Previously, we identified a therapy-resistant role of IL-34 in an immune checkpoint blockade in murine models. To investigate whether a similar mechanism is applicable in human tumors as well, we used this protocol for the selection of IL-34-neutralizing antibody and transplanting human tumor tissue expressing both IL-34 and PD-L1 as a patient-derived xenograft in immunologically humanized mice. This model helps to determine the effect of IL-34 neutralization along with the immune checkpoint blockade in human tumors.
STAR Protocols

Protocol
An optimized protocol for patient-derived xenograft in humanized mice to evaluate the role of IL-34 in immunotherapeutic resistance

Nanumi Han,1,3 Hye Yoon Jang,2 Naoki Hama,1 Takuto Kobayashi,1 Ryo Otsuka,1 Haruka Wada,1 and Ken-ichiro Seino1,4,*

1Division of Immunobiology, Institute for Genetic Medicine, Hokkaido University, Kita-15 Nishi-7, Sapporo 060-0815, Japan
2DNA Link, Inc., Seoul National University College of Medicine, Biomedical Science Building 117, 103 Daehakro, Jongro-gu, Seoul 03080, South Korea
3Technical contact
4Lead contact
*Correspondence: seino@igm.hokudai.ac.jp
https://doi.org/10.1016/j.xpro.2021.100460

SUMMARY
Previously, we identified a therapy-resistant role of IL-34 in an immune checkpoint blockade in murine models. To investigate whether a similar mechanism is applicable in human tumors as well, we used this protocol for the selection of IL-34-neutralizing antibody and transplanting human tumor tissue expressing both IL-34 and PD-L1 as a patient-derived xenograft in immunologically humanized mice. This model helps to determine the effect of IL-34 neutralization along with the immune checkpoint blockade in human tumors.

For complete details on the use and execution of this protocol, please refer to Hama et al. (2020).

BEFORE YOU BEGIN
Figure 1 illustrates the selection procedure of how we chose a clone of anti-human IL-34 monoclonal antibody for tumor treatment. The procedure consists of three steps: isolation of peripheral blood mononuclear cells (PBMCs), collecting CD14+ cells via magnetic separation, and culturing the isolated CD14+ cells with recombinant human IL-34 (rhIL-34) and the above-mentioned antibody.

Note: All steps to be performed under sterile conditions.

Isolation of PBMCs from healthy volunteers

© Timing: 1 h

1. All experiments should be performed with the approval of the Institutional Ethics Committee.
2. Prepare 15 mL Histopaque®-1077 in a sterile 50 mL conical centrifuge tube for collecting white blood cells according to the manufacturer’s protocol. Coat the inside of the blood-collecting tube with heparin to prevent coagulation.
3. Collect 35 mL of venous blood using a 23-gauge needle.
4. Carefully layer the collected blood onto the Histopaque®-1077 solution (Figure 1A).

Note: Do not mix the two layers since the purity would deteriorate upon mixing. To avoid mixing, slowly release the blood from the pipette.
A. Isolation of peripheral blood mononuclear cells

- Slowly and Touching tube wall
- Centrifuge
- Plasma
- PBMCs (Cloudy layer)
- Histopaque®-1077
- RBCs
- Remove plasma
- Collect PBMCs

B. Collecting CD14 positive cells using MACS

- Anti-CD14 Microbeads
- Binding CD14 and Microbeads
- Column
- Collecting CD14 positive cells

C. Culture the cells with recombinant IL-34 and antibody

- Collected cells
- NS
- IL-34
- IL-34 (Anti-Human IL-34 antibody)
- Observation
- 5 days
- Proper inhibitor
- Improper inhibitor
- In vivo experiment

D. Non-stimulated vs. Stimulated with Recombinant IL-34 (100 ng/mL) and E0330E8 (10 µg/mL)

- Non-stimulated
- Recombinant IL-34 (100 ng/mL)
- Recombinant IL-34 (100 ng/mL) + E0330E8 (10 µg/mL)
5. Centrifuge at 400 x g at 22°C–26°C for 30 min.

▲ CRITICAL: Acceleration and deceleration of the centrifuge should be set at a low speed to prevent mixing of the layers. For example, in the TOMY AX-310 centrifuge, it takes 1 min 31 s to reach 400 x g and 1 min 38 s to stop under the “slow” setting. On the contrary, it takes 24 s to reach 400 x g and 25 s to stop under the general (“fast”) setting.

6. Carefully take the tube out of the centrifuge. There should be four distinct layers; namely, plasma, PBMCs (cloudy), Histopaque®-1077, and red blood cells (RBCs) (top to bottom) (Figure 1A).

7. Discard the top layer slowly using a 10 mL serological pipette (Figure 1A).

8. Carefully harvest the cloudy second layer (PBMCs) with a P1000 pipette and transfer to a fresh 50 mL conical tube (Figure 1A).

9. Wash the PBMCs with 10 mL PBS and centrifuge at 250 x g at 22°C–26°C for 10 min. You can now set high speed for acceleration and deceleration.

10. Discard the supernatant and add 5 mL PBS to wash the pelleted cells. Centrifuge the tube at 250 x g at 22°C–26°C for 10 min.

11. Resuspend the PBMCs in 0.5 mL PBS and count the cells.

Collecting CD14+ cells from the isolated PBMCs using MACS kit

© Timing: 1 h

12. Centrifuge whole PBMCs at 300 x g at 4°C for 10 min.

13. Aspirate the supernatant and resuspend the pellet in 80 µL of MACS buffer per 10^7 cells. Further, add 20 µL of CD14 MicroBeads per 10^7 cells (Figure 1B).

14. Mix and incubate the tube at 4°C for 15 min.

15. Add 1 mL of MACS buffer per 10^7 cells and centrifuge at 300 x g at 4°C for 10 min. This is the washing step.

16. Aspirate the supernatant and resuspend the pellet in 500 µL of MACS buffer.

17. Place the column in a magnetic field and rinse with 500 µL of MACS buffer (Figure 1B).

Note: The column and magnet are included in the Mini & MidiMACSTM Starting Kit.

18. Apply cell suspension onto the column such that the magnetic bead-bound CD14+ cells adhere to the magnetic field (Figure 1B).

19. Wash with 500 µL of MACS buffer thrice to discard the flow-through.

20. Place a 1.5 mL microcentrifuge tube or 15 mL conical tube below the column and remove the column from the magnetic field (Figure 1B).

21. Add 1 mL of MACS buffer to the column and collect the isolated CD14+ cells pushing plunger of the Mini & MidiMACSTM Starting kit.

22. Count the cells.

Note: 2–5 x 10^6 CD14+ cells from 2 x 10^7 PBMCs (20 mL of blood) can be expected.
Alternatives: It is possible to collect CD14+ cells using flow cytometry if you use fluorescent-labeled anti-human CD14 antibody (Boiers et al., 2018).

Culturing CD14+ cells with rIL-34 and anti-IL-34 antibody

☑ Timing: 30 min

23. Centrifuge the isolated CD14+ cells at 300 g at 4°C for 10 min. Resuspend the pelleted cells in RPMI-1640 medium containing 10% FBS, 1% penicillin/streptomycin (10,000 U/mL, 1 x), and 1% MEM non-essential amino acid solution (1 x).

24. Seed 4 x 10^6 cells in 2 mL per well in a 6-well plate and add 100 ng/mL of rIL-34 (Figure 1C). The rIL-34 can be stored at −20°C for up to three months.

25. Add 10 μg/mL (1:50 dilution) of anti-human IL-34 antibodies (E033B8 and E0320E7, BioLegend). Do not add either antibodies to the control well (Figure 1C). The storage temperature for antibodies is 4°C.

26. Culture the cells in an incubator set in 37°C with 5% CO2 for five days with no media-change. Observe with inverted microscope every day for any changes in cell morphology.

27. Select the antibody that inhibits differentiation the most.

Note: The rIL-34 significantly changed the cell morphology in the present study (Figure 1D). However, the addition of E0330E8 or E0320E7 to the rIL-34-treated cells showed reduced change in morphology, almost similar to that in the non-stimulated group. Among them, many dead cells (dull, non-refractile, round) were observed in the E0330E8 group, which was not the case with the E0320E7 group (Figure 1D). Therefore, we selected E0320E7 as the IL-34 inhibitor in the following in vivo experiment.

Determination of IL-34-limitation frequency in mouse immunotherapy model

☑ Timing: 1 h (tumor challenge), 30 min (injection antibody), 20 min (observation)

This part describes how we determined the frequency of anti-IL-34 antibody treatment in immunotherapy using a murine tumor model and anti-mouse IL-34 antibody.

28. All animal experiments must be performed with the approval of an appropriate Animal Care Committee.

29. We developed a mouse tumor model using CT26, a colon cancer cell line. Harvest the cells and prepare them for injecting as follows:
   a. Remove RPMI-1640 media from the dish (10 cm diameter) culturing CT26 cells.
   b. Wash the cells with 10 mL of PBS (1 x).
   c. Add 1 mL of trypsin EDTA and incubate at 37°C for approximately 5 min to detach the adherent cells. The trypsin EDTA can be stored at 4°C for up to one month.
   d. After detaching the cells, add 5–10 mL of RPMI-1640 media to inactivate trypsin. Collect the detached cells and centrifuge at 440 g at 4°C for 5 min.
   e. Discard the supernatant and resuspend the pelleted cells in 1 mL of RPMI-1640 media.
   f. Count the cells and prepare a suspension of 2 x 10^5 cells per tube. Please note that the tubes should be prepared according to the number of mice enrolled in the study.

30. After preparing the cells for injection, remove hair from the right flank of the six to eight-week-old wild-type female BALB/c mice using an electric clipper for subcutaneous injection.

31. Remove the supernatant from the tubes using a pipette. Add ice-cold 10 μL of Matrigel® matrix and mix well using a pipette while the tubes are on ice. The storage temperature of Matrigel® matrix is −20°C.
CRITICAL: Matrigel® matrix should be added when on ice and the mixing step should be performed quickly since the matrix gels at 22°C–26°C.

32. Fill a 30-gauge syringe with the Matrigel®–cell suspension and inject subcutaneously into the right flank of the mouse (day 0).

33. Measure tumor growths with a caliper and inject antibodies (anti-mouse CTLA-4: 250 μg (12.5 mg/kg), anti-mouse IL-34: 200 μg (10 mg/kg) per mouse) intraperitoneally following the indicated schedule (Figure 2) from day 5 (The antibodies are stored at 4°C).

a. Calculation: Tumor size (mm³) = (length × width × height)

Note: Generally, the size of tumors reaches over 5 mm in diameter on day 5.

Note: In this experiment, the results of four groups were compared to determine the frequency of anti-IL-34 antibody treatment. The experimental group with three times treatment
Selection of IL-34- and PD-L1-expressing human tumor tissues from an online database for patient-derived xenograft (PDX) model

© Timing: 30 min

34. We searched online databases to retrieve IL-34-expressing human tumor tissues. Consequently, we found some tumors that expressed IL-34 in the DNA Link, Inc. repository (Figure 3A, PDX Link [https://www.dnalink.com/pdx-about.html]). Please note that various cancer types are available on the homepage of this database (Figure 3A).

Note: The PDX models were generated from various types of patients who underwent surgery as primary treatment at Samsung Medical Center (Seoul, Korea) and were cryopreserved in liquid nitrogen.

The database provides information on gene mutation, expression, and copy number variation of the tumors.

35. Among the IL-34-expressing tumors, we found several tumors that co-expressed PD-L1 (CD274) with various levels (Figure 3A).

Confirmation of IL-34 and PD-L1 protein expression in the selected tumors with immunohistochemistry

© Timing: 2 days

36. In our case, formalin-fixed paraffin-embedded (FFPE) sections of the selected tissues were provided by DNA Link, Inc.

Use normal human skin as positive control for IL-34 and the human lung squamous carcinoma cell line NCI-H226 as positive control for PD-L1. Furthermore, use purified mouse IgG1, κ isotype ctrl antibody as negative control for IL-34 and purified rabbit polyclonal isotype ctrl antibody as negative control for PD-L1.

Note: Because the database provides only transcript expression, it is necessary to check protein expression before proceeding with in vivo experiments. The method for staining PD-L1 was DAB while that for IL-34 was indirect fluorescent. We have described both these methods separately.

37. DAB staining for PD-L1
   a. Deparaffinize the tissue sections with three changes of xylene and rehydrate the slides with 100%, 90%, and 80% ethanol for 5 min each.
   b. Immerse the slides in PBS and prepare for the antigen retrieval step.

Note: There are two methods of antigen retrieval; heat-induced and enzyme-induced. In this experiment, we conducted the former one.
   i. Add 500 μL Immunosaver (antigen retrieval reagent) to 100 mL ultrapure water (1:200 dilution) and mix well.
   ii. Transfer the mix to a staining vat and immerse the sections in it.
   iii. Place the staining vat in a kettle.
c. Heat the kettle for 40 min (95°C–100°C). After it cools down to 22°C–26°C, rinse the slides with PBS for 5 min in a Coplin staining jar. While rinsing, add 3 mL of 100% Triton-X in 1 L of PBS to prepare 0.3% Triton-X–PBS (PBS–T) that will be used for the subsequent washing steps.
**Note:** Preparing 1 L of PBS–T is for soaking the slides. Depending upon your experimental size and number of sections, you can adjust (increase/decrease) the final volume of PBS–T.

d. For blocking endogenous peroxidase, use 3% H$_2$O$_2$ solution in methanol at 22°C–26°C for 10 min before blocking with serum. Add the solution to avoid drying out tissue during reaction times.

e. Apply 1–2 drops of the blocking buffer (normal horse serum, 2.5% of ImmPRESS Universal Polymer Kit) onto the sections at 22°C–26°C and incubate for 1 h. To prevent drying out, place the slides in a humidity chamber. Cover the tissue with a parafilm strip cut according to its size. During the blocking step, dilute the primary and control antibodies (1:100 dilution) with PBS–T according to the size and number of sections. In addition, prepare a 100-fold dilution of rabbit anti-human CD274 (PD-L1) antibody using PBS–T.

f. Remove the blocking buffer, apply 100 μL of the diluted primary antibody over the sections, and cover with parafilm as described in step (e). Incubate at 4°C for 8–12 h in the humidity chamber.

**Pause point:** Primary antibody reaction can be carried out for up to 12 h with samples safely kept at 4°C.

g. Immerse the slides in a Coplin staining jar containing PBS–T for 5 min for washing the antibodies. Repeat this washing step twice.

h. Apply 1–2 drops of horseradish peroxidase (HRP)-polymer (ImmPRESS Universal Polymer Kit) onto the sections, incubate at 22°C–26°C for 20 min, and cover with parafilm as described in step (e).

i. Immerse the slides in the Coplin staining jar containing PBS–T for 5 min for washing the secondary antibody; repeat this washing step twice. During or before washing, prepare 0.05% DAB–0.015% H$_2$O$_2$ in Tris-HCl substrate solution (dissolve 1 mg DAB in 5 mL Tris-HCl for DAB staining. Dispense 1 mL of the solution to each tube and add 5 μL of 3% H$_2$O$_2$ into the tube just before applying).

j. Apply the substrate solution to the sections at 22°C–26°C until the appearance of brown color. After confirmation of dye, apply hematoxylin in the undiluted solution at 22°C–26°C for 5 min.

k. Rinse the slides under running tap water for 15 min and dehydrate the tissues with 100% ethanol for 5 min. After dehydrating, immerse the slides in xylene for 5 min and coverslip using a mounting solution (Malinol).

**Pause point:** The coverslipped samples can be stored at 22°C–26°C semi-permanently.

l. Observe the tissues under a bright-field microscope (recommended magnification: 200×–400×) and assess the expression of CD274 (PD-L1).

38. Fluorescent staining for IL-34

a. Deparaffinize the tissue sections with three changes of xylene and rehydrate the slides with 100%, 90%, and 80% ethanol for 5 min each.

b. Immerse the slides in PBS and prepare for the antigen retrieval step.

**Note:** There are two methods of antigen retrieval; heat-induced and enzyme-induced. In this experiment, we conducted the former one.

i. Add 500 μL Immunosaver (antigen retrieval reagent) to 100 mL ultrapure water (1:200 dilution) and mix well.

ii. Transfer the mix to a staining vat and immerse the sections in it.

iii. Place the staining vat in a kettle.

c. Heat the kettle for 40 min (95°C–100°C). After it cools down to 22°C–26°C, rinse the slides with PBS for 5 min in a Coplin staining jar. Prepare a blocking buffer for 100 μL per slide with 5% goat serum added to PBS–T.
d. Apply 100 μL of the blocking buffer onto sections at 22°C–26°C and incubate for 1 h. To prevent drying out, place the slides in a humidity chamber. Cover the tissue with a parafilm strip cut according to its size. During the blocking step, dilute the primary and control antibodies (1:200 dilution) with PBS–T according to the size and number of sections. Prepare a 200-fold dilution of mouse anti-human IL-34 antibody using PBS–T to apply 100 μL of the dilution onto two sections.

e. Remove the blocking buffer apply 100 μL of the diluted primary antibody over the sections, and cover with parafilm as described in step (d). Incubate at 4°C for 8–12 h in the humidity chamber.

Pause point: Primary antibody reaction can be carried out for up to 12 h with samples safely kept at 4°C.

f. Immerse slides in a Coplin staining jar containing PBS–T for 5 min for washing the primary and control antibodies. Repeat this washing step twice. Prepare 1:200 dilution of Alexa Fluor 488 conjugated-anti-mouse goat IgG secondary antibody using PBS–T during or before washing step.

g. Apply the secondary antibody onto sections and cover with parafilm as described in step (d).

Note: You can choose another fluorescent dye.

h. Immerse the slides in the Coplin staining jar containing PBS–T for 5 min to wash the secondary antibody. Repeat this washing step twice. Meanwhile, prepare a solution of Hoechst diluted 1000 times using PBS–T for counterstaining.

i. Apply the Hoechst solution onto the sections at 22°C–26°C for 5 min.

j. Rinse the sections once with ultrapure water and coverslip using a mounting solution (VECTASHIELD Mounting medium).

Pause point: The coverslipped samples can be stored at 4°C for up to two days. However, there are limitations in observation because fluorescent signals are faded as days go by.

k. Observe the tissues under a fluorescent microscope and assess the expression of IL-34.

Note: In this experiment, we first selected three PDX tissues from the online database as shown in Table 1. Of them, we chose LU-TM-0007 and LU-TM-0112 for immunohistochemical staining because IL-34 expression was low in LU-CO-0003. After detecting IL-34 and PD-L1, LU-TM-0007 was finally chosen as the PDX model owing to the sufficient amounts of protein expression in it (Figure 3C).

### KEY RESOURCES TABLE

| Reagent or resource | Source | Identifier |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Biotin anti-human IL-34 (E033B8) | BioLegend | Cat#361401; RRID: AB_2563034 |
| Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Thermo Fisher Scientific | Cat#A-11001; RRID: AB_2534069 |
| ImmPRESS Polymer Reagent | Vector Laboratories | Cat#MP-7500 |

(Continued on next page)
| Reagent or resource | Source | Identifier |
|---------------------|--------|------------|
| LEAF™ Purified anti-mouse IL-34 Antibody anti-IL-34 (C054-35) | BioLegend | Cat#147201 |
| Macs beads anti-human CD14 MicroBeads | Miltenyi Biotec | Cat#130-050-201 |
| Purified Mouse IgG1, κ Isotype Ctrl Antibody | BioLegend | Cat#401401 |
| Purified Rabbit Polyclonal Isotype Ctrl Antibody | BioLegend | Cat#910801 |
| Purified anti-human CD274 (E1L3N) | CST | Cat#13684; RRID: AB_2687655 |
| Purified anti-human IL-34 (1D12) | Millipore | Cat#MABT493 |
| Purified anti-human IL-34 (E0320E7) | BioLegend | Cat#361301; RRID: AB_2563032 |
| Purified anti-human PD-1 (monoclonal) | Selleckchem | Cat#A2002; RRID: AB_2810223 |
| Purified anti-mouse CTLA-4 (UC10-4F10) | Dr. Hideo Yagita (Juntendo University, Tokyo) | N/A |

| Biological samples |
|--------------------|
| PDX tissue derived from colon cancer patient (model ID #LU-CO-0003) | DNA Link, Inc. | N/A |
| PDX tissue derived from lung cancer patient (model ID #LU-TM-0007) | DNA Link, Inc. | N/A |
| PDX tissue derived from lung cancer patient (model ID #LU-TM-0112) | DNA Link, Inc. | N/A |

| Chemicals, peptides, and recombinant proteins |
|-----------------------------------------------|
| 10% Formalin solution | Sigma | Cat#HT-5014 |
| 2.5 g/L-Trypsin/1 mmol/L-EDTA solution, with phenol red | Nacalai Tesque | Cat#32777-15 |
| 30% Hydrogen peroxide | Dojindo Laboratories | Cat#D847-00904 |
| Defined fetal bovine serum | Sigma-Aldrich | Cat#F7524 |
| Ethanol | Merck | Cat#609-0770-4 |
| Hematoxylin | Merck | Cat#131-09665 |
| Histopaque®-1077 | Merck | Cat#10771-500ML |
| Hoechst 33342 | Thermo Fisher Scientific | Cat#62249 |
| Immunosaver | Nissin | Cat#333 |
| MEM Non-Essential Amino Acid Solution (100×) | Nacalai Tesque | Cat#06344-56 |
| Malinol | Muto Pure Chemicals Co., Ltd | Cat#2009-2 |
| Matrigel® Matrix | Coming | Cat#354234 |
| Methanol | Fujifilm | Cat#13701923 |
| Normal goat serum | Fujifilm | Cat#143-06561 |
| Paraffin | Leica | Cat#39601006 |
| Penicillin-Streptomycin Mixed Solution (10,000 U/mL) | Nacalai Tesque | Cat#26253-84 |
| RPMI-1640 with L-Glutamine and Phenol Red | Fujifilm Wako Pure Chemical Industries | Cat#189-02025 |
| Recombinant human IL-34 (carrier-free) | BioLegend | Cat#77902 |
| Triton™ X-100 | Merck | Cat#X100-100ML |
| Ultrapure water | Millipore | N/A |
| VECTASHIELD Mounting medium | Vector Laboratories | Cat#H-1000 |
| Xylene | Fujifilm | Cat#8143-06561 |
| autoMACS Rinsing Solution | Miltenyi Biotec | Cat#130-091-222 |

| Experimental models: Cell lines |
|-------------------------------|
| CT26 | American Type Culture Collection (Manassas, VA, USA) | Cat#ATCC® CRL-2638™ |

| Experimental models: Organisms/strains |
|----------------------------------------|
| BALB/c | Japan SLC, Inc. | N/A |
| Humanized Mouse (NOD.Cg-Prkd<scid> Il2rg<tm1Wjl>/SzJ) | The Jackson Laboratory-JAX West | N/A |

| Others |
|--------|
| 13 gauge Trocar needle | Jeungdo | Cat#JD-S-128 |
| 15 mL Conical tube | TrueLine | Cat#TR2001 |
| 23 gauge Butterfly needle | BD vacutainer | Cat#367364 |
| 30 gauge Syringe | Nipro | Cat#08277 |
| 50 cc Syringe | Terumo | Cat#SS50-ESz |
| 50 mL Conical tube | TrueLine | Cat#TR2004 |

(Continued on next page)
MATERIALS AND EQUIPMENT

RPMI-1640 with L-Glutamine and Phenol Red Medium (store at 4°C, the maximum time for storage is 4 weeks.)

+ 10% Fetal Bovine Serum

+ 1% Penicillin/streptomycin

+ 1% MEM Non-Essential Amino Acid Solution

Note: This is used for culturing PBMCs and CT26 cells.

STEP-BY-STEP METHOD DETAILS

Implantation of tumor tissue in humanized mouse

© Timing: 40 min

We purchased humanized mice from Jackson Laboratory.

Note: Following is the description of how humanized mice were generated at the Jackson laboratory. Immunocompromised mice (NOD.Cg-Prkdc<scid> Il2rg<tm1Wjl>/SzJ, NSG mice) were engrafted human hematopoietic stem cells (HSCs) (Shultz et al., 2005). CD34+-purified HSCs from human fetal liver were purchased from Stem Express and intravenously injected into 3-week-old female NSG mice (10⁵ cells per mouse) 4 h after total body irradiation (140 cGy) using RS-2000 irradiator (Rad Source). The engraftment levels of human CD45⁺ cells were determined 12 weeks post-HSC transplantation by flow cytometric quantification of peripheral blood. Humanized NSG mice that had over 25% of human CD45⁺ cells in the peripheral blood were considered as engrafted and humanized. Twelve weeks after engraftment, mice were delivered in two shipments and raised in the animal facility of DNA Link, Inc. Immune cell component of the humanized mice was confirmed from the Jackson Laboratory at arrival (Table 2).

Note: All items must be autoclaved or sterile to maintain an environment for immunocompromised mice and prevent any infection. Using sterile surgical and protective equipment is essential.
1. Remove hair from the dorsal flank (implantation site) of the mouse with an electric clipper.

2. Place mice in the anesthesia chamber. Prepare a mixture of oxygen/isoflurane with approximately 2% isoflurane in a gas stream.

3. Prepare 100 mL of serum-free and 1% penicillin/streptomycin RPMI-1640 media.

   **Note:** Depending upon your experimental size and number of sections, you can adjust (increase/decrease) the final volume of media. The media can be stored at 4°C for up to twelve months.

4. Thaw the cryopreserved tumor tissues in a heating bath at 37°C for approximately 2 min. Mix the thawed contents with 10 mL of serum-free RPMI-1640 medium.

5. Place the tissue in a sterile cell culture dish (10 cm diameter) on ice. Add 5 mL of 1% penicillin/streptomycin RPMI-1640 medium to keep the tissue moist and prevent it from shrinking.

6. Mince the tissue into approximately 1 mm³ with a sterile blade such that the tissue pieces can be transferred to a 13-gauge trocar needle (Figure 4A).

7. Anesthetize the humanized mice by isoflurane in the anesthesia chamber.

8. After anesthesia, pierce ears with ear-punch for identification.

---

**Table 2. Details about shipment of humanized mice from the Jackson laboratory**

| Mouse ID   | hCD45* Total % | hCD19* B cell % of hCD45 | hCD3* T cells % of hCD45 | hCD33* myeloid % of hCD45 | hCD45 Total % |
|------------|----------------|---------------------------|---------------------------|---------------------------|---------------|
| First shipment |                |                           |                           |                           |               |
| 2490-001   | 42.7           | 86.1                      | 5.0                       | 4.1                       | 57.3          |
| 2490-002   | 57.4           | 80.8                      | 9.0                       | 4.7                       | 42.6          |
| 2490-003   | 49.7           | 85.9                      | 6.0                       | 4.1                       | 50.3          |
| 2490-004   | 40.4           | 71.1                      | 18.0                      | 4.3                       | 59.6          |
| 2490-005   | 57.4           | 80.1                      | 6.0                       | 7.9                       | 42.6          |
| 2490-011   | 47.9           | 89.0                      | 3.0                       | 3.2                       | 52.1          |
| 2490-012   | 57.6           | 81.1                      | 3.0                       | 6.5                       | 42.4          |
| 2490-013   | 73.1           | 65.6                      | 22.0                      | 2.2                       | 26.9          |
| 2490-014   | 47.7           | 81.9                      | 10.0                      | 4.0                       | 52.3          |
| 2490-015   | 54.1           | 86.1                      | 4.0                       | 4.2                       | 45.9          |
| Average    | 52.8           | 80.77                     | 8.6                       | 4.52                      | 47.2          |
| Deviation  | 8.50           | 6.58                      | 5.88                      | 1.45                      | 8.50          |

| Mouse ID   | hCD45* Total % | hCD19* B cell % of hCD45 | hCD3* T cells % of hCD45 | hCD33* myeloid % of hCD45 | hCD45 Total % |
|------------|----------------|---------------------------|---------------------------|---------------------------|---------------|
| Second shipment |                |                           |                           |                           |               |
| 5797-006   | 45.9           | 82.3                      | 10.0                      | 5.1                       | 54.1          |
| 5797-007   | 50.2           | 85.5                      | 8.0                       | 4.3                       | 49.8          |
| 5797-009   | 39.0           | 89.6                      | 4.0                       | 3.9                       | 61.0          |
| 5797-010   | 49.8           | 83.2                      | 10.0                      | 4.4                       | 50.2          |
| 5797-011   | 43.5           | 90.2                      | 4.0                       | 3.2                       | 56.5          |
| 5797-013   | 43.3           | 87.2                      | 7.0                       | 4.2                       | 56.7          |
| 5797-016   | 50.7           | 88.4                      | 6.0                       | 4.1                       | 49.3          |
| 5797-017   | 52.9           | 88.6                      | 6.0                       | 3.6                       | 47.1          |
| 5797-018   | 66.8           | 79.5                      | 8.0                       | 10.3                      | 33.2          |
| 5797-019   | 58.1           | 89.7                      | 4.0                       | 4.3                       | 41.9          |
| Average    | 50.02          | 86.42                     | 6.7                       | 4.74                      | 49.98         |
| Deviation  | 7.27           | 3.31                      | 2.09                      | 1.82                      | 7.27          |
9. Place the minced tumor pieces in the syringe of the 13-gauge trocar needle using a pair of small iris forceps (Figure 4B).

10. Sterilize the entire shaved area with 70% ethanol to the extent that the hair around it gets wet.

11. Insert the trocar needle subcutaneously 1.5 to 2 cm into the mouse flank using a pair of large straight forceps (Figure 4C).

△ CRITICAL: The trocar needle should be inserted in one direction smoothly. If you move the needle during insertion, the hemisphere shape is likely to be scattered.

12. Push the plunger of the trocar needle to engraft the tumor fragments and slowly pull out the needle. After this, hold the skin with a pair of large straight forceps so that the tumor pieces placed in “proper position” (Figure 4D). The proper position is the point of 1.5 cm ahead from the insertion of the needle. Make the tissue approximately 0.5 cm diameter to prevent it from scattering.
13. Disinfect the skin of the tumor-implanted site with 70% ethanol transfer the mouse to a warm pad, and observe until the mouse wakes up from anesthesia (Figure 4E).
14. Measure the size of tumor volume using caliper after 2 weeks of engraftment, and passage the tumor to another mouse when tumor volume reaches about 500 mm³. Repeat steps 1 to 13 for passaging a tumor.
15. Engraft the PDX tumor to 14 mice by following steps 6 to 11, and make groups for antibody treatment in two weeks.

**Anti-IL-34 antibody and anti-PD-1 antibody treatment in PDX model**

**Timing:** 4 weeks

16. Begin antibody treatment when the passaged tumor volumes reach 70–120 mm³ (Figure 4F).
   a. Intraperitoneally inject 12.5 mg/kg (250 µg) of anti-human IL-34 antibody (E0320E7, Bio-Legend; as mentioned above) and combination of anti-IL-34 antibody and anti-PD-1 antibody to the respective groups three times a week for 4 weeks.
   b. Further, intraperitoneally inject 10 mg/kg (approximately 200 µg) of the anti-PD-1 antibody as the first dose and inject 5 mg/kg every 5 days until the study endpoint.
   c. In addition, intraperitoneally inject saline twice a week to the control group until the study endpoint. The volume is the same as the antibody solution.
17. Measure the tumor size with a caliper twice a week until the endpoint.
   a. Calculation: Tumor size (mm³) = (length × width²) / 2
18. Collect the tumors with resection at day 28 after transplantation.

**Sample preparation for histopathological analysis**

**Timing:** 2 days

19. After the endpoint, dissect tumor tissues and fix in 10% formalin solution for 12 to 24 h at 22°C–26°C for histological analysis. Fixation volume should be 5 to 10 times of the tissue volume.
20. Wash the sample with PBS once for 30 min.
21. Dehydrate the sample as follows:
   a. Soak in 70% ethanol two times at 22°C–26°C for an hour each.
   b. Soak in 90% ethanol one time at 22°C–26°C for an hour.
   c. Soak in 95% ethanol one time at 22°C–26°C for an hour.
   d. Soak in 99% ethanol one time at 22°C–26°C for an hour.
   e. Soak in 100% ethanol three times at 22°C–26°C for an hour each.
   f. Soak into xylene three times at 22°C–26°C for an hour.
   g. Infiltrate with 60°C paraffin four times for an hour.
22. Embed the tissues into paraffin blocks.
23. Trim the paraffin blocks and cut at 4 µm using a microtome and blade. We skip the detailed description of the sectioning step in this protocol.
24. Place the paraffin ribbon in a water bath at approximately 40°C–45°C and mount the sections onto individual slides.
25. Air dry the slides for 30 min and place them in an oven at 45°C–50°C for 8–12 h.

**EXPECTED OUTCOMES**

It takes over 2 weeks until the passage following tumor implantation. Further, it takes another 2 weeks for preparing groups for the antibody treatment. Overall, it takes over a month until the first antibody injection.
The tumor size and body weight should be measured at least twice a week until the endpoint. The survival result and body weight are measures to assess the toxicity of the drug treatment. Therefore, it is recommended to check the conditions of the mice every day.

In this experiment, every treatment group [single or combination (anti-human PD-1 and/or anti-human IL-34)] included three mice, where one mouse was the non-treated control (Figures 4F and 5B). The combination treatment tended to show an anti-tumor effect. Each collected sample was used for pathological examination including estimation of the immune cell infiltration after the treatment. One tumor with severe necrosis in the combination group was hard to be collected, thereby rendering it impossible to further analyze the infiltration of immune cells (Figure 5B). We considered that the necrosis occurred due to an immune response caused by the combination treatment, not by a transplant rejection, since every engraftment before the treatment was stable. We, therefore, consider that five or more (if possible) mice should be included in the study to observe a significant difference.

LIMITATIONS
Origin of the cancer tissue (the organ type) could be a limitation for the generation of PDX model. Although we could successfully generate a PDX model using human lung cancer tissues in this study, it is known that some other cancer tissues, such as those from the breast or ovary, are relatively difficult for establishing PDX. It is important to appropriately select tissues that express molecule(s) you are interested in.

TROUBLESHOOTING
Problem 1
A mouse does not wake after the implantation experiment (step 13).
Potential solution
Adjust the concentration of isoflurane. Confirm the media is serum free and the warm pad is working.

Problem 2
No tumors formed (step 14).

Potential solution
Ensure that the tumor tissue is fresh and confirm the necrotic areas of the tumor. If necrotic areas are found, scrape out or cut the areas.

Problem 3
The engrafted tissues are not similar in size before treatment (step 16).

Potential solution
Ensure engrafted tissues are gathering in one place without scattering in step 12.

Problem 4
A mouse has suddenly died in the middle of the treatment (step 17).

Potential solution
There is a possibility to injure organs in the injection step. Confirm that the injection site is correct.

Problem 5
There is a tendency but no clear treatment effect (step 17).

Potential solution
If there is no change in parameters such as survival and body weight, adjust the dose and concentration at which the effect occurs.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ken-ichiro Seino (seino@igm.hokudai.ac.jp).

Materials availability
This study did not generate any new unique reagents.

Data and code availability
This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS
This work was supported in part by the Japan Agency for Medical Research and Development (AMED); Practical Research for Innovative Cancer Control (K.S.). We would like to thank Editage (www.editage.com) for English language editing.

AUTHOR CONTRIBUTIONS
K.S. designed the study. N. Han, H.J., N. Hama, and T.K. performed the experiments. All authors analyzed data and discussed the results. N. Han, N. Hama, T.K., R.O., H.W., and K.S. contributed to manuscript preparation. All authors approved the final version of this manuscript.
DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES

Hama, N., Kobayashi, T., Han, N., Kitagawa, F., Kajihara, N., Otsuka, R., Wada, W., Lee, K.Y., Rheee, H., Hasegawa, Y., et al. (2020). Interleukin-34 limits the therapeutic effects of immune checkpoint blockade. iScience 23, 101584.

Boiers, C., Richardson, S.E., Laycock, E., Zriwil, A., Turati, V.A., Brown, J., Wray, J.P., Wang, D., James, C., Herrero, J., et al. (2018). A human IPS model implicates embryonic B-myeloid fate restriction as developmental susceptibility to B acute lymphoblastic leukemia-associated ETV6-RUNX1. Dev. Cell 44, 362–377.

Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., Mangada, J., et al. (2005). Human lymphoid and myeloid cell development in NOD/LtSz-scid Il2Rγc null mice engrafted with mobilized human 131 hemopoietic stem cells. J. Immunol. 174, 6477–6489.