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TITLE:

A Xenograft Mouse Model to Assess Efficacy of Therapeutic Agents for Human Acute Leukemia

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KEYWORDS:

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SUMMARY:

Mouse (*Mus Musculus*) models are being widely used to develop xenografts using human leukemia cells. These models provide a comparable biological system to study drug efficacy, pharmacodynamics, and pharmacokinetics. Modeling acute myeloid leukemia in immunocompromised mice is described in detail using the U937 cell line xenograft as an example.

ABSTRACT:

Preclinical evaluation of therapeutic agents using an appropriate animal model is a critical step and a requirement for selecting drugs worth testing in humans. Therapeutic agents such as small molecule inhibitors, biological agents, immune checkpoint inhibitors, and immunotherapy each have unique mechanisms of action and call for careful selection of in vivo systems in which their efficacy can be tested. The purpose of this article is to describe in detail development of one such leukemia xenograft model for testing the therapeutic efficacy of novel agents. Using an immunocompromised (NRG) murine model that lacks B, T, and NK cells helps engraftment of transplanted leukemia cells and provides an acceptable microenvironment to study the therapeutic efficacy of small molecule inhibitors and some biological agents. This article describes the development of leukemia murine xenografts for in vivo drug testing using an acute myeloid leukemia (AML) cell line murine model treated with the cytotoxic drugs daunorubicin and cytarabine as an example. Treatment response can be assessed during therapy using several noninvasive and minimally invasive methods. Bioluminescence imaging can be used to measure leukemia burden over time when luciferase prelabeled leukemia cells are used to develop xenografts. Peripheral blood count analysis provides vital information about side effects such as myelosuppression (e.g., cytopenia) and therapeutic effect (e.g., blast count or differentiation). These techniques help track differences in the development of leukemia or decrease in tumor burden at various time points during the drug treatment without scarifying the study animals. Secondary methods such as immunophenotyping using flow cytometry are applied to confirm differences in the leukemia burden among treated and untreated groups. The methods described here can be tailored and used for developing xenografts of other types of leukemia (e.g., acute lymphoblastic leukemia).

INTRODUCTION:

Acute myeloid leukemia (AML) is a clonal disorder arising from a malignantly transformed multipotent hematopoietic stem cell that acquires consecutive genomic alterations, eventually advancing into clinically overt disease. It is a highly complex disease with significant genetic, epigenetic, and phenotypic heterogeneity[1]. The uncontrolled proliferation and impaired differentiation of myeloid precursor cells (i.e., blasts) is one of the hallmarks of AML, leading to anemia, thrombocytopenia, and eventually death[2]. According to the American Cancer Society, in 2019, ~21,450 new cases of AML will be diagnosed, and ~10,920 people will succumb to the disease[3]. Standard therapeutic options include cytarabine-based chemotherapy and hematopoietic stem cell transplantation (HSCT). The 5-year overall survival (OS) of patients younger than 60 years old is around 40%, and for those older than 60 years it is only 10−20%[4].

Novel drug discovery and drug development is a formidable challenge for the scientific community as well as the pharmaceutical industry. On average, the development of a novel drug costs ~$2.6 billion and takes over 10 years[5]. Drug discovery for anticancer drugs is an inefficient and cumbersome process with 89% of drugs failing in preclinical testing to gain FDA approval[6]. Flawed preclinical research is one of the reasons for drug failures[7]. Although multiple models of cell culture and in vitro studies are useful and important for testing potential therapies, the drawback of cell line models is that the synthetic nature of their culture conditions means they do not necessarily reflect the behavior of the original cancer cells in patients[8]. Also, it is

impossible to fully recapitulate the complexity of the whole organism and the bone marrow microenvironment in cell culture[8]. As a result, cell line-derived xenograft tumor mouse models were generated through the transplantation of well-established cancer cell lines into immunocompromised mice. The major advantage of the cell line-derived xenograft mouse models is that they more closely simulate the tumor's microenvironment and pathophysiological conditions[9].

Here, we provide a comprehensive protocol explaining the steps to generate leukemia xenograft mouse models using a stable AML cell line labeled with a luciferase reporter (U937-Luc-tdTomato). Details of lentiviral transduction of the leukemia cell line will not be explained[10,11]. We also describe a detailed protocol to monitor leukemia progression using bioluminescence imaging (BLI). Statistical analysis and reporting of the differences observed in leukemia progression in mice treated with control, with a single standard of care drug (i.e., cytarabine or daunorubicin), or a combination of both, will be detailed. The first part of this protocol (sections 1, 2, and 3) are devoted to selection of the mouse strain and generation of the xenograft model, where we describe the transplantation procedure followed by imaging, randomization, and drug treatment of mice. Later, we detail steps to collect leukemia cells and perform flow cytometric analysis using antibodies directed against intracellular and surface hematopoietic markers to determine their phenotype. Also included is the Wright-Giemsa staining of bone marrow and spleen cells, which was done to show blast number or structural differences following drug treatment.

This protocol is robust and highly reproducible. The data shown here will aid investigators in testing the efficacy of novel therapeutic drugs. The immunocompromised RAG deleted mouse model used here is an established model for these kinds of studies, where tumor burden and survival rates can be monitored during the treatment regimen. Additionally, this method can be used to provide information on the proliferation and survival of leukemic cells and other hematopoietic cell populations throughout diagnosis.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the Pennsylvania State University College of Medicine.

1. Selection of animals for the study

1.1. Maintain 18 healthy 10−12-week old male NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}/SzJ (NRG) mice in a barrier environment under pathogen-free conditions.

NOTE: The common source for the right mouse strain for the study is peer-reviewed publications. The recommended age for mice is between 6 and 12 weeks-old, depending on the study. If there is no gender preference for the experiment, it is best to include both genders to avoid any gender-specific variations. Considerations about number and biological variables are explained in the discussion section.

2. Transplantation of leukemia cells

2.1. Count the cultured luciferase labeled leukemia cells and calculate the total cell number needed based on the desired cell number/mouse (10,000 U937-Luc-tdTomato cells per mouse is needed to achieve sufficient engraftment).

NOTE: Cultured cells should not be overgrown. The density should be between 5 x 10^4–1 x 10^6 cells/mL.

2.2. Harvest the calculated cells in cell growth medium (RPMI-1640 with 10% fetal bovine serum [FBS]) in a 50 mL tube and centrifuge at 360 x *g* for 5 min at 4 °C.

2.3. Dump off the supernatant and resuspend cells in Hank's balanced salt solution (HBSS) (150 µL per mouse). If 10,000 cells per mouse are desired and there are 10 mice total, then resuspend 100,000 cells in 1,500 µL (1.5 mL) of HBSS. Place the cellular suspension on ice.

NOTE: When counting the cells, consider only viable cells, not total cells. Also, prepare cells for some extra mice. For example, if 10 mice are needed for injection, prepare cells for 12−15 mice.

2.4. Fill 28 G insulin syringes with 150 µL of cell suspension. Place the mouse in a restrainer. Rub the tail gently with a 70% alcohol pad to clean the injection site.

NOTE: The mouse restrainer should be thoroughly cleaned with disinfectant (**Table of Materials**).

2.5. Hold the distal tail to dilate the vein. Inject the cells into the tail vein once the vein is dilated enough.

NOTE: Heat lamps can be used to dilate the tail veins. Cells settle down at the bottom of the tube, so mix cells before each injection for consistency.

2.6. After the injection, dispose of the syringe and needle into a sharps container. Press the tail to secure clotting and prevent bleeding. Lead the mouse into the cage and monitor for a few seconds to make sure there is no bleeding.

3. Imaging, randomization, and initialization of the treatment

3.1. Depending on the cell line and animal model, perform the first animal imaging 3−12 days post-engraftment.

3.2. Transfer the animals to the imaging room following the IACUC protocol. Record the whole bodyweight of each animal before the anesthesia.

3.3. Turn on the anesthesia system and charge the anesthesia chamber. Set an anesthesia wheel

following the manufacturer's instructions for healthy mice (2.5%). However, set it lower (i.e., 1−1.5%) for sicker mice to deliver less anesthetic.

3.4. Take the mice and inject them with 5 µL/g of 30 mg/mL stock luciferin intraperitoneally with a 28 G insulin syringe. Determine the amount of luciferin per mouse from the bodyweights recorded on the sheet (e.g., 24 g mouse = 120 µL, 27 g mouse = 130 µL).

NOTE: Always round the drug volume down (e.g., if the study has a 27 g mouse and a 26 g mouse, they both will be dosed at 130 µL).

3.5. As the mice are injected, move them into the anesthesia induction chamber. Once they are immobile, move them one by one into the imaging cabinet to deliver a maintenance dose of isoflurane to the mice through nosecones in the imager.

NOTE: Induction in a chamber generally requires 5% isoflurane, and maintenance (chamber or nose cone) requires 1−2% (flow rate = 0.5−1 L/min).

3.6. Position mice on a ventral position for imaging and take the images within 7−15 min of the luciferin delivery.

NOTE: Optimization of imaging time is recommended if the model is being tested for the first time. Do not open the door of the imager while an image is being taken. This might cause major issues with the machine, and the whole program may need to be restarted and reinitialized.

3.7. After acquiring the images, remove the mice from the imaging chamber and place them back into their home cage. Lay them gently on the top rack of the cage to recover from the anesthesia. Lid the cage while they recover so that they do not stumble off the cage and fall to the floor, resulting in injury or death. Once recovered and awake, place the mice gently back inside of their home cage.

3.8. Analyze the whole-body bioluminescence images of the mice using the imaging software (**Table of Materials**) to free draw around the entire mouse body, excluding the tail. Region of interest (ROI) values will be automatically provided after drawing free lines surrounding each mouse. Rank the mice depending on their ROI signal levels. Evenly disperse mice among all groups based on their ROI to yield as even a group average as is possible for the entire study.

3.8.1. Exclude the mice with signals that are either too high or too low. Mark (e.g., ear punch) each mouse in order to follow up the individual mouse in each study group.

3.9. Start the treatment by following the study's planned treatment regimen (e.g., see **Figure 1C**).

NOTE: Care must be taken to follow manufacturer's instructions for the solubility, storage, and handling of each treatment agent. For example, daunorubicin (DNR) was reconstituted in saline and aliquots were stored at 4 °C. Cytarabine (Ara-C) was reconstituted in saline and stored at 25

°C. DNR (1 mg/kg) was delivered intravenously (IV) and Ara-C (25 mg/kg) was delivered intraperitoneally (IP). Store the drugs in daily-based volumes to avoid freeze-thaw cycles and maintain their stability.

3.10. Image the mice throughout the study to evaluate the treatment response following steps 3.2–3.8.

4. Blood collection (tail vein puncture)

NOTE: Blood collection should be done once per week during the study to monitor abnormal blast cells as well as complete blood count (CBC) levels for toxicity.

4.1. Place the mouse tail first into the restrainer and place the restraint plug in the tube, flush with the mouse's nose so that the mouse becomes immobile without being crushed. Tighten the plug so that the mouse cannot move.

NOTE: It may be useful to dip the tail in warm water to increase vasodilation prior to starting.

4.2. Wipe the tail with an alcohol pad to remove any debris. Locate one of the lateral tail veins.

4.3. Insert a 25 G needle attached to a 1 mL syringe into the lateral tail vein, approximately 1/3 of the way down the tail from the base of the tail. Insert the needle at a ~5° angle, almost parallel to the tail, with the beveled end pointed up.

4.4. Remove the needle and place the capillary tube up against the blood as it wells up. Depending on the blood flow, it could take several seconds for the blood to travel down the capillary into the tube. Once the blood begins to drop into the tube, periodically flick the tube gently to mix with EDTA and prevent the blood from clotting.

4.5. Collect at least 50 µL of blood.

NOTE: Collecting less blood will make it difficult to perform the CBC counts. If blood flow is too slow, try puncturing the tail vein with the needle higher up towards the base of the tail. It is also effective to try the other lateral tail vein. Do not puncture the tail more than 3x in one sitting to prevent undue stress.

4.6. Once the blood is collected, take the capillary top off and close the lid of the tube. Gently flick the tube for 30 s to further mix and prevent clotting. If the blood clots, it cannot be used for CBC counts. Apply pressure to the tail at the puncture location for 30 s to stop the bleeding.

4.7. Unscrew the plug and remove the mouse from the restrainer. Monitor the mouse to make sure there is no further bleeding from the tail. If bleeding continues, apply styptic powder to the puncture site.

NOTE: Clean the restrainer when changing mice to limit pheromone-induced stress and contamination. If desired, submandibular or saphenous vein bleeding can also be used[10].

5. Euthanasia

NOTE: Follow the institution-approved standard procedure for mice euthanasia.

5.1. When mice exhibit distinct signs of being moribund (i.e., hind limb paralysis, lethargy, inability to right itself, more than 10% weight loss, hunching, immobility), perform euthanasia.

NOTE: This will occur typically around day 22 postengraftment for the U937-Luc-dsRed mouse model.

5.2. For euthanasia by CO_2, place the mice in the cage and place the lid attached to the CO_2 regulator onto the top of the cage. Ensure that the cage is sitting flush with the top of the cage, and the air vent holes are not blocked. Open the valve on the CO_2 tank to start the flow. Adjust the CO_2 regulator to 1−2 L/min.

NOTE: Allow the empty chamber to charge for 2 min before placing the mice in.

5.3. Once the mice have become unconscious, as indicated by lack of movement and rapid breathing, increase the CO_2 regulator knob to 4−5 L/min. Continue to monitor the mice until 1−2 min after the last visual breath. Turn the CO_2 regulator valve to 0 and close the CO_2 tank valve. Take the mice out of the chamber.

5.4. Perform a secondary form of euthanasia (e.g., cervical dislocation, cardiac puncture, or removing major organs) to each mouse to ensure death.

5.4.1. To perform cervical dislocation, place two fingers on either side of the mouse's neck. While applying pressure to the neck, pull the tail up at a 45° angle until a separation of the joints in the neck is felt.

6. Leukemia cell isolation from organs (bone marrow and spleen)

6.1. Prepare the work area by spraying the cork or lid of a polystyrene foam box surface with ethanol, covering with paper towels, and spraying with more 70% ethanol. Place a bench pad underneath the surface to limit depositing debris on the surrounding areas.

6.2. If blood is needed for CBC and serum analysis, perform a cardiac puncture immediately after CO_2 euthanasia.

6.2.1. Place the mouse on its back. Place the 25 G needle attached to a syringe bevel up between the ribs on the left side of the mouse, at the level of the elbow, approximately 5 mm into the chest cavity, to puncture the heart.

6.2.2. Gently pull back the plunger. If the needle is in the heart, blood will flow into the syringe. If there is no blood, pull the needle out slowly until blood starts to flow into the syringe. Keep the needle still once the blood starts to flow into the syringe.

NOTE: If unable to collect blood after several attempts, open the body cavity as described below to access the chest cavity to collect blood.

6.2.3. Once the blood has filled the syringe, pull the plunger back again slowly. The heart will need time to fill back up, and it may take a few seconds to get the blood flowing again. Place approximately 50 µL of blood into an EDTA tube and gently flick the tube to prevent clotting.

NOTE: The rest of the blood can be placed in a microcentrifuge tube for serum analysis. The blood for serum analysis can be left to clot.

6.3. Prepare 6 well plates by adding 5 mL of cold phosphate-buffered saline (PBS) to each well with a 0.45 µm cell strainer. Place the 6 well plates on ice.

6.4. Place the mouse on its back and spray the dorsal surface with 70% ethanol until the fur is wet. Secure the mouse to the surface by placing a 20 G needle through the tail, ~1 cm from the base of the tail, and one needle through the neck of the mouse, just below the jaw, to allow access to the internal organs, as well as the leg bones, for bone marrow collection.

6.5. Using forceps, pull the fur up and make a 1 cm cut through the first layer of skin using scissors. There will be another layer of fascia visible just underneath the cut.

6.6. Pull up on the second layer of fascia and make an incision to expose the internal organs. While pulling up on the skin, make a midline cut through both layers of fascia towards the head, ending at the bottom of the sternum. Make a similar cut towards the tail of the mouse, ending in line with the hips.

6.7. At the end of the caudal cut, make a 1 cm cut down the inside of the thigh on each leg. At this point, it may be helpful to pin back any excess skin to better visualize the internal organs. The spleen is located on the mouse's left flank, underneath the stomach, next to the kidney. To remove the spleen, gently lift it using the forceps and cut away any connective tissue.

6.8. Place the spleen in the cell strainer and using the back of a 1 mL syringe plunger, mash the spleen into the strainer until it becomes a single cell suspension. Mash the spleen until there is no red tissue left in the strainer, only white tissue.

6.9. While the spleen suspension is on ice, start to remove the bone marrow by taking the previous incision on the thigh, from step 6.7, down to the ankle of the mouse. Continue that incision around the ankle and pull the skin away from the leg muscle to expose the upper thigh.

6.10. Cut the muscle from the back of the leg bones starting from the ankle up to the pelvis. Find the head of the femur and feel for the separation between the pelvis and the head of the femur. Cut through the separation to detach the leg from the body.

NOTE: If more cells are needed for analysis, the forelimbs, spine, and iliac crest may also be harvested.

6.11. Remove the tissue from the tibia by twisting the ankle and gently pull up to remove the ankle and tissue from the tibia. For the remaining tissue, use a gauze pad or paper towel to gently pull away from the tissue without breaking the bones. Place the tibia and femur into the 6 well plate and repeat on the other leg.

6.12. Cut the tips of the bones off and take up a few milliliters of PBS in the dish into a syringe attached to a 25 G needle. Guide the needle tip into the opening of the bone and flush out the bone marrow. Continue to flush the bones until water flushes through the bone cavity without resistance. Small pieces of bone marrow or flakes will be visible in the collection tube. Transfer the spleen and bone marrow contents to respective 15 mL conical tubes and fill to 15 mL with cold PBS.

NOTE: With advanced engraftment, there will be very little red in the bone marrow. In this case, thoroughly scrape the inside of the bones to remove as many cells as possible.

6.12.1. Alternatively, crush the bones with a mortar and pestle to remove the cells. Before transferring the bones to the 15 mL conical tube, thoroughly resuspend the cells, and pipette through nylon mesh to remove any bone particles. Rinse the bones with 2 mL of cold PBS to remove any cells left in the mortar, and pipette through the strainer. Fill the conical tube to 15 mL with cold PBS.

6.13. Centrifuge at 360 x *g* for 5 min. Aspirate the supernatant and resuspend the pellet thoroughly in 3 mL of 1x red blood cell lysis buffer (**Table of Materials**).

6.14. Incubate the suspension for 8 min at room temperature (RT), then dilute by filling the tube with cold PBS up to 15 mL. Centrifuge again at 360 x *g* for 5 min. The pellet should be white, with no red blood cells left.

6.15. Aspirate the supernatant and resuspend the pellet in 1 mL of cold PBS or fluorescence-activated cell sorting (FACS) buffer (**Table of Materials**).

7. Cell surface and intracellular immunofluorescence staining of isolated animal cells

7.1. Cell surface antigen staining

7.1.1. Count out 0.5−1 x 10^6 cells per sample collected in step 6.15 and suspend them in 3 mL of FACS buffer in a 5 mL 12 x 75 mm FACS tube. Wash the cells 2x in 3 mL of FACS buffer and

centrifuge at 360 x *g* for 5 min at 4 °C.

7.1.2. Discard the supernatant (i.e., with a vacuum pump/Pasteur pipette or simply by washing off the pellet) and resuspend the pellet in its residual volume by gently tapping on the benchtop. Then add 1 µL of human Fc block (mouse anti-human Fc receptor antibody, 1:100) and 1 µL of mouse Fc block (rat anti-mouse CD16/32 antibody, 1:100) per sample to block nonspecific Fc-gamma receptor (FcgammaR)-mediated binding of antibodies. Mix the tubes and incubate them on ice for a minimum of 15 min.

NOTE: Always keep tubes on ice while working to prevent low viability. No protection from light is needed up to this step.

7.1.3. Prepare an antibody cocktail containing anti-mouse CD45, anti-human CD45, anti-human CD33, viability dye (i.e., 7-aminoactinomycin D [7-AAD] or fixable viability dye [FVD]), and fluorescence of cells (i.e., tdTomato or yellow fluorescent protein [YFP]) for the cell surface staining in the FACS buffer (**Table 1**).

NOTE: Use brilliant stain buffer when two or more different staining reagents conjugated with brilliant fluorescent polymer dyes (e.g., BV421 anti-human CD33 and BV605 anti-mouse CD45) need to be used. It provides better data interpretation and helps to prevent any fluorescent dye interactions that may cause staining artifacts. An example panel for U937-tdTomato animal studies is shown in **Table 1**. When making the flow cytometry antibody panel, it is important to consider the fluorescence of the cells to be tested, if any. Viability dye plays a crucial role in excluding dead cells to avoid any false positive or negative results.

7.1.4. Add the antibody cocktail and incubate the samples on ice or at 4 °C for 30 min in the dark (i.e., cover with aluminum foil).

NOTE: The protection of samples from light becomes very important after this step. Working in a dark environment is ideal. If that is not possible, minimize the light exposure by covering the tubes with aluminum foil in between steps, for example.

7.1.5. Add 0.5−1 mL of FACS buffer and centrifuge the tubes at 300 x *g* at 4 °C for 5 min. Dump the supernatant and repeat this step.

NOTE: If intracellular staining is desired, use PBS (azide and serum/protein-free) instead of FACS buffer.

7.1.5.1. Calculate the engraftment percentage by obtaining the ratio of human CD45 (white blood cells) positive cells to whole CD45 positive cells (human CD45+ and mouse CD45+ cells) using the following formula:

$$Engraftment = 100\ x\ \frac{Human\ CD45\ positive\ cells}{Human\ CD45\ positive\ cells + \ Mouse\ CD45\ positive\ cells}$$

7.1.6. Resuspend the stained cells in 300 µL of cold FACS buffer (for 7-AAD) or PBS (for FVD) and 0.1−0.5 µL/mL of viability dye (FVD or 7-AAD). Incubate for 5−10 min on ice or at RT while protected from the light.

NOTE: If intracellular staining is desired, use FVD instead of 7-AAD in PBS (azide and serum/protein-free).

7.1.7. Continue with the intracellular immunofluorescence staining protocol if desired. Otherwise, perform flow cytometry in 1 h.

7.2. Intracellular immunofluorescence staining

NOTE: First, perform cell surface antigen staining as described in section 7.1, then follow the steps below to stain intracellular antigens.

7.2.1. Fixation of the cells

7.2.1.1. Warm the fixation buffer (**Table of Materials**) in a water bath. Aliquot 0.5 mL of buffer per 1 x 10^6 cells and warm to 37 °C. After two washes in PBS (azide and serum/protein-free) as in step 7.1.5, dump off the supernatant and resuspend the pellet in 0.5 mL/tube prewarmed fixation buffer for 20 min at RT in the dark.

7.2.1.2. Centrifuge at 350 x *g* for 5 min at 4 °C or 25 °C and discard the supernatant.

NOTE: The cells are now fixed. If short- or long-term storage is desired, the experiment can be stopped at this point. For short-term storage, wash cells 1x with FACS buffer, resuspend in FACS buffer, and store at 4 °C. For long-term storage, resuspend in freezing medium (90% FBS and 10% dimethyl sulfoxide [DMSO]) at -80 °C. Otherwise, continue with the permeabilization protocol.

7.2.2. Permeabilization of cells

7.2.2.1. Dilute 10x intracellular staining permeabilization wash buffer (**Table of Materials**) to 1x in distilled water.

7.2.2.2. Resuspend the fixed cells in 1 mL of intracellular staining permeabilization wash buffer and centrifuge at 350 x *g* for 5 min. Repeat this step. After the second wash, make “Unstained” or “Isotype” control tubes. Label control tube as “Unstained” or “Isotype” and resuspend in FACS buffer. Control tube is now ready, no further staining step is needed. Put the control tube on ice.

7.2.3. Intracellular antigen staining

7.2.3.1. Resuspend fixed, permeabilized cells in residual intracellular staining permeabilization wash buffer and add the antibody of interest (i.e., PE-anti-pSTAT3-tyr705 or PE-anti-BTK) into the

tubes and incubate for 20 min in the dark at RT. Wash 2x with 1 mL of intracellular staining permeabilization wash buffer and centrifuge at 350 x *g* for 5 min at 4 °C.

7.2.3.2. Resuspend fixed, permeabilized, and intracellularly labeled cells in 300 µL of FACS buffer and analyze with appropriate controls.

8. Wright-Giemsa staining of bone marrow and spleen cells

8.1. Proceed with the Wright-Giemsa staining protocol once the bone marrow and spleen cells are suspended (step 6.15).

8.2. Preparation of cells for cytocentrifuge

8.2.1. Prepare a cell suspension of no more than 2−5 x 10^5 cells in 200 µL of PBS or PBS with 2% FBS in 1.5 mL tubes. Prelabel the microscope slides (i.e., Control, U937, bone marrow [BM], NSG, Day 20, and date frozen).

8.2.2. Load the slide clip with a filter card and sample chamber, followed by loading the cell suspension into the chamber.

NOTE: Make sure the chamber and filter card are aligned. Add the cell suspension after the slide, chamber, and slide clip set-up has been placed in the cytocentrifuge.

8.2.3. Cytocentrifuge the chambers at 600 x *g* for 5 min.

8.2.4. Remove the slide from the chamber carefully by holding the slide clip firmly and pushing the spring against the slide to release it from the hooks. Then move the spring away from the hooks and allow the sample chamber to be released from the clip. Air-dry the slides and proceed with the Wright-Giemsa staining protocol.

8.3. Wright-Giemsa staining of slides

8.3.1. Stain slides with Wright-Giemsa staining solution (i.e., dip in staining solution or flood slides with staining solution on a staining rack) for 1 min.

NOTE: If staining on a rack is preferred, add sufficient Wright-Giemsa solution to cover the entire surface.

8.3.2. Dip the slides in distilled water for 2 min (or longer for darker staining). Rinse the slides with distilled water, and air-dry.

8.3.3. Mount the slides with a coverslip using the mounting medium and examine the slides under the microscope.

REPRESENTATIVE RESULTS:

We developed a model to study the standard of care chemotherapeutic regimen for AML in a mouse model. Luciferase and tdTomato-expressing U937 cells were cultured to allow a few passages. The luciferase activity of the cells was checked using the BLI system and found to be highly active (**Figure 1A**). Cells were observed under a fluorescence microscope to confirm the tdTomato expression (**Figure 1B**). NRG mice were injected intravenously with U937-Luc-tdTomato cells, and the transplantation was confirmed by BLI at day 6 (post-engraftment). Animals with equivalent bioluminescent signals were randomized into groups and were treated intraperitoneally with vehicle control (DMSO), intravenously with Daunorubicin (DNR, 1 mg/kg), Cytarabine (Ara-C, IP, 25 mg/kg), or a combination of DNR and AraC as illustrated in **Figure 1C**. The drug doses were selected based on the literature and the experience in the laboratory[12-15].

DNR monotherapy had no impact on the disease burden during the study. On the other hand, AraC monotherapy showed significant efficacy beginning at the early stages of the study that continued throughout (**Figure 2A**,**B**). Notably, combination treatment suppressed the leukemia progression more effectively than the single-drug treatments, as revealed by BLI (**Figure 2A**–**C**). **Figure 2C** shows detectable leukemia BLI signals of animals on day 22. Animals from DNR and AraC monotherapy groups showed 2.8-fold ($P < 0.001$) and 48.3-fold ($P < 0.0001$) lower signals compared to control animals, respectively. Remarkably, animals treated with a combination of DNR and AraC exhibited 102-fold lower signals compared to control animals ($P < 0.0001$) (**Figure 2C**). To monitor the adverse effect of the drugs, the total bodyweight and overall appearance of the animals were monitored throughout the study, and no significant changes in the bodyweight or abnormal appearance were observed (**Figure 2D**). Bodyweight loss above 10% compared to Day 0, lethargy, piloerection, diarrhea, behavioral changes, respiratory distress, or neurological abnormalities can be signs of drug toxicity if not disease-related. Investigators need to be careful, because >15% bodyweight loss or any severe forms of the signs mentioned above would meet the humane endpoint criteria described by the IACUC.

On day 22, single cell suspensions of bone marrow and spleen tissues were prepared to analyze the leukemia engraftment by flow cytometry. **Figure 3A** shows the gating strategy followed to quantify the percent engraftment in the tissues tested (i.e., bone marrow and spleen). Briefly, debris was excluded with the first gating, followed by a single-cell gating (i.e., FSC-A vs. FSC-W). Only live cells were analyzed, because dead cells were excluded with FVD. Percent engraftment was calculated by the formula shown in the protocol. Flow cytometry data showed 29% human CD45-positive (hCD45+) leukemia infiltration in the bone marrow of a control mouse (**Figure 3B**). DNR monotherapy partially inhibited leukemic infiltration, with 17% hCD45+ cells remaining in the bone marrow (**Figure 3B**). AraC monotherapy was more effective at reducing leukemia cells, with only 2.8% hCD45+ residual cells (**Figure 3A**). Notably, the combination of DNR and AraC resulted in near-complete elimination of hCD45+ cells in the bone marrow with 0.05% leukemia remaining (**Figure 3B**). In spleen, the control group had 5.8% leukemic infiltration, as demonstrated by the number of hCD45+ cells (**Figure 3C**). DNR monotherapy resulted in no reduction of hCD45+ cells in the spleen (7.5%), whereas the AraC monotherapy reduced the number of hCD45+ (0.6%) cells (**Figure 3C**). Strikingly, the combination of DNR and AraC eliminated almost all hCD45+ leukemic cells from the spleen (0.05%) (**Figure 3C**). Cells that were

positive for hCD45 (white blood cell marker) were also tested for human CD33 (myeloid cell marker) and found to be positive over 90% in all groups (**Figure 3B**,**C**).

The spleen is the largest lymphoid organ and a common site for the extramedullary hematopoiesis and leukemia invasion in AML. Therefore, enlargement of the spleen (splenomegaly) is commonly observed in AML models and contributes to the progression of the disease. On day 28, spleens from all groups were harvested and grossly examined for their size and weight. The sizes and weights of the spleens were significantly different between the control and the drug treatment groups (**Figure 4A**). The spleen size of mice cotreated with DNR and AraC were smaller than all single-arm groups (**Figure 4A**). Single-cell suspensions of spleens were prepared and analyzed by flow cytometry to quantify the engraftment as described above. The control group exhibited 85% hCD45+ cells in the spleen, which was significantly higher than the day 22 results of the same study (**Figure 4B**). DNR alone showed 79% and AraC alone 24% hCD45+ cells (**Figure 4B**). Once again, the combination treatment with DNR and AraC revealed improved efficacy over the single-drug treatments, with 13% hCD45+ cells remaining in the spleen (**Figure 4B**). Bone marrow and spleen cells were collected on day 28 and stained with Wright-Giemsa solution to examine the morphology. Cytological analysis of the bone marrow and spleen showed an elevated percentage of blasts with a typical morphology in control bone marrow (**Figure 5A**) and spleen (**Figure 5B**) samples. However, lower blast cells but higher spared normal-looking mouse monocytes and granulocytes were observed in specimens of DNR, AraC, or animals treated with a combination of both (**Figure 5A**,**B**). These morphological features correlate with the BLI and flow cytometry data and confirm the efficacy of the treatments.

FIGURE AND TABLE LEGENDS:

Figure 1: Functional characterization of U937-Luc-tdTomato cells. (**A**) Luciferase activity of cultured U937-Luc cells. Bar graphs show quantification of luciferase activity. (**B**) Bright and fluorescent micrographs (20x objective) of tdTomato positive U937 cells in growth medium. Scale = 100 µm. (**C**) Schematic representation of the study. Luciferase and tdTomato-labeled human AML U937 cells in NRG mice. The engraftment was confirmed by BLI and randomized into treatment groups based on whole-body bioluminescence. Treatment with vehicle control, daunorubicin (DNR, 1 mg/kg), cytarabine (AraC, 25 mg/kg), or a combination of DNR and AraC started on day 7. Animals were monitored by BLI throughout the study. Bone marrow or spleen was collected to analyze the frequency, phenotype, and morphological changes of U937 cells after the treatment.

Figure 2: Monitoring the efficacy of treatment with the BLI system. Luciferase and tdTomato-expressing U937 (U937-Luc-tdTomato)-bearing NRG mice were treated with either vehicle control, daunorubicin (DNR, 1 mg/kg), cytarabine (AraC, 25 mg/kg), or a combination of DNR and AraC. (**A**) Inhibition of leukemia progression with single-drug or combination treatment monitored by BLI. Data = mean ± SEM. (**B**) Bioluminescence images of the study animals. (**C**) Efficacy of DNR, AraC, or the combination treatment on day 22 compared to the control. Data = mean ± SEM analyzed by one-way ANOVA. (**D**) Body weight changes of study animals throughout the study. Data = mean ± SEM analyzed by one-way ANOVA. ***P < 0.001, ****P < 0.0001, one-way ANOVA.

Figure 3: Quantitative analysis of leukemia in tissues of AML-bearing animals by flow cytometry. Bone marrow or spleen cells were isolated from the animals treated either with vehicle control, daunorubicin (DNR, 1 mg/kg), cytarabine (AraC, 25 mg/kg), or the combination of DNR and AraC on day 22, and analyzed by flow cytometry. (**A**) Gating strategy used to quantify the engraftment percentage in the tissues. (**B**,**C**) Percent human CD45 positive (hCD45+) and human CD33 positive (hCD33+) cells in the bone marrow (**B**) and spleen (**C**) of study animals.

Figure 4: Analysis of spleen. (**A**) Spleen sizes harvested from the study animals treated either with vehicle control, daunorubicin (DNR, 1 mg/kg), cytarabine (AraC, 25 mg/kg), or the combination of DNR and AraC on day 28. The lower panel shows the weight of the spleens. (**B**) Chimerism of leukemic cells (hCD45+) in the spleen at day 28 after transplantation. Lower panel shows percentage of human CD33 (hCD33) cells in the spleen.

Figure 5. Morphological analysis of bone marrow and spleen. Morphological appearance of Wright-Giemsa-stained bone marrow (**A**) and spleen (**B**) cells isolated from mice treated either with vehicle control, daunorubicin (DNR, 1 mg/kg), cytarabine (AraC, 25 mg/kg), or the combination of DNR and AraC 28 days after the transplantation of U937-luc-tdtTomato cells into NRG mice. Scale = 100 μm.

Table 1: Flow cytometry panel for U937-tdTomato cells.

DISCUSSION:

Critical steps in the protocol

Characterization of the mouse model: Each cell line and primary cell-derived murine xenograft has a unique disease and host-specific factors that influence the time and characteristics of engraftment. Time to engraftment is usually defined as time taken for the bone marrow to have 25% blast cells or have bioluminescence signaling at least two logs higher than background or non-tumor bearing mice. It is critical to characterize the mouse model prior to starting an experiment to look for the therapeutic efficacy of a novel agent, even if the same model has been used and published by others.

Viability of the cells: Engraftment depends on the condition of cells, cell cycle stage, and number of the leukemia cells being transplanted. If the viability of the cells is <80%, use density gradient medium to separate dead cells. Luciferase labeling of the leukemia cells should be checked prior to injection (>95%). If the source of cells is a patient or previously engrafted mouse, perform flow cytometry to know the exact percent of leukemia cells (CD13 and CD33+ myeloid blast of total CD45+ cells).

Authentication of cells: Cell authentication (e.g., ATCC STR method) is recommended, especially if the cells have been passaged several times.

Transplantation technique: It is critical to ensure that all animals in each group receive the same cell dose, especially in the case of non-luciferase labeled cells. The individual most experienced

in this procedure should perform the injection. An alternate method that can be used for cell transplantation is retro-orbital injection of cells. However, there is a risk of cell accumulation or formation of tumor clumps within the orbital cavity, which would stress the animals.

Timing of tissue collection: Choosing the appropriate time for tissue collection is critical in animal models where primary cells or unlabeled cell lines are used. Pilot experiments to characterize the progression of leukemia in each of the models will help determine how rapidly leukemia will progress, leading to demise of the animal. Several factors determine the timing of leukemia development and progression, including the drug used, aggressiveness of the cells used (initial vs. relapsed refractory leukemia cells), and the dose of the cells used (e.g., 50,000 vs. 1 x 10^6 cells per mice). Serial imaging as well as monitoring of the general status of the mice will help determine the optimal time to collect tissue. Typically, 4–6 weeks of drug treatment after engraftment is sufficient to demonstrate drug efficacy[16,17].

Humane conditions: Mice should be sacrificed regardless of engraftment if signs of moribund illness are noted. Animals should be carefully monitored and euthanized if treatment interferes with normal feeding, drinking, and grooming behavior or if they show signs of morbidity such as loss of weight, imbalance, lack of feeding/drinking, crouching or hunching up, or difficulty breathing. In studies where there is potential for pain or distress from either test material administration or procedures, humane endpoints to avoid or terminate unrelieved pain or distress should be employed whenever possible.

Number and gender preference: Group size should be carefully determined to minimize the number of animals needed to give statistically significant results. For example, power analysis for a one-way ANOVA shows that the animal numbers used in each group is sensitive enough to detect a minimum effect size of .46 with $\alpha = 0.05$, $\beta = 0.2$, $F = 3.20$ (calculated using G*Power 3.1.9.2). The number should be adjusted for the rate of engraftment (e.g., some cells may only have a 60–80% engraftment rate) and an additional 10% to account for intervention-unrelated death and illness. The preferred age for xenograft studies is between 6 to 12 weeks old. We recommend investigators use both genders (50% each) for all studies where comparisons will be made across studies. Otherwise, sex-dependent differences in response to treatment cannot be excluded, especially for the therapeutic testing.

Cell dose for transplant: The number of cells used for intravenous injection differs depending on the type of cell line. An initial study to determine the correct dose necessary to obtain the desired engraftment within a reasonable time has to be performed prior to starting the efficacy experiment. For example, injecting 50,000, 100,000, 200,000, and 500,000 luciferase labeled cells to three mice each and measuring the luciferase signal can determine the engraftment rate, progression pattern, and signal detection. Choose the appropriate cell dose based on the findings.

Modifications and troubleshooting of the method

Selection of mouse strain: It is worth noting that even within the same class of leukemia, such as AML, the rate of engraftment differs based on cell characteristics and host factors. Start with the

right genetic background: Special consideration should be given for the selection of a mouse strain based on the cell line and the drug being used. NRG and NRG strains are preferred over NSG or NSG-S strains of mice. NRG mice are more resistant to toxicity caused by irradiation and DNA-damaging drugs. NSG-S or NRG-S/SGM are the strains of mice best suited for myeloid leukemia xenografts[13,18]. Talking to a mice vendor about various available strains would be worthwhile prior to beginning expensive experiments.

Imaging: If BLI does not show increased signaling, but the mice are expected to have engrafted, it is possible that the luciferase substrate injection and dosing are not appropriate. Double-check the dose and proper injection. It is important to characterize each model for imaging time. Time of injection of luciferin substrate and distribution of substrate throughout circulation and peak signal time may be different with each cell line. Generally, the peak signal is between 2-4 min after the injection of substrate. Exact timing of the peak signal can be determined by doing serial imaging of one sentinel animal and use the same time interval for all animals for the rest of the experimental duration. Acquire images consistently after the same amount of time (2 or 4 min) for all animals of the same group. The maximum bioluminescence intensity detectable from imaging also depends on each cell line (e.g., polyclonal vs. monoclonal), method used for luciferase labeling. Although BLI is a useful and sensitive method to study leukemia in animals, it is recommended to pair it with flow cytometry to compare and correlate them. This is especially useful when certain drugs interfere with BLI.

Secondary method to track response and engraftment: Check 1–2 mice from each group for engraftment using a secondary method like bone marrow flow cytometry. NOD SCID mice are very susceptible to infections. Sterile precautions while handling, acidified drinking water, and sterile technique should be practiced while handling mice.

Drug administration techniques: Depending on the route of drug administration (e.g., oral gavage, intravenous, or intraperitoneal), keep in mind possible immediate and late adverse effects that may harm the animal, such as drug delivery into the lung cavity, puncture of the intestine during IP injection, damage to the esophagus during gavage, spasm of blood vessels during multiple vein injections, etc.

Limitations of the method

Lack of leukemia-immune interaction and tumor microenvironment: The biggest limitation of xenograft models using immunocompromised mice is the lack of interaction between immune components and cancer and the therapeutic agent. As we learn more about the role of the immune system and immunotherapy, this model is not appropriate to study drugs that may interact or work by modulating the immune system. Newer syngeneic, humanized models are being developed to study immunotherapeutics.

Expenses: The cost associated with the purchase of mice, handling, and housing is high. Maintaining breeding colonies may help mitigate some of the cost, but it is important to know the proper breeding techniques. Working closely with your institutional animal facility to maintain sterile conditions and handling techniques is of utmost importance, because common

contamination can compromise entire experiments.

Significance and future direction

Despite some limitations, cell line and patient cell-derived murine models are widely accepted, relatively simple xenograft models that can quickly provide valuable insight regarding in vivo efficacy of novel therapeutic agents in a short span of time (6–8 weeks) for proof of concept projects. The Patient Derived Xenograft (PDX) model is an animal model in which the primary leukemia cells of patients are transplanted directly into immunodeficient mice. Development of PDX leukemia models needs some additional considerations that are not discussed in this protocol. There are large repositories of well-characterized PDX and cell line xenograft models that are published and available for the researcher to obtain and use. Many of these mouse models also have cytogenetics, immune phenotype, and genome sequencing information available that makes it easy to choose a model that is most appropriate for the targeted agent being investigated. Many academic research institutes have core facilities that provide assistance to investigators for the use of xenograft models. Xenograft models are not only an excellent system to study therapeutic efficacy but also to understand drug-host interactions in tumor-bearing animals, pharmacodynamics, pharmacokinetics, and dose-limiting toxicities of the test compound.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare that they have no competing interests. Mark Kester is the Chief Medical Officer of Keystone Nano, Inc. Thomas P. Loughran, Jr. is on the Scientific Advisory Board and has stock options for both Keystone Nano and Bioniz Therapeutics. There are no conflicts of interest with the work presented in this manuscript.

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Figure 1

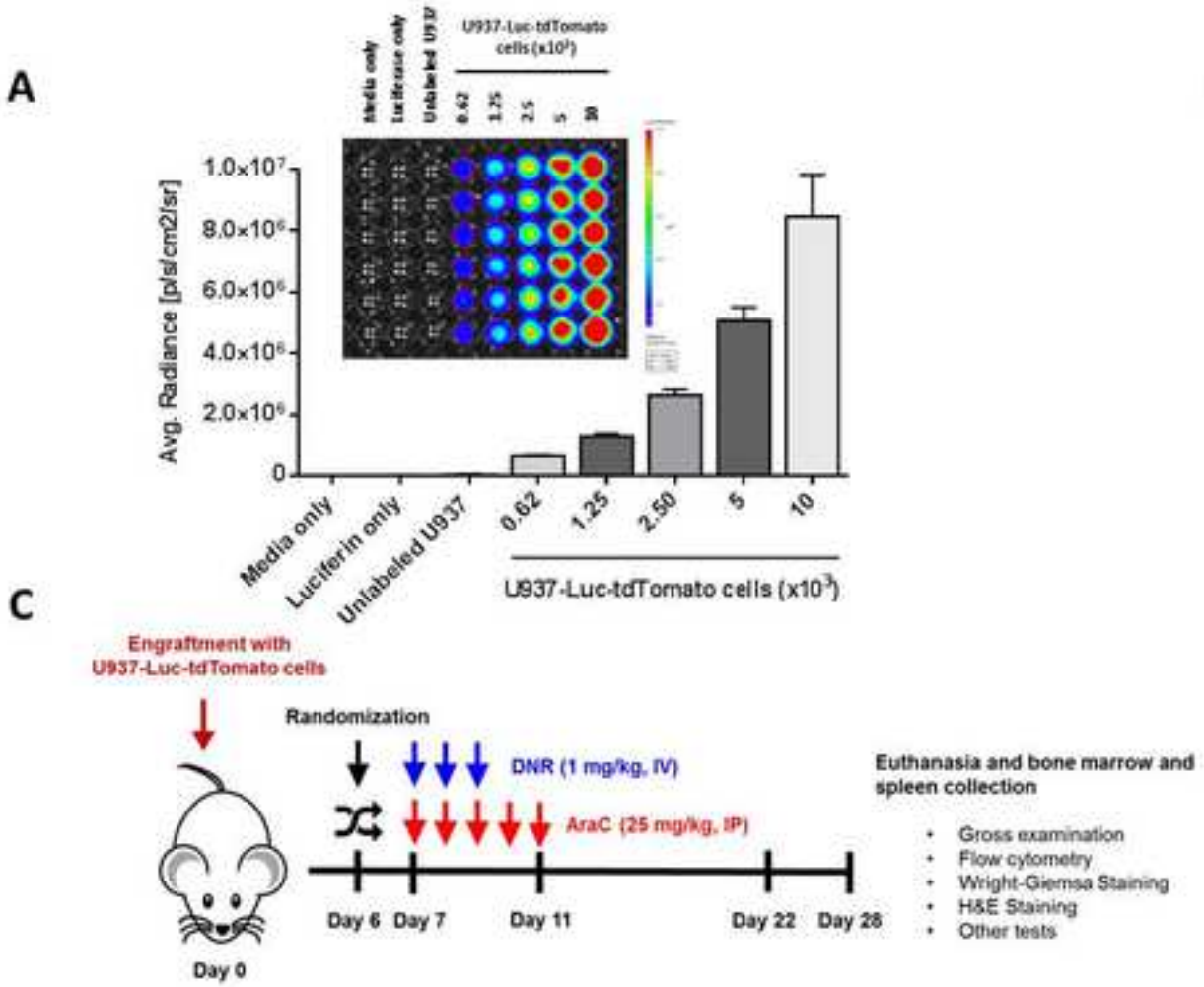

Figure 2

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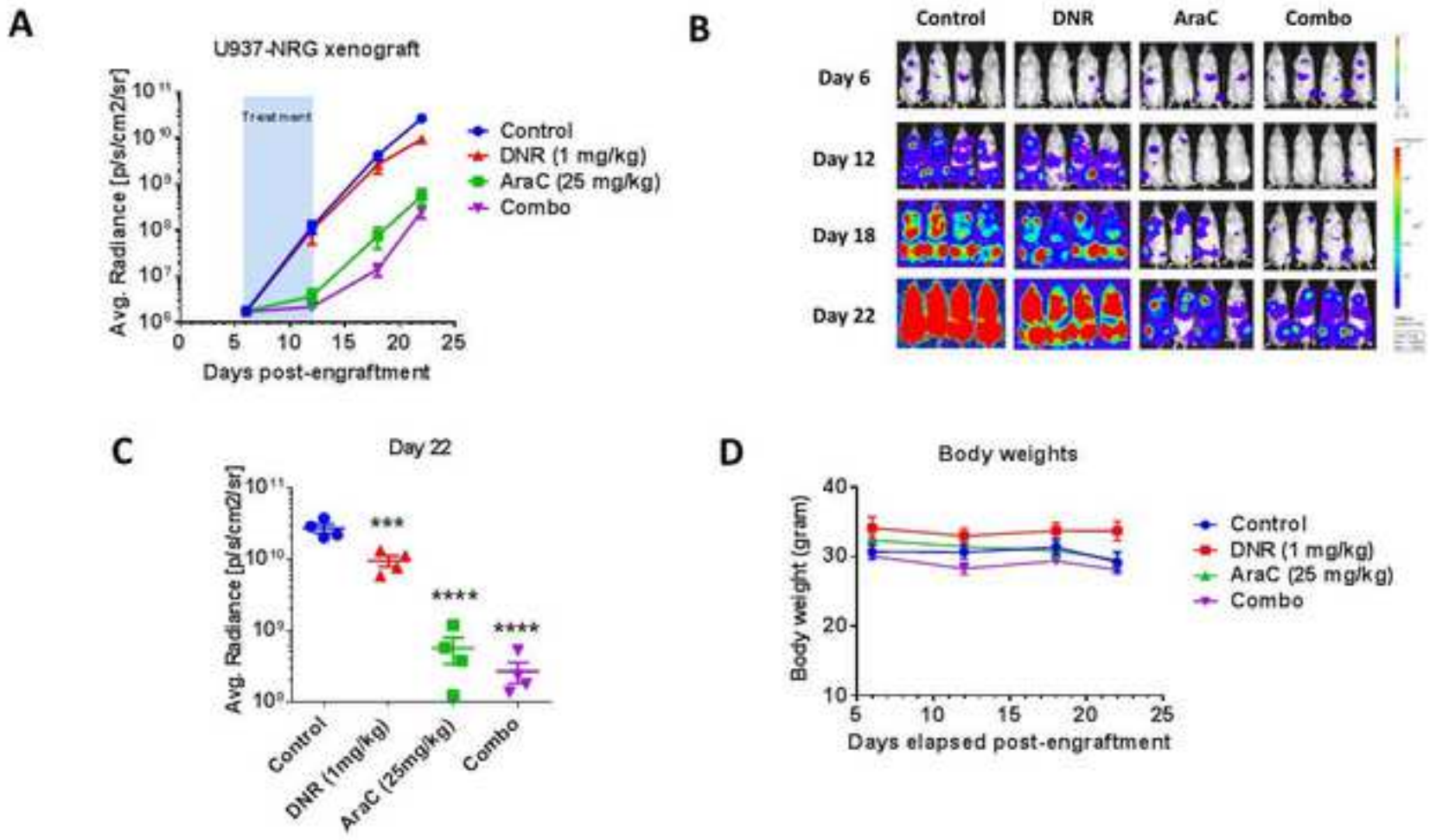

Figure 3

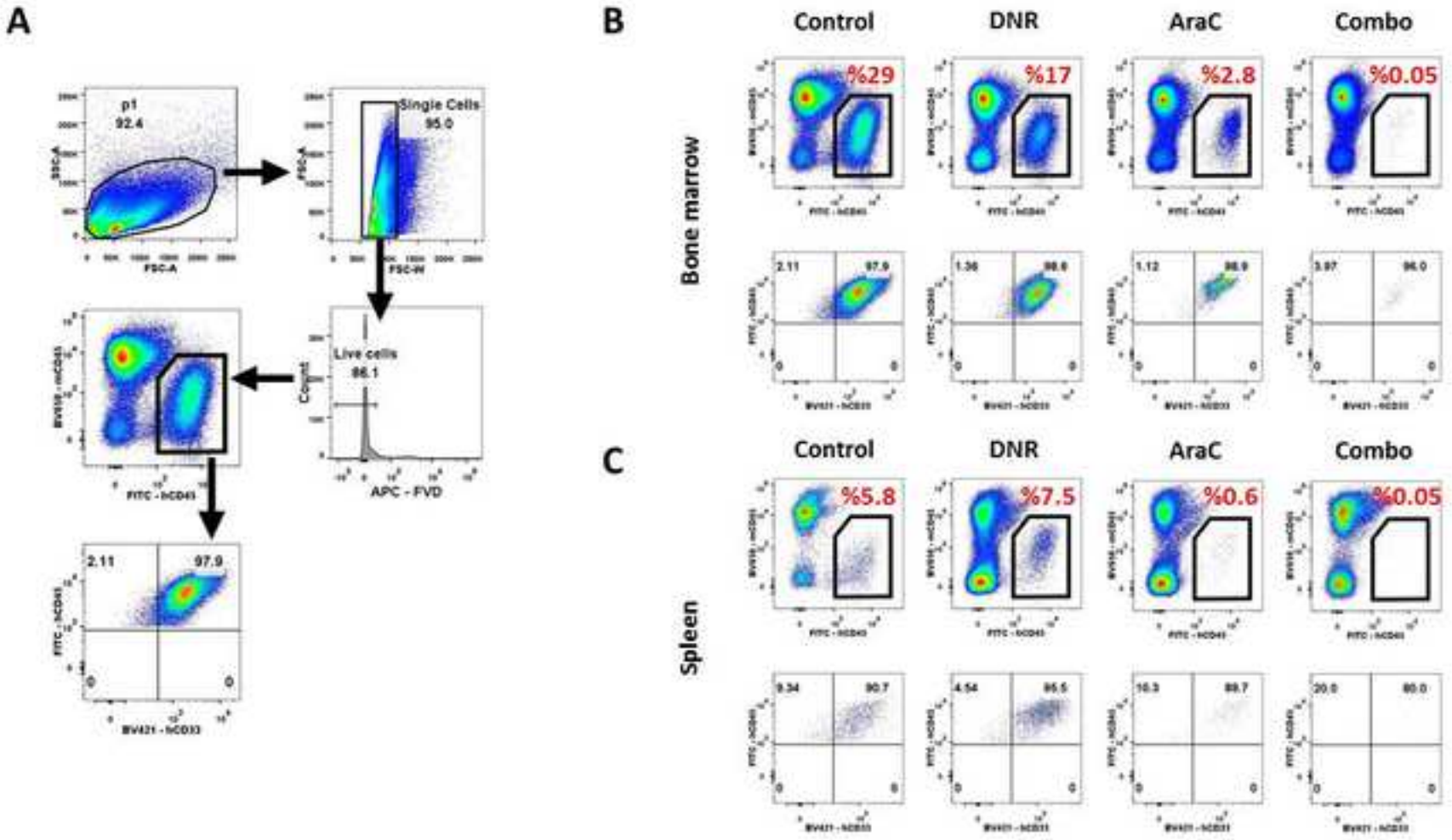

Figure 4

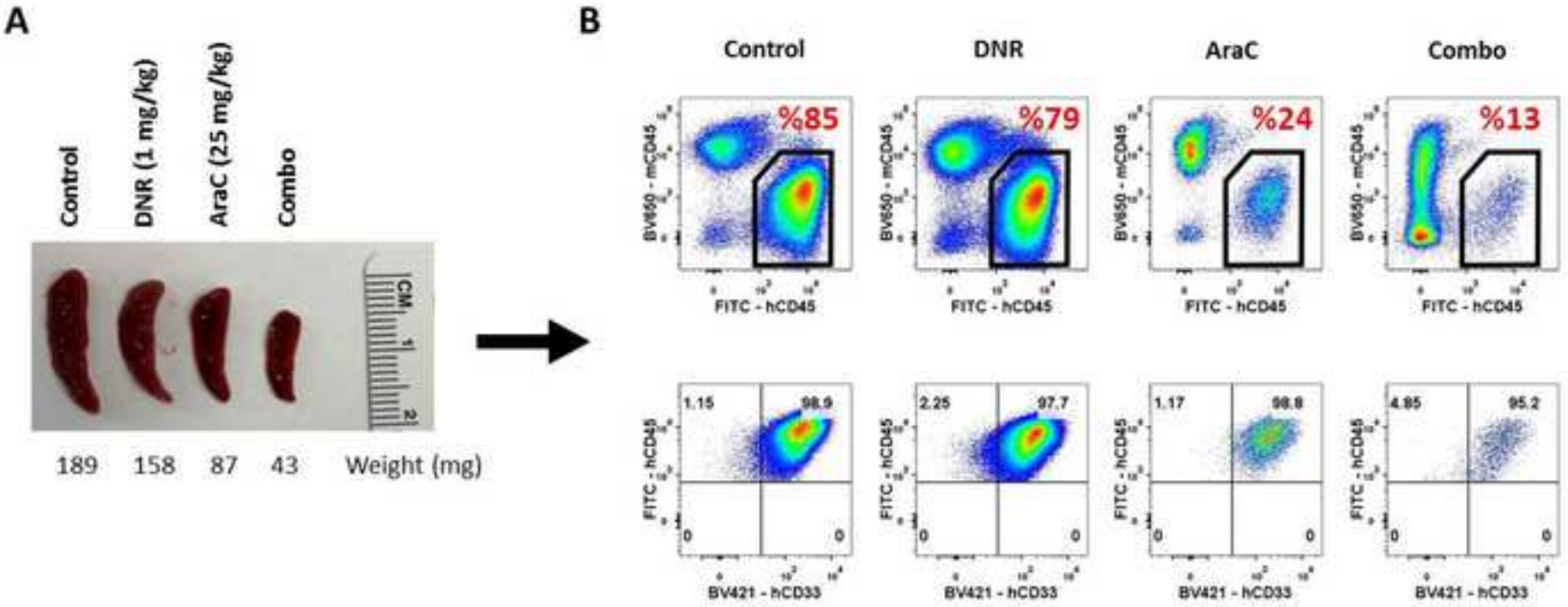

Figure 5

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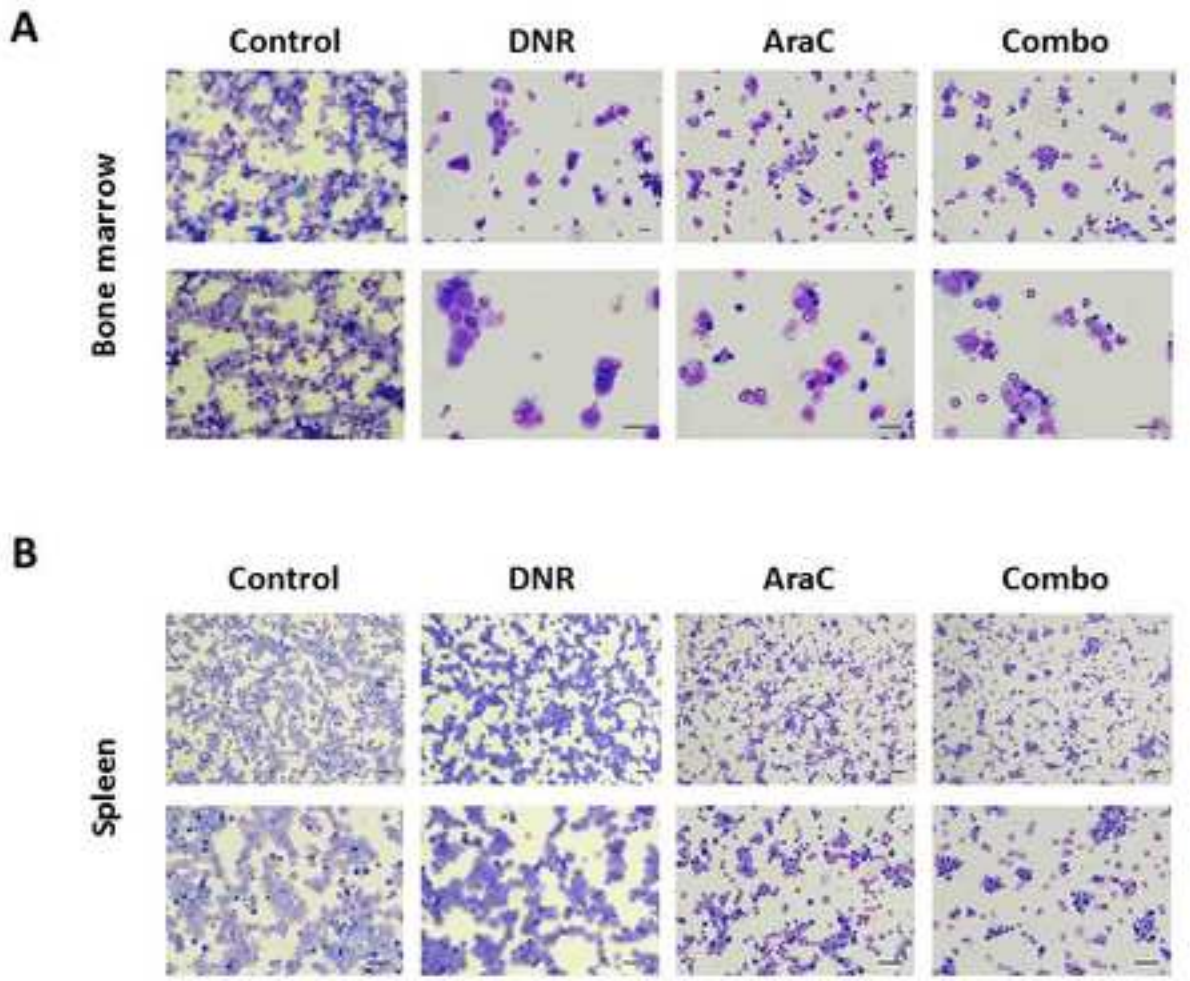

Table

Antibody or dye	Fluorochrome	Laser Excitation	Detection Filter	Clone	Amount in 300 µL sample (µL)
mouse CD45	BV-650	Violet 405nm	670 nm	300-F11	0.3
human CD45	FITC	Blue 488nm	525 nm	HI30	2.5
human CD33	BV421	Violet 405nm	450 nm	P67.6	2.5
Fixable Viability dye (FVI	eFlour 660 (APC)	Red 635nm	670 nm	N/A	0.5
U937 cells	tdTomato (PE)	Green 532nm	582 nm	N/A	N/A

Table of Materials

Click here to access/download;Table of Materials;60980_Table of materials.xlsx

Name	Vendor name	Catalog/Part/Stock number
1 mL Syringe	Fisher Scientific	309659
1.5 inch short bevel 20 g needle	Fisher Scientific	305179
1.5 mL microcentrifuge tube	Fisher Scientific	02-682-002
13 mm Single Ring slide	Fisherbrand	22-037-241
15 mL Polypropylene Conical Tube	FALCON	352097
1X RBC Lysis Buffer	Fisher Scientific	501129751
2 mL microcentrifgue tube	Fisher Scientific	05-408-138
4.5 inch blunt/straight tip scissors	Fisher Scientific	28251
4.5 inch serated straight forceps	VWR	82027-440
5 mL, 12x75 mm round bottom test tubes (flow tubes)	Corning	352008
5/8 inch sterile 25 g needle	Fisher Scientific	305122
6-well Non Treated Cell Culture Plate	USA Scientific	CC7672-7506
7AAD	Biolegend	420404
Alcohol Prep	COVIDIEN	6818
Aluminum Foil	VWR	89107-726
AutoFlow IR Water-Jacketed CO2 Incubator	NUAIRE	Model no. NU-8700
Blood Collection	RAM Scientific	76011
Brilliant Stain Buffer	BD Biosciences	563794
BV421-human CD33	Biolegend	366622
BV-650 mouse CD45	BD Biosciences	563410
Cell Analyzer	EMD Millipore Corparation	N/A
Cell Strainer	FALCON	352350
Centrifuge machine	BECKMAN COULTER	605168-AC
Count & Viability Kit	EMD Millipore Corparation	MCH100102
Cytocentrifuge	Fisher Scientific	A78300003
Cytoclip Slide Clip	Fisher Scientific	59-910-052
Dimethyl Sulfoxide (DMSO)	EMD Millipore Corparation	67-68-5
Disposable Centrifuge Tube	Fisher Scientific	05-539-8
D-Luciferin-Sodium Salt	GoldBio	LUCNA-1G
FACS buffer (PBS with 2% FBS (Heat-inactivated))	N/A	N/A
Filter Cards for Cytospin	Fisher Scientific	22-030410

FITC-human CD45	Biolegend	304014
Fixable Viability Dye	Thermo Fischer	65-0864-14
Fixation Buffer	Biolegend	420801
Flow cytometer	BD Biosciences	N/A
Flow Cytometry analysis software	FlowJo, LLC	Version 10
Freezing Medium (90% FBS + 10% DMSO)	N/A	N/A
Graduated Tips	USASCIENTIFIC	10/20 µL (1110-3700), 200 µL (1111-1700), and 1000 µL (1111-2720)
Hank's Balanced Salt Solution (HBSS)	Gibco	14025092
Heat Inactivated Fetal Bovine Serum	Atlanta Biologicals	H17112
In Vivo Imaging system	Perkin Elmer	CLS136331
Insulin Syringes	BD	329461
Inverted Microscope	Olympus	CKX31
Isoflurane	VEDCO	NDC 50989-150-15
LABGARD CLASS II TYPE A2 BIOLOGICAL SAFETY CABINET	NUAIRE	Model no. NU-425-400
Living Image Software – IVIS Lumina Series	Perkin Elmer	128110
Low Flow CO2 Regulator	E-Z Systems	EP-1305
MB-10 tablets, sterilant	Quip Laboratories	MBTAB75BX
Micro cover glass	VWR	48366 205
Mounting medium	Fisher Scientific	SP15-100
Mouse anti-human Fc receptor antibody	BD Biosciences	564220
Mouse cage lid for euthanasia	E-Z Systems	E-20028
NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}/SzJ (NRG)	Jackson laboratory	7799
ntracellular Staining Permeabilization Wash Buffer (10X)	Biolegend	421002
PE-anti-BTK	Biolegend	558528
PE-anti-pSTAT3-tyr705	Biolegend	651004
Penicilllin Streptomycin Solution, 100X	CORING	30-002-CI
Phosphate-Buffered Saline (PBS)	CORNIG cellgro	21-040-CV
Pipet controller	DRUMMOND Scientific	109883
Pipette	Eppendord Research	2.5 (O24694B), 10 (O31418B), 20 (O24694B), 100 (O337778), 200 (O26279B) and 1000 (O40665B)
Rat anti-mouse CD16/32 antibody	BD Biosciences	553142
RBC Lysis Buffer (10X)	Biolegend	420301
Refrigerated Centrifuge	NuAire	NU-C200R

Reusable sample chamber	Fisher Scientific	5991040
RPMI-1640 medium	CORING	10-040-CV
Serological pipet	FALCON	5mL (357543), 10mL (357551), 25mL (357535)
Styptic Powder	Fisher Scientific	NC1577028
Tailveiner for mouse	Agnthos	TV-150
Trypan Blue Solution	Corning	25900051
Wipes	Fisher Scientific	34155
Wright-Giemsa Stain	VWR	10143-106

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December 20, 2019

Dr. Xiaoyan Cao, PhD,
Review Editor
JoVE

Dear Dr. Cao,

We are pleased to submit a revised version of our manuscript ID# JoVE60980, entitled “A xenograft mouse model to assess efficacy of therapeutic agents for human acute leukemia.” for consideration for publication in Journal of Visualized Experiments.

We thank the Editor and Reviewer’s for the valuable comments and suggestions, which have helped us to strengthen the current version of the manuscript. We have incorporated reviewer’s suggestions, and we hope that the revised version meets with your and the reviewers’ approval for publication. Suggestions have been addressed as follows:

Reviewers' comments:

Reviewer #1:

Manuscript Summary: The authors provided a detailed protocol of using immunodeficient mice and cell line engraftment as in vivo model for leukemia study. They described how to establish the xenografts and the methods to monitor and evaluate disease progression. They also showed the example that using this model to investigate the efficacy of therapy. The protocol is mainly comprehensive and most key points are covered; I only have a few minor comments:

1. It would be nice if the authors can comment on the number of mice to use, such as how many mice are needed to have sufficient statistic power in general.

   Author comment: Authors thank reviewer for an important suggestion. In the revised version we have stated in the discussion the number of mice typically used in this type of study. Briefly, group size should be carefully determined to minimize the number of animals needed to give statistically significant results. For example, power analysis for a one-way ANOVA shows that animal numbers used in each group will be sensitive enough to detect a minimum effect size of .46 with $\alpha = 0.05$, $\beta = 0.2$, $F = 3.20$; calculated using G*Power 3.1.9.2. Number should be adjusted for rate of engraftment (some cells may only have 60-80% engraftment rate) and additional 10% to account for intervention unrelated death and illness.

2. The authors should discuss the number of cells needed for transplantation. Although for their model 10k cells are sufficient, it is common much more cells are needed to establish engraftment, especially for PDXs.

   Author comment: Authors thank reviewers for the comments. We have discussed some considerations to determine optimal cell dose for transplantation. In summary, cell dose for transplant differs depending on the type of cell line. Initial study to determine the correct dose to obtained desired engraftment within a reasonable time has to be determined prior to starting the efficacy experiment. For example, inject 50K, 100K, 200K and 500K luciferase labeled cells to 3 mice each and measure luciferase signal to determine, the engraftment rate, progression pattern, and signal detection. Choose appropriate cell dose based on the findings.

3. It is useful to mention that spine can also be collected when more bone marrow cells are needed, although with spine only mortar and pestle can be used for extracting bone marrow cells.

   Author comment: We have noted that other bones may also be used in addition to those mentioned.

4. It is practical to take a serial of pictures during BLI, thus the image with the peak signaling can be chosen for analysis.

   Author comment: Authors thank reviewers for pointing this out. We clarified in the discussion that serial imaging of one sentinel animal would be ideal. Exact timing of the peak signal can be determined by doing serial imaging of one sentinel animal and use the same time interval for all animals for the rest of the experimental duration.

5. It is helpful that the authors provide some references that characterize/compare different mouse strains.

   Author comment: Authors thank reviewers for their comments. We have added two relevant references to help readers understand the selection of an appropriate strain of mouse for their experiment.

6. For the Representative Results:
   a. Please clarify that whether the data described in Figure 3 and Figure 4 are from the same cohort of mice, or from different experiments? **b.** Do the authors have BLI data and flow cytometry data from the same set of mice? Can the authors show how well these data are correlated? It is very useful for the reader to know the similarity or potential difference between these two methods, which can guide the experiment design.

   Author comment: **a.** Data presented in Figures 3 and 4 were obtained from the same cohort of animals but different individual mice. **b.** The authors have collected BLI data up to day 22 (post-engraftment) for all animals in the study (Figure 2). However, authors collected flow cytometry data of only a mouse (mouse #4) from each cohort, and kept other animals for other assays. BLI data and flow cytometry data from the same set of mice correlate well. We suggest using both the methods to obtain a reproducible and more powerful data set.

Reviewer # 2:

As xenograft models of leukemia continue to become more widespread, there are more and more investigators attempting these experiments for the first time. Therefore, complete step by step protocols would certainly be of value to the field. There are several approaches and methods to generating, treating, and monitoring leukemic mice. The current manuscript focuses on using a BLI model of a cell line (U937) derived xenograft, which is a common and useful xenograft approach.

While this protocol is likely to be of interest to the JoVE audience, I feel that there are areas for improvement. The manuscript could benefit significantly from adjusting focus of each of the protocols to what is unique to xenograft modeling specifically. There are many opportunities to offer insights into these points, however the manuscript falls short in this regard.

Specific comments:

1. This model requires a luciferase+ cell line. It would be a nice addition to include the process of creating such a line so that a reader could start from the beginning. Transduction with human trophic envelopes, selection of positive clones (bulk sort or single cell cloning).

   Author comment: We agree with the comment. However, this is out of scope for this article.

2. The abstract says NOD/SCID mice are used, but NRG mice are used. This should be corrected. NOD/SCID should not be used as a generic term for immune deficient mice.

   Author comment: We apologize for the oversight and error is corrected in the revised version of the Manuscript.

3. The summary (and a couple of other places throughout) indicates that the protocol models liquid tumors in immunocompromised mice using cell line xenograft as an example. However, I don't think the methods presented here would be so readily translated to non-AML cell line modeling such as primary patient material. In my opinion, the claims of utility of the protocol should be limited to AML cell lines with BLI.

   Author comment: We agree with the reviewer. We have made it clear in summary and in discussion that current manuscript focuses on AML cell line xenografts only.

4. Protocol 1.1.1 - "depending on the study" -- it would be helpful to describe what type of studies require young mice and when is it acceptable to use 20-week-old mice. Please expand on how to select strain, sex, age, etc.

   Author comment: Reviewer is thanked for the comment. Mice used in the present studies were between ages of 10-12 weeks. We apologize for the typographical mistake. Age of the mice is important to consider based on the disease you are trying to model. We have consistently used mice between 6-12 weeks of age which has shown consistent AML cell engraftment. Recent studies indicate that there is a difference in the extent of cancer cell engraftment between male and female xenograft mice and recommended that investigators should use one sex (female or male) for all studies where comparisons will be made across studies. Some other studies have shown that female mice engraft better for hematopoietic studies. Specially for the therapeutic testing, we cannot exclude sex dependent differences in response to treatment. We have elaborated this in the discussion section.

5. In the note for this point, I would allow for the possibility that some cell lines might engraft better in one Sex or the other and latencies could be different. This should be determined beforehand for each model. Maybe this is what is meant by "If there is no gender preference", but I took this to mean that the preference was held by the investigator.

   Author comment: Reviewer's interpretation is correct that it is investigators' final decision to wisely select gender based on the literature or simply availability of animals if there is no gender preference. Ideally using both genders mixed in the cohorts is beneficial, which would also help to compare the genders.

6. Protocol 2.1 - Include a typical number of cells transplanted per mouse for a cell line, or at least the specific number of U937 cells used for the example experiment. Is that a typical number for an AML cell line?

   Author comment: We thank Reviewer for the comment. We have included the number of cells transplanted. AML is a heterogeneous disease and AML cell lines behave differently. So each cell line needs different cell numbers to achieve successful engraftment depending on their growth rate and kinetics. Optimizing cell number to transplant (inject) is highly recommended if experimental cell line is not commonly used and/or data is not available in the literature.

7. Protocol 3.4 - What is the concentration of Luciferin used? 5uL/ gram of what concentration?

   Author comment: The concentration used was 30mg/mL of Luciferin. This information is now added in protocol 3.4.

8. 3.5 - For the U937 model, are mice positioned on their backs or on their fronts? Or Both?

Author comment: Mice were positioned on ventral (belly) side. This information is now added in protocol 3.5 in new version of manuscript.

9. 3.6 - Why this step? Why not just put the mice back in the home cage for recovery?

Author comment: These steps were followed to comply with Penn State IACUC protocol and regulations. Steps are further clarified in the article.

10. 3.7.1 - Ear punching should be done earlier - at the time of leukemia injection or when taking weights for the first time.

Author comment: Ear punches are permanent, so it would be better to punch their ears once mice are randomized and cohort groups are ready to get the treatment.

11. No section for drug treatments. How is dauno/ara-c handled, stored, diluted, injected, etc.

Author comment: Thank you for bringing this up. Information has been added in protocol 3.7.2.

12. 5 -- Different institutes have unique SOPs regarding euthanasia. This could be added to the first paragraph of the discussion.

Author Comment: Thank you for pointing that out. We have addressed that in the comment section.

13. 6.13 - Leukemic mice with advanced engraftment often do not have any red in the bone marrow cavity. There should be a better way to describe the flushing procedure that accounts for this likely possibility.

Author comment: It has been added that bones are to be flushed multiple times. Also a note has been added that red in the bone marrow may not be visible with advanced engraftment.

14. Representative results. (Line 450-3). The fold changes in BLI signal are informative, but not necessarily a linear measure of AML burden. The authors should not claim that BLI fold changes equal AML burden fold changes. Actually, the flow data below this line confirms this is the case in comparing day 22 BLI and bone marrow flow data. For example, the Ara-C alone has ~10 fold less AML burden, not ~48 fold as predicted by BLI. This would be a nice point to carefully explain to the reader.

Author comment: We agree with reviewer's comment on use of "Leukemic burden" carefully in the manuscript. Since it was identified using Bioluminescence Imaging (BLI) system, we have replaced "Leukemic burden" either with "BLI signals" or "signals" throughout the manuscript.

15. Line 453-5. What would constitute significant (meaningful) change in body weight? What should the investigator following your advice be looking for? What abnormal appearance could one expect from chemotherapy? What would indicate overdose? These are the type of insights that make a protocol/methods paper more useful.

Author comment: We have carefully described drug or disease related signs or findings in the result section.

16. The doses used for both daunorubicin and cytarabine are quite low compared to other in vivo studies. It would be helpful to discuss how this particular dose was determined and how applicable it might be for other cell lines or PDX models utilizing primary samples

Author comment: Authors have decided the daunorubicin and cytarabine doses based on literature and the experience in the lab (PMID: 30125602, 26130514, 30540936 and 24244429).

17. Line 489 "normal-looking monocytes and granulocytes". These are mouse cells as opposed to differentiated or normalized human cells. I suggest the authors make this point clear.

Author comment: We thank reviewer for an excellent comment and agree that normal-looking cells would refer to both mouse and human cells (might happen due to differentiation). Wright-Giemsa staining of slides showed granulocytes with a ring-shaped nucleus, which is typical morphology of murine neutrophils, whereas a more horseshoe or “banded” nucleus is expected in human immature granulocytes (PMID: 23423530). Mature human granulocytes have four or more segments, but mouse mature granulocytes would have a more cloverleaf-like pattern.

18. Line 509-511. CD33 staining is not sufficient to quantify the exact number of blast cells in a patient sample. I suggest avoiding trying to claim the model is applicable to other xenograft applications. It works nicely for cell lines with luciferase, and that is enough.

Author comment: We have discussed this in the discussion section. Briefly, Staining with CD markers for AML (hCD45+, CD13+ and CD33+) is useful in non-luciferase labelled cell line or primary cell line models and sometimes as a secondary method of leukemia burden measurement in luciferase labelled cell line xenograft along with BLI but is not necessary.

19. Discussion is lacking many references that could and should be made to support statements. "But it is important to know the proper breeding techniques." Some discussion of this point would be helpful.

Author comment: Thank you for comments. We have added several relevant references.

Reviewer #3:

Manuscript Summary:
This is a well written manuscript, which might be very useful to assess efficacy of therapeutic agents for human acute leukemia in a xenograft mouse model

Major Concerns:

1. Figure 1 A In my opinion it is also important to show stability of the detected signal over time, here over 22 days.

Author comment: Thank you for bringing up the question about stability of luciferase activity. In our hands we have seen repeatedly that multiple passaging several time (~15-20 passage) retain the same activity as previous passages in vitro. This is also true in animals; the stability of luciferase can be noticed by progressive increase in BLI signals in control animal group. Since Control group represents the no therapeutic treatment arm, it gives an excellent idea of stability of Luciferase expression in animals (Figures 2A and B).

2. Is the used cell line a monoclonal cell line? If not, please check out the following paper to see the different circumstances, which have influence on the signal: Bioluminescence imaging of leukemia cell lines in vitro and in mouse xenografts: effects of monoclonal and polyclonal cell populations on intensity and kinetics of photon emission. Christoph S, Schlegel J, Alvarez-Calderon F, Kim YM, Brandao LN, DeRyckere D, Graham DK. J Hematol Oncol. 2013 Jan 23;6:10. doi: 10.1186/1756-8722-6-10. If this a polyclonal cell line, these should be discussed.

Author comment: Thank you for the comment. We have added relevant discussion and reference address the issue.

Minor Concerns:

1. Figure 1 A shows that the detected signal increases with the number of cells. To make the correlation more clear I would recommend to add a graph.

   Author comment: Thank you so much your suggestion to make the manuscript strong. We have added new data that shows the BLI numbers in bar graphs (Figure 1A).

2. Tumor xenograft model derived from leukemia patients is an animal model in which the leukemia cells or primary cell lines of patients are transplanted directly into immunodeficient mice. This should be also part of the discussion.

   Author comment: Since this protocol is focused on cell line derived AML xenograft, we briefly mentioned PDX models and discussed that several steps are different and the details of development of AML PDX models is not completely discussed in this protocol.

The Editor and Reviewers are once again thanked for the thorough review of our manuscript and hope that the revised version will be suitable for publication in the Journal of Visualized Experiments.

Changes to be made by the author(s):

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

Author comment: Manuscript has been thoroughly proofread.

2. Please revise lines 382-385 to avoid textual overlap with previously published work.
Author comment: Lines 382-385 have been revised.

3. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

Author comment: Authors provided an ethical statement at the beginning of each protocol section.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: BD Horizon, Styrofoam, Eppendorf, Cytoclip, IVIS, etc.

Author comment: Authors have addressed the all concerns/questions regarding the commercial/trade/generic names.

5. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.
Author comment: Authors have replaced all personal pronouns and colloquial phrases.

6. Please use imperative tense throughout the protocol as if directing someone how to do your experiment. Please be as specific as you can with respect to your experiment providing all necessary details.
Author comment: Authors have considered the comment and revised the manuscript accordingly.

7. 2.2: Please provide the composition of cell growth medium. What happens after centrifugation, remove supernatant?

Author comment: We have added clarification ----- “Dump off the supernatant…” in the sentence.

8. 4: When is the blood collection done?
Author comment: We have added a statement that blood collection is to be done once per week (4.1).

9. 5. When are mouse euthanized?
Author comment: We have added a statement that mice are to be euthanized when mice exhibit signs of being moribund (5.1).

10. 6.3: Please specify the mesh size of the cell strainer used here.
Author comment: 0.45um strainers are to be used, and have been added to 6.3.

11. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Author comment: Some steps throughout the protocol have been combined for clarity.

12. Please include single line spacing between each numbered step or note in the protocol.
Author comment: Revised manuscript text has been lined with single line spacing.

13. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.
Author comment: We have highlighted the text to be featured in the video.

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.
Author comment: We have highlighted the text to be featured in the video.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Author comment: We have highlighted complete protocols to be included in the script.

16. Please do not highlight any steps describing anesthetization and euthanasia.
Author comment: We did not highlight any anesthetization and euthanasia steps.

17. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.
Author comment: We have uploaded figures in .JPG format.

18. Figure 1B and Figure 5: Please include a scale bar, ideally at the lower right corner, for all microscopic images to provide context to the magnification used. Define the scale in the appropriate figure Legend.
Author comment: Authors have inserted scales into microscopic images.

19. Table of Materials: Please remove any ™/®/© symbols. Please sort the materials alphabetically by material name.
Author comment: All symbols have been removed now.

20. Table S1: Please remove commercial language (BD LSRII).
Author comment: We have removed all commercial names.

21. A minimum of 10 references should be cited in the manuscript. For instance, please include applicable references to previous studies when describing advantages over alternative techniques. Please do not abbreviate journal titles; use full journal name. – will add more references to discussion section
Author comment: A tenth reference has been added in regards to alternative methods for blood collection (4.8).

Sincerely,

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