CD56 as a marker of an ILC1-like population with NK cell properties that is functionally impaired in AML

Manuscript ADVANCES/2018/030478 by Salomé et al.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL METHODS

Human cell collection

Buffy coats were obtained from healthy donors (HD: n=128) at the local Blood Transfusion Center, Lausanne, Switzerland; peripheral blood samples were obtained from AML patients at diagnosis (n=80) or remission (n=14), from healthy children, from patients with severe combined immunodeficiency (SCID) (EMATC-2013-01, EC 11-09-2006, and 1237-25090, Study 105, 17971-2015). Fresh tissues were obtained from recently deceased Healthy Donors (HDs) (VD431/13). Fresh skin, fresh bone marrow fine needle aspirations, cord blood and thymus samples, fetal bone marrow, liver and thymus samples were obtained under the EC approvals: VD2797; 265/12; EM186/2017/0. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation and frozen at -80°C (extracellular flow cytometry analysis for an evaluation of the ILC proportions and phenotype characterization) or immediately used (fluorescence activated cell sorting FACS, metabolism assays, intracellular flow cytometry analysis, CD107a degranulation assays). For the AML patients, we selected samples for the *in vitro* and *ex vivo* assays exclusively according to cell viability/counts.

Preparation of CD3-CD56+ -enriched PBMC fractions

The CD3 depletion and CD56 enrichment were consecutively executed using anti-CD3 and anti-CD56 microbeads (Miltenyi), to obtain the CD3-CD56+ fractions of HD PBMCs as previously described in NK adoptive transfers for patients[1–4]. The CD3-CD56+ fractions were analyzed *ex vivo* by flow cytometry.

Flow cytometry analysis

The cells were stained in FACS buffer. The following lineage markers were used for ILC/NK cell identification: FITC-anti-CD3, -CD4, -CD8, -CD14, -CD15, -CD19, -CD20,

-CD33, -CD34, -CD203c, and -FcεRIα for the NK cells, anti-CD16 being added for the ILC staining. A reduced lineage cocktail was used for metabolism assays: APC-anti-CD15, -CD33, Krome Orange-anti-CD3, -CD4, -CD8, -CD14 with/without addition of ECD-anti-CD16. Anti-CD127, -CRTH2, -cKit and -CD56 antibodies were added for the ILC/NK cell staining. As indicated, antibodies directed against specific markers of interest were added.

Second, the cells were stained with the viability dye VIVID (Life technologies). For intracellular analyses, the cells were additionally fixed and permeabilized and stained with antibodies directed against intracellular markers.

For metabolic studies, the cells were incubated with 0.5 µM fatty acids (BIODIPY 500/510 C1,C12) or 100 nM MitoTracker dyes (MitoTrracker Green FM, MitoTracker Deep Red FM, Thermo Fisher). For the glucose analogue uptake, the cells were incubated in Krebs-Ringer Hepes buffer. 2-NBDG (Thermo Fisher) was then added at 100 µM for 10 minutes at 37°C.

For the AICL staining, the cells were incubated with Fc receptor blocking reagent (Miltenyi), anti-AICL mouse IgG1 7F12[21] or purified mouse IgG1 (Beckman Coulter) was added, cells were then washed and stained with a secondary APC anti-mouse IgG1.

For the HLA-E staining, the CD33+ fraction of PBMCs obtained from AML patients was incubated with an anti-HLA-E antibody.

A Gallios flow cytometer (Beckman Coulter), a LSRFortessa (BD Biosciences) and FlowJo software (Treestar) were used for flow cytometry analyses. The ILCs were sorted using MoFlo Astrios (Beckman Coulter) or BD FACSAria III or II (BD Biosciences). An ImageStream X Mark II imaging flow cytometer (Amnis) was used for imaging.

Antibodies used for flow cytometry analyses

Antibodies directed against lineage markers	Anti-CD3	UCHT1	FITC	Beckman Coulter
			Krome Orange	
	Anti-CD4	13B8.2	FITC	Beckman Coulter
			Krome Orange	
	Anti-CD8	LT8	FITC	AbD Serotec
		B9.11	Krome Orange	Immunotech
	Anti-CD14	RMO52	FITC	Beckman Coulter
		RMO52	Krome Orange	
	Anti-CD15	80H5	FITC	Beckman Coulter
		HI98	APC	BD Biosciences
	Anti-CD16	3G8	Krome Orange	Beckman Coulter
			FITC	Beckman Coulter
			Brilliant Violet 510	BioLegend
			ECD	Beckman Coulter
	Anti-CD19	J3-119	FITC	Beckman Coulter
	Anti-CD20	2H7	FITC	BioLegend
	Anti-CD33	HIM3-4	FITC	BioLegend
		WM-53	APC	BD Biosciences
	Anti-CD34	561	FITC	BioLegend
	Anti-CD203c	NP4D6	FITC	BioLegend
	Anti-FcεRIα	AER-37	FITC	BioLegend
Antibodies used for ILC and NK subset discrimination	Anti-CD127	A019D5	Brilliant Violet 421	BioLegend
		A019D5	PEDazzle	BioLegend
	Anti-CRTH2	BM16	Brilliant Violet 421	BD Biosciences
			PE-CF594	BD Biosciences
			PE	BioLegend
			Brilliant Violet 711	BioLegend
			PerCPCy5.5	BioLegend
	Anti-cKit	104D2	PE	BioLegend
		YB5.B8	APC	BD Biosciences
		104D2	Brilliant Violet 605	BioLegend
		104D2	APC/Fire 750	BioLegend
	Anti-CD56	QA17A16	PerCPCy5.5	BioLegend
		HCD56	Alexa 700	BioLegend
		5.1H11	Brilliant Violet 785	BioLegend
		CMSSB	APC-eFluor 780	eBioscience
Antibodies directed against specific markers of interest	Anti-TRAIL	RIK-2	PE	BioLegend
			APC	BioLegend
	Anti-DNAM-1	11A8	PerCPCy5.5	BioLegend
	Anti-NKp30	P30-15	PE	BioLegend
		REA823	PE-Vio615	Miltenyi Biotec
		P30-15	PE-Cy7	BioLegend
	Anti-NKp44	P44-8	APC	BioLegend
			PerCPCy5.5	BioLegend
	Anti-NKp46	9E2	PerCPCy5.5	BioLegend
			PC7	BD Biosciences
	Anti-NKp80	5D12	PE	BioLegend
		REA845	APC-Vio770	Miltenyi Biotec
	Anti-CD57	HNK-1	PerCPCy5.5	BioLegend
	Anti-CD94	HP-3B1	PE	Immunotech

		DX22	PerCPCy5.5	BioLegend
	Anti-NKG2A	REA110	PE-Vio770	Miltenyi Biotec
	Anti-NKG2C	REA205	APC	Miltenyi Biotec
	Anti-HLA-A, -B, -C	W6/32	PerCPCy5.5	BioLegend
	Anti-CD158a/h	11PB6	PE	Miltenyi Biotec
	Anti-CD158b	DX27	PE	Miltenyi Biotec
	Anti-CD158e	DX9	PE	Miltenyi Biotec
	Anti-CD158i	JJC11.6	PE	Miltenyi Biotec
	Anti-CD69	FN50	APC	BD Biosciences
	Anti-CD103	Ber-ACT8	PE-Cy7	BioLegend
	Anti-CD49a	SR84	PE	BD Biosciences
	Anti-HLA-E	3D12	PE-Cy7	BioLegend
Antibodies used for intracellular stainings	Anti-granzyme A	CB9	Alexa-700	BioLegend
	Anti-granzyme B	CLB-GB11	PE	Hölzel Diagnostica
	Anti-granzyme K	G3H69	PerCP-eF710	eBioscience
	Anti-granzyme M	4B2G4	eFluor660	eBioscience
	Anti-perforin	dG9	APC	BioLegend
	Anti-granulysin	DH2	PE	eBioscience
	Anti-Tbet	O4-46	PerCPCy5.5	BD Biosciences
	Anti-Eomes	WD1928	eFluor660	eBioscience
	Anti-RORγt	Q21-559	PE	BD Biosciences
	Anti-PLZF	R17-809	Alexa Fluor 647	BD Biosciences
	Anti-GATA3	L50-823	PE-Cy7	BD Pharmingen

IFNγ and CD107a degranulation assays

The PBMCs were depleted of leukemic blasts using anti-CD33 microbeads (Miltenyi) prior to the enrichment when from AML patients. The cells were then enriched with ILC and NK cells using a Human NK Cell Enrichment Kit (STEMCELL Technologies). For the CD107a assay, the enriched cells were stimulated overnight with IL-2 (200 IU/mL) and then cocultured during 4 hours with the K562 or U937 lines, or autologous blasts in the presence of Brilliant-Violet 510-anti-CD107a (BioLegend) and Golgi stop (BD Biosciences). When precised, the AhR agonist FICZ (Sigma Aldrich) was added during the overnight culture at 0.3 nmol/mL. For the IFNγ assay, the enriched cells were cultured with the AhR agonist FICZ at 0.3 nmol/mL or DMSO during 48h. IL-12, IL-15, and IL-18 (each cytokine being at 5 ng/mL) were added after 24h and BrefeldinA (BioLegend) was added during the last 4 hours.

Cell culture

The tumor cell lines BJAB, U937, K562, Wild Type (WT) 721.221 and HLA-E-transfected 721.221 were maintained in 10% fetal calf serum medium.

mRNA sequencing

Pure cell subsets were sorted in RNAlater (Thermo Fisher) and frozen (-80°C). The mRNA was extracted (RNeasy Microkit, Qiagen), cDNA was prepared (SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing, Clontech) and the quality verified by Qubit and Fragment Analyzer (Nextera XT DNA Library Preparation Kit, Illumina; Advanced Analytical Technologies) prior and after the cDNA preparation for sequencing. The samples were single-read sequenced on an Illumina HiSeq 2500 device at the Lausanne Genomic Technology Facilities at a read-length of 100 base pairs.

The raw sequencing reads were trimmed to remove the adapters and filtered for quality (Cutadapt v..1..3[5]). The reads were aligned (STAR v.2.4.2a[1–46]) against the human genome (*Homo sapiens.GRCh38.82)* and their number was summarized (htseq count v.0.6.1). Further analyses were performed using R software v.3.3.2 and packages for R. Only the genes presenting more than 3 raw counts per sample were retained in the analysis (n=29,684). Raw counts were normalized into log2-counts per million (logCPM) using the voom function in the limma package[7]. The differential expression of a gene was considered at a p-value<0.05 The p-values were adjusted

using the Benjamini-Hochberg method[8]. Dimensionality reduction was performed using multidimensional scaling. The pheatmap package was used to create heatmaps of the z-scores of the gene expression levels.

Gene expression analysis of TCGA data of AML patients

We associated the HTSeq count data from TCGA (The Cancer Genome Atlas) with clinical data of AML patients (TCGA database used: "TCGA-LAML" project, Data Release 12.0-June 13, 2018). The count data were normalized (Trimmed Mean of M values normalization using the edge R package v3.22.5) and transformed into logCPM using the voom function implemented in the limma package 3.36.5 for R. We removed deceased patients with missing clinical data and patients with the acute promyelocytic leukemia (APL). The remaining patients (n=125) were divided into 2 groups depending on their TGFB1 expression level. The difference in survival between the two groups was determined using a Cox proportional hazards regression (survival package v2.42-6 for R).

Chromium release assays

The targets were loaded with ^{51}Chromium (^{51}Cr) (PerkinElmer) for 1 h at 37°C, while the effectors were incubated with rh-TRAIL R2-Fc-404 (clone 293[22]), anti-DNAM-1 (R&D systems), anti-NKp30 (F252) or anti-NKp80 (5D12[21]). Then, 500 targets were incubated with effectors at the indicated ratio during 4 h at 37°C, supernatant was transferred into LumaPlates and radioactivity counted using a TopCount (Perkin Elmer).

Stimulation of PBMCs from AML patients

PBMCs from patients were depleted of leukemia blasts using anti-CD33 microbeads (Miltenyi). Then, the CD33- fraction was incubated for 24 h in 8% human serum medium.

Impairment of NK receptors on HD PBMCs

HD PBMCs were enriched with ILC and NK cells with an EasySep Human NK Cell Enrichment Kit (STEMCELL Technologies). The enriched fraction was incubated for 24 h with hr-TGFβ1 (Peprotech) at 5 ng/mL.

Analysis of the serum TGF-β concentration

Sera were analyzed to determine the hr-TGFβ1 concentration using a LEGENDplex human FreeActive/Total TGFβ1 assay (BioLegend).

Humanized mouse model

Humanized mice (HIS mice) were generated by injecting 100 000 human CD34+ hematopoietic stem cells (Lonza) intrahepatically into NSG mice (NOD.Cg-$Prkdc_{scid}Il2rg_{tm1Wjl}$/SzJ; Jackson Laboratory stock numer 005557) that were maintained under pathogen-free conditions (License VD2797).

Luminex

Highly-pure ILC and NK cell subsets were FACS-sorted and cultured at a concentration of 5000 cells/mL in a 96-well plate with hr-IL12, -IL15, -IL18 (each at 50ng/mL). After 72h, supernatants were collected and frozen. Luminex assays were conducted using the 45-Plex Human ProcartaPlex™ Panel 1, according to the manufacturer's recommendations.

CD56+ ILC1-like cell culture on OP9 stromal cells

Highly-pure CD56+ ILC1-like cells were FACS-sorted as described in Fig.S2A and cultured in 96-well plates with OP9 stromal cells (3000 cells/well, kindly provided by Dr. Hergen Spits, Amsterdam, The Netherlands) in 8% human serum medium supplemented with hr-IL-2 (20I U/mL) and hr-IL-7 (5 ng/mL) with or without hr-IL-15 (50ng/mL). The phenotype was analyzed after 10 days by flow cytometry.

Composition of the media and buffers

- FACS Buffer : PBS, 50 µM EDTA, 0.2% BSA
- 10% fetal calf serum medium : RPMI-1640 (Gibco 61870-010) with 10% fetal calf serum, penicillin-streptomycin (1%, Gibco 15140-114), arginine, asparagine, L-glutamine, ciproxin (5 µg/mL, Bayer infusion CAT), and Hepes buffer (1 M, Gibco 15630-056).
- 8% human serum medium : RPMI-1640 (Gibco 61870-010) supplemented with 8% human serum, 100 U hr-IL-2/mL, nonessential amino acids (1%, Gibco 11140-035), sodium pyruvate (1%, Gibco 11360-039), penicillin-streptomycin (1%, Gibco 15140-114), L-glutamine (1%, Gibco 25030-024),

kanamycin (0.1 mg/mL, Gibco 15160-054) and 2β-mercaptoethanol (0.1%, Sigma-Aldrich M7522).

t-SNE and principal component analysis

t-SNE analysis was run using the R software and the R package “cytofkit”. Ggplot2 package was then used to represent the data. PCA component analysis was conducted using the R software and the “prcomp” function. The scatterplot 3D package was then used to visualize the results.

Statistics

GraphPad Prism 7 software was used to perform the statistical tests (multiple Holm-Sidak t-tests, Mann-Whitney unpaired t-tests and Wilcoxon paired t-tests). The p-values are represented as : * <0.05, ** <0.01, *** <0.001, and **** <0.0001.

For original data, please contact the corresponding author: camilla.jandus@gmail.com.

Fig.S1. Peripheral blood CD56+ ILC1-like cells do not express CD49a. Extracellular flow cytometry analysis of the CD49a expression by conventional ILC/NK subsets and CD56+ ILC1-like cells. **(A)** Representative results; **(B)** Summary of the results.

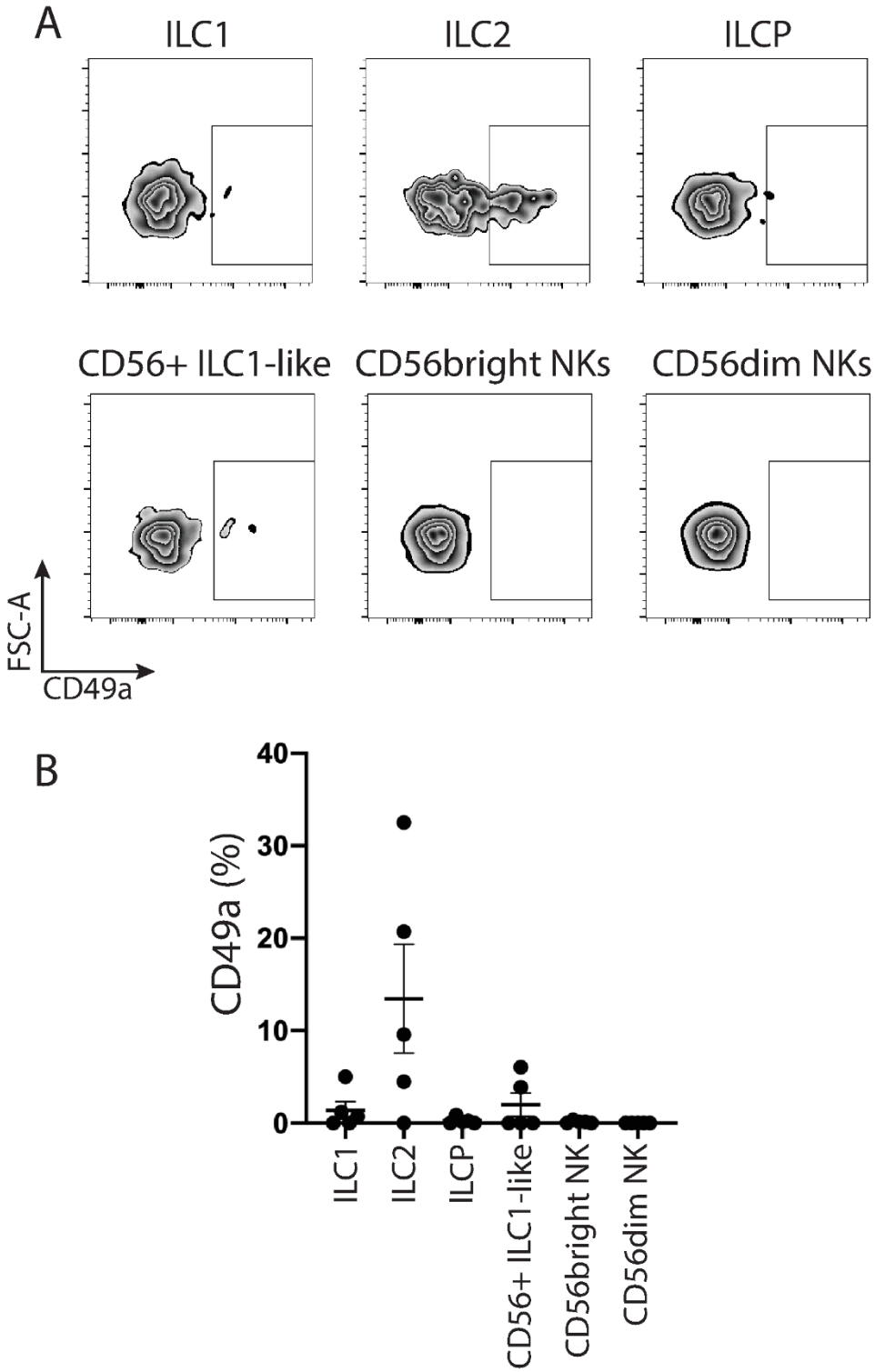

Fig.S2. CD56+ ILC1-like cell cytokine profile. (A-B) Quantification of soluble mediators secreted by CD56+ ILC1-like cells and cNKs was analyzed by Luminex assay after 72h culture with IL-12, IL-15 and IL-18 (50 ng/mL each). Multiple Holm-Sidak t-tests were used in A.

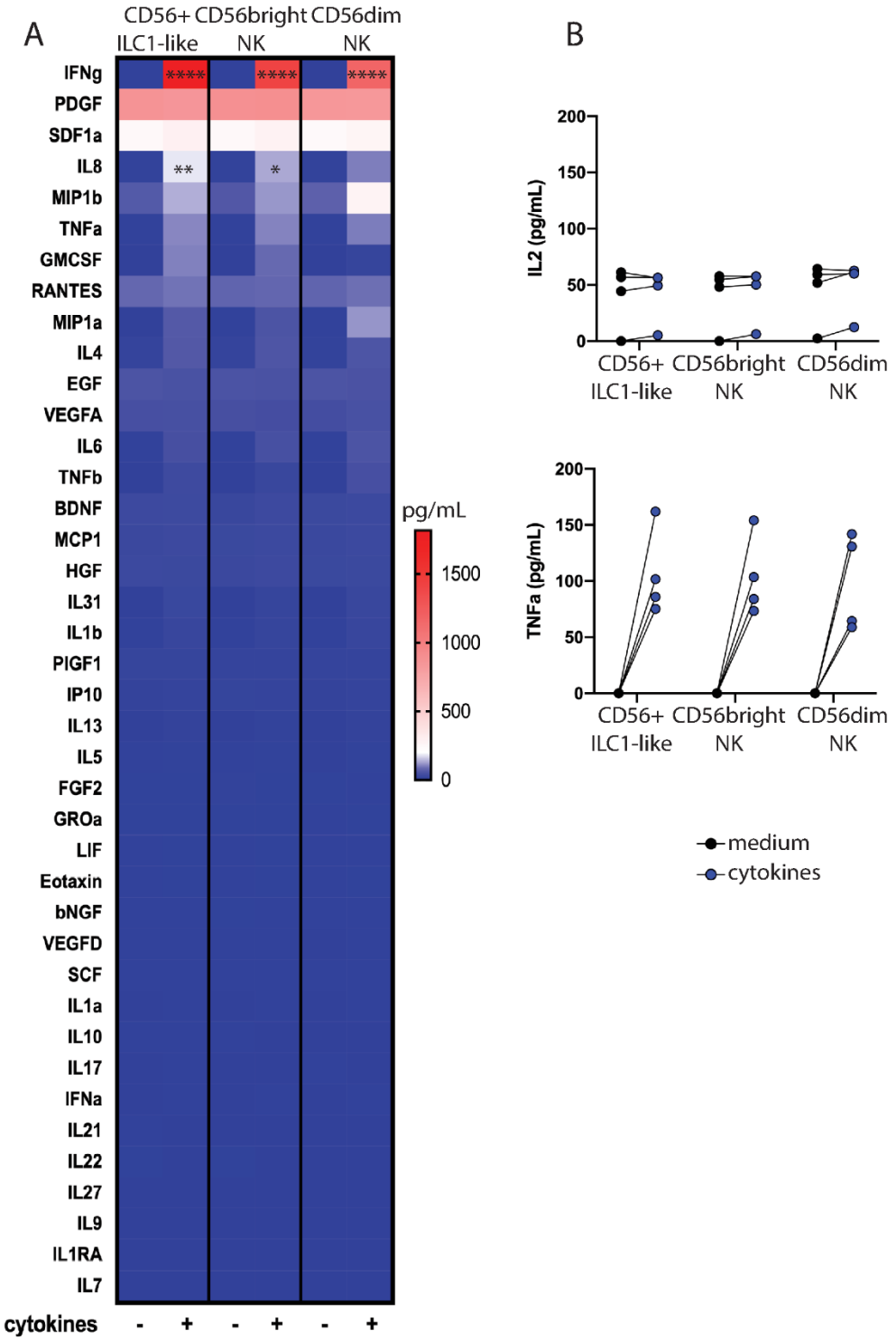

Fig.S3. Transcriptomic signature of *ex vivo* CD56+ ILC1-like cells in peripheral blood from healthy donors. (A) ILC and conventional NK cell subsets were sorted by fluorescence activated cell sorting from peripheral blood mononuclear cells of HDs (n=3) as schematically represented. Purity of *ex vivo* sorted cells (>95%) was assessed. Cell imaging was performed by ImageStream technology. **(B**) Heatmap of z-scores of the expression levels of genes encoding ILC/NK nomenclature markers (n=3). Variance explained (**C**) and component loading (**D**) for each component of the PCA analysis detailed in Figure 2A. (**E**) Intracellular flow cytometry analysis of transcription factors in CD56+ ILC1-like cells was performed and results used to conduct a principal component analysis on the ILC and NK subsets. Component loading (**F**) and variance explained (**G**) for each component of the PCA analysis presented in panel **E**. (**H**) T-bet and Eomes expression in ILCs and NK cells at protein-level, as assessed by intracellular flow cytometry.

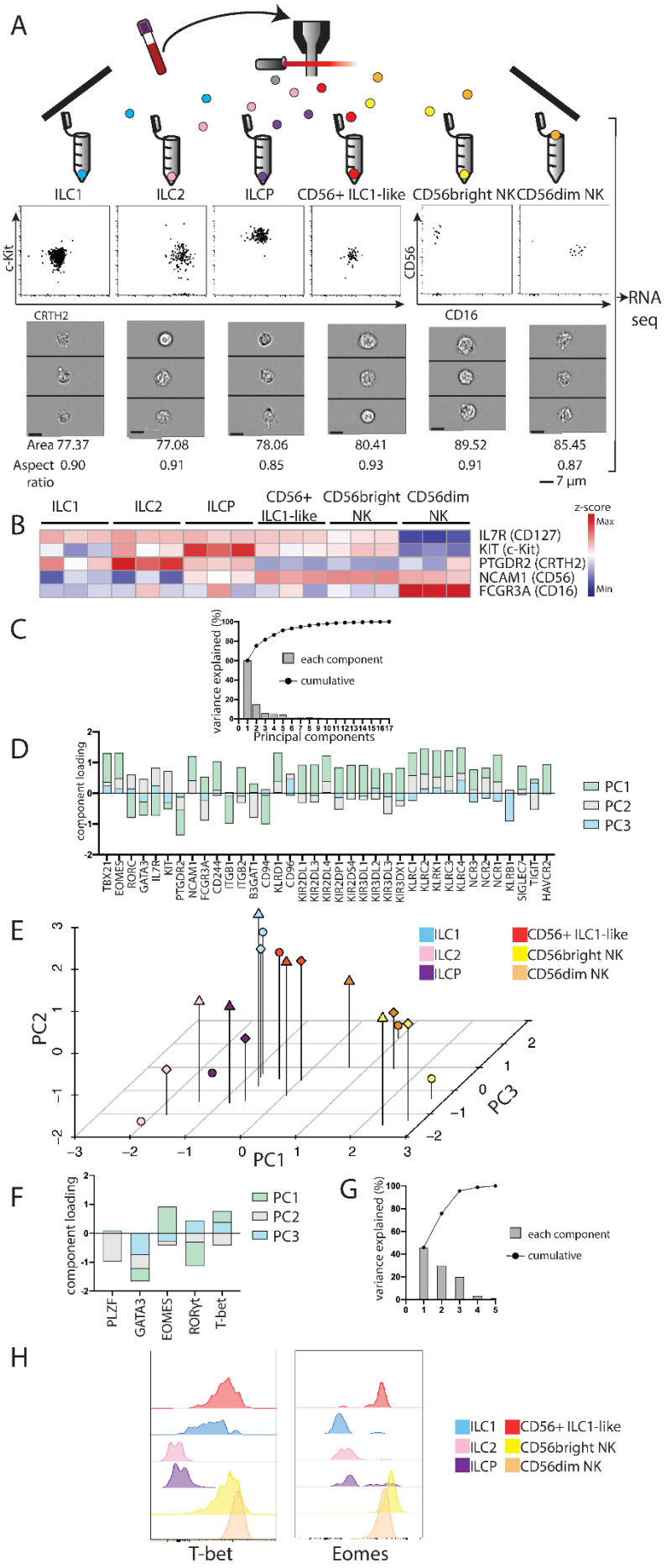
A
ILC1
ILC2
ILCP
CD56+ ILC1-like
CD56bright NK
CD56dim NK
c-Kit
CD56
RNA seq
CRTH2
CD16
Area 77.37 77.08 78.06 80.41 89.52 85.45
Aspect ratio 0.90 0.91 0.85 0.93 0.91 0.87
7 µm
B
ILC1
ILC2
ILCP
CD56+ ILC1-like
CD56bright NK
CD56dim NK
z-score
Max
Min
IL7R (CD127)
KIT (c-Kit)
PTGDR2 (CRTH2)
NCAM1 (CD56)
FCGR3A (CD16)
C
variance explained (%)
each component
cumulative
Principal components
D
component loading
PC1
PC2
PC3
TBX21 EOMES RORC GATA3 IL7R KIT PTGDR2 NCAM1 FCGR3A CD244 ITGB1 ITGB2 B3GAT1 CD94 KLRD1 CD96 KIR2DL1 KIR2DL3 KIR2DL4 KIR2DP1 KIR2DS4 KIR3DL1 KIR3DL2 KIR3DL3 KIR3DX1 KLRC1 KLRC2 KLRK1 KLRC3 KLRC4 NCR3 NCR2 NCR1 KLRB1 SIGLEC7 TIGIT HAVCR2
E
ILC1
ILC2
ILCP
CD56+ ILC1-like
CD56bright NK
CD56dim NK
PC1
PC2
PC3
F
component loading
PC1
PC2
PC3
PLZF GATA3 EOMES RORγt T-bet
G
variance explained (%)
each component
cumulative
H
ILC1
ILC2
ILCP
CD56+ ILC1-like
CD56bright NK
CD56dim NK
T-bet
Eomes

Fig.S4. CD56+ ILC1-like cells are metabolically distinct from conventional NK cells. PBMCs were incubated with glucose analog 2-NBDG, fatty acids (FA), MitoTracker deep red and MitoTracker green, and the dye uptake was analyzed by flow cytometry. **(A)** Representative histograms of dye uptake by HD total ILCs. (**B**) Summary of the results of the dye uptake by HD ILC and NK subsets. Dunn's corrected multiple comparison t-test was used for this analysis. (2NBDG: n=20, FA: n=19, MitoTracker dyes: n=17). B: Each dot represents 1 sample.

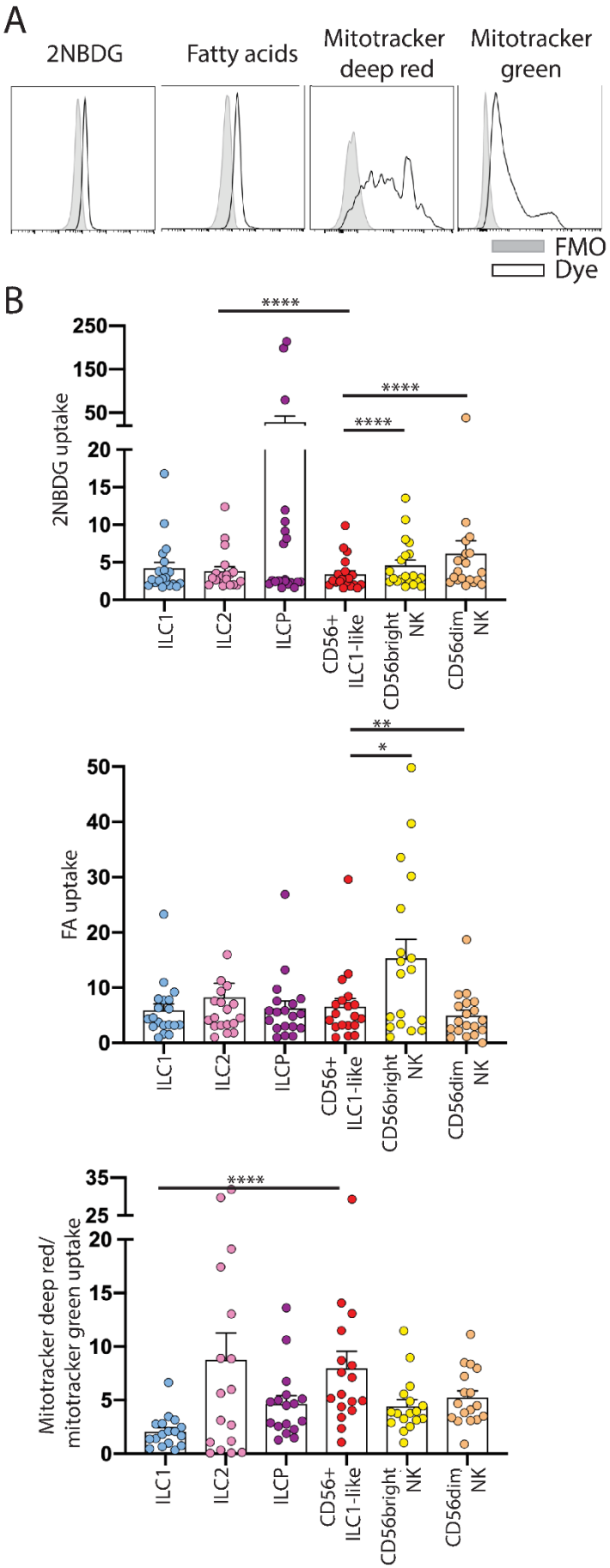
A
2NBDG
Fatty acids
Mitotracker deep red
Mitotracker green
FMO
Dye
B
2NBDG uptake
FA uptake
Mitotracker deep red/ mitotracker green uptake
ILC1
ILC2
ILCP
CD56+ ILC1-like
CD56bright NK
CD56dim NK
****
**
*

Fig.S5 CD56+ ILC1-like cell cytotoxic potential. Representative histograms of the flow cytometry analysis of **(A)** the intracellular expression of granyzmes, perforin and granulysin by CD56+ ILC1-like cells, ILC1 and NK cells. **(B)** Representative histogram of HLA-A, -B, and -C expression in the K562, BJAB and U937 tumor cell lines. (**C**) AICL expression in the K562 and U937 cell lines. (**D**) HLA-E expression in the Wild-type (WT) and HLA-E-transfected 721.221 tumor cell lines.

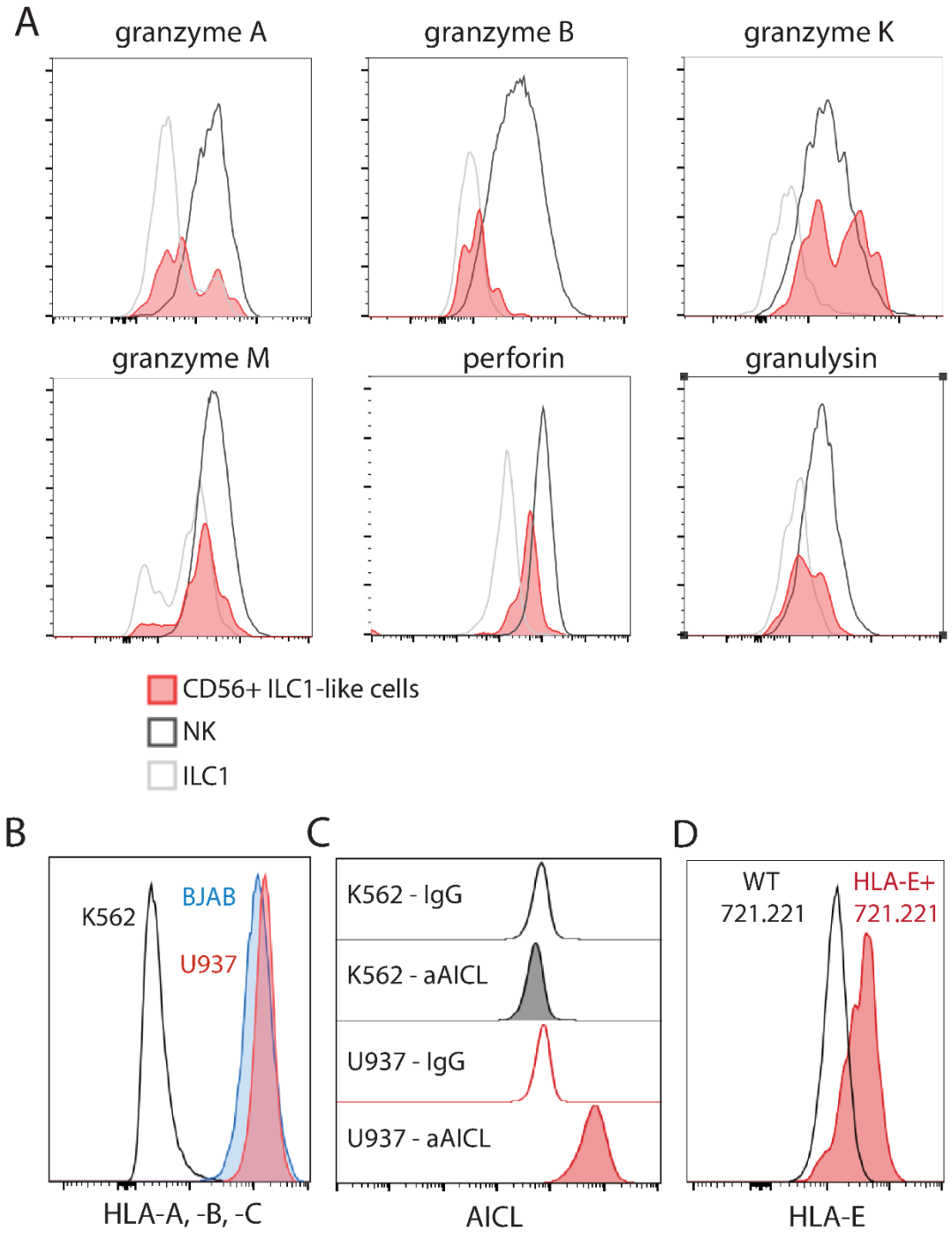

Fig.S6. CD56+ ILC1-like cell development. CD56+ ILC1-like cells were FACS-sorted and their phenotype analyzed after 10 days of culture on OP9 stromal cells in the presence of IL-7, with or without IL-15 (n=3). (**A**): Representative graphs of the phenotype after co-culture. (**B**) Summary of the data in the 3 donors upon different culture conditions. **(C)** CD56+ ILC1-like cell relative frequencies in human cord blood and fetal organs (n=2-9)**.** (**D**) Representative density plots of the extracellular flow cytometry panel used to identify ILCs in blood from humanized HIS mice. (**E**) Summary of the results of the ILC subset and conventional NK cell proportions in blood from HIS mice at 8, 12, or 16 weeks post-HSC injection. (**F**) ILC and NK cell subset frequencies among lymphocytes in peripheral blood of SCID patients. (**G**) t-SNE analyses of the ILC and NK compartments in peripheral blood of SCID patients based on CD127, CD56, CD16, CRTH2, c-Kit expression.

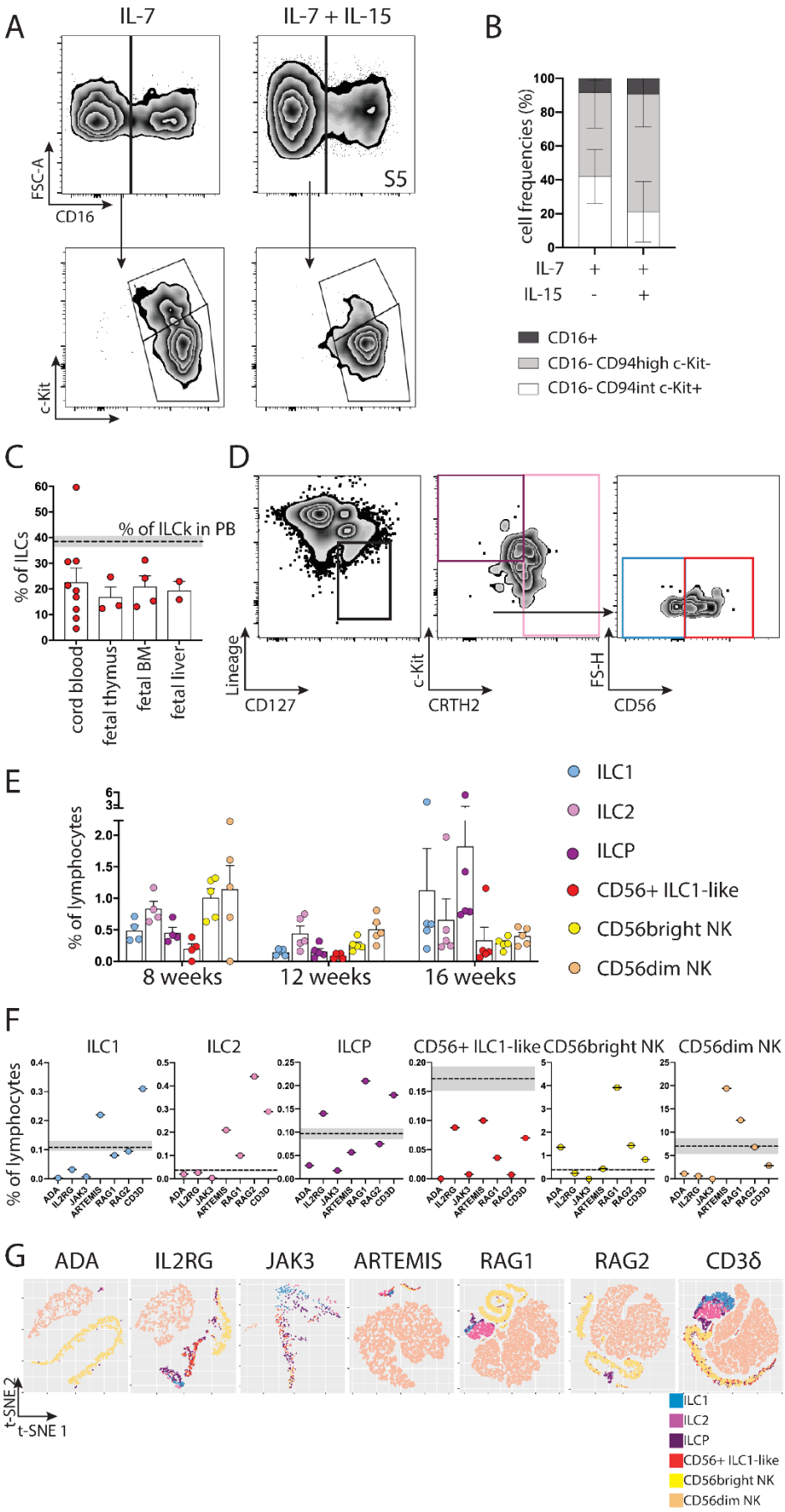
A
IL-7
IL-7 + IL-15
FSC-A
CD16
S5
c-Kit
B
cell frequencies (%)
IL-7
IL-15
CD16+
CD16- CD94high c-Kit-
CD16- CD94int c-Kit+
C
% of ILCs
% of ILCk in PB
cord blood
fetal thymus
fetal BM
fetal liver
D
Lineage
CD127
c-Kit
CRTH2
FS-H
CD56
E
% of lymphocytes
8 weeks
12 weeks
16 weeks
ILC1
ILC2
ILCP
CD56+ ILC1-like
CD56bright NK
CD56dim NK
F
ILC1
ILC2
ILCP
CD56+ ILC1-like
CD56bright NK
CD56dim NK
% of lymphocytes
ADA
IL2RG
JAK3
ARTEMIS
RAG1
RAG2
CD3D
G
ADA
IL2RG
JAK3
ARTEMIS
RAG1
RAG2
CD3δ
t-SNE 2
t-SNE 1
ILC1
ILC2
ILCP
CD56+ ILC1-like
CD56bright NK
CD56dim NK

Fig.S7. AhR agonist effect on CD56+ ILC1-like cell function. NK/ILC-enriched PBMCs were cultured overnight with IL-2 in the presence or absence of FICZ. CD56+ ILC1-like and cNK cell degranulation potential was evaluated after 4h co-culture with K562 in the presence of an anti-CD107a antibody and monensin (n=8) (**A**). (**B**) NK/ILC-enriched PBMCs were cultured during 36h in the presence or absence of FICZ. IL-12, IL-15 and IL-18 were added during the last 16 hours at a concentration of 5ng/mL and intracellular flow cytometry was performed to analyze IFN-γ production (n=9). Wilcoxon paired t-tests were used.

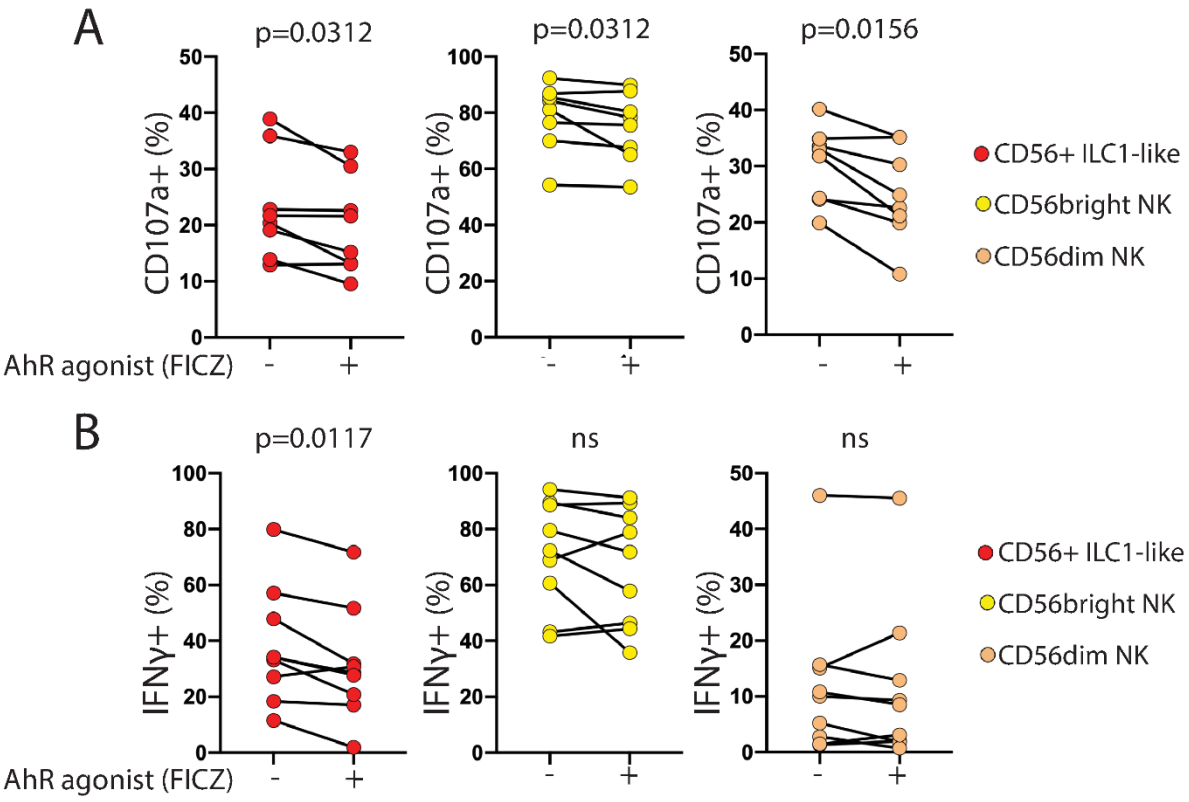

Table S1. AML patients' characteristics. IQR=Interquartile range. We excluded patients presenting a t(15;17) translocation, i.e., patients with acute promyelocytic leukemia as classified according to the 2017 European LeukemiaNet recommendations[9], from our cohort since this condition represents a distinct AML pathophysiological entity.

Patient information		Patients at diagnosis	Patients at remission
Number of patients		80	12
Age median, IQR (year)		64, 57-64	57, 50-71
Sex (n)	Male	46	6
	Female	34	6
Cytogenetic risk (n)	Low	9	2
	Intermediate	42	8
	High	22	2
	unknown	7	0
AML type (n)	M0	5	0
	M1	24	3
	M2	10	0
	M4	17	3
	M5	11	2
	M6	2	0
	M0/M1	3	0
	M4/M5	1	0
	M1/M2	2	1
	M2/M4	3	2
	unknown	2	1

SUPPLEMENTARY REFERENCES

1. Curti A, Ruggeri L, Parisi S, et al. Larger Size of Donor Alloreactive NK Cell Repertoire Correlates with Better Response to NK Cell Immunotherapy in Elderly Acute Myeloid Leukemia Patients. *Clin Cancer Res*. 2016;22(8):1914–21.
2. Rubnitz JE, Inaba H, Ribeiro RC, et al. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2010;28(6):955–9.
3. Bachanova V, Cooley S, Defor TE, et al. Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. *Blood*. 2014;123(25):3855–3863.
4. Shaffer BC, Le Luduec JB, Forlenza C, et al. Phase II Study of Haploidentical Natural Killer Cell Infusion for Treatment of Relapsed or Persistent Myeloid Malignancies Following Allogeneic Hematopoietic Cell Transplantation. *Biol Blood Marrow Transplant*. 2016;22(4):705–709.
5. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*. 2011;17(1):10–12.
6. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England)*. 2013;29(1):15–21.
7. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic acids research*. 2015;43(7):e47.
8. Yoav Benjamini YH. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistic Society. Series B (Methodological)*. 1995;57(1):289–300.
9. Dohner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129(4):424–447.