in this model is the lack of GVHD development. This could be due to reduced viability of PBMC which can be addressed by checking viability of PBMC by counting cells with trypan blue. If trouble with cell viability is encountered, the cells can be put in PBS supplemented with 2% FBS to improve survival. Also, fewer mice can be injected at a time to reduce the time cells sit at room temperature. The efficacy of the retro-orbital injection can be the problem. Mice that are injected with PBMC but do not develop GVHD can be euthanized and immune cells isolated from their spleens can be analyzed for presence of human cells via dPCR or flow cytometry. If there is poor engraftment, then there is likely a problem with injection technique. As a test of injection technique, mice can be injected retro-orbitally with 200 µL of Evans blue dye. If the injection is successful, the ears, paws and tail of the mouse will turn blue.

The xenoGVHD model closely mimics human acute GVHD disease pathogenesis and progression[30]. Unlike the murine MHC mismatch GVHD model, the xenoGVHD model allows testing of the effect of immunosuppressive therapies, including human cell therapies, on human cells rather than murine cells. This reduces the variation due to species differences when applying research results to the clinic. The xenoGVHD model can also serve as an in vivo suppression assay in other fields of T cell research. Thus, results from experiments using the xenoGVHD model can be applied to any human T cell-mediated inflammatory disease in addition to GVHD.

There are limitations of the xenoGVHD model. These include experimental variability and possible differences in GVHD treatment compared to the clinic. Experimental inconsistencies can stem from differences in mouse strain, sites of PBMC injection, radiation dose and microbial environment[30,31]. Thus, laboratories using this model should attempt to standardize these parameters to ensure consistent outcomes. In this protocol, we describe scoring methods that help reduce variability in scoring. Factors that may limit comparability of xenoGVHD data to clinical outcomes include the lack of control groups treated with GVHD prophylactic drugs and the use of irradiation as the only source of conditioning in the xenoGVHD model[31]. Additionally,

the mechanism of xenoGVHD does not completely recapitulate the underlying pathogenesis in human GVHD. For example, it is donor APCs rather than host APCs that activate human T cells in the xenoGVHD model, whereas host APCs play a significant role in human GVHD[7]. Thus, as with most pre-clinical models, there are inconsistencies and incompatibilities between xenoGVHD and human GVHD that may limit the application of data generated from the xenoGVHD to the clinic.

ACKNOWLEDGMENTS:

We would like to acknowledge the laboratory of Lane Christenson for providing the digital PCR machine used in these experiments and for the technical support provided. We would also like to thank Dr. Thomas Yankee for his guidance and mentorship. These studies were supported by the Tripp Family Foundation.

DISCLOSURES:

No conflicts of interest declared.

REFERENCES:

1 Jagasia, M. et al. Risk factors for acute GVHD and survival after hematopoietic cell transplantation. *Blood.* **119** (1), 296-307, (2012).

2 Bolanos-Meade, J. et al. Phase 3 clinical trial of steroids/mycophenolate mofetil vs steroids/placebo as therapy for acute GVHD: BMT CTN 0802. *Blood.* **124** (22), 3221-3227; quiz 3335, (2014).

3 Gooley, T. A. et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. *New England Journal of Medicine.* **363** (22), 2091-2101, (2010).

4 Hahn, T. et al. Significant improvement in survival after allogeneic hematopoietic cell transplantation during a period of significantly increased use, older recipient age, and use of unrelated donors. *Journal of Clinical Oncology.* **31** (19), 2437-2449, (2013).

5 Khoury, H. J. et al. Improved survival after acute graft-versus-host disease diagnosis in the modern era. *Haematologica.* **102** (5), 958-966, (2017).

6 King, M. A. et al. Human peripheral blood leucocyte non-obese diabetic-severe combined immunodeficiency interleukin-2 receptor gamma chain gene mouse model of xenogeneic graft-versus-host-like disease and the role of host major histocompatibility complex. *Clinical & Experimental Immunology.* **157** (1), 104-118, (2009).

7 Lucas, P. J., Shearer, G. M., Neudorf, S., Gress, R. E. The human antimurine xenogeneic cytotoxic response. I. Dependence on responder antigen-presenting cells. *Journal of Immunology.* **144** (12), 4548-4554, (1990).

8 Ito, R. et al. Highly sensitive model for xenogenic GVHD using severe immunodeficient NOG mice. *Transplantation.* **87** (11), 1654-1658, (2009).

9 Kawasaki, Y. et al. Comprehensive Analysis of the Activation and Proliferation Kinetics and Effector Functions of Human Lymphocytes, and Antigen Presentation Capacity of Antigen-Presenting Cells in Xenogeneic Graft-Versus-Host Disease. *Biology of Blood and Marrow Transplantation.* **24** (8), 1563-1574, (2018).

10 Ito, R. et al. A Novel Xenogeneic Graft-Versus-Host Disease Model for Investigating the Pathological Role of Human CD4(+) or CD8(+) T Cells Using Immunodeficient NOG Mice. *American Journal of Transplantation.* **17** (5), 1216-1228, (2017).

11 Trotta, E. et al. A human anti-IL-2 antibody that potentiates regulatory T cells by a structure-based mechanism. *Nature Medicine.* **24** (7), 1005-1014, (2018).
12 Dijke, I. E. et al. Discarded Human Thymus Is a Novel Source of Stable and Long-Lived Therapeutic Regulatory T Cells. *American Journal of Transplantation.* **16** (1), 58-71, (2016).
13 Huang, F. et al. Human Gingiva-Derived Mesenchymal Stem Cells Inhibit Xeno-Graft-versus-Host Disease via CD39-CD73-Adenosine and IDO Signals. *Frontiers in Immunology.* **8** 68, (2017).
14 Stockis, J. et al. Blocking immunosuppression by human Tregs in vivo with antibodies targeting integrin alphaVbeta8. *Proceedings of the National Academy of Sciences of the United States of America.* **114** (47), E10161-E10168, (2017).
15 Vogelstein, B. & Kinzler, K. W. Digital PCR. *Proceedings of the National Academy of Sciences of the United States of America*. **96** (16), 9236-9241, (1999).
16 Quan, P. L., Sauzade, M. & Brouzes, E. dPCR: A Technology Review. *Sensors (Basel).* **18** (4), (2018).
17 Sykes, P. J. et al. Quantitation of targets for PCR by use of limiting dilution. *Biotechniques.* **13** (3), 444-449, (1992).
18 Ma, H. et al. Evaluation of Bacterial Contamination in Goat Milk Powder Using PacBio Single Molecule Real-Time Sequencing and Droplet Digital PCR. *Journal of Food Protection.* 10.4315/0362-028X.JFP-17-535 1791-1799, (2018).
19 Vitale, S. R. et al. An optimized workflow to evaluate estrogen receptor gene mutations in small amounts of cell-free DNA. *Journal of Molecular Diagnostics.* 10.1016/j.jmoldx.2018.08.010, (2018).
20 Gorgannezhad, L., Umer, M., Islam, M. N., Nguyen, N. T. & Shiddiky, M. J. A. Circulating tumor DNA and liquid biopsy: opportunities, challenges, and recent advances in detection technologies. *Lab Chip.* **18** (8), 1174-1196, (2018).
21 Yardeni, T., Eckhaus, M., Morris, H. D., Huizing, M. & Hoogstraten-Miller, S. Retro-orbital injections in mice. *LabAnimal.* **40** (5), 155-160, (2011).
22 Machholz, E., Mulder, G., Ruiz, C., Corning, B. F. & Pritchett-Corning, K. R. Manual restraint and common compound administration routes in mice and rats. *Journal of Visualized Experiments.* 10.3791/2771 (67), (2012).
23 Cooke, K. R. et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. *Blood.* **88** (8), 3230-3239, (1996).
24 Weisdorf, D. J. et al. Prospective grading of graft-versus-host disease after unrelated donor marrow transplantation: a grading algorithm versus blinded expert panel review. *Biology of Blood and Marrow Transplantation.* **9** (8), 512-518, (2003).
25 Nervi, B. et al. Factors affecting human T cell engraftment, trafficking, and associated xenogeneic graft-vs-host disease in NOD/SCID beta2mnull mice. *Experimental Hematology.* **35** (12), 1823-1838, (2007).
26 Leon-Rico, D. et al. Comparison of haematopoietic stem cell engraftment through the retro-orbital venous sinus and the lateral vein: alternative routes for bone marrow transplantation in mice. *LabAnimal.* **49** (2), 132-141, (2015).
27 Ali, N. et al. Xenogeneic graft-versus-host-disease in NOD-scid IL-2Rgammanull mice display a T-effector memory phenotype. *PLoS One.* **7** (8), e44219, (2012).

28 van Rijn, R. S. et al. A new xenograft model for graft-versus-host disease by intravenous transfer of human peripheral blood mononuclear cells in RAG2-/- gammac-/- double-mutant mice. *Blood.* **102** (7), 2522-2531, (2003).
29 Wunderlich, M. et al. OKT3 prevents xenogeneic GVHD and allows reliable xenograft initiation from unfractionated human hematopoietic tissues. *Blood.* **123** (24), e134-144, (2014).
30 Schroeder, M. A. & DiPersio, J. F. Mouse models of graft-versus-host disease: advances and limitations. *Disease Models & Mechanisms.* **4** (3), 318-333, (2011).
31 Zeiser, R., Blazar, B. R. Preclinical models of acute and chronic graft-versus-host disease: how predictive are they for a successful clinical translation? *Blood.* **127** (25), 3117-3126, (2016).

Figure 1

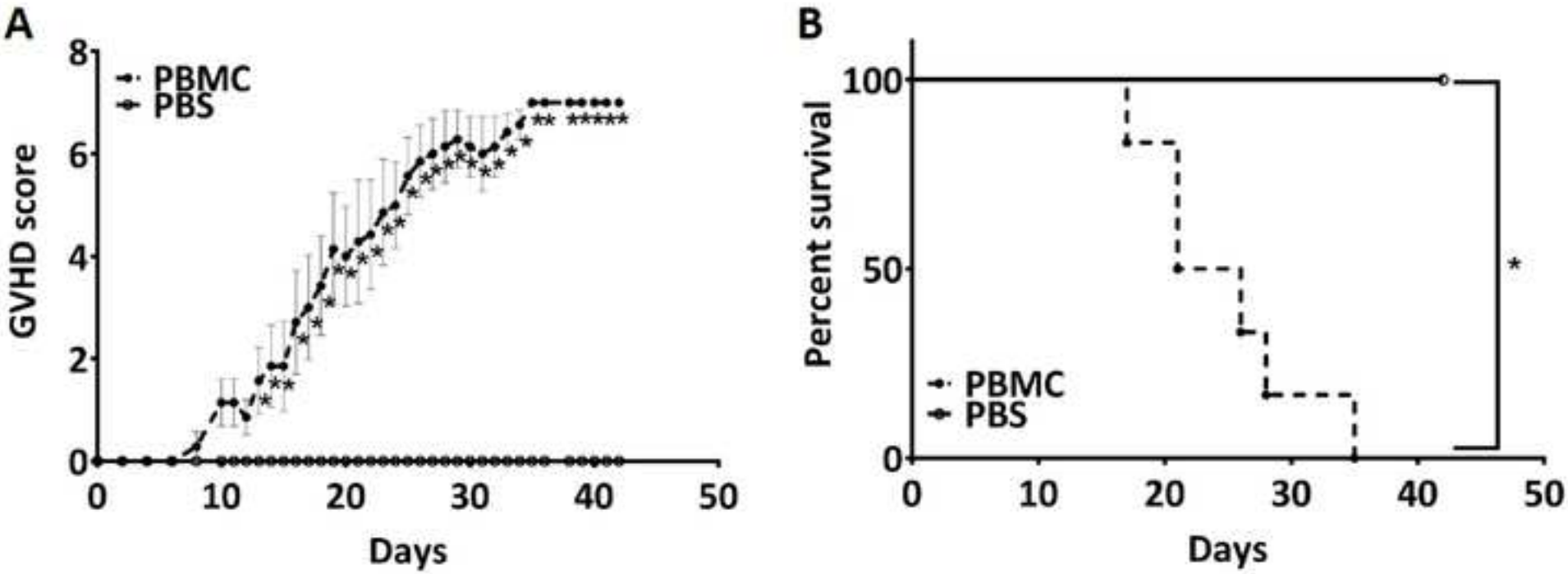
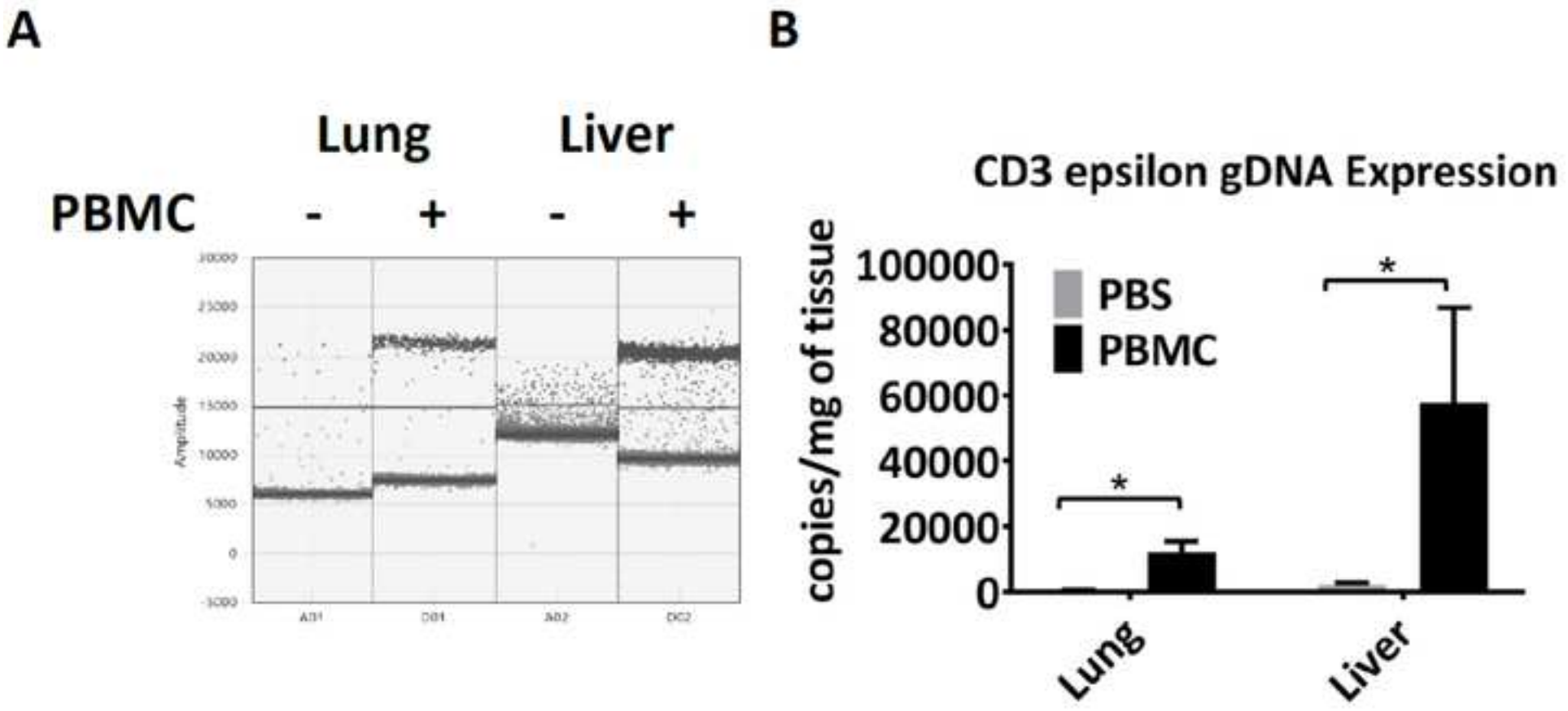


Figure 2



Name of Material/ Equipment	Company	Catalog Number
1.5 mL eppendorf tubes	Fisher	05-408-129
10 mL serological pipet	VWR International	89130-898
10mL BD Vacutainers - Green capped with Sodium Heparin	Becton Dickinson	366480
250 µL Ranin pipette tips	Rainin	17001118
50 mL conical tube	VWR International	89039-656
96-Well ddPCR plate	Bio-Rad	12001925
ACK (Ammonium-Chloride-Potassium) Lysing Buffer	Lonza	10-548E
Alcohol Wipes	Fisher Scientific	6818
Anesthesia Chamber	World Precision Instruments	EZ-178
Anesthesia Machine	Parkland Scientific	PM1002
BD Vacutainer Safety-Lok Blood Collection Set	Becton Dickinson	367281
DG8 Cartridges and Gaskets for QX100/QX200 Droplet Generator	Bio-Rad	1864007
DNAse and RNAse free Molecular Grade H2O	Life Technologies	1811318
Ethyl alcohol, Pure,200 proof, for molecular biology	Sigma-Aldrich	E7023-500ML
Fetal Bovine Serum	Atlanta Biologicals	S11150
Ficoll	Fisher Scientific	45001750
Insulin Syringe	Fisher Scientific	329424
Isoflurane	Sigma-Aldrich	CDS019936
Liquid nitrogen	N/A	N/A
Mouse Irradiator Pie Cage	Braintree Scientific, Inc.	MPC 1
Nexcare Gentle Paper Tape (a.k.a. 3M Micropore Surgical Tape / 3/4")	Fisher Scientific	19-027-761
P1000 pipetman	MidSci	A-1000
P200 pipetman	MidSci	A-200
Pierceable Foil Heat Seal	Bio-Rad	1814040
Pipetaid Gilson Macroman	Fisher Scientific	F110756

Pipet-Lite Multi Pipette L8-200XLS+	Rainin	17013805
Qiagen DNeasy Blood and Tissue Kit	Qiagen	69506
qPCR plates	VWR International	89218-292
QX200 Droplet Digital PCR System	Bio-Rad	12001925
QX200 Droplet Generation Oil for EvaGreen	Bio-Rad	1864006
QX200 ddPCR EvaGreen Supermix	Bio-Rad	1864033
RNase and DNase-free plate seal	Thermo Scientific	12565491
RPMI Advanced 1640	Life Technologies	12633012
Sterile Gauze Pads (2" x 2", 12-Ply)	Fisher Scientific	67522
Sterile Phosphate Buffered Saline	Fisher Scientific	21040CV
Sterile reservoir	VWR International	89094-662
Surgial Scissors	Kent Scientific	INS600393-4
Surgical Forceps	Kent Scientific	INS650914-4

Comments/Description

Do not use other pipettes or pipet tips for droplet generation

Optional

Provided by animal facility

Provided by animal facility

Provided by animal facility

Holds up to 11 mice

Do not use other pipettes or pipet tips for droplet generation

Includes droplet generator, droplet reader, laptop computer, software, associated component consumables, for EvaGreen or probe-based digital PCR applications

jove VISUALIZED
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Induction and clinical scoring of GVHD in a Xenogenic animal model and quantification of human T cells in mouse tissues using digital PCR

Author(s): Amara Seng and Mary A. Markiewicz

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove VISUALIZED JOURNAL OF EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Mary Markiewicz
Department:	Microbiology, Molecular Genetics + Immunology
Institution:	University of Kansas Medical Center
Title:	Assistant Professor
Signature:	[signature]
Date:	9/14/18

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

JoVE submission JoVE59107

Reviewers' comments:

Reviewer #3: Thank you for your comments. Please, find an answer to each point below. Please, note that line references correlated to the unmarked, final version of the revised manuscript. All changes in the revised document are underlined.

The manuscript written by Amara Seng and Mary Markiewicz propose a method to score severity of GvHD using multiple parameters in a xenograft model using human PBMC in NSG mice. As there is no single standardized method to score xenogeneic GvHD and standardizing the readouts as suggested in the manuscript can be helpful in comparing results from studies conducted by different groups. However, there are few points to be addressed in the current form of manuscript:

1. In the abstract author states " Thus, there is ongoing research to provide an acute GVHD therapy that can effectively target host T cells and reduce side effect". It was not clear why host T cells are targeted instead of the donor T cells.

- The reviewer is correct and this should read as “donor T cells” rather than “host T cells.” The text has been amended accordingly. Please, refer to line 36.

2. In section 4, author states that monitoring the movements of the mice is one of the parameters. "Movement" can be better defined, as movement can subjective in a way, and minimizing subjectivity is important in developing a standardized scoring method.

- Movement in section 4 has been specified as walking. Please, refer to lines 169-171.

3. Labelling the first paragraph as 4.1, next, 4.2, and 4.3 … will be easier to follow. Also consider separate each parameters looked as subsections, instead of having multiple parameters in the current section 4.1

- Sections were numbered and multiple steps were combined as advised by the editors in earlier submitted versions of the protocol.

4. Author states that body weight loss of <10% is score of 1, and 10-25% is score of 2. Given that humane endpoint for mice is ~20-25% body weight loss in many animal facilities, and that author states total score of 7 as end point, many of the mice may need to be sac'ed before reaching end point 7, due to the body weight loss.

- 25% is the cutoff for the scoring system recommended by Cooke et al. [1] and has been used in multiple published studies [2-4]. Our IACUC approved the weight cutoff and a note was added to explain that additional justification may be needed to get this protocol approved by IACUC boards. Please, refer to lines 185-187.

1. Cooke, K. R. et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. Blood. 88 (8), 3230-3239, (1996).
2. Dijke, I. E. et al. Discarded Human Thymus Is a Novel Source of Stable and Long-Lived Therapeutic Regulatory T Cells. Am J Transplant. 16 (1), 58-71, (2016).

3. Toubai, T., et al., Mitochondrial Deacetylase SIRT3 Plays an Important Role in Donor T Cell Responses after Experimental Allogeneic Hematopoietic Transplantation. J Immunol, 2018. 201(11): p. 3443-3455.
4. Wolf, D., et al., Superior immune reconstitution using Treg-expanded donor cells versus PTCy treatment in preclinical HSCT models. JCI Insight, 2018. 3(20).

5. Can paleness of paw and ear be used as one of the read out? Xenogeneic GvHD is known to cause anemia and ear and paw colors are one of easier symptoms to look at.

- This would be possible. However, this is outside the scope of this protocol as the goal was to define a standardized procedure for an existing scoring system that has been used in previous publications.

6. In section 5.1, it will be helpful to describe the method to freeze isolated tissues in Liquid nitrogen. Also just spell out liquid nitrogen for simplicity as it is only used once in the manuscript.

- Method to freeze isolated tissues was described. Liquid nitrogen was spelled out as recommended. Please, refer to lines 196-197.

7. Quantifying CD3 can be used as a readout for infiltrating T cells, but not all infiltrating T cells are pathogenic. For example, increased infiltration of regulatory T cells may mean better outcome for GvHD patients. This need to be discussed.

- A note was added to explain that some infiltrating T cells may be regulatory T cells and that these T cells could be quantified using primers for regulatory T cell markers. Please, refer to lines 213-214.

8. Figure 2, describe when the tissue was harvested. Is it after full blown GvHD or at the start of GvHD?

- The phrase “when mice reached a GVHD score of ≥7 or 42 days post injection“ was added to describe when tissues were collected. Please refer to lines 247-248.

9. How many times has the studies described in Fig 1 and 2 performed? Indicate the replicate.

- Please refer to Figure 1 and 2 legends for replicate N values. The number of experiments was also added to clarify replicates. Please, refer to lines 239 and 248-249.

Reviewer #4: We appreciate your comments. Our answers to your points are as follows. Please, note that line references correlated to the unmarked, final version of the revised manuscript. All changes in the revised document are underlined.

Manuscript Summary:
The manuscript, "Induction and scoring of graft-versus-host disease in a xenogenic murine model and quantification of human T cells in mouse tissues using digital PCR" by Seng and Markiewicz presents a clear and thorough protocol for a highly relevant and useful model for

graft versus host disease (GVHD). The xenogenic transplant model in NSG mice enables in vivo experimentation on the human immune system, particularly as related to GVHD. The protocol is clearly written, providing end-to-end method for the transplantation and analysis of GVHD experiments in this model. The sample data are clearly presented and demonstrate biologically relevant outcomes. Overall, this is an excellent method paper with only a few minor questions.

Major Concerns:
None

Minor Concerns:

1. Section 4.1. The scoring for weight loss is clear, though may be problematic for some IACUC protocols- many institutions (including all 3 that I've been at as a trainee and faculty member) recommend euthanizing mice who lose > 20% of their body weight. What is the rationale for 25% as the cutoff for the stage 3 scoring?

- 25% is the cutoff for the scoring system recommended by Cooke et al. [1] and has been used in multiple published studies [2-4]. Our IACUC approved the weight cutoff and a note was added to explain that additional justification may be needed to get this protocol approved by IACUC boards. Please, refer to lines 184-187.

1. Cooke, K. R. et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. Blood. 88 (8), 3230-3239, (1996).
2. Dijke, I. E. et al. Discarded Human Thymus Is a Novel Source of Stable and Long-Lived Therapeutic Regulatory T Cells. Am J Transplant. 16 (1), 58-71, (2016).
3. Toubai, T., et al., Mitochondrial Deacetylase SIRT3 Plays an Important Role in Donor T Cell Responses after Experimental Allogeneic Hematopoietic Transplantation. J Immunol, 2018. 201(11): p. 3443-3455.
4. Wolf, D., et al., Superior immune reconstitution using Treg-expanded donor cells versus PTCy treatment in preclinical HSCT models. JCI Insight, 2018. 3(20).

2. Section 5. "about the size of a grain of rice" seems like it may not be the most clear instruction. Perhaps a recommendation to something more along the lines of "3 mm x 0.5 mm"? Just a suggestion.

- The phrase "about the size of a grain of rice" was changed to “3 mm x 0.5 mm." Please, refer to lines 191-192.

Reviewer #5: Thank you for taking the time to provide comments on our manuscript. Our responses are as follows. Please, note that line references correlated to the unmarked, final version of the revised manuscript. All changes in the revised document are underlined.

Manuscript Summary:
The protocol is well described.

Minor Concerns:

1. Please describe time and frequency of scoring (including the time of first scoring) after retro-orbital injection in the methods section.

- A description of the frequency of scoring was added to section 4. Please, refer to lines 166-167.

2. Although it's a standard protocol, the authors may want to describe the genomic DNA digestion in a little more detail.

- The genomic DNA digestion is carried out according to manufacturer's instructions in a kit as described in the protocol (now stated in lines 198-199). Since different labs may use different genomic DNA isolation kits, the manufacturer's instructions may vary.