Video Article

Expression of Exogenous Cytokine in Patient-derived Xenografts via Injection with a Cytokine-transduced Stromal Cell Line

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Abstract

Patient-derived xenograft (PDX) mice are produced by transplanting human cells into immune deficient mice. These models are an important tool for studying the mechanisms of normal and malignant hematopoiesis and are the gold standard for identifying effective chemotherapies for many malignancies. PDX models are possible because many of the mouse cytokines also act on human cells. However, this is not the case for all cytokines, including many that are critical for studying normal and malignant hematopoiesis in human cells. Techniques that engineer mice to produce human cytokines (transgenic and knock-in models) require significant expense before the usefulness of the model has been demonstrated. Other techniques are labor intensive (injection of recombinant cytokine or lentivirus) and in some cases require high levels of technical expertise (hydrodynamic injection of DNA). This report describes a simple method for generating PDX mice that have exogenous human cytokine (TSLP, thymic stromal lymphopoietin) *via* weekly intraperitoneal injection of stroma that have been transduced to overexpress this cytokine. Use of this method provides an *in vivo* source of continuous cytokine production that achieves physiological levels of circulating human cytokine in the mouse. Plasma levels of human cytokine can be varied based on the number of stromal cells injected, and cytokine production can be initiated at any point in the experiment. This method also includes cytokine-negative control mice that are similarly produced, but through intraperitoneal injection of stroma transduced with a control vector. We have previously demonstrated that leukemia cells harvested from TSLP-expressing PDX, as compared to control PDX, exhibit a gene expression pattern more like the original patient sample. Together the cytokine-producing and cytokine-negative PDX mice produced by this method provide a model system that we have used successfully to study the role of TSLP in normal and malignant hematopoiesis.

Video Link

The video component of this article can be found at https://www.jove.com/video/55384/

Introduction

Patient-derived xenografts (PDX) are a powerful *in vivo* model for studying the production of normal and malignant hematopoietic cells in a 'native' mammalian environment. Most often, PDX are produced by injecting or transplanting human cells into immune deficient mice. The production of PDX using normal human hematopoietic stem cells allows *in vivo* studies of normal human blood and immune cell development. PDX produced from leukemia or other cancer cells make it possible to study oncogenic mechanisms and to identify effective therapies in context of the range of genetic landscapes and mutations present in the human population.[1] Consequently, PDX are the current gold standard for translational biomedical research to identify effective therapies and an important tool for understanding mechanisms of cancer progression. PDX models are an essential tool to aid research into health disparities diseases due to specific genetic lesions, or any disease in which the variations of a patient's genetic landscape can substantially contribute to oncogenesis and treatment outcome.

Mouse-human PDX models are possible because many mouse cytokines adequately mimic their human analogs in activating the cytokine receptors of human cells while they are inside the mouse. For example, interleukin-7 (IL-7) provides a critical signal for human B cell development.[2] In this case, mouse IL-7 has sufficient homology with human IL-7 that the mouse cytokine stimulates signaling pathways in human B cell precursors.[2,3,4] However, this is not the case for thymic stromal lymphopoietin (TSLP),[5,6] which among other cytokines (IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF),[7] is important for the production of normal and malignant human hematopoietic cells. When mouse and human cytokines show low homology the mouse cytokines do not activate their respective receptors on human cells. To overcome this obstacle, a number of strategies have been used to engineer expression of human cytokines in PDX mice. These include injection of recombinant human cytokines, hydrodynamic injection of DNA, lentiviral expression, transgenic expression and knockin gene replacement.[7] This report describes a method for engineering PDX to produce human cytokine *via* stromal-mediated cytokine delivery (**Figure 1**).

In the method demonstrated here, PDX mice are engineered to express the human cytokine, TSLP, or to serve as cytokine-negative controls. TSLP-expressing PDX are achieved by weekly intraperitoneal injections of stromal cells that have been transduced to express high levels of human TSLP. Cytokine-negative PDX "control" mice are similarly engineered; though control stroma are transduced with a control vector. This method achieves normal physiological levels of human TSLP in PDX mice injected with the TSLP+ stroma. No detectable TSLP is observed in PDX mice receiving the cytokine-negative stroma. We selected the human stromal cell line HS-27A for our studies because it grows robustly in culture and shows very low level of cytokine production that does not support proliferation of isolated progenitor cells in cocultures.[8] For human TSLP expression, stroma were transduced with an advanced generation self-inactivating lentiviral vector derived from a previously described backbone,[9] and includes the cPPT/cts element and the woodchuck hepatitis post-transcriptional regulatory element (WPRE) to increase transgene expression. The human TSLP gene was constructed into this vector under the control of the elongation factor-1 (EF-1) alpha promoter to achieve robust, constitutive, and long-term expression.

The engineering of this human-cytokine enhanced PDX model consists of 4 major steps. First, transduced stroma are expanded *in vitro* and assessed by enzyme-linked immunosorbent assay (ELISA) for stable, high level cytokine production. Second, the activity of human cytokine produced by the transduced stromal cells (and lack of cytokine activity from control stroma) is verified using phospho-flow cytometry. Cell lines known to be responsive to cytokine of interest (in this instance,TSLP) are incubated with stromal cell supernatant and assayed for cytokine-induced phosphorylation. Third, mice are injected with transduced human stroma and then mouse plasma is assessed by ELISA for levels of human cytokine on a weekly basis. Fourth, human hematopoietic cells are transplanted and the *in vivo* functional effects of the human cytokine is evaluated on a known target (*e.g.* cell population).

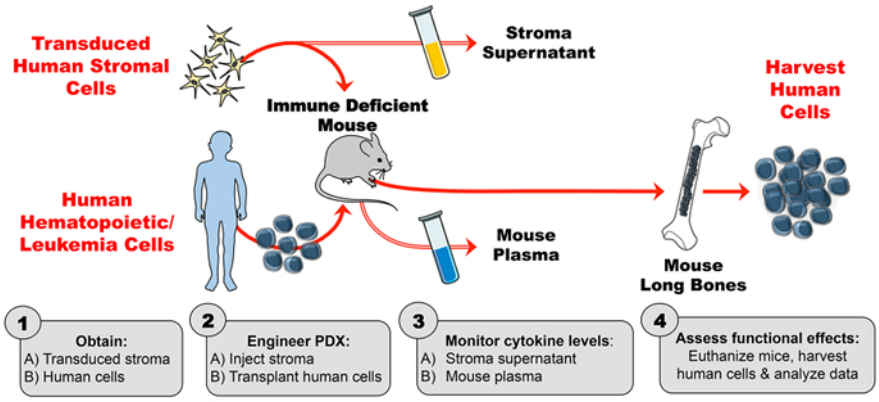

Figure 1: PDX Model Engineered to Produce Exogenous Human Cytokine in Mice. (**1A**) Design experiment and obtain transduced human stromal cells (**1B**) Obtain human cells (hematopoietic stem cells, leukemia cells, *etc.*) to generate PDX (patient-derived xenograft) mice. (**2A**) Inject engineered stroma and (**2B**) transplant human cells into immune deficient mice according experimental schedule. (**3A-B**) Monitor cytokine concentrations in the stroma supernatant and the mouse plasma by ELISA. (**4**) Harvest human cells and assess the *in vivo* functional effects of the human cytokine present in the PDX. Please click here to view a larger version of this figure.

Delivery of human cytokine *via* stromal cells offers both advantages and disadvantages when compared to other methods of delivering/producing human cytokines in PDX mice.[7] Compared to injection of recombinant human cytokine, stroma-mediated delivery is generally less expensive (cost of stromal cell culture is less than cost of recombinant cytokine) and less labor intensive (one injection per week versus multiple injections per week). The issue of short cytokine half-life is also mitigated since stroma continually produce the exogenous cytokine. Delivery of cytokine *via* hydrodynamic injection of DNA may be less expensive than delivery *via* stroma. However, it is similarly transient and may require more technical skill than the simple weekly intraperitoneal injection required for stroma-mediated delivery. Lentiviral gene expression in the mouse may provide a less transient method of cytokine delivery; however, in our hands physiological TSLP levels were not achieved. Additionally, this method is labor intensive, requiring continuous production of lentiviral vector. Transgenic or knock-in mice offer stable long-term expression of cytokine and can be engineered for tissue specific expression, which can be an advantage. On the other hand, the transgenic expression of the human cytokine gene on the immune deficient mouse background required for PDX mice, necessitates an immense investment of resources before the value of the model has been established. Furthermore, transgenic models do not generally allow for the option of varying the timing of cytokine initiation or level of *in vivo* cytokine production. These can be achieved with stroma-mediated delivery by simply changing the time point for initiation of stromal cell injection or the dose of stromal cells injected.

The stromal-cell mediated cytokine delivery method detailed here was used to develop PDX for evaluating the role of TSLP in normal human B cell development[4,6] and high risk B-cell acute lymphoblastic leukemia.[6] This method provides an alternative cytokine delivery method for use in generating similar models with human cytokines other than TSLP. This model can also be useful for generating preliminary data that can help determine whether the value of a cytokine transgenic or cytokine knock-in PDX model would be worthy of the substantial time and money investment.

Protocol

Studies were conducted in accordance with Loma Linda University's Institutional Animal Care and Use Committee (IACUC) and Institutional Review Board (IRB) approved protocols and according to all federal guidelines.

CAUTION: PDX and human tissues should be handled in accordance with safety procedures to prevent the transmission of blood borne pathogens.

1. Culture and Expansion of Cytokine-Transduced Stromal Cells

NOTE: Each time stroma are passaged, harvest the culture supernatant (1 mL aliquot) and store at -80 °C for future quantification of cytokine level *via* ELISA assay.

1. Prepare R10 culture medium and freezing medium as described in the Table of Materials.
2. Generate[10,11] or obtain control stroma (transduced with empty vector) and cytokine-producing (TSLP+) stroma.[6] Store in liquid nitrogen (LN_2) until use. Thaw stromal cells as described[12] and plate them in 5 mL of R10 medium in separate T-25 cell culture flasks. Culture cells in an incubator at 37 °C with 5% CO_2. This is post-thaw "passage #1".
3. Once stroma are confluent (24-48 h), passage cells by trypsinizing using 1× trypsin EDTA (0.25%) and re-plating in a T-150 culture flask. This is post-thaw "passage #2".
4. **When stroma achieve confluency in the T-150 flasks (3-4 days later), passage the cells by trypsinizing. Use this cell suspension for experimental needs.**
    1. Cell expansion to multiple flasks
        1. Split the harvested cells between three T-150 flasks and culture until confluent. Repeat this step if stroma expansion is necessary. Typical human stromal (HS-27A) cell count at confluence in a T-150 flask is ~5 x 10^6.
    2. Cell storage
        1. Transfer the harvested cell suspension to a conical tube and centrifuge at 500 x g for 5 min, decant the supernatant and resuspend the cells in freezing medium. Freeze and store in LN_2 ~1.5 x 10^6 cells per vial.
    3. For intra-periotoneal stroma injections into mice, proceed to step 4.1.

2. ELISA Assays to Monitor *In Vitro* Cytokine Production from Transduced Stroma

1. Purchase commercially available ELISA assay kit to detect the cytokine of interest.
2. Thaw stromal cell supernatant from control and cytokine-producing (TSLP+) stroma harvested at early, middle and late passages (**Figure 2**).
3. Determine the TSLP concentration in supernatant samples using an ELISA kit according to manufacturer's instruction.[13] Compile these serial data to monitor the stability of cytokine production during cell culture (**Figure 2A-B**).
    1. Identify and discard cytokine-producing stroma with reduced or suboptimal cytokine expression, as well as any corresponding vials in storage (**Figure 2A-B**).
    2. Identify and maintain cytokine-producing stroma that demonstrate stable, high level cytokine concentrations. Use these thaws for experiments.
4. Use the ELISA kit to routinely monitor stroma throughout the duration of the cell culture (at least 1 time during early, middle and late post-thaw passages) to assure stable cytokine production or, in the case of control stroma, the absence of cytokine production.

3. Phospho-flow Cytometry Assays to Evaluate the Activity of Cytokine Produced by Stroma

NOTE: Assess the supernatant from engineered stroma before the first experiment to verify the relevant bio-activity of the stroma-generated cytokine for individual experiment.[6] Once the engineered stroma are validated, further testing is not necessary unless stroma are introduced that were produced with a new vector.

1. Purchase MUTZ5 and/or MHH-CALL4 leukemia cell lines (TSLP responsive),[14] recombinant human cytokine, and antibodies approved for use in phospho-flow cytometry assays to detect appropriate downstream phosphorylation targets.
2. Thaw harvested supernatant from control and cytokine-expressing stroma.
3. Plate the leukemia cell lines in R20 medium (see **Table of Materials**) at a concentration of 0.2 x 10^6 cells per well in a 96 well tissue culture plate. Plate 3 wells per condition to allow for triplicate assays. Incubate for 2 h at 37 °C. This time allows for the loss of any phosphorylation that occurred during prior culture conditions.
   NOTE: 12 wells per cell line provides triplicate assays for each experimental condition shown in step 3.4.
4. **After 2 h in culture, transfer the cells from each well to separate 4 mL tubes and centrifuge at 500 x g for 5 min, at 4 °C. Decant the supernatant.**
    1. Re-suspend the cells in 200 µL for each of the following experimental conditions (perform assays in triplicate): 1) negative control-media only, 2) positive control-media with recombinant cytokine at saturating concentration(s) (TSLP at 15 ng/mL, *etc.*), 3) control stroma-supernatant from control stroma, and 4) cytokine-stroma-supernatant from cytokine-producing stroma.
5. Incubate the cells for the duration specified by specific commercial phospho-assay (*e.g.* 30 min for phospho-STAT5 in MUTZ5 or MHH-CALL4 in response to TSLP).
6. Centrifuge at 500 x g for 5 min at 4 °C and decant the supernatant.
7. Perform phospho-flow cytometry staining per manufacturer's protocol and collect flow cytometry data.[15]
8. **Analyze flow cytometry data.**[15]
    1. Gate on intact cells based on forward (FSC, indicates cell size) and side (SSC, indicates cell granularity) light scatter.
    2. Determine the the median fluorescent intensity (MFI) of the intact cells in each sample. Compare the average MFI obtained from the triplicate values of stromal cell supernatant to that of positive and negative media controls.

4. Injection of Cytokine-Transduced Stroma into Mice

NOTE: Culture HS-27A stroma for a minimum of 3 post-thaw passages prior to injecting them into mice to ensure healthy cells and adequate cytokine production.

1. **Preparation of stroma**
    1. Transfer the harvested stromal cell suspension to a conical tube and aliquot 10 µL for hemocytometer count; obtain live cell count using trypan blue.[16] Centrifuge the remaining cell suspension at 500 x g for 5 min.
    2. Aspirate the supernatant from the pelleted cells and gently resuspend the cells in the necessary volume of sterile phosphate-buffered saline (PBS) for mouse injection (depending on the number of mice to inject).
    NOTE Typical stromal cell concentration for mouse injection: 0.5 x 10^6-5 x 10^6 cells per mouse in 200 µL PBS.
    3. Keep the stromal cell suspension at 4 °C (or on ice) until immediately before the injection. Ensure that the cell suspension is at least at room temperature (RT) (~25 °C) for mice injection.

2. **Stromal cell injection**
    1. Disinfect the bio-safety hood, assemble materials for injections and then place the mouse cage inside.
    2. Gently mix the cell suspension by inversion prior to drawing up 200 µL into a tuberculin syringe.
    3. Using standard intra-peritoneal injection techniques,[17] restrain the mouse and administer the 200 µL cell suspension into the peritoneal cavity. Details have been previously demonstrated by Machholz *et al.*[18]

5. Serial Blood Collection and Plasma Monitoring for Exogenous Human Cytokine in Mice

1. **Blood collection *via* tail snip**
    1. Disinfect the biosafety hood and assemble mouse restrainer, scissors and other tools/supplies for the procedure.
    2. Place the mouse in a restrainer and secure the tube to minimize mouse movement.
    3. Disinfect the tail and surgical scissors with isopropyl alcohol. Apply topical anesthetic cream on the tail.
    4. Using very sharp surgical scissors snip off about 0.5 - 1 mm of the tip of the mouse's tail. Collect approximately 80 µL of peripheral blood using heparinized capillary tubes.
    NOTE: If blood will be collected with this method more than six times, tail removal should be conservative with respect the amount of tail removed. As snips progress up the tail the diameter increases, it hemorrhages faster, and healing takes longer.

2. **Plasma separation**
    1. Transfer the blood using a bulb syringe (to expel it from the capilliary tubes) into a labeled K_2EDTA microtainer tube; invert 20 times to prevent coagulation.
    NOTE: Typically blood clotting from tail nip is fast. However, if hemorrhage persists longer than ~ 2 min use a styptic pencil/powder to aid coagulation.
    2. Centrifuge blood collection tubes according to manufacturer's instruction and aliquot the plasma for storage (-20 °C) until ready for the ELISA assay.
    3. If mouse-human cell chimerism data is needed from the mouse blood, then treat the remaining pellet as described in step 6.3.1. Otherwise, discard the blood cell pellet.

3. **Modified ELISA for micro-volumes of mouse plasma**
NOTE: The manufacturer's ELISA procedure was modified from the recommended 100 µL sample volume to a 40 µL volume to accommodate the micro-volumes collected from mice. It is not possible to run the *in vivo* mouse plasma samples in triplicate with these limited volumes. At the end of the experiment ~500 µL of post-mortem blood is collected via cardiac puncture. Cytokine concentrations evaluated in 100 µL of euthanasia plasma are used to validate the 40 µL *in vivo* data for each mouse.
    1. Thaw frozen mouse plasma samples.
    2. Serially dilute ELISA standards and plate them in triplicate at 40 µL per well.
    3. Add 40 µL of each mouse plasma sample to the ELISA plate.
    NOTE: If a mouse sample is less than 40 µL, then bring the volume up to 40 µL with ELISA buffer. Record this diluent volume and use it to calculate a dilution factor for each diluted sample. When analyzing the ELISA data multiply the diluted sample concentration by the sample's dilution factor to determine the actual cytokine concentration in original sample.
    4. Complete ELISA assay according to manufacturer's instructions.

6. Transplantion of Hematopoietic Cells into Mice

1. **Sublethal irradiation to condition bone marrow for transplant**
NOTE: Loma Linda University (LLU) uses a Cobalt-60 radiation source. Animals are placed in a pie restrainer which is positioned 80 cm from the radiation source in a 20 x 20 cm field and with a 0.5 cm layer of Lexan on top of the restrainer. Exposure time is calculated to achieve a 225 cGy dose based on most recent calibration of source (currently 2-3 min for this source).
    1. Sub-lethally irradiate mice (total body irradiation dose of 225 cGy maximum for immune deficient mice).
    2. Transplant hematopoietic cells ~ 24 h later *via* intravenous tail vein injection.

2. **Intra-venous hematopoietic cell transplant**
    1. Prepare human cell suspensions for transplant in a volume of 200 µL sterile PBS per mouse: CD34+ hematopoietic stem cells (HSC) : 1 x 10^5 to 5 x 10^5 cells per mouse and leukemia cell lines/patient samples: 1 x 10^6 to 5 x 10^6 cells per mouse.
    2. Keep cells at 4 °C (transport on ice) until immediately prior to transplant. Ensure cells are at least at RT prior to transplant.

3. Disinfect the bio-safety hood and prepare the hood area for transplant injections.
4. Place the mouse in warming cage for at least 5 min to allow dilation of tail veins.
5. Gently mix the cell suspension and draw up 200 µL into a tuberculin syringe. Safely set the needle aside where it will remain sterile.
6. Place the mouse in a restraint and disinfect the tail with isopropyl alcohol.
7. Using standard intravenous injection techniques,[17] inject the human cell suspension into mouse tail vein.
   NOTE: Tail vein injections may take several attempts, especially for a novice animal handler. Kovacscics and Raper's JoVE video[19] provides detailed demonstration of this animal technique.
8. Monitor mouse's recovery for ~5 min. Record any adverse events during the injection (*e.g.* spilled cells, major hemorrhage, excessive tail trauma).

3. **Analysis of human cell chimerism in mouse peripheral blood**
   1. Obtain the blood cell pellet remaining in microtainer tube after plasma separation (step 5.2.3).
   2. For red blood cell (RBC) lysis, resuspend the remaining blood pellet in a volume of PBS equal to plasma removed (to replace plasma volume) and mix well (vortex/pipette).
   3. Transfer each re-suspended blood sample to a labeled 4 mL tube and then add RBC lysis buffer to each tube in a 1:9 ratio (900 µL RBC lysis buffer for 100 µL re-suspended blood). Incubate for 5 min at RT. Centrifuge (5 min, 500 x g) and decant.
   4. Perform second RBC lysis buffer incubation (5 min at RT). Centrifuge and decant as before.
   5. Wash the remaining cells in ~ 1 mL PBS. Centrifuge and decant as before.
   6. Resuspend the pellet in a volume of PBS appropriate for standard flow cytometry staining (this varies according to manufacturer and individual lab protocols).[20] Use immunophenotyping antibodies validated for flow-cyotmetery to identify mouse cells (mouse CD45+) and human cells (human CD45+)[6,21] as well as additional human leukocyte markers specific to the cell lineage of interest.
      NOTE: It is recommended to take a small volume (5 µL-20 µL) from each mouse sample to create "pooled samples" to use for unstained, viability, and isotype control samples. It is best practice to have unstained and isotype controls for each experimental condition.

7. Functional Evaluation of *In Vivo* Cytokine Activity

1. **Mouse euthanasia and tissue harvest**
   1. Euthanize mice *via* CO_2 asphyxiation at designated experimental time point.
   2. Harvest blood *via* cardiac puncture and collect plasma as in steps 5.2.1-5.2.2.
      NOTE: Collect the blood prior to other tissues because it begins coagulating immediately after death.
   3. Process the mouse blood, aliquot and store plasma as in step 5.2.2.
      1. At a later time, assess exogenous cytokine concentration in plasma obtained at euthanasia *via* ELISA according to the manufacturer's protocol. Evaluate and compare exogenous cytokine concentrations in the mouse plasma from the serial *in vivo* blood collection and the euthanasia plasma samples (described in section 5.3).
   4. Add PBS to remaining blood sample to replace plasma volume and process as described in steps 6.3.1 and 6.3.2. Perform flow cytometry assays immediately or resuspend cells in freezing medium for LN_2 storage.
2. Harvest bone marrow (BM) and spleen from PDX mice and prepare single cell suspensions as previously described.[22]
3. Obtain cell counts for each PDX mouse tissue sample using 3% acetic acid with methylene blue (lyses cell membranes and RBCs leaving intact nuclei of living cells) in a 1:1 dilution and count cells using a hemocytometer. Tabulate total cell counts for bone marrow and spleen samples for each animal.
4. Centrifuge (500 x g, 15 min) cell suspensions and decant the supernatant. Perform flow-cytometery assays of bone marrow and/or spleen cells immediately or resuspend in freezing medium for LN_2 storage.
   NOTE: Freeze 10 BM aliquots (for future biochemical assays) and ~5 spleen aliquots (for future PDX transplants) per mouse.
5. **Immunophenotyping to identify *in vivo* functional effects**
   1. Prepare one master-mix (MM) of flow cytometry antibodies to immunophenotype hematopoietic population(s) responsive to the cytokine of interest and a second MM of isotype control antibodies (**Figure 5** and Table of Materials).
   2. Thaw PDX tissue aliquots (37 °C bead bath) prepared in step 7.4.
   3. Wash the thawed cells by transfering them to a conical tube and adding least 3x volume of PBS. Centrifuge (500 x g, 5 min) and decant the supernatant.
   4. Repeat the wash step (7.5.3).
   5. Create pooled aliquots by taking ~10 µL-20 µL cells from each PDX sample for the unstained control, viability control, and the isotype controls (see step 6.4.6 note). Stain all samples, except the unstained control, with a fixable viability dye (dead cell marker), incubate and wash as per manufacturer's protocol.
   6. Stain each PDX cell sample with phenotyping-MM according to standard flow cytometry staining protocol; similarily stain the pooled isotype-control sample(s) with the isotype-MM. Incubate all samples, wash with PBS, and fix samples by re-suspending in 1% paraformaldehyde.
   7. Collect flow cytometry data and analyze data using a gating strategy appropriate for the cell population(s) of interest (**Figure 5**). A typical gating is as follows:
      1. Define intact cells by drawing a FSC and SSC gate that excludes debris.
      2. Identify the living cells population by gating on cells that are negative for the dead cell marker (this is the "total living cells").
      3. Use sub-gates within the "total living cells" to define the cell popultions with desired immunophenotype (see Figure 5).
      4. Obtain the frequency of subset cell populations within the total living cell count from flow cytometry analysis.[6,21]
   8. Calculate the number of target cells in each tissue by multiplying the frequency of the B cell subset, within total living cells (obtained in step 7.5.6.4) by the total living cell counts per tissue obtained in step 7.3 (% B cell subset x "hemocytometer cell count"; see **Table 1**)

9. Compare the cell counts of target cell populations between control PDX mice and cytokine-expressing (TSLP+) PDX mice to determine *in vivo* functional effects of the exogenous human cytokine (**Table 1**).

Representative Results

An overview of the model is shown in **Figure 1**. Once cytokine-producing (TSLP+ stroma) and control stroma have been obtained they are expanded in culture. Prior to stroma injection into mice, stromal cell supernatant is assessed by ELISA to verify cytokine production (**Figure 2**) and phospho-flow cytometry (Protocol #3) is used to verify the activity of the cytokine produced by the stroma. Supernatant is collected from confluent stromal cell cultures (control stroma and TSLP+ stroma) when cells are passaged. ELISA is used to monitor concentration of TSLP in supernatant at least once during early, middle, and late passages. Representative data from control stroma are shown in **Figure 2A**. Control stromal cell cultures that show TSLP expression (and any vials frozen down from them) should be discarded. Control cultures with undetectable TSLP are expanded and frozen down for future use. As shown in **Figure 2B** cultures of TSLP+ stroma showing low-level TSLP production (and vials frozen down from them) are discarded. TSLP+ stroma showing stable, high-level production of the cytokine are selected for expansion and storage for use in future experiments. Average levels of TSLP in supernatant from multiple cultures of control and TSLP+ stroma are shown in **Figure 2C**.

The experimental timeline for the production of control PDX mice with human cytokine is shown in **Figure 3**. Stromal cell cultures are initiated 3 weeks prior to transplantation and ELISA assay of *in vitro* TSLP production in culture supernatant is performed as described in **Figure 2.** *In vivo* human TSLP in the mouse plasma is monitored weekly (**Figure 4**) using the "modified ELISA for micro-volumes of mouse plasma" described in Protocol #4. Peripheral blood is collected concurrently with plasma and is assayed as described in Protocol #6 under "Analysis of human cell chimerism in mouse peripheral blood." PDX injected with control stroma consistently showed plasma human TSLP levels that are below the threshold for detection (**Figure 4A**). The plasma level of TSLP in PDX mice injected with TSLP+ stroma is proportional to the number of TSLP + stromal cells injected during the preceding 1 - 2 weeks. The plasma levels of TSLP rapidly drop if stromal cell injections are discontinued.[6] As seen in **Figure 4B**, weekly injections of 0.5 x 10^6 TSLP+ stroma (producing on average > 1,000 pg/mL of TSLP in culture supernatant) gave plasma TSLP levels near the lower boundary of physiological levels (~ 5-10 pg/mL). Weekly injection of 5 x 10^6 TSLP+ stroma in PDX mice resulted in plasma TSLP levels that reach high physiological levels (~ 35 pg/mL) as shown in **Figure 4C**. Weekly assays of plasma TSLP using the modified ELISA gives results that are, in general, consistent over time (**Figure 4D**). It should be noted that this assay is modified and performed in simplicate, thus individual data points taken alone, are unlikely to be reliable indicators of plasma cytokine levels. For example in **Figure 4B** it seems unlikely that the plasma TSLP level of 60 pg/mL at week 2 for one animal is an accurate assessment, particularly given the much lower value for all other animals and in the same animal at other time points. The unmodified triplicate ELISA assay of plasma TSLP concentrations performed at euthanasia provides a valuable validation of weekly assessments.

TSLP has been shown to increase the production of normal human B cell precursors.[23] Thus, to evaluate the *in vivo* function of TSLP we compared the production of normal human B cell precursors in control and TSLP+ PDX mice transplanted with hematopoietic stem cells as shown in **Figure 5** and in **Table 1**. **Figure 5A** shows the flow cytometry gating for immunopheneotyping used to identify subsets of human B lineage cells. Sample calculations for enumerating the number of cells in each subset for one control PDX and one TSLP+ PDX are shown in **Table 1**. As shown in Figure 5B, B lineage cells are significantly increased in TSLP+ as compared to control PDX and this increase begins with the earliest B cell precursors (pro-B cells). The number of non-B lineage cells was not significantly different between control and TSLP+ PDX (data not shown). This assay provides a way of verifying that the human TSLP produced *in vivo* in TSLP+ PDX exerts *in vivo* functional effects on a target cell population.

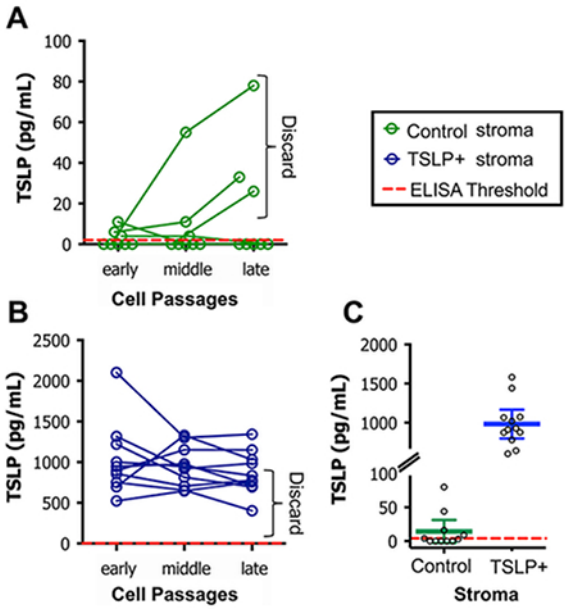

Figure 2: ELISA to Monitor TSLP Cytokine Concentrations in Stroma Supernatant. ELISA assays were used to measured TSLP concentrations in the supernatant from control stroma (transduced with control vector) and TSLP+ stroma (transduced to express human TSLP) at least once during the following cell passages: early (passage 1-5), middle (passages 6-12), and late (passages 13-20). The detection threshold for the human TSLP ELISA assay used here is 1.9 pg/mL (red dashed line). (**A**) Control stroma produce minimally detectable human TSLP concentrations; these levels are generally below the ELISA detection threshold for the duration of the experiment. Control stroma in culture that show increasing TSLP concentrations (>15 pg/mL) are discarded along with all aliquots frozen from that culture. (**B**) Human TSLP production varies between cultures generated from different thawed vials of TSLP+ stroma (*n* = 9) and throughout the culture period. TSLP stroma that show decreased or unstable cytokine production (TSLP <1,000 pg/mL) are also discarded. (**C**) Average TSLP concentrations for control (green) and TSLP+ (blue) stroma (means and 95% confidence intervals). Please click here to view a larger version of this figure.

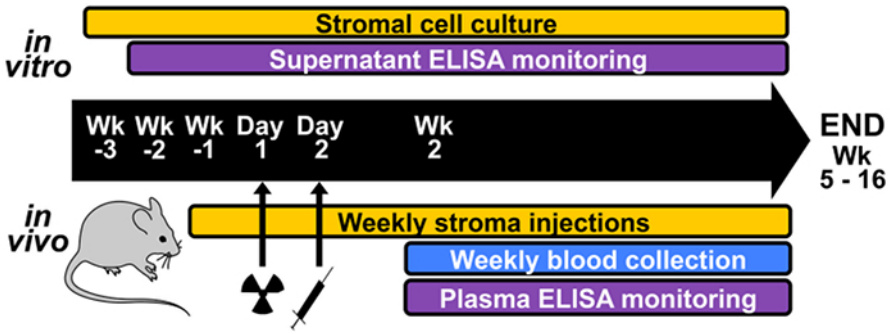

Figure 3: Experimental Timeline for Generating PDX Mice with Exogenous Human Cytokine Production. *In vitro* and *in vivo* portions of the experiment progress concurrently. Control and +TSLP stroma cell culture is initiated 2-3 weeks prior to hematopoietic cell transplant, which ensures a minimum of 3 cell passages prior to the first (at Wk -1) of the weekly stromal cell injections. This ensures that the stromal cells are healthy, proliferative and producing adequate cytokine levels. Supernatant aliquots are collected when cells are passaged and ELISA assays are used to determine TSLP concentration (see **Figure 2**) in the stroma supernatant. Animals are irradiated at day 0, one day prior to human hematopoietic cell transplant on day 1. Weekly blood collection begins 1 to 2 weeks after first stroma injections. At this time, exogenous human cytokine concentrations should be detectable in the mouse plasma using modified ELISA assays (**Figure 4**). The experiment endpoint is 5-16 weeks after human cell transplant; this depends on the amount of human cell chimerism detected in mouse peripheral blood. Normal hematopoiesis is typically well established by week 5-7, leukemia cell chimerism varies between cell lines and primary samples (3-16 weeks). PDX transplant success and progression also depend on the number of human cells injected at transplant. Please click here to view a larger version of this figure.

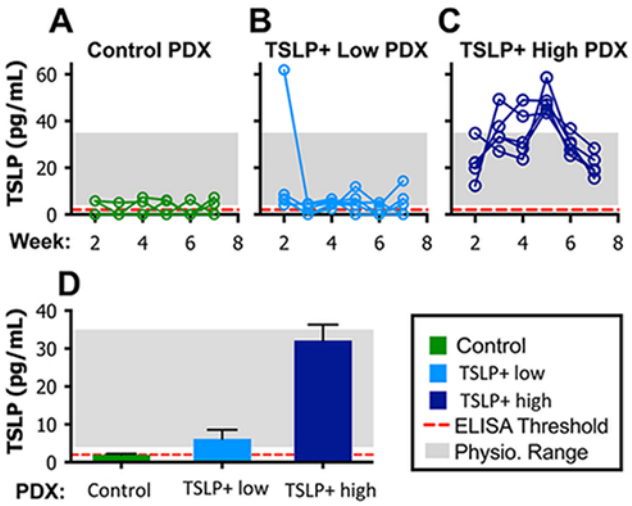

Figure 4: Achieving Physiological Human TSLP Cytokine Concentrations in Mouse Plasma. Mice receive weekly intraperitoneal injections (200 µL) of transduced cells and their plasma is collected to evaluate human TSLP concentrations (ELISA assay) *in vivo* in the mice over time. **"**Control" mice received 5 x 10^6 control stroma cells, while "TSLP+ low" (low TSLP stroma dose) mice received 0.5 x 10^6 TSLP+ stroma cells, and "TSLP+ high" (high TSLP stroma dose) mice received 5 x 10^6 TSLP+ stromal cells each week. The human TSLP ELISA assay detection threshold is 1.9 pg/mL (red dashed line) and the human physiological range of TSLP is ~5 to 35 pg/mL (grey shading).[24] (Reference 11 and Coats unpublished data) (**A**) "Control" mouse plasma consistently has TSLP levels <5 pg/mL or below the ELISA detection threshold. (**B**) "TSLP + low" mouse plasma shows low physiological levels of TSLP; whereas (**C**) "TSLP+ high" mouse plasma shows high physiological levels. (**D**) Average TSLP concentrations for each experimental group (mean ± SEM of all mice and timepoints) show that TSLP levels detected in mouse plasma is proportional to stroma dose received; control mouse plasma levels are below the ELISA detection threshold and "TSLP+ high" plasma levels are four fold greater than the "TSLP+ low" mouse plasma levels. Please click here to view a larger version of this figure.

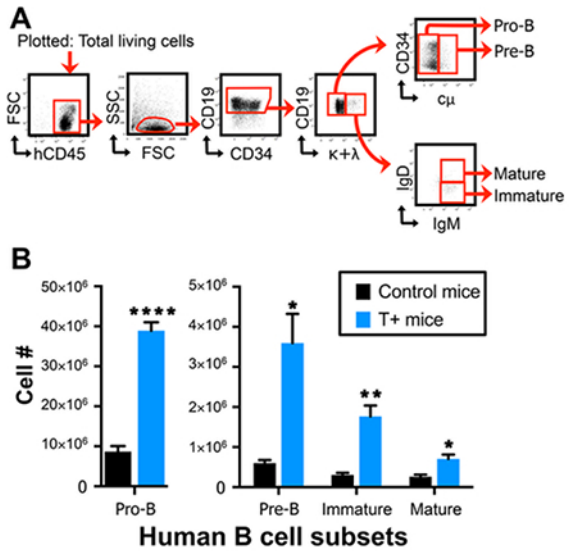

Figure 5: Assay of Normal Human B Cell Populations Validate *In Vivo* Functional Effects of Exogenous Human TSLP in PDX Mice. PDX mice were engineered with control and TSLP+ stroma and transplanted with human CD34+ cells isolated from umbilical cord blood. Immuno-phenotyping by flow cytometry was used to identify subsets of human B cell precursors in bone marrow harvested from control and TSLP+ PDX as follows: harvested bone marrow, was thawed and stained with fixable viability dye, and stained for flow cytometry to detect anti-human CD45, CD19, CD34, *κ & λ* light chain, and either surface IgD and IgM or intracellular IgM (cμ). (**A**) Total living cells were gated based on a viability marker. Successive plots show subsequent gates to identify developmentally sequential B lineage subsets (data shown is from TSLP+ PDX). (**B**) Frequency of each subset within total living cells was determined by flow cytometry software and numbers of cells in each B cell subset were calculated. (See Table 1). Cell counts for each B cell subset in control (black, *n* = 3) and TSLP+ (blue, *n* = 5) PDX mice are graphed. (mean ± SEM, **p* <0.05, ***p* <0.01, *****p* <0.0001). Please click here to view a larger version of this figure.

Human Cell Population	Freq. of Total Living Cells	Living Cell Count in BM	# Cells in Population
Control Mouse			
Total living cells	100.00%	39.5×10^6	39.5×10^6
hCD45+	98.50%	39.5×10^6	38.9×10^6
Total CD19+	27.20%	39.5×10^6	10.7×10^6
Pro-B	24.20%	39.5×10^6	9.56×10^6
Pre-B	1.70%	39.5×10^6	0.68×10^6
Immature B	0.83%	39.5×10^6	0.33×10^6
Mature B	0.68%	39.5×10^6	0.27×10^6
TSLP+ Mouse			
Total living cells	100.00%	72.0×10^6	72.0×10^6
hCD45+	99.30%	72.0×10^6	71.5×10^6
Total CD19+	65.10%	72.0×10^6	46.8×10^6
Pro-B	60.20%	72.0×10^6	43.3×10^6
Pre-B	2.70%	72.0×10^6	1.92×10^6
Immature B	1.60%	72.0×10^6	0.11×10^6
Mature B	0.40%	72.0×10^6	0.29×10^6

Table 1: Frequency and Counts of Normal Human B Cell Subsets in PDX Bone Marrow. The frequency (%) of each B cell subset within the total living cells was determined by flow cytometry analysis (see Figure 5A for gating strategy). Representative bone marrow (BM) data from one control PDX mouse (received control stroma injections, 5×10^6 cells/week) and one TSLP+ PDX mouse (received TSLP stroma injections, 5×10^6 cells/week). The total living BM cell counts were recorded at euthanasia and immune-phenotyping flow cytometry was used to assess subset frequency (%) and calculate the size of each B cell subset population (subset cell count = "total living cells" x "subset frequency").

Discussion

This manuscript describes a simple, quick, and relatively cost effective method for engineering PDX to express exogenous human cytokine. The strategy described here is based on weekly intraperitoneal injections of a stromal cell line transduced to express the human cytokine, TSLP. Prior to performing the methods described here, stroma engineered to express high levels of the cytokine of interest (TSLP) and similarly engineered control stroma were generated. In the protocols presented here, stroma are expanded in culture and screened for the ability to provide stable, high level cytokine production over time (or for the absence of cytokine production in the case of control stroma). Assays to verify the activity of stroma-generated cytokine were performed and experimental timelines for stromal cell injection were developed to produce PDX with physiological human TSLP (and control mice that lack human TSLP). Procedures for monitoring plasma levels of human TSLP and for verifying its *in vivo* functional effects on cytokine responsive cell populations were performed.

Several factors should be considered in selecting stromal cells and vectors that will be used in the protocols presented here. Stromal cell lines should not endogenously produce high levels of cytokine, and should not be responsive to the cytokine of interest. For studies here, the human bone marrow stromal cell line, HS-27A, was selected because it grows robustly in culture with low-level cytokine production.[8] The method described here also includes "control" PDX engineered with the same stroma as TSLP+ PDX, but without overexpression of TSLP. Comparisons of results in TSLP+ and control PDX allow us to control for any effects due to endogenous stromal cell cytokine production. Transduction of stromal cells with vectors that include fluorescent proteins or other selectable markers can be helpful for isolating cells that are likely to overexpress the cytokine of interest. However, it is essential to assay cytokine supernatant to determine the level of cytokine produced by the stromal cells because the *in vivo* plasma cytokine levels in mice correlate with the cytokine concentration in stromal cell supernatant,[6] as well as the number of stroma injected on a weekly basis (**Figure 4**).

It is important that the activity of the cytokine produced by stroma is verified prior to PDX studies. We used phospho-flow cytometry to assay for molecules phosphorylated downstream of TSLP stimulation in cells that are responsive to TSLP. TSLP activates the JAK-STAT5 and PI3/AKT/mTOR pathways. The MUTZ5 and MHH-CALL4 leukemia cell lines express high levels of the TSLP receptor components and show downstream STAT5, AKT and ribosomal protein S6 phosphorylation following TSLP stimulation.[14] Here we used phopho-flow cytometry to evaluate phosphorylation of STAT5 (pSTAT5) induced downstream of TSLP stimulation. In previous work we assessed for additional TSLP-stimulated phosphorylation events: phospho-AKT (pAKT) and phospho-ribosomal protein S6 (pS6, downstream of mTOR). Results showed that the pAKT assay is less sensitive and the pS6 more sensitive than the pSTAT5 assay.[6] When phospho-assays are performed, responsive cells should be cultured with saturating levels of recombinant human TSLP as a positive control and with media only (no cytokine) as a negative control. Supernatant from control stroma should be assayed along side that from TSLP+ stroma as a second means of verifying (in addition to ELISA) that TSLP is not produced by control stroma. This also serves as a control to assure that endogenous cytokine production is not responsible for the phosphorylation observed in assays of TSLP+ cells. Ideally phosphorylation induced from cytokine-producing stroma samples should be similar to that observe with recombinant cytokine condition, although it may be lower if the concentration of cytokine in the supernatant

is lower than in the saturating, positive control. A positive control with recombinant TSLP levels that match the levels observed by ELISA for TSLP+ stroma are a good alternative.

Identifying known functional indicators of *in vivo* cytokine activity can be a challenge. Assays of phosphorylation may not be possible because phosphorylation induced by the exogenous human cytokine *in vivo* can be rapidly lost during tissue harvest. Since TSLP has been shown to increase the production of human B cell precursors,[2,3] the functional effect of TSLP in PDX was verified by assaying for *in vivo* increases in the human B cell precursor population using flow cytometry immunophenotyping. An alternative strategy could be assaying for specific cytokine-induced changes in gene expression and comparing expression in cells harvested from cytokine-expressing PDX to cells from control PDX.[6]

The ultimate goal of human cytokine expression in PDX is to generate a preclinical model that more closely models the *in vivo* environment present in patients. This was tested by comparing whole genome expression in human tissues isolated from control PDX and from cytokine-expressing (TSLP+) PDX to the original patient samples. These studies showed that gene expression in patient samples is significantly closer to leukemia cells from TSLP+ PDX than controls.[6] However, our studies focused on lymphopoiesis. A number of myeloid-promoting cytokines produced in the mouse do not activate their human receptor counterparts. There are human cytokine knockin mice (NSGS mice and others) that address this issue. Indeed, one value of the model we describe here is that it can be used in NSGS mice to create a model that more closely models the human *in vivo* environment by expressing TSLP in addition to myeloid-promoting human cytokines. On the other hand, a cytokine +/- system could be generated in NSG mice using the method described here for each of these myeloid-promoting cytokines. This model may be used to study the precise *in vivo* role of each of them in myelopoieis and/or myeloid leukemia.

Engineered PDX that produce physiological levels of human TSLP provides a preclinical model that more closely mimics the *in vivo* environment present in patients than classic PDX.[4] The production of control PDX mice that are similarly engineered, are also described. Together control and cytokine-producing PDX create a human TSLP+/- model system that we have successfully used to evaluate the role of TSLP in the *in vivo* production of normal and malignant human B cells.[4,6] This model is highly relevant to studies of a particular high risk form of B-cell acute lymphoblastic leukemia (B-ALL) that is characterized by genetic alterations leading to overexpression of CRLF2. CRLF2 is a receptor for the TSLP cytokine and thus TSLP-induced CRLF2 signaling likely contributes to oncogenesis and progression of CRLF2+ B-ALL. The use of PDX to study this disease is particularly critical because PDX allow us to study leukemia in context of the patient's genetic landscape. Genetic landscape as a disease contributor is strongly implicated in CRLF2+ B-ALL, which occurs five times more often in Hispanic/Latino children than others and makes up half of all B-ALL cases in Down Syndrome patients. PDX models expressing human TSLP such as the one describe here will be an important tool in identifying therapies and understanding disease mechanisms of CRLF2+ B-ALL as well as for understanding normal hematopoiesis.

Disclosures

The authors have nothing to disclose.

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