Video Article

Using the BLT Humanized Mouse as a Stem Cell based Gene Therapy Tumor Model

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URL: http://www.jove.com/video/4181
DOI: doi:10.3791/4181

Keywords: Cancer Biology, Issue 70, Stem Cell Biology, Immunology, Biomedical Engineering, Medicine, Bioengineering, Genetics, Oncology, Humanized mice, stem cell transplantation, stem cells, in vivo animal imaging, T cells, cancer, animal model

Date Published: 12/18/2012

Citation: Vatakis, D.N., Bristol, G.C., Kim, S.G., Levin, B., Liu, W., Radu, C.G., Kitchen, S.G., Zack, J.A. Using the BLT Humanized Mouse as a Stem Cell based Gene Therapy Tumor Model. *J. Vis. Exp.* (70), e4181, doi:10.3791/4181 (2012).

Abstract

Small animal models such as mice have been extensively used to study human disease and to develop new therapeutic interventions. Despite the wealth of information gained from these studies, the unique characteristics of mouse immunity as well as the species specificity of viral diseases such as human immunodeficiency virus (HIV) infection led to the development of humanized mouse models. The earlier models involved the use of C. B 17 scid/scid mice and the transplantation of human fetal thymus and fetal liver termed thy/liv (SCID-hu) [1, 2] or the adoptive transfer of human peripheral blood leukocytes (SCID-huPBL) [3]. Both models were mainly utilized for the study of HIV infection.

One of the main limitations of both of these models was the lack of stable reconstitution of human immune cells in the periphery to make them a more physiologically relevant model to study HIV disease. To this end, the BLT humanized mouse model was developed. BLT stands for bone marrow/liver/thymus. In this model, 6 to 8 week old NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ (NSG) immunocompromised mice receive the thy/liv implant as in the SCID-hu mouse model only to be followed by a second human hematopoietic stem cell transplant [4]. The advantage of this system is the full reconstitution of the human immune system in the periphery. This model has been used to study HIV infection and latency [5-8].

We have generated a modified version of this model in which we use genetically modified human hematopoietic stem cells (hHSC) to construct the thy/liv implant followed by injection of transduced autologous hHSC [7, 9]. This approach results in the generation of genetically modified lineages. More importantly, we adapted this system to examine the potential of generating functional cytotoxic T cells (CTL) expressing a melanoma specific T cell receptor. Using this model we were able to assess the functionality of our transgenic CTL utilizing live positron emission tomography (PET) imaging to determine tumor regression (9).

The goal of this protocol is to describe the process of generating these transgenic mice and assessing *in vivo* efficacy using live PET imaging. As a note, since we use human tissues and lentiviral vectors, our facilities conform to CDC NIH guidelines for Biosafety Level 2 (BSL2) with special precautions (BSL2+). In addition, the NSG mice are severely immunocompromised thus, their housing and maintenance must conform to the highest health standards (http://jaxmice.jax.org/research/immunology/005557-housing.html).

Video Link

The video component of this article can be found at http://www.jove.com/video/4181/

Protocol

A. Generation of BLT mice

The generation of BLT mice is divided into three (3) parts: (1) processing of fetal tissue and preparation of CD34 human hematopoietic stem cells, (2) transplantation of human tissue, and (3) secondary transplant. For all the protocols, we have provided **Table 1** with reagent information.

1. Tissue Processing and Isolation of CD34 Human Hematopoietic Stem Cells

Fresh fetal tissue or tissue shipped overnight on ice from various organ procurement agencies can be used. Often fetal tissue is not sterile, as evidenced by the production of 1,000 bacterial colonies on blood agar per milliliter of surrounding medium. We routinely wash the tissue twice in 40 ml of sterile PBS. While this does not remove all bacteria, it can make a difference between a successful transplant series and an outcome in

which most of the recipient mice succumb to bacterial infection. As an added step, to further disinfect the tissue, we culture the cell suspension in the presence of antibiotics.

1.1 Processing of Fetal Thymic Tissue

1. Wash thymus by pouring off the medium into a beaker of bleach and using scalpel to prevent the tissue from sliding into the bleach. Fill up the tube with PBS. Cap and invert a few times to wash the thymus. Decant supernatant into bleach. Repeat.
2. Place the thymus tissue in 8 ml of RPMI + 10% Fetal Calf Serum in a 60-mm dish and shear into 1.5 to 2 mm pieces with two scalpels. Shearing with scalpels minimizes tearing and damaging the tissue bits. Damaged tissue tends to become swollen, sticky, and difficult to draw into the implant needle. The number of bits obtained is the first upper limit on transplantable mice.
3. Transfer the suspended bits with a 25 ml pipet (to minimize tissue damage) into a T25 flask. Add 70 µl of piptazo (Zosyn) to flask and culture overnight, **at 37 °C, 5% CO_2**. This considerably reduces contaminating bacteria. If you wish to do tissue typing or any other phenotyping assays, take out 1 ml of cells.

1.2 Processing of Fetal Liver Tissue

1. Wash the liver as in step 1.1.1.
2. Add 10 ml of Iscove's medium and decant everything into a 100 mm Petri dish.
3. With two scalpels, dice the liver into small (~3 mm) pieces. If you run into white connective tissue, scrape the liver from it and discard it in the bleach.
4. Homogenize the tissue by sucking it into a 12 ml syringe fitted with a 16 gauge blunt needle 3 or 4 times and transferring to a 50 ml tube.
5. Prepare the enzymes for tissue digestion as follows: To 10 ml of Iscove's in a 50 ml tube, add 200 µl of each: collagenase type IV (1 mg/ml), hyaluronidase (1 mg/ml), DNase I (2 U/ml).
6. Draw enzymes into a 12 ml syringe and fit it with a 0.22 µ filter. Filter the enzyme/medium directly into the cell suspension.
7. Cap and seal the cells with Parafilm. Rotate at 37 °C for 90 min.
8. Set up a 50 ml tube with a 100 µ cell strainer. Pipet the cell digest through this strainer.
9. Add PBS to the cells to bring the volume up to 50 ml. Split this into two tubes of 25 ml.
10. Underlay the cells with 10 ml of Ficoll. Spin at 2,400 rpm for 20 min without brake.
11. The interface should be thick. Suck it out with a 5 ml pipet and transfer to another tube. You should have two tubes of interface. Add 50 ml of PBS to each and spin at 1,200 rpm for 10 min.
12. Combine pellets into one tube and wash three more times with PBS containing 2% FCS.
13. Finally suspend in 50 ml of RPMI + 10% FCS and count cells using trypan blue. Depending on the size of the tissue, you can get from 10^8 to 10^9 total hepatocytes.
14. Transfer the cells to a T150 flask and add 30 ml of RPMI + 10% FCS and 0.8 ml of piptazo.
15. Incubate for 1 to 1.5 hr at 37 °C to remove adherent cells.

1.3 Sorting and Transduction of CD34 Hematopoietic Stem Cells

1. After attachment of cells in step 1.2.15, agitate the flask back and forth gently for a minute to dislodge loose cells. There will be a lot of fibroblasts and such stuck to the surface.
2. Count again using a 1 to 20 dilution and record the total number of cells. Save a few tenths of a ml for later staining. You should have approximately 30% fewer cells from your initial cell counts (step 1.2.13).
3. Spin down and wash two times with 40 ml of MACS buffer.
4. CD34 cells are sorted using Miltenyi Biotech's CD34 Direct Kit. We follow exactly the manufacturer's protocol using LS columns provided by the same company.
5. Recovered cells will be in 5 ml. Count and calculate total cells. Your yield of CD34+ cells should be 3-5% of your total cell counts. This varies with the age of the fetal liver. Younger fetal livers tend to give a higher yield of CD34+ cells.
6. Divide the number of cells by $1x10^6$. This gives the second upper limit on transplantable mice.
7. Count the CD34- fraction (flow through and washes) and reserve 6 x 10^6per mouse to be transplanted. Culture these cells overnight in 50 to 80 ml of RPMI + 10% Fetal Calf Serum + 1% piptazo.
8. Transduce the CD34+ cells overnight with your lentiviral vector. We use retronectin by Takara following exactly the manufacturer's instructions.
9. The next day, harvest the CD34- cells, the transfected CD34+ cells and count.
10. Split the CD34+ cells in half: Part A and Part B.
11. Spin down Part A CD34+ transduced cells and suspend in 4 to 6 ml of freezing medium (RPMI + 10% FCS + 10% DMSO, 0.22 µ sterile filtered). Put 1 ml aliquots into freezing vials and label with "huFetalCD34", the number of cells in the vial, the date, and your initials.
12. For each mouse mix $0.5x10^6$Part B CD34+ transduced cells and $4.5x10^6$CD34- cells in a sterile screw-cap microfuge tube, pellet and keep on ice. Also thaw a tube of Matrigel (BD Biosciences) on ice. All these tubes must be kept on ice until used (go to step 2.10).

2. Tissue Transplantation

The goal of this step is to transplant a human fetal thymus/liver organoid under the NSG mouse kidney capsule. This organoid better mimics the process of human T cell selection and maturation processes as the human hematopoietic stem cells will use the human thymus and not the mouse as the site for their differentiation to different T cell and other lymphoid lineages. The use of CD34- cells in the transplantation process is to re-generate the fetal liver stroma and allowing for better transplantation and growth of the implant. The age of NSG mice used is 6-8 weeks old.

1. Preparation of drugs for surgery:

a. Isoflurane: Roll up a 2 inch gauze pad and stuff it into a 15 ml screwcap centrifuge tube. Pour in about 1 ml of isoflurane directly from the bottle.
b. Ketamine/zylazine: Add 0.52 ml of ketamine (Ketaset) (100 mg/ml) and 0.52 ml of xylazine (Anased) (100 mg/ml) to a 20 ml vial of saline for injection. Label with the expiration date of the earliest expiring component and keep frozen at -20 °C until needed.
c. Carprofen (Rimadyl). Dilute this just before a surgical procedure. Dilute 1:100 by withdrawing 0.1 or 0.2 ml from the vial with a 1 ml syringe and a 25G needle. Inject into a 10 or 20 ml of saline for injection. Label and date. Keep in the fridge for one day only. Load into 3 ml syringes with 25G needles.

2. Set up the table for surgeries. Cover it with sterile blue pads, and a sterile towel for each surgeon. Each surgeon gets from their box of sterilized instruments: 4" scissors, curved blunt forceps, needle-nose forceps, hemostat, 16G blunted trochar, and wound clip applicator. In addition, they need a curved-needle Vicryl suture, a stack of isopropanol wipe packets, a 35 mm Petri dish of PBS, and a 1 ml syringe filled with PBS.
3. Set in the middle of the table a glass Coplin jar with 2 cm of Betadine and 2 cotton swabs. Pour the thymus bits into a 60 mm Petri dish.
4. The anesthesia is performed on a separate table or in a hood covered with sterile blue pads. Set up two small racks and an electronic balance with a beaker to hold the mice. Use one rack to hold 0.5 ml X 28G insulin syringes loaded with ketamine/xylazine and the other to hold the 3 ml syringes of carprofen. Also move the biohazard waste container near to hold the shaved fur.
5. Weigh all the mice (6-8 week old) in a cage to get the average weight and load syringes with a dose of 15 µl/g of ketamine/xylazine. Inject all the mice in the cage.
6. After the mice become sluggish shave their left side from hip to shoulder between the center of the back and the belly with an Oster clipper with No. 40 blade. Record their weight, and punch their ears for numbering.
7. Inject 0.3 ml carprofen subcutaneously over the shoulder.
8. Check the anesthesia level of the mouse by squeezing a paw. If the mouse flinches, administer isoflurane from a tube for about 12 sec. Then try the paw squeeze again. Keep giving short shots of isoflurane until the paw reflex disappears. Lay the mouse on its right side facing left.
9. A 16-gauge cancer implant needle, with the tip filed round is used to insert the tissue. Flush out the trochar a few times in the dish of PBS. Holding it horizontal with the rod just inside the tip. Add a piece of thymus with the needlenose forceps and suck it in.
10. A helper adds 5 µl of cold Matrigel to one tube of cells (step **1.3.12**) with an Eppendorf positive displacement pipet, and mixes by gentle stirring. The surgeon holds the trochar horizontally and pulls the rod back slowly as the helper pipets the Matrigel mix into the trochar. The mix gels at room temperature.
11. Swab the bare skin with Betadine. Then wipe this off with an isopropanol wipe. Repeat.
12. Locate the spleen under the skin. It is the darkest spot. The mouse's kidney is located about 5 mm dorsal to the spleen, which can be seen through the shaved skin.
13. Pick up the skin over this spot with the blunt forceps and make a cut parallel to the spleen about 15 mm long. Make a similar cut in the peritoneum muscle layer below. In males, the kidney should be directly visible and you simply have to squeeze the abdomen to pop it out. In females, you may have to pick up the ovary with the hemostat and drag out the kidney. This may help retain the kidney outside of the body. For males, you will have to keep some pressure on the abdomen with your left hand to keep the kidney exposed.
14. Pluck a little hole in the posterior end of the kidney capsule. It may bleed a little.
15. Slide the trochar into this hole and along the kidney until the orifice of the trochar is completely covered by the kidney capsule. Then using the little finger of your right hand, inject the tissue.
16. Push the kidney back in gently with the closed hemostat. Put one stitch in the peritoneum tied with a double know. Squeeze the skin up like a purse and put in two wound clips.
17. Put a drop of PBS on each eye and lay the mouse on its side on the cage bedding. Make sure all the mice have returned to laying on their bellies before leaving them.
18. Next day, give each mouse another shot of carprofen.

3. Secondary Transplantation

The goal of this step in the generation of BLT mice is to populate the moue bone marrow with human hematopoietic stem cells. The transplanted implant from procedure (2) is not sufficient to support full reconstitution of the human immune system in these mice. During the secondary transplant, we sub-lethally irradiate the mice to deplete murine bone marrow cells thus generating "space" for the implantation of the human CD34+ cells.

1. Four to six weeks later irradiate the mice with 2.7 Gy. The mice are injected the same day with Part A CD34+ transduced cells. The timeframe is based on the establishment and growth of the thy/liv implant transplanted in step 2. Typically, during this time the implant has grown significantly allowing us to proceed to the next steps.
2. Thaw out the Part A CD34+ transduced cells, wash and suspend in medium to a concentration of $5x10^6$/ ml.
3. Load 0.5 ml X 28G insulin syringes with 0.1 ml of cells (10^5 to $5x10^5$ cells per 0.1 ml per mouse) for one cage of mice (each cage contains a maximum of 5 mice) and lay them on a rack facing away.
4. Anesthetize a mouse with an isoflurane tube by scruffing it and holding its nose in the tubes for about 20 sec. Count to keep track of the time.
5. Then face it to the right, and pull the skin around its neck tight with the thumb and forefinger of the left hand. Press under its jaw with your third finder so that its right eye bulges out. Holding the syringe parallel to the long axis of the mouse's head, slip the needle behind the eye and slide it gently until it stops. Don't press any further, but inject the load over about 2 sec.
6. Two to three months later mice are bled retroorbitally using EDTA coated glass pipettes. You can only take a maximum of 200 µl of blood per month per mouse. 50-100 µl of blood should be sufficient for FACS analysis. Alternative bleeding strategies include facial vein bleeds and tail vein bleeds. The latter will give you very small volumes of blood that may not be sufficient for multiple assays.
7. Blood samples are stained for the expression of the human lymphocyte marker CD45 to assess reconstitution. Blood samples are added into 1 ml red blood cell lysis buffer (160 mM NH_4Cl, 100 mM $KHCO_3$, 0.01 mM EDTA) and incubated for 5 min at room temperature.
8. Wash the cells in lysis buffer, 5 min at 1,200 rpm, and repeat if necessary.
9. Centrifuge cells as in 3.8 and wash in PBS supplemented with 3% fetal calf serum (FACS-PBS).
10. Remove supernatant and re-suspend pellets in 100 µl FACS-PBS.

11. Add antibodies to assess human lymphocyte reconstitution. We typically include the following CD45 (human lymphocyte marker), CD8, CD4, CD14 and CD16 for macrophages and monocytes, CD19 for B cells and CD56 for NK cells.
12. Incubate at 4 °C for 30 min.
13. Wash as in step 3.9 and re-suspend cells in 2% paraformaldehyde for FACS analysis.
14. If mice are reconstituted, we proceed with tumor injections. Reconstitution levels of human lymphocytes vary, from less than 1% to 40% of total blood cells. The levels and types of lymphocyte lineages are comparable to what one would expect from healthy human blood donors. However, do expect fluctuations in the number of non-T cell lineages. If the numbers are not acceptable within the 12-week timeframe you could repeat the secondary transplant procedure from step 3.1.
15. Tumor cell lines are cultured according to manufacturer's or laboratory recommendations. Cells are harvested, washed and resuspended in matrigel at a 1:1 ratio (50 µl cells/50 µl matrigel). Samples are kept on ice at all times.
16. Mice are anesthetized and shaved at the area of injection. The area is sterilized using an isopropanol pad. A helper loads cell suspensions in pre cooled 23G 1 ml syringes. Tumor cells are injected subcutaneously. Treat the site of injection with antibiotic ointment. Tumors should establish and begin growing in 4 weeks.

B. *In vivo* Positron Emission Tomography (PET)

For our studies we examine glucose uptake ([^{18}F]-fluorodeoxyglucose ([^{18}F]FDG) thus mice are fasted 4-6 hr prior to imaging. MicroPET/CT scans are done using the microPET Inveon scanner (Siemens Preclinical Solutions) and MicroCATII CT scanner (Siemens PreclinicalSolutions). Image analysis is done using OsiriX (Pixmeo, Switzerland) software. The goal is to measure the metabolic activity of the tumor and ultimately to use PET imaging as an alternative to physically measuring tumor regression. As seen in **Figure 2**, we have encountered tumors that based on physical appearance and size are not targeted but live PET imaging revealed extensive tissue necrosis. This methodology can serve as a more sensitive and accurate indicator of tumor regression.

1. Designate one chamber for anesthetizing (2% isofluorane) the mice, and one chamber as the 60 min uptake of FDG before PET scanning ("waiting chamber"). Place blue pads in both chambers.
2. As animals are anesthetized over a long periods, they should be kept warm at 30 °C through the use of water filled gloves that are warmed in a microwave and placing the cages on heat pads.
3. Turn the isoflurane flow for this chamber on. Allow the isoflurane to fill the anesthetizing chamber for approximately 5-10 min before placing any mice into the chamber. Make sure the lid is locked and closed tightly to avoid leaking isoflurane.
4. Place the first mouse into the anesthetizing chamber and lock the lid tightly. Allow 5-10 min or until the mouse is unconscious.
5. Remove the mouse from the anesthetizing chamber, label the tail of the mouse (different color per cage) and inject intraperitoneally the mouse with [^{18}F]FDG (80 µCi). Take note of the time the mouse was injected. The mouse will be anesthetized again 50 min from this timepoint, allowing for 10 min to set up for PET scanning (for the total of 60 min [^{18}F]FDG uptake before PET scanning).
6. Place the injected mouse into the waiting chamber, allowing [^{18}F]FDG uptake for 1 hr before PET scanning. Do not lock the lid. Leave the lid loose to allow airflow.
7. Immediately place the next mouse for FDG injection into the anesthetizing chamber. Subsequent mice injections should be spaced 10 min apart, because the process of PET scanning is 10 min. Immediately after one mouse is scanned, the 60 min FDG uptake for the next mouse to be scanned will be ready exactly 10 min apart.
8. Continue anesthetizing and injecting mice as stated in steps 7-9.
9. After 50 min from the first mouse injection timepoint, place the first injected mouse into the anesthetizing chamber again (while still continuing to anesthetize and inject mice).
10. Connect the mouse bed to the oxygen and isoflurane lines, and turn the isoflurane flow on for the bed. Place a blue pad on the bed.
11. Place the mouse ready for PET scanning onto the bed, stretching the arms and legs and holding this position with tape. Place lubricant over the eyes. This step is the most crucial since the positioning of the animal can affect the quality of your imaging.
12. Disconnect the isoflurane and oxygen lines, and quickly mount the bed on the PET scanning machine. Place the isoflurane and oxygen lines. Make sure the isoflurane flow is on for the PET scanning machine. Connect the PET scanning cable and begin the scan.
13. Once the PET scan is complete then move the animal to the CT scanner. Following this scan move the animal to its respective cage.

Representative Results

A flow chart of the transplantation process is shown in **Figure 1A**. A picture of the thy/liv implant is shown in **Figure 1B**. The thymic tissue develops normally and has a physiological distribution of human CD4 and CD8 T cells. Following reconstitution, the animals carry a human immune system with normal distribution of CD4, CD8 T cells and other immune cell lineages.

The discrepancy between tumor size and live tissue is shown in **Figure 2**. While the CT scan (grey area) indicated a large tumor growth, *in vivo* PET imaging showed that it was mostly necrotic and scar tissue (**Figure 2**). This underscores the utility of PET imaging as a more sensitive and accurate way to assess tumor regression and clearance.

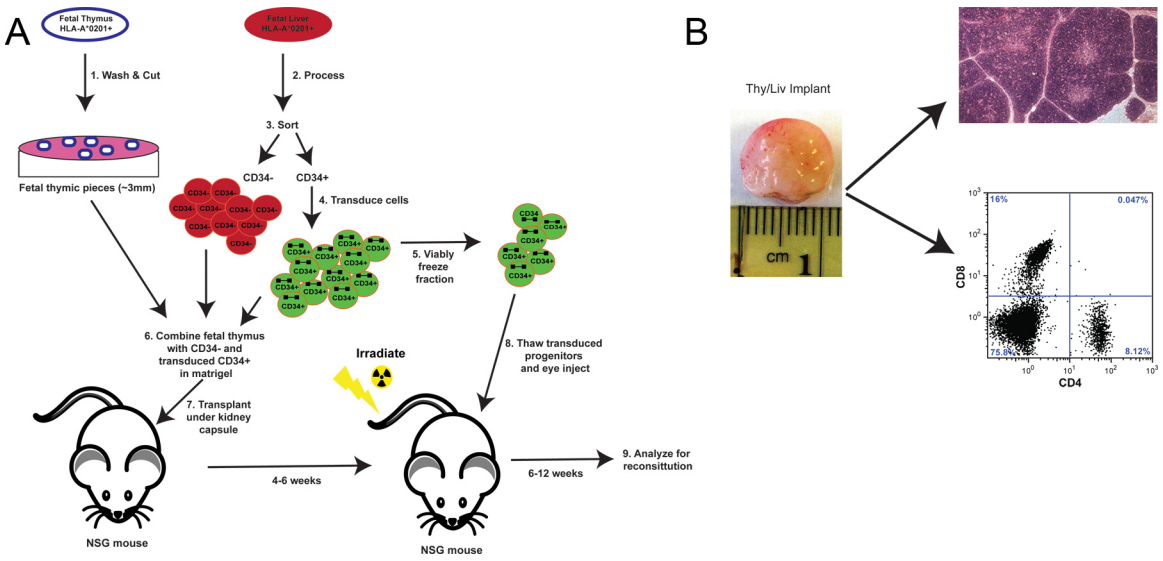

Figure 1. (A) A schematic diagram on the modified BLT model used in these studies for the generation of chimeric mice carrying MART-1 specific T cells. The Thy/Liv implant was reconstructed from transduced and non-transduced CD34 cells isolated from an autologous fetal liver. A fraction of the transduced cells is frozen and injected into the irradiated mice 4-6 weeks later. (B) A representative image of the thy/liv implant in humanized mice. The implants have a physiological tissue distribution and CD4/CD8 ratios as shown by the IHC and flow cytometry figures. Click here to view larger figure.

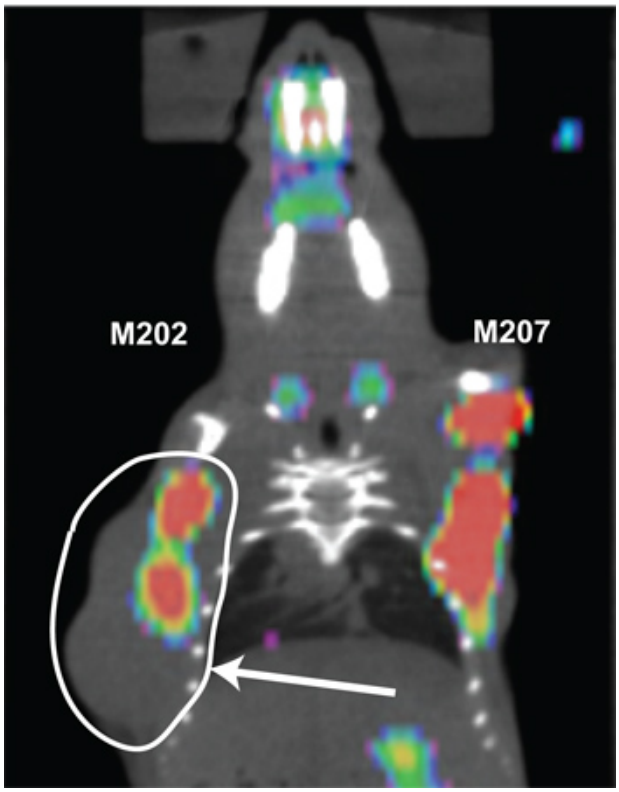

Figure 2. A PET and CT image of a mouse carrying a melanoma tumor. The grey area indicates the physical size of the tumor while the red color indicates the tumor's metabolic activity, which is very limited.

Discussion

The modified BLT humanized mouse model coupled with *in vivo* PET imaging are powerful tools to study chronic human diseases. This system takes the BLT mouse model and advances it beyond the limited scope for HIV research. In addition, it is a great system in which we can examine

various gene therapy protocols as well as diagnostic techniques before they can reach the clinical setting. The latter coupled with the low cost of using mice versus primates makes this a very useful model.

The PET imaging technology allowed us to assess the efficacy of our approach. If we relied exclusively on physical measurements of the tumor, we would have underestimated the potency of the antitumor response generated by our transgenic T cells. The extensive scarring and necrotic tissue gave the appearance of a large tumor, which in reality was dead tissue.

In conclusion, the utility of the modified BLT mouse model can be extended to other disease models. While some disadvantages still persist such as the shorter lifespan of mice, this can be a very strong tool to *in vivo* assess many aspects of human immunity, test and develop novel therapeutic interventions.

Disclosures

No conflicts of interest declared.

Acknowledgements

We would like to thank Alvin Welch and Larry Pang for their technical assistance. This work was funded in part by the National Institutes of Health (NIH) award P50 CA086306, the California Institute for Regenerative Medicine (CIRM) grants RC1-00149-1 and RS1-00203-1; CIRM New Faculty Award RN2-00902-1, the Caltech-UCLA Joint Center for Translational Medicine, UCLA Center for AIDS Research (CFAR) NIH/NIAID AI028697, the UCLA AIDS Institute, the CIRM Tools and Technology Award RT1-01126, and the UC Multicampus Research Program and Initiatives from the California Center for Antiviral Drug Discovery (number MRPI-143226).

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