SUPPLEMENTAL METHODS

***Mass Cytometry Analysis*:** Lymphocyte subsets were identified using FlowSOM (Supplemental Table 1; Live cells; CD11b, CD127, CD14, CD16, CD19, CD25, CD3, CD34, CD4, CD45, CD56, CD8; hierarchical consensus, 10 metaclusters, 100 clusters, 10 iterations, random seed; scales not normalized).[1] Metaclusters were manually annotated, based on lineage marker expression (Fig. 2B), and NK cell metacluster was used for subsequent phenotypic analysis. FlowSOM gated NK cells for each patient/donor pair were analyzed individually using viSNE (CD11b, CD127, CD137, CD16, CD25, CD27, CD45, CD56, CD57, CD62L, CD69, CD8, CD94, DNAM1, Granzyme B, Ki-67, KIR2DL1/S1, KIR2DL2/2DL3, KIR2DL5, KIR3DL1, NKG2A, NKG2C, NKG2D, NKp30, NKp44, NKp46, Perforin, TRAIL; proportional sampling, 1000 iterations, perplexity = 30, theta = 0.5.).[2] The viSNE NK cell subsets were then identified using FlowSOM (tSNE1, tSNE2; all events, hierarchical consensus, 20 metaclusters, 196 clusters, 15 iterations, random seed, scales not normalized). Subsequent metaclusters were annotated and binned based on previously described phenotypes, including those defined from previous allogeneic ML NK cell trials where donor- or recipient- specific HLA was used to confirm the expanding ML NK cell population were the adoptive transferred IL-12/15/18 activated NK cells.[2,3] **CD56^{bright} NK cells**: CD56^{hi}, CD16$^{lo/-}$ CD45^{lo}, KIR^{-}, NKG2A^{+}, CD57^{-}, NKp46^{hi}, Perforin^{low}, Granzyme B^{lo}; **CD56^{dim} NK cells**: CD56^{dim}, CD16^{lo+} CD45^{hi}, KIR$^{+/-}$, NKG2A$^{+/-}$, CD57$^{+/-}$, NKp46^{+}, Perforin^{+}, Granzyme B^{+}, CD11b^{lo}; **ML NK cells**: CD56^{hi}, NKp46^{hi}, NKp30^{hi}, DNAM-1^{hi}, CD62L^{+}, NKG2A^{+}, Perforin^{+}, Granzyme B^{+}, CD11b^{lo}.[2,3] Data were analyzed using Prism v9.2.0 and Cytobank.[4]

Serum cytokines: Serum was collected from red top vacutainers at least 1 hour after harvest. Clots were clarified from serum at 544 x g for 5 minutes and further clarified at 10,000 x g at 4C for 5 minutes and then banked at -80C until multiplex analysis was performed. Serum was quick

thawed, clarified and then MILLIPLEX Human Cytokine/Chemokine/Growth Factor Panel A (Cat. # HCYTA-60K, MillporeSigma) was performed in duplicate as recommended by the manufacturer. Quantitation was performed using MILLIPLEX Analyst v5.1 software.

***scRNA-seq Analysis*:** Resulting FASTQ files were aligned to the GRCh38 reference (P-ML008) or combined GRCh38 and mm10 reference (P-ML002), using CellRanger (default settings, v.6.0). The CellRanger filtered barcode matrix for each sample was imported into the *R*-based package, Seurat (v.3.99/4.0) for normalization, clustering, visualization, and differential expression by patient as previously described.[5,6] Briefly, low-quality, dying, or doublet cells were removed from downstream analysis according to the following QC metrics: P-ML008: expressing less than 200 features, ≥ 10% mitochondrial genes; P-ML002: human cells defined as previously described expressing less than 200 features, ≥ 10% mitochondrial genes, or UMIs from both mouse and human.[6,7] An initial round of clustering was performed in order to remove any non-NK cell populations including log-normalization, variable gene selection, gene expression scaling regressing out cell cycle score. Principle component analysis (PCA) was run on the most variable genes and significant PCs for clustering were selected based on the inflection of the Elbow Plot, Jackstraw Analysis, and Dimensionality Heatmaps (P-ML002: PC15; P-ML008: PC35). The following non-NK or low-quality clusters were removed for P-ML002: murine cells (2074 cells), myeloid (347 cells), proliferating with high counts (107 cells), red blood cells (47 cells), and plasma cells (97 cells) with a cluster resolution of 1.0; for P-ML008: doublets (766 cells), B cells (237 cells), dying cells (816 cells), proliferating with high counts (457 cells), and T cells (91 cells) with a cluster resolution of 0.6. Remaining cells (P-ML002: 27,506; P-ML008: 25, 952) were then re-clustered repeating the above steps after first removing the top 0.8% of cells per 1000 cells in each cluster individually based upon the 10x Genomics reported doublet rate. Data was then

renormalized, new human variable features were found, and scaling and dimensional reduction was performed as described above (P-ML002: PC14, res 0.8; P-ML008: PC21, res 0.6). Clusters were then classified into either CD56^{bright} (*FCGR3A*$^{neg/lo}$, *TCF7*^{hi}), CD56^{dim} (*FCGR3A*^{pos}), ML (*KLRC1*^{hi}, *SELL*^{hi}, *FCGR3A*^{hi}), adaptive (*KLRC2*^{hi}, *KLRC1*^{lo}), or dying cells (high mitochondrial percent and low counts). Additional CD56^{bright} and CD56^{dim} markers confirming the above identifications were derived using a Wilcoxon Rank-Sum test implemented in Seurat FindAllMarkers.[8,9] Percentages of CD56^{bright}, CD56^{dim}, ML, and adaptive NK cells were calculated as the proportion of each NK cell type in live NK cells at each time point as described in the figure legends.

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SUPPLEMENTAL TABLE LEGENDS

Supplemental Table 1: NK cell phenotypic and functional mass cytometry panels. The metal isotope tag, marker name, antibody clone, source and clustering usage are shown for the mass cytometry functional (F) or phenotypic (P) panels. Clustering demarcates which channels were used for generating the lymphocyte subsets or NK cell viSNE plots (lymph and NK, respectively) in the phenotypic panel. The asterisk (*) included after the source indicates antibodies that were custom-conjugated using Fluidigm antibody labeling kits, per manufacturer's instructions. NA, not applicable.

Supplemental Table 2: Antibodies for negative selection of NK cells by FACS. The fluorescent tag, marker name, and source are shown for the antibodies used for negative selection of NK cells for the scRNA-seq analyses.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: ML NK cell product characteristics. A. ML NK cell product was assessed by flow cytometry prior to infusion. Graph shows percentage of cells in the final cellular product that were viable and expressed the indicated markers. **B and C.** Total number (B) and dose (cells/kg; C) of ML NK cells infused into each patient. Each colored symbol represents an individual patient. Mean and SE are shown.

Supplemental Figure 2: Overall survival of patients treated with DLI + ML NK cell immunotherapy. Kaplan-Meier curve of overall survival data for all patients treated with ML NK cells is presented.

Supplemental Figure 3. NK cells expand in vivo in patients treated with donor-derived ML NK cells. Peripheral blood samples from patients were analyzed with flow cytometry. **A.** Percentage of NK cells (CD56) by flow cytometry over time are shown for each patient at indicated time points post NK cell infusion. **B.** NK cell (CD56) numbers (cells/μL) over time are shown for each patient at various time points. Numbers calculated by multiplying percentage measured in A by absolute lymphocyte count determined by peripheral blood complete blood count. **C** and **D.** Summary of mean percentage (C) and number (D) of NK cells in peripheral blood of all patients at indicated time points as measured in panels A and B, respectively. Data are mean ± SEM. P-ML004 is not shown or included in summary data due to disease progression prior to day 7 and correlative sample collection.

Supplemental Figure 4. ML NK cells are distinct from CD56^{dim} NK cells at D28. Patient peripheral blood NK cells analyzed as in Figure 2. ML NK and CD56^{dim} compared from the same sample. **A**. Density viSNE plot of NK cells from patient peripheral blood at day 28 post NK cell

administration. **B.** Overlay viSNE from A. **C**. Representative histograms of indicated markers on CD56dim- and ML NK-gated NK cells from patient peripheral blood at day 28. **D** and **E**. Summary of median (**D**) and percentage positive (**E**) on $CD56^{dim}$ compared to ML NK cells. For panels D and E, data are presented as mean and SEM from patients P-ML002, P-ML003, P-ML005, P-ML008, P-ML009, and P-ML0011. Data were tested for normal distribution (Shapiro-Wilk) and then compared using paired T or Wilcoxon matched-pairs signed rank test. P-value is indicated (n.s. = not significant).

Supplemental Figure 5. Serum IL-2 levels are not altered after DLI and NK cell infusion. IL-2 in patient serum collected at the indicated timepoints was measured by MILLIPLEX Human Cytokine/Chemokine/Growth Factor Panel A, per manufacturer's directions. Each line represents an individual patient. Samples were run in duplicate and error bars represent range.

Supplemental Figure 6. ML NK cells are transcriptionally distinct from conventional NK cells. Patient peripheral blood was analyzed as in Figure 4. scRNA-seq was performed on enriched NK cells and subpopulations were identified by unsupervised cluster analysis. Data is shown for P-ML002. **A**. UMAP visualization of NK cells at the indicated timepoints. Panels are colored by timepoint overlaid on composite data from all timepoints (gray). ML NK cells are designated by blue dashed lines on UMAPs. **B.** UMAP of indicated NK cell populations within composite data from all timepoints in A. **C**. Expression of key NK cell population identifying genes. Cells are ordered on the UMAP by expression level. Black gate denotes ML NK cell population. **D**. Percentage of $CD56^{bright}$, $CD56^{dim}$, and ML NK cells in the donor product (baseline, pre-infusion) and from the patient at days 28 and 50 post-infusion.

Supplemental Table 1: NK cell phenotypic and functional mass cytometry panels.

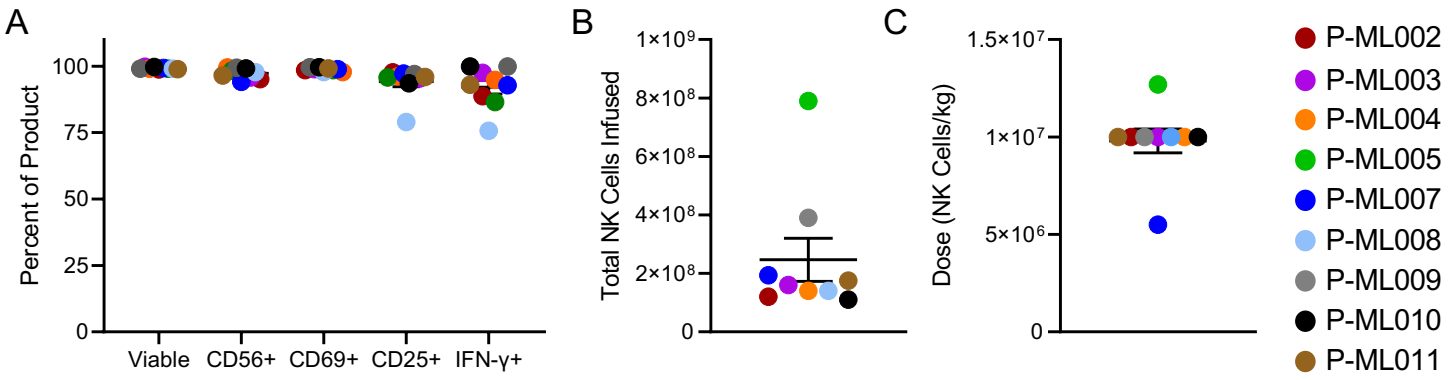
Tag	Antibody	Clone	Source	Panel	Clustering
89 Y	CD45	HI30	Fluidigm	F/P	Lymph
141 Pr	CD14	M5E2	BD Pharmingen*	F/P	Lymph
142 Nd	CD19	HIB19	Fluidigm	F/P	Lymph
143 Nd	KIR3DL1	DX9	R&D*	F/P	NK
145 Nd	KIR2DS4	FES172	Beckman Coulter*	F/P	NK
146 Nd	KIR2DL1/2DS1	EB6B	Beckman Coulter*	F/P	NK
147 Sm	NKG2D	1D11	R&D*	F/P	NK
148 Nd	KIR2DL2/2DL3	CH-L	BD Pharmingen*	F/P	NK
149 Sm	CD127	AO19D5	Fluidigm	P	Lymph
149 Sm	T-Bet	4B10	BD Pharmingen*	F	NA
150 Nd	CD4	OKT4	Miltenyi*	P	Lymph
150 Nd	Mip1a	93342	R&D*	F	NA
151 Eu	TRAIL	RIK-2	Biolegend*	P	NK
151 Eu	CD107a	H4A3	Fluidigm	F	NA
152 Sm	CD8	SK1	eBioscience*	P	Lymph
152 Sm	TNF	Mab11	Fluidigm	F	NA
153 Eu	CD62L	DREG-56	Fluidigm	F/P	NK
154 Sm	KIR2DL5	UP-R1	Beckman Coulter*	F/P	NK
155 Gd	CD27	L128	Fluidigm	F/P	NK
156 Gd	PDL1/PDL2	29E.2A3	Fluidigm	P	NA
158 Gd	CD137	4B4-1	Fluidigm	F/P	NK
159 Tb	NKG2C	134591	R&D*	F/P	NK
160 Gd	CD69	FN50	Biolegend*	F/P	NK
161 Dy	NKp30	P30-15	Biolegend*	F/P	NK
162 Dy	KI-67	B56	Fluidigm	P	NK
162 Dy	LAG3	11C3C65	Biolegend*	F	NA
163 Dy	CD94	DX22	Biolegend*	F/P	NK
164 Dy	FoxP3	PCH101	Invitrogen*	P	NA
164 Dy	Tim-3	F38-2E2	Biolegend*	F	NA
165 Ho	CD16	3G8	Fluidigm	F/P	Lymph, NK
166 Er	NKG2A	ZI99	Beckman Coulter*	F/P	NK
167 Er	NKp44	P44-8	Biolegend*	F/P	NK
168 Er	DNAM1	DX11	Miltenyi*	P	NK
168 Er	IFN-γ	B27	Fluidigm	F	NA
169 Tm	CD25	2A3	Fluidigm	P	Lymph, NK,
169 Tm	Eomes	WD1928	Invitrogen*	F	NA

170 Er	CD34	581	Invitrogen*	F/P	Lymph
171 Yb	Granzyme B	GB11	Fluidigm	P	NK
171 Yb	PD-1	EbioJ105	Invitrogen*	F	NA
172 Yb	CD57	HCD57	Fluidigm	F/P	NK
173 Yb	CD3	UCHT1	BD Pharmingen*	F/P	Lymph
174 Yb	NKp46	9E2	R&D*	F/P	NK
175 Lu	Perforin	B-D48	Fluidigm	F/P	NK
176 Yb	CD56	NCAM16.2	Fluidigm	F/P	Lymph, NK
209 Bi	CD11b	209Bi	Fluidigm	F/P	Lymph, NK

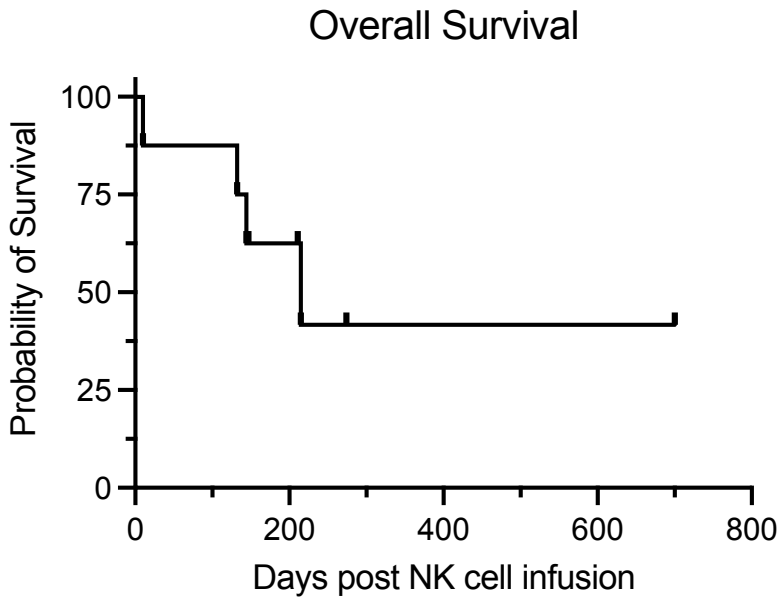
Supplemental Table 2: Antibodies for negative selection of NK cells by FACS.

Tag	Antibody	Source
	Zombie NIR viability dye	Biolegend (Cat: 423106)
BV510	CD14	Biolegend (Cat: 301842)
ECD	CD19	Beckman Coulter (Cat: IM2708U)
FITC	CD33	BD Pharmingen (Cat: 555626)
PE	CD3	eBioscience (Cat: 12-0038-42)

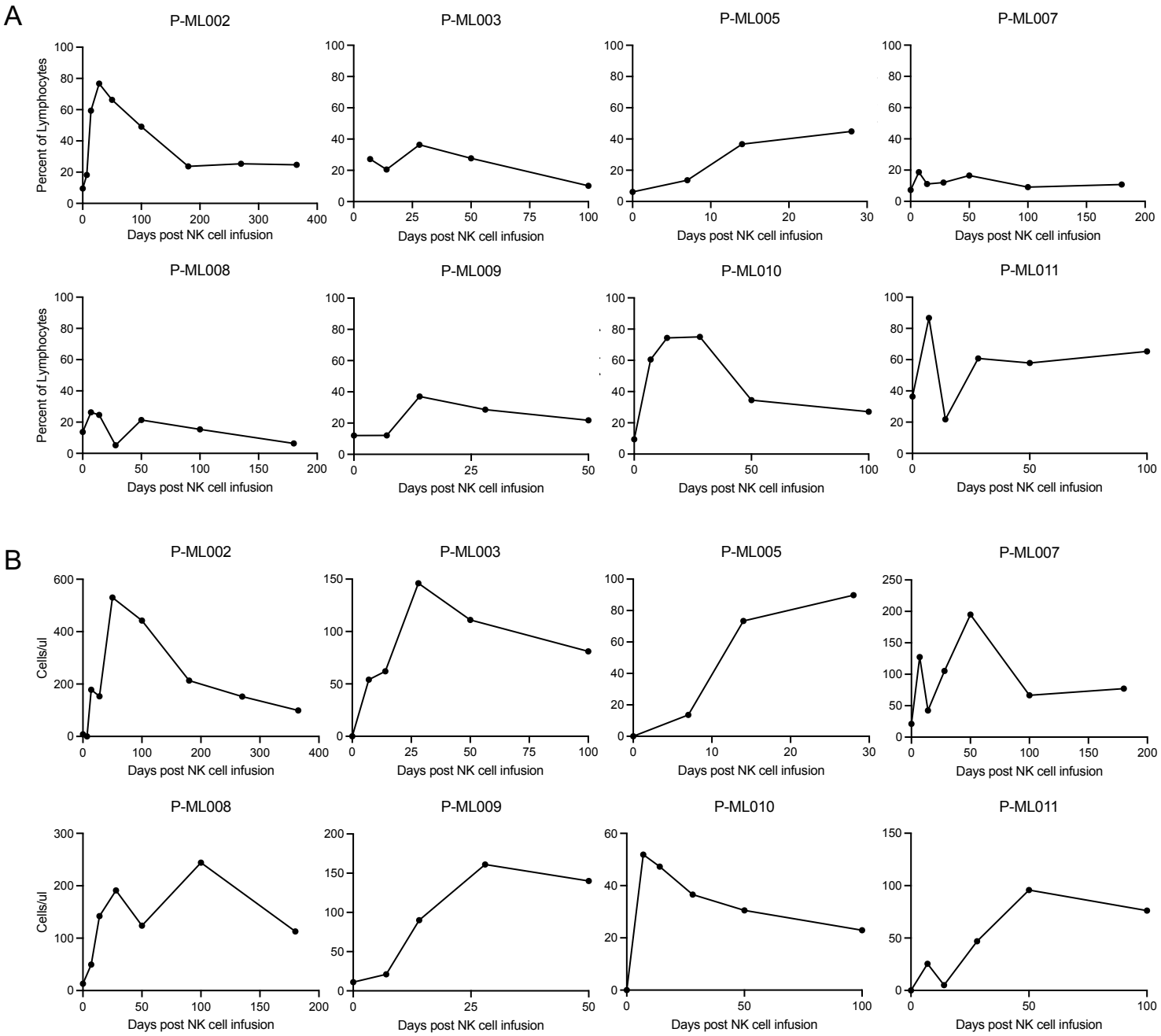
Supplemental Figure 1



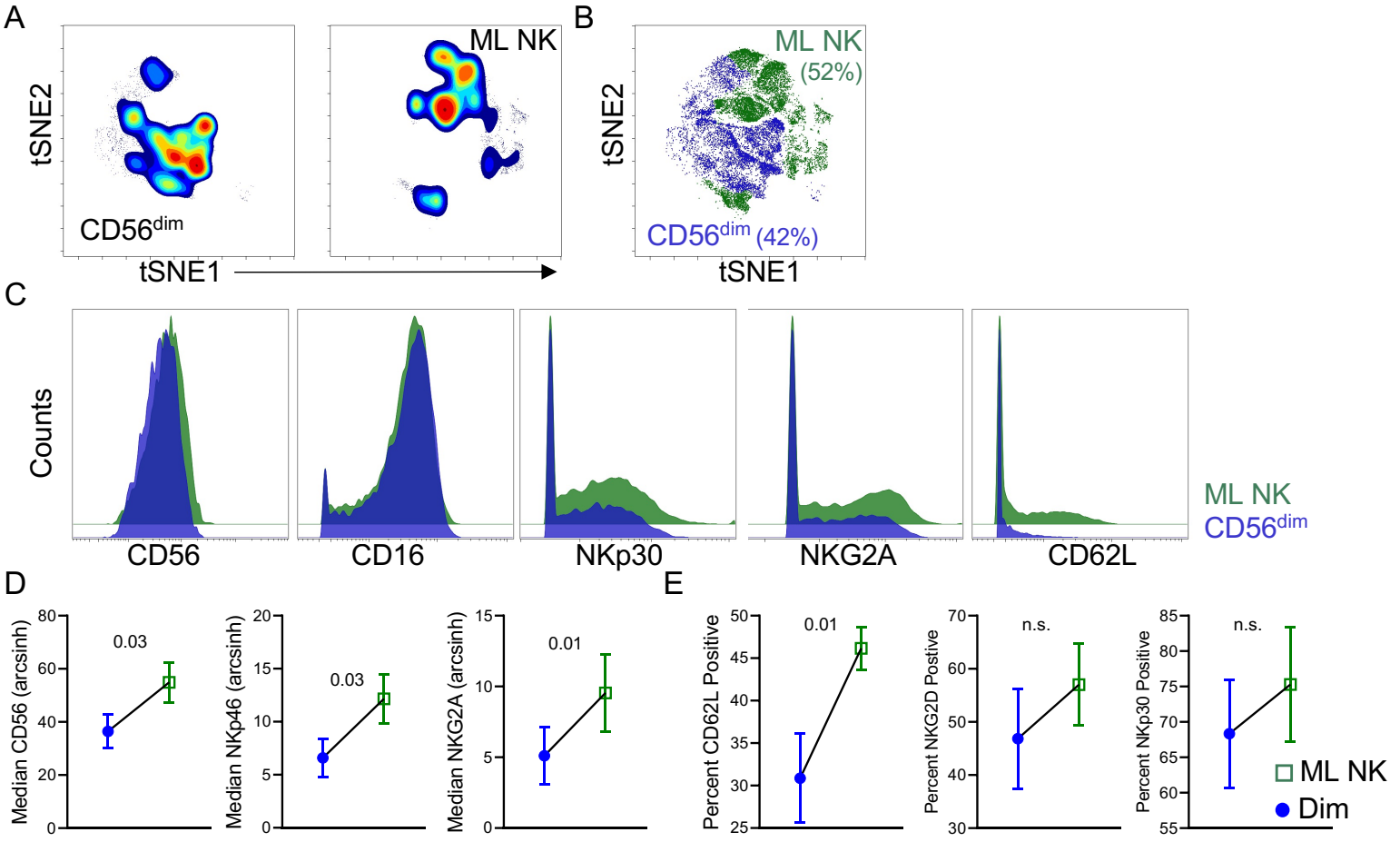
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



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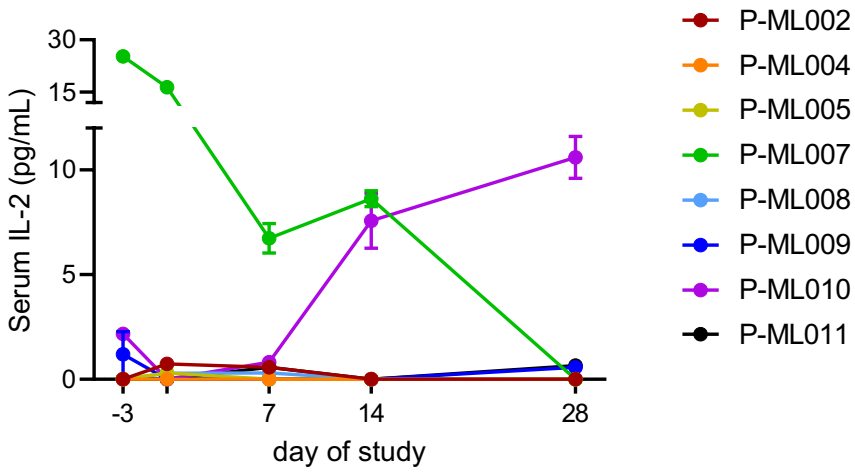
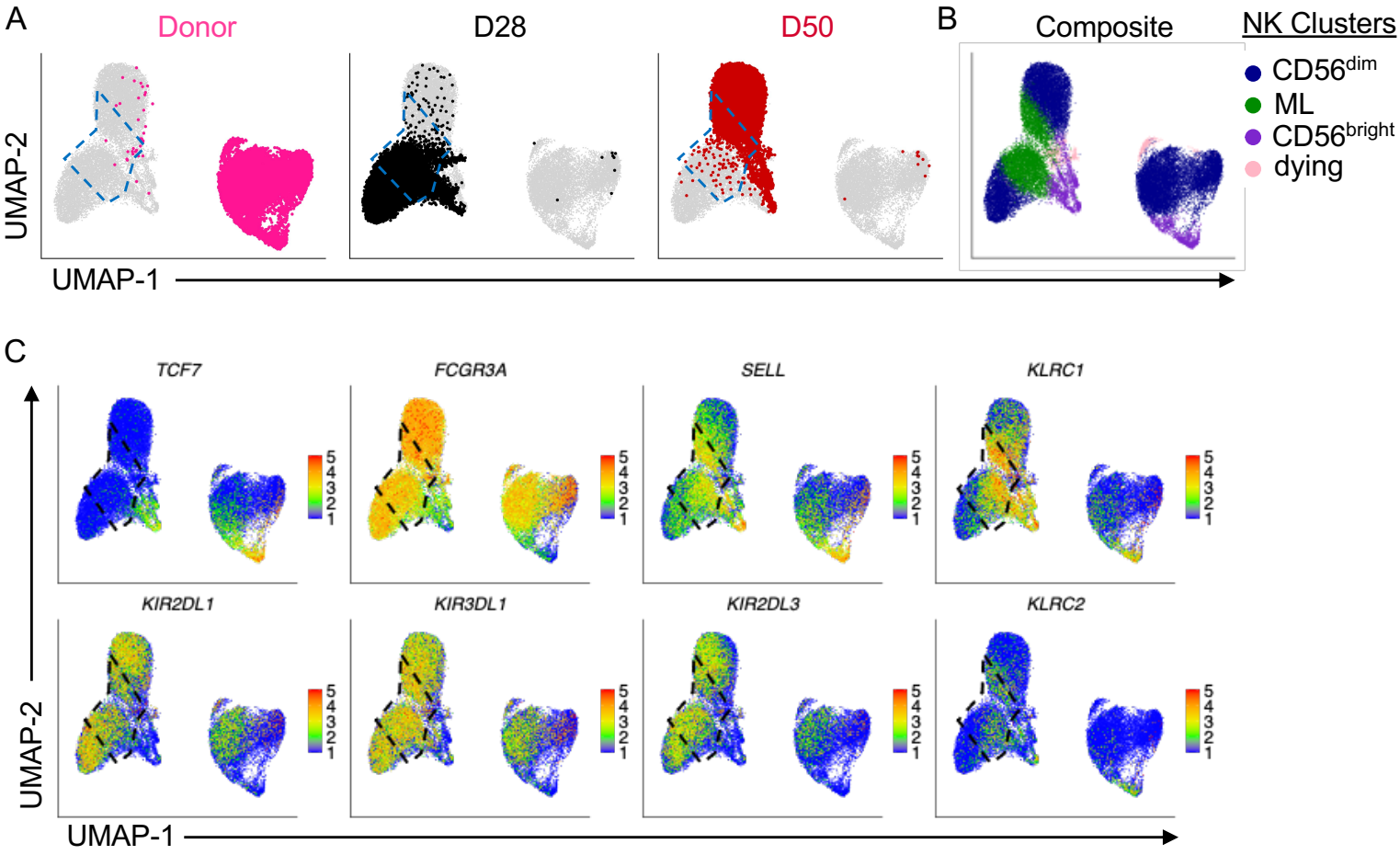


Supplemental Figure 6



SUPPLEMENTAL APPENDIX

***Clinical trial synopsis*:**

Title: A Pilot Study of Cytokine Induced Memory-like NK Cell Adoptive Therapy for Relapsed AML after Allogeneic Hematopoietic Cell Transplant in Children and Young Adults

Clinicaltrials.gov: NCT03068819

Institutional IRB protocol #: 201709041

Patient inclusion criteria:

- Relapsed AML after HLA-matched or HLA-mismatched related or unrelated allogeneic hematopoietic cell transplant
- ≥1 and ≤30 years of age
- Available original donor from initial stem cell transplant that is eligible for collection
- Patients with known CNS AML disease are eligible provided that they have been treated and CSF is clear for at 2 weeks prior to enrollment into the study
- Karnofsky performance status > 60 %
- Adequate organ function as defined below:
    - Total bilirubin < 2 mg/dl
    - AST(SGOT)/ALT(SGPT) < 3.0 x IULN
    - Creatinine within normal institutional limits OR creatinine clearance > 60 mL/min/1.73 m^2 by Cockcroft-Gault Formula
    - Oxygen saturation ≥90% on room air
- Not currently requiring systemic corticosteroid therapy (10 mg or less of prednisone) or any other immune suppressive medications
- Women must have a negative pregnancy test within 28 days
- Ability to understand and sign an IRB approved written informed consent document

Patient exclusion criteria:

- Acute or chronic GVHD with ongoing active systemic treatment
- Circulating blast count >30,000/μL by morphology or flow cytometry
- Uncontrolled bacterial or viral infections
- Cardiac abnormalities
- New or progressive pulmonary infiltrates
- Known hypersensitivity to one or more of the study agents
- Received any investigational drugs within the 14 days prior to CIML NK cell infusion date
- Pregnant and/or breastfeeding

Donor inclusion criteria:

- At least 2 years of age
- Donor weight must be at least 15 kg
- Same donor as used for the allo-HCT

- In general good health, and medically able to tolerate leukapheresis
- Ability to understand and sign an IRB approved written informed consent document

Donor exclusion criteria:
- Active hepatitis, positive for HTLV, or HIV on donor viral screen
- Pregnant and/or breastfeeding

Salvage chemotherapy:
Patients received standard of care salvage chemotherapy with FLAG (fludarabine, cytarabine and filgrastim) 2-4 weeks prior to receiving the ML NK cell infusion. Fludarabine (30 mg/m2 per dose) was given daily for a total of 5 doses administered as an IV infusion over 30 minutes. Cytarabine (2000 mg/m2 per dose) was given daily for a total of 5 doses administered as IV infusion over 3 hours, starting the same day as fludarabine. Filgrastim (5 mcg/kg per dose to a maximum of 300 mcg) was given subcutaneously daily for a total of 5 doses starting the same as fludarabine and cytarabine above. Standard supportive care was given as needed.

Donor cell graft:
Within 24 hours of planned cellular therapy, original donor underwent standard non-mobilized PBSC collection via standard 3x volume leukapheresis. Collection goal was 2 x 10^{10} total nucleated cells.

Donor lymphocyte infusion:
Following collection, patient immediately received DLI with 1 x 10^6 $CD3^+$ T cells/kg per standard of care institutional guidelines. DLI was infused without a filter or pump, slowly by gravity per standard of care.

Donor ML NK cell generation and infusion:
After DLI infusion, remaining apheresis product was enriched for NK cells by CD3 depletion and CD56 positive selection using Miltenyi's CliniMACS®. NK cell fraction ($CD3^-CD56^+$ cells) was activated for 12-18 hours with rhIL-12 (10 ng/ml), rhIL-15 (50 ng/ml) and rhIL-18 (50 ng/ml) under GMP conditions. ML NK cells were infused on day 0 (approximately 24 hours after DLI). Maximum number of ML NK cells infused was capped at 10 x 10^6/kg (minimum: 0.5 x 10^6/kg). ML NK cells were infused without a filter or pump, slowly by gravity over at least 15-30 minutes. Patients were premedicated with acetaminophen and diphenhydramine within 1 hour before and 4 hours after cell infusion. Demerol was given as needed for chills/rigors during infusion. Standard supportive care was given as needed. Vital signs are obtained before infusion and every 15 minutes during the infusion, and then 30 minutes and 1 hour after the infusion.

Disease assessment and correlative studies:
Peripheral blood was collected at screening, day -3, 0, 7, 14, 28, 50, 100, 6 months, 9 months, and 12 months. Bone marrow aspirate was collected at screening, day 7, 14, 28, and 100 and any clinically indicated bone marrow collection.

Adverse events:
Adverse events were grade as defined by the CTCAEv4.0.

Response criteria:
Clinical response was defined by IVG criteria.[50]

- *Complete remission (CR)*: Morphologically leukemia free state and absolute neutrophil count ≥1000 /μL and platelets ≥100,000 /μL. Patient must be independent of transfusions.
- *Complete remission with incomplete count recovery (CRi)*: Meets criteria for CR except that absolute neutrophils <1000 /μL or platelets <100,000 /μL in the blood.
- *Partial response (PR)*: Decrease of at least 50% in the percentage of blasts to 5% to 25% in the bone marrow and recovery of absolute neutrophils and platelets as for CR.
- *Treatment failure (TF)*: Patient survives > 28 days from the ML NK cell infusion with persistent leukemia in the peripheral blood or bone marrow (>25% blasts), or with persistent extramedullary disease, but without further clinical progression of leukemia.
- *Progressive disease (PD*): Increase of blast population in bone marrow or peripheral blood by >10% or aggravation or new development of extramedullary disease or further deterioration or death due to leukemia.