# CML hematopoietic stem cells expressing IL-1RAP can be targeted by chimeric antigen receptor (CAR)-engineered T cells

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# Abstract

Chronic myeloid leukemia (CML) is a chronic disease resulting in myeloid cell expansion through expression of the BCR-ABL1 fusion transcript. Tyrosine kinase inhibitors (TKI) have significantly increased survival of CML patients, and deep responders may consider stopping the treatment. However, more than 50% of patients relapse and restart TKI, subsequently suffering unknown toxicity. Because CML is a model immune system-sensitive disease, we hypothesize that chimeric antigen receptor (CAR) T cells targeting interleukin-1 receptor-associated protein (IL-1RAP) in quiescent CML stem cells may offer an opportunity for a permanent cure. In this study, we produced and molecularly characterized a specific monoclonal anti-IL-1RAP antibody from which fragment antigen-binding nucleotide coding sequences were cloned as a single chain into a lentiviral backbone and secured with the suicide gene iCASP9/rimiducid system. Our CAR T cell therapy exhibited cytotoxicity against both leukemic stem cells and, to a lesser extent, monocytes expressing IL-1RAP, with no apparent effect on the hematopoietic system, including CD34+ stem cells. This suggests IL-1RAP as a tumor-associated antigen for immunotherapy cell targeting. IL-1RAP CAR T cells were activated in the presence of IL-1RAP+ cell lines or primary CML cells, resulting in secretion of pro-inflammatory cytokines and specifically killing in vitro and in a xenograft murine model. Overall, we demonstrate the proof of concept of a CAR T-cell immunotherapy approach in the context of CML that is applicable for young patients and primary TKI-resistant, intolerant, or allograft candidate patients.

## Statement of significant

Findings present the first characterization and proof of concept of a chimeric antigen receptor (CAR) directed against IL-1RAP expressed by leukemic stem cells in the context of CML.

## Introduction

Chronic myeloid leukemia (CML), characterized by a p210 BCR-ABL1 oncoprotein expression results in myeloid cell expansion (1). From radiotherapy and cytoreduction using conventional chemotherapy to targeted therapies with tyrosine kinase inhibitors (TKIs) and allogenic stem cell transplantation (allo-SCT) associated with interferon (IFN)$\alpha$ (2), CML treatment has progressed to the point that most patients have a normal predicted life expectancy (3,4).

Nevertheless, TKI discontinuation rates can be substantial (5), in part because of intolerance and toxicity, potential risk in pregnancy, and medico-economic reasons (6). The results of the pivotal Stop Imatinib (STIM) study (7) have been confirmed by many others (8) and with the second-generation TKIs (9), making it possible to stop treatment in approximately 40% of patients who have undetectable minimal residual disease. The remaining portion of patients relapse at the level superior of a molecular response (RM3.0), even if TKI reintroduction allows a return to a deep molecular response.

Results of TKI discontinuation studies indicate that TKIs may cure the disease in up to half of CML patients, as current TKIs are more of a suppressive than a curative therapy, requiring continuous long-term administration with unexpected and unknown adverse effects. Moreover, long-term TKI administration in young CML patients may present some unforeseen future challenges (10).

Detection of the BCR-ABL1 breakpoint fusion gene by long-range or reverse PCR (11) and cell division studies (12)] has revealed that a quiescent primitive CML stem cell compartment persists after TKI treatment by remaining insensitive, presenting a source of relapse. The next challenge is to cure CML disease and avoid the need for continuous TKI treatment. Moreover, additional treatment should be provided for CML patients with a suboptimal response to all available TKIs, but also to CML patients in the accelerated or blastic phase in order to bridge or substitute the allograft.

A graft-versus-leukemia immunological effect of allo-SCT and the efficacy of donor lymphocyte infusion allow for durable disease remission, if not a cure, despite treatment-related mortality (13). Thus, persistent TKI-resistant quiescent CML precursors need to be eliminated by new approaches while avoiding allo-SCT as much as possible. Signaling pathways that regulate the maintenance and self-renewal of CML stem cells, mainly Alox5, sonic hedgehog, Wnt/$\beta$-catenin, JAK/STAT, transforming growth factor-$\beta$/forkhead box O, and mammalian target of rapamycin (mTOR), are potential targets (14).

In addition to the well-known graft-versus-leukemia effect of allo-SCT, other features point to CML as an immune-sensitive disease. These characteristics include immune surveillance evasion after downregulation of major histocompatibility complex (MHC)-II expression by CML cells (15), BCR-ABL fusion region peptides that elicit CML-specific T-cell responses (16), the potential for autologous dendritic cell vaccination, the role of natural

killer (NK) cells (17), the anti-BCR-ABL efficacy of T-helper or cytotoxic T lymphocytes (18,19), and the restoration of immune control associated with programmed cell death-1 (PD-1) inhibition in molecular (RM3.0) or deep-response CML patients (20). Thus, CML is a candidate for new immunotherapies. Among the options are T cell lymphocytes genetically modified to express a chimeric antigen receptor (CAR) directed against a cell surface tumor-associated antigen, which have shown unexpected and unprecedented success not only in refractory/relapse acute lymphoblastic leukemia (ALL) (21) and chronic lymphoblastic leukemia (22), but also solid tumors (23) and many other promising preclinical studies in the field of hematology, mainly multiple myeloma (CD38, CD44v6, or CS1), acute myeloid leukemia (AML; CD33 or CD123), T-cell malignancies (CD5), and lymphomas (CD30) (24).

In CML and AML (25), gene expression profiling studies (26,27) have revealed a cell surface biomarker, interleukin-1 receptor accessory protein (IL-1RAP, IL-1R3), that is expressed by the leukemic but not the normal $CD34^{+}/CD38^{-}$ hematopoietic stem cell (HSC) compartment. In vitro studies with $CD34^{+}$ cord blood cells retrovirally transduced by major (e13-or e14-a2) BCR-ABL1 transcripts have confirmed upregulation of IL-1RAP (26). Moreover, IL-1RAP expression has been correlated with tumor burden and the clinical phase of CML (28,29).

The IL-1RAP protein is a co-receptor of the IL-1 and IL-33 receptor involved in IL-1 signaling, activating different signaling pathways implicated in inflammation and proliferation (30). The tumor cell surface expression makes IL-1RAP an ideal candidate to target and eradicate AML or CML HSCs, which are thought to be the origin of relapse. Antibody-dependent cellular cytotoxicity (ADCC) evaluated using an IL-1RAP antibody not only selectively kills HSCs in vitro (25,26), but also in a xenograft murine model of CD34+/CD38- AML (31) or CML (32) HSCs.

Thus, IL-1RAP is a promising cell surface tumor-associated antigen for targeting with lower toxicity and higher efficacy in immunotherapy approaches, such as CAR T-cells. We hypothesized that T cells expressing a CAR directed against IL-1RAP may eliminate leukemic stem cells. Here, we describe the proof of concept and the preclinical evaluation of an IL-1RAP CAR in the context of CML.

# Materials & Methods

Additional methods are provided in the supplementary materials.

### Patient samples, healthy donor blood samples, cell lines

Samples were collected from CML patients at diagnosis and follow-up after TKI treatment within a non-interventional trial (ClinicalTrials.gov: NCT02842320). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient density centrifugation using Ficoll-Paque (Velizy-Villacoublay, France) with anonymous blood samples collected from healthy donors at a French blood center (Besançon, France). Patients and donors provided written informed consent, the study was conducted in accordance with the ethical guideline (declaration of Helsinki) and approved by the local ethical the CPP-Est committee (France). Human tumor KU812 (CRL-2099) and K562 (CCL-243), epithelial 239T (CRL-3216), HT1080 (CCL-121), and H-MEC-1 (CRL-3243) cell lines were obtained from the America Type Culture Collection and stored in a master cell bank.

### Monoclonal antibody production, selection, and molecular characterization

A mouse anti-hIL-1RAP mAb was generated by the standard hybridoma technique. Briefly, BALB/c mice were immunized via the foot pad (n=3) or intraperitoneally (n=5) with a recombinant fusion protein consisting of the extracellular portion of IL-1RAP and the Fc portion of human immunoglobulin (Ig)G1 (R&D Systems, Lille, France). Lymph node or spleen cells and blood samples were harvested and the cells fused with the mouse myeloma cell line, then screened by FACS analysis against positive (KU812) and negative (Raji, KG1) cell lines. Anti-human IL-1RAP mAb #89412 (R&D) was used as a reference. The selected antibody (clone #A3C3) was more deeply characterized by Western blotting, enzyme-linked immunosorbent assay (ELISA) against recombinant IL-1RAP protein, immunohistochemistry (IHC), confocal microscopy, and using primary samples from CML patients. Molecular characterization was performed by Sanger sequencing of cloned and amplified PCR products obtained using degenerate primers specific for the FR1 and constant regions of the VH and VL chains (33). V-D-J-C gene rearrangement and the CDR3 region were identified after alignment of consensus nucleotide sequences against the IMGT® database using the V-QUEST online tool (34).

### Lentiviral constructs, supernatant production, and genetically modified CART cell preparation

The CAR lentiviral construct (pSDY-iC9-IL-1RAPCAR-dCD19) was prepared by cloning the synthetically produced single-chain fragment variable (scFv; derived from the #A3C3 IL-1RAP hybridoma) into the SIN-pSDY backbone (kindly provided by Dr. Mateo Negroni, CNRS-UPR

9002, Strasbourg). Lentiviral vector supernatant stock was harvested at 48 and 72 h from subconfluent transfected 293T cells and overnight soft spin centrifugation. Titration was then performed to adjust the multiplicity of infection (MOI). Activated T cells were established from healthy donors or patients and transduced with lentiviral supernatant encoding the IL-1RAP CAR or Mock sequence (missing the CAR sequence) according to the procedure described in Figure S1. Briefly, a 9-day process comprised activation (CD3/CD28 beads), IL-2 transduction (on day 2), and magnetic selection for CD19 cell surface expression. CD3+/CD19+ gene-modified T cells (GMTCs), mainly expressing CAR, were evaluated by flow cytometry.

**Western blotting, subcellular fractioning, immunohistochemistry, tissue microarray, confocal microscopy, and IL-1RAP mRNA expression**

Whole-cell, subcellular, or secreted protein fractions were obtained after cells were sonicated and suspended in RIPA buffer supplemented with a protease inhibitor cocktail. Proteins were transferred to membranes and probed overnight with primary IL-1RAP #A3C3 mAb (diluted 1:$10^3$), CD3ζ, or β-actin for IL-1RAP, CAR, or β-actin expression, respectively. Detection was performed with a camera and Bio-1D software (Vilber-Lourmat, Collegien, France). A US Food and Drug Administration standard frozen tissue array including 90 tissue cores (30 organs) from three individual donors per organ (US Biomax, Rockville, MD, United States) was incubated under the same conditions. Confocal microscopy was performed with KU812 and Raji cells concentrated on slides (Superfrost™ Plus, 4951PLUS4, ThermoFisher Scientific) by Cytospin. IHC was performed on paraffin-embedded or frozen IL-1RAP–positive or –negative cell pellets. Relative IL-1RAP mRNA expression was determined by RT-qPCR using the Hs_00895050_m1 Taqman qPCR gene expression assay (ThermoFisher Scientific) targeting the mRNA variant codon for the cell surface protein.

**Flow cytometry**

HSCs from CML patients were tracked using a panel containing CD45, CD34, CD38, CD33, CD133, and CD117 and including our murine Alexa Fluor 488–labeled IL-1RAP mAb, clone #A3C3. Transduced cells were stained using a panel of antibodies including CD3, CD4, CD8, and CD19 to differentiate helper or cytotoxic GMTCs. Naïve, central, and memory T cell subsets were analyzed using a panel of CD45RA, CD62L, CD95, and CCR7 mAbs. Cells were collected by a CANTO II cytometer (BD Biosciences, Le Pont-de-Claix, France) and analyzed by DIVA 6.1 software (BD Biosciences, Le Pont-de-Claix, France). The mAbs used for phenotyping, intracellular staining, and cytometry, and other cytometry reagents are described in Table S1.

**In vitro cytotoxicity assay functionality, including iCASP9 safety**

T-cell–mediated cytotoxic activity was analyzed by a CD107 degranulation assay. The cytotoxicity of CART cells against live tumor cells was assessed by incubation for 20–24 hours at different effector:target (E:T) ratios. Effector cells were distinguished from targets by previous labeling with e-Fluor and gated by CD3/CD19 and anti-IL-1RAP staining. The percentage of Annexin-V/7-amino actinomycin D (7-AAD+) target cells was measured. Activation of the chemical-inducible caspase 9 (iCASP9) safety switch was performed in vitro and in vivo with 20 nM of dimerizer (AP1903), and the induction of apoptosis was monitored 24 or 48 h later using 7-AAD. Cell death was quantified using Trucount tubes based on 5000-fluorescent-bead cytometry acquisition (35).

**IFNγ intracellular staining and cytokine release within culture supernatants by multiplex analysis**

K562-IL-1RAP-variants 1 or 5, KU812, or patient primary cells were mixed with 2×10E6 T or CART cells at a predefined E:T ratio of 1:5 to study effector IFNγ synthesis by intracellular staining. For the cytokine profile of CART cells, we used the human Th1/Th2/Th17 Cytokines Bead Array Kit (BD Biosciences) allowing quantification of human IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, IFN-γ, and IL-17A secretion. CART cell proliferation was analyzed by measuring the carboxyfluorescein succinimidyl ester (CFSE) dye dilution proportional to cell division in target cells (ratio 1:1).

**Xenograft murine models**

A xenograft NSG murine model was used to study CART cell cytotoxicity and iCASP9/rimiducid suicide gene efficiency . NOG mice (Taconic Biosciences) engrafted with human cord blood CD34+ cells (Transcure Bioservices SAS, Archamps, France) were used to assess autologous CART cell toxicity against healthy hematopoietic stem cells and immune competent cells .

NSG mice were transplanted (i.p. or i.v.) with clonal or bulk Luc+, IL-1RAP+, GFP+ tumor and CML KU812 cells, with or without injection of effector CART cells. Circulating CART cells and tumor burden were analyzed every week by either cytometry or bioluminescence.

Animal protocols were performed under control of the animal care and use committee of the University of Besançon. Mice were followed until the animals in the untreated group reached a moribund health state and signs of leukemia manifested (i.e., weight loss >15%, decreased activity, and/or hind limb paralysis). Mouse experimentations were approved by the local ethical committees (CELEAG and protocol 11007R, Veterinary Services for Animal Health & Protection, respectively for hu-NOG and NSG models).

# Results

### Anti-IL-1RAP mAb clone #A3C3 exhibits specific recognition of human IL-1RAP antigen

Selection of mAb subclones that discriminated IL-1RAP–positive cells (KU812 or KG-1 corresponding to AML or Phi$^{+p210}$ CML, respectively) from negative cells (Tom-1, NALM-20, Jurkat, or Raji corresponding to Phi$^{+\ p190}$ B-ALL, Phi$^{-}$ B-ALL, T-ALL, or Burkitt's lymphoma, respectively) with a ratio fluorescent intensity (RFI) >2 (Figure 1A) was performed. Use of #A3C3 mAb in Western blot also identified KU812 cells (Figure 1A). Flow cytometry and IHC confirmed the specificity of #A3C3 mAb (Figure 1B, left). Confocal microscopy clearly showed cell surface staining corresponding to IL-1RAP expression (Figure 1B). Finally, integration of the #A3C3 mAb into a panel of antibodies allowed discrimination of IL-1RAP$^{+}$ leukemia-expressing CD34$^{+}$CD38$^{+}$ or CD34$^{+}$CD38$^{-}$ stem cell subpopulations in BM or PB from CML patients at diagnosis and until 6 months post-TKI treatment (Figure 1C). These results are in agreement with the mRNA quantification, in which CML samples were positive but healthy donor samples lacked IL-1RAP mRNA expression (Figure 1D). We also confirmed by ELISA that the #A3C3 mAb recognizes the IL-1RAP recombinant protein (Figure S1).

### Efficient generation of IL-1RAP CAR-expressing T cells using lentiviral gene transfer

Based on Sanger sequencing of VDJ or VJ rearrangements and CDR3 nucleotide sequence determination, we designed a self-inactivating lentiviral construct carrying the iCASP9 safety cassette, the scFv of mAb #A3C3 (patent pending B17-4492EPLM/VHA for sequence. GenBank Submission ID: 2156936), and a cell surface–expressed marker (ΔCD19) for monitoring and potential cell selection. All three transgenes were separated by 2A peptide cleavage sequences and under control of the elongation factor 1 alpha (EF1) promoter plus the SP163 enhancer sequence (Figure 2A). The in vitro production process (Figure S2A) with lentiviral supernatant (SN) allowed for 82.4% to 78.4% transduction of primary T cells at an MOI of 2 (n=6; 1.56×10E6 and 1.98×10E6 infectious viral particles/mL for Mock or IL-1RAP CAR, respectively; Figure 2B). Our GMTC manufacturing process using CD3/CD28 beads and IL-2 (500 U/mL) for the activation step allowed for preservation of the initial CD4+/CD8+ ratio found in PBMCs (mean 55.25±7.89 vs. 57.98±13.58 and 34.28±10.1589 vs. 42.68±8.66, for CD4$^{+}$ and CD8$^{+}$ cells, respectively; n=4; Figure S2B).

Western blotting of subcellular fractions demonstrated that IL-1RAP CAR is associated with CD3ζ signaling (signal at 55 kDa compared to the expected endogenous CD3ζ signal at 16 kDa; Figure 2C). Additional analysis using serial dilution of biotinylated IL-1RAP protein (from 20 ng to 2.4 pg/mL) and FACS analysis detected IL-1RAP CAR-transduced CEM T cells or primary T cells. A single experiment showed that different amounts of recombinant protein (1.25 ng and 0.15 ng) were required to recruit the maximum CEM (85.8%) or primary (68.5%)

GMTCs (Figure S3). Moreover, the addition of equivalent amounts of sIL-1RAP (up to 500 pg/mL) to E:T co-culture did not affect the cytotoxicity of IL-1RAP CART cells, but a higher amount (from 125 ng/mL to 10 μg/mL) of cold recombinant IL-1RAP protein led to significant inhibition of effector cytotoxicity (Figure S4A). K562v5 Cell culture supernatant containing secreted sIL-1RAP didn't affect the CART-cell cytotoxicity (Figure S4B). Overall, these results confirmed that CAR is present at the cell surface and that soluble IL-RAP does not affect IL-1RAP/IL-1RAP CART cell binding.

**IL-1RAP–CART cells secured by an iCASP9 safety switch have no major deleterious effect on healthy hematopoietic cells**

For off-target toxicity prediction, we used the #A3C3 mAb to investigate IL-1RAP expression using a tissue macroarray (TMA) of 30 normal human tissues. Staining was detected at various intensity levels, excluding inflammatory or necrotic elements, in only six tissues: lymph node, prostate, skeletal muscle, stomach, colon and small intestine, and pancreas (Figure S5A and Table S2). Interestingly, the microvascular HMEC-1 endothelial cell line is not recognized by our #A3C3 IL-1RAP mAb (Figure S5B), whereas the R&D IL-1RAP mAb clearly detects cell surface expression, suggesting recognition of a different epitope.

Regarding targeting of the healthy hematopoietic system, if mAb #A3C3 did not detect HSCs in bone marrow (RFI<1.2, n=5) from healthy donors (Figure 3A, B) or normal cord blood (Figure 3B, C), we noted weak staining (RFI<2) of the monocyte subpopulation in PB (2/5) and BM (3/5) from healthy donors (Figure 3A). Thus, we studied in vitro sensitivity of monocytes by co-culturing PBMCs with autologous CART cells at various E:T ratios. At ratio of 1:1, only some of the monocytes are targeted, leaving 41.45% of monocytes alive (Figure 3D, Table S3), whereas lymphocytes, granulocytes, and the K562 IL-1RAP-negative cell line are not affected (Figure 3D), even at superior E:T ratios. Interestingly, at this E:T ratio, 94.77% of leukemic cells are killed (Figure 3E). These results were confirmed in-vivo, in an hCD34-engrafted murine model (hu-NOG) (Figure S6A) receiving functional IL-1RAP CART-cells (Figure S6B), in which we demonstrated that, although monocytes decreased on day 15 (41 ± 25%, n=3, p=n.s), that other human immunocompetent cells derived from hCD34+ cells are not affected by CART cells (Figure S6C). Hematopoietic stem cell culture assay after in vitro co-culture of healthy CD34+ cord blood HSCs with autologous CART cells (n=3) confirmed that HSCs are not affected (Figure S7). These results agree with IL-1RAP CART cell immunotherapy being associated with few side effects on the hematopoietic system.

The Functionality of the safety switch of the iCASP9/AP1903 suicide system cassette was evaluated after chemical inducer dimerizer (CID; 10 nM) exposure. First, using optical microscopy, 293T cell culture transduced by IL-1RAP CAR was sensitive to the CID (Figure S8A, top). Cytometric analysis showed that, in a mixed population of CD19+ and CD19- IL-

1RAP CART cells, only the CD19- CD3+ cells persisted after 24 hours of CID exposure (Figure S8A, bottom). More precisely, in a quantitative assay of apoptosis, 84.11% and 88.93% of IL-1RAP CART cells were eliminated after 24or 48h of CID exposure, respectively, compared to nontransduced T cells (C0) (1.28% and 6.13% at 24 or 48 hours, respectively; $p<0.001$, n=3; Figure 3F). Finally, in-vivo evaluation of the safety switch in the NSG murine model showed that 87 ± 7.32% ($p<0.01$, n=3) of IL-1RAP CART cells can be eliminated after i.p. AP1903 administration but were not affected after PBS administration, whereas control T cells (C0) are not affected by either treatment (Figure 3G and S8B).

**IL-1RAP–dependent proliferation and cytokine secretion of IL-1RAP CAR-expressing T cells**

To analyze the proliferative and functional properties of IL-1RAP CART cells, in addition to the KU812 cell line naturally expressing IL-1RAP, we generated a deficient MHC class I cell line, K562, expressing either the membrane (isoform 1) or soluble (isoform 3) forms of IL-1RAP translated from variant 1 (v1) or 5 (v5) transcripts, respectively (Figure S9A, B, C). Interestingly, we showed that IL-1RAP expression was higher in transfected K562-v1 cells than KU812 cells (RFI=10.57 vs 33.46; Figure S9D).

To determine the Proliferative capability of IL-1RAP CART cells triggered by IL-1RAP target-expressing cells was determined by co-cultured (E:T=1:1) CFSE-stained (C0), Mock, or IL-1RAP CART cells in the presence of K562, K562-v1, -v5, or KU812 cells. Effector IL-1RAP CART cells divided significantly only in response to the presence of cell surface IL-1RAP–expressing K562-v1 (76.1%±10.9) and KU812 cells (81.6%±6.16), and divided at a lowest levels against K562-v5 (27.3%±9.03) or medium only (18.8%±7.02) (Figure 4A; $p<0.001$, n=4).

IL-1RAP CART CD8+ or CD8- cells, but not C0 or Mock cells (E:T ratio of 1:5), produced IFN$\gamma$ and exclusively against IL-1RAP–expressing target cells K562-v1 (CD8+, 23.7±0.71%; CD8-, 14.8±3.58%) and KU812 (CD8+, 22.3±2.39%; CD8-, 13.1±2.79%; $p<0.001$, n=4). No response was found against K562 alone or K562-v5 (Figure 4B).

Finally, Co-culturing of target cells with C0, Mock, or CART cells (E:T=1:1). Showed that only cell surface IL-1RAP–expressing K562-v1 and KU812 cells could trigger cytokine secretion with robust IFN$\gamma$ and IL-2 secretion, moderate TNF$\alpha$, and low IL-4, IL-6, and IL-10, but not IL-17 secretion, indicating a specific Th1 profile (Figure 4C and Figure S10).

**IL-1RAP–dependent CAR cytotoxicity and lysis of IL-1RAP–expressing tumor target cells**

The CD107a&b degranulation assay applied to IL-1RAP CART cells co-cultured at an E:T ratio of 1:5 against IL-1RAP+ (K562-v1, KU812) target cells demonstrated specific and significant cell surface mobilization of CD107a&b in both the CD8- (mainly CD4+) and CD8+

compartments of IL-1RAP–specific T cells. ($p<0.001$, n=4; Figure 5A). IL-1RAP-dependent cytolytic potency of IL-1RAP CAR-expressing T cells in vitro, was determined using fluorescent (eFluor) and 7-AAD staining to discriminate CART cells and living cells, respectively. As expected, co-culture at an E:T ratio of 1:1 and compared to C0 or Mock-T cells revealed significant lytic activity characterized by the disappearance of cells in the 7-$AAD^-$/$eFluor^-$ gate between IL-$1RAP^+$ (K562-v1 and KU812) target cells and IL-$1RAP^-$ (K562, K562-v5) target cells ($p<0.001$, n=4; Figure 5B).

**Xenograft murine model**

In a tumor xenograft murine model (Figure 6A), following tumor engraftment (D4), IL-1RAP CART cells (E:T=1:1) were allowed to target K562-v1 IL-1RAP+/Luc+ (i.p., Figure 6B) or CML KU812/Luc+ (i.v., Figure 6C) tumors until a decrease in size (D4 to D9) was noted, leading to complete elimination (>D9, i.p.). In contrast, we noticed tumor progression in untreated or Mock-T–treated mice, leading to death (2/3 in both groups, respectively, at D28), but no mice died in the CART cell–treated group. Notably, tumors continued to grow in the absence of CART cells in surviving mice in the untreated and Mock-T-treated groups (Figure 6B, C). In a second animal experiment, mice (n=6/group) were grafted with bulk tumor cells (97.3% K562-v1 IL-1RAP+/GFP+/Luc+) containing residual (2.7%) IL-1RAP-/GFP-/Luc+ cells (Figure S11A). After the first objective response (D7, in 5/6 mice), and despite a second CART-cell injection, we noted the recurrence of Luc+ cells (D24) (Figure S11B).Flow cytometric analysis of human (hCD45+) tumor cells sorted revealed that cells are IL-1RAP-(GFP-) cells originated from initial bulk injection (Figure S11C).

**In vitro cytotoxicity against primary IL-1RAP-expressing cells from CML patients**

From a primary TKI-resistant CML patient (always with BCR-ABL(IS) ratio > 10%) to five lines with four TKIs (Figure 7A) treatment for a period of 4 years, we were able to produce CART cells with a transduction efficiency of 95.5% (Figure 7A). IL-1RAP CART cells exhibited dose-dependent cytotoxic activity against IL-1RAP+ KU812 cells with 95% efficiency at an E:T ratio of 3:1 compared to an allo-reactive cytotoxicity of 18% and 21% for C0 or Mock-T cells, respectively (Figure 7B). Co-culture of autologous IL-1RAP CART cells against CML patient PBMCs exhibited specific lysis ($76.65 \pm 9.2\%$ for IL-1RAP CART cells compared to $4.16 \pm 4.3\%$ and $2.78 \pm 1.72\%$ for C0 or Mock-T cells, respectively) of IL-1RAP+/CD34+ cells after 24 h (Figure 7C).

Moreover, autologous IL1-RAP CART cells produced (transduction efficiency: 85.33 ± 8.8%) from CML patients (n=3) under long-term treatment, including TKIs, or free of treatment (TableS4), and directed against their respective initial long-term cryopreserved

(>20 years) peripheral blood stem cell autograft, killing the CD34+/IL-1RAP+ cells with an efficiency of 79.78 ± 10.7% (Figure 7D and Figure S12).

## Discussion

Immunotherapy approaches using gene-modified T cells expressing a CAR have emerged as powerful tools for patients with solid or liquid tumors. Evident positive results have been obtained in hematology (24), particularly with CART cells directed against CD19 antigen in patients with relapsed/refractory ALL, resulting in a high rate of long-lasting remission (36).

Based on the identification of a cell surface biomarker, IL-1RAP, that allows discrimination of CML from normal HSCs (26,27,29) and measurement of CML tumor burden (28), we investigated a cell-based killing strategy to target CML HSCs. Despite the very high efficiency of TKI treatments in CML, we need to provide new approaches to eradicate this disease.

IL-1RAP is an interesting target because it is a rare cell surface marker that is upregulated in all leukemic CML but not normal HSCs, whereas the other CML HSC markers, CD25 and CD26, are expressed only in a portion of BCR-ABL1$^+$ cells (27). Of additional interest is that IL-1RAP is overexpressed in AML and high-risk MDS and correlates, in this case, with poor clinical outcome (37). Anti-IL-1RAP antibody has been demonstrated to kill primary cells from AML patients in vitro (25) and in a xenograft AML murine model (31). Thus, IL-1RAP is an additional target for new cell immunotherapy in myeloid malignancies in addition to CD123, CD33, or CD44v6 targeting.

Despite the impressive success of CART cell immunotherapies in R/R ALL, LNH, CLL, or MM treatment (38), this approach is frequently associated with toxicity, such as cytokine release syndrome, neurotoxicity, B cell depletion, immunosuppression, or tissue toxicity in the case of targeting CD19 or TAA expressed by healthy tissues or cells (on-target, off-tumor), or tumoral lysis syndrome when applied in the context of a high tumor burden (39). Poor or bad hematopoietic reconstitution may also occur when the CART cells target HSCs expressing cell surface antigens. We have shown that IL-1RAP is not expressed by normal CD34+ HSCs, which our #A3C3 mAb does not recognize microvascular endothelial cell, but it does stain monocyte cells, though not in all healthy peripheral blood or bone marrow samples. In vitro co-culture of autologous IL-1RAP CART cells demonstrated that, at a 1:1 E:T ratio, <50% of monocytes were targeted, whereas >90% of leukemic cells were killed. To the best of our knowledge, depletion of a portion of the monocyte population does not represent a major clinical problem. Moreover, IL-1 secreted by monocyte/macrophages was recently reported to be involved in cytokine release syndrome (CRS) and neurotoxicity after CART-cell infusion (40). Thus, partial targeting of this population, as shown in our IL-1RAP CART-cell model may contribute to limiting these deleterious effects. Finally, an absence of significant #A3C3 immunostaining in healthy TMA, though it does not constitute formal and final proof, is also an argument that IL-1RAP targeting may be associated with limited side effects. This is in favor of continuing investigations of IL-1RAP CART-cell targeting, especially toxicity studies, at the preclinical level, until the first use in humans.

Use of an anti-IL-6 receptor antagonist (tocilizumab) and/or corticosteroid will help manage and reduce these complications. Moreover, the presence of the suicide cassette in our CAR lentiviral construct makes CART-cell depletion possible if complications occur. To limit potential adverse events, other tools should be explored, such as new designs of viral constructs, integrating our IL-1RAP CAR in bispecific/dual (tanCAR), inducible, inhibitory (iCARs), conditional (UniCAR), safety (sCAR) backbones (41).

With the success of TKI treatments, TKIs clearly remain the best, safest, and easiest-to-manage first-line therapy for CML in the clinical setting, though they do not eradiate residual CML stem cells and do not biologically cure the disease, though most CML patients are considered clinically cured. CML disease is highly susceptible to the allogenic immune system, as indicated by allo-SCT (42) and donor lymphocyte infusion (DLI) (43), making advanced phases of CML, high-risk disease, or disease in the chronic phase that is resistant or intolerant to TKIs the ideal candidate for allogenic donor-derived CART cell immunotherapy (DLI administration). In this context, matched human leukocyte antigen donor T cells may be used as a source of GMTCs in sequential association with TKIs or allo-SCT. This may circumvent CART cell manufacturing failures arising from the use of T cells from heavily treated patients (44). Especially in CML, the used of these donor CART cells will enhance the anti-leukemic effects by combining the alloreactivity and specific cytotoxicity of IL-1RAP antigen targeting. The major concern when infusing allogenic T cells is potentially inducing graft-versus-host disease, which may be controlled by activating the suicide iCASP9/AP1903 system (45) using our lentiviral construct. The suicide system may also be activated in the case of adverse events, such as cytokine release syndrome, cerebral nervous system toxicity, off-target toxicity, or tumor lysis syndrome (46).

IL-1RAP ADCC eliminates CML stem cells (26,32) and primary AML cells (31) through IL-1/IL-1RAP signaling blockade via proliferation and expansion of the suppression mechanism of cells responsive to IL-1. The same work highlights this lysis mechanism by IL-1RAP and ADCC, which recruits effector NK cells to activate the immune response. In CML, TKI treatment has a different effect, with a significant increase or decrease in the immune NK effector population after dasatinib or nilotinib treatment, respectively (47). However, even if NK cells play an important role in mediating treatment-free remission in CML, half of patients who cease TKI therapy relapse due to a failure of the immune system (48). In this context, a defect in the NK cell population will reduce ADCC efficiency, and these patients will need alternative immunotherapy approaches, such as CART cells, to achieve a cure. Here, we provided a new therapeutic approach with direct recognition of the IL-1RAP cell surface protein and without the need for accessory immunocompetent NK cells.

In addition to the transmembrane form of IL-1RAP, alternative splicing results in an mRNA encoding a secreted and soluble (sIL-1RAP) form characterized by the extracellular

domain of the protein. The soluble form may interfere with membrane IL-1RAP–expressing tumor cells by saturating the CAR. In healthy people, circulating levels of soluble IL-1RAP are detected at a mean concentration of 200 pg/mL (49). We demonstrated in vitro that a higher concentration (2400 pg/mL) of IL-1RAP recombinant protein did not result in staining of IL-1RAP CAR-expressing T cells (Figure S3). Moreover, both recombinant IL-1RAP protein < 125 ng/mL (Figure S4A) and the culture supernatant of K562-v5, secreting sIL-1RAP (Figure S4B) did not affect CART cytotoxicity against IL-1RAP–expressing targets. Overall, these findings suggest that sIL-1RAP would not interfere with IL-1RAP tumor cell recognition, though an accurate serum sIL-1RAP concentration needs to be identified in a cohort of CML or AML patients for precise quantification of expression.

This pre-clinical work demonstrates for the first time the whole production and validation process of CART cells directed against IL-1RAP–expressing CML stem cells, from the development and characterization of the mAb to the final in vitro and in vivo functional studies of gene-modified CART cells. We also demonstrated that multi-TKI treatment over a 4-year period does not affect transduction efficiency or otherwise improve it, even if it remains to be demonstrated formally. The cytotoxic activity of autologous CART cells has also been demonstrated in CML patients, even if the CML model is a limited application field in the TKI area, and would be beneficial to TKI non-responders or TKI-intolerant CML patients and young or accelerated/blastic phase CML patients, who are all candidates for allograft. Because AML leukemic cells express IL-1RAP (37), this promising CART cell immunotherapy approach may be applied in clinically critical acute hematological disease. In this context (CML), use of IL-1RAP CART allogenic cells would constitute a bridge towards allograft. This new potentially curative immunotherapy for CML could also help with medico-economic considerations.

Taken together, these findings based on the CML model make IL-1RAP an interesting TAA for immunotherapy cell targeting using CART cells. In-depth studies are required to determine and/or reduce potential toxicities and side effects before phase I clinical trials.

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# Figure Legends

**Figure 1. IL-1RAP mAb production and characterization.**

**(A)** Top, Screening of IL-1RAP mAb using different cell lines. An IL-1RAP (R&D) murine mAb (IgG1 Clone #89412) was used for staining comparison. Bottom, Western blotting of different hematopoietic cell lines with the #A3C3 IL-1RAP antibody (1:20). A transfected HT1080 cell line with IL-1RAP cDNA variant 1 (IL-1RAPv1) was used as a control. Actin was revealed as a protein loading control. **(B)** #A3C3 mAb immunostaining and confocal microscopy on frozen IL-1RAP$^+$ (KU812) or IL-1RAP$^-$ (Raji) cells. Confocal of KU812 and Raji cells stained with fluorescence mAbs. Left, anti-murine Fc-IgG; middle, IL-1RAP (#A3C3). **(C)** Representative flow cytometric analysis of primary cells from a single CML patient's bone marrow (BM) at diagnosis or 3 or 6 months) after imatinib treatment (400 mg; IM400; left) and the CML patient cohort (right). *p<0.05, ** p<0.01. IL-1RAP (#A3C3) was used in combination with CD34$^+$ and CD38$^-$ fluorescent staining. (○): CD34+/CD38-, (□): CD34+/CD38+. **(D)** RT-qPCR of IL-1RAP mRNA expression. Relative gene expression in whole blood samples from CML patients (n=5) at diagnosis, PBMCs from healthy donors (HD, n=3), CD14+ sorted monocytes from HD, IL-1RAP variant 1 or 5 cDNA-transfected K562 cells, or other cell lines (lymphoma, ALL, n= 4) is provided compare to calibrator KU812 cell line.

**Figure 2. Generation of IL-1RAP CAR–expressing gene-modified T cells.**

**(A)** Schematic overview of the IL-1RAP CAR lentiviral vector. The construct carries three different parts: the suicide safety cassette iCASP9 (50), the IL-1RAP CAR, and the cell surface selection marker, ΔCD19 (CD19 truncated at the intracellular part to avoid signaling). A mock construct missing the CAR sequence was used as a control construct. **(B)**. Lentiviral transduction efficiency of donor T-cells measured by flow cytometry (top). MOI was deducted from SN titration according to the number of starting cells. Representative Cytometry plot after CD3$^+$/CD19$^+$ staining of nontransduced (C0), Mock-T, and IL-1RAP CART cells (bottom left).Results are presented as Mean ± standard deviation (SD) of 6 independent transductions of 6 different donor PBMCs (bottom right) using 100X concentrated Supernatant. **(C)** Western blot analysis of IL-1RAP CAR expression using a mouse anti-human CD3ζ antibody. Sizing allowed discrimination of CD3ζ associated with CAR (55 kDa) and the endogenous form (16 kDa). Additional probing with CD45, lamin, and GAPGH antibodies confirmed fraction enrichment. See also supplementary data.

**Figure 3. Effect of IL-1RAP CAR T cells on healthy hematopoietic cells and efficiency of the safety suicide gene iCASP9 cassette.**

**(A)** IL-1RAP cell surface expression on peripheral blood (left) or bone marrow (right) cells from healthy donors (n=5). SSC-A/CD45+ allowed discrimination of subpopulations as lymphocytes (SSC-A low), monocytes (CD33+), granulocytes (SSC-A high), or HSCs (CD33-/CD34+). RFI is provided in each window. **(B)** IL-1RAP-positive cells among CD34+ cells in cord blood (CB, n=5) or bone marrow (BM) from healthy donors (n=5) compared to CD34+ cells from the BM (n=10) or peripheral blood (PB, n=10) from CML patients. **(C)** Representative IL-1RAP staining of whole human cord blood cells. IL-1RAP staining is provided for whole CD34+, CD34+/CD38-, and CD34+/CD38+ HSC cord blood subpopulations.

**(D)** Left, Dot plot of granulocyte (G), monocyte (M), and lymphocyte (L) subpopulations cultured in the presence of different effector:target (E:T) ratios of autologous nontransduced T cells, Mock or IL-1RAP CART cells. Right, Relative percentage of alive cells among lymphocytes (square), monocytes (circle), and granulocytes (triangle), normalized to nontransduced autologous T cells (C0) co-cultured 24 h with autologous Mock-T cells (dashed line) or IL-1RAP CART cells (solid line). **(E)** Comparison of IL-1RAP CAR killing between monocytes and KU812 leukemic cell line Relative percentage of alive cells among the monocyte (square), KU812 (circle), or K562 (triangle) subpopulations in the presence of different E:T ratios of Mock (black, dashed line) or IL-1RAP CAR T cells (white, solid line). Percentages were calculated using absolute cell number determined using Trucount tubes (cytometry acquisition of 5000 fluorescent-beads). **(F)** Left, Gating strategy and analysis for absolute count of CID AP1903-induced cell death. Nontransduced (C0) or IL-1RAP CAR T cells were exposed to medium alone or medium +CID (20 nM, 24 h). The quantification was performed after acquiring 5000 fluorescent beads. Killing efficiency was normalized to control cells (untreated cells). Cell killing was calculated as follows: % Dead cells= [1−(absolute number of viable cells in AP1903-treated cells/absolute number of viable cells in untreated cells)] × 100. Right, Absolute percentage of mortality. 24 h or 48 h C0 or IL-1RAP CAR (gated on CD3+/CD19+) T cell CID exposure. Right, Means from three independent experiments. ** p<0.001. See also supplementary data. **(G)** Absolute quantification of IL-1RAP CAR T cells injected in a tumor (CML KU812, i.v.) xenograft NSG model 24 h after i.p. AP1903 (white bars) treatment (n=3 mice/group). Mice infused with control T-cells (C0) were used as controls (n=2 mice/group). **p<0.01. Number of cells is provided per ml of PB.

**Figure 4. IL-1RAP CAR T cells proliferate in co-culture with IL-1RAP–expressing target cells secreting and releasing IFNγ and pro-inflammatory cytokines. (A)** Top, Gating strategy for flow cytometry CFSE dilution analysis. Nontransduced (C0) and Mock or IL-1RAP CAR transduced T-cells were cultured in medium alone, in contact with IL-1RAP$^-$, cell surface (K562-v1 and KU812), or soluble (K562-v5) IL-1RAP$^+$–expressing target cells at an effector:target (E:T) ratio of 1:1. Effectors were labeled with 0.5 μM CFSE. After 48 or 96 h of co-culture without IL-2 supplementation, measure of CFSE dye dilution allow to assessed the division of live CD3$^+$/CD19$^+$ gated cells. Bottom, Percentage of total dividing CFSE-positive cells. Mean ± SD of four independent experiments. *** p<0.001. **(B)** Left, Gating strategy for intracellular IFNγ cytokine detection. C0, Mock-T, or IL-1RAP CAR-T cells were co-cultured at least 16 h at an E:T of 1:5 in the presence of target cells expressing or not expressing IL-1RAP at the surface. Cells stimulated with PMA/ionomycin were used as positive controls. Fluorescent IFNγ signal was detected after gating CD3$^+$/CD19$^+$/CD8$^+$or /CD8$^-$ populations. Right, Percentage of total intracellular IFNγ-producing cells. Mean ± SD of four independent experiments for CD8$^+$ and CD8$^-$ (mainly CD4$^+$) cells. *** p<0.001. **(C)** Cytokine-binding assay was used to capture and dose secreted IFNγ, IL-2, TNF, IL-4, IL-6, IL-10, and IL-17 cytokines within the culture medium. See also supplementary data.

**Figure 5. Lysis function analysis of effector cells. (A)** Left, CD107a&b degranulation assay. Transduced (Mock or IL-1RAP CAR) and non-transduced T cells were co-cultured at an E:T ratio of 1:5 for 6h with target cells expressing or not expressing IL-1RAP. After 5h, CD3$^+$/CD19$^+$/CD8$^+$ cells were analyzed by flow cytometry for CD107a and CD107b staining. Right, Percentage of total CD107a&b-positive CD8$^+$ or CD8$^-$ cells. Mean ± SD of four

independent experiments. *** p<0.001. **(B)** Left, Efficacy of IL-1RAP CAR T cells at lysing cell surface IL-1RAP–expressing cells. Effectors,labeled with e-Fluor were cultured at an E:T of 1:5 in the presence/ absence of IL-1RAP+ target cells . Right, Percentage of total killed target cells. Mean ± SD of four independent experiments. *** p<0.001. See also supplementary data.

**Figure 6. NSG murine xenograft tumor model experiment. (A)** Mice were sub-lethally irradiated at a dose of 2 Gy (n=3/group) 24 h before tumor transplantation. 5×10E6 clonal cells of K562-v1, an IL-1RAP$^{+}$–, luciferase$^{+}$–, and green fluorescent protein (GFP)-positive cell line (K562-v1$^{IL\text{-}1RAP+/GFP+/Luc+}$) or CML KU812$^{Luc+}$ cell line were respectively transplanted via intraperitoneal (i.p.) or intravenous (i.v.) injection into 6 to 8-week-old NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice . At an effector:target ratio of 1:1, Mock-T or IL-1RAP CAR T cells were injected i.p. or i.v. 4 days after tumor injection. Controls: A group of tumor engrafted mice not treated with T cells. **(B)** BLI analysis of mice of different groups from days 2 to 28. (x): dead mice, (⊗): euthanized mice, (➡): the time of gene-modified T cell (IL-1RAP CAR or Mock-T cells) injection. See also supplementary data. **(C)** Top, BLI analysis of mice of different groups from days 3 to 19. (➡) the time of gene-modified T cell (IL-1RAP CAR- or Mock-T cells) injection. Bottom, Radiance of the the in vivo bioluminescent signal (radiance p/s/cm$^2$/sr) harvested using the IVIS Illumia III (Perkin Elmer). (⬇) indicates time of IL-1RAP CAR T-cell injection. * p<0.05, ** p<0.01.

**Figure 7. In vitro toxicity against primary IL1-RAP+ circulating cells from a CML patient**. (**A)** Left, Kinetic quantification of the BCR-ABL1 transcript ratio (% on International Scale) according to the Europe Against Cancer (EAC) method. RM3.0, RM4.0, RM4.5, and RM5.0 represent molecular response levels corresponding to a decrease of 3, 4, 4.5, and 5 Log, respectively. IM400: imatinib 400 mg/day, DAS100: dasatinib 100 mg/day, BOS400: bosutinib 400 mg/day, NIL600: nilotinib 600 mg/day. Right, Transduction efficiency of PBMCs from the CML patient. **(B)** Left, Dot plot of cytometry of effector cells, C0, Mock-T, or CAR T cells, labeled with eFluor and co-cultured with KU812 cells at various E:T ratios. Right, Graphical representation of persisting viable KU812 cells within the FSC+/7-AAD- gate. **(C)** Left, Lysis efficiency of autologous IL-1RAP CAR T cells against IL-1RAP–expressing cells from a TKI-resistant CML patient. Right, Percentage of total killed target calculated from duplicate experiments. Results are presented as mean ± SD. **(D)** Cytotoxicity of IL-1RAP CAR or Mock-T cells against their respective CML autografts at various (E:T) ratios. Aggregate results of three independent experiments (three different CML patients). The percentage of remaining viable CD34+/IL-1RAP+ cells calculated from control cells (C0) is provided. **p<0.01.

Figure 1

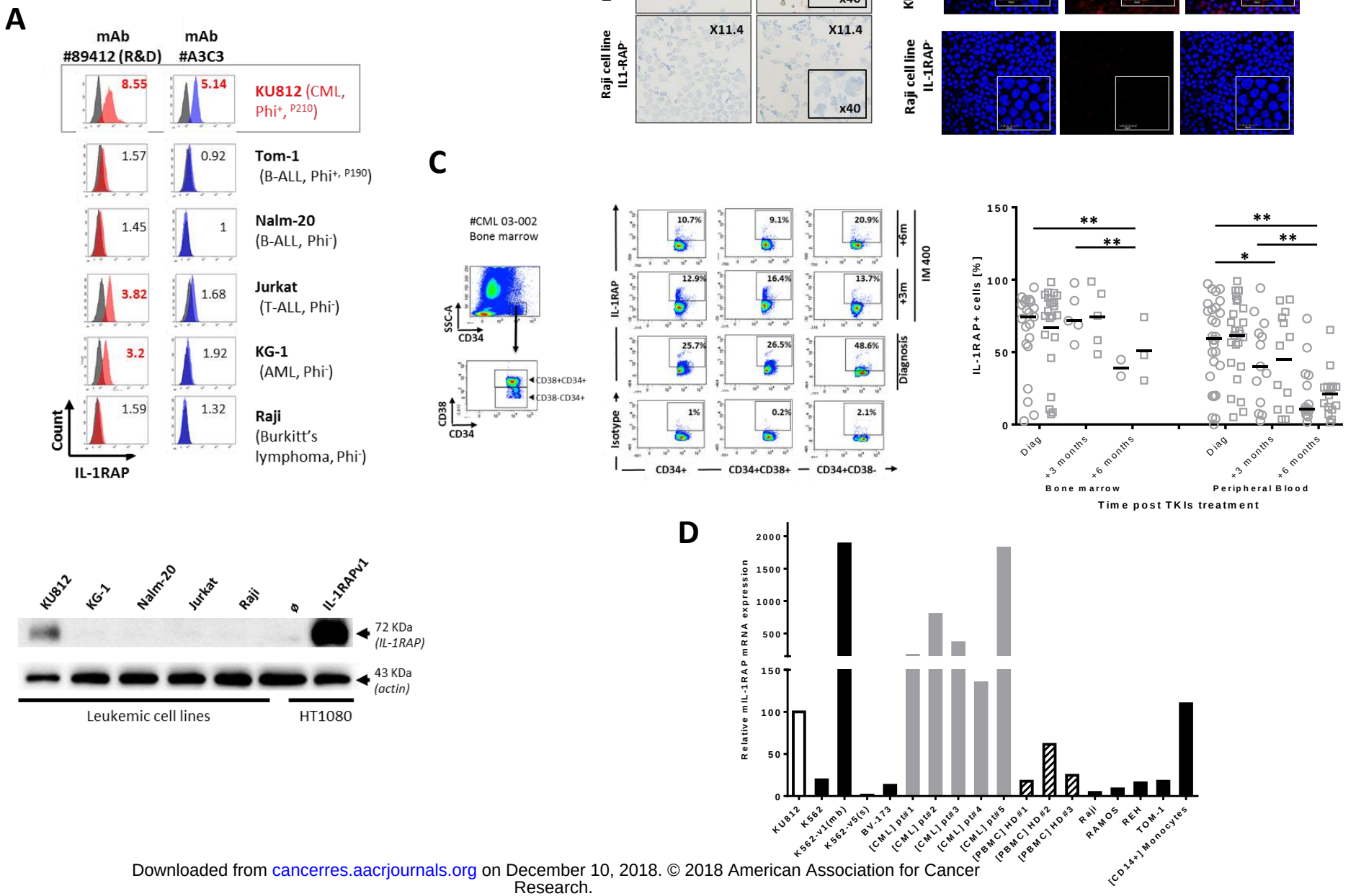
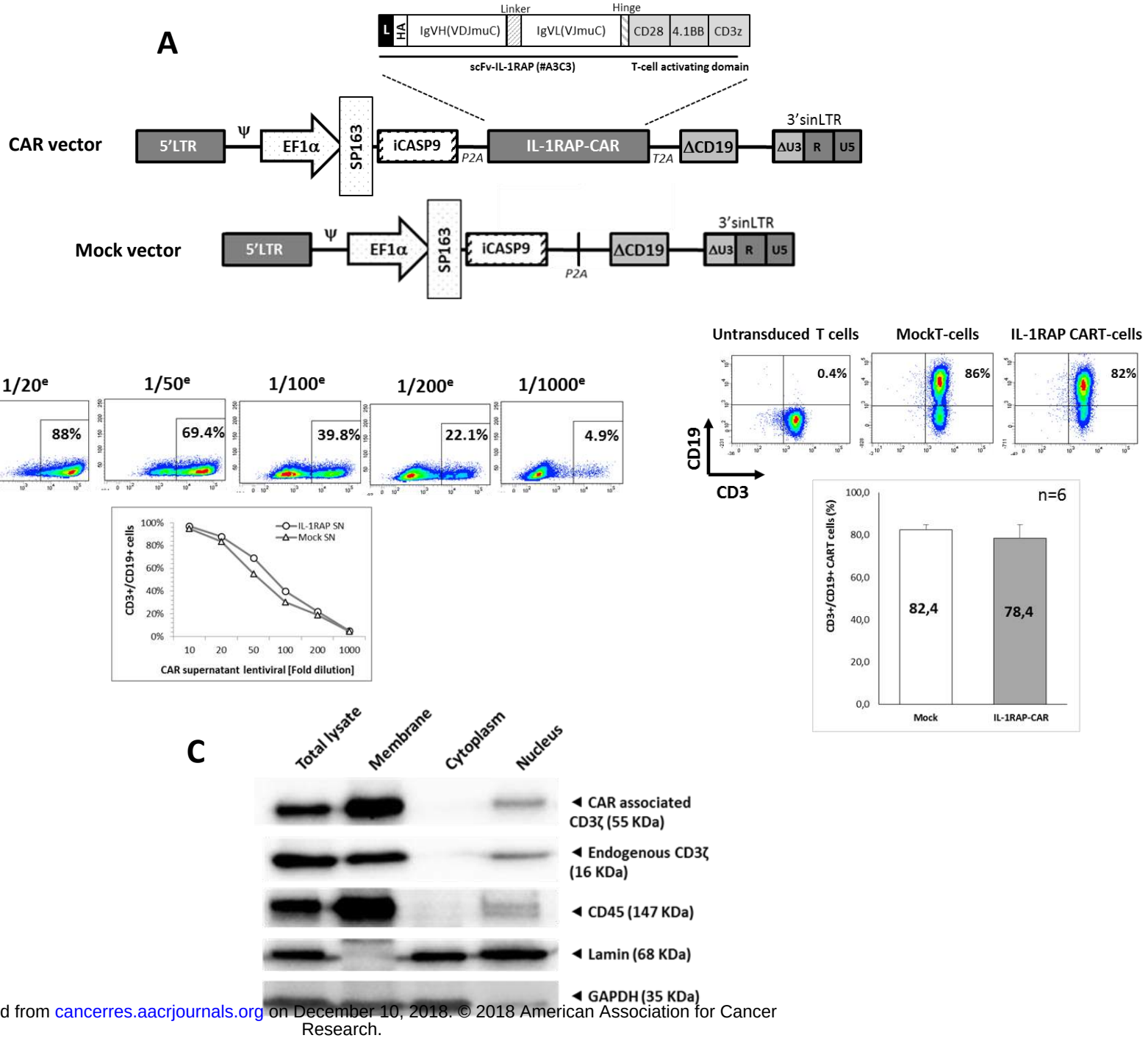


**Figure 2**



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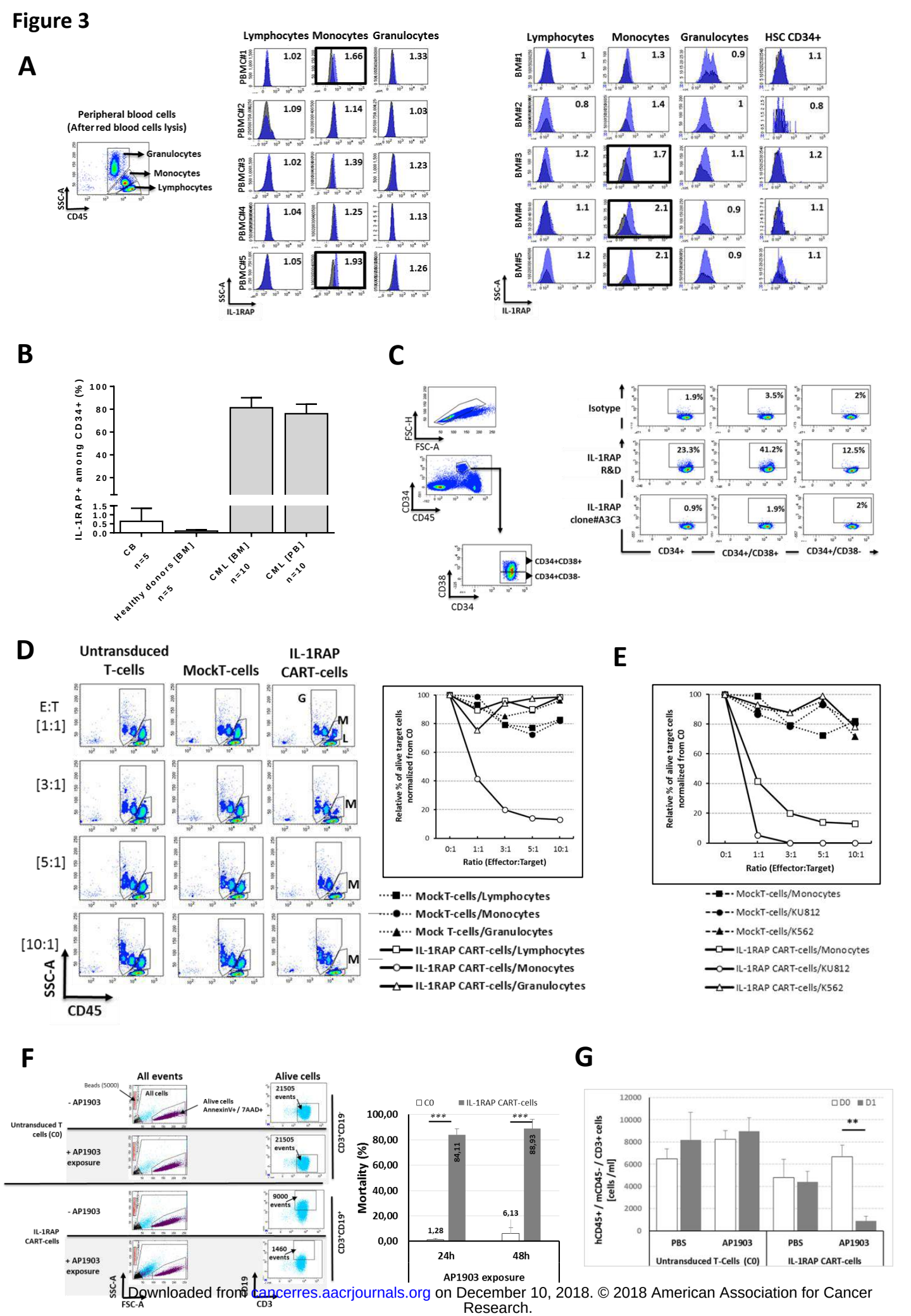

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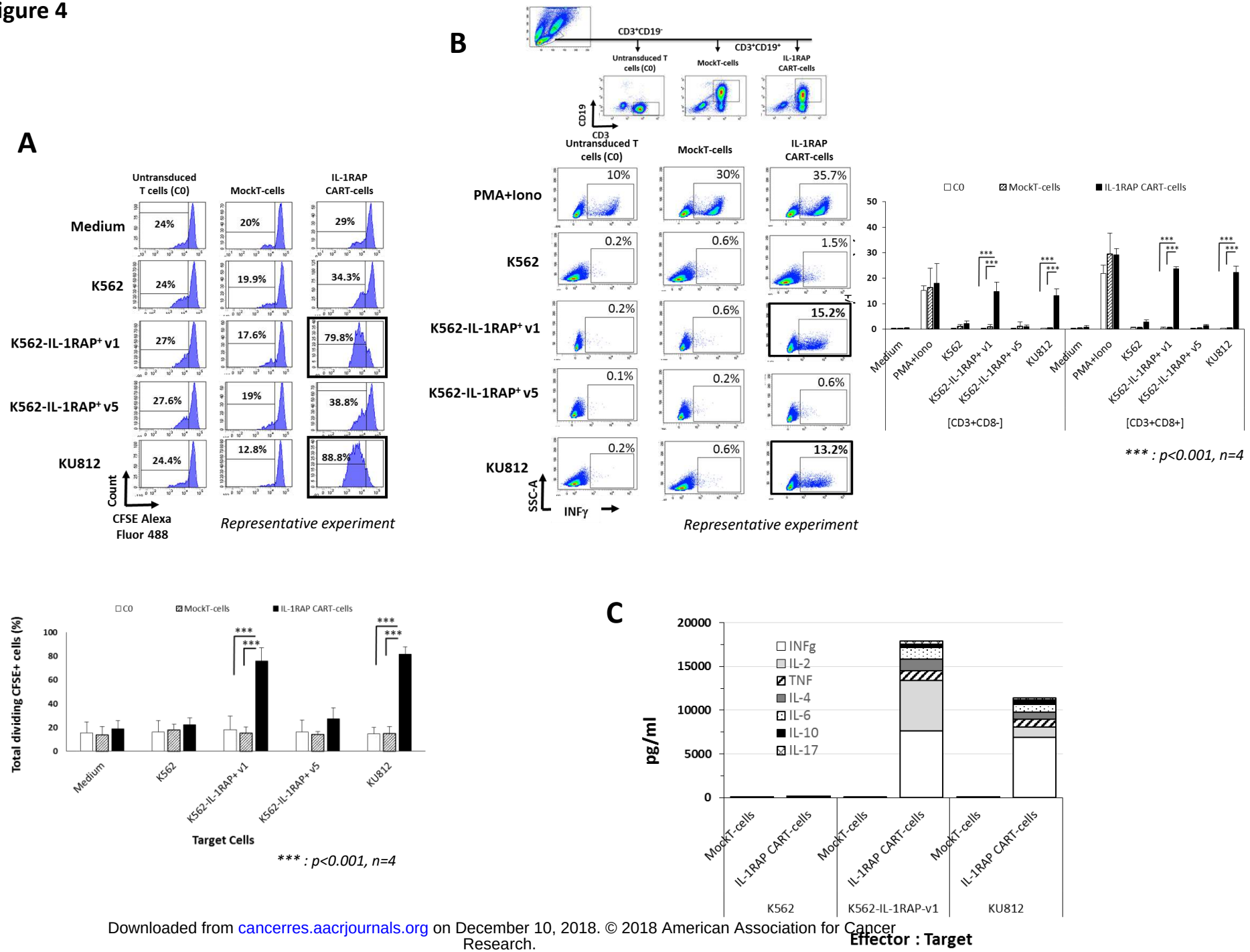

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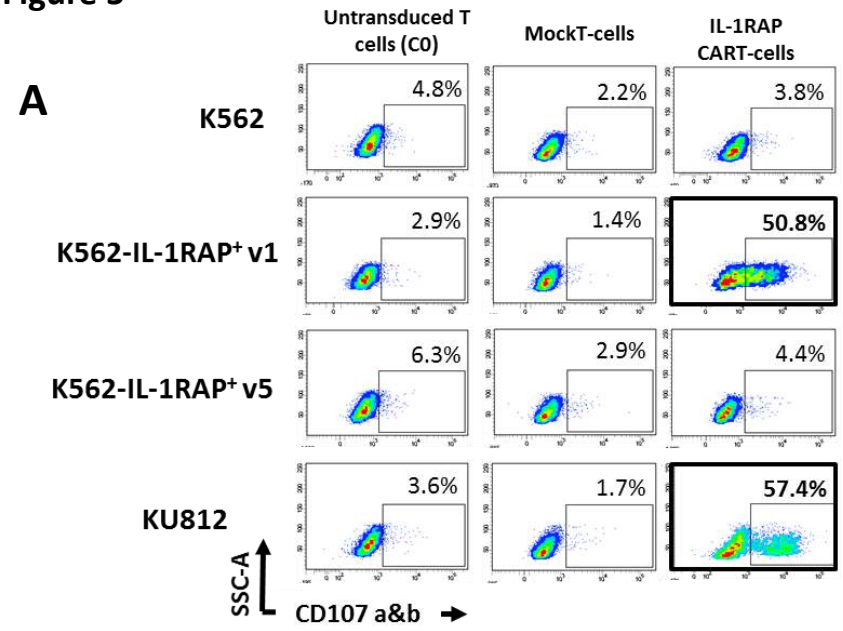
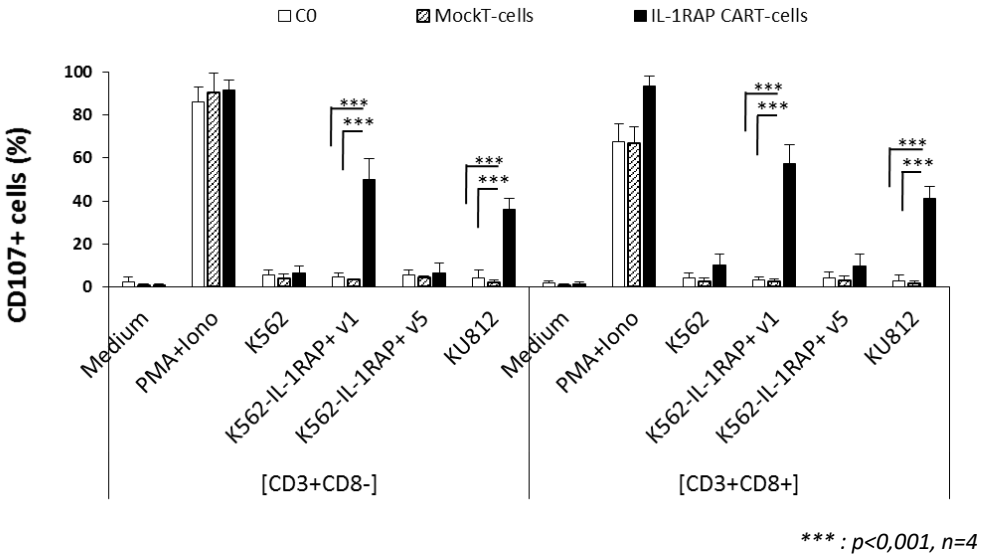
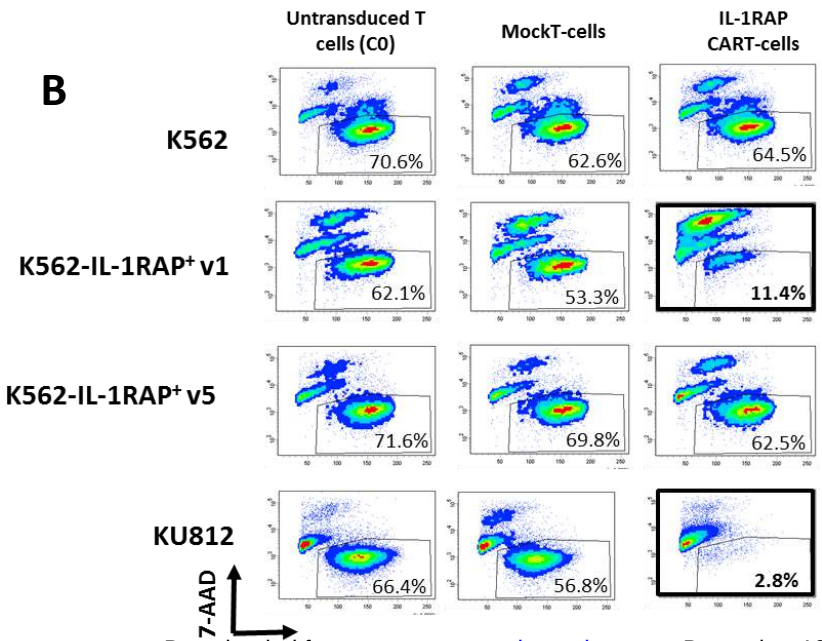
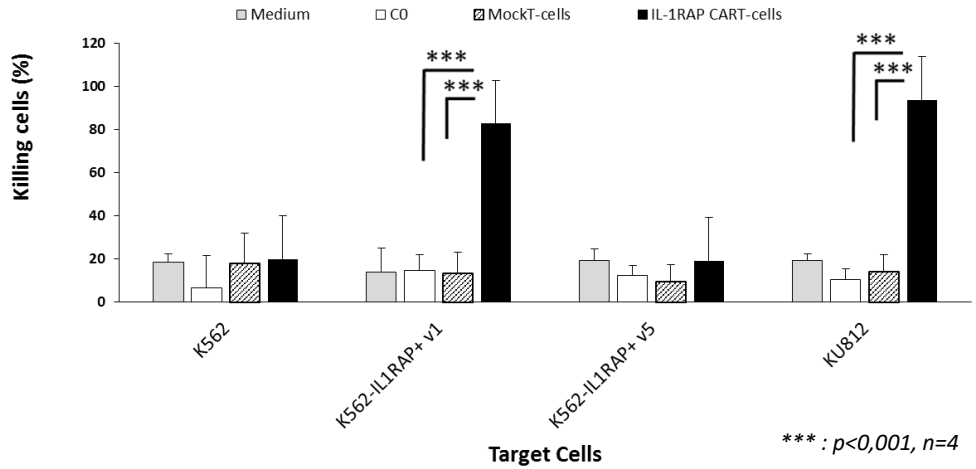



*Representative experiment*

Figure 6

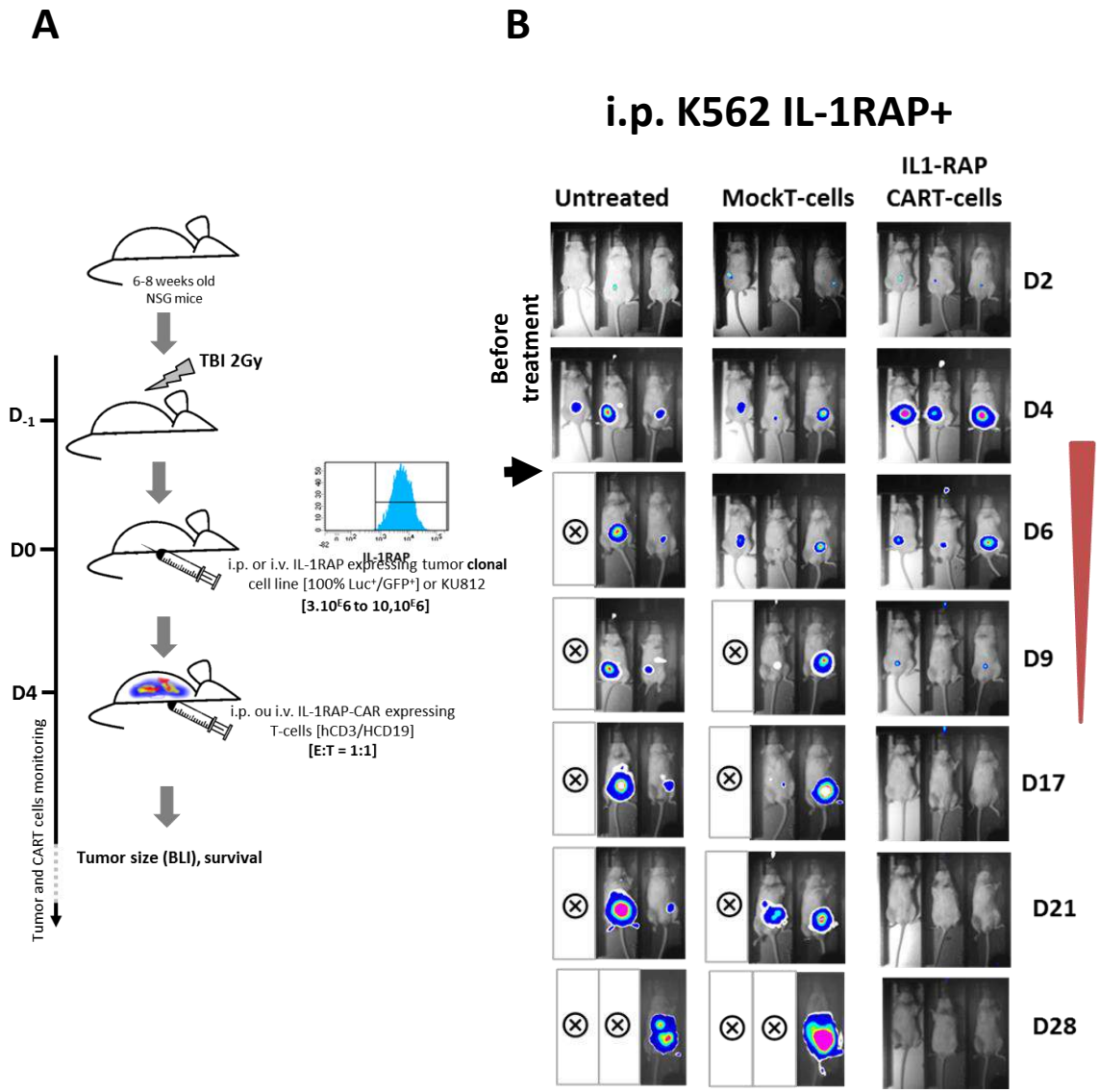
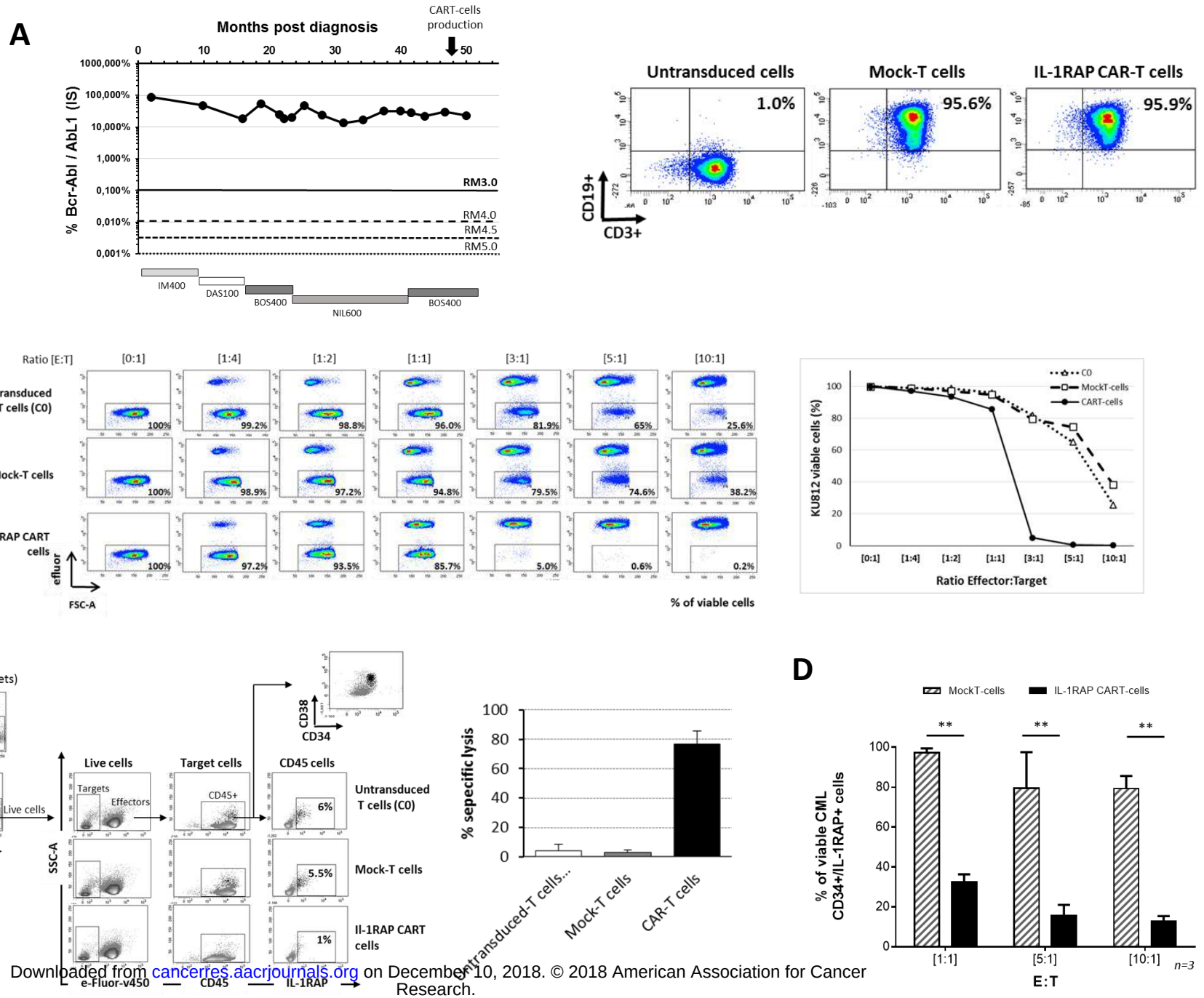


**Figure 7**



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The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

# CML hematopoietic stem cells expressing IL-1RAP can be targeted by chimeric antigen receptor (CAR)-engineered T cells

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