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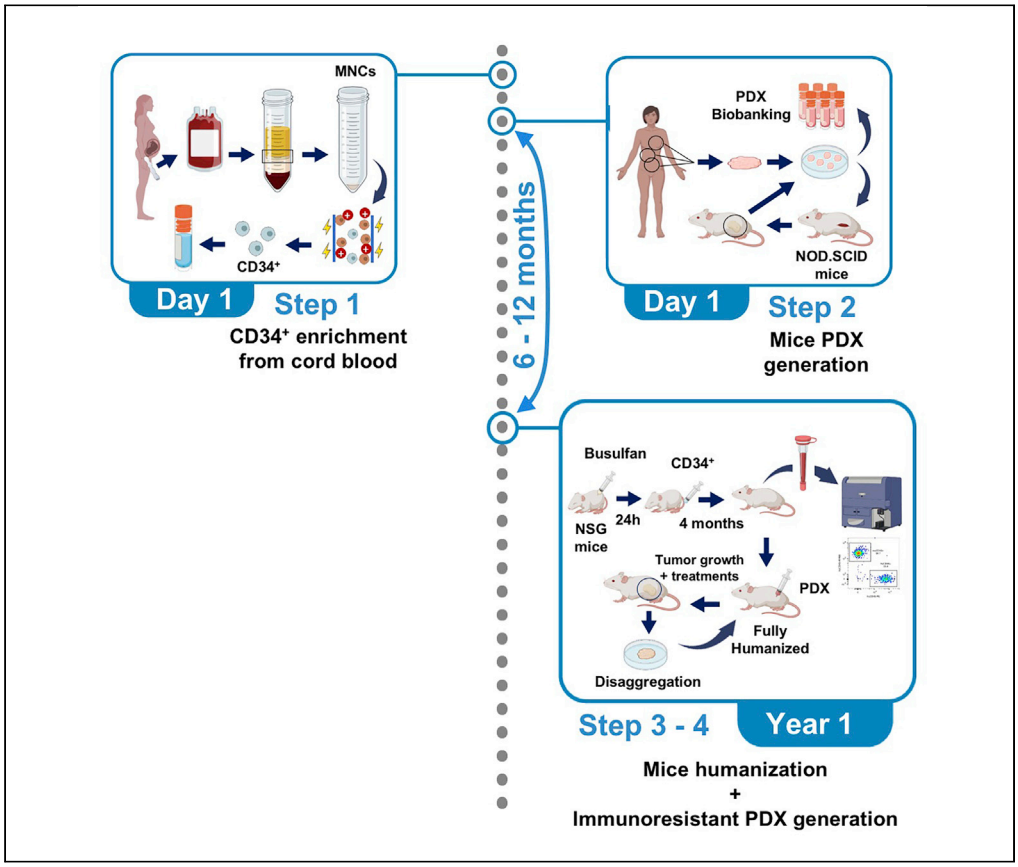
Protocol to generate a patient derived xenograft model of acquired resistance to immunotherapy in humanized mice



Immunotherapy has revolutionized cancer treatment, but preclinical models are required to understand immunotherapy resistance mechanisms underlying patient relapse. This protocol describes how to generate an acquired resistance humanized *in vivo* model to immunotherapies in patient-derived xenografts (PDX). We detail steps to inject human CD34$^+$ cells into NSG mice, followed by generation of immunoresistant PDX in humanized mice. This approach recapitulates the human immune system, allowing investigators to generate preclinical resistance models to different immunotherapies for identifying the resistant phenotype.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Human CD34$^+$ hematopoietic stem cells isolation from cord blood

Patient-derived xenograft (PDX) generation and maintenance

Generation of fully humanized mice with CD34$^+$ cells for longterm experiments

Generation of immunoresistant PDX in humanized mice to study mechanisms of resistance

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Protocol to generate a patient derived xenograft model of acquired resistance to immunotherapy in humanized mice

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SUMMARY

Immunotherapy has revolutionized cancer treatment, but preclinical models are required to understand immunotherapy resistance mechanisms underlying patient relapse. This protocol describes how to generate an acquired resistance humanized *in vivo* model to immunotherapies in patient-derived xenografts (PDX). We detail steps to inject human CD34$^+$ cells into NSG mice, followed by generation of immunoresistant PDX in humanized mice. This approach recapitulates the human immune system, allowing investigators to generate preclinical resistance models to different immunotherapies for identifying the resistant phenotype.

For complete details on the use and execution of this protocol, please refer to Martínez-Sabadell et al., 2022 and Arenas et al. (2021).

BEFORE YOU BEGIN

1. CD34$^+$ cells can be commercially purchased, even though this is less cost-effective than isolating them from good-quality cord blood units.
2. This protocol is optimized to humanize NSG mice, which should be purchased in advance.
3. The entire protocol should be performed under sterile conditions, excepting PDX passages and tumor cell implantation, performed in the animal facility.
4. NSG immunodeficient mice should be kept in Specific-Pathogen Free (SPF) facilities in the animal facility of the investigator's research center to avoid infections that can alter the results.
5. Before starting each one of the steps, investigators should prepare the solutions specified in the "materials and equipment" paragraph.
6. Investigators should note that flow cytometry is a technique that requires experience. The panels described here should be prepared by the researcher in the first CD34$^+$cells isolation and humanization phenotyping. Additionally, this protocol is performed with BD Bioscience FACSCanto II or FACSCelesta.

Institutional permissions

Protocols for the isolation of human tumor cells from patient derived xenografts and the use of human cord blood requires the approval by the Institutional Review Board (IRBs). All human samples specified in this protocol were obtained following institutional guidelines under protocols approved by the IRBs at Vall d'Hebron Hospital.

Tumor or immune cell implantation in mice requires an approval by the Ethical Committee for the Use of Experimental Animals. Animal work in this protocol was performed according to protocols approved by the Ethical Committee for the Use of Experimental Animals at the Vall d'Hebron Institute of Oncology.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
huCD45-PE (1:300 diution)	BioLegend	Cat# 304008, RRID: AB_314396
huCD34-AlexaFluor488 (1:300 diution)	STEMCELL Technologies	Cat#60013AD
moCD45-AlexaFluor488 (1:300 diution)	BioLegend	Cat# 103122, RRID: AB_493531
huCD4-Brilliant Violet 421 (1:300 diution)	BioLegend	Cat# 317434, RRID: AB_2562134
huCD8-PE/Cy7 (1:300 diution)	BioLegend	Cat# 344712, RRID: AB_2044008
Biological samples		
Umbilical cord blood. Healthy female adult donors (18–45 years old).	Blood and Tissue Bank of Catalonia (BST)	N/A
Patient tumor samples. Breast cancer: Female adults (>18). Rest of cancer origins: Adults (>18) from both sexes.	Vall d'Hebron Hospital	N/A
Chemicals, peptides, and recombinant proteins		
PBS	Biowest	Cat#L0615
Ficoll-Paque Premium	Cytiva	Cat#17-5442-02
Red Blood Cell (RBC) Lysis Buffer	Invitrogen	Cat#00-4333-57
Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat#A9647
CryoStor CS10	STEMCELL Technologies	Cat#07959
Horse Serum	Gibco	Cat#26050
Propidium iodide	Sigma-Aldrich	Cat#81845
Bambanker	NIPPON Genetics	Cat#BB02
DMEM:F12 Medium	Gibco	Cat#21331
Penicillin-Streptomycin	Sigma-Aldrich	Cat#P4333
Methocel 2%	OmniVision	N/A
17-β-estradiol	Sigma-Aldrich	Cat#E8875
Busulfan	Tillomed	N/A
DNAse I from bovine pancreas	Sigma-Adrich	Cat#D4263
Fetal Bovine Serum (FBS)	Gibco	Cat#10270106
Ketolar 50 mg/mL (Ketamine)	Pfizer	N/A
Xilagesic 20 mg/mL (Xilacine)	Laboratorios Calier	N/A
Collagenase	Sigma-Aldrich	Cat#C2674
Hyaluronidase	Sigma-Aldrich	Cat#H3506
EDTA	Calbiochem	Cat#324503
Critical commercial assays		
EasySep Human Progenitor Cell Enrichment Kit with Platelet depletion	STEMCELL Technologies	Cat#1956
Zombie Aqua™ Fixable Viability Kit	BioLegend	Cat#423101
CryoStor CS10	STEMCELL Technologies	Cat#07930
Matrigel	Corning	Cat#356235

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mouse: NOD.SCID (NOD.CB17-Prkdcscid/J) female mice for breast cancer; female and male mice for the rest of cancers; NOD/ShiLtJ background; 5–6 weeks	Charles River	Cat#634
Mouse: NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) female mice for breast cancer; female and male mice for the rest of cancers; NOD/ShiLtJ background; 5–6 weeks	Charles River	Cat#614
Software and algorithms		
BD FACSDiva software	BD Biosciences	https://www.bdbiosciences.com/en-eu
FlowJo version 10.2	Tree Star	https://www.flowjo.com/
Other		
Centrifuge 5810 R	Eppendorf	N/A
Microcentrifuge Sorvall Legend Micro 21R	Thermo Scientific	N/A
EcoShield Eco Nitrile PF 250	SHIELD Scientific	Cat#625123
OPS Essential Surgical Gown	Medline	N/A
14 mL polystyrene round-bottom tubes	Falcon	Cat#352057
EasySep magnet	STEMCELL Technologies	Cat#18001
Mr. Frosty	Thermo Scientific	Cat#5100-0001
BD FACSCanto	BD Biosciences	N/A
BD FACSCelesta	BD Biosciences	N/A
Microvette 200 LH, heparinized tubes	Sarstedt	Cat#201292
V-bottom shape 96-well-plate	Greiner Bio-One	Cat#651201
Carbon Steel scalpels	B. Braun Surgical	Cat#BB524
Large finger loops straight 10 cm	F.S.T.	Cat#14040-10
Narrow Pattern Forceps	F.S.T.	Cat#11003-12
Absorbable Synthetic Sutures	DH Material SL	Cat# 32093-36U

MATERIALS AND EQUIPMENT

- Recipes are provided to a specified final volume, but investigators may choose to prepare different volumes depending on the number of samples processed.

PBS-EDTA			
Reagent	Final concentration	Stock concentration	Add to 500 mL
PBS	1×	1×	498 mL
EDTA	2 mM	0.5 M	2 mL

Note: Store at 4°C for 1 month.

Recommended medium			
Reagent	Final concentration	Stock concentration	Add to 50 mL
PBS	1×	1×	49.8 mL
Bovine Serum Albumin (BSA)	0.5%	100%	250 mg
EDTA	2 mM	0.5 M	200 µL

Note: Store at 4°C for 1 month.

FACS Buffer			
Reagent	Final concentration	Stock concentration	Add to 500 mL
PBS	1×	1×	472.5 mL
EDTA	2.5 mM	0.5 M	2.5 mL
BSA	1%	100%	5 g
Horse Serum	5%	100%	25 mL

Note: Store at 4°C for 1 month.

Defreeze media			
Reagent	Final concentration	Stock concentration	Add to 50 mL
PBS	1×	1×	48 mL
Heat inactivated FBS	2%	100%	1 mL
Penicillin-Streptomycin	100 U/mL	10.000 U/mL	500 µL
DNAse I	10 U/mL	1,000 U/mL	500 µL

Note: Store at 4°C for 1 month. To Heat-inactivate FBS, put it for 30 min at 56°C in a water bath.

Ketamine-xilacine mix			
Reagent	Final concentration	Stock concentration	Add to 10 mL
PBS	0.6 ×	1 ×	6 mL
Ketamine	75 mg/kg	50 mg/kg	3 mL
Xilacine	10 mg/kg	20 mg/kg	1 mL

Note: Store at RT (20°C–25°C) for 1 month.

Enzymatic digestion mix			
Reagent	Final concentration	Stock concentration	Add to 50 mL
DMEM:F12	1×	1×	40 mL
Collagenase	300 U/mL	3,000 U/mL	5 mL
Hyaluronidase	100 U/mL	1,000 U/mL	5 mL

Note: Use freshly prepared.

Alternatives: Regular laboratory reagents such as PBS, BSA, or horse serum can be obtained from other sources than the specified in the key resources table. Alternatively to the $CD34^+$ isolation step, this cell population can be commercially purchased from different companies.

STEP-BY-STEP METHOD DETAILS

$CD34^+$ cells isolation from cord blood

Timing: 6–8 h

Human $CD34^+$ cells are isolated from human cord blood and cryopreserved in order to be used whenever mice humanization is required.

Note: Cord blood unit quality varies. Investigator should order cord bloods <36 h post-partum and with a weight greater than 100 g.

⚠ CRITICAL: Cord blood units, being recently post-partum, are usually not tested for infectious virus presence such as HIV or hepatitis. For this reason, it is crucial that the researcher works in sterile conditions and with appropriate PPE (Personal Protection Equipment).

1. Dilute cord blood 1:2 with PBS-EDTA.
2. Isolate mononuclear cells.
   a. Add 15 mL of Ficoll-Paque Premium to 50 mL Falcon tubes.
   b. Layer 30 mL of the diluted blood carefully on top of the Ficoll.

⚠ CRITICAL: Add the diluted blood at the minimum speed with the pipette controller and touching the wall of the falcon tube, avoiding the mixing of the blood and the Ficoll. Do not let the blood lie on the Ficoll for more than 10 min or it will start mixing. Additionally, when going to the centrifuge, take care not to disturb the gradient.

   c. Centrifuge tubes at 400 g for 30 min at 21°C without acceleration or brake in a swing-bucket rotor centrifuge.
   d. Aspirate the upper yellow layer (plasma), leaving the white thin layer (mononuclear cells) undisturbed. Harvest the white layer with a 5 mL pipette and transfer it to a new 50 mL tube, taking care of taking as little Ficoll as possible (transparent layer under the mononuclear cells). Top up with PBS-EDTA diluting it at least 4 times.
   e. Centrifuge at 700 g for 10 min at RT. Aspirate supernatant.
   f. Put together the pellets in two 50 mL Falcon tubes per cord blood and centrifuge at 700 g 5 min.
3. Lyse red blood cells.
   a. Add 5 mL of warm 1× RBC Lysis Buffer per falcon tube. Incubate at RT for 4 min, gently shaking them for the first 2 min. Top up with PBS-EDTA.
   b. Centrifuge at 600 g for 5 min. Aspirate supernatant and check if the pellet is white. If not, repeat step 3a. one more time for a maximum of two RBC Lysis Buffer incubations.
4. Count mononuclear cells.
   a. Resuspend and mix both pellets together in 40 mL of PBS 1×. Count cells with the desired method.
   b. Transfer $0.5*10^6$ cells to an Eppendorf tube and keep on ice. This will be the "Pre-enrichment sample". Always use the same counter to have more reproducible results.
   c. Centrifuge at 600 g for 5 min the rest of the cells.
5. Purify $CD34^+$ cells.
   a. Prepare a cell suspension at $50*10^6$ cells/mL in recommended medium and transfer it to a 14 mL polystyrene round-bottom tube.

⚠ CRITICAL: Investigators are strongly encouraged to use polystyrene tubes, as they have been proven to work much better than other material tubes for magnetic separation.

   b. Add the EasySep Human Progenitor Cell Enrichment Cocktail with Platelet depletion at 50 µL/mL of cells. Mix well and incubate 15 min at RT with overhead shaking.
   c. Vortex the EasySep D Magnetic Particles for 30 s and add 100 µL/mL of cells. Mix well and incubate 15 min at RT with overhead shaking, avoiding sedimentation.
   d. Place the tube without cap in a suitable magnet for the round bottom tube. Set aside for 10 min.

Note: This protocol is performed with a negative magnetic enrichment of $CD34^+$ cells. There are alternatives of positive enrichment of $CD34^+$ cells commercially available, although is preferably to leave the desired cell population undisturbed.

Note: This protocol is performed with a specific magnet and specific polystyrene tubes that fit into, but investigators could perform the magnetic separation with different material.

e. Harvest the supernatant by inverting the tube without getting it out of the magnet.

⚠ CRITICAL: Do not collect the supernatant with a pipette or you will collect magnetic particles bound to undesired cell types. Supernatant has to be collected by inversion.

f. Centrifuge at 600 g for 5 min. Aspirate supernatant and resuspend in 2 mL PBS 1×.
g. Count cells with desired method. Take a note of the number of $CD34^+$ cells obtained after the purification.
h. Transfer 20,000 cells to an Eppendorf tube and keep on ice. This will be the "Post-enrichment sample"

6. If not going to be used the same day, cryopreserve purified $CD34^+$ cells.
   a. Centrifuge the cells at 600 g for 5 min. Aspirate supernatant.
   b. Resuspend pellet in 500 μL of CryoStor CS10 freezing medium (one vial per cord blood unit). Use Mr. Frosty and store at −80°C for 24 h.
   c. Transfer vials to liquid nitrogen for long term storage.
7. Evaluate enrichment of the sample by flow cytometry.
   a. Add 500 μL of FACS buffer to the Pre and Post-enrichment samples for 20 min at 4°C to block membrane proteins.
   b. Centrifuge at 400 g for 5 min, discard supernatant, and add anti-huCD45-PE and anti-huCD34-AF488 at a 1/300 dilution in FACS buffer, 100 μL per sample. Incubate 30 min at 4°C, protected from light.
   c. Centrifuge, discard supernatant, and add the viability marker propidium iodide (PI) at a 1/500 dilution in PBS 1×, 300 μL per sample.

Note: Different viability markers can be used, such as DAPI, Zombie Aqua, etc. If changed, the investigator should take into account the possible overlap with the other fluorophores.

   d. Analyze the enrichment of $CD34^+$ cells in the Pre and Post-enrichment samples in the cytometer, by means of double positive $CD45^+$ and $CD34^+$ cells (Figure 1).

Note: This protocol has been performed with BD Bioscience cytometers, the BD FACSCanto II and the BD FACSCelesta. Other cytometers could be used if they allow the analysis of the specified panel. Analysis was performed using FlowJo software.

Note: Comparison between Pre and Post-enrichment sample will give the investigator the efficacy of the enrichment of $CD34^+$ cells performed. This can vary between samples.

PDX generation

Timing: 6–12 months

This step describes the process to generate and expand PDXs from both primary and metastatic tumor samples from breast, pancreatic, colorectal or gastric cancer origin.

Optional: Biopsies can be frozen in Bambanker before implantation, although it is not recommended.

8. Collect the sample from the clinicians.

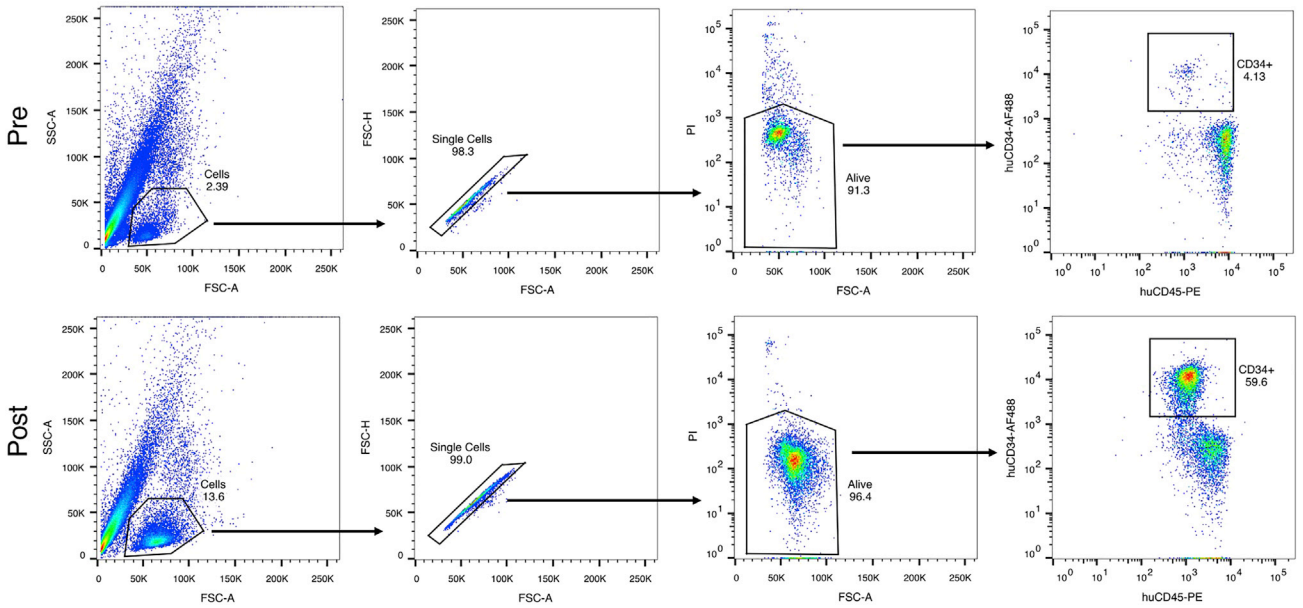

Figure 1. Gating strategy for the number determination of CD34⁺ cells pre and post-enrichment

Flow cytometry plots displaying a representative analysis of $CD34^+$ cells in a cord blood unit in the pre and post-enrichment samples. Arrows represent the steps of the gating strategy used in this protocol.

Note: It is crucial to have a good circuit of biopsies and surgery samples between clinicians and researchers, so that samples get to the investigator in the best possible conditions.

Note: This protocol is optimized for fresh samples from either surgery pieces or core-needle breast biopsy (CNB), and implanted in female immunodeficient mice, such as NOD.SCID or NSG.

9. Transfer the tumor sample to a 50 mL falcon tube with 10 mL DMEM:F12 media supplemented with 1% penicillin-streptomycin.
10. In sterile conditions under laminar flow hood, place the tumor sample in a sterile plastic dish and mechanistically dissociate it with scalpels in fragments of approximately 3 × 3 mm.
11. For mice orthotopical implantation of breast cancer tumor pieces, these steps should be followed (Figure 2).
    a. Anesthetize the female mouse by injecting intraperitoneally the ketamine-xylazine mix. Put a drop of methocel to each mouse eye to prevent eye drying and consequent blindness.
    b. Shave the ventral area of the mouse.
    c. Expose the 4^{th} pair of mammary glands (abdominal ones) with a minimal incision.
    d. Place a fragment subcutaneously of top of the exposed mammary gland. One tumor piece per flank. Pieces should not be larger than 3 × 3 mm.
    e. Close the wound using surgical absorbable suture.
    f. For breast cancer PDXs, maintain mice with 17-β-estradiol at 1 μM in the drinking water.

Note: Ketamine-Xilacine mix should not be stored for more than 1 month at RT (20°C–25°C).

12. For mice subcutaneous implantation of pancreatic, colorectal and gastric cancer tumor pieces these steps should be followed (Figure 3).
    a. Anesthetize the mouse by injecting intraperitoneally the ketamine-xylazine mix. Put a drop of methocel to each mouse eye to prevent eye drying and consequent blindness.
    b. Shave the back area of the mouse.

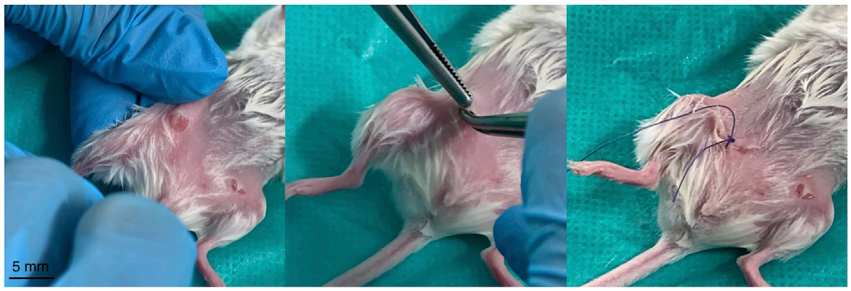

Figure 2. Orthotopic implantation of a breast cancer PDX in the mice mammary fat pad
Images showing the process of the PDX implantation. From left to right, incision and exposure of the mammary fat pad, PDX implantation and suture of the wound. Scale bar represents 5 mm.

c. Expose left and right flanks with a minimal incision.
d. Place each fragment subcutaneously. One tumor piece per flank. Pieces should not be larger than 3 × 3 mm^3.
e. Close the wound using surgical absorbable suture.

13. Monitor tumor growth by measuring once a week the volume of the PDX with calipers and using the formula: (length × (width)*2)/(π/6).
14. Before tumors reach a volume of 1,000 mm^3, mice should be euthanized and tumors resected and cut in pieces to be expanded for the next PDX passage into new mice. Follow steps 11 or 12 to implant them again.

Optional: The rest of the excised tumor pieces that are not implanted into new mice should be kept as a cryopreserved material using Bambanker cryopreservation media or as Formalin Fixed Paraffin Embedded (FFPE) blocks. These preservations will allow the researcher to recover old PDX passages and further study its biology. Ideally, after every PDX passage a piece of the tumor should be assayed for a complete pathogen infection (in particular *C. Bovis*). This can be performed by different companies with a variety of pathogen analysis by PCR. Our group uses IMPACT Mouse Profiles from IDEXX Bionalytics.

Optional: After the first passage, PDXs could be digested and implanted as steps 19 and 20.

Note: We recommend female mice for usage with all PDX generation, as male mice tend to be more aggressive and mouse death occur more often.

Mice humanization

Timing: 5 months

Immunodeficient NSG mice are humanized with $CD34^+$ hematopoietic stem cells in order to generate a large variety of immune cell types mimicking what would be a human immune system.

15. Myeloablate NSG mice by injecting 15 mg/kg busulfan intraperitoneally in PBS 1× 24 h before $CD34^+$ cells injection (200 µL per mouse).

Optional: Mice myeloablation can be done by irradiation. Investigator should decide the best strategy. In our case, busulfan was decided because it does not affect the animals' health.

16. Defreeze the $CD34^+$ cells. When using cells purified the same day, this step is not necessary.
a. Put the frozen vials in a 37°C water bath until CS10 frozen media is liquid.
b. Transfer the cells to 15 mL falcon tubes containing 10 mL of defreeze media. Troubleshooting 1.

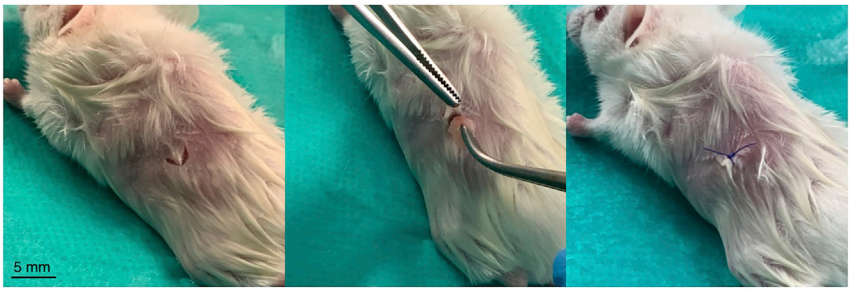

Figure 3. Subcutaneous implantation of a pancreatic cancer PDX in mice

Images showing the process of the PDX implantation. From left to right, incision of the back of the mouse, PDX implantation and suture of the wound. Scale bar represents 5 mm.

c. Centrifuge at 400 g for 10 min and resuspend in 500 μL of defreeze media.
d. Count cells with the desired method.
e. Take 20,000 cells for staining and follow step 7.
f. Calculate cells for injection, considering cell number, viability and $CD34^+$ staining.

17. Inject 10^5 $CD34^+$ cells per mouse in 100 μL PBS into the tail vein, using 27G needles and 1 mL syringes.

Note: Preferably use vials with >0.5 × 10^6 of $CD34^+$ cells for humanization, increasing the number of mice humanized with the same unit and avoiding variability.

⚠ CRITICAL: Humanization must be performed in young NSG mice, up to 6 weeks.

18. After 20 weeks of $CD34^+$ cells injection, analyze humanization levels in peripheral blood.
    a. Collect a minimum of 50 μL of blood per mouse from the facial vein using Microvette heparinized tubes.
    b. Transfer 50 μL of blood to 15 mL falcon tubes and add 2 mL of RBC Lysis Buffer to each tube. Incubate for 4 min at RT with gently shaking for the first 2 min.

⚠ CRITICAL: Mice erythrocytes are harder to lyse; therefore, the sample should be incubated with at least 2 mL of RBC lysis buffer.

    c. Centrifuge at 400 g for 5 min and aspirate supernatant. Repeat step 18b once again if the pellet is still red, to a maximum of two RBC Lysis Buffer incubations. Pellet should be light orange.
    d. Resuspend pellet in 200 μL and transfer all the volume to a well of a V-bottom shape 96-well-plate.
    e. Centrifuge the plate at 400 g for 5 min and remove supernatant throwing it directly to the bin or sink with a single move. Add 200 μL of FACS buffer per well and incubate 20 min at 4°C.
    f. Centrifuge the plate, remove supernatant, and add primary antibodies in FACS buffer. Add 100 μL per well and incubate at 4°C protected from light for 30 min. The antibody cocktail, all at 1/300 dilution, consists of:
        i. muCD45-AF488.
        ii. huCD45-PE.
        iii. huCD4-BV421.
        iv. huCD8-PE/Cy7.

Note: This protocol is optimized for this antibody cocktail, if other antibodies are used, investigators should titrate them.

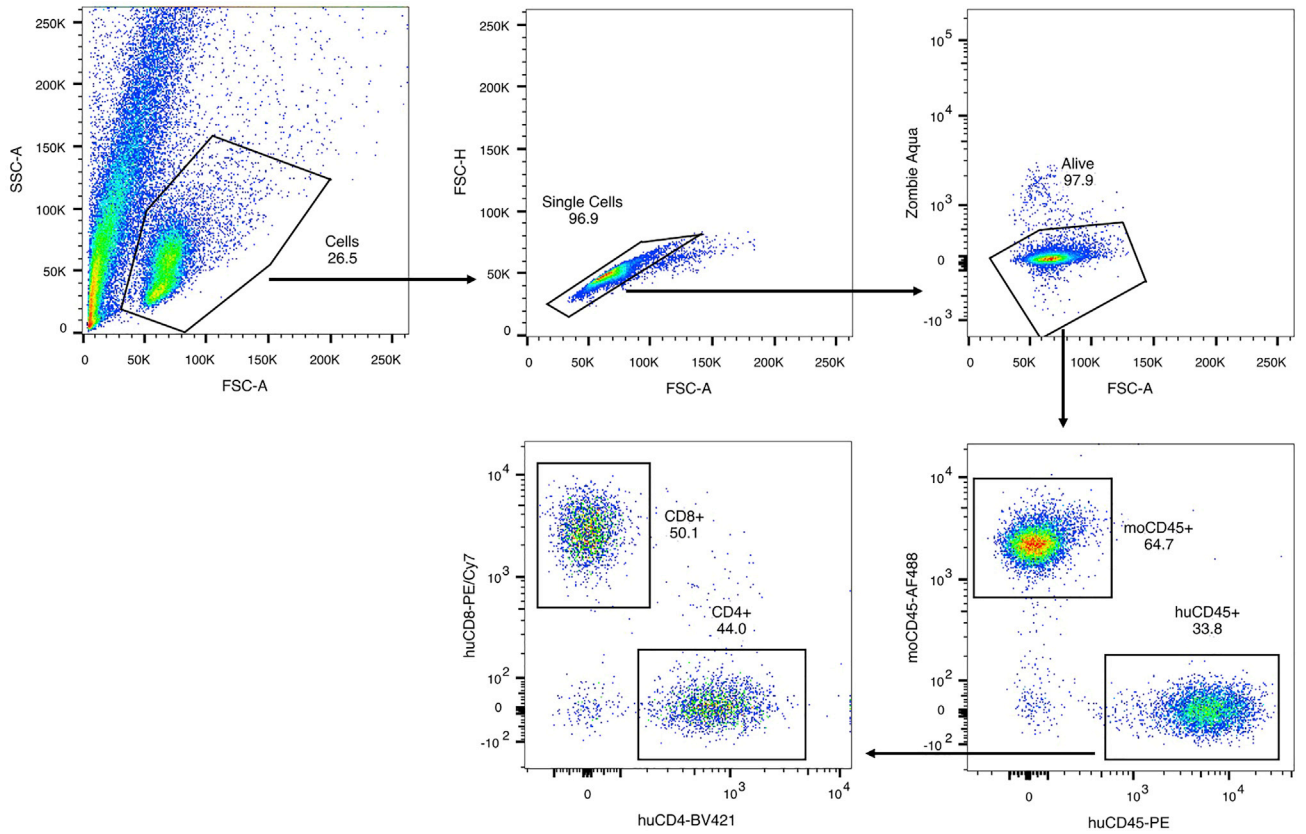

Figure 4. Gating strategy for the percentage determination of huCD45$^+$ cells in humanized mice blood

Flow cytometry plots showing a representative analysis of mice humanization in peripheral blood. Arrows represent the steps of the gating strategy used in this protocol.

g. Centrifuge the plate and resuspend pellet in 120 μL PBS 1× with 1/500 viability marker Zombie Aqua.

h. Analyze in the cytometer, acquiring 80 μL per well in the V-bottom 96wp with the HTS mode from the cytometer (Figure 4).

⚠ CRITICAL: A minimum of 20% human CD45$^+$ cells in the peripheral blood of the mice is required to consider it humanized. Investigators should use more mice and cord blood units than expected to assure the availability of humanized mice when desired. Usually, around 30% of CD34$^+$ cells are lost after thawing. The ratio of success of mice humanization after 5 months is around 70%.

Generation of an acquired immunoresistance model in PDX

Timing: Around 1 year

This step describes the process to generate a model of acquired immunotherapy resistance to T Cell Bispecific antibodies (TCBs) or Chimeric Antigen Receptor T cells (CAR Ts) with PDX in humanized mice.

Note: This protocol could be used to generate models of resistance to other immunotherapies such as immune checkpoint inhibitors.

19. Digest the PDX sample.
    a. Excise the tumor from the mice bearing it before it reaches 1,000 mm^3.
    b. In sterile conditions under laminar flow hood, place the tumor sample in a sterile plastic dish and mechanistically dissociate it with scalpels until you obtain a tumor mass.
    c. Collect the sample with 10 mL of enzymatic digestion mix and put it into 50 mL falcon tubes. This enzymatic mix has to be freshly prepared everytime.
    d. Place the tubes horizontally in a shaker at 37°C and 70 rpm (10 g) for 1 h.
    e. Filter the mixture with 100 μm cell strainers into new 50 mL falcon tubes. If the sample does not go through, you can force it with the back of a sterile syringe.
    f. Centrifuge 5 min at 400 g and aspirate supernatant.
    g. Add 5 mL of warm 1× RBC Lysis Buffer per falcon tube. Incubate at RT for 4 min, gently shaking them for the first 2 min. Top up with PBS 1×.
    h. Centrifuge at 400 g for 5 min. Aspirate supernatant and check if the pellet is white. If not, repeat step 3a. one more time for a maximum of two RBC Lysis Buffer incubations.
    i. Resuspend cells with PBS 1× and count cell number.

Note: Digestion of the tumor is recommended for a more homogenous mix of the original tumor, as heterogeneity of the previous tumor passage can mask the differential response of the tumors.

20. Implant cells into new humanized mice.
    a. Resuspend the desired number of cells in a 1:1 mixture of PBS 1× and Matrigel and place the sample into Eppendorf tubes in ice.
    b. Inject 100 μL of cells orthotopically or subcutaneously depending on the tumor origin as in steps 11 or 12. Cell number should be assayed by researcher as it depends on the PDX, but normally 10^6 should be sufficient.
21. Monitor tumor growth by measuring once a week the volume of the PDX with calipers and using the formula: (length × (width)*2)/(π/6). When tumor is palpable, measure should be done twice a week.
22. When tumors reach 300 mm^3, start the treatment with increasing concentrations.
    a. When treating with a TCB, depending on the tumor expression of the targeted antigen and the potency of the TCB, a wide range of concentrations from 0.1 to 1 mg/kg have been observed by our group to be ideal. Treatments are performed twice a week (Martínez-Sabadell et al., 2022; Arenas et al., 2021; Rius Ruiz et al., 2019).
    b. When treating with CAR T cells, 3 × 10^6 have been observed to be efficacious regardless the targeted antigen. Treatments are performed once a week or once every 14 days.

Note: A pilot in vivo experiment with the desired treatment is recommended to decide the starting dose of the treatment for immunoresistant PDX generation. A dose in which tumor complete response is not achieved is recommended. Tumors should reach a 90% of tumor response with a volume around 100 mm^3 in order to be transplanted.

23. When tumor regresses, stop the treatment and let the tumor grow. When regrown, treatment can be restarted again. This process can be sustained for up to 5 months, as lymphocytes get exhausted after chronic treatment. Troubleshooting 2.

Note: Mice humanized with $CD34^+$ cells do not present graft vs host symptoms, which gives the advantage that mice can last for more than 180 days after PDX implantation. Ideally, investigators should assay mice humanization every 2–3 months, as immune cells can get exhausted and the mice loose its humanization phenotype due to treatment.

24. Passage the tumor digesting it as in steps 19 and 20.

Note: Investigators should cryopreserve a tumor pellet in Bambanker after every passage so that the model is not lost due to technical problems in the future.

Note: In our experience, resistance to TCBs or CAR Ts is achieved after 3 passages. Each immunoresistant PDX model should be treated independently. This will allow to have a greater number of immunoresistant PDX models, and therefore strength the identified mechanism of resistance.

25. Confirm the acquired resistance phenotype of the generated model by performing an in vivo experiment with the parental PDX and the newly generated resistant PDX.

Note: Mice should be randomized before implantation according to levels of $CD34^+$ cells in peripheral blood, and it should be noted which mice belongs to which cord blood unit.

⚠ CRITICAL: For downstream studies such as immunohistochemistry, protein isolation or RNA isolation tumor pieces are used, and a minimum number of 6 replicates should be assayed, as PDX tumor pieces can be heterogenous.

EXPECTED OUTCOMES

Investigators will have generated an in vivo acquired resistant model to immunotherapy in a very complex model that recapitulates the different immune populations present in a patient. These models are powerful tools to answer several hypotheses and to identify novel mechanisms of resistance by using omics such as scRNA-Seq, proteomics, CyTOF, etc., to have a better insight into the biology of the tumors, the stroma, and the response to the immunotherapies. These findings could become a translational finding, with clinical applications in the future such as the identification of a biomarker or response or a dual targeting therapy to improve the existing one.

LIMITATIONS

This protocol involves 4 different processes, each one with its own limitations.

For the $CD34^+$ cell isolation, the quality of the sample and the number of isolated cells highly vary between donors, and some cord bloods will give very low number of $CD34^+$ cells.

In the case of the PDX generation, many patient samples do not graft into the mouse and will never grow, and other PDXs do not grow when implanted after cryopreservation. In general, immunodeficient mice, and particularly NSG mice, are highly sensitive to pathogen infection. These pathogen infections will have a direct impact in the health of the mice and the results obtained, particularly in the response to therapies. Of note, *C. Bovis* is the major problem in this regard.

Regarding $CD34^+$ mice humanization, some cord blood samples have been seen to fail to humanize mice, obliging the investigator to use more cord blood units and mice than desired.

In the case of the in vivo acquired immunotherapy resistant model generation, being a preclinical model, it could happen that it does not mimic what happens in the patient.

This protocol is time consuming (takes years to achieve) and requires a high economical expense.

Mechanistic studies such as gain and loss of function and functional assays are difficult to perform in tumor samples, contrary to in vitro models of acquired resistance.

TROUBLESHOOTING

Problem 1

A mucosal mix of cells appear when thawing $CD34^+$ cells.

When thawing $CD34^+$ cells, a mucus can be formed either when resuspended with the defreeze media or with the PBS. Step 16.

Potential solution

If the mucus is formed, it is difficult to save the sample. Investigator should resuspend the sample with a 1 mL pipette, which will disaggregate the sample a bit, but the viability will decrease.

For the next $CD34^+$ cell cryovial thawed, investigator should shorten the time of thawing in the water bath and increase the volume of defreeze media in which is resuspended. Additionally, thawing step should be done as fast as possible.

Problem 2

Mice show unhealthy shape due to pathogens or graft vs host disease.

Some mice can exhibit bad shape due to humanization process or pathogen infection during the immunoresistant PDX generation, as NSG mice are more sensitive to infections. Step 23.

Potential solution

It is very difficult to elucidate if the mouse is unhealthy due to pathogen infection or graft vs host disease. For this reason, animal should be euthanized, and the PDX passed to another humanized animal. Nonetheless, the receptor mouse should be put in quarantine and the PDX should be assayed for pathogen infection. In case it is infected with difficult pathogens such as *C. Bovis*, that PDX should be discarded.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Enrique J. Arenas (earenas@vhio.net).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate a dataset or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.M.S., J.A., E.J.A.

Investigation & methodology, A.M.S., P.O.R., E.J.A.

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Supervision, J.A., E.J.A.

DECLARATION OF INTERESTS

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