

Acute myeloid leukemia as a candidate for IL-1RAP CAR T cell immunotherapy

Chimeric antigen receptor T-cells targeting IL-1RAP: a promising new cellular immunotherapy to treat acute myeloid leukemia

Trad et al

Supplemental Materials and methods

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Supplemental material and Methods

Transcriptomic and RNAseq *in silico* analyses

Gene expression profiling (GEP) was performed using Human Genome U133 Plus 2.0 arrays (Affymetrix/ThermoFisher, Santa Clara, CA). Gene expression data available in the Gene Expression Omnibus (24) database were included for 32 peripheral blood samples from healthy donors (GSE48060: GSM1167102-21 and GSM1167123; GSE54992: GSM1327541,42,44,46,49,50, GSE20489: GSM514766,71,76,81,86) and 26 bone marrow samples from healthy donors (GSE3526: GSM80576, 577, 602-604; GSE18674: GSM463920; GSE32725: GSM813068-71; GSE33075: GSM818813-19,23,24; GSE41130: GSM1008985-91).

RNAseq dataset *in silico* analysis

Total single-strand RNAseq data were available for sorted samples from healthy donors (n= 9, 32 samples) and high risk AML patients (n=12, 49 samples) representing in total 81 samples.

RNAseq data: For the sorted RNAseq samples, data were downloaded from the GEO portal (accession number GSE74246). Raw data were aligned to the human reference genome (GRCh38) using Bowtie2. Transcripts were counted using HTSeq with Gencode v33 annotation, normalized to transcripts per kilobase million (TPM) values and log2+1 transformed. Differential expression analysis was performed using DESeq2 on the raw counts. TCGA LAML RNAseq raw counts and log2 RSEM+1 normalized counts were downloaded from the UCSC Xena Browser (<https://xenabrowser.net/datapages/> last access: March 2020).

Gene set enrichment analysis: For gene set enrichment analysis (GSEA), TCGA raw counts were normalized using DESeq2. The whole cohort (n=152) was then divided into 3 terciles according to IL1RAP expression. GSEA was performed using all human pathways from the Enrichment Map repository (http://download.baderlab.org/EM_Genesets/current_release/Human/symbol/, last access: May 2020) on a filtered cohort to compare the low versus high terciles of IL1RAP expression (n=101). An enrichment map was then plotted using Cytoscape v3.8.0 and EnrichmentMap v3.2.1. (PLoS One, 2010 Nov 15;5(11): e13984) with custom filtering parameters (cutoffs: p-value 0.001, FDR Q-value: 0.05, overlap: 0.5, normalized enrichment score > 2 or < -2). Common pathways were first annotated using AutoAnnotate v1.3.3 (F1000Res, 2016; 5: 1717.) and then manually annotated. The few remaining isolated nodes with no other relations were discarded.*

Survival analysis: The TCGA cohort was filtered to include only intensively treated patients (n=139), divided at the median according to IL1RAP expression and separated into a high and low expression group. Kaplan-Meier survival curves and multivariate Cox models were generated using the survminer and survival R packages.

All statistical analyses were performed using R software v 3.6.0.

*Acute myeloid leukemia as a candidate for IL-1RAP CAR T cell immunotherapy***Lentiviral vector construction**

A mouse anti-hIL-1RAP mAb was generated with the standard hybridoma technique using BALB/c mice immunized with human IL-1RAP recombinant protein. The selected antibody (clone #A3C3) was characterized by western blotting, enzyme-linked immunosorbent assay (ELISA) against recombinant IL-1RAP protein, immunohistochemistry, and confocal microscopy and using primary samples from patients with CML. Molecular characterization was performed by Sanger sequencing.

We designed a CAR plasmid (pSDY-IC9-IL-1RAPCAR-dCD19) using a self-inactivating lentiviral backbone carrying the scFv of mAb #A3C3, an iCASP9 safety cassette and a cell surface-expressed marker (Δ CD19) for monitoring and potential cell selection (Figure S1A). The three transgenes were all separated by peptide cleavage sequences (P2A and T2A) and under the control of the elongation factor 1 alpha (EF1) promoter in addition to the enhancer sequence SP163. The mock plasmid carried the same construct but lacking the gene sequence encoding the whole CAR including the specific scFv of IL-1RAP and the T-cell signaling sequence (CD28/4.1BB/CD3z).

Determination of IL-1RAP mRNA expression and western blotting

IL-1RAP mRNA was extracted from AML cell lines and primary cells from AML patients (5×10^6 cells lysed in RLT buffer) following the manufacturer's protocols (RNAasy Mini Kit, Qiagen, Cat n°: 74106). The relative IL-1RAP mRNA expression was determined by RT-qPCR using the Hs_00895050_m1 TaqMan qPCR gene expression assay (Thermo Fisher Scientific) targeting the mRNA variant codon for the intracellular domain of the cell surface protein. PCR was performed using a CFX96 TM Real Time System (C1000TM Thermal Cycler, BioRad) according to the following program: enzymatic activation at 95°C within 20 sec-3 min (1 cycle), denaturation at 95°C within 1-3 sec (40 cycles), and pairing/elongation/acquisition at 60°C for 15 sec (40 cycles). The results were analyzed using BioRad CFX Manager Software.

For western blotting, AML cell lines (5×10^6 cells) were sonicated and suspended in RIPA buffer supplemented with a protease inhibitor cocktail. Proteins were transferred to membranes and probed overnight with primary antibodies targeting IL-1RAP: #A3C3 mAb (clone A3C3, 8423A3C3, Diaclone) (diluted 1:3e3) and anti- β -actin antibody (clone AC15, #A5441, Sigma-Aldrich, St Louis, MO, USA) (diluted 1:10e3) as an internal loading control. For immunodetection, an HRP-anti-mouse IgG was added (#515-035-062, Jackson ImmunoResearch, USA). Detection was performed by chemiluminescence using a camera and Bio-1D software (Vilber-Lourmat).

Functional tests in vitro

Effector C0 (untransduced), mock and IL-1RAP CAR T-cells (1×10^6 cells/mL) were labeled with Cell Proliferation Dye e-Fluor-V450 (ref: 65-0842-85, eBioscience) following the manufacturer's protocol. Labeled cells were cultured in a 96-well plate at an appropriate E:T ratio with target-cells at 37°C for the needed time. The final

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volume/well was of 200 μ L. After coculture, cells were labeled with 7-AAD (Cat N°: 51- 68981E, BD) and anti-CD3-PE, anti-CD19-APC (for evaluating T-cells) and anti-IL-1RAP (for evaluating target-cells) antibodies. A CD107a-PE degranulation assay (Clone H4A3, BD Biosciences) and an intracellular IFN- γ -PE expression assay (BD Cytotfix/Cytoperm™ Plus Kit, ref: 555028) were performed according to the manufacturers' protocols.

Xenograft murine models and the patient-derived xenograft murine model

AML cell lines used for the in vivo tests were transduced with a luciferase lentiviral vector (pLenti CMV V5-Luc Blast vector, Addgene) and blasticidin-selected. Six- to 8-week-old NSG-S (triple transgenic NSG-SGM3 mice expressing human IL3, GM-CSF and SCF, Jackson Laboratory, Sacramento, CA, USA) were sublethally irradiated (250 cGy) on day 4 prior to tumor injection. One day later, each mouse was injected intravenously, with 1.10^6 HL-60 (IL-1RAP^{Low}), Molm-13 (IL-1RAP^{Int}) or Mono-Mac-6 (IL-1RAP^{High}) luciferase-expressing AML cells suspended in 300 μ L of PBS or primary cells from AML patients. Following AML cell engraftment (day 0), mice were treated intravenously with MockT or IL-1RAP CAR T-cells (10.10^6 cells in 300 μ L of PBS) and assessed for leukemia progression on days 3, 5, 10, 14, 17 and 21 by BLI measurements or determination of the percentage of human CD45+/IL-1RAP+ cells in mouse blood for the PDX mice. Mice received 3 mg of luciferin intraperitoneally (VivoGlo 150 Luciferin, Promega, Fitchburg, WI, USA) within 10 min of imaging using the IVIS® Lumina III system (PerkinElmer). Untreated mice and mice treated with CO T-cells or MockT-cells were used as controls.

In the PDX models, mice were treated earlier on D5 after AML blast engraftment. At D32, mice were sacrificed, and cells from organs were collected and identified by FCM staining (hCD45+/CD3-/CD34+).

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Supplemental Table

Supplemental Table 1: Monoclonal antibodies, reagents and kits used to characterize cells by flow cytometry.

Fluorochrome	Antibody	Provider	Reference
FITC	Mouse anti-human IL-1RAP (BR-58)	Diaclone	DSP-FF190327
FITC	Mouse IgG-1 isotype control (B-Z1)	Diaclone	857.071.010
FITC	Mouse anti-human IL-1RAP (BL-43)	Diaclone	8423A3C3
BV421	Mouse anti-human CD34	BD Biosciences	562577
APC	Mouse anti-human CD38	BD Biosciences	345807
V500	Mouse anti-human CD45	BD Biosciences	560777
APC-H7	Mouse anti-human CD14	BD Biosciences	641394
PerCP-Cy5.5	Mouse anti-human CD33	BD Biosciences	333146
Vio-Blue	Mouse anti-human CD3	Miltenyi Biotec	130-094-363
APC	Mouse anti-human CD19 (LT19)	Miltenyi Biotec	130-113-165
APC	Mouse anti-human CD34 (581 RUO)	BD Biosciences	555824
PE	Mouse anti-human IFN- γ (27 RUO)	BD Biosciences	559327
FITC	Mouse anti-human CD8 (B-Z31)	Diaclone	854.961.010
PE-Cy7	Mouse anti-human CD123	Biolegend	306010
PE-Cy7	Mouse IgG-1 isotype control	Biolegend	400-126
PE	Mouse anti-human CD3 (B-B11)	Diaclone	954.012.010
Pacific-Blue	Mouse anti-human CD3	BD Biosciences	558117
PerCP-Cy5.5	Mouse anti-human CD45	BD Biosciences	317428
BV510	Mouse anti-human PD-1	BD Biosciences	563076
BV510	Mouse IgG-1 isotype control	BD Biosciences	562946
FITC	Mouse anti-human LAG-3	Biolegend	369308
FITC	Mouse IgG-1 isotype control (MOPC-1)	Biolegend	400110
APC-H7	Mouse anti-human TIM-3	Biolegend	345026
APC-H7	Mouse IgG-1 isotype control	Biolegend	400128
BV650	Rat anti-mouse CD45	BD Biosciences	563410
BV500	Mouse anti-human CD45	BD Biosciences	560777
BV421	Mouse anti-human CD3 (UCHT1)	BD Biosciences	562426
BV510	Mouse anti-human CD8 (SK1)	BD Biosciences	563919
PE	Mouse anti-human CD95 (DX2)	BD Biosciences	555674
PE	Mouse IgG-1 isotype control (MOPC-21)	BD Biosciences	555749
PECy7	Mouse anti-human CD45RA (HI100)	Sony	RT2120630
PerCP-Cy5.5	Mouse anti-human CD45RO (UCHL1)	Sony	RT2121110
FITC	Mouse anti-human CCR7 (150503)	R&D Systems	FAB197F-100
FITC	Mouse IgG-2a isotype control (20102)	R&D Systems	IC003F
PE	Streptavidin	BD Biosciences	5150605051

Reagent	Provider	Reference
7-AAD	BD Biosciences	51-68981E
Cell proliferation dye e-fluor-V450	Thermo Fisher	65-0842-85
e-Fluor fixable viability dye	eBiosciences	65-0865-18

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Supplemental Table 2: Clinical, cytogenetic and molecular information of AML patients from the Filo cohort.

AML patients	Sexe	% Blastosis	Groupe	Karyotype	NPM1	FLT3
1	Female	21	Favorable	Normal	Mutated	Wild type
2	Female	53	Favorable	Normal	Mutated	Wild type
3	Male	42	Favorable	Normal	Mutated	Wild type
4	Female	29	Favorable	Normal	Mutated	Wild type
5	Male	25	Favorable	Normal	Mutated	Wild type
6	Male	80	Favorable	Normal	Mutated	Wild type
7	Male	43	Favorable	Normal	Mutated	Wild type
8	Female	30	Favorable	Normal	Mutated	Wild type
9	Female	54	Favorable	Normal	Mutated	Wild type
10	Male	80	Favorable	Normal	Mutated	Wild type
11	Female	63	Intermediate	Normal	Mutated	Mutated
12	Male	81	Intermediate	Normal	Wild type	Mutated
13	Male	53	Intermediate	Normal	Wild type	Wild type
14	Male	39	Intermediate	Normal	Mutated	Mutated
15	Male	95	Intermediate	Normal	Mutated	Mutated
16	Male	99	Intermediate	Normal	Wild type	Wild type
17	Female	84	Intermediate	Normal	Wild type	Mutated
18	Male	84	Intermediate	Normal	Wild type	Mutated
19	Female	99	Intermediate	Normal	Mutated	Mutated
20	Male	22	Intermediate	Normal	Wild type	Wild type
21	Female	53	Adverse			
22	Male	93	Adverse			
23	Female	82	Adverse			
24	Female	72	Adverse			
25	Male	70	Adverse			
26	Male	75	Adverse			
27	Female	65	Adverse			
28	Male	34	Adverse			
29	Female	82	Adverse			
30	Male	53	Adverse			

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Supplemental Figures

Figure S1

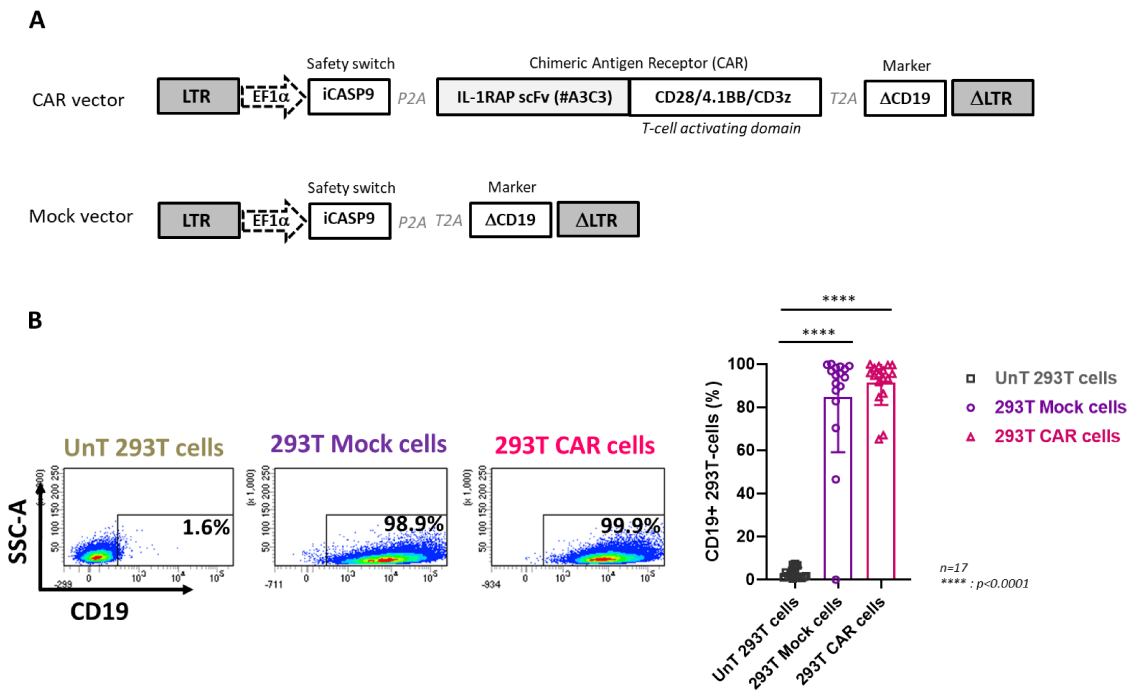


Figure S1: Production of IL-1RAP CAR lentiviral vector. **A/** Schematic construct of the IL-1RAP lentiviral vector (above) and the control Mock lentiviral vector (below). **B/** 293T-cells were transfected with pMDG, pPAX2 and transgene plasmids to produce IL-1RAP CAR and mock supernatants. The efficiency of the transfection was measured by flow cytometry by gating on CD19⁺ cells (left, representative experiment). The transfection efficiencies were 90.19 ± 13.99 and 91.54 ± 10.5 for 293T mock cells and 293T CAR cells, respectively. Untransfected (UnT) 293T-cells were used as a control. The results are also presented in bar histograms with the mean ± standard deviation (SD) from n=17 independent experiments (right).

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Figure S2

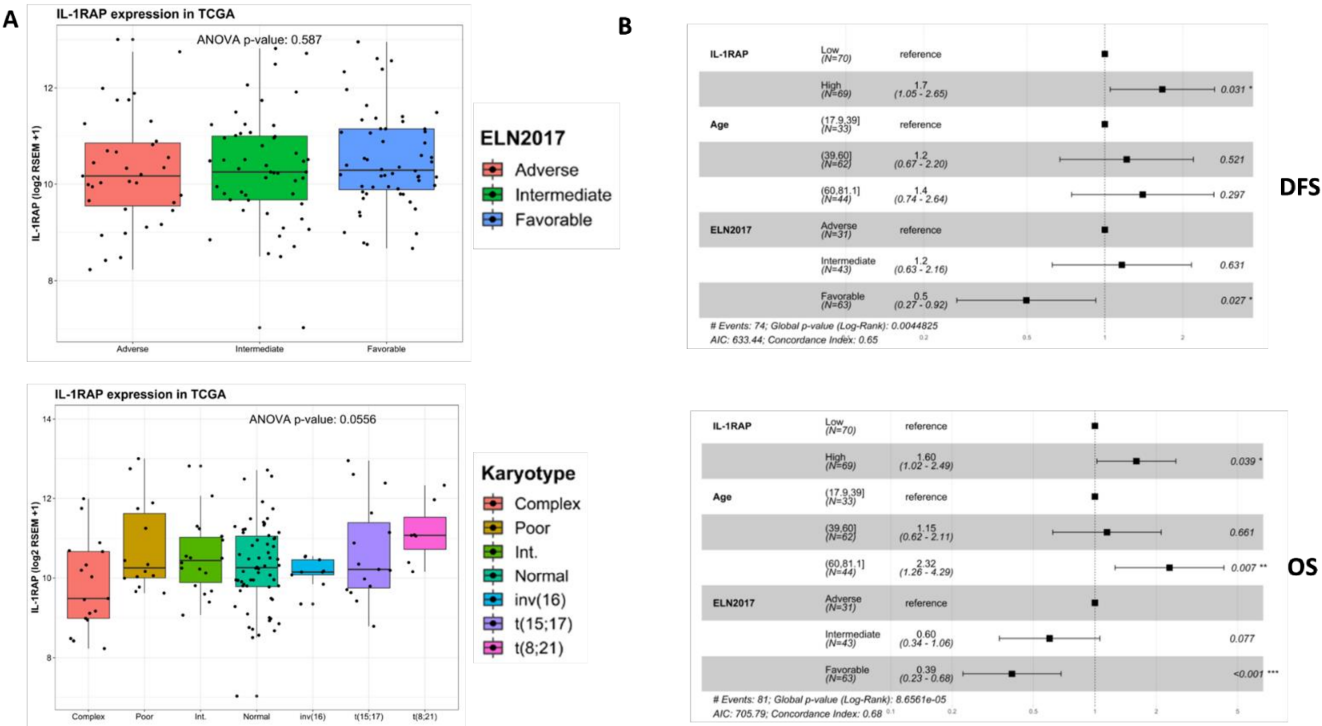


Figure S2: Prognostic role of IL-1RAP. A/ IL-1RAP mRNA expression from the TCGA cohort according ELN2017 prognosis AML classification groups (upper) and regarding the Karyotype, including Core Binding Factor (CBF) AML groups. There is no statistical differences in IL-1RAP expression between the different genetic and molecular subtypes of AML. **B/** The multivariate Cox model using age and ELN2017 stratification as confounding variables confirmed the negative prognostic impact of high IL-1RAP expression on both DFS (HR 1.60, p-value: 0.037) and OS (HR 1.60, p-value: 0.014).

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Figure S3

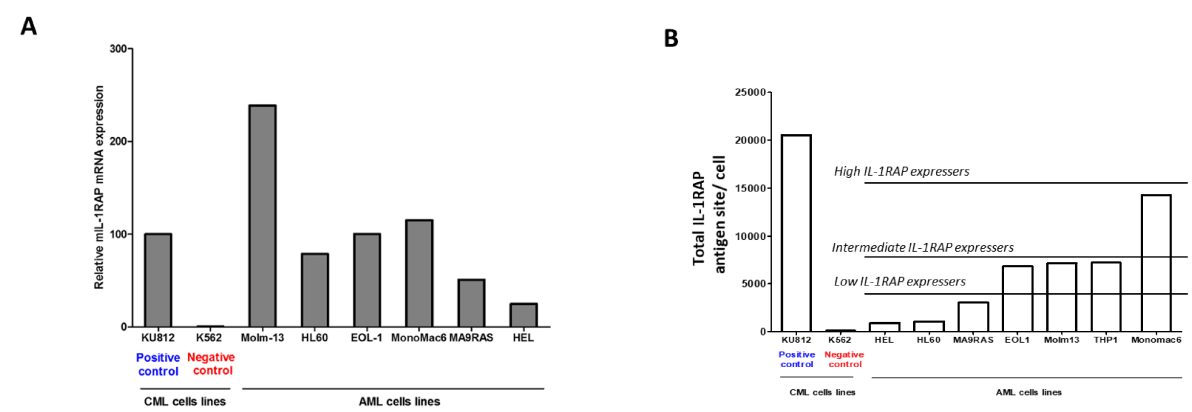


Figure S3: A/ Quantification of IL-1RAP mRNA by RT-qPCR in AML cell lines. RT-qPCR was performed on AML cell lines. **B/ Determination of the total absolute number of IL-1RAP antigenic sites** expressed per cell at the surface of AML cell lines. The KU812 and K562 CML cell lines were used as positive and negative controls, respectively.

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Figure S4

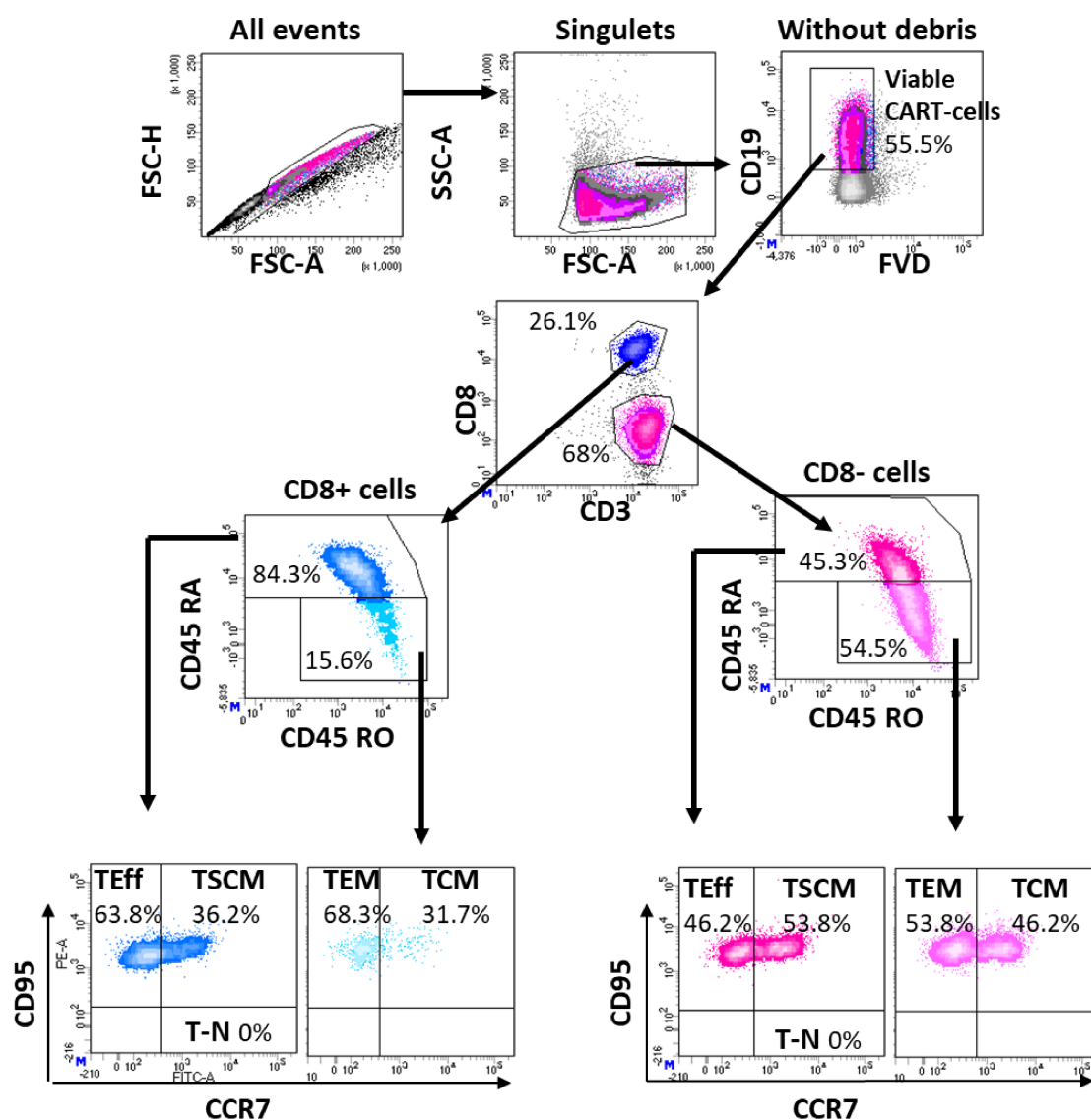


Figure S4: Gating strategy of T-cells subpopulations. Viable CART-cells are gated using CD8 and CD3 from FVB/CD19 cells. CD4+ transduced T-cells are deduced as CD8- cells. From either CD8+ or CD8- gates, we discriminate CD45RA (naïve) and CD45RO (memory). CCR7 and CD95 staining help to determine effector T-cells (TEff) (CD45RA/CD95+/CCR7-), Stem Central Memory T-cells (TSCM) (CD45RA/CD95+/CCR7-), Effector Memory T-cells (TEM) (CD45RO/CD95+/CCR7-) and central memory T-cells (TCM) (CD45RO/CD95+/CCR7-) subpopulations, TN : naïve T-cells.

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Figure S5

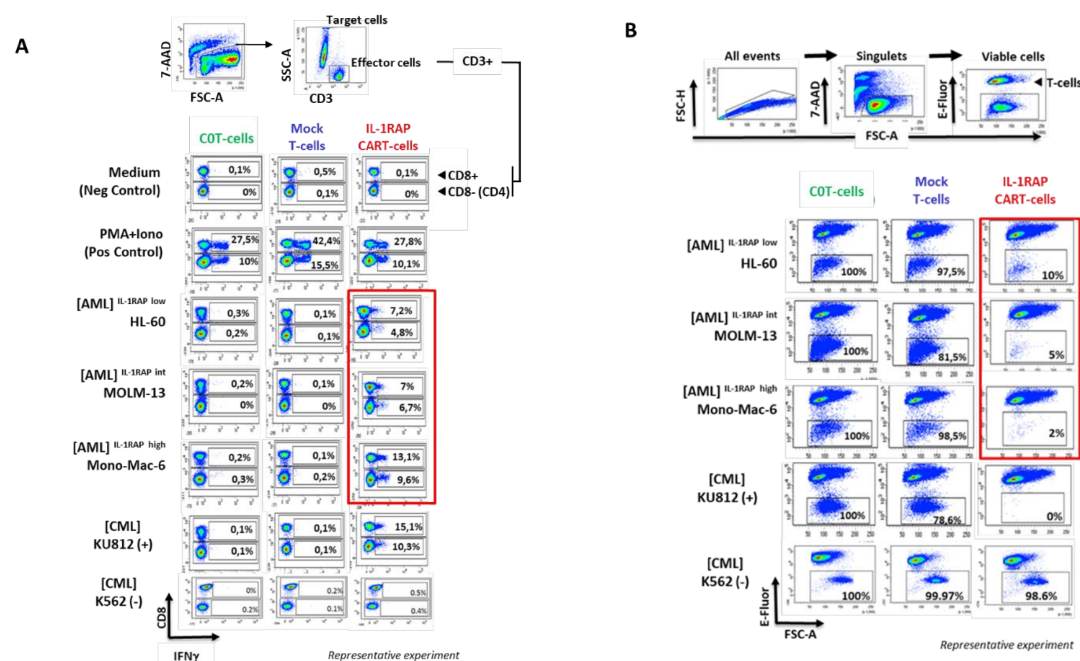


Figure S5: A/ Representative Intracellular IFN̳ plot analysis by flow cytometry gating of CD8+ (CD3+/CD8+) or CD4+ (strictly CD3+/CD8-) subpopulations of C0T-cells, mock T-cells and IL-1RAP CAR T-cells cocultured overnight with AML cell lines with various levels of IL-1RAP cell surface expression at an E:T ratio of 1:5. Cultures with medium alone and with PMA/ionomycin were used as negative and positive controls, respectively. The K562 and KU812 cell lines were used as targets and as negative and positive IL-1RAP expressers, respectively. B/ Representative cytometry plot of cell cytotoxicity. C0 T-cells, MockT-cells and IL-1RAP CAR T-cells generated via genetic modification of healthy donor PBMCs were cultured at different E:T ratios for 24 h, with target AML cell lines expressing different levels of IL-1RAP. The K562 and KU812 cell lines were used as targets and as negative and positive IL-1RAP expressers, respectively. Viable cells were gated based on 7-AAD labeling via flow cytometry, and T-cells were distinguished from tumor cells by eFluor labeling. The percentage of remaining tumor cells within the eFluor negative gate is provided. C0T-cells : CD3/CD28 beads activated / untransduced / 9 days cultured. MockT-cells CD3/CD28 beads activated / Mock-transduced / 9 days cultured

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Figure S6

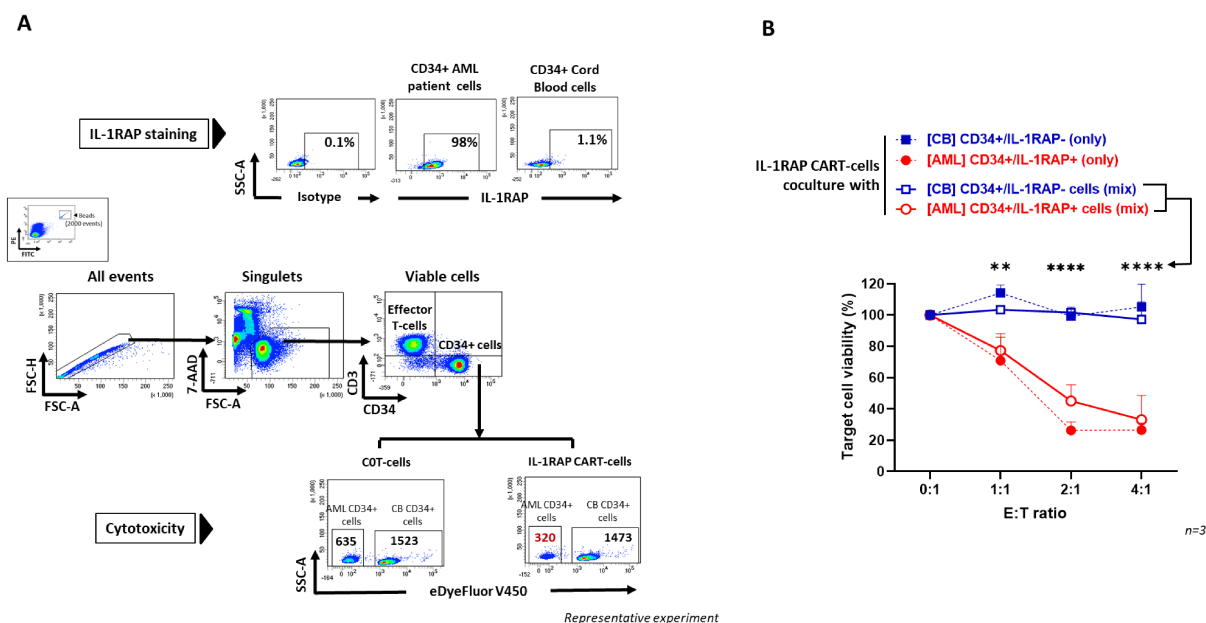


Figure S6: In vitro evaluation of IL-1RAP CAR-T cells toxicity against Healthy HSC

Healthy CD34+ HSCs were purified from cord blood (CB) and CD34+ HSCs were selected from the Bone Marrow (BM) mononuclear cells of AML patients, using human CD34 Microbead kit (Milteny, Germany). **A/ Gating strategy** for IL-1RAP cell surface staining and for effector cytotoxicity. For cytotoxicity assay 2000 fluorescent beads were acquired for each samples in order to have an absolute cell counts. Effector T-cells (either T-cells or IL-1RAP CAR T-cells; CD3+, CD34-) were distinguished from target cells (CD3-, CD34+) by staining with CD3-PE (Dialone, B-B11), CD34-APC (Biolegend, 581 clone). IL-1RAP cell surface expression was analyzed after staining using IL-1RAP-FITC (BR-58) antibodies and 7-AAD (living cells). Dye eFluor™ 450 labelling of healthy CD34+ CB HSC allows for discriminating them AML CD34+ HSC in a 50:50 pooled mixed population. Analysis was performed with a CANTO II cytometer. COT-cells: CD3/CD28 beads activated / untransduced / 9 days cultured. **B/ IL-1RAP CART-cell effectors cytotoxicity.** Healthy CD34+ CB HSC (solid or open squares respectively for alone or mix population), AML CD34+ HSC (solid or open circles respectively for alone or mix population) or the mixed 50:50 population (open symbols) were cocultured 6 hours with either activated/untransduced/cultured- (C0) or IL-1RAP CAR-T cells. IL-1RAP CART-cells cytotoxicity has been reported on the graph after normalisation to the cytotoxicity of C0, to subtract alloreactivity. The viability of target cells was quantified using a TruCount tubes (Viability percentage=Absolute number of viable target cells after co-culture with IL-1RAP CAR-T cells*100/Absolute number of target cells after co-culture with untransduced-T cells). Mean values \pm SEM calculated from three independent experiments (3 different CB were mixed with 3 different AML patient's BM) are shown.

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Figure S7

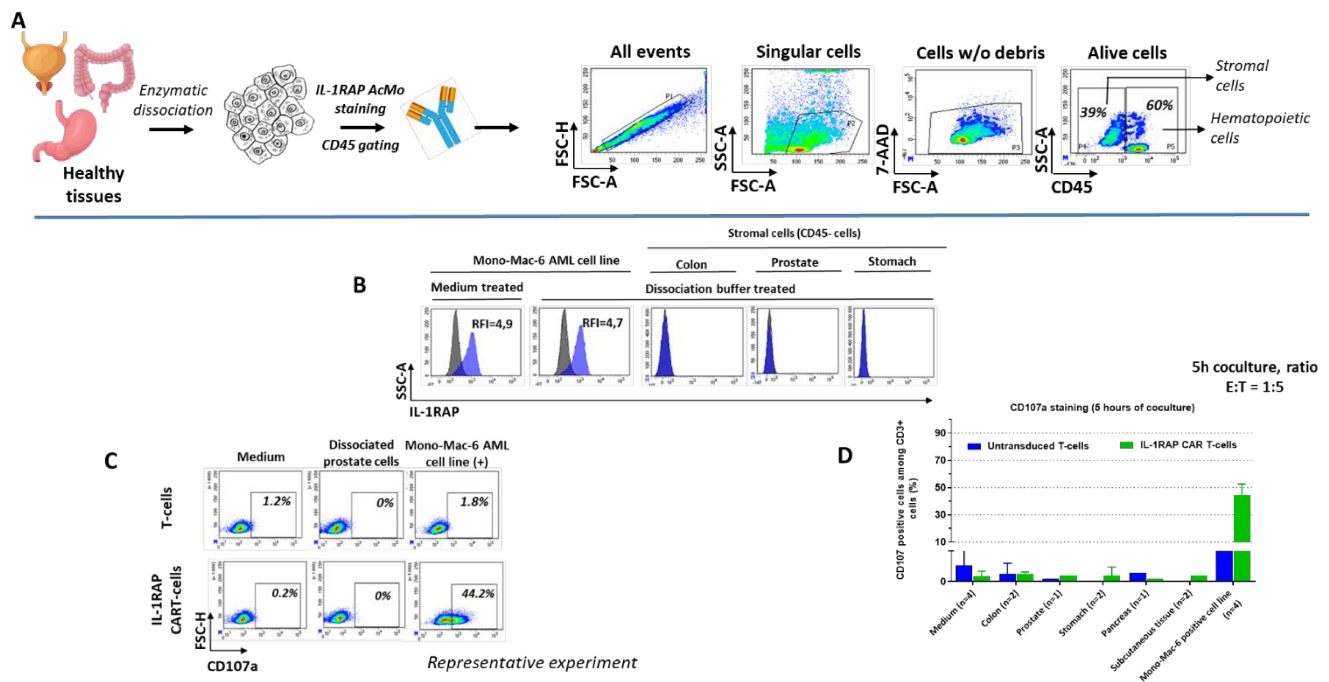


Figure S7: Coculture of IL-1RAP CAR T-cells with healthy dissociated tissues harvested close or at the periphery of different solid tumors. Selection's section is made by an experimented cytopathologist. **A/** Briefly, healthy tissues (1 to 3 mm³ pieces) were enzymatically dissociated in DTTD (BD Tissue and Tumor Dissociation; BD Biosciences) either 2 or 16 hours, then stained with CD45 monoclonal antibody in order to discriminate stromal cells (CD45 negative). **B/ IL-1RAP staining of healthy tissues.** Mono-Mac-6 DTTD treated or not, are used as control staining. **C/ Representative experiment of CD107 staining** of IL-1RAP CAR T-cells after co culture (5h, E:T ratio =5) with dissociated prostate healthy tissue and AML IL-1RAP+ cell line **D/** CD107 staining among IL-1RAP CAR T-cells (CD3+/CD19+)

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Figure S8

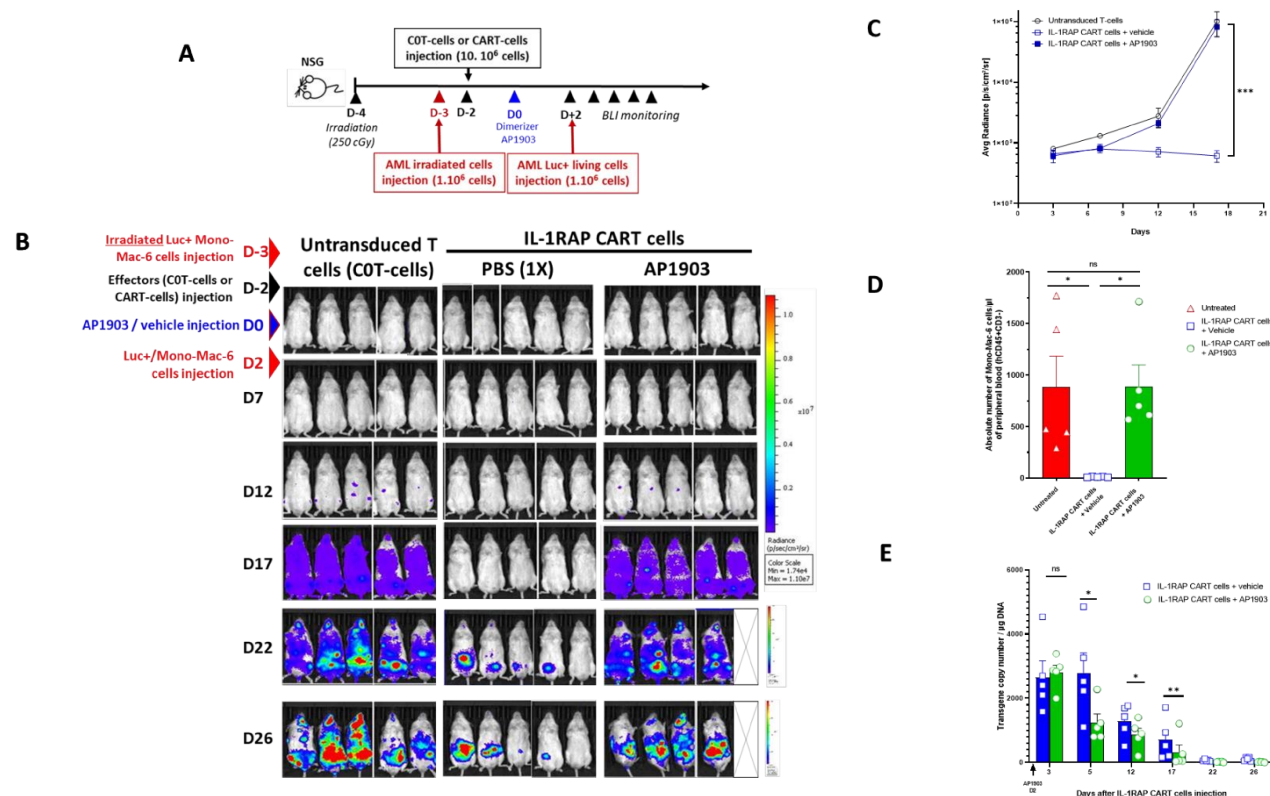


Figure S8: In vivo safety switch experiment. **A/** Synopsis of the murine safety switch experiment. Irradiated (250 cGy, D-4) NSG mice were previously injected with irradiated Mono-Mac-6 AML cell line (1.10E6/mice, D-3), then one day later (D-3) with Effector T- or CART-cells (10.10E6/mice). Two day after (D0), mice were treated with vehicle (PBS) or AP1903 dimerizer (10nM), then with Luc+ Mono-Mac-6 AML cell line (1.10E6/mice, D2). COT-cells: CD3/CD28 beads activated / untransduced / 9 days cultured. **B/** By bioluminescence imaging, we notice the first signs of tumors increase, at day 17 in mice group treated with AP1903 whereas mice treated with vehicle harbored very low level of tumor. **C/** The luminescence measurement confirmed a significant increase of tumor at day 17 (average radiance 100320 ± 43896 and 81460 ± 25822.1 for untreated and CART-cells/AP1903 treated mice vs 611.6 ± 131.7 for CART treated mice/vehicle, (n=3 mice/group) p<0.001 (***)). **D/** Flow cytometry absolute count of circulating Mono-Mac-6 AML cell line (gated as hCD45+/CD3-). Level of AML cell line is higher for untreated (885.5 ± 673.2 cells/μl blood) compare to vehicle treated mice (12.5 ± 3.8 cells/μl blood) showing that IL-1RAP CART-cells are able to eliminate AML cell line. For AP1903 treated mice, the level (888.3 ± 473.7 cells/μl blood) is comparable to untreated mice, demonstrating that IL-1RAP CART-cells, eliminated by AP1903 treatment are not able to control AML cell line. (mean ± SD, n=3 mice / group), p<0.05 (*). **E/** Circulating CAR T cells were kinetically

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quantify by dPCR in both vehicle and AP1903 treated mice. Taken together, these results show that IL-1RAP CAR T-cells can be efficiently controlled by the safety switch. At D5 post IL-1RAP CART-cell injection (D3 post AP1903 treatment) the transgene copy number / μg DNA is 2793.1 ± 1388.3 and 1238.5 ± 609.9 respectively for vehicle or AP1903 treatment. $p < 0.05$ (*).

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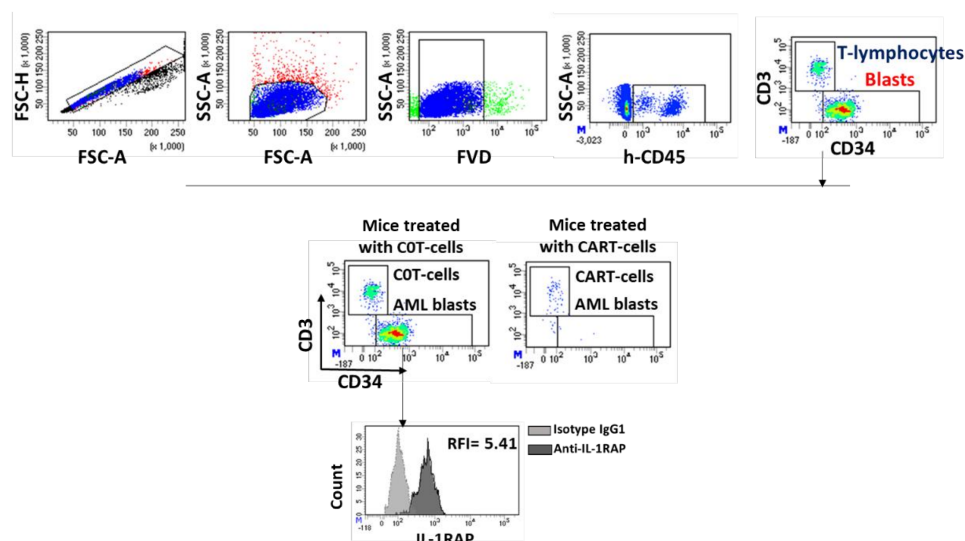
Figure S9

Figure S9: Gating strategy for human AML blasts from murine bone marrow samples of AML PDX mouse models. Human AML primary cells with hCD45+/CD34+/IL-1RAP+ labeling were detected by flow cytometry. T-cells were gated as CD3+. An IgG-1 isotype (light gray peak) was used as a control for IL-1RAP staining. COT-cells : CD3/CD28 beads activated / untransduced / 9 days cultured.

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Figure S10

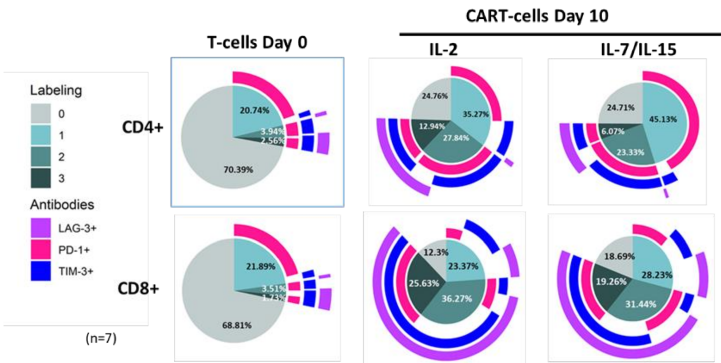


Figure S10 : Evaluation of the exhaustion marker expression on the surface of CAR T-cells (CD4+ or CD8+ T cell subpopulations) produced from samples from AML patients (n=7). The expression of PD-1, TIM-3 and LAG-3 was measured by flow cytometry prior to production (day 0) and at the end of the CAR T cell production (day 10) using either IL-2 or a combination of IL-7/IL-15. The percentage of cells expressing 0, 1, 2 or 3 checkpoint inhibitors (PD-1, LAG-3, and TIM-3) among CD3+CAR+ cells (CD4+/CD8+) is provided.

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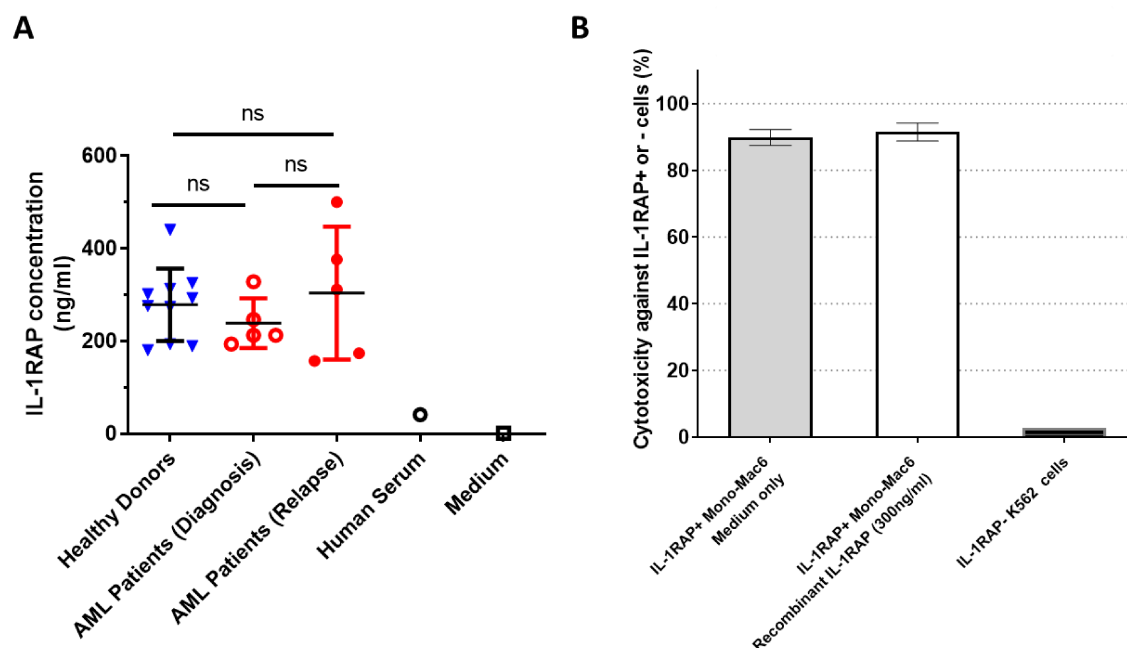
Figure S11

Figure S11: ELISA quantification of the soluble IL-1RAP form. **A/** Quantification of soluble IL-1RAP form was performed by ELISA (R&D systems kit) in the context of AML for patients at time of diagnosis (D, n=5) and relapse (R, n=5) but also for comparison for Healthy Donors (HD, n=10). We did not see not any differences between HD (278.5 ± 78.3 ng/ml) and AML D (238.8 ± 53.5 ng/ml) or R (303.8 ± 143.6 ng/ml) patients. **B/** A concentration of 300ng/ml of IL-1RAP soluble recombinant protein, (mimicking that quantified in AML patients and healthy donor) do not inhibit cytotoxicity of IL-1RAP CART-cells against AML positive Mono-Mac-6 cell line. Cytotoxicity of $89.86 \pm 2.37\%$ and $91.50 \pm 2.70\%$ respectively without or with recombinant IL-1RAP protein). Ratio E:T = 1:1. Negative control: $2.67 \pm 2.08\%$ when the CART-cells are cultured against K562 IL-1RAP negative cell line.

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Figure S12

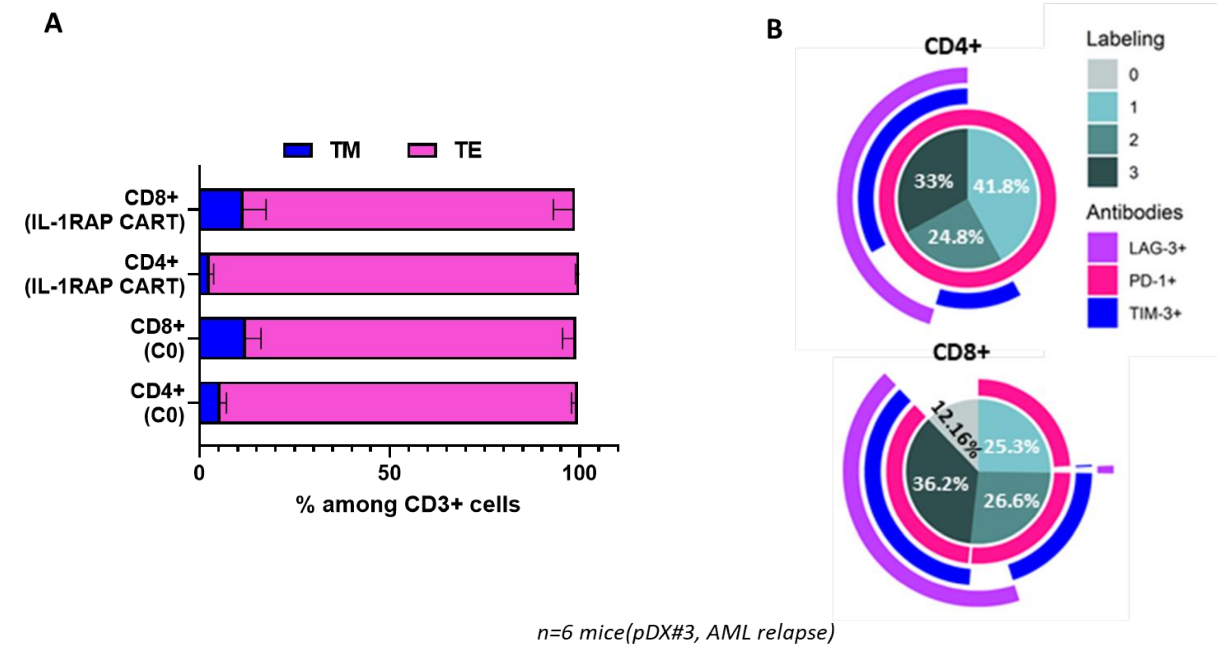


Figure S12: Characterization of circulating effectors IL-1RAP CART-cells in AML PDX mice. **A/** Percentage of effector (TE, pink bars) and memory (TM, blue bars) CAR T-cells (gated on CD3+/CD19+ and CD4+ or CD8+) harvested from the spleen at the time of sacrifice of mice with xenografted tumors from a R/R AML patient (n=6 mice, from PDX#3 AML relapsed patient) treated with either untransduced control T-cells (C0) or IL-1RAP CAR T-cells. **B/** Number of checkpoint markers (PD-1, LAG-3, and TIM-3) expressed on CD3+/CAR+ cells (CD4+ or CD8+) in the spleen of six mice PDX#3 (with tumors derived from a R/R AML PDX#3 patient) after sacrifice and percentage expression of cells expressing the respective markers.