SUPPLEMENTARY APPENDIX

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 4ºC and washed. For AML patient samples, cells were pre-incubated with human Fc block (BD Biosciences) for 10 min at 4ºC prior to surface staining. Cells were then stained with antibodies against surface markers in FACS buffer (PBS with 2% FCS) for 30 min at 4ºC. Cells were then washed and fixed in 2% paraformaldehyde (PFA, ThermoFisher) for 15 min at 4ºC, then resuspended in FACS buffer before acquisition on a FACSymphony (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 4ºC, then washed and stained with anti-PVRIG-PE in Permeabilization Buffer (eBioscience) for 30 min at 4ºC. 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.[1] Briefly, target cells were labelled with 100 μCi Chromium-51 (^{51}Cr, PerkinElmer) for 1 hr at 37ºC, washed, then co-cultured 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}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.

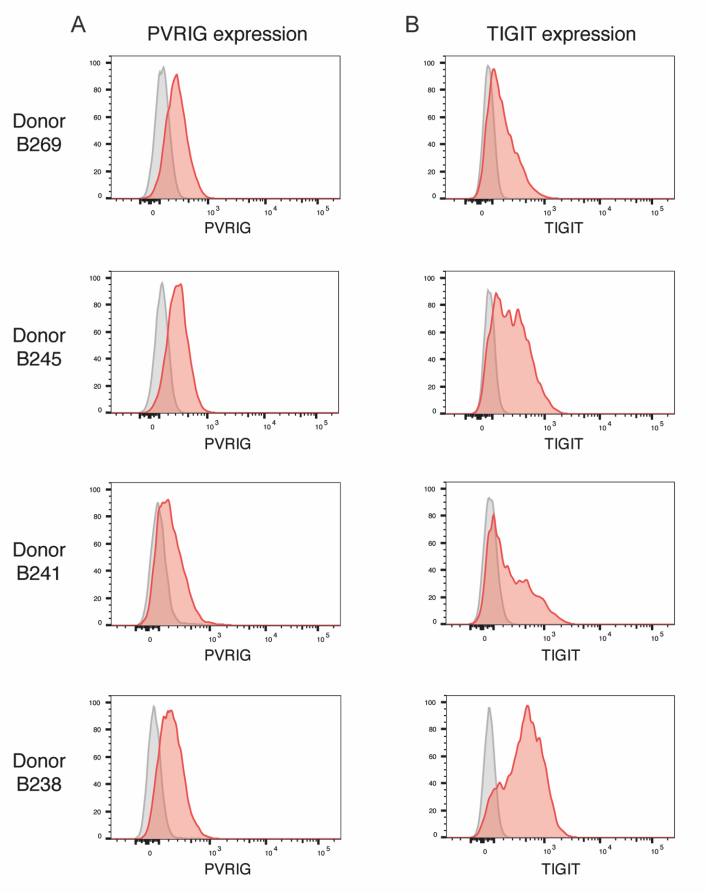
Marker	Fluorochrome	Clone	Source
PVRIG	PE	-	Compugen
TIGIT	APC	MBSA43	eBioscience
DNAM-1	BV711	DX11	BD
PVRL2	PECy7	TX31	Biolegend
PVR	BV421	TX24	BD
CD3	BUV496	UCHT1	BD
CD8	BUV805	SK1	BD
CD11b	BV785	ICRF44	Biolegend
CD14	APC-H7	MϕP9	BD
CD16	BUV395	3G8	BD
CD19	APC-Cy7	SJ25C1	Biolegend
CD33	BV480	HIM3-4	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 isotype control	PE	-	Compugen
Mouse IgG1 isotype control	APC	MOPC-21	Biolegend
Mouse IgG1 isotype control	BV711	MOPC-21	Biolegend
Mouse IgG1 isotype control	PECy7	MOPC-21	Biolegend
Mouse IgG2a isotype control	BV421	MOPC-173	Biolegend

Supplementary Table S2. AML patient clinical characteristics.

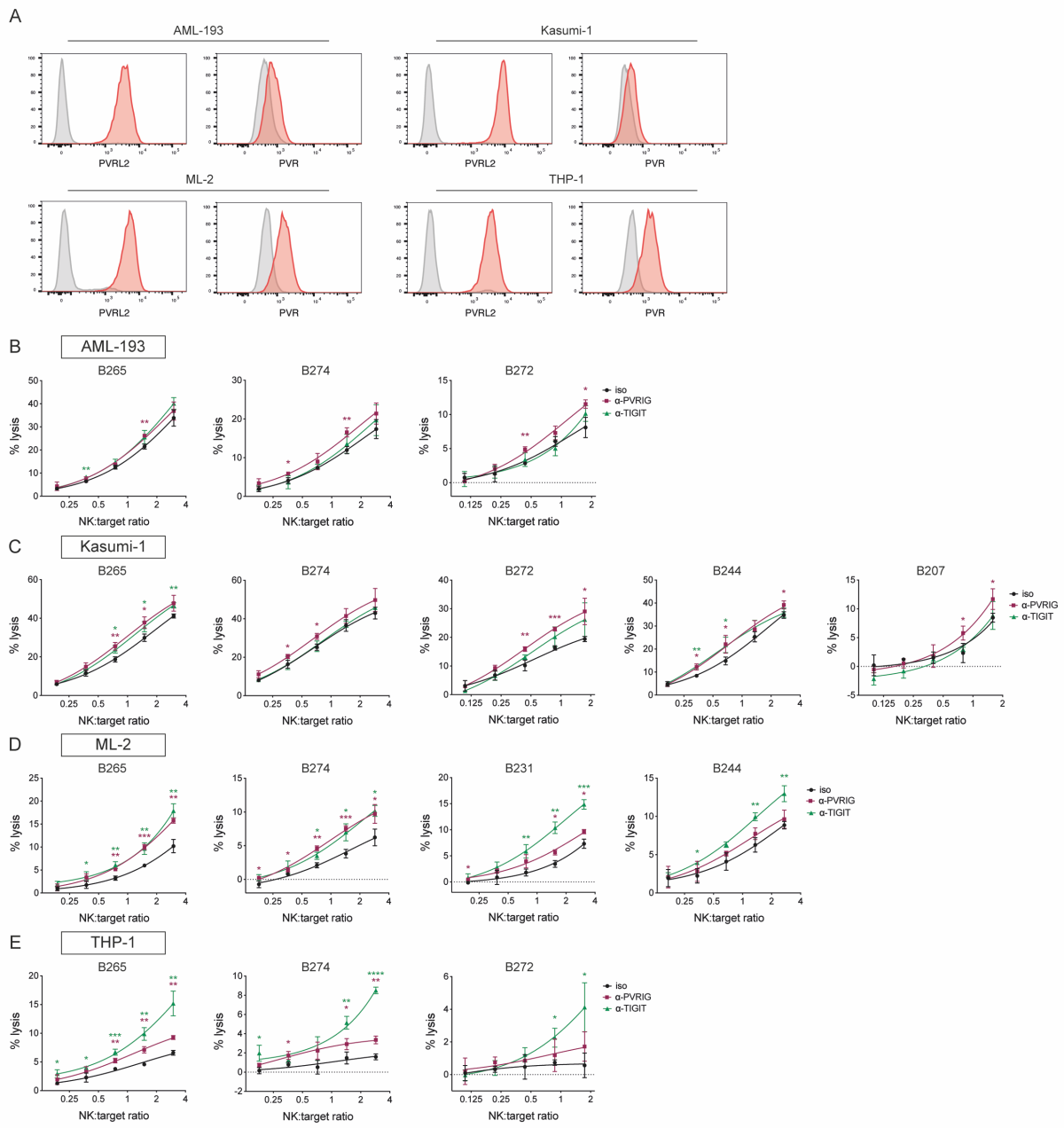
Patient ID	Age (years)	Sex	WHO classification[2]	Molecular	Cytogenetic
1470	77	M	M5	No mutation	Cytogenetic abnormalities not classified as favourable or adverse
1472	75	M	M2	No mutation	-5, -7 Complex Karyotype
1482	55	F	M1	No mutation	-7, Complex Karyotype
1533	67	M	AMLMRC	NA	Normal Karyotype
1592	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	F	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

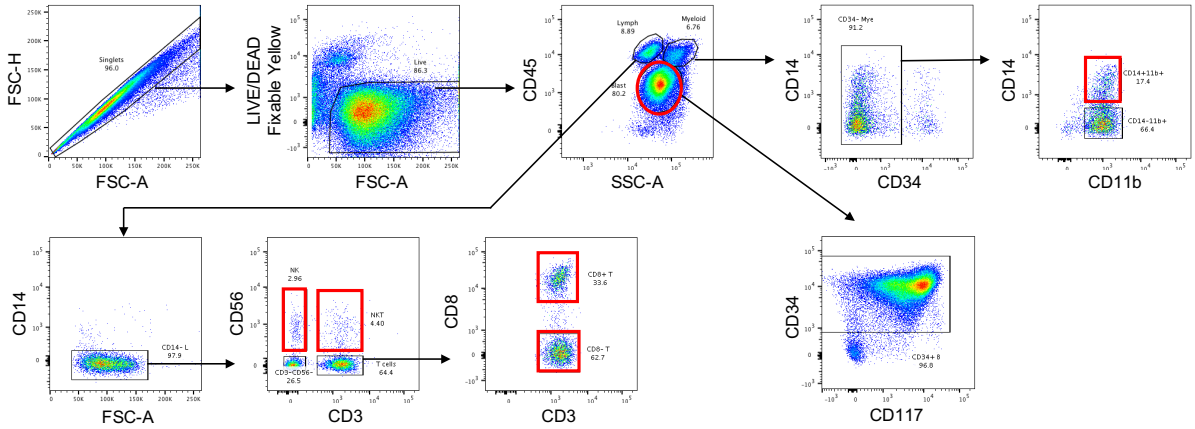


Supplementary Figure S1. Expression of PVRIG and TIGIT on NK cells from healthy donors. (A) PVRIG and (B) TIGIT expression on NK cells from healthy donor PBMCs. Histograms of test (red) and isotype control stains (grey) from four representative donors are shown (each row is one donor).

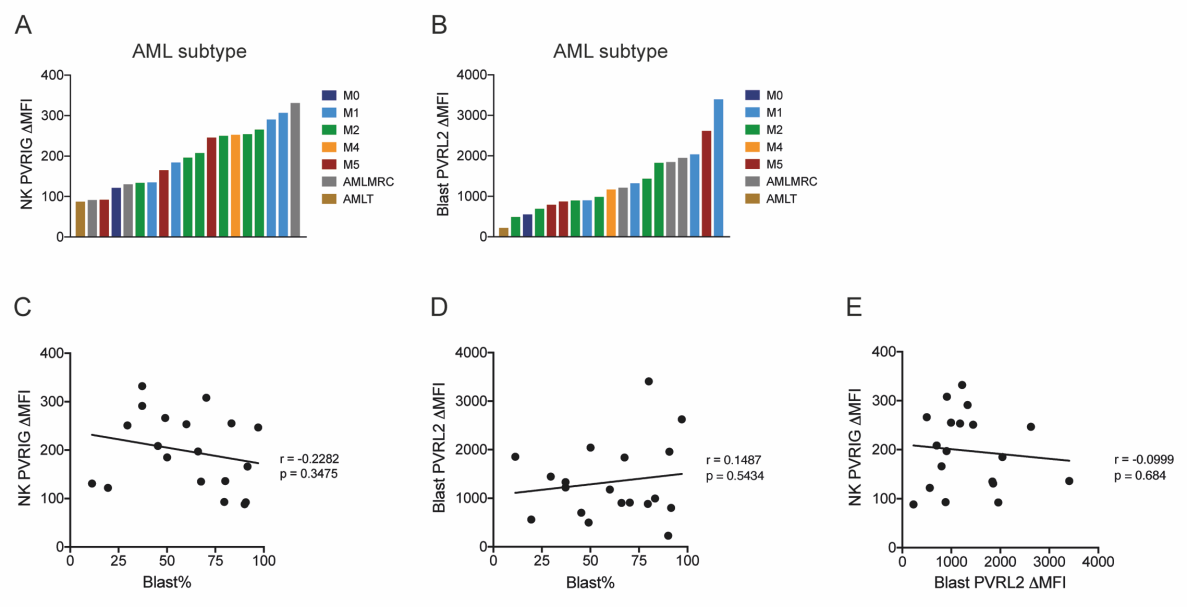


Supplementary Figure S2. Relative capacity of PVRIG or TIGIT blockade to enhance NK cell killing is related to target cell PVR expression. (A) Expression of PVRL2 and PVR (red histograms) on AML-193, Kasumi-1, ML-2 and THP-1 cells compared with isotype control stain (grey histograms). Percentage lysis 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

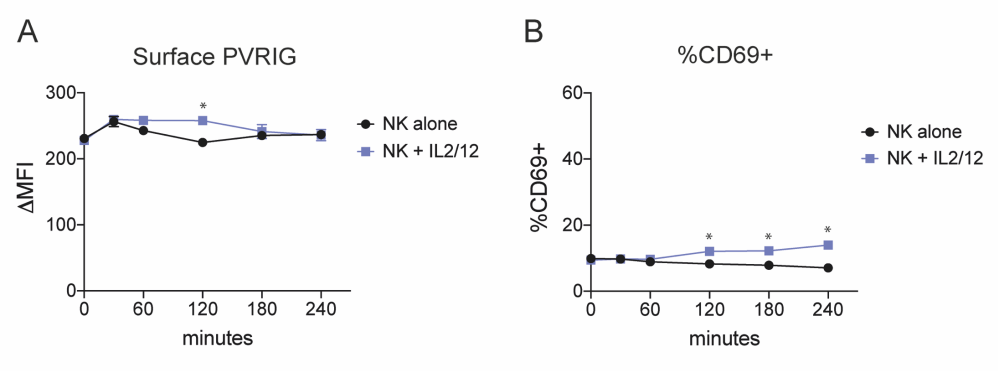
calculated using % of NK cells in PBMC as determined by flow cytometry. Significance determined by Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$; red = anti-PVRIG vs iso, green = anti-TIGIT vs iso.



Supplementary Figure S3. Gating strategy for AML bone marrow. AML blasts were identified as $CD45^{lo}SSC^{int}$, along with various combinations of the markers CD33, CD34, CD117, depending on the patient. Mature myeloid cells ($CD45^{hi}SSC^{hi}$) could be further subdivided into $CD14^{+}CD11b^{+}$ or $CD14^{-}CD11b^{+}$ cells. The $CD14^{-}CD11b^{+}$ 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 ($CD45^{hi}SSC^{lo}$), NK cells ($CD56^{+}CD3^{-}$), NKT cells ($CD56^{+}CD3^{+}$), $CD8^{+}$ T cells ($CD3^{+}CD56^{-}CD8^{+}$) and $CD8^{-}$ T cells ($CD3^{+}CD56^{-}CD8^{-}$) could be identified. For healthy donor BM, the same gating strategy was employed, with $CD45^{lo}SSC^{int}$ 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 37ºC 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.

Supplementary References

1. Hsu AK, Quach H, Tai T, et al. The immunostimulatory effect of lenalidomide on NK-cell function is profoundly inhibited by concurrent dexamethasone therapy. Blood. 2011;117(5):1605-1613.
2. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-2405.