PVRIG is a novel natural killer cell immune checkpoint receptor in acute myeloid leukemia

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Supplementary Data

PVRIG is a novel NK cell immune checkpoint receptor in acute myeloid leukemia

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Supplementary Methods

Flow cytometry staining

In all experiments, cells were first labelled with LIVE/DEAD Fixable Yellow (ThermoFisher) in phosphate buffered saline (PBS) for 20 min at 4°C and washed. For AML patient samples, cells were pre-incubated with human Fc block (BD Biosciences) for 10 min at 4°C prior to surface staining. Cells were then stained with antibodies against surface markers in FACS buffer (PBS with 2% FCS) for 30 min at 4°C. Cells were then washed and fixed in 2% paraformaldehyde (PFA, ThermoFisher) for 15 min at 4°C, then resuspended in FACS buffer before acquisition on a FACSsymphony (BD). For total (intracellular + surface) PVRIG staining, after staining for surface markers (CD56, CD16 and CD69) cells were washed and fixed using Fixation Buffer (eBioscience) for 20 min at 4°C, then washed and stained with anti-PVRIG-PE in Permeabilization Buffer (eBioscience) for 30 min at 4°C. Where indicated, ΔMFI of each marker was calculated as geometric mean of test – iso.

Chromium release assay

Chromium release assay was performed as described previously. Briefly, target cells were labelled with 100 µCi Chromium-51 (\(^{51}\text{Cr}\), PerkinElmer) for 1 hr at 37°C, washed, then cocultured with PBMCs in triplicate wells at effector:target ratios from 32:1 to 2:1. Blocking or isotype control antibodies were each added at a final concentration of 10 µg/ml. EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid, Sigma) was added at a final concentration of 4 mM. Wells with targets alone (spontaneous release) and targets with 10% Triton X 100 (Sigma, maximum release) were included as controls. After 4 hr, supernatants were collected and the amount of \(^{51}\text{Cr}\) released detected using a gamma
counter (Wallac Wizard). The % specific lysis was calculated by [(experimental release – spontaneous release)/ (maximum release – spontaneous release)] * 100. NK:target ratio was calculated from the percentage of NK cells found in PBMCs, determined by flow cytometry.

Degranulation assay

Target cells were labelled with Cell Trace Violet (ThermoFisher) in PBS for 10 min at 37ºC, washed, then co-cultured with PBMCs in triplicate wells at the specified effector:target ratios. Blocking or isotype control antibodies were each added at a final concentration of 10 µg/ml, and anti-CD107a AF488 was included during the co-culture period. Wells with targets alone or PBMCs alone were included as controls. After 4 hr, cells were washed and stained with LIVE/DEAD Fixable Yellow followed by antibodies against CD56, CD16, CD3 and CD69. Due to variation in baseline CD69 levels between donors, CD69 MFI was normalised as a percentage of the isotype control treated group.
Supplementary Tables

Supplementary Table S1. Antibodies used for flow cytometry.

| Marker        | Fluorochrome | Clone | Source       |
|---------------|--------------|-------|--------------|
| PVRIG         | PE           | -     | Compugen     |
| TIGIT         | APC          | MBSA43| eBioscience  |
| DNAM-1        | BV711        | DX11  | BD           |
| PVRL2         | PECy7        | TX31  | Biolegend    |
| PVR           | BV421        | TX24  | BD           |
| CD3           | BUV496       | UCHT1 | BD           |
| CD8           | BUV805       | SK1   | BD           |
| CD11b         | BV785        | ICRF44| Biolegend    |
| CD14          | APC-H7       | MφP9  | BD           |
| CD16          | BUV395       | 3G8   | BD           |
| CD19          | APC-Cy7      | SJ25C1| Biolegend    |
| CD33          | BV480        | HIM3-4| BD           |
| CD34          | BV605        | 581   | Biolegend    |
| CD38          | FITC         | HIT2  | Biolegend    |
| CD45          | AF700        | HI30  | Biolegend    |
| CD56          | BUV563       | NCAM16.2| BD     |
| CD56          | BUV737       | NCAM16.2| BD     |
| CD69          | BV786        | FN50  | BD           |
| CD107a        | AF488        | H4A3  | Biolegend    |
| CD117         | PE/Dazzle    | 104D2 | Biolegend    |
| HLA-DR        | BUV395       | G46-6 | BD           |
| Human IgG4    | PE           | -     | Compugen     |
| isotype control|              |       |              |
| Mouse IgG1    | APC          | MOPC-21| Biolegend   |
| isotype control|              |       |              |
| Mouse IgG1    | BV711        | MOPC-21| Biolegend   |
| isotype control|              |       |              |
| Mouse IgG1    | PECy7        | MOPC-21| Biolegend   |
| isotype control|              |       |              |
| Mouse IgG2a   | BV421        | MOPC-173| Biolegend  |
| isotype control|              |       |              |
Supplementary Table S2. AML patient clinical characteristics.

| Patient ID | Age (years) | Sex | WHO classification | Molecular | Cytogenetic |
|------------|-------------|-----|--------------------|-----------|-------------|
| 1470       | 77          | M   | M5                 | No mutation | Cytogenetic abnormalities not classified as favourable or adverse |
| 1472       | 75          | M   | M2                 | No mutation | -5, -7 Complex Karyotype |
| 1482       | 55          | F   | M1                 | No mutation | -7, Complex Karyotype |
| 1533       | 67          | M   | AMLMRC             | NA        | Normal Karyotype |
| 1592       | 46          | M   | M2                 | NA        | Normal Karyotype |
| 1609       | 20          | M   | M2                 | NA        | Normal Karyotype |
| 1673       | 16          | M   | M2                 | Wild-type NPM1 with FLT3-ITD low | Cytogenetic abnormalities not classified as favourable or adverse |
| 1703       | 80          | M   | M2                 | No mutation | NA |
| 1734       | 75          | F   | M1                 | Mutated NPM1 without FLT3-ITD | Normal Karyotype |
| 1739       | 33          | M   | M1                 | No mutation | Complex Karyotype |
| 1744       | 68          | F   | M1                 | No mutation | Cytogenetic abnormalities not classified as favourable or adverse |
| 1921       | 62          | M   | M5a                | No mutation | Normal Karyotype |
| 1939       | 25          | M   | M2                 | No mutation | Normal Karyotype |
| 1993       | 57          | M   | M0                 | NA        | Normal Karyotype |
| 2062       | 51          | F   | M5                 | NA        | Normal Karyotype |
| 2084       | 65          | M   | AMLT               | NA        | Monosomal Karyotype |
| 4897       | 58          | NA  | AMLMRC             | NA        | NA |
| 5032       | 25          | M   | M2                 | No mutation | Normal Karyotype |
| 5098       | 48          | M   | AMLMRC             | NA        | Complex Karyotype |
| 5100       | 48          | F   | M4                 | Mutated NPM1 with FLT3-ITD high | Normal Karyotype |

NA: data not available; M0: AML with minimal differentiation; M1: AML without maturation; M2: AML with maturation; M4: Acute myelomonocytic leukemia; M5a/b: Acute monoblastic/monocytic leukemia; AMLT: therapy related AML; AMLMRC: AML with myelodysplasia related changes.
Supplementary Figures

**Supplementary Figure S1. Expression of PVRIG and TIGIT on NK cells from healthy donors.**

(A) PVRIG and (B) TIGIT expression on NK cells from healthy donor PBMCs. Histograms of test (red) and isotype control stains (grey) from four representative donors are shown (each row is one donor).
Supplementary Figure S2. Relative capacity of PVRIG or TIGIT blockade to enhance NK cell killing is related to target cell PVR expression. (A) Expression of PVRL2 and PVR (red histograms) on AML-193, Kasumi-1, ML-2 and THP-1 cells compared with isotype control stain (grey histograms). Percentage lysis of (B) AML-193, (C) Kasumi-1, (D) ML-2 or (E) THP-1 cells after 4 hr co-culture with PBMCs in the presence of anti-PVRIG, anti-TIGIT, or isotype antibodies, measured by $^{51}$Cr release assay. Each graph shows mean ± SD of triplicates for an individual healthy donor (donor ID labelled above each graph), with NK:target ratios.
calculated using % of NK cells in PBMC as determined by flow cytometry. Significance determined by Student’s t-test, * p < 0.05, ** p < 0.01, *** p < 0.01; red = anti-PVRIG vs iso, green = anti-TIGIT vs iso.
Supplementary Figure S3. Gating strategy for AML bone marrow. AML blasts were identified as CD45<sup>lo</sup>SSC<sup>int</sup>, along with various combinations of the markers CD33, CD34, CD117, depending on the patient. Mature myeloid cells (CD45<sup>hi</sup>SSC<sup>hi</sup>) could be further subdivided into CD14<sup>+</sup>CD11b<sup>+</sup> or CD14<sup>+</sup>CD11b<sup>-</sup> cells. The CD14<sup>-</sup>CD11b<sup>+</sup> subset was not present in all patients, and was absent in all healthy donors, therefore was not included in further analysis. Within the lymphoid population (CD45<sup>hi</sup>SSC<sup>lo</sup>), NK cells (CD56<sup>+</sup>CD3<sup>-</sup>), NKT cells (CD56<sup>+</sup>CD3<sup>+</sup>), CD8<sup>+</sup> T cells (CD3<sup>+</sup>CD56<sup>-</sup>CD8<sup>+</sup>) and CD8<sup>-</sup> T cells (CD3<sup>+</sup>CD56<sup>-</sup>CD8<sup>-</sup>) could be identified. For healthy donor BM, the same gating strategy was employed, with CD45<sup>lo</sup>SSC<sup>int</sup> immature myeloid cells used as an analogous population to compare with AML blasts.
Supplementary Figure S4. No correlation between expression of PVRIG or PVRL2 in AML patient bone marrow and AML subtype or blast percentage. Expression of (A) PVRIG on NK cells or (B) PVRL2 on blasts in AML patients coloured by disease subtype (WHO classification; AMLT: therapy related acute myeloid leukemia, AMLMRC: acute myeloid leukemia with myelodysplasia related changes). Pearson correlation between (C) NK cell PVRIG expression or (D) blast PVRL2 expression with bone marrow blast percentage, or (E) NK cell PVRIG expression with blast PVRL2 expression.
Supplementary Figure S5. NK cells are not strongly activated by IL-2 and IL-12 stimulation within 4 hours. (A) PVRIG and (B) CD69 expression on isolated NK cells incubated alone or with 100 U/ml IL-2 and 10 ng/ml IL-12 at 37°C for the indicated timepoints. Mean ± SD of duplicates from 1 experiment is shown. Significance determined by multiple t-tests with Holm-Sidak’s correction, * p < 0.05 compared with NK alone.
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