

Poznanski SM *et al.* Supplemental Information

Supplemental Information for:

Expanded human NK cells from lung cancer patients sensitize patients' PDL1-negative tumours to PD1-blockade therapy

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Poznanski SM *et al.* Supplemental Information**SUPPLEMENTARY METHODS****Cell lines and reagents**

K562 myelogenous leukemia cells that express membrane-bound IL-21 (K562-mb-IL21; Clone 9) described previously (18) were a kind gift in 2012 from Dr. Dean A. Lee at Nationwide Children's Hospital (Ohio State University Comprehensive Cancer Center, USA). K562-mb-IL21 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% hepes, 1% penicillin-streptomycin, and 1% L-glutamine. Cells from the A549 human lung carcinoma cell line, MDA-MB-231 triple-negative breast cancer cell line and OVCAR8 high-grade ovarian serous adenocarcinoma cell line were obtained from the National Cancer Institute. A549 cells were cultured in α MEM supplemented with 10% FBS, 1% hepes, 1% penicillin-streptomycin, and 1% L-glutamine. OVCAR8 and MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS, 1% hepes, 1% penicillin-streptomycin, and 1% L-glutamine.

Recombinant human IFN γ , IL-15, and IL-2 cytokines were purchased from Peprotech (Rocky Hill, NJ, USA). Collagenase Type IV and DNase I were purchased from ThermoFisher (Waltham, MA, USA).

Cell Staining for flow cytometry

All cell staining was conducted in light-sensitive conditions and incubations carried out at 4°C. For viability staining, fixable viability stain was diluted 1000x in PBS. For extracellular and intracellular antibody stains, fluorescent minus one wells that contained corresponding isotype controls were used as controls to determine population gating.

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The following fluorescently conjugated anti-human antibodies were used for cell staining. From BD Biosciences (San Jose, CA, USA): CD45-APCR700 clone HI30, CD56-BV421 clone NCAM16.2, CD56-PECF594 clone NCAM16.2, CD3-APC-H7 clone SK7, CD14-PE-Cy7 clone M5E2, CD107a-APC clone H4A3, IFN γ -BV421 clone 4S.B3, PDL1-APC clone MIH1, CD69-PECF594 clone FN50, NKp46-BV786 clone 9E2, KIR2DL1-APC clone HP-3E4, KIR3DL1-Alexa700 clone NKB1. From BioLegend (San Jose, CA, USA): CD45-Alexa488 clone HI30, CD3-PerCp/Cy5.5 clone UCHT1, NKG2D-PerCp/Cy5.5 clone 1D11, NKp30-APC clone P30-15, NKp44-PE clone P44-8, KIR2DL2/L3-PE clone NKAT2, PD1-PerCp/Cy5.5 clone EH12.2H7. From Miltenyi Biotec (Bergisch Gladbach, Germany): NKG2A-PE-Vio770 clone REA110.

Killing Assays

For all killing assays, NK cell killing was calculated by percent specific lysis of tumour cells. In assays using CFSE-labeled A549 cells, tumour cells were gated on CFSE+ events. For killing assays against patient tumours, tumour cells were gated as CD45- events. Viability was then assessed on the tumour cell gate. Control wells with tumour cells alone were used to enumerate basal death.

$$\% \text{ specific lysis} = (\% \text{ dead} - \% \text{ basal death}) / (100 - \% \text{ basal death}) \times 100.$$

Degranulation Assay

For degranulation assays and IFN γ expression against patient tumours, anti-human CD107a was added at the start of incubation and golgi stop (BD Biosciences) was added following the first hour of incubation. At 5 hours, cells were stained with fixable viability stain

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and fluorescently conjugated anti-human CD45, CD56, CD3 (extracellular) and IFN γ (intracellular). NK cells were gated on as CD45+CD56+CD3- cells. CD107a and IFN γ expression were assessed on the NK cell gate.

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Figure S1. NK cells can be expanded from lung cancer patient blood, pleural effusions, and tumours and show a highly activated phenotype post-expansion. NK cells were expanded *ex vivo* from the peripheral blood (pbNK), pleural effusions (peNK), or tumours (taNK) of lung cancer patients (LCP) or peripheral blood of healthy donors (HD). **(A)** Representative NK cell gating strategy. Sample shown is the pre-expansion NK cell population in a patient tumour. Fold expansion and purity of NK cells from **(B)** donor-matched blood and pleural effusions, or **(C)** matched blood and tumours of lung cancer patients compared to the peripheral blood of healthy donors. **(D)** Quantification and representative flow plots of viability of NK cells from PDL1+ and PDL1- lung cancer patient tumours following 28 days of expansion. **(E)** Quantification and representative flow plots showing the proportion of CD56^{dim}, CD56^{bright}, and CD56^{superbright} NK cells post-expansion. exNK cell expression of the **(F)** activation receptors CD69, NKG2D, NKp30, NKp44, and NKp46, and **(G)** inhibitory receptors NKG2A and KIR2DL1, KIR2DL2/L3, and KIR3DL1 assessed via flow cytometry. Data show means \pm SEM of three to six replicates per condition. B,C, and E, analyzed via two-way ANOVA; D analyzed via unpaired t-test; F and G, analyzed via one-way ANOVA. ****p<0.0001.

Poznanski SM *et al.* Supplemental Information**Figure S2. Representative gating strategies for NK cell killing and degranulation assays.**

(A-B) exNK cells were incubated with CFSE-labelled A549 cells for 5 hours at 1:1, 5:1, or 10:1 effector-to-target ratios (E:T). After 5 hours, cells were stained with fixable viability stain. (A) Schematic shows experimental design. Flow plots show representative gating strategy to identify dead tumour cells. Doublets were discriminated via FSC and SSC height (H) and width (W) and live/dead cells were enumerated on the CFSE+ tumour cell population. (B) Representative flow plots of tumour cells death following incubation with expanded HD pbNK, LCP pbNK, and LCP taNK cells at the 1:1, 5:1, and 10:1 E:T ratios. Basal tumour cell death is also shown. (C) exNK cells were incubated with patient tumours for 5 hours. Flow plots show representative gating for NK cell killing and degranulation. For NK cell tumour killing, tumour cell death was assessed via viability stain on the CD45⁻ cell population. For assessment of NK cell degranulation and IFN γ expression, NK cells were identified as live CD45⁺CD56⁺CD3⁻ cells.

Figure S3. exNK cells increase lung tumour PDL1 expression. A549 cells, OVCAR8 cells, MDA-MB-231 cells, or patient tumours were seeded on apical and basolateral surfaces of a transwell and treated with exNK cells in the apical chamber or left untreated (control). PDL1 expression on live basolateral tumour cells was assessed following 48 hours. (A) Schematic shows experimental design and flow plots show representation of gating strategy on A549 cells. Quantification and representative flow plots or histogram of percent PDL1⁺ cells and PDL1 MFI on (B) A549, (C) OVCAR8, or (D) MDA-MB-231 cells. (E) Representative gating strategy of PDL1 expression on patient tumours. Data show means \pm SEM of three replicates per condition. Results analyzed via unpaired t-test. ****p<0.0001, ***p<0.001, **p<0.01.

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Patient	Baseline tumour PDL1 status	
	Diagnostic IHC	Flow Cytometry
1	P (HI, >50%)	P (HI, >50%)
2	P (LOW, 1-49%)	P (LOW, 1-49%)
3	P (LOW, 1-49%)	P (LOW, 1-49%)
4	N (<1%)	N (<1%)
5	P (LOW, 1-49%)	P (LOW, 1-49%)
6	NA	N (<1%)
7	N (<1%)	N (<1%)

Table S1. Patient baseline PDL1 status assessment via diagnostic IHC and flow cytometry.

Diagnostic IHC; determined clinically via immunohistochemistry. High-positive; TPS \geq 50%.

Low-positive; TPS \geq 1% and \leq 49%. Negative; TPS <1%.