

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

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ABSTRACT

Background Acute myeloid leukemia (AML) remains a very difficult disease to cure due to the persistence of leukemic stem cells (LSCs), which are resistant to different lines of chemotherapy and are the basis of refractory/relapsed (R/R) disease in 80% of patients with AML not receiving allogeneic transplantation.

Methods In this study, we showed that the interleukin-1 receptor accessory protein (IL-1RAP) protein is overexpressed on the cell surface of LSCs in all subtypes of AML and confirmed it as an interesting and promising target in AML compared with the most common potential AML targets, since it is not expressed by the normal hematopoietic stem cell. After establishing the proof of concept for the efficacy of chimeric antigen receptor (CAR) T-cells targeting IL-1RAP in chronic myeloid leukemia, we hypothesized that third-generation IL-1RAP CAR T-cells could eliminate AML LSCs, where the medical need is not covered.

Results We first demonstrated that IL-1RAP CAR T-cells can be produced from AML T-cells at the time of diagnosis and at relapse. In vitro and in vivo, we showed the effectiveness of IL-1RAP CAR T-cells against AML cell lines expressing different levels of IL-1RAP and the cytotoxicity of autologous IL-1RAP CAR T-cells against primary cells from patients with AML at diagnosis or at relapse. In patient-derived relapsed AML xenograft models, we confirmed that IL-1RAP CAR T-cells are able to circulate in peripheral blood and to migrate in the bone marrow and spleen, are cytotoxic against primary AML cells and increased overall survival.

Conclusion In conclusion, our preclinical results suggest that IL-1RAP CAR T-based adoptive therapy could be a promising strategy in AML treatment and it warrants the clinical investigation of this CAR T-cell therapy.

INTRODUCTION

Despite several successive improvement in treatment, acute myeloid leukemia (AML), a

KEY MESSAGES

- ⇒ Unmet medical needs remain in the acute myeloid leukemia (AML) treatment landscape, because of heterogeneity of the disease and because of lack of well-characterized, truly tumor-specific surface antigens.
- ⇒ Interleukin-1 receptor accessory protein (IL-1RAP) is a cell surface expressed by AML leukemic hematopoietic stem cells and primary blasts and poorly expressed by healthy tissues and thus might be an interesting target for cell immunotherapies.
- ⇒ Our preclinical data demonstrated that a therapeutic chimeric antigen receptor T-cells IL-1RAP, secure by a safety suicide switch, may be an interesting novel option for the treatment of patients with refractory/relapsed AML.

hematological malignancy of leukemic stem cells,¹ remains difficult to treat and to cure.²

The current conventional initial ‘7+3’ treatment consists of an induction phase with high doses of cytarabine and anthracycline (daunorubicin) chemotherapy followed by a consolidation phase of chemotherapy or allogeneic stem cell transplantation (SCT) for high-risk patients. With the knowledge of the molecular mutation landscape of AML leukemogenesis, other targeted therapy options have emerged with lower toxicity, such as Fms-like tyrosine kinase 3 (FLT3) inhibitors like midostaurin and gilteritinib, the isocitrate dehydrogenase 1/2 (IDH1/2) mutant inhibitors ivosidenib and enasidenib, and the BCL2 inhibitor venetoclax in combination with hypomethylating agents.³

Immunotherapy targeting leukemia-specific AML antigens has been explored to improve the outcome of patients with AML,

such as conjugated monoclonal antibodies (CD33-GO, gemtuzumab ozogamicin, and antibodies targeting CD44, CD123 or CD47),⁴ bispecific T-cell engager (BiTE) antibodies (targeting CD3/CD33 or CD3/CD123), and immune checkpoint inhibitors (targeting programmed cell death protein 1 (PD-1)/programmed cell death ligand 1, anti-T-cell immunoglobulin and mucin containing protein-3 (TIM-3), and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4)).⁵

Since SCT is not suitable for every patient with AML and based on the success and subsequent approval of cellular gene therapy CD19-targeted immunotherapies,⁶ these technologies, particularly chimeric antigen receptor (CAR) T-cells, are being translated into robust anti-AML therapies for refractory/relapsed (R/R) patients.⁷ Several AML cell surface targets have been explored, like CD33, CD123, CD44v6, CLL-1, and B7H6⁸; however, they have potential toxicities due to their frequent expression on healthy hematopoietic stem cells (HSCs) or progenitors⁹ and can lead to ablation of all myeloid progeny, thus requiring investigation of other targets.

Interleukin receptor accessory protein (IL-1RAP) forms a complex with the IL-1 α , IL-1 β , and IL-33 receptors on the surface of hematopoietic cells. The IL-1RAP protein has been shown to be overexpressed on the surface of leukemic stem cells (LSCs) of AML, myelodysplastic syndrome and chronic myeloid leukemia (CML) but not on normal HSCs^{10,11} and to be an interesting minimal residual disease markers.¹² In a previous work, we have confirmed absence of IL-1RAP detection of normal HSC, and functionally, we have shown, *in vitro*, by colony-forming cell assay and *in vivo* using an immunosafety cord blood engrafted murine model, that autologous IL-1RAP cocultured with HSC or infused in mice with partial human hematopoiesis do not target normal HSC and thus do not affect hematopoiesis recovery, except lower recovery of monocytes.¹³ It is a proinflammatory protein that has an oncogenic effect in AML via the FLT3 and C-kit pathways¹⁴ that promotes leukemic proliferation over normal hematopoiesis¹⁵ and has been shown to be related to some solid tumors.¹⁶

Currently, IL-1RAP is targeted only by monoclonal antibodies,^{17–19} which are still under clinical evaluation (ClinicalTrials.gov: NCT03267316 and NCT04452214) in solid tumors.²⁰ The goals of such antibodies are to block the interaction with the IL-1 receptor, impair tumor progression, and enhance antibody-dependent cellular cytotoxicity by recruiting natural killer (NK) cells. In both cases, the antibodies did not generate a persistent memory effect compared with CAR T-cells.

Based on our previous work demonstrating the proof of concept that third-generation IL-1RAP CAR T-cells^{13,21} are an interesting and robust immunotherapy approach in CML that does not affect healthy HSCs, in this work, we evaluated this innovative cell immunotherapy in AML to demonstrate its feasibility for a future first phase I clinical trial.

METHODS

Additional details of the materials and methods section are available in the online supplemental methods.

Transcriptomic and RNA-sequencing *in silico* analysis

Gene expression profiling was performed using Human Genome U133 Plus 2.0 arrays (Affymetrix/ThermoFisher, Santa Clara, California, USA), as previously described.²² A total of 244 acute leukemia samples were subjected to transcriptomic analysis, among which 74 AML samples included different French-American-British subtypes: 28 AML0, 11 AML1, 17 AML2 including t(8;21), 12 AML4 including t(inv16), 6 AML5, 60 T-ALL, 24 B-ALL, and 13 blastic plasmacytoid dendritic cell neoplasm samples. Gene expression intensity values were log-transformed and normalized using the robust multiarray average algorithm with DNA-Chip Analyzer DCHIP software.²³ Gene expression data from 32 peripheral blood (PB) samples from healthy donors available in the Gene Expression Omnibus (GEO) database were included.

Raw RNA-sequencing data of sorted normal bone marrow (BM) and peripheral blood mononuclear cells (PBMCs) (n=49 samples from 9 healthy donors) and leukemic BM cells (n=32 samples from 12 patients with AML) were retrieved from the NCBI GEO portal under the accession number GSE74246.²⁴ Statistical analysis was performed using R software V.3.6.0 (see online supplemental methods).

Tumor cell lines, primary AML cells, and patient sample collection

The human AML tumor cell lines Molm-13 (ACC-554), HL-60 (CCL-240), EOL-1 (ACC-386), HEL (ACC-11), Mono-Mac-6 (ACC-124), THP-1 (TIB-202), and Ma9RAS (MLL/AF9), the CML cell lines KU812 (CRL-2099) and K562 (CCL-243), and the human embryonic kidney epithelial cell line 239T (CRL-3216) were obtained from ATCC and stored in a master cell bank. Cells derived from working cell banks were used for the present study. PBMCs were isolated by Ficoll gradient density centrifugation using Ficoll-Paque (Velizy-Villacoublay, France) from anonymous blood samples collected from healthy donors at a French blood center (Besançon, France). Cord Blood CD34+ cells were purchased from Lymphobank Besançon, France. AML primary cell collection from patients with AML was performed at the time of diagnosis.

Determination of IL-1RAP mRNA expression, western blot analysis, and determination of the number of IL-1RAP antigenic sites

Relative IL-1RAP mRNA expression was determined by real-time quantitative reverse transcription-PCR (RT-QRT-PCR) using the Hs_00895050_m1 TaqMan qPCR gene expression assay (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) targeting the mRNA variant codon for the intracellular domain of the cell surface protein. IL-1RAP protein expression analysis was

performed by western blot analysis on AML cell lines (5×10^6 cells) using our own primary antibody targeting IL-1RAP (#A3C3 monoclonal antibody (mAb); BL-43, Diaclone, France; (diluted 1000 \times) and an antibody targeting β -actin (clone AC15, #A5441, Sigma-Aldrich, St Louis, Missouri, USA) (diluted 1000 \times) as an internal loading control.

The number of IL-1RAP antigenic sites in AML cell lines and primary cells from 30 patients with AML (FILO collection) was determined by flow cytometry (FCM) using the #A3C3 mAb and an antimouse IgG-1 mAb according to manufacturer's recommendations (CELL-QUANT Calibrator, Biocytex, Marseille, France)

Production of lentiviral constructs supernatant and genetically modified CAR T-cells

The CAR construct and the self-inactivating lentiviral (LV) vector have been previously described.¹³ Briefly, the extracellular receptor domain, derived from the IL-1RAP mAb (#A3C3), is linked to a third-generation intracellular T-cell activating domain (CD28/4-1BB/CD3 ζ). The vector (online supplemental figure S1A) also carries a safety switch consisting of a suicide gene (inducible caspase 9 (iCASP9) and a truncated CD19 gene (Δ CD19) encoding the cell surface marker). LV supernatant was harvested from tritransfected 293 T-cells (pMDG, pPAX2, and transgene plasmids) (online supplemental figure S1B). Cells taken from healthy donor or AML patient samples, after initial T-cell selection and activation using anti-CD3/CD28 magnetic beads and IL-2, were transduced with either IL-1RAP CAR or mock (controls, lacking the CAR sequence) LV supernatant. After 6 days, genetically modified CD3 $^+$ /CD19 $^+$ T cells were evaluated by FCM and expanded for 9 days. All over this work, control cells prepared from the same respective donor or patient are either CD3/CD28 beads activated/untransduced/9 days cultured (C0) or CD3/CD28 beads activated/Mock-transduced/9 days Co (MockT-cells).

Flow cytometry immunophenotyping

Patient primary AML cell immunophenotyping was performed using a panel of mAbs targeting human CD45, CD34 (stem cells), CD38 (progenitor cells), CD33 (AML blasts), CD14 (monocytes), and CD123 (AML blasts), including our own murine FITC-labeled IL-1RAP mAb (#A3C3, clone BL-43, from which the CAR was derived) and/or #H6E11 (clone BR-58; Diaclone, Besançon, France). Transduced T-cells were stained using both anti-CD3 and anti-CD19 antibodies. T lymphocytes from patients with AML were stained using an immunophenotyping panel containing mAbs targeting CD3, CD8, CCR7, and CD95 and a checkpoint inhibitor panel containing mAbs targeting Programmed cell Death protein 1 (PD-1), Lymphocyte-activation gene 3 (LAG-3), and T-cell immunoglobulin and mucin containing protein-3 (TIM-3). Stained cells were collected by a CANTO II cytometer (BD Biosciences, Le Pont-de-Claix, France) or an LSR Fortessa flow cytometer and analyzed using DIVA software

(BD Biosciences). Suitable matched isotype controls were used for analysis. The relative fluorescence intensity ratio (RFI) was calculated by dividing the mean fluorescence intensity (MFI) of IL-1RAP staining by that of the isotype control mAb. Cells were considered positive for IL-1RAP expression at an RFI >1. The mAbs used for phenotyping, intracellular staining, cytometry, and other cytometry reagents are described in online supplemental table S1.

In vitro cellular analysis: cell activation, interferon- γ intracellular expression, and cytotoxicity analysis

IL-1RAP CAR T-cells were cocultured with target tumor cells (AML cell lines or primary AML blasts) overnight at an effector:target (E:T) ratio of 1:5 for the detection of interferon (IFN)- γ intracellular expression. An IL-1RAP CAR T-cell proliferation assay was performed after 3 days of coculture (E:T ratio=1:3) and assessed by FCM after V450 e-Fluor staining. The cytotoxicity of IL-1RAP CAR T-cells against AML leukemic cells was assessed after incubation for 24 hours at different E:T ratios by FCM using trucount Absolute Counting tubes (BD Biosciences). Cell death was evaluated by 7-amino actinomycin D (7-AAD $^+$) labeling. Effector cells were distinguished from target cells with a previously described V450 e-Fluor labeling method. Gating on CD3 $^+$ /CD19 $^+$ cells and on IL-1RAP $^+$ cells allowed discrimination of CAR T-cells from IL-1RAP $^+$ tumor AML cells. In the case of allogeneic mismatch, alloreactivity was taken into account by subtracting the cytotoxicity of untransduced T-cells (C0) or MockT-cells at the respective E:T ratio. C0 and MockT-cells were used as controls.

Xenograft murine models and patient-derived xenograft murine model

In all the manuscript, NSG and NSGS mice were used for cell line or patient-derived xenograft (PDX) murine model, respectively. NSG mice aged 6–8 weeks (Jackson Laboratory, Sacramento, California, 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 $^{\text{Low}}$), Molm-13 (IL-1RAP $^{\text{Int}}$), or Mono-Mac-6 (IL-1RAP $^{\text{High}}$) luciferase-expressing AML cells or primary cells from patients with AML (taken at diagnosis or relapse). Following AML cell engraftment (day 0), mice were treated intravenously with MockT-cells or IL-1RAP CAR T-cells (10×10^6 cells) and assessed for leukemia progression on days 3, 5, 10, 14, 17, and 21 by in vivo bioluminescent imaging or determination of the percentage of human CD45 $^+$ /IL-1RAP $^+$ cells in mouse blood for the PDX model.

For the in vivo safety switch efficiency study, irradiated (250 cGy, D4) NSG mice were previously injected with irradiated Mono-Mac-6 AML cell line (1×10^6 /mice, D3), then 1 day later (D3) with effector T-cells or CAR T-cells (10×10^6 /mice). Two days after (D0), mice were treated with vehicle (phosphate-buffered saline) or AP1903 dimerizer (10 nM), then with Luc+Mono-Mac-6 AML cell line (1×10^6 /mice, D2). Mice were in-vivo

Bioluminescence imaging (BLI) monitored, circulating CAR T-cells quantified by digital PCR (dPCR) and AML circulating cell line analyzed by cytometry.

In the PDX model, at D32 and D71, NSGS mice (triple transgenic NSG-SGM3 mice expressing human IL-3, granulocyte macrophage-colony-stimulating factor and Stem Cell Factor (SCF, Jackson Laboratory) were sacrificed, and BM or spleen cells were collected and identified by FCM staining (hCD45+/CD3-/CD34+). Monitoring of CAR T-cells was performed either by FCM early after infusion or by sensitive dPCR for long-term monitoring and for distribution within solid organs, as previously described.²⁵ CAR T-cells effector/memory phenotype was assessed by the expression of CD95/CCR7 among CD45RO+/CD45RA- or CD45RO-/CD45RA+ cells.

Statistical analysis and graph constructions

The results are expressed as the mean (minimum–maximum) for MFI and RFI ratio and as the mean±SD for all other results. All statistical analyses in this study were performed using GraphPad Prism V.7 (GraphPad Software, San Diego, California, USA). Comparisons between two groups were assessed by analysis of variance and t-tests. A p value <0.05 was considered statistically significant. Other analyses were performed with Anaconda and R software. Some data were plotted with R using the ggplot2 and ggnewscale packages. The code is available on request from the authors.

RESULTS

IL-1RAP expressed at cell surface of AML primary blasts is a promising marker for immunotherapy approaches

In silico analysis of IL-1RAP expression was performed with a public dataset of 81 samples of sorted BM and PB cells from 9 healthy donors and 12 high-risk patients with AML.²⁴ First, IL-1RAP mRNA expression was compared with that of the other current AML cell surface targets in ongoing CAR T-cell studies.²⁶ Differential gene expression analysis between AML cells (LSCs or blasts) and normal hematopoietic progenitor and stem cells revealed significant overexpression of IL-1RAP in LSCs and blasts (fold changes of 21.7 and 15, respectively), with a significant false discovery rate-adjusted p value (~1.10E-20). The IL-1RAP marker was highly discriminatory compared with common AML cell surface targets, such as TIM-3, CLEC12A (CLL-1), IL3RA (CD123), MICA/MICB (NKG2DL), CD44, CD33, and FLT3 (figure 1A).

As reported in figure 1B, comparison of IL-1RAP expression between total normal PB/BM and AML cells clearly showed a higher level in blasts and LSCs. Importantly, IL-1RAP is not expressed in B, T (CD4+ or CD8+) or NK cells but is known to be expressed in monocyte PB subpopulations. In addition, all BM progenitor subpopulations expressed IL-1RAP mRNA at very low level, inferior to those of monocytes. Using TCGA AML cohort (n=139), we did not find statistical differences between European LeukemiaNet (ELN) 2017 prognosis classification groups

as well as regarding the karyotype subgroups classification (online supplemental figure S2A). Interestingly, we found that a high IL-1RAP mRNA expression was associated with a trend of poor disease-free survival (DFS (low vs high expression): 65.1% vs 49.9% and 46.6% vs 35.3%, respectively at 12 and 24 months, median 12.1 vs 20.8 months, p=0.066) and significant overall survival (OS (low vs high expression): 63.2% vs 50.8% and 51.7% vs 38.4%, respectively at 12 and 24 months, median: 14.5 vs 25.8 months, p=0.036) (figure 1C).

This association stood significant in a multivariate Cox model using age and ELN 2017 group as confounding variables (HR 1.60 for high vs low IL-1RAP expression level for both DFS and OS, p value=0.037 and 0.014, respectively) (online supplemental figure S3B). Finally, Affymetrix transcriptional profiling of the IL-1RAP mRNA encoding the membrane expressed isoform (mRNA variant 1) showed that it was overexpressed in all AML FAB subtypes compared with the soluble IL-1RAP isoform (variant 5) (figure 1D).

IL-1RAP is expressed at various levels on the cell surface of AML cell lines and primary AML cells

By RT-qPCR, we detected mRNA in different AML cell lines (n=6) (online supplemental figure S3A). IL-1RAP protein was also detected by western blot analysis in different AML cell lines (figure 2A). Cell surface expression at various levels was identified in seven AML cell lines (2<RFI<4.3) (figure 2B) as well as in primary AML blasts and LSCs (1.8<RFI<3.6, n=30), while it was, as expected expressed on monocytes from patients with AML (1.6<RFI<2.3, n=30) (figure 2C). No expression of IL-1RAP was detected on normal hematopoietic cells and normal monocytes. Clinical informations of patients with AML are provided in online supplemental table 2. Absolute quantification of the number of IL-1RAP antigen sites confirmed the different levels of expression in different antigen cell lines (n=7) (online supplemental figure S3B) as well as in primary AML blasts (n=11) (figure 2D,E). The absolute number of IL-1RAP antigen sites was not statistically different according to ELN group (figure 2F). To model IL-1RAP cell surface expression for further analysis, we classified three AML cell lines regarding antigen sites amount and defined them as ‘low (HL-60)’, ‘intermediate (int) (Molm-13)’, and ‘high (Mono-Mac-6)’ IL-1RAP expressers.

Efficient generation of IL-1RAP CAR T-cells from T-cells taken from patients with AML at either diagnosis or relapse

Using our LV vector,¹³ we were able to efficiently transduce T-cells from healthy donors with either the mock (75.29%±22.7%) or IL-1RAP CAR (74.06%±16.48%) T-cell supernatant (n=24). More interestingly, T-cells from patients with AML taken either at diagnosis (D) or at the time of relapse (R/R) could be transduced with high efficiency (90.4%±6.58% and 88.1%±9.28%, respectively, for mock or IL-1RAP CAR T-cell supernatant, n=12) without noticeable differences from T-cells from healthy donors and between D and R/R AML samples (figure 3A). We then identified the ratio of CD4+ and CD8+ cells before CAR T-cell production (D0) and at the end of the process (D9) using

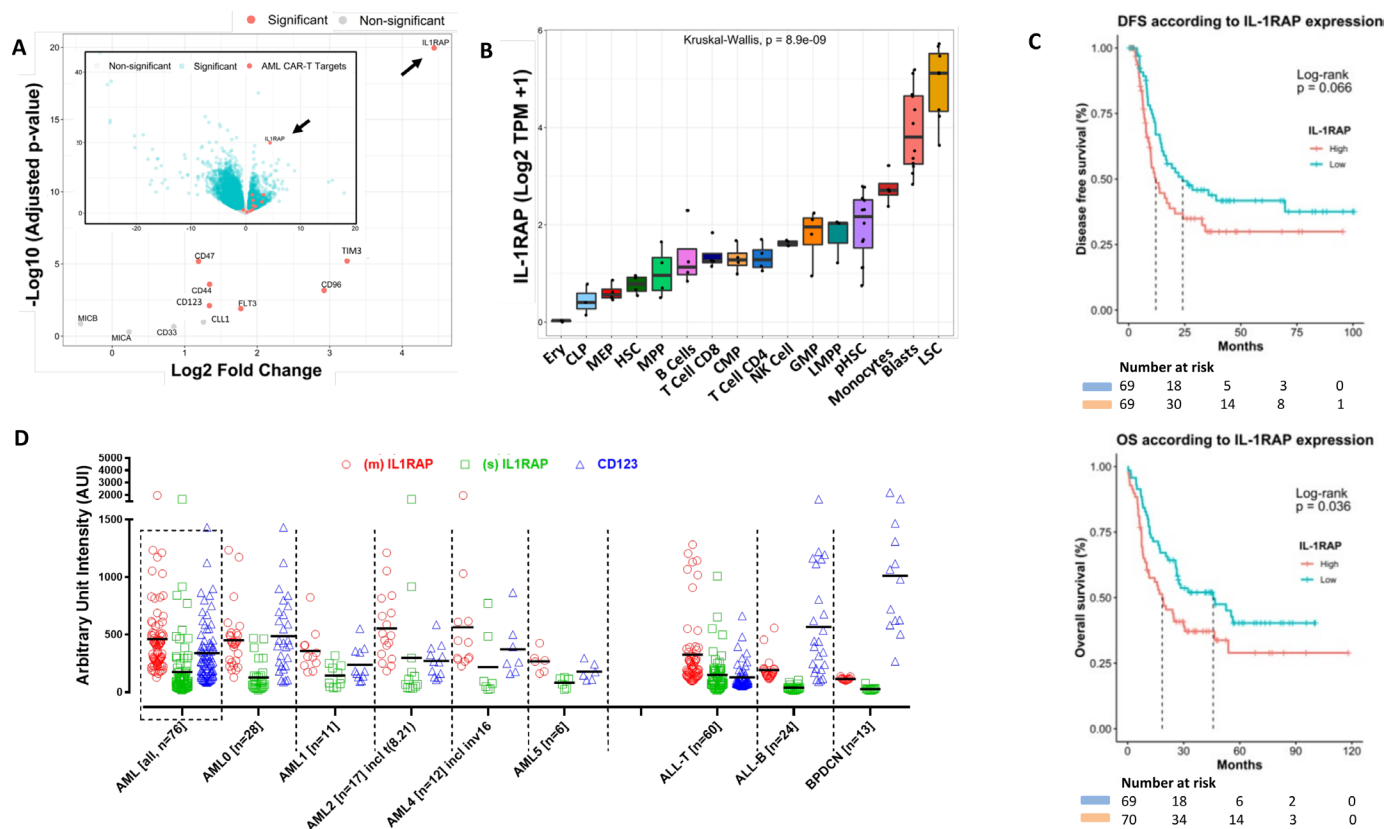


Figure 1 In silico analysis of IL-1RAP mRNA expression in an AML public database and Affymetrix gene expression profiling. (A) Volcano plot showing differentially expressed genes in AML LSCs compared with normal HSCs. Light green or red dots represent significant AML markers. The fold change is presented on the x-axis (expressed as log₂), and the p value (expressed as the $-\log_{10}$ adjusted p value) is presented on the y-axis. Genes with log₂ fold change > |1| and adjusted p value < 0.05 and with log₂ mean expression > -5 were tagged as significant. (B) IL-1RAP gene expression (expressed as the log₂ TPM +1 value, a standard unit used in RNA-sequencing analysis corresponding to log-transformed TPM. TPM are often log-transformed to normalize their distributions, and 1 count is added to handle 0 values) averaged over the replicates of each sample across AML blasts and LSCs compared with normal peripheral blood or bone marrow cells. (C) DFS and OS of intensively treated patients with AML from the TCGA cohort (n=139) according to IL-1RAP expression (high or low; divided at the median). Univariate analysis with Kaplan-Meier survival estimates showed worse OS with a tendency for worse DFS with high IL-1RAP expression. (D) Transcriptome analysis of the IL-1RAP mRNA transcripts encoding the cell surface (m) and soluble (s) IL-1RAP isoforms in patients with AML classified according to FAB classification. CD123 mRNA expression is given for comparison. ALL, acute lymphoid leukemia; AUI is, Arbitrary Unit Intensity of Fluorescence; BPDCN, blastic plasmacytoid dendritic cell neoplasm; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DFS, disease-free survival; Ery, erythroid progenitor; GMP, granulocyte macrophage progenitor; HSC, hematopoietic stem cell; IL-1RAP, interleukin-1 receptor accessory protein; LMPP, lymphoid-primed multipotent progenitor; LSC, leukemic stem cell; MEP, megakaryocyte/erythrocyte progenitor; MPP, multipotent progenitor; NK, natural killer; OS, overall survival; pHSC, hematopoietic stem cell progenitor; TPM, transcript per million.

either IL-2 or IL-7/IL-15 as cytokines in the culture medium. As reported in [figure 3B](#), similar to IL-1RAP CAR T-cells from healthy donors, IL-1RAP CAR T-cells produced from patients with AML conserved the CD4⁺/CD8⁺ ratio during the culture. Ultimately, the IL-1RAP CAR T-cells (either CD4⁺ or CD8⁺) produced from T-cells taken from patients with AML (D or R/R) showed the same profile of memory phenotype that was observed with T-cells taken from healthy donors with a conservation of this phenotype during culture with either IL-2 or IL-7/IL-15 ([figure 3C](#) and online supplemental figure S4 for gating strategy).

IL-1RAP CAR T-cells are able to specifically proliferate, secrete IFN- γ , and kill AML cell lines independent of IL-1RAP cell surface expression levels

The HL-60 (IL-1RAP^{Low}), Molm-13 (IL-1RAP^{Int}), and Mono-Mac-6 (IL-1RAP^{High}) IL-1RAP⁺ AML cell lines expressing respectively 1258, 7156, and 14 267 antigen sites at their cell surface were used for in vitro and in vivo functional analysis. For the proliferation test, IL-1RAP CAR T-cells were cocultured for 3 days with the three AML cell lines at a 1:3 E:T ratio. Compared with culture in medium only, we demonstrated that 71.5% \pm 18.09%, 81.58% \pm 6.4%, and 80.3% \pm 15.3% IL-1RAP CAR T-cells divided when cultured with respectively the low, int, and high IL-1RAP cell surface expression cell lines (n=4),

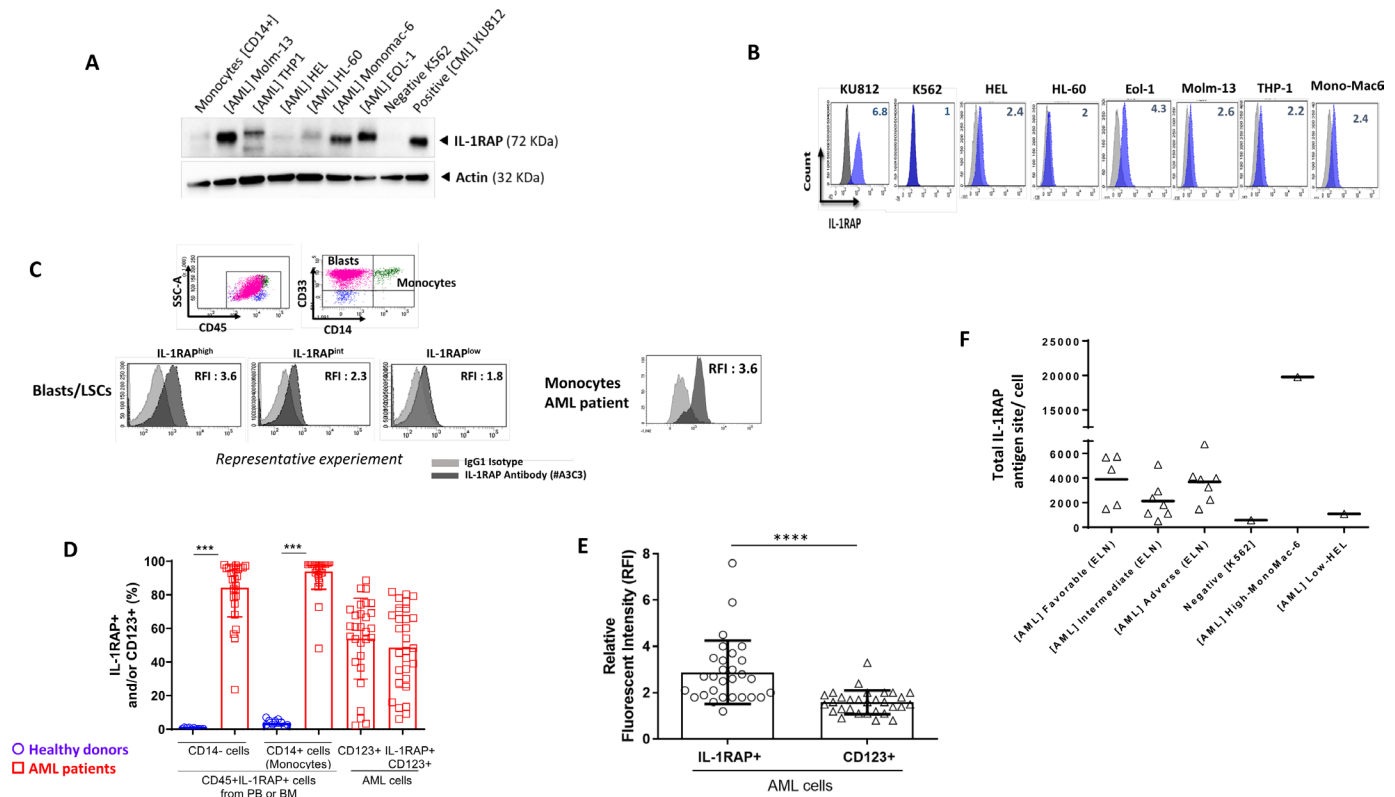


Figure 2 Expression of the IL-1RAP protein in AML primary blasts. (A) Western blot analysis of IL-1RAP in different AML cell lines and monocyte subpopulations derived from sorted primary CD14⁺ cells. The membrane was hybridized with the #A3C3 IL-1RAP mAb. The membrane isoform of IL-1RAP was detected at the expected size (72 kDa). Protein load was assessed by actin detection at 43 kDa. The K562 and KU812 cell lines served as negative and positive controls, respectively. (B) Flow cytometry detection of IL-1RAP cell surface expression (blue histogram) compared with that of the IgG-1 isotype (gray histogram). The calculated RFI is provided. (C) Representative gating strategies for primary AML blasts (CD45⁺/CD14⁻/CD33⁻) and monocytes (CD45⁺/CD14⁺/CD33⁺) and representative histograms obtain from different AML patients samples, showing different IL-1RAP cell surface expression levels (dark gray) on primary blasts (CD14⁻) and monocytes (CD14⁺) compared with the IgG-1 isotype levels (light gray). The RFI was calculated by comparing the IL-1RAP and isotype signals. (D) Percentages of IL-1RAP⁺ cells and/or CD123⁺ (y-axis) in AML blasts (PB) and AML LSCs (BM) as well as in CD14⁺ monocytes in AML patient samples (squares, red lines) compared with healthy (circles, blue lines) hematopoietic cells (CD14⁻) and monocytes (CD14⁺). The percentages of CD123⁺ and double CD123⁺/IL-1RAP⁺ cells are also provided (***p<0.01; ****p<0.001). Percentage of cells (y-axis) refers to the population reported in the x-axis, gated as illustrated in C. (E) IL-1RAP and CD123 RFIs in all CD45⁺/CD14⁻ AML primary blasts (n=29) regardless of ELN classification. (F) Quantification of the absolute number of total IL-1RAP antigen sites per cell present at the surface according to ELN classification using the #A3C3 IL-1RAP monoclonal antibody. K562 cells served as a negative control. The Mono-Mac-6 and HEL AML cell lines show high and low IL-1RAP expression, respectively. AML, acute myeloid leukemia; BM, bone marrow; IL-1RAP, interleukin-1 receptor accessory protein; LSC, leukemic stem cell; mAb, monoclonal antibody; PB, peripheral blood; RFI, relative fluorescence intensity.

independently of the IL-1RAP expression level. These results were significantly different from those achieved with coculture of IL-1RAP⁻ targets or with coculture of IL-1RAP⁺ targets with untransduced T-cells (C0) or MockT-cells (figure 4A,B). Similarly, in the presence of IL-1RAP-positive targets, CD4⁺ (27.23%±7.5% (low), 41.63%±12.2% (int), and 58.25%±12.9% (high)) or CD8⁺ (26.8%±6.7% (low), 38.73%±3.7% (int), and 71.75%±17.2% (high)) cells are able to express IFN- γ (figure 4C and online supplemental figure S5A for gating strategy). Finally, IL-1RAP CAR T-cell cytotoxicity was evaluated at different E:T ratios against the three AML cell lines and the negative IL-1RAP cell line K562. After coculture, at a high E:T ratio=10:1, in contrast to MockT-cells or IL-1RAP CAR T-cells that were unable to

kill negative control IL-1RAP⁻ K562 cells (10.5%±1.3%), IL-1RAP CAR T-cells were able to statistically eliminate positive control CML KU812 IL-1RAP⁺ cells (99%±0.1%) and all AML cell lines ((95.3%±1.52% (low); 99%±0.1% (int); 99.3%±0.57% (high), p<0.001, n=3). This is also true for lower E:T ratio until 1:2 (p<0.001) and until 1:16 (p<0.01) (figure 4D and online supplemental figure S5B for gating strategy).

IL-1RAP CAR T-cells target AML CD34⁺ HSC but do not affect healthy CD34⁺ HSC and healthy tissues harvested at the periphery of some solid tumors

We have previously shown in vivo and in vitro that IL-1RAP CAR T-cells do not affect healthy HSC. We reported now in the context of AML that CD34⁺ healthy

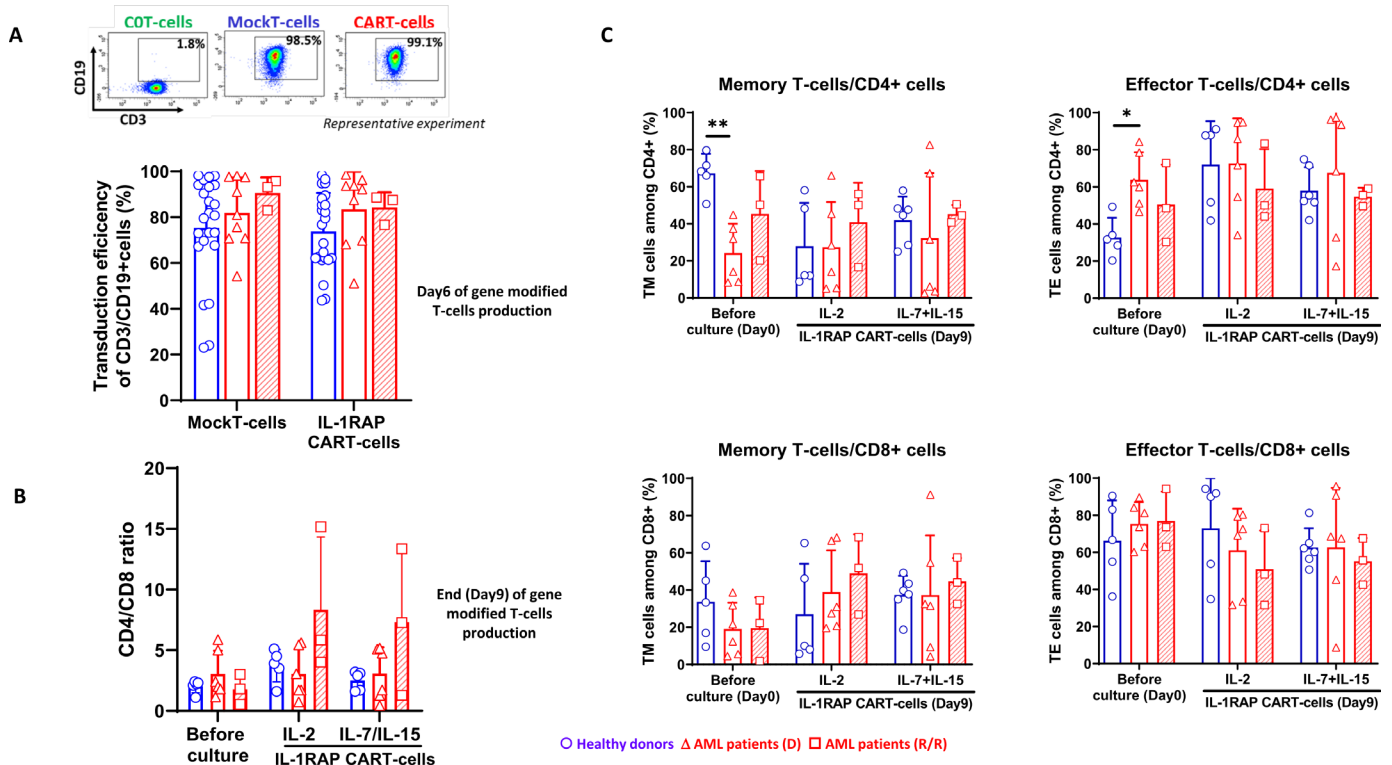


Figure 3 Production and characterization of IL-1RAP CAR T-cells taken from healthy donors or patients with AML at diagnosis or relapse. (A) Generation of mock or IL-1RAP CAR gene-modified T-cells using 30× concentrated supernatant. Upper: representative gating strategy after staining with anti-CD19 and anti-CD3 mAbs. Lower: genetically modified T-cells obtained from healthy donors (n=24, circles, blue lines) and from patients with AML at diagnosis (n=9, triangles, red lines), or relapse (n=3, squares, red lines, hatched column) all from different independent experiments. Transduction efficiency is analyzed at day 6 out of 9 of the CAR T-cells production process. C0T-cells: CD3/CD28 beads activated/untransduced/9 days cultured. MockT-cells CD3/CD28 beads activated/Mock-transduced/9 days cultured. (B) Identifying the CD4+/CD8+ ratio of IL-1RAP CAR T-cells produced from healthy donors (circles, blue lines) and from samples from patients with AML at diagnosis (triangles, red lines) or at time of relapse (squares, red lines, hatched column) and cultured in medium with IL-2 or IL-7/IL-15 cytokines. (C) Effector (TE) (CD95+/CCR7−)/memory (TM) (CD95+/CCR7+) phenotyping perform at day 9 of CAR T-cells (CD4+ or CD8+) generated from T-cells (day 0, starting material) taken from healthy donors (circles, blue lines) or from patients with AML at diagnosis (triangles, red lines) or at relapse (squares, red lines, hatched column) and cultured in medium with IL-2 or IL-7/IL-15 (*p<0.01, **p<0.01). The results are presented as the mean±SD. AML, acute myeloid leukemia; CAR, chimeric antigen receptor; IL-1RAP, interleukin-1 receptor accessory protein; mAb, monoclonal antibody.

CB HSC (IL-1RAP negative) mixed with CD34+ BM cells of patients with AML (IL-1RAP positive) are not targeted (2.79±11.2%) by IL-1RAP CAR T-cells in contrast with CD34+ AML HSC (66.9±15.6%), p<0.0001 (****), n=3, at E:T=4:1. It is also true for E:T ratio of 1:1 (p<0.001 (**)) and 2:1 (p<0.0001 (****)) (online supplemental figure S7). We confirmed also specificity of IL-1RAP CAR T-cells by coculturing them with different healthy tissues, harvested at the periphery of solid tumors (in an inflammatory context suggesting promoting IL-1RAP expression). As reported in online supplemental figure S8, we did not detect any IL-1RAP cell surface expression in the dissociated tissues. IL-1RAP CAR T-cells did not show degranulation against these healthy tissues.

IL-1RAP CAR T-cells clear AML tumor cell lines, decrease the tumor burden, and can be controlled by the safety switch in an in vivo xenograft murine model

To test the efficacy of IL-1RAP CAR T-cells in vivo in murine xenograft models, we generated three luciferase-positive

AML cell lines to be injected intravenously into immunodeficient NSG mice (figure 5A). Tumor engraftment was confirmed by appearance of the first luminescence signals before CAR T-cells injection. By bioluminescence imaging, we notice the first signs of tumors decrease, 4 days after CAR T-cells injection in IL-1RAP CAR T-cells-treated mice compared with untreated mice, whereas AML tumors (formed from cells with low, int or high IL-1RAP cell surface expression) progressed to very high tumor burdens or until mouse death (day 21) in the untreated group and group treated with C0T-cells (figure 5A). The luminescence measurement confirmed, for all three animals models (HL-60 IL-1RAP^{Low}, MOLM-13 IL-1RAP^{Int}, MONO-MAC-6 IL-1RAP^{High} engrafted mice), a significant decrease at day 21 with an average >10e5 p/s/cm²/sr for untreated or T-cell-treated mice versus <10e3 p/s/cm² for IL-1RAP CAR T-cell-infused mice (p<0.001, n=3 mice/group). Taken together, these results show that IL-1RAP CAR T-cells efficiently controlled AML cell line growth in

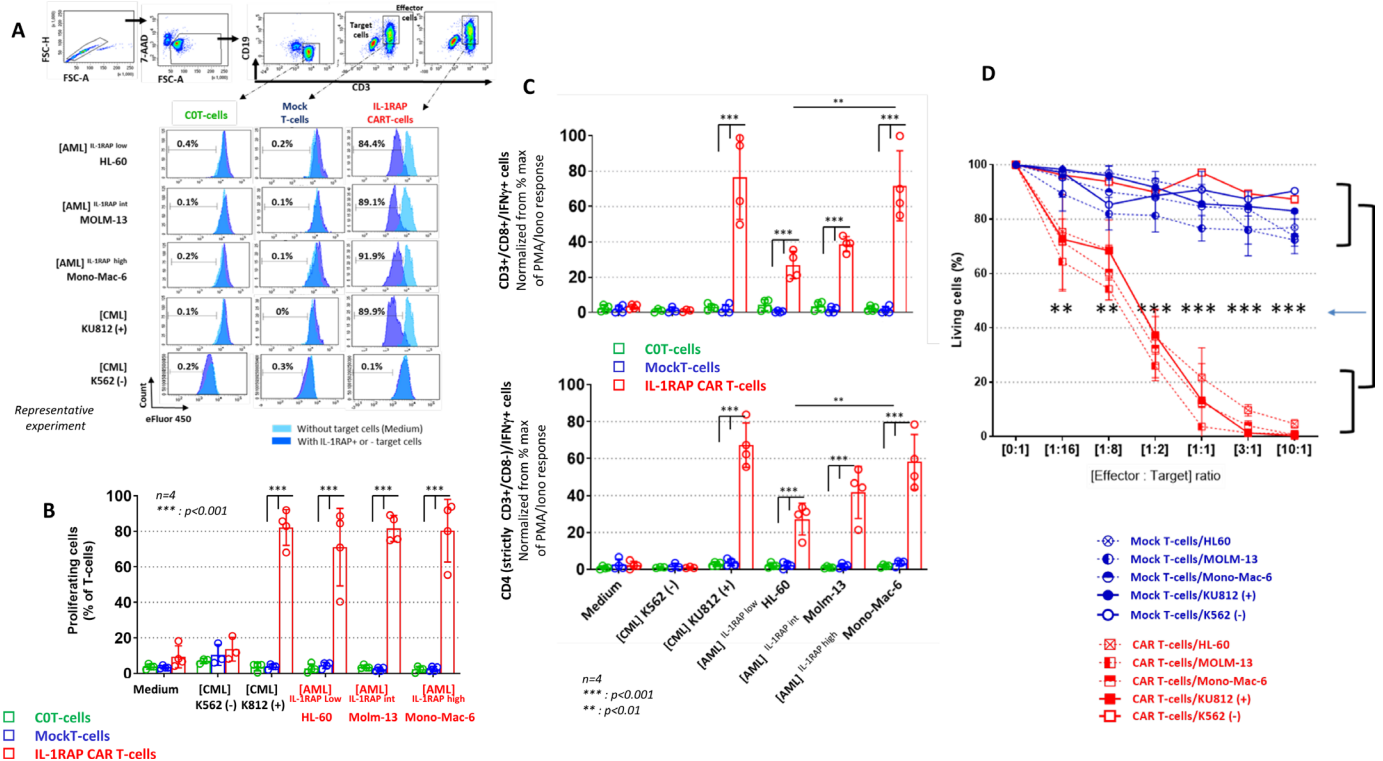


Figure 4 In vitro proliferation assay, determination of the IFN- γ expression and cytotoxicity of IL-1RAP CAR T-cells following coculture with AML cell lines. (A) Cytometry histograms of a representative experiment. COT-cells (CD3/CD28 beads activated/untransduced/9 days cultured) and MockT-cells (CD3/CD28 beads activated/Mock-transduced/9 days cultured) were used as controls. T-cells were transduced with either mock (controls for transduced cells) or the IL-1RAP CAR were labeled with eFluor 450 diluted at 1:1000 and cocultured (72 hours at an E:T ratio of 3) or not with three AML cell lines with different levels (antigen sites/cells: 1258, 7156, and 14,267, respectively for HL-60 (low), Molm-13 (intermediate) and Mono-Mac-6 (high)) of IL-1RAP cell surface expression. The K562 and KU812 cell lines were used as targets and respectively as negative and positive IL-1RAP expressers. The gating strategy (doublets/living cells/CD19/CD3/eFluor) allows discriminating effectors (MockT-cells or IL-1RAP CAR T-cells) from target cells. Dilution of the eFluor level enabled estimation of effector cell division and thus proliferation after contact with target cells (dark blue histogram), which was compared with the division and proliferation of cells cultured without target cells (light blue histogram). The percentage of eFluor450 cells, which represent the cells that have undergone one or more rounds of division, compared with cells cultured in medium only. (B) Percentage of proliferative IL-1RAP CAR T-cells. Mean \pm SD of four independent experiments. (C) Intracellular IFN- γ analysis by flow cytometry of CD8 (CD3+/CD8+) or CD4 (strictly CD3+/CD8-) subpopulations of UnT-cells, MockT-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. Percentage of intracellular IFN- γ (relative to PMA/ionomycin) expressed by stimulated CD8+ and CD4+ (CD8-) IL-1RAP CAR T-cells in response to the IL-1RAP antigen (lower) is provided. Mean \pm SD of n=4 independent experiments. (D) COT-cells, MockT-cells and IL-1RAP CAR T-cells generated by genetic modification of healthy donor PBMCs were cultured at different E:T ratios for 24 hours, 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. Percentage of living cells within the tumor cell population after coculture at different E:T ratios with MockT-cells (blue circle, dotted line) or IL-1RAP CAR T-cells (red square, dotted line) is provided. The results are from n=3 independent experiments. Solid lines (blue or red) represent coculture with IL-1RAP+ or IL-1RAP- targets. **P<0.01; ***p<0.001. 7-AAD, 7-amino actinomycin D; AML, acute myeloid leukemia; CAR, chimeric antigen receptor; E:T, effector:target; IL-1RAP, interleukin-1 receptor accessory protein; IFN, interferon; PBMC, peripheral blood mononuclear cell; PMA, phorbol myristate acetate.

vivo (figure 5B), although more partially for MOLM-13 AML cell line (IL-1RAP int). We also confirm, in an in vivo murine model (online supplemental figure S8A), in the context of AML, that the activation of the safety switch iCASP9/AP1903 allows controlling circulating IL-1RAP CAR T-cells (online supplemental figure S8B-E).

IL-1RAP CAR T-cells generated from healthy donors or patients with AML (D or R/R) are efficient against primary AML cells (taken from patients at D or R/R)

We then produced IL-1RAP CAR T-cells from PBMCs of healthy donors or harvested from patients with AML at the time of diagnosis or at the time of relapse. At the end of

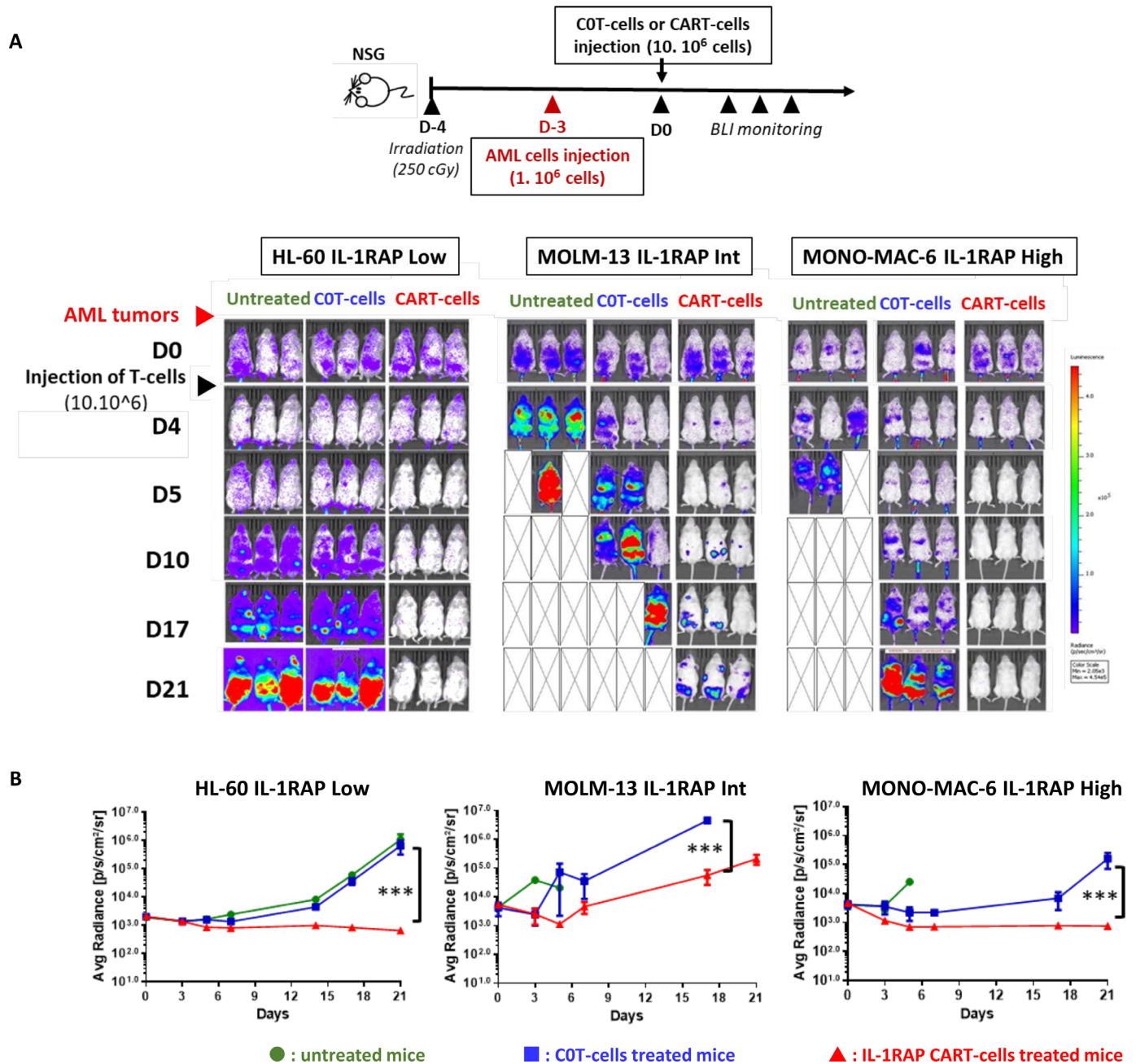


Figure 5 In vivo cytotoxicity of IL-1RAP CAR T-cells against AML cell lines in an AML xenograft NSG mouse model. (A) Synopsis of the murine experiment is provided. NSG mice aged 6–8 weeks were irradiated at a dose of 250 cGy ($n=3/\text{group}$) before tail vein (intravenous) injection of 1×10^6 HL-60, Molm-13, or Mono-Mac-6 luciferase+/IL-RAP+ cells (which have low, intermediate, and high expression of IL-RAP, respectively). After tumor engraftment, confirmed by appearance of the first luminescence signals (D0), 10×10^6 T-cells or IL-1RAP CAR T-cells were intravenously injected. Mice engrafted with AML tumor cell lines but not treated or treated with C0T-cells as controls. C0T-cells: CD3/CD28 beads activated/untransduced/9 days cultured. (B) BLI was performed until the tumor load was too high or the mice died (X) in the control groups. The radiance of the in vivo bioluminescent signal (radiance p/s/cm²/sr) collected using the IVIS Illumina III (Perkin Elmer) is shown. (▶) and (▸) indicates respectively the time of AML tumor cell lines and effectors (controls T-cells or CAR T-cell) injection. ** $P < 0.01$; *** $p < 0.001$. AML, acute myeloid leukemia; CAR, chimeric antigen receptor; IL-1RAP, interleukin-1 receptor accessory protein.

the production process, characterization of the IL-1RAP CAR T-cells produced from patients with AML showed that they acquired the expression of exhaustion markers such as PD-1, TIM-3, and LAG-3 (online supplemental figure S9 for gating and online supplemental figure S10). We evaluated the ability of allogeneic (from a donor or

a patient with AML) or autologous (from a patient with AML) CAR T-cells (figure 6A) to kill primary AML blasts (taken at diagnosis or relapse) after 24 hours of coculture at different E:T ratios (0:1, 1:2, 1:1, 3:1, 5:1, and 10:1). As reported in figure 6B, we confirmed that IL-1RAP CAR T-cells prepared from healthy donor T-cells were able to

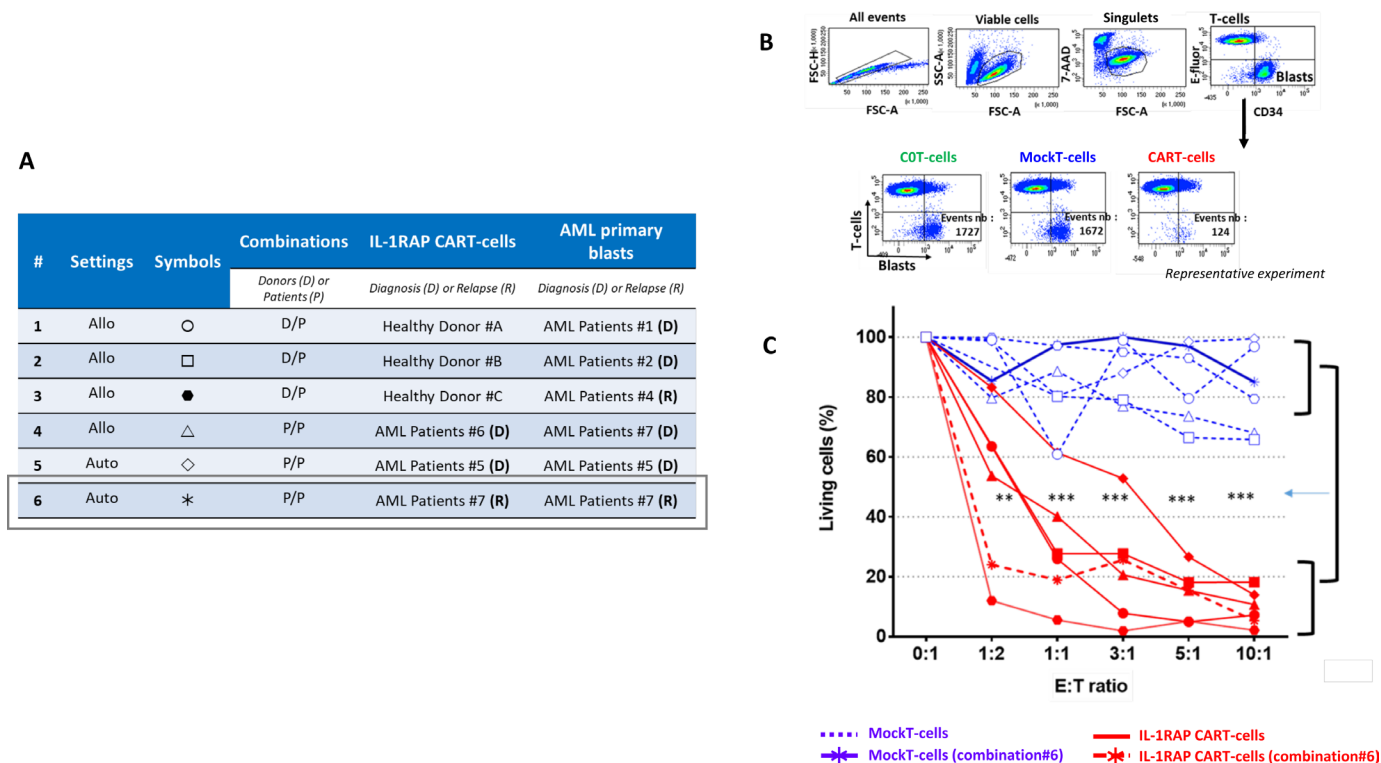


Figure 6 IL-1RAP CAR T-cells are cytotoxic against AML primary cells taken from patients at diagnosis or at relapse in vitro and in vivo in an AML PDX NSGS mouse model. (A) Primary AML blasts taken from patients at diagnosis or relapse were cocultured at different E:T ratios with effector T-cells (untransduced (UnT and C0) or transduced with mock or the CAR lentiviral vector) harvested from healthy donors or patients with AML at diagnosis or relapse. (*) Patient with R/R AML previously treated by several lines of treatment as chemotherapy, midostaurin, CD33-GO, sorafenib, and quizartinib. Percentage alloreactivity was accounted by subtracting the untransduced T-cell (C0T-cells: CD3/CD28 beads activated/untransduced/9 days cultured). Coculture value for all cytotoxicity tests in allogeneic settings. MockT-cells: CD3/CD28 beads activated/Mock-transduced/9 days cultured. The absolute number of events within the AML tumor cell gate is provided. (B) Gating strategy for discrimination of effector T-cells (cells stained with eFluor) from primary AML blasts (human CD34+ cells). (C) The percentages of viable CD34+/IL-1RAP+ cells after coculture with MockT-cells (blue line) or IL-1RAP CAR T-cells (red line) for n=6 independent donor: patient combinations are also reported. Solid lines represent coculture of effector T-cells (mock or IL-1RAP CAR T-cells) generated from PBMCs taken from a patient with AML at the time of relapse with the same patient's own R/R primary AML blasts. Solid blue line and dotted red line with both symbol (*) distinguished the combination (#6 boxed) of autologous IL-1RAP CAR T-cells produced from T-cells at relapse against their respective R/R AML primary blasts. Percentage of living cells was calculated from absolute cell count using trucount tubes as followed: (absolute number of target cells after coculture with CAR T-cells or MockT-cells)/(absolute number of target cells after coculture with T-cells)×100. **P<0.01; ***p<0.001. AML, acute myeloid leukemia; CAR, chimeric antigen receptor; E:T, effector:target; IL-1RAP, interleukin-1 receptor accessory protein; PBMC, peripheral blood mononuclear cell; PDX, patient-derived xenograft; R/R, refractory/relapsed.

effectively kill primary AML blasts extracted at diagnosis or relapse at as low as a 1:1 E:T ratio. Moreover, IL-1RAP CAR T-cells produced from T-cells taken from patients with AML at D or after relapse were also cytotoxic against primary AML blasts in allogeneic and autologous settings also at a low E:T ratio of 1:1. Finally, autologous IL-1RAP CAR T-cells produced from PBMCs taken from a patient with relapsed AML (patient #7, figure 6A, boxed) with FLT3+/NPM1+/CD33+ disease (following several lines of treatment: chemotherapy, midostaurin, CD33-GO, sorafenib, and quizartinib) eliminated primary AML blasts taken at relapse at as low as a 1:2 (76% of blasts vs 14.6% elimination respectively for IL-1RAP CAR T-cells and MockT-cells, p<0.01) to 10:1 (94.7% blasts elimination vs 15%, respectively for IL-1RAP CAR T-cells and MockT-cells, p<0.001) E:T ratios. Hence, CAR T-cells

obtained from HDs or newly diagnosed or even patients with R/R AML had comparable cytotoxic effects in eliminating AML targets in autologous or allogeneic settings. We also demonstrate, after determination of the mean value of soluble IL-1RAP protein in patients with AML plasma (D or R/R) (online supplemental figure S11A), that in vitro IL-1RAP CAR T-cells cytotoxicity against AML cell line is not affected by the soluble form (online supplemental figure S11B).

R/R primary AML blasts are targeted in BM and successfully eliminated by IL-1RAP CAR T-cells in an in vivo AML PDX murine model

The cytotoxicity of healthy donor IL-1RAP CAR T-cells against R/R patient primary AML cells in vitro was next confirmed in an AML PDX murine model (figure 7A).

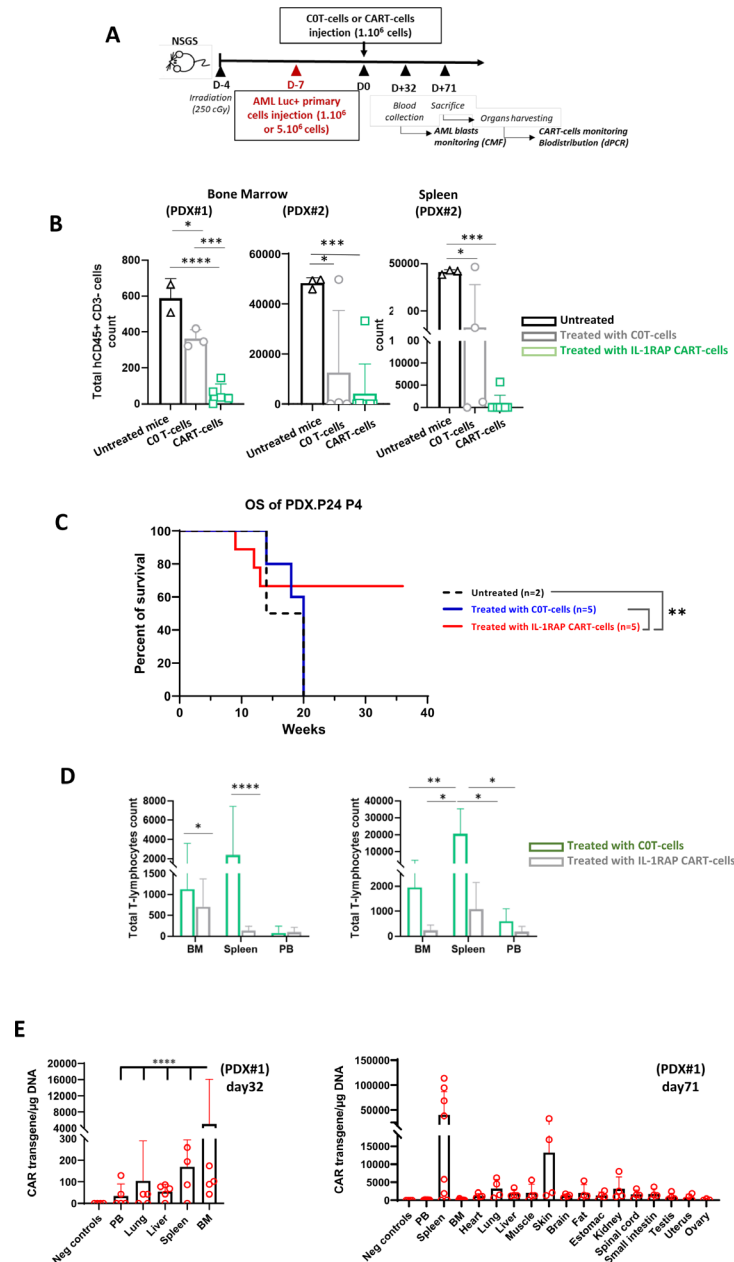


Figure 7 IL-1RAP CAR T-cells are cytotoxic against AML primary cells taken from a R/R patient in vivo in an AML PDX NSGS mouse model. (A) NSGS mice were injected with either 5×10^6 (PDX#1 and #2, passage 2) or 1×10^6 (PDX#3, passage 4) AML blasts/mice, taken respectively from diagnosis or patients with R/R AML treated with multiple AML therapies. One week after that the first human AML blasts appeared in the PB, the mice were treated with 1×10^6 CAR T-cells or with untransduced COT-cells (CD3/CD28 beads activated/untransduced/9 days cultured). Mice were sacrificed at day 32 (PDX#1, for antileukemic effect study) or 71 days (PDX#2, for CAR T-cell persistence and biodistribution analysis) after treatment, and leukemic cells and human T-cells (untransduced or CAR-transduced T-cells) levels in PB or harvested organs were monitored. In B–D: left graphs represent PDX#1 and right graphs represent PDX#2, representing two different PDXs. (B) Total human blast count (mCD45–/hCD45+/CD3–) in the BM and the spleen of untreated mice (black bars, triangle, $n=3$), mice treated with control untransduced COT-cells (gray bars, circle, $n=4$ mice) and mice treated with IL-1RAP CAR T-cells (green bars, square, $n=8$ mice) quantified by flow cytometry ($*p<0.05$, $***p<0.01$, $****p<0.001$). Mean \pm SD. (C) Kaplan-Meier survival curve representing the survival of untreated or treated mice with untransduced COT-cells or CAR T-cells (week 0). Effector cells were infused when patient AML blast cells (PDX#3: 1×10^6 cells/mice, passage 4) started to be detectable in PB. Statistical significance was calculated with the Mental-Cox (log rank) test, $**p<0.01$. The number of animals is indicated in each groups. (D) Total T lymphocyte counts within the BM, spleen and PB assessed by flow cytometry. Mean \pm SD of four mice. (E) IL-1RAP CAR transgene copies per μ g of DNA quantified by dPCR (targeting the 4.1BB/CD3z junction) in different types of organs (PCR was performed in duplicate for $n=4$ mice) ($****p<0.0001$). Negative controls represent quantification on organs from untreated mice. Statistical significance was calculated with the Mental-Cox (log rank) test, $*p<0.01$. Mean \pm SD of four independent experiments. AML, acute myeloid leukemia; BM, bone marrow; CAR, chimeric antigen receptor; CMF, cytometry; dCPR, digital PCR; IL-1RAP, interleukin-1 receptor accessory protein; PB, peripheral blood; PDX, patient-derived xenograft; R/R, refractory/relapsed.

After engraftment of fully refractory AML primary blasts and human T-cell treatment (control or IL-1RAP CAR T-cells), the experiment was stopped and animal sacrificed for ethic purposes when the tumor burden is too high to be supported by animals. We performed FCM analysis of AML blasts (online supplemental figure S9 for gating strategy) and statistically showed that the number of AML primary blasts remained higher in the BM of untreated mice than in the BM of mice treated with either C0 ($p < 0.05$) or IL-1RAP CAR T-cells ($p < 0.001$). Importantly, R/R AML blasts were more effectively eliminated by IL-1RAP CAR T-cells than by C0 T-cells ($p < 0.01$). The difference between untreated and C0 T-cells treated animals reflects the alloreactivity (figure 7B). In another AML PDX murine model, in which primary AML blasts harvested at diagnosis from a patient in the favorable ELN group (NPM1+ and FLT3wt) were engrafted 3 months before treatment, we showed a higher OS of PDX mice treated with IL-1RAP CAR T-cells ($n=5$) than of untreated animals ($n=2$) or C0 T-cell-treated ($n=5$) (240 days vs 98 and 126 days for untreated mice and C0 T-cell-treated mice, respectively; $n=5$ mice per group; $p < 0.01$) (figure 7C).

From two independent PDX experiments, we detected human T-cells at various levels in the BM (where the AML blasts home), and spleen in C0T-cells as well as in IL-1RAP CAR T-cells-treated mice (figure 7D). Interestingly, by dPCR quantification of the CAR transgene at the day of sacrifice, we quantified a high level of IL-1RAP CAR T-cells homing in the BM or spleen respectively in PDX#1 (day 32) and PDX#2 (day 71) with a higher copy number in the BM than in all other organs ($p < 0.0001$, $n=3$) for PDX#1. From PDX#3, a R/R AML case ($n=6$ mice), either IL-1RAP CAR or C0 T-cells harvested from the spleen and the BM, had a more effector than a memory phenotype (online supplemental figure S12A) and had an exhausted status, as evidenced by expression of three different checkpoints, PD-1, LAG-3, and TIM-3 (online supplemental figure S12B). Additionally, we studied by dPCR the biodistribution of IL-1RAP CAR T-cells in AML PDX #2 mice, assessed on different types of organs, showed a distribution of IL-1RAP CAR T-cells in a high level in the spleen of mice, with however no detection in the BM, and in the skin and in lower levels in other types of organs approximately 2 months after injection (figure 7E).

DISCUSSION

AML is a hematological malignancy, which remains with a poor outcome, especially for high-risk patients, despite improvements in hematopoietic SCT and several other treatments. Indeed, only approximately 40%–45% of young patients and 10%–20% of elderly patients can be cured, suggesting the necessity of new alternatives for treating patients.²⁷ Immunotherapy using CAR T-cells, particularly CD19 CAR T-cells, which have shown remarkable results in acute lymphoid leukemia and lymphoma²⁸ as well as in multiple myeloma,²⁹ is an interesting strategy

to explore in AML. Several cell surface AML antigen targets, mainly CD123, CD33, CLL1, FLT3, and CD44v6, are under investigation at preclinical stages; however, none are truly perfect targets due to their hematopoietic toxicity (eg, they can induce profound myeloablation).³⁰ Thus, there is a need to continue investigating new AML markers targetable by CAR T-cells.

In this work, we investigated IL-1RAP, a novel and unexplored AML cell surface marker expressed by leukemia cells but not by the healthy HSCs or the myeloid progenitors, not by the T and B lymphocytes, although it is expressed by circulating monocytes.^{11 13} IL-1RAP, the accessory protein of the IL-1 receptor, plays a crucial role in immunity, inflammation, and cancer. It is involved in a tumor microenvironment that favors leukemia proliferation,^{15 16} potentiates multiple oncogenic signaling pathways and oncogenic pathways, and contributes to AML oncogenesis.^{14 31} As showed by Lv *et al*,¹⁶ in TGCA-stomach adenocarcinoma, we confirm in this work, that IL-1RAP mRNA high expressers have also a poor EFS and OS in TGCA-AML, making IL-1RAP an interesting prognosis marker. The need for IL-1RAP for tumorigenesis and its overexpression in cancer makes it an interesting target for avoiding further selection and escape because of antigen loss after CAR T-cell immunotherapy, as occurs with CD19³² or BCMA³³ CAR T-cell immunotherapies. Michaud *et al*³⁴ demonstrated that soluble IL-1RAP is present at a low level in healthy people, and Warda *et al*¹³ showed that high concentrations of soluble IL-1RAP did not result in staining of IL-1RAP CAR-expressing T-cells and did not affect CAR T cytotoxicity against IL-1RAP+ target cells. We confirm here, *in vitro*, that the IL-1RAP soluble form do not inhibit IL-1RAP CAR T-cells cytotoxicity against AML cell line (online supplemental figure S10) at a mean concentration quantified in patients with AML at diagnosis or relapse.

Currently, only an mAb targeting IL-1RAP (nidanilimab) is under investigation in phase I/II clinical trials (NCT03267316 and NCT04452214, www.clinicaltrials.gov) in pancreatic and triple-negative breast cancer. This antibody is used as a blocking agent in association with pembrolizumab or to enhance chemotherapy¹⁸ and has shown a manageable safety profile.³⁵ Regarding toxicity, in addition to our previous work,¹³ where we showed absence of targeting of the healthy HSCs, we give here another argument in favor of IL-1RAP targeting by showing absence of degranulation of IL-1RAP CAR T-cell against healthy peritumoral tissues from different solid tumors, thus in an inflammatory context.

We demonstrated that IL-1RAP mRNA is expressed in all AML FAB subtypes and that the IL-1RAP protein is expressed at different levels on the cell surface of AML blasts independent of ELN classification. Interestingly, we showed in 30 AML patient cohorts that most primary IL-1RAP+ blasts coexpressed CD123, making this target suitable for bispecific CAR T-cell targeting, which will increase specificity via 'and/or' CAR activation signaling and presumably without increasing toxicity.³⁶ Indeed, a

dissociated T-cell activating signal as CD123/CD28 and IL-1RAP/CD3z can reduce CD123 toxicity and enhance IL-1RAP efficacy while better targeting AML blasts.

Following our previous work in CML demonstrating a proof of concept for targeting IL-1RAP,¹³ we here showed in a preclinical study that this new alternative treatment has efficacy in AML using an innovative approach: targeting the cell surface marker IL-1RAP, which is expressed by both leukemic stem cells and primary AML blasts. The fact that the cytotoxicity and efficacy of IL-1RAP CAR T-cells were the same against both cells with low IL-1RAP and cells with high IL-1RAP expression (primary AML cells and AML cell lines) suggests their efficacy.

Interestingly, while chemotherapy and other targeted therapies are recognized as early line treatments currently, it is important to demonstrate that T-cells from patients with R/R AML can be used for CAR T-cell generation. Indeed, non-responder patients with AML are eligible for targeted therapies following chemotherapy, and some of these patients receive FLT3 tyrosine kinase inhibitor drugs³⁷ that may interact with intrinsic tyrosine kinases³⁸ involved in CAR transduction signals, such as Fyn, ZAP-70, and lck.³⁹ We were able to transduce T-cells taken from patients with AML at diagnosis and at relapse with high efficiency, and we demonstrated that autologous CAR T-cells are effective *in vitro* against primary R/R AML blasts. Moreover, the presence of a suicide gene within our LV construct will protect against adverse events in cases of unexpected leukemic cell transduction, as previously shown.²¹ This mechanism will also help in the case of persistent adverse events affecting monocytes by functioning as a safety switch in order to eliminate CAR T-cells and allow a mature myeloid cell recovery. The functionality of the safety switch system has been evaluated in killing persistent IL-1RAP CAR T-cells.¹⁸

Characterization of the IL-1RAP CAR T-cells at the end of the production process and then in PDX mice showed that they acquire the expression of exhaustion markers such as PD-1, TIM-3, and LAG-3. Interestingly, the expression of these markers did not affect the function of IL-1RAP CAR T-cells in killing AML blasts. Further studies are needed in order to evaluate the expression of these markers' ligands (such as PDL1) on the surface of AML cells for a possible combination of IL-1RAP CAR T-cells therapy with checkpoints inhibitors.⁴⁰ Importantly, the persistence of IL-1RAP CAR T-cells in the BM and the spleen of PDX mice was confirmed by dPCR for approximately 2 months after treatment. This persistence may be due to the co-stimulation molecule 4-1BB present in the CAR construct.

In conclusion, this work clearly confirms the potential of IL-1RAP as a target in AML and the strong antileukemic effects of strategies targeting this marker both *in vitro* and *in vivo*, laying the foundation for a promising future first-in-human clinical trial in R/R AML. IL-1RAP CAR T-cells have not yet been evaluated in humans, and doing so will help determine the optimal IL-1RAP CAR

T-cell dose and evaluate the efficacy and safety of this approach.

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Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study was approved by the local ethics committee of the Cohort French Innovative Leukemia Organization (FILO) Cell Biobank; AML collection (N° BB-0033-00073, declaration 2009-944, and authorization AC 201261739) AML-CAR Collection, N° CPP2019-03-022a/2018-A03300-55, harvested from Besançon and Dijon clinical centers. Patients and donors provided written informed consent, and the study was conducted in accordance with Declaration of Helsinki. Mouse experiments were approved by the local ethical committees (CELEAG and protocol 11 007R, Veterinary Services for Animal Health and Protection).

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