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Human killer cell immunoglobulin-like receptor, three Ig domains and long cytoplasmic tail (KIR3DL3) is expressed on natural killer (NK) cells and is a newly identified inhibitory receptor for B7 family member HERV-H LTR-associating protein 2 (HHLA2). Here, we summarize the isolation and expansion of KIR3DL3+ human NK cells, and in vitro functional characterization of in-house anti-KIR3DL3 monoclonal antibody (mAb). We also describe a human NK cell-based xenogeneic lung tumor model for testing the therapeutic activity of KIR3DL3 blockade in vivo.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol for evaluating antitumor activity of KIR3DL3 blockade in an NK cell-based xenogeneic lung tumor model

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SUMMARY
Human killer cell immunoglobulin-like receptor, three Ig domains and long cytoplasmic tail (KIR3DL3) is expressed on natural killer (NK) cells and is a newly identified inhibitory receptor for B7 family member HERV-H LTR-associating protein 2 (HHLA2). Here, we summarize the isolation and expansion of KIR3DL3+ human NK cells, and in vitro functional characterization of in-house anti-KIR3DL3 monoclonal antibody (mAb). We also describe a human NK cell-based xenogeneic lung tumor model for testing the therapeutic activity of KIR3DL3 blockade in vivo. For complete details on the use and execution of this protocol, please refer to Wei et al. (2021).

BEFORE YOU BEGIN
Our previous studies demonstrate that KIR3DL3 is a receptor for HHLA2 and that KIR3DL3-HHLA2 is an immunosuppressive pathway via inhibition on human NK and CD8 T cell cytotoxicity against HHLA2+ human tumors (Wei et al., 2021; Zang, 2017). Blockade of KIR3DL3-HHLA2 pathway promotes NK-based anti-tumor immunity in vitro and in vivo. The protocol below describes the specific methods for isolation and expansion of KIR3DL3+ NK cells, evaluation of the anti-tumor activity of KIR3DL3 blockade in vitro and in vivo using a human lung cancer line HCC827 cells transduced with a luciferase reporter enzyme (HCC827-Luc2). In addition, we provide steps to prepare and analyze samples for experiments including fluorescence-activated cell sorting (FACS), fluorescence-based flow cytometry, flow cytometry-based-cytotoxicity assay, and in vivo imaging system (IVIS). The methods developed by us enable a researcher to demonstrate the therapeutic efficacy of immune checkpoint inhibitors in human NK cell-based humanized mouse models.

Institutional permissions
Experiments described in this protocol utilize human blood samples and live mice. In this case, human peripheral blood samples from healthy donors were obtained from the New York Blood Center (New York, NY, USA). For in vivo experimental work with mice, approval from the Institutional Animal Care and Use Committee (IACUC) must be obtained prior to beginning.
experiments. In this case, all work was approved by the IACUC of Albert Einstein College of Medicine in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals.

**Thaw and culture HCC827-Luc2 target cells**

⏱️ **Timing:** 15 min for thaw, 1 week for growth

1. Thaw HCC827-Luc2 cells quickly while shaking gently by hand in 37°C water bath.
2. Wash once with HCC827 cell media to remove cryoprotectant.
   a. Dilute thawed cells to 10 mL in HCC827 cell media in a 15 mL conical tube.
   b. Centrifuge at 300 × g for 5 min at 20°C–22°C. Aspirate supernatant.
   c. Resuspend cells in 10 mL of fresh HCC827 cell media and transfer to 10 cm cell culture dish.
3. Grow cells in humidified incubator at 37°C and 5% CO₂.
4. Split cells every 2–3 days to maintain 70%–80% cell confluency.

**Note:** HCC827-Luc2 cells grow slowly. To increase the cell growth rate, replace with fresh media on day 2 after splitting if 70%–80% cell confluency is not reached.

**Isolating peripheral blood mononuclear cells (PBMCs)**

⏱️ **Timing:** 2.5 h

5. Obtain 50 mL of human buffy coat and gently mix blood with two volumes of RPMI 1640 media (1:2 ratio).
6. Isolating PBMCs by density gradient centrifugation with Ficoll-Paque™ PLUS.
   a. Gently layer 30 mL of diluted buffy coat on top of 15 mL Ficoll-Paque™ PLUS in 50 mL conical tubes. Take care not to mix the buffy coat with the density gradient medium.
   b. Centrifuge at 1,200 × g for 25 min at 20°C–22°C (acceleration and brake set to 1 and 0, respectively) to separate the PBMC fraction.
   c. Carefully remove the top plasma layer and transfer the PBMC layer between the plasma and the Ficoll layer to new 50 mL conical tubes using 25 mL disposable polystyrene serological pipets.
   d. Add RPMI 1640 media to a final volume of 50 mL and centrifuge at 500 × g for 8 min, 4°C (acceleration and brake set back to 9).
   e. Aspirate and discard the supernatant leaving the cell pellet.
   f. Resuspend the pellet in 50 mL of RPMI 1640 media for a second wash. Centrifuge at 500 × g for 8 min, 4°C.
   g. Count cells and determine viability.

**Optional:** If there is red blood cell (RBCs) contamination, remove red blood cells using RBC lysis buffer (Biolegend, see key resources table) following the manufacturer’s instructions.

**Note:** At this stage, cells are ready for downstream applications. Alternatively, freeze and store 10–30 × 10⁶ cells per 1 mL of freezing media in liquid nitrogen for later use.

⚠️ **CRITICAL:** Donors vary in the frequency of KIR3DL3⁺ NK cells (10%–40%) (Wei et al., 2021). To obtain a higher input cell number for expansion, it’s recommended to prepare at least 2 donors initially to account for donor variability and identify donors with relatively higher percentage of KIR3DL3⁺ cells.
### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Goat anti-mouse IgG PE (polyclonal antibody), 1:200 dilution | BioLegend | Cat# 405307; RRID: AB_315010 |
| Mouse IgG1 isotype (clone HKSP), used as 10 μg/mL | Leinco Technologies | Cat# 1-536; RRID: AB_2737545 |
| Anti-human CD3-APC (clone HIT3a), 1:300 dilution | BioLegend | Cat# 300312; RRID: AB_314048 |
| Anti-human CD56-FITC (clone 5.1H11), 1:200 dilution | BioLegend | Cat# 362546; RRID: AB_2565964 |
| Anti-human KIR3DL3-PE (clone 26E10), 1:300 dilution | Zang Lab | Wei et al. (2021) |
| Purified anti-human CD3 antibody (clone OKT3), used as 10 ng/mL | BioLegend | Cat# 317326; RRID: AB_11150592 |
| **Biological samples** |        |            |
| Human buffy coats from healthy donors | New York Blood Center, USA | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Trypan Blue solution, 0.4% | Corning | Cat# 25-900-CI |
| DAPI | BioLegend | Cat# 422801 |
| 7-AAD viability staining solution | eBioscience | Cat# 00-6993-50 |
| Recombinant human IL-2 | BioLegend | Cat# 589108 |
| Recombinant human IL-15 | BioLegend | Cat# 570308 |
| D-luciferin | Gold Biotechnology | Cat# LUCNA-100 |
| Ficoll-Paque™ PLUS | GE Healthcare | Cat# GE17-1440-02 |
| RBC lysis buffer (10x) | BioLegend | Cat# 420301 |
| Fetal bovine serum (FBS) | R&D | Cat# S11510 |
| RPMI 1640 medium | Corning | Cat# 10-040-CV |
| Eagle’s Minimum Essential Medium (MEM) medium | ATCC | Cat# 302003 |
| OpTmizer™ T Cell Expansion SFM | Gibco | Cat# A1048501 |
| Human AB serum | Sigma-Aldrich | Cat# H4522 |
| L-Glutamine solution (200 mM) | Corning | Cat# 25-005-CI |
| Penicillin-Streptomycin solution (100×) | Corning | Cat# 30-002-CI |
| 2-Mercaptoethanol (1000×, 55 mM) | Gibco | Cat# 21985-023 |
| Non-essential Amino Acid Solution (NEAA), 100× | HyClone | Cat# HS023801 |
| Sodium pyruvate solution (100 mM) | Corning | Cat# 25-000-CIR |
| HEPES solution (1 M) | Corning | Cat# 25-060-CI |
| Dimethyl sulfoxide (DMSO) | Fisher Bioreagents | Cat# BP231-100 |
| Human FcR Blocking reagent | Miltenyi Biotec | Cat# 130-059-901 |
| PBS | Corning | Cat# 21-040-CV |
| 0.5 M EDTA solution | Thermo Fisher Scientific | Cat# BP2482-500 |
| Isolurane | Covetru | Cat#1169-B-6777-2 |
| Mouse serum | MP Biomedical | Cat# 021522805 |
| **Critical commercial assays** |        |            |
| Human NK Cell Isolation Kit | Miltenyi Biotec | Cat# 130-092-657 |
| PKH26 Red Fluorescent Cell Linker Kit | Sigma-Aldrich | Cat# MIDI26 |
| **Experimental models: Cell lines** |        |            |
| HCC827 cells lentivirally transduced a pCDH-EF1-Luc2-P2A-tdTomato plasmid (HCC827-Luc2) | Zang Lab | Wei et al. (2021) |
| **Experimental models: Organisms/strains** |        |            |
| 6–10 weeks old, male or female NSG mice | The Jackson Laboratory | Cat# 005557; RRID: IMSR_JAX:005557 |
| **Software and algorithms** |        |            |
| GraphPad Prism version 8.0.2 | GraphPad Software | [https://www.graphpad.com/scientific-software/prism/](https://www.graphpad.com/scientific-software/prism/) |
| FlowJo version 10.5 | FlowJo LLC | [https://www.flowjo.com/solutions/flowjo](https://www.flowjo.com/solutions/flowjo) |
| **Other** |        |            |
| IVIS Spectrum In Vivo Imaging System | PerkinElmer | N/A |
| Irradiator | JL Shepherd & Associates | Mark I Series, Cs^{137} |
**MATERIALS AND EQUIPMENT**

**HCC827 media**

| Reagent                                      | Final concentration | Amount       |
|----------------------------------------------|---------------------|--------------|
| RPMI 1640 media                              | × 1                 | 440 mL       |
| Heat inactivated FBS                        | 10%                 | 50 mL        |
| L-Glutamine solution                        | 2 mM                | 5 mL         |
| Penicillin-Streptomycin solution (100×)      | × 1                 | 5 mL         |
| Total                                        | N/A                 | 500 mL       |

**Freezing media**

| Reagent                                      | Final concentration | Amount       |
|----------------------------------------------|---------------------|--------------|
| Heat inactivated FBS                        | 90%                 | 45 mL        |
| DMSO                                         | 10%                 | 5 mL         |
| Total                                        | N/A                 | 50 mL        |

**PBMC media**

| Reagent                                      | Final concentration | Amount       |
|----------------------------------------------|---------------------|--------------|
| RPMI 1640 media                              | × 1                 | 424.5 mL     |
| Heat inactivated FBS                        | 10%                 | 50 mL        |

**Note:** Heat inactivate the FBS by incubation at 56°C for 45–60 min. Store HCC827 media at 4°C for up to 3 months.

**Alternatives:** RPMI 1640 can be substituted with DMEM media.

**Note:** Prepare freezing media freshly prior to use.

⚠️ **CRITICAL:** DMSO can be absorbed through skin, wear gloves for protection.

(Continued on next page)
Note: Store PBMC media at 4°C for up to 3 months.

△ CRITICAL: 2-Mercaptoethanol can be toxic if inhaled, add to media in fume hood for safety.

### NK cell basal media

| Reagent                                      | Final concentration | Amount  |
|----------------------------------------------|---------------------|---------|
| OpTmizer™ T-Cell Expansion Basal Media       | × 1                 | 1,000 mL|
| OpTmizer™ T-Cell Expansion Supplement       | × 1                 | 26 mL   |
| L-Glutamine solution                         | 2 mM                | 10.5 mL |
| Penicillin-Streptomycin solution (100×)      | × 1                 | 10.5 mL |
| Total                                        | N/A                 | 1,047 mL|

Note: Store NK cell basal media at 4°C for up to 2 months.

### NK cell expansion media

| Reagent                                      | Final concentration | Amount  |
|----------------------------------------------|---------------------|---------|
| NK cell basal media                          | × 1                 | 489.5 mL|
| Recombinant human IL-2 (50 μg/mL stock)      | 40 ng/mL            | 0.4 mL  |
| Recombinant human IL-15 (50 μg/mL stock)     | 10 ng/mL            | 0.1 mL  |
| Human AB serum                               | 2%                  | 10 mL   |
| Total                                        | N/A                 | 500 mL  |

Note: Store NK cell expansion media at 4°C for up to 2 weeks.

### NK cell feeding media

| Reagent                                      | Final concentration | Amount  |
|----------------------------------------------|---------------------|---------|
| NK cell expansion media                      | × 1                 | 50 mL   |
| Purified anti-human CD3 antibody (clone OKT3, 10 μg/mL) | 10 ng/mL            | 50 μL   |
| Total                                        | N/A                 | 50 mL   |

Note: Store NK cell feeding media at 4°C for up to 2 weeks.

Note: Dilute 1 mg/mL of anti-human CD3 antibody stock to 10 μg/mL with PBS prior to use.

### PKH26 working solution

| Reagent                                      | Final concentration | Amount  |
|----------------------------------------------|---------------------|---------|
| PKH26 dye (1 mM)                             | 3 μM                | 3 μL    |
| Diluent C (DC) buffer                        | × 1                 | 997 μL  |
| Total                                        | N/A                 | 1 mL    |
**Note:** Prepare immediately prior to use and keep protected from light.

### MACS or FACS buffer

| Reagent                  | Final concentration | Amount |
|--------------------------|---------------------|--------|
| PBS                      | × 1                 | 488 mL |
| Fetal bovine serum       | 2%                  | 10 mL  |
| 0.5 M EDTA solution      | 2 mM                | 2 mL   |
| Total                    | N/A                 | 500 mL |

**Note:** Sterilize with 0.22 μm filter and store at 4°C for up to 3 months.

### Mixture of antibody and cytokine for *in vivo* treatment

| Reagent                                | Final concentration | Amount |
|----------------------------------------|---------------------|--------|
| mIgG1 or 26E10 (4 mg/mL)               | 2 mg/mL             | 250 μL |
| Recombinant human IL-2 (50 μg/mL stock)| 10 μg/mL            | 100 μL |
| Recombinant human IL-15 (50 μg/mL stock)| 10 μg/mL           | 100 μL |
| PBS                                    | × 1                 | 50 μL  |
| Total                                  | N/A                 | 500 μL |

**Note:** Prepare immediately prior to use.

### D-luciferin solution

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| D-luciferin | 15 mg/mL          | 1 g    |
| DPBS    | × 1                 | 66.7 mL|
| Total   | N/A                 | 66.7 mL|

**Note:** Aliquot and keep D-luciferin solution protected from light at −20°C for up to 3 months.

### STEP-BY-STEP METHOD DETAILS

#### Enriching NK cells from PBMCs

**Timing:** 12–16 h for recovering frozen PBMCs, 1.5 h for NK cell enrichment

This section describes how to enrich NK cells from frozen PBMCs. We use a MicroBead-based human NK cell isolation kit generally following the recommendations of Miltenyi Biotec with some modifications.

1. Thaw frozen PBMCs.
   a. Thaw PBMCs quickly with gentle agitation by hand in 37°C water bath.
   b. Dilute thawed cells to 10 mL with PBMC media in a 15 mL conical tube.
   c. Centrifuge at 500 × g for 5 min at 20°C–22°C. Aspirate supernatant.
   d. Resuspend cells with fresh PBMC media and transfer to a T-75 flask at a cell density of 5–10 × 10⁶ cells/mL.
   e. Maintain cells in humidified incubator at 37°C and 5% CO2 for 12–16 h.

**△ CRITICAL:** We have found that 12–16 h incubation of PBMCs can resolve cell clumps formed during thawing, which may improve the number of recovered cells.
2. Collect PBMCs.
   a. Run cells through a 40 μm cell strainer into a 50 mL conical tube.
   b. Count cells, centrifuge for 5 min at 500 x g, 4°C.
   c. Wash cells once with MACS buffer, spin cells down for 5 min at 500 x g, 4°C.

△ CRITICAL: Before NK cell enrichment, reserve 1/10 autologous PBMCs as feeder cells for subsequent KIR3DL3+ NK cell expansion. These cells can be maintained in PBMC media before use.

3. Enrich NK cells with MicroBead-based human NK cell isolation kit.
   a. Resuspend cell pellet in 40 μL MACS buffer per 10⁷ cells.
   b. Add 5 μL NK cell Biotin-Antibody Cocktail per 10⁷ cells.
   c. Mix well and incubate for 10 min at 4°C.
   d. Add an additional 30 μL MACS buffer per 10⁷ cells.
   e. Add 10 μL NK cell MicroBead Cocktail per 10⁷ cells.
   f. Mix well and incubate for 20 min at 4°C.
   g. Place a LS column onto QuadroMACS™ Separator attached to MACS MultiStand, rinse column with 3 mL MACS buffer.
   h. Apply cells onto the column.
   i. Collect flow-through containing NK cells in a 15 mL conical tube.
   j. Wash column twice with 3 mL buffer and collect in the same tube.
   k. Count cells, spin cells down for 5 min at 500 x g.
   l. Resuspend at 5 x 10⁶ cells/mL in MACS buffer.

Note: We have found that a comparable NK cell purity can be achieved when using half of the recommended amount of reagents and extending the incubation time. If desired, assess NK cell purity by flow cytometry staining with CD3 and CD56 antibodies. The purity should be >95%.

Note: If the total amount of PBMCs exceeds 1 x 10⁸, use one LS column per 1 x 10⁸ PBMCs.

Pause point: Enriched NK cells can be used immediately to proceed to subsequent KIR3DL3+ cell sorting, or maintained in PBMC media at 3–5 x 10⁶ cells/mL in the incubator for 1 d.

Sorting and expanding KIR3DL3+ NK cells

⏱ Timing: 3.5 h for sorting, 2–3 week for cell expansion

In this section, we describe how to sort KIR3DL3+ NK cells from enriched NK cells and expand cells for in vivo establishing a human NK cell-based mouse model. This method is adapted from previous research (Granzin et al., 2017; Lim et al., 2013; Siegler et al., 2010; Zhuang and Long, 2019) and developed by us (Wei et al., 2021). After cell sorting, KIR3DL3+ NK cells are co-cultured with irradiated feeder cells (autologous PBMCs) for 5–6 days. Feeder cells are irradiated to impede their growth in culture. Afterward, cells are expanded without feeder cells for additional 1–2 weeks to obtain desired amount of cells.

4. Resuspend cells at 5 x 10⁶ cells/mL in MACS buffer.
5. Block NK cells in a 15 mL conical tube with human FcR blocking reagent diluted 1:10 in MACS buffer for 10–15 min at 20°C–22°C.
6. Without washing, add PE anti-KIR3DL3 mAb 26E10 (1:300) and APC anti-human CD3 (1:300), stain cells for 30–40 min at 4°C.
Note: Prepare corresponding fluorophore-conjugated isotype controls to determine the accurate cell population for sorting.

7. Wash cells twice with MACS buffer.
   a. 1st wash – Add 10 mL buffer to stained cells, spin down at 500 × g for 5 min at 20°C–22°C, aspirate supernatant.
   b. 2nd wash – Resuspend cell pellet in 10 mL FACS buffer, spin down at 500 × g for 5 min at 20°C–22°C, aspirate supernatant.
8. Resuspend at 5 × 10⁶ cells/mL in FACS buffer and transfer to a 5 mL tube with cell strainer cap.
9. Collect CD3⁻ KIR3DL3⁺ cells based on the gating strategy (Figure 1A).
   a. We use the BD FACSAria for cell sorting.
10. Prepare feeder cells.
    a. Collect reserved feeder cells (autologous PBMCs) from step 2 in a 15 mL conical tube.
    b. Irradiate feeder cells (30 Gy) with Cesium-137 radioactive source.
    c. Wash irradiated feeder cells with PBMC media once.
11. Expand KIR3DL3+ NK cells.
   a. Mix sorted CD3- KIR3DL3+ cells with irradiated feeder cells at a ratio of 1:10–20 (NK:feeder cells).
   b. Spin cell mixture down at 500 × g for 5 min at 20°C–22°C, aspirate supernatant.
   c. Resuspend at 2–3 × 10⁶ cells/mL in NK cell feeding media and culture in the incubator.
   d. After 5–6 days, transfer cells to a larger flask and culture in NK cell expansion media without feeder cells for additional 1–2 week.
   e. Check cell number and viability twice a week.

   **Note:** Replenish fresh NK cell expansion media every 1–2 d to maintain cells at a density of 2–3 × 10⁶ cells/mL during remainder of expansion. The number of NK cells is expected to be doubled every 2–3 d starting from day 6. The cell viability should be > 95%.

**Pause point:** Alternatively, the expanded KIR3DL3+ NK cells can be frozen and stored in liquid nitrogen on days 10–14 after sorting. Typically, we store 10–30 × 10⁶ cells per mL of freezing media.

### Validating KIR3DL3 expression following NK cell expansion

© Timing: 2 h

This section will describe the examination of KIR3DL3 expression on expanded NK cells by flow cytometry on day 10 after sorting.

12. Collect NK cells and place 0.1–0.5 × 10⁶ cells per well in a 96-well round bottom plate.
13. Wash cells once with 200 μL of FACS buffer.
14. Spin cells down at 650 × g for 2 min at 4°C.
15. Resuspend cells with 50 μL of human FcR blocking reagent diluted 1:10 in FACS buffer, incubate for 10 min at 20°C–22°C.
16. Without washing, add PE anti-KIR3DL3 mAb 26E10 (1:300), FITC anti-human CD56 (1:200) and APC anti-human CD3 (1:300).

   **Note:** Prepare corresponding fluorophore-conjugated isotype controls to determine the cell population when analyzing by flow cytometry.

17. Incubate for 30–40 min at 4°C.
18. Wash twice with 200 μL of FACS buffer.
19. Resuspend cells in 200–300 μL of FACS buffer supplemented with DAPI (1:1,000).
20. Proceed to flow cytometry.
   a. We use BD LSRII for flow cytometry.

   **Note:** A representative example of the analysis is shown in Figure 2.

### Functional characterization of anti-KIR3DL3 blocking mAb in vitro

© Timing: 7–8 h

This section describes the evaluation of the effect of KIR3DL3 blockade on NK cell function by a flow-cytometry based NK cytotoxicity assay (Kim et al., 2007; Wei et al., 2021). Basically, target cells are
prelabeled with PKH26 red fluorescent cell membrane dye to differentiate from effector NK cells. By gating on PKH26+ target cells and using a live/dead stain 7-AAD, the specific lysis of the target cells is quantified. This methodology is translatable to multiple target cell types. Here we describe the assay using HCC827 cells.

21. Pre-incubate NK cells with isotype control mIgG1 or anti-KIR3DL3 mAb 26E10.
   a. Harvest 2 × 10⁶ expanded KIR3DL3+ NK cells into a 15 mL conical tube, centrifuge cells for 5 min at 500 × g at 20°C–22°C, discard supernatant.
   b. Resuspend cells in HCC827 media at 1 × 10⁶ cells/mL. Divide cells into 3 parts and add 10 μg/mL of mIgG1 or 26E10, or equal volume of PBS to each part.
   c. Incubate for 45 min at 20°C–22°C.
   d. Move forward without washing cells.

22. Label HCC827 cells with PKH26 dye.
   a. Harvest HCC827 cells.
   b. Wash cells with PBS once, centrifuge cells for 5 min at 500 × g at 20°C–22°C, discard supernatant.
   c. Resuspend cells in Diluent C buffer (in PKH26 Red Fluorescent Cell Linker Kit) at a concentration of 5–10 × 10⁶ cells/mL.
   d. Add an equal volume of PKH26 working solution to HCC827 cell suspension and incubate for 2 min at 20°C–22°C.
   e. Add 10 × volume of HCC827 media and incubate 3–5 min at 20°C–22°C.
   f. Wash cells with 5 mL of HCC827 media three times, centrifuge for 5 min at 500 × g, 20°C–22°C.
   g. Resuspend cells with HCC827 media at a cell density of 1 × 10⁵ cells/mL.
   h. Protect labeled cells from light.
Alternatives: Other lipophilic membrane dyes such as PKH67 green fluorescent dye (Sigma-Aldrich) can be used in place of PKH26.

△ CRITICAL: To increase the cell viability before cytotoxicity assay, cells should be passaged the day prior to experimental setup.

23. Co-culture PKH26-labeled HCC827 cells with NK cells (Figure 3).
   a. Seed prepared cells at indicated effector:target (E:T) ratios into a 96-well round bottom plate in duplicate (Table 1).
   b. Mix cells by gently pipetting.
   c. Centrifuge plate for 2 min at 110 g.
   d. Incubate plate in the incubator for 4–5 h.

24. Analyze by flow cytometry.
   a. Centrifuge plate for 2 min at 650 g at 20°C–22°C after incubation.
   b. Resuspend cells in 200 µL/well with FACS buffer supplemented with 7-AAD (1:150).
   c. Proceed to flow cytometry.

Note: The fluorescence of PKH26 and 7-AAD have an excitation/emission spectrum of 551/567nm and 546/647nm, respectively. Choose proper detection channels for PKH26 and 7-AAD according to the flow cytometer used. In the case of the BD LSR II cytometer, the fluorescence maxima of PKH26 and 7-AAD is detected in PE and PerCP/Cy5.5 channel, respectively.

Note: A representative example of the analysis is shown in Figure 4.

Evaluating therapeutic effect of KIR3DL3 blockade in NK cell-reconstituted NSG mouse models of human lung cancer

① Timing: 3–4 weeks

This method adapted from previous studies (Dong et al., 2019; Kamiya et al., 2019) determines in vivo anti-tumor activity of KIR3DL3 blockade with mAb 26E10 in a challenging intravenously (i.v.) xenograft model of human lung cancer, established using HCC827 tumor cells (Figure 5).
Note: 6–10 weeks old NSG mice are used for the experiment. Group size should be determined prior to the experiment. In general, equal numbers of male and female mice should be used.

**CRITICAL:** Prepare and expand KIR3DL3+ NK cells ahead to ensure there are enough cells for treatment. Determine the timings to obtain required number of NK cells according to NK expansion data (Figure 1B) from previous experiments in step 10. In general, at least $500 \times 10^6$ cells can be generated from $1 \times 10^6$ KIR3DL3+ NK cells over two weeks.

Day 0.

25. Establish HCC827-Luc2 human lung tumor xenograft in NSG mice.
   a. Trypsinize and harvest HCC827-Luc2 cells at approximately 80%–90% confluency into 50 mL conical tubes.
   b. Centrifuge cells for 5 min at 500 $\times$ g, 4°C.
   c. Wash cells 3 times with sterile PBS, centrifuge cells for 5 min at 500 $\times$ g, 4°C.
   d. Resuspend cells in PBS at a cell density of 2.5 $\times$ $10^6$/mL. Prepare an excess of 20% of cells. Keep cells on ice until inoculation.
   e. Mix the cell suspension and inject i.v. 200 mL of cells ($5 \times 10^5$ cells) into the tail vein of each mouse using 1 mL syringe with 30-gauge needle.

   **Note:** To ensure enough cells prior to the inoculation, tumor cells should be expanded one week ahead. It is recommended to use tumor cells that have been passaged less than 20 times.

   **CRITICAL:** Filter resuspended cells through a 40 $\mu$m cell strainer before counting cells to ensure single cell suspension. Mix the cell suspension before each injection.

Day 4.

26. Perform bioluminescence imaging (BLI) of mice.
   a. Place mice into an anesthetic induction chamber (isoflurane 2.0%, with flow rate 2 L/min) until fully anesthetized.
   b. Inject mice i.p. with 150 mg/kg D-luciferin.
   c. Three minutes after the D-luciferin injection, image the mice with an IVIS Spectrum Imaging platform (Perkin Elmer) every 3 min for a total of 15 min with Living Image software, using the serial imaging setting and auto-exposure function.
   d. Determine total flux (p/s) by creating a region of interest (ROI) over the lungs of each mouse.
   e. After imaging, return the mice to their cages and monitor until fully recovery from anesthesia.
   f. Allocate mice to 26E10 and mlgG1 treatment groups with similar average tumor burden.

Day 5–9.

27. Treat tumor-bearing mice with KIR3DL3+ NK cells and mlgG1 or 26E10.
   a. Collect KIR3DL3+ NK cells into 50 mL conical tubes, centrifuge cells for 5 min at 500 $\times$ g, 4°C.

### Table 1. Cell dilutions for indicated E:T ratios

| E:T ratio | Volume of NK cells (μL) | Volume of HCC827 cells (μL) | Additional media (μL) |
|-----------|--------------------------|-----------------------------|-----------------------|
| 0:1       | 0                        | 100                         | 100                   |
| 5:1       | 25                       | 100                         | 75                    |
| 10:1      | 50                       | 100                         | 50                    |
| 20:1      | 100                      | 100                         | 0                     |
b. Wash cells 3 times with sterile PBS, centrifuge cells for 5 min at 500 g, 4°C.
c. Resuspend cells in PBS at a cell density of 7–8 x 10^7/mL. Prepare an excess of 20% of cells. Keep cells on ice until inoculation.
d. Prepare mixture of antibody and cytokine for in vivo treatment (see materials and equipment).
e. Add equal volume of mixture of antibody and cytokine (1 μg rhIL-2, 1 μg rhIL-15, and 200 μg 26E10 or mIgG1 per mouse) to cell suspension (7–8 x 10^6 cells/mouse), mix well and inject i.v. 200 μL into the tail vein of each mouse using 1 mL syringe with 30-gauge needle.
f. Repeat treatment on day 7 and day 9.

Day 10–24.

28. To monitor tumor status, bi-weekly bioluminescence imaging is performed for the duration of the study.

⚠ CRITICAL: Prior to starting in vivo experiments, it’s highly recommended to determine the D-luciferin kinetic curve for the tumor models of interest by following PerkinElmer protocols (https://resources.perkinelmer.com/labsolutions/resources/docs/SOP_Determine_Luciferin_Kinetic_Curve.pdf, accessed January 2nd, 2022). Optimal timings between D-luciferin injection and imaging should be determined before starting. Three min after D-luciferin injection, we perform serial imaging every 3 min for a total of 15 min.
EXPECTED OUTCOMES

Following the isolation of NK cells from PBMCs and sorting for KIR3DL3+ NK cells, feeder autologous PBMCs and cytokines (IL-2 and IL-15) will induce rapid and robust proliferation of NK cells. This method for NK cell expansion is anticipated to yield up to 900 times of initial cell amount and about 98% of NK cells remain KIR3DL3+ over 3 weeks after sorting (Figure 1), which should be sufficient for testing one NK cell-reconstituted in vivo mouse model.

We and others identified KIR3DL3 as an inhibitory receptor for HHLA2 (Wei et al., 2021; Bhatt et al., 2021). HHLA2 is aberrantly expressed in various human cancers, and its overexpression is often associated with poor prognoses (Janakiram et al., 2015). HCC827 cells express endogenously HHLA2, and are therefore resistant to NK cell killing by exploiting the KIR3DL3-HHLA2 inhibitory pathway. When blocking KIR3DL3-HHLA2 engagement with anti-KIR3DL3 blocking mAb, the cytolytic activity of NK cells is expected to be enhanced in vitro (Figure 4B) and in vivo (Wei et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

The raw data generated from the flow cytometry-based cytotoxicity assay are analyzed using Flowjo. Below is the formula used to calculate specific lysis (%) of target cells:

\[
\text{Specific Lysis (\%)} = \frac{\text{PKH26}^+ \text{ target cells}}{\text{PKH26}^- \text{ target cells}} \times 100
\]

The specific lysis (%) data shown by mean of the duplicate wells of each condition are then transferred into GraphPad Prism for statistical analysis using a multiple unpaired t test.

LIMITATIONS

Donors vary in the frequency of NK cells and KIR3DL3+ cells, which may impact the starting number of KIR3DL3+ NK cells for subsequent expansion. Prior to setting up experiments, it is necessary to screen donors with higher percentage of KIR3DL3+ cells to ensure rapid and robust expansion of cells.

NK cells are difficult to maintain in culture and sensitive to passage number, cell density, and IL-2/IL-15 quality. These are crucial to maintain NK cell viability which will directly affect consistency in cytotoxicity assays.
Moreover, human NK cell-based humanized NSG mice lack other immune cells, particularly T cells that play an important role in the tumor microenvironment (TME) and anti-tumor immunity. Furthermore, KIR3DL3 is also expressed on human T cells, particularly on terminally differentiated effector memory CD8+ T (CD8+ TEMRA) cells (Wei et al., 2021). Thus, a comprehensively humanized mouse models would be more suitable for studying the effect of KIR3DL3 on anti-tumor immune responses in TME.

TROUBLESHOOTING

Problem 1
Low yield of expanded KIR3DL3+ NK cells in Section “sorting and expanding KIR3DL3+ NK cells (step 10)”.

Potential solution
The number of input KIR3DL3+ NK cells affects the yield greatly. Select donors that have higher frequency of KIR3DL3+ cells. In addition, we have found that using freshly isolated instead of frozen PBMCs as the source of NK cells and feeder cells can increase the yield significantly.

Problem 2
Low NK cell viability in Section “sorting and expanding KIR3DL3+ NK cells (step 10)”.

Potential solution
NK cells proliferate rapidly in the presence of IL-2 and IL-15 during expansion. Check cells and replenish fresh media every 1–2 days to prevent overgrowth i.e., > 3 × 10^6 cells/mL.

Problem 3
High variability in readouts of cytotoxicity assay in Section “functional characterization of anti-KIR3DL3 blocking mAb in vitro”.

Potential solution
Accuracy in cell counting before coculture accounts for the variability significantly. In addition, use tumor cells with low passage numbers (< 20) and passage cells a day prior to coculture to increase cell viability. If there are cells attached to plate after 4 h coculture, trypsinize to collect all cells.

Problem 4
The mouse dies quickly after tail vein injection of tumor cells and NK cells in Section “evaluating therapeutic effect of KIR3DL3 blockade in NK cell-reconstituted NSG mouse models of human lung cancer (steps 24 and 26)”.

Potential solution
Cell suspension may contain aggregated cells or air bubbles, which form an embolus and occlude vessels in the mice, ultimately leading to lethality. Filter cell through a 40 μm cell strainer to ensure a single cell suspension and remove air bubbles in the syringes before i.v. injection.

Problem 5
False negative BLI emission values following i.p. injection of D-luciferin in Section “evaluating therapeutic effect of KIR3DL3 blockade in NK cell-reconstituted NSG mouse models of human lung cancer (steps 24–27)”.

Potential solution
This may be caused by improper i.p. injection of D-luciferin. Repeat D-luciferin injection in affected mice. If a positive readout is obtained, the false negative values should be excluded from data analysis.
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Xingxing Zang (xingxing.zang@einsteinmed.edu).

Materials availability
HCC827-Luc2 and anti-KIR3DL3 mAb 26E10 described in this study will be made available from the lead contact for academic/noncommercial research purposes on request under appropriate materials transfer agreement.

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

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AUTHOR CONTRIBUTIONS
X.R. and D.C. designed and conducted experiments, analyzed data, and wrote the first draft of the manuscript. X.Z. reviewed and edited the manuscript, acquired funding, and supervised the study.

DECLARATION OF INTERESTS
X.R. and X.Z. are inventors of a pending patent (KIR3DL3 is an inhibitory receptor of the immune system and uses thereof). X.Z. is an inventor of two granted patents (HHLA2 as a novel inhibitor of human immune system and uses thereof; TMIGD2 and its derivatives as blockers or binders of cancer-expressed HHLA2 for immunotherapies). X.Z. is a scientific co-founder of NextPoint Therapeutics.

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