Human T-ALL Xenografts

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Abstract

Intense chemotherapy regimens of patients diagnosed with T cell acute lymphoblastic leukemia (T-ALL) have proved successful for improving patient's overall survival, especially in children. But still T-ALL treatment remains challenging, since side effects of chemotherapeutic drugs often worsen patient's quality of life, and relapse rates remain significant. Hence, the availability of experimental animal models capable of recapitulating the biology of human T-ALL is obligatory as a critical tool to explore novel promising therapies directed against specific targets that have been previously validated in in vitro assays. For this purpose, patient-derived xenografts (PDX) of primary human T-ALL are currently of great interest as preclinical models for novel therapeutic strategies toward transition into clinical trials. In this chapter, we describe the lab workflow to perform PDX assays, from the initial processing of patient T-ALL samples, genetic in vitro modifications of leukemic cells by lentiviral transduction, inoculation routes, monitoring for disease development, and mouse organ examination, to administration of several treatments.

Keywords

T-ALL, Patient-derived xenograft (PDX), Lentiviral transduction, Immunodeficient mice, Bone marrow aspiration, Bioluminescence imaging

1. Introduction

T cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological malignancy, which represents 10–15% of childhood and 20–25% of adulthood cases of ALL [1]. T-ALL originates from oncogenic transformation of T cell precursors within the thymus and results in colonization of both lymphoid and nonlymphoid organs.

Traditionally, T-ALLs are classified based on their immunophenotype, which ultimately relies on similarities to discrete stages of human T cell development [2]. In addition, improvement of diagnostic genetic techniques in the last years has contributed to the knowledge of the prevalent genomic lesions that accumulate in T-ALLs [3], which have proved useful as prediction parameters to evaluate the outcome of the patients [4–6]. Consequently, these advances have led to the redefining of T-ALL classification based on their genomic lesions and immunophenotype [7].

Intensive chemotherapy regimens using standard drugs such as VxL (Vincristine, Dexamethasone and L-asparaginase) have led to a relatively encouraging prognosis for T-ALL patients. Event-free and overall survival have increased to 70 and 80%, respectively, in the last decades, compared to the 1960s, when only 5–10% of patients were cured [8]. However, relapsed T-ALL is still high and presents a dismal outcome (~20% overall survival), especially in adults [9]. Additionally, secondary effects derived from massive chemotherapy worsen patient recovery. Therefore, the efforts of the scientific community have been lately focused on identifying leukemia initiating or stem cells (LICs or LSCs), which are the cells that accumulate the initial mutations that contribute to the leukemogenic process and the ultimate responsible for relapses.

Primary human T-ALL cells can be functionally studied by ex vivo coculture with murine stromal cells [10]. However, patient-derived xenograft (PDX) models of T-ALL have become a gold standard approach to study leukemia biology, to identify LIC characteristics, and to assay potential new therapies, since these models better preserve the genomic integrity and tumor heterogeneity observed in patients and are able to recapitulate the genetic and epigenetic landscape of T-ALLs [11, 12]. Serial transplantation of PDXs is essential to identify LICs as cells capable of leukemia initiation in new host mice and to study T-ALL clonal evolution and chemotherapy resistance. Leukemia PDXs provide a great advantage over xenografts of solid tumors since disease development can be tracked in vivo without mice euthanasia. The possibility of aspirating the T-ALLs colonizing the bone marrow (BM) has led to significative advances in the knowledge of the interactions of leukemic cells with their niches, and has allowed the study of the mechanisms that contribute to the novo generation of human T-ALL [13].

In recent years, advancements in molecular imaging have emerged as a noninvasive and sensitive tool for continuous monitoring of biological processes within living organisms [14, 15]. Implementation of bioluminescent imaging has allowed to study migration and proliferation and to evaluate therapeutic drug efficacy in preclinical animal models of leukemia [16, 17].

In this chapter, we describe how to perform different types of T-ALL PDXs using immunodeficient mice as hosts, from the initial processing of patient T-ALL samples, genetic in vitro modifications of leukemic cells by lentiviral transduction, inoculation routes (i.e., intravenous, subcutaneous), monitoring of disease development (i.e., bone

marrow aspiration, bioluminescence), and mouse organ examination, to administration of alternative treatments (Fig. 1).

2. MATERIALS

2.1. Leukemic blasts enrichment by density centrifugation.

1. Lymphoprep (STEMCELL Technologies).
2. 15-ml conical tubes.
3. RPMI 1640 without L-glutamine
4. Trypan Blue solution. To prepare 10 ml of working solution, mix 2 ml of 1.06 % Trypan Blue powder diluted in distilled H_2O and 8 ml of 1 % NaCl diluted in distilled H_2O.
5. Neubauer chamber.
6. Inverted microscope.

2.2. Phenotypic characterization by flow cytometry.

1. Flow cytometry antibodies (Table 1): CD2-APC (clone RPA-2.10), CD3-APC (clone UCHT1), CD5-FITC (clone L17F12), CD7-BV421 (clone M-T701), CD8-PE-Cy7 (clone RPA-T8), CD11b-PE (clone ICRF44), CD13-PE (clone WM15), CD19-PE-Cy7 (clone SJ25C1), CD45-V450 (clone 2D1), CD117-APC (clone YB5.B8), HLA-DR-FITC (clone G46-6), streptavidin (sa)-APC-Cy7 (BD Biosciences), CD4-PE-Cy5 (clone 13B8.2), CD33-PE-Cy5 (clone P67.6), CD34-FITC (clone 581), CD56-PE-Cy5 (clone N901-NKH1) (Beckman Coulter), CD1a-biotin (clone Na1/34-HLK) (AbD Serotec) and CD7-PE (clone CD7-6B7).
2. FACS (Fluorescence-Activated Cell Sorting) staining buffer (SB): 10 % bovine serum albumin 1 % fetal bovine serum and 0.01 % sodium azide in PBS.
3. Blocking solution: 10 % normal mouse serum and 10 % CD16/CD32 antibodies commercial mix (Fc-Block) in SB.

4. 5-ml Polystyrene tubes.
5. Assay plate, 96 well no lid V-bottom, non-treated polystyrene.
6. Flow cytometer.

2.3. Cryopreservation.

1. Freezing-medium: FBS + 10 % Dimethyl sulfoxide (DMSO).
2. Isopropyl alcohol freezing container.
3. 1.8-ml cryogenic tubes.

2.4. Lentiviral transduction.

All solutions and materials used for cell culture must be sterile.

1. T-ALL culture medium: RPMI 1640 supplemented with 10 % FBS, 2 % human AB serum, 10 U/ml Penicillin/Streptomycin (P/S), 2 mM L-glutamine and 10 mM Hepes.
2. OP9 culture medium (α-MEM + 20 % FBS): Use 1 L graduate cylinder and add 1 pkg. alpha-MEM powder and 2.2 g $NaHCO_3$ and make up to 1 L with milliQ H_2O while stirring. Do not use the cylinder for any other purpose and do not clean it with any detergent. Filter under sterile conditions (0.22 µm Millipore Stericup unit). Add 10 U/ml P/S (Gibco) and store at 4ºC. Supplement with 20 % FBS when used.
3. Dulbecco's PBS (DPBS) 1X [-] $CaCl_2$, [-] $MgCl_2$.
4. 0.25 % Trypsin: dilute 1 ml of 2.5 % Trypsin in 10 ml DPBS 1X.
5. 100-mm tissue culture-treated cell culture dish.
6. Mycoplasma-free 293T packaging cells (ATCC).
7. 293T culture medium: Dulbecco's Modified Eagle Medium (DMEM), 10 % FBS, 10 U/ml P/S, 2 mM L-glutamine and 10 mM Hepes.
8. jetPEI reagent and 150 mM NaCl (Polyplus-transfection).

9. Second-generation lentiviral packaging system: pHRSIN (lentiviral backbone) (***18***), psPAX2 (gag/pol/rev/tat), pMD2G (VSV-G envelope). psPAX2 and pMD2G can be obtained from Addgene (***19***).
10. 5-ml syringes.
11. 0.22 μm syringe-driven filter unit.
12. 1.5-ml tubes.
13. BD Cytofix/Cytoperm kit (BD Biosciences).
14. 0.05 % Trypsin/EDTA.
15. PBS.
16. 30 μm cup filters.
17. RetroNectin® (Recombinant Human Fibronectin Fragment, Takara): 1 mg/ml in PBS. Store 100 μl aliquots at -20ºC. Dilute each aliquot to 50 μg/ml in PBS and store at 4ºC for the duration of the experiment.
18. Polybrene (hexadimethrine bromide, 8 mg/ml in PBS).
19. PBS + 2 % BSA.
20. Recombinant human IL-7 (rhIL7, National Institute for Biological Standards and Control, NIBSC). Resuspend each ampoule (100000 units) in 1 ml of PBS + 0.05 % BSA (Final concentration 100 U/μl). Prepare 100 μl-aliquots and store at -70ºC. Working aliquot can be kept at 4ºC for short periods.
21. 24-well polystyrene clear flat bottom not treated cell culture plate, with lid.
22. 48-well clear flat bottom tissue culture-treated multiwell cell culture plate.
23. Parafilm M.
24. Humidified 37°C, 5% CO_2 incubator.
25. Water bath.

2.5. Mouse immunodeficient strains and irradiation.

Immunodeficient mice are used for xenotransplantation of human T-ALL cells. For housing and manipulation, an individually ventilated caging system and specific pathogen-free (SPF) barrier areas must be used in the animal facility. Animal procedures are performed using Type IIB2 biosafety cabinets.

1. 6-10 weeks-old NSG (NOD.Cγ-Prkdcscid Il2rγtm1Wjl/SzJ).
2. 6-8 weeks-old RAG-$2^{-/-}$ $\gamma c^{-/-}$ (B6; 129-$Rag2_{tm1Fwa}$ll$2rg_{tm1Rsky}$/DwlHsd) (***20***).
3. 6-10 weeks-old CB17 SCID (CB17/Icr-*Prkdc*^{scid}/IcrIcoCrl).
4. IBL-437C (137Cs) gamma irradiator.

2.6. Cell inoculation.

1. T-ALL cellular preparations in PBS.
2. 70 μm cup filters.
3. 1.5-ml tubes.
4. Ear punch.
5. 70% ethanol-soaked cotton pieces.

2.6.1. Retro-orbital injection.

1. See subheading 2.6, list 1-5.
2. Isoflurane-soaked cotton pieces.
3. 0.5-ml 0.30 mm (30G) x 8 mm insulin syringes.
4. Warming blanket.
6. Isoflurane 1000 mg/g.
7. Plexiglas® chamber.

2.6.2. Intravenous injection.

1. See subheading 2.6, list 1-5.
2. 0.5-ml 0.30 mm (30G) x 8 mm insulin syringes.

3. Heat lamp.
4. Rotating tail injector restrainer.

2.6.3. Subcutaneous injection.

1. See subheading 2.6, list 1-5.
2. 1-ml 0.33 mm (29G) x 12.7 mm insulin syringes.

2.7. Therapeutic treatment of T-ALL xenotransplanted mice.

2.7.1. Intraperitoneal administration.

1. Drugs or antibody solutions.
2. PBS.
3. 1-ml 0.33 mm (29G) x 12.7 mm insulin syringes.
4. Electronic weighing scale.

2.7.2. Oral gavage administration.

1. Compound solutions.
2. Reusable metal gavage straight needle 22G x 25 mm (2 mm tip diameter).
3. 1-ml syringes (BD Biosciences).
4. Electronic weighing scale.

2.8. Xenograft monitoring and mouse analysis.

2.8.1. Body weight measurement.

1. Electronic weighing scale.

2.8.2. Peripheral blood extraction.

1. Heparin sodium solution for injection (1000 IU/ml).
2. 1.5-ml tubes.
3. 23G 1” – Nr.16 0.6 x 25 mm sterile needles.
4. 70% ethanol-soaked cotton pieces.

5. Erythrocyte lysis buffer (ELB-I): 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in distilled H_2O, pH 7.2-7.4.
6. PBS or SB (see subheading 2.2).

2.8.3. Bone marrow aspiration.

1. Isoflurane 1000 mg/g.
2. PBS.
3. 1.5-ml tubes.
4. 1-ml 0.33 mm (29G) x 12.7 mm insulin syringes.
5. Warming blanket.
6. 70% ethanol-soaked cotton pieces.
7. Anesthesia equipment McKinley 2 (Everest Veterinary Technology) including an induction chamber and a funnel-shaped cone.

2.8.4 Bioluminescence imaging (IVIS).

1., 15 mg/ml stock solution of D-Luciferin (Firefly, potassium salt) in PBS. Filter sterilize through a 0.22 μm filter. Store at -20ºC.
2. 1-ml 0.33 mm (29G) x 12.7 mm insulin syringes.
3. Warming blanket
4. IVIS Lumina II imaging system (Caliper Life Sciences).
5. Living imaging 3.2 Software (Caliper Life Sciences).
6. Anesthesia equipment McKinley 2 (Everest Veterinary Technology) coupled to IVIS Lumina II imaging System, including an induction chamber.

2.8.5 Subcutaneous tumor measurement and analysis.

1. Digital (or manual) caliper.
2. Tweezers and scissors.
3. 40 μm cell strainers.

4. 5-ml syringes.
5. 6-well clear flat bottom tissue culture-treated multiwell cell culture plate.
6. 70 μm cup filters.
7. PBS.

2.8.6 Mice euthanasia and organ extraction.

1. Tweezers and scissors.
2. 40 μm cell strainers.
3. 5-ml syringes.
4. 6-well clear flat bottom tissue culture-treated multiwall cell culture plate.
5. 70 μm cup filters.
6. PBS.
7. Erythrocyte lysis buffer (ELB-II): 9 vols. 0.83 % NH_4Cl, 1 vol. 0.06 % TrisHCl pH 7.6, final pH 7.2.

3. METHODS

This chapter describes the protocol used to establish xenografts of primary human T-ALL cells. Human T-ALL derived cell lines can be used as well for xenograft assays (some guidelines are included at some steps of the Methods section).

3.1. T-ALL sample preparation, characterization and lentiviral transduction.

3.1.1. Enrichment of leukemic blasts from peripheral blood or bone marrow samples by density centrifugation.

T-ALL samples provided from hospitals were obtained from peripheral blood or bone marrow aspirates of pediatric patients at diagnosis, after informed consent was provided (*see* **Note 1**).

1. For T-ALL cell isolation, dilute the sample (2-5 ml) up to 10 ml in RPMI.

2. Load 3 ml of Lymphoprep solution into 15-ml conical tubes and add 10 ml of T-ALL diluted sample drop-by-drop over Lymphoprep (*see* **Note 2**).

3. Centrifuge at 800 x *g* for 30 min at RT and without brake. After centrifugation, recover the layer of cells that remains at the interface between Lymphoprep solution (colorless) and plasma (yellow) (Fig.1). This layer contains all mononucleated cells from the sample, including both leukemic blasts and healthy hematopoietic cells (i.e. monocytes, B and T cells).

4. Wash the recovered cells with RPMI by centrifugation at 500 x *g* for 10 min twice to eliminate residual Lymphoprep solution.

5. Resuspend the cellular pellet in RPMI and count the cells under an inverted microscope using Trypan Blue solution and a Neubauer chamber. Proceed to phenotypic characterization by flow cytometry.

3.1.2. Phenotypic characterization by flow cytometry.

Determination of the proportion of T-ALL blasts in the sample and characterization of their immunophenotype according to EGIL/WHO is routinely performed by flow cytometry. Antibodies used in our laboratory and their combinations are listed in Table 1 (*see* **Note 3**).

1. For each staining, use 0.5-1 x 10^5 of total mononucleated cells obtained in subheading 3.1.1. and resuspend in SB. Cell labelling is performed in V-bottom p96-well plates. Add up to 200 µl of the cell suspension into the well and centrifuge the plate at 300 x *g* for 5 min at 4ºC.

2. Discard the supernatant by performing a gentle but firm swing with plate upside-down into a sink. To avoid non-specific binding of primary antibodies to Fc receptor-expressing cells present in the sample, perform a blocking step prior to antibody incubation. Add 20 µl of blocking solution (Fc-Block) to each well pellet and mix gently (by vortex or by pipetting). Incubate on ice for 15 min.

3. Wash with 150 µl of cold SB and centrifuge the plate as indicated above. Discard supernatant and add the corresponding antibody mix to each well. Incubate 20-30 min on ice and in the dark.

4. Wash with 150 µl of cold SB and centrifuge the plate as indicated above. Resuspend the cells in 150-200 µl of SB and transfer to 5-ml polystyrene tubes containing 150-200 µl of SB. Keep the tubes on ice and in the dark. Proceed to analyze in a flow cytometer (*see* **Note 4**).

3.1.3. Cryopreservation.

T-ALL samples can be stored by cryopreservation in liquid N_2 for years. After phenotypic characterization, centrifuge the T-ALL cell suspension, discard supernatant and carefully resuspend the cellular pellet in freezing-medium. Cell concentration can vary depending on total cell numbers obtained but ranges between 5 to 20 x 10^6 cells/ml.

1. Distribute the sample in cryogenic tubes (0.5-1 ml/tube) and freeze them using an isopropyl alcohol freezing container.

2. Place the container inside a -70ºC freezer for 24 h and then transfer the cryogenic tubes to liquid N_2 containers.

3.1.4. Lentiviral transduction.

T-ALL samples can be modified *in vitro* by lentiviral transduction in order to express some genes of interest, microRNAs, short hairpin RNAs (shRNAs) or markers commonly used to trace transduced cells *in vivo* (luciferase, GFP).

Co-culture with stromal cell lines, such as OP9 (***21***), could be beneficial to favor survival of primary T-ALL during lentiviral transduction (Fig. 1). For transduction of T-ALL cell lines, OP9 co-cultures are not necessary and experiment can be started at day 3, as described below. Cells are cultured inside a humidified incubator at 5% CO_2 and 37ºC.

For the production of lentiviral (LV) supernatants, culture 293T packaging cells for at least one week before starting the experiment:

Day 1: Split 293T cells into 100-mm culture dishes in 10 ml DMEM + 10 % FBS. Generate one plate for each lentiviral plasmid. Ideally, cells should be at 50-70 % confluence on the day of transfection.

Day 2: Change the medium 5-6 h before transfection. Mix 250 µl 150 mM NaCl with the three different plasmids (6.6 µg of pHRSIN, 4.8 µg of psPAX2 and 1.44 µg of pMD2G) in a 1.5-ml tube, vortex for 10 sec and spin down briefly. Mix 250 µl of 150 mM NaCl and 26 µl of jetPEI in a different tube, vortex for 10 sec and spin down briefly. Add the jetPEI solution to DNAs solution drop-by-drop, vortex immediately for 10 sec and spin down. Incubate 30 min at RT. Add the jetPEI/DNA mixture drop-wise into the cells, gently swirl the plate and return to the incubator. Keep for 16-20 h.

Day 3: Discard the medium and add 4.5 ml of pre-warmed T-ALL culture medium to each plate. Return to the incubator for 24 h.

Day 4: Recover the LV supernatant into 15-ml conical tubes by filtering using 5-ml syringes and 0.22 µm syringe filters. Store at 4ºC to use it on the same day or in 1 ml-aliquots at -70ºC for future experiments. Add 4.5 ml of pre-warmed T-ALL culture medium to each plate. Return to the incubator for 24 h.

Day 5: Repeat steps from day 4 on (*see* **Note 5**).

For the transduction of T-ALL cells with LV vectors:

Day 1: Plate OP9 cells (*see* **Note 6**) in 10 ml of OP9 culture medium in 100-mm culture dishes. OP9 must be growing in culture for 3-4 days before the onset of the experiment.

Day 2: Thaw T-ALL samples stored in liquid N_2 by introducing the cryogenic tube into a 37ºC-water bath and rapidly transfer the volume to a 15-ml conical tube containing 10 ml of T-ALL culture medium and centrifuge at 350 x *g* for 10 min. Discard supernatant and resuspend the cell pellet in the desired volume and count under the microscope using Trypan blue and a Neubauer chamber. Plate T-ALL cells (10^6/ml) onto OP9 cells in T-ALL culture medium supplemented with rhIL-7 (200 U/ml) (when T-ALL cells express IL-7R) (***22***). Incubate at 37ºC and 5 % CO_2 for 2 days.

Day 3: Prepare RetroNectin®-coated plates by adding 300 µl/well of a 50 µg/ml RetroNectin® solution to not treated 24-well plates and incubate O/N at RT (*see* **Note 7**). In case T-ALL cell lines are used, transduction is performed in the presence of polybrene, instead of RetroNectin®. Add polybrene (8 µg/ml final concentration) simultaneously with T-ALL cells and LV supernatants (see day 4).

Day 4: Carefully recover T-ALL cells by pipetting from co-culture plates, trying not to detach the OP9 monolayer, and filter through 30 µm cup filters. Plate the flow-through medium containing T-ALL cells in new 100-mm dishes to allow attachment of OP9 cell contaminants for 2 h. Repeat twice this step and finally resuspend T-ALL cells in an adequate volume for transduction (see below).

Remove RetroNectin® from 24-wells and perform a blocking step by adding 1 ml PBS + 2 % BSA to each well and incubating at RT for 30 min. Wash three times with 1 ml warm PBS and keep with PBS until use.

LV supernatants (freshly-prepared or frozen) must be warmed to RT. Discard PBS from RetroNectin®-coated 24-wells, plate the T-ALL cells resuspended in T-ALL culture

medium (1-2 x 10^5 cells in 0.5 ml per 24-well) and add the required volume of LV supernatant (*see* **Note 8**). Adjust to a final volume of 1.5 ml with T-ALL culture medium and guarantee that FBS and HNS are present at 10 % and 2 % final concentration, respectively. Supplement with 200 U/ml of rhIL-7 if desired. In case of transduction of T-ALL cell lines, add polybrene (8 μg/ml). Cover the plate with parafilm to avoid lid opening during centrifugation. Centrifuge the plate at 750 x *g* for 2 h, at RT without brake and incubate at 37ºC and 5 % CO_2 for 24 h.

Day 5: After transduction, recover T-ALL cells by gentle pipetting, transfer them into 15-ml conical tubes containing T-ALL culture medium, centrifuge for 5 min and resuspend cellular pellet in a volume of PBS appropriate for mice injection (*see* Subheading 3.2). Keep a small aliquot of transduced-T-ALL cells *in vitro* to analyze transduction efficiency after 2-3 days of culture. When possible, washed transduced cells should ideally be cultured onto OP9 cells for two additional days to check for transduction efficiency prior to mice injection.

3.2. T-ALL xenotransplantation.

The procedures described below should be performed in accordance to guidelines approved by the Animal Experimentation Ethics Committee of the Institution where animal procedures are carried out.

3.2.1. Mouse immunodeficient strains and irradiation.

Mouse shipping and accommodation should be performed 2-3 weeks before irradiation and transplantation. Irradiation doses will depend on the immunodeficient mouse strain used (*see* **Note 9**). NSG is the strain of choice for conventional T-ALL PDX assays (***23***). However, NSG mice have impaired antibody-mediated complement dependent cytotoxicity (CDC) (***24***), and both NSG and RAG2$^{-/-}\gamma c^{-/-}$ mice are deficient in NK cells (***25***, ***26***). Therefore, either RAG2$^{-/-}\gamma c^{-/-}$ and SCID mice, or exclusively SCID mice, must

be used instead of NSG mice for antibody administration approaches analyzing involvement of CDC or antibody-dependent cellular cytotoxicity (ADCC), respectively. NSG and RAG2$^{-/-}\gamma c^{-/-}$ are commonly subjected to a single 1.5 Grays (Gy) irradiation dose and CB17 SCID mice to 2 Gy. Cellular inoculation is recommended within the next 24 h (*see* **Note 10**).

3.2.2. Cell inoculation.

For animal identification, ear punching is performed before cell inoculation following the standard ID methods (***27***).

For retro-orbital injection:

1. T-ALL cells are resuspended in PBS at 0.5-5 x 10^6 cells/ml, filtered through 70 µm cup filters to avoid clamps, collected in 1.5-ml tubes and kept on ice until injection.

2. Load the syringe with 200 µl of the suspension (10^5-10^6 cells/mouse) (*see* **Note 11**).

3. Mice to be subjected to retro-orbital injection should be anesthetized. Because of its rapid induction and recovery times, isofluorane inhalation is the anesthetic method currently chosen. Place the mouse in a Plexiglas® chamber and use an isoflurane-soaked cotton.

4. When anesthetized, remove the mouse from the chamber. For injection into the right retro-orbital sinus, place the mouse in left lateral recumbency with its head facing to the right (*see* **Note 12**). Then, partially protrude the mouse's right eyeball from the eye socket by applying gentle pressure to the skin dorsal and ventral to the eye (***28***).

5. For injection, carefully introduce the needle, bevel down, at an angle of approximately 30°, and slowly and smoothly inject the cell preparation (***28***). After injection is complete, the needle is slowly and smoothly withdrawn (*see* **Note 13**) and the mouse is placed back into its cage for recovery.

For intravenous injection:

1. Prepare the cells as described in subheading 3.2.2.1 at 10^6-10^7 cells/ml.

2. Warm the mouse under a heat lamp or other heating device, being sure not to overheat the animal (*see* **Note 14**). This procedure dilates the vein and makes injection easier (*see* **Note 15**). Meanwhile, load the syringe with 100 µl of the cellular preparation (10^5-10^6 cells/mouse) (*see* **Note 16**).

3. Place the animal in a rotating tail injector restrainer and stabilize the tail between the thumb and forefinger of the hand that will not manipulate the syringe (*see* **Note 17**).

4. Clean the tail with ethanol-soaked cotton.

5. Attempt the injection starting at the middle or slightly distal part of the tail. With the tail under tension, insert the needle, bevel up, approximately parallel to the vein and insert the needle at least 3 mm into the vein. Inject the material in a slow, fluid motion (*see* **Note 18**). An illustrative video on how restraining and intravenous injection are performed can be visualized in (***26***).

6. Remove the needle slowly and apply a piece of dry cotton to the puncture site to block the loss of the sample.

7. After injection is complete, place the mouse back into its cage.

For subcutaneous injection, mice do not need to be irradiated nor anesthetized:

1. Resuspend primary or cultured T-ALL cells in PBS at 5-25 x 10^6 cells/ml.

2. Load the syringe with 200 µl (1-5 x 10^6 cells/mouse) (*see* **Note 19**).

3. Restrain the mouse by the scruff and wash softly the area of injection, normally the right or left flank of the mouse or both (i.e. control cells on one flank and treated/modified cells on the other flank) with ethanol-soaked cotton. Make a tent of skin on one flank using your thumb and forefinger.

4. Insert the needle under the skin at the base of the tent, directed toward the posterior of the mouse. Inject the whole volume. Proceed to the other flank if required. An illustrative video can be visualized here (***29***).

3.3. Therapeutic treatment of T-ALL xenotransplanted mice.

Drug treatment is normally started when human cell engraftment in peripheral blood is > 0.5 % (*see* Subheading 3.4.2).

3.3.1. Intraperitoneal administration.

1. Load the syringe with up to 200 µl of the desired compound suspension. For monoclonal antibody (mAb) treatment, endotoxin-free solutions of purified mAb (1 mg/ml in PBS) sterilized by filtration should be used (10 mg/Kg) (*see* **Note 20**).

2. Restrain the mouse by the scruff method. Expose the ventral side of the animal, tilting the head down at a slight angle. Insert the sterile needle at a 30º angle, bevel up, in the lower right or left quadrant of the abdomen and inject the material as shown in (***29***).

3.3.2. Oral gavage administration.

Gavaging is used to dose an animal with a specified volume of material directly into its stomach.

1. Weigh the animal and calculate the maximum volume that can be administered orally (up to 10 ml/kg).

2. Measure the distance from the oral cavity to the end of the xiphoid process (caudal point of the sternum) with the feeding needle/tube on the outside of the restrained animal. This will be the distance the needle will be inserted into the esophagus. Mark this distance on the needle using a permanent marker (*see* **Note 21**).

3. Fill the syringe with the appropriate volume of material and attach the needle.

4. Restrain the animal by the scruff.

5. Place the tip of the needle into the animal mouth and slide gently past the back and left of the tongue. The needle should slide easily down the esophagus, if properly placed. DO NOT FORCE. If any resistance is met, remove the needle and reinsert.

6. Once the needle is properly placed, administer the material.

This procedure is detailed in UBC animal care guidelines (***30***).

3.4. Xenograft monitoring and mouse analysis.

3.4.1. Body weight measurement.

Body weight loss is used as an indicator of disease progression in T-ALL xenotransplanted mice. The endpoint of the experiment is established once 15-20 % weight losses are reached compared with measurements at the starting point. Measure body weight by placing each mouse independently on an electronic weighing scale once a week at the beginning of the experiment. When weight loss is starting to be appreciated, more frequent measurements (every 2-3 days) are recommended.

3.4.2. Peripheral blood extraction.

Mouse peripheral blood is obtained from the submandibular vein by using needles instead of razors, without the use of anesthesia (***31***).

1. Prepare 1.5-ml tubes containing 100 µl of heparin sodium solution and one sterile needle per mouse.

2. Quickly, poke the mouse in the submandibular vein with the needle and collect blood into the tube. When enough blood is obtained, press the vein with a small piece of ethanol-soaked cotton to stop bleeding. Mix blood with heparin to avoid coagulation by inverting the tube 4-5 times. Keep at RT. Alternative bleeding methods such as retro-orbital, distal tail or cardiac puncture can also be used (***32***).

3. Mouse erythrocyte depletion is required for further flow cytometry analysis. Add 1 ml of ELB-I to each blood-containing tube and incubate 5 min at RT. Centrifuge

at 5000 x *g* for 10 min at 4ºC. Discard supernatant and wash twice with 1 ml of cold PBS or SB. Resuspend in 200 µl of SB, transfer to a p96-well plate and proceed to label the sample for flow cytometry (*see* Subheading 3.1.2).

3.4.3. Bone marrow aspiration.

Serial sampling of mouse tibia BM facilitates longitudinal studies of BM composition and T-ALL engraftment over time without requiring mouse sacrifice.

1. Place the mouse in the induction chamber connected to anesthesia equipment that vaporizes isoflurane at 3-4% in O_2.

2. When completely anesthetized, remove the mouse from the induction chamber and place it with its face upside down and its nose inside a funnel-shaped cone, which functions as an anesthesia mask, connected with the equipment. For maintenance, isoflurane concentrations should be 1.25 -1.75% (*see* **Note 22**). Anesthetized animal must be closely monitored during the procedure to assure that it is maintained in the proper anesthetic plane (*see* **Note 23**).

3. Most common anesthetic complication is hypothermia. Keep the mouse on a warming blanket to prevent hypothermia during the procedure. A protected warming device can be also placed in the cage.

4. Disinfect the entire leg containing the tibia that will undergo aspiration with 70% ethanol-soaked cotton pieces.

5. Fill the syringe with 200-500 µl of PBS and reserve.

6. Keep the tibia bent from the femur by pushing the tibia with either the ring finger or the fifth finger in a 45º angle. This allows to exposure the proximal end of the tibia. The syringe is held using the thumb and the index finger (Fig.2).

7. Insert the needle through the trabecular bone and turn it clockwise and counterclockwise while pushing it slowly into the epiphysis cavity. Confirm the correct positioning of the needle by gently moving the syringe laterally (Fig.2).

8. Inject 50 µl of PBS and gently pull the needle plunger back, creating negative pressure, while moving the needle back and forth within the tibia cavity. Successful aspiration will be confirmed visually by the appearance of blood in the top of the needle in the base of the syringe (*see* **Note 24**).

9. Remove the needle and syringe and transfer the aspirated sample to a 1.5-ml tube prefilled with 500 µl of PBS. For most applications, BM samples should be kept on ice until further processing. For flow cytometry, resuspend in 200 µl of SB, transfer to p96-well plates and proceed as indicated in subheading 3.1.2.

10. Following completion of the aspiration procedure, remove the mouse from the anesthesia and keep it on a warming blanket until fully recovered (*see* **Note 25**). Ensure it is able to ambulate and reach food and water. Observe the mouse for signs of distress or infection post procedure in the next 24 h. Signs include: constant bleeding, anemia or lethargy. If any of these signs are seen post procedure, the animal(s) should be euthanized.

11. Analgesic ibuprofen is administered in the drinking water at 0.2 mg/ml, providing a daily dose of approximately 40 mg/kg ibuprofen with average daily water consumption of 5-6 ml for two days (*see* **Note 26**). An illustrative example for femoral BM aspiration in live mice can be visualized in (***33***).

3.4.4. Bioluminescence imaging (IVIS).

This protocol allows monitoring T-ALL cells in live animals using bioluminescent images (summarized in Fig.3). T-ALL cells should be transduced with a luciferase-reporter vector (*see* Subheading 3.1.4) (Fig. 3a, b) that leads to production of the

luciferase enzyme (Firefly), which oxidases the substrate D-Luciferin with the consequent emission of photons (*see* **Note 27**).

1. Before starting mouse manipulation, initialize the IVIS imaging system and allow the CCD camera to reach the operating temperature (see manual instructions of IVIS Lumina II Caliper Life Science) (***34***).

2. Load the syringe with D-Luciferin solution (15 mg/ml) and inject 150 mg/Kg mouse body weight (10 ml/Kg) intraperitoneally (*see* Subheading 3.3.1).

3. After 5 min, place the mouse in a dark anesthesia chamber connected to anesthesia equipment that vaporizes isoflurane at 3-4% in O_2.

4. Remove anesthetized mice from the chamber and place them inside the IVIS machine on the warming tray with their heads inside the funnel-shaped cone (Fig. 3c). Keep a constant isoflurane concentration of 1.5 % during imaging procedures.

5. Take images after 5 min of exposures starting face up, and then, 5 more min in the exposure face down (*see* **Note 28**).

6. After capturing and saving the images, remove mice from the anesthesia and keep on a warming blanket until fully recovered.

7. The IVIS imaging system expresses the bioluminescent signal in photons per seconds and displays it as an intensity map. Draw a region of interest (ROI) of identical size over each mouse (Fig. 3d). The photon flux emitted by the luciferase-expressing cells is measured as an Average radiance (photons/sec/cm^2/sr) (Fig. 3e). Imaging analysis is performed using the Living Image® Software 3.2 (***34***).

3.4.5. Subcutaneous tumor measurement and analysis.

To establish tumor growth, monitor tumor appearance weekly and, once tumors are detectable, measure every 2-3 days. Use a digital caliper when available (or a manual one in its place).

1. Annotate both the width (shorter measure) and length (longer measure) of the tumor. Tumor volume is calculated using the modified ellipsoidal formula: Tumor volume = 1/2(length x width^2). Mice should be sacrificed when tumors reach a diameter > 17 mm.

2. Isolate the tumor removing as much skin as possible using scissors and tweezers. Cut it in small pieces and transfer to 40 µm cell strainers inserted into a 6-well plate well.

3. Rinse with PBS. Mechanically disaggregate using tweezers and 5-ml syringe embolus. Recover flow-through, dilute with PBS and filter using 70 µm cup filcons.

4. Centrifuge at 500 x *g* for 5 min, discard supernatant and resuspend cellular pellet in an adequate volume for cell counting. Proceed to flow cytometry analysis (*see* Subheading 3.1.2).

3.4.6. Mice euthanasia and organ extraction for flow cytometry analysis.

Mice should be euthanized when they present advanced symptoms of disease and reach established humane endpoints (i.e. tumor diameter > 17mm, body weight loss > 15-20 %, labored respiration, etc.) (*see* **Note 29**). Carbon dioxide chamber is the most common method of euthanasia for rodents. T-ALL cells usually colonize BM and peripheral lymphoid (thymus, spleen, lymph nodes) and non-lymphoid organs (liver, brain, kidney). Organ extraction must be done under sterile conditions as grafting T-ALL cells can be used for serial transplantations. Protocols and videos for the extraction of different organs can be found in (***35***). The protocol described below is indicated for flow cytometry analysis of T-ALL cells colonizing different mice organs. Alternatively, immunohistochemistry techniques are useful to characterize leukemic grafting cells.

1. Extract the organ of interest, cut it into small pieces using tweezers and scissors and transfer to 40 μm cell strainers inserted into a 6-well plate well. Proceed as described in subheading 3.4.5 for isolated subcutaneous tumors.

2. Highly irrigated organs such as spleen and liver should be depleted of erythrocytes prior to flow cytometry. Thus, after centrifugation, resuspend the cellular pellet in ELB-II and incubate at RT for 30 sec-1 min (*see* **Note 30**). Dilute 10-fold with cold PBS, filter through 70 μm cup filcons and centrifuge at 500 x *g* for 5 min. Discard supernatant and resuspend in an adequate volume for cell counting. Proceed to flow cytometry analysis (*see* Subheading 3.1.2).

4. NOTES

1. Frozen T-ALL samples are kept in liquid N_2 containers until use.
2. See also Lymphoprep manufacturer´s instructions for different blood sample volumes. Lymphoprep solution must be protected from light and pre-warmed to RT (i.e. 15-25ºC) prior to use. Centrifugation must be done at RT (i.e. 20ºC), with no brake in order to avoid density gradient disruption.
3. The antibody combinations shown in Table 1 are recommended for analysis in a FACS Canto II cytometer (BD Biosciences). Alternative antibody combinations may be used for other flow cytometers.
4. We highly recommend flow cytometry of fresh samples because T-ALL cells are quite sensitive to fixation.
5. Lentiviral supernatants must be titrated to determine the number of transducing units/ml (***36***), which would be beneficial for an optimal T-ALL transduction.
6. We normally use OP9-GFP (***21***) or OP9-DL4 (***37***) stromal cells as feeders for T-ALL cell cultures. OP9 cell lines must be cultured as indicated (***38***).

7. RetroNectin® can also be incubated for 2 h at 37ºC the same day of transduction. We use 1-2 x 10^5 T-ALL cells per RetroNectin®-coated well of 24-well plates. Retronectin®-coated 6-well plates are used for higher T-ALL cell numbers (0.5-1 x 10^6 cells/well).
8. For transduction of primary T-ALL cells, we routinely use a multiplicity of infection (MOI) of 10-20 (10-20 transducing units/cell), but optimal transduction doses should be determined according to the sensitivity or resistance of leukemic cells to lentivirus infection. Using too high MOIs may lead to increased cell death. This protocol normally results in 10-40 % of primary T-ALL transduced cells. When a 100 % transduced population is required for PDX assays, cell sorting must be performed prior to transplantation. For T-ALL cell lines, a MOI of 5-10 can lead to a >80% transduction efficiency.
9. Dose-survival curves should be performed to determine the maximal sublethal dose that facilitates BM T-ALL engraftment. An important consideration is that NSG and SCID mice display the *scid* side effect (radiation sensitivity and increased toxicity with genotoxic drugs) (***23***).
10. Proliferating mouse cells are sensitive to death within the next 24 h after irradiation and they recover afterwards, thus preventing human cell engraftment in the BM.
11. Injection of > 10^6 cells/mouse could result in local tumor growth. No less than 10^5 cells/mouse is recommended for injection of thawed cells, owing to decreased viability. Injected volume should not exceed 200 μl.
12. In addition to using the inhalant anesthetic, we recommended to place a drop of ophthalmic anesthetic (0.5% proparacaine hydrochloride ophthalmic solution,) on

the eye that will receive the injection. This provides additional procedural and post-procedural analgesia.

13. Slow needle withdrawing prevents the injectable to follow the needle path out. There should be little or no bleeding.
14. Temperature should not exceed 25-30º C at the level of the animal. Remove the mouse from the heat source immediately should any change in respiration rate or excessive salivation is observed.
15. For tail vein injection, mice should be older than 6 weeks because at younger ages the vessels are not thick enough for injection.
16. Injected volume should not exceed 100 µl. Cell numbers will depend on the engraftment potential and viability of the particular T-ALL sample.
17. Since injection needs accurate manipulation of the needle, holding the mouse is a critical part of the procedure. Several restraint devices are useful for holding mice for longer periods of time. We recommend a rotating injector device (Braintree Scientific) in which the tail can be rotated. This feature provides for an easy access to lateral vein in the tail.
18. DO NOT ASPIRATE, as it will cause the vein to collapse. If any swelling at the injection site or resistance to injection occurs, remove the needle and reinsert it slightly above the initial injection site. Penetration caused by excessively deep insertion is quite common, because the vessel wall is located just beneath the skin surface. Once the needle tip is under the skin, it is very important to pull back the syringe slightly during insertion to confirm the blood will flow back and then start the injection without moving the needle tip.
19. Cell numbers will depend on the injected T-ALL cell line. For leukemia cell lines such as Jurkat or HPB-ALL, 1-2 x 10^6 cells/mouse should develop subcutaneous

tumors with a latency of 2-3 wks. When cell lines with unknown latency periods are required, initial cell numbers must be previously assayed.

20. If the antibody is commercial, it should be azide-free. Laboratory purified antibodies should be prepared in endotoxin-free (< 0.25 EU/ml) PBS.
21. If the needle used is too long, there is a greater chance of injuring the animal by damaging the esophagus. Using a needle that is too short may cause the animal to aspirate the substance.
22. Inhalant anesthetics must be used with scavenging devices. One acceptable scavenging method is the use of Type IIB2 biosafety cabinets, which are vented to the outside.
23. If the anesthetic plane is too light, the animals may start to move or struggle. If the anesthetic plane is too deep, the animals may die. Once the anesthesia has been administered and enough time has elapsed for it to take effect, the anesthetic plane can be assessed by pinching the toe, tail or ear of the animal. Any reaction from the animal indicates that the anesthesia is too light and that additional anesthesia should be given. An increased respiration rate is a sign that the anesthesia is too light. A deep, shallow, decreased or irregular respiration rate is indicative of anesthesia that is too deep.
24. If no blood is seen in the syringe it is likely that a small bone or tissue fragment is stuck in the needle. Remove the syringe and use a new needle and syringe (again wet the syringe with 200-500 µl of PBS).
25. In some cases, if the procedure takes too long and the respiration rate is irregular, the mouse may stop breathing and cardiopulmonary resurrection procedure is needed. Use a 5-ml syringe to pump oxygen directly in its mouth along with cardiac massage.

26. BM aspiration can be repeated weekly in alternate tibia to prevent repeated trauma to the same leg.
27. Since dark skin pigmentation or color can attenuate bioluminescence, we recommend SCID or NSG mice.
28. Considering that D-Luciferin uptake by T-ALL cells is time-dependent, we recommend running sequential images of mice every minute until luminescence saturation is reached.
29. Different humane endpoints can be found in online guidelines from animal care institutions and should also be provided by the corresponding animal facility. A general guide example can be found in (***39***).
30. The volume and incubation time required for erythrocyte lysis depends on the size of the cellular pellet. Some examples: for spleens add 1-2 ml of ELB-II and incubate 1 min at RT; for bone marrow add 300 µl and incubate 30 sec at RT.

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REFERENCES

1. Bene MC, Castoldi G, Knapp W et al (1995) *Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL)*. Leukemia 9:1783-1786

2. Liu Y, Easton J, Shao Y, et al (2017) The genomic landscape of pediatric and young adult T-lineage acute lymphoblastic leukemia. Nat Genet 49(8):1211-1218
3. Brammer JE, Saliba RM, Jorgensen JL et al (2017) Multi-center analysis of the effect of T-cell acute lymphoblastic leukemia subtype and minimal residual disease on allogeneic stem cell transplantation outcomes. Bone Marrow Transplant 52(1):20-27
4. Kataoka K, Iwanaga M, Yasunaga JI, et al (2018) Prognostic relevance of integrated genetic profiling in adult T-cell leukemia/lymphoma. Blood 11;131(2):215-225
5. Petit A, Trinquand A, Chevret S, et al (2018) Oncogenetic mutations combined with MRD improve outcome prediction in pediatric T-cell acute lymphoblastic leukemia. Blood 131(3):289-300
6. Swerdlow SH, Campo E, Harris NL et al (eds) (2008) WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues: World Health Organization Classification of Tumors. International Agency for Research on Cancer (IARC): Lyon, France
7. Hunger SP, Mullighan CG (2015) *Acute lymphoblastic leukemia in children*. N Engl J Med 373:1541-1552
8. Pui CH, Evans WE (2013) A 50-year journey to cure childhood acute lymphoblastic leukemia. Semin Hematol 50(3):185-96
9. Karrman K, Johansson B (2017) Pediatric T-cell acute lymphoblastic leukemia. Gene Chromosome Can 56(2):89-116
10. Chiu PL, Jiang H, Dick JE (2010) Leukemia-initiating cells in human T-lymphoblastic leukemia exhibit glucocorticoid resistance. Blood 116:5268-5279

11. Krivtsov A, Wang X, Farnoud NR et al (2014) Patient Derived Xenograft (PDX) Models Recapitulate the Genomic-Driver Composition of Acute Leukemia Samples. Blood 124(21):286
12. Wang K, Sanchez-Martin M, Wang X et al (2017) Patient-derived xenotransplants can recapitulate the genetic driver landscape of acute leukemias. Leukemia 31(1):151-158
13. García-Peydró M, Fuentes P, Mosquera M et al (2018) The NOTCH1/CD44 axis drives pathogenesis in a T cell acute lymphoblastic leukemia model. J Clin Invest 128(7):2802-2818
14. Contag CH, Spilman SD, Contag PR et al (1997) Visualizing gene expression in living mammals using a bioluminescent reporter. *Photochem Photobiol* 66(4): 523-531
15. Contag PR, Olomu IN, Stevenson DK et al (1998) Bioluminescent indicators in living mammals. *Nat Med* 4(2): 245-47
16. Bhadri VA, Cowley MJ, Kaplan W et al (2011) Evaluation of the NOD/SCID xenograft model for glucocorticoid-regulated gene expression in childhood B-cell precursor acute lymphoblastic leukemia. BMC Genomics 12,565
17. Nijmeijer BA, Willemze R, Falkenburg JH (2002) An animal model for human cellular immunotherapy: specific eradication of human acute lymphoblastic leukemia by cytotoxic T lymphocytes in NOD/scid mice. Blood 100(2):654-660.
18. Garaulet G, Alfranca A, Torrente M et al (2013) IL10 released by a new inflammation-regulated lentiviral system efficiently attenuates zymosan-induced arthritis. Mol Ther 21:119-130
19. Didier Trono Lab Plasmid. http://www.addgene.org/Didier_Trono/ Accessed 20 Sept 2019

20. van Rijn RS, Simonetti ER, Hagenbeek A et al (2003) A new xenograft model for graft-versus-host disease by intravenous transfer of human peripheral blood mononuclear cells in RAG2-/- gammac-/- double-mutant mice. Blood 102:2522-31
21. Schmitt TM, de Pooter RF, Gronski MA et al (2004) Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. Nat Immunol 5(4):410-417
22. González-García S, Mosquera M, Fuentes P et al (2019) IL-7R is essential for leukemia-initiating cell activity and pathogenesis of T-cell acute lymphoblastic leukemia. Blood, Sept 17
23. The Jackson Laboratory (2006) Choosing an immunodeficient mouse model. https://www.jax.org/news-and-insights/2006/march/choosing-an-immunodeficient-mouse-model. *Accessed 25 Sept 2019*
24. Baxter AG, Cooke A (1993) Complement lytic activity has no role in the pathogenesis of autoimmune diabetes in NOD mice. Diabetes 42:1574-1578
25. Christianson SW, Greiner DL, Hesselton RA et al (1997) Enhanced human CD4+ T cell engraftment in beta2-microglobulin-deficient NOD-scid mice. J Immunol 158(8):3578-3586
26. DiSanto JP, Müller W, Guy-Grand D et al (1995) Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. Proc Natl Acad Sci USA 92(2):377-381
27. Dickie MM (1975) Universal mouse numbering system. https://www.jax.org/jax-mice-and-services/customer-support/technical-support/breeding-and-husbandry-support/mouse-identification

28. Yardeni T, Eckhaus M, Morris HD et al (2011) Retro-orbital injections in mice. Lab animal 40(5):155-160
29. Machholz E, Mulder G, Ruiz C et al (2012) Manual restraint and common compound administration routes in mice and rats. J Vis Exp 26;(67)
30. Jones K (2012) Oral Dosing (Gavage) in Adult Mice and Rats SOP. https://animalcare.ubc.ca/sites/default/files/documents/ACC-2012
31. Golde WT, Gollobin P, Rodriguez LL (2005) A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. Lab Anim (NY) 34(9):39-43
32. Hoggatt J, Hoggatt AF, Tate TA et al (2016) Bleeding the laboratory mouse: Not all methods are equal. Exp Hematol 44(2):132-137
33. Chung YR, Kim E, Abdel-Wahab O (2014) Femoral bone marrow aspiration in live mice. J Vis Exp 5;(89)
34. Living Image Software: User´s manual Version 4.0 (2002-2010) https://bcf.technion.ac.il/wp-content/uploads/2015/10/IVIS-200-Living_Image_40_User_Manual-8589.pdf. Accessed 25 Sept 2019
35. JoVE Science Education Database. Lab Animal Research. (2019) Sterile Tissue Harvest. JoVE, Cambridge, MA. Accessed 25 Sept 2019
36. Salmon P, Trono D (2007) Production and titration of lentiviral vectors. Curr Protoc Hum Genet 54:12.10.1-12.10.24
37. Ayllón V, Bueno C, Ramos-Mejía V et al (2015) The Notch ligand DLL4 specifically marks human hematoendothelial progenitors and regulates their hematopoietic fate. Leukemia 29(8):1741-1753
38. de Pooter RF, Schmitt TM, Zúñiga-Pflücker JC (2006) In vitro generation of T lymphocytes from embryonic stem cells. Methods Mol Biol 330:113-121

39. Richardson C (2014) Humane endpoints. https://www.nc3rs.org.uk/humane-endpoints. Accessed 28 Aug 2019

FIGURE LEGENDS

Figure1. Schematic diagram summarizing sequential steps of the T-ALL patient-derived xenograft (PDX) assay. T-ALL cells isolated by density centrifugation from PB or BM samples from T-ALL patients are classified according to EGIL by flow cytometry phenotyping and they are then either transplanted into immunodeficient mice or stored in liquid N_2. Before genetic manipulation (i.e. lentiviral transduction), either freshly-isolated or frozen T-ALL cells are cultured *in vitro* onto feeder (OP9) cells for 1-3 days. Mice are subjected to sublethal (1.5-2 Gy) gamma-irradiation prior to transplantation, which is performed following inoculation routes described in subheading 3.2. Xenografts are monitored according to procedures described in subheading 3.4.

Figure 2. Schematic representation of intra-tibial bone marrow aspiration procedure. The needle is introduced through the trabecular bone, in the proximal end of the tibia and it is slowly placed into the epiphysis cavity. Successful aspiration is confirmed visually by the appearance of blood in the top of the needle in the base of the syringe.

Figure 3. Schematic representation of sequential steps of T-ALL xenograft *in vivo* bioluminescence imaging. (**a**) Generation of stable luciferase-expressing human T-ALL cells by transduction with lentiviral vectors containing the Firefly luciferase gene. (**b**) Intravenous injection of luciferase-expressing T-ALL cells into sublethally-irradiated immunodeficient mice. (**c**) Image acquisition is performed using an IVIS Lumina II spectrum imaging system. (**d**) Pseudo-color images of mice transplanted with luciferase-

expressing human T-ALL cells at the indicated weeks post-transplant. (**e**) Quantification of bioluminescent signals from ventral and dorsal mouse exposures expressed as Average Radiance (Avg Rad: photons/sec/cm^2/sr) at the indicated weeks post-transplant. Imaging analysis is performed using the Living Image® Software 3.2.

Table 1. Antibodies used for T-ALL phenotyping using a FACS Canto II (BD Biosciences) flow cytometer.

Detector	Excitation laser line (nm)	Filter	Mix 1	Mix 2	Mix 3	Mix 4
FL1-A	488	530/30	CD5-FITC	CD5-FITC	HLA-DR-FITC	CD34-FITC
FL2-A	488	582/15	CD7-PE	TCRαβ-PE	CD11b-PE	CD13-PE
FL3-A	488	670LP	CD4 PE-Cy5	TCRγδ-PE-Cy5	CD56-PE-Cy5	CD33-PE-Cy5
FL4-A	488	780/60	CD8-PE-Cy7	CD8-PE-Cy7	CD19-PE-Cy-7	CD8-PE-Cy7
FL5-A	633	670/30	CD3-APC	CD3-APC	CD117-APC	CD2-APC
FL6-A	633	780/60	CD1a-biotin + saAPC-Cy7	CD1a-biotin + saAPC-Cy7	-	-
FL7-A	405	450/40	CD45-V450	CD45-V450	CD7-BV421	CD7-BV421

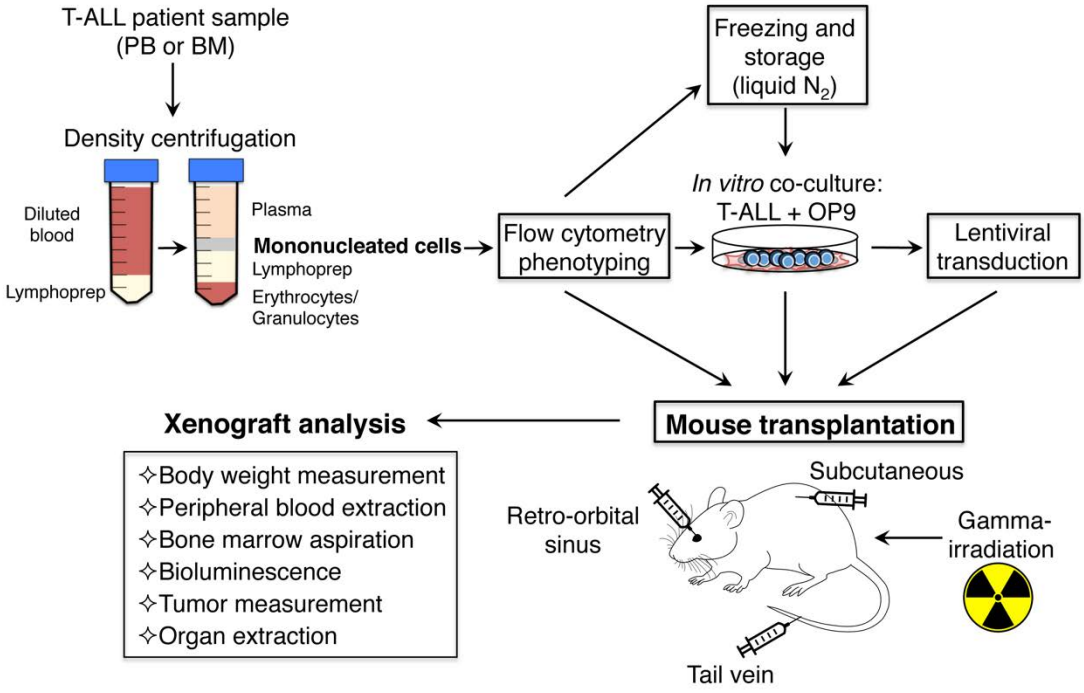
T-ALL patient sample
(PB or BM)
Density centrifugation
Diluted blood
Lymphoprep
Plasma
Mononucleated cells
Lymphoprep
Erythrocytes/
Granulocytes
Freezing and storage (liquid N_2)
Flow cytometry phenotyping
In vitro co-culture:
T-ALL + OP9
Lentiviral transduction
Mouse transplantation
Xenograft analysis
✧Body weight measurement
✧Peripheral blood extraction
✧Bone marrow aspiration
✧Bioluminescence
✧Tumor measurement
✧Organ extraction
Subcutaneous
Retro-orbital sinus
Gamma-irradiation
Tail vein

Figure 1

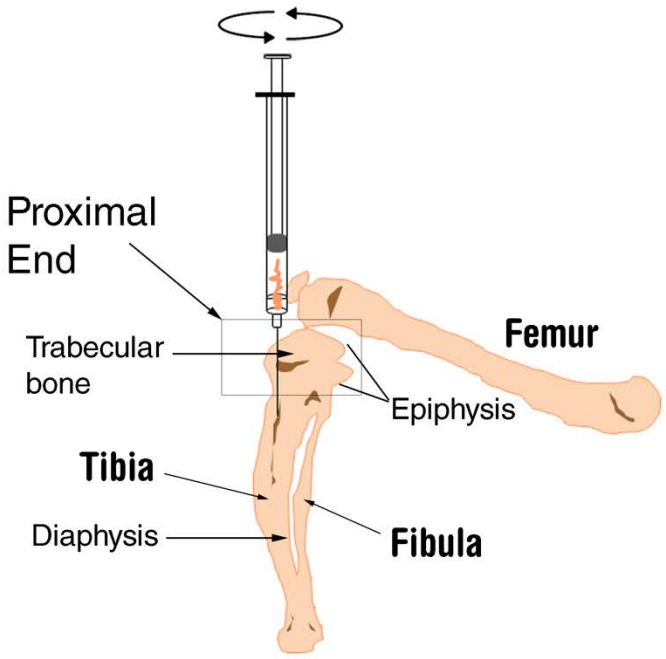
Proximal
End
Trabecular
bone
Femur
Epiphysis
Tibia
Diaphysis
Fibula

Figure 2

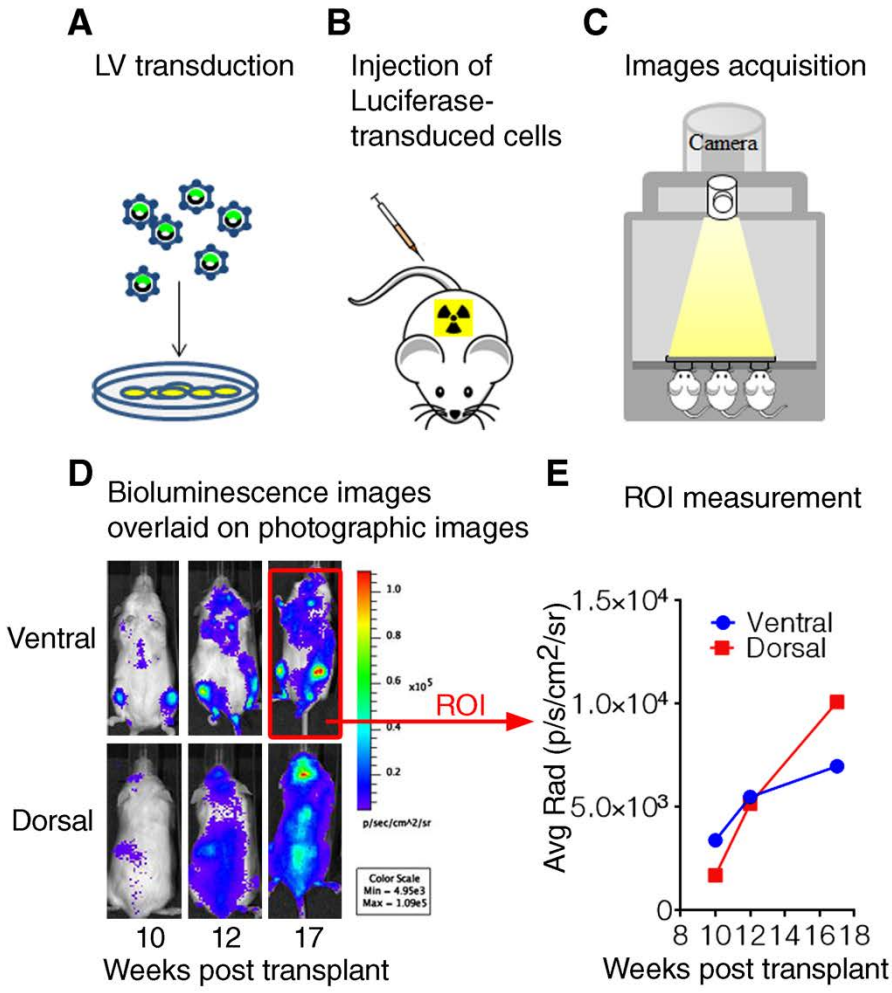
A
LV transduction
B
Injection of Luciferase-transduced cells
C
Images acquisition
Camera
D
Bioluminescence images overlaid on photographic images
Ventral
Dorsal
ROI
1.0
0.8
0.6
x10^5
0.4
0.2
p/sec/cm^2/sr
Color Scale
Min = 4.95e3
Max = 1.09e5
10
12
17
Weeks post transplant
E
ROI measurement
Avg Rad (p/s/cm2/sr)
1.5×10^4
1.0×10^4
5.0×10^3
0
Ventral
Dorsal
8 10 12 14 16 18
Weeks post transplant

Figure 3