Cancer Research

CML Hematopoietic Stem Cells Expressing IL1RAP Can Be Targeted by Chimeric Antigen Receptor–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 patients with CML, 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 IL1 receptor-associated protein (IL1RAP) 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-IL1RAP 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 IL1RAP, with no apparent effect on the hematopoietic system, including $CD34^+$ stem cells. This suggests IL1RAP as a tumor-associated antigen for immunotherapy cell targeting. IL1RAP CAR T cells were activated in the presence of $IL1RAP^+$ cell lines or primary CML cells, resulting in secretion of proinflammatory 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.

Significance: These findings present the first characterization and proof of concept of a chimeric antigen receptor 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 (TKI) and allogenic stem cell transplantation (allo-SCT) associated with IFNα (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 relapses 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 patients with CML, 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 patients with CML 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 patients with CML with a suboptimal response to all available TKIs, but also to

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-18-1078

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patients with CML in the accelerated or blastic phase in order to bridge or substitute the allograft.

A graft-versus-leukemia immunologic effect of allo-SCT and the efficacy of donor lymphocyte infusion (DLI) 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/β-catenin, JAK/STAT, transforming growth factor-β/forkhead box O, and 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 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; ref. 21) and chronic lymphoblastic leukemia (22), but also in 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; ref. 24).

In CML and AML (25), gene expression profiling studies (26, 27) have revealed a cell-surface biomarker, IL1 receptor accessory protein (IL1RAP, IL1R3), 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 IL1RAP (26). Moreover, IL1RAP expression has been correlated with tumor burden and the clinical phase of CML (28, 29).

The IL1RAP protein is a coreceptor of the IL1 and IL33 receptor involved in IL1 signaling, activating different signaling pathways implicated in inflammation and proliferation (30). The tumor cell-surface expression makes IL1RAP 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 IL1RAP antibody selectively kills HSCs not only *in vitro* (25, 26), but also in a xenograft murine model of $CD34^+/CD38^-$ AML (31) or CML (32) HSCs.

Thus, IL1RAP 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 IL1RAP may eliminate leukemic stem cells. Here, we describe the proof of concept and the preclinical evaluation of an IL1RAP CAR in the context of CML.

Materials and Methods

Additional methods are provided in the Supplementary Materials.

Patient samples, healthy donor blood samples, and cell lines

Samples were collected from patients with CML at diagnosis and follow-up after TKI treatment within a noninterventional trial (ClinicalTrials.gov: NCT02842320). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient density centrifugation using Ficoll–Paque with anonymous blood samples collected from healthy donors at a French blood center. Patients and donors provided written-informed consent, and the study was conducted in accordance with the ethical guideline (declaration of Helsinki) and approved by the local ethics of 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 ATCC and stored in a master cell bank.

Monoclonal antibody production, selection, and molecular characterization

A mouse anti-hIL1RAP 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 IL1RAP and the Fc portion of human immunoglobulin (Ig)G1 (R&D Systems). 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 IL1RAP mAb #89412 (R&D) was used as a reference. The selected antibody (clone #A3C3) was more deeply characterized by Western blotting, ELISA against recombinant IL1RAP protein, IHC, confocal microscopy, and using primary samples from patients with CML. 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 variable heavy and variable light 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 CAR T-cell preparation

The CAR lentiviral construct (pSDY-iC9-IL1RAPCAR-dCD19) was prepared by cloning the synthetically produced single-chain fragment variable (scFv; derived from the #A3C3 IL1RAP hybridoma) into the SIN-pSDY backbone (kindly provided by Dr. Mateo Negroni, CNRS-UPR 9002). Lentiviral vector supernatant (SN) stock was harvested at 48 and 72 hours 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 SN encoding the IL1RAP CAR or Mock sequence (missing the CAR sequence) according to the procedure described in Supplementary Fig. S1. Briefly, a 9-day process comprised activation (CD3/CD28 beads), IL2 transduction (on day 2), and magnetic selection for CD19 cell-surface expression. $CD3^+/CD19^+$ gene-modified T cells (GMTC), mainly expressing CAR, were evaluated by flow cytometry.

Western blotting, subcellular fractioning, IHC, tissue microarray, confocal microscopy, and IL1RAP 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 IL1RAP #A3C3 mAb (diluted 1:10^3), CD3ζ, or β-actin for IL1RAP, CAR, or β-actin expression, respectively. Detection was performed with a camera and Bio-1D software (Vilber-Lourmat). An FDA standard frozen tissue array including 90 tissue cores (30 organs) from three individual donors per organ (US Biomax) 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 IL1RAP-positive or -negative cell pellets. Relative IL1RAP 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 patients with CML were tracked using a panel containing CD45, CD34, CD38, CD33, CD133, and CD117 and including our murine Alexa Fluor 488–labeled IL1RAP 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) and analyzed by DIVA 6.1 software (BD Biosciences). The mAbs used for phenotyping, intracellular staining, and cytometry, and other cytometry reagents are described in Supplementary 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 CAR T cells against live tumor cells was assessed by incubation for 20 to 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-IL1RAP 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 hours 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-IL1RAP variants 1 or 5, KU812, or patient primary cells were mixed with 2 × 10E6 T or CAR T cells at a predefined E:T ratio of 1:5 to study effector IFNγ synthesis by intracellular staining. For the cytokine profile of CAR T cells, we used the human Th1/Th2/Th17 Cytokines Bead Array Kit (BD Biosciences) allowing quantification of human IL2, IL4, IL6, IL10, TNFα, IFNγ, and IL17A secretion. CAR T-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 CAR T-cell cytotoxicity and iCASP9/rimiducid suicide gene efficiency. NOG mice (Taconic Biosciences) engrafted with human cord blood CD34$^+$ cells (Transcure Bioservices SAS) were used to assess autologous CAR T-cell toxicity against healthy HSCs and immune competent cells.

NSG mice were transplanted (i.p. or i.v.) with clonal or bulk Luc$^+$, IL1RAP$^+$, GFP$^+$ tumor, and CML KU812 cells, with or without injection of effector CAR T cells. Circulating CAR T 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-IL1RAP mAb clone #A3C3 exhibits specific recognition of human IL1RAP antigen

Selection of mAb subclones that discriminated IL1RAP-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 lymphoma, respectively) with a ratio fluorescent intensity (RFI) >2 (Fig. 1A) was performed. Use of #A3C3 mAb in Western blot also identified KU812 cells (Fig. 1A). Flow cytometry and IHC confirmed the specificity of #A3C3 mAb (Fig. 1B, left). Confocal microscopy clearly showed cell-surface staining corresponding to IL1RAP expression (Fig. 1B). Finally, integration of the #A3C3 mAb into a panel of antibodies allowed discrimination of IL1RAP$^+$ leukemia-expressing CD34$^+$CD38$^+$ or CD34$^+$CD38$^-$ stem cell subpopulations in bone marrow (BM) or peripheral blood (PB) from patients with CML at diagnosis and until 6 months after TKI treatment (Fig. 1C). These results are in agreement with the mRNA quantification, in which CML samples were positive, but healthy donor samples lacked IL1RAP mRNA expression (Fig. 1D). We also confirmed by ELISA that the #A3C3 mAb recognizes the IL1RAP recombinant protein (Supplementary Fig. S1).

Efficient generation of IL1RAP 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 (Fig. 2A). The *in vitro* production process (Supplementary Fig. S2A) with lentiviral 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 IL1RAP CAR, respectively; Fig. 2B). Our GMTC manufacturing process using CD3/CD28 beads and IL2 (500 U/mL) for the activation step allowed for preservation of the initial CD4$^+$/CD8$^+$ ratio

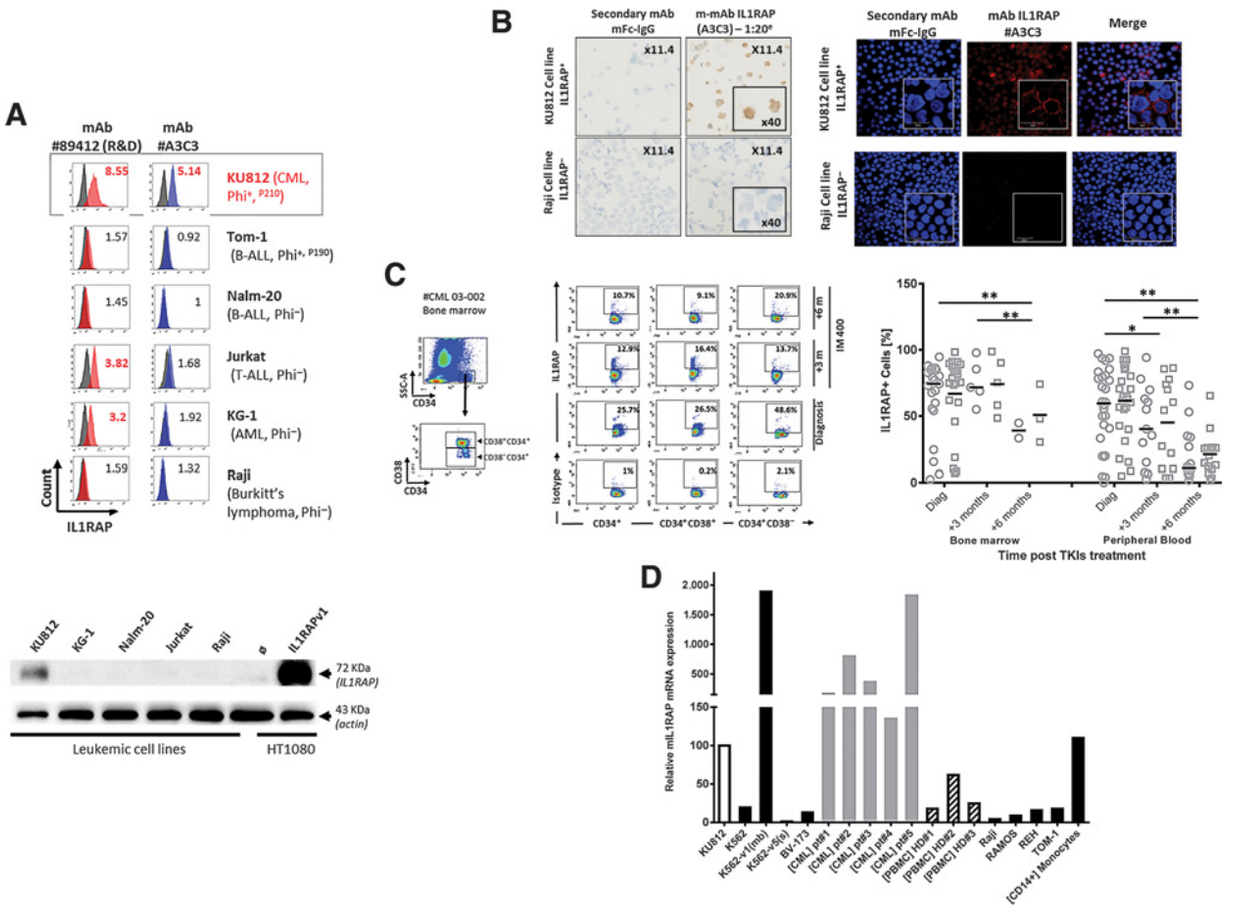

Figure 1.

IL1RAP mAb production and characterization. **A,** Top, screening of IL1RAP mAb using different cell lines. An IL1RAP (R&D) murine mAb (IgG1 Clone #89412) was used for staining comparison. Bottom, Western blotting of different hematopoietic cell lines with the #A3C3 IL1RAP antibody (1:20). A transfected HT1080 cell line with IL1RAP cDNA variant 1 (IL1RAPv1) was used as a control. Actin was revealed as a protein loading control. **B,** #A3C3 mAb immunostaining and confocal microscopy on frozen IL1RAP$^+$ (KU812) or IL1RAP$^-$ (Raji) cells. Confocal of KU812 and Raji cells stained with fluorescence mAbs. Left, anti-murine Fc-IgG; middle, IL1RAP (#A3C3). **C,** Representative flow cytometric analysis of primary cells from a single CML patient's BM at diagnosis or 3 or 6 months after imatinib treatment (400 mg; IM400; left) and the CML patient cohort (right). *, $P < 0.05$; **, $P < 0.01$. IL1RAP (#A3C3) was used in combination with CD34$^+$ and CD38$^-$ fluorescent staining. ○, CD34$^+$/CD38$^-$; □, CD34$^+$/CD38$^+$. **D,** RT-qPCR of IL1RAP mRNA expression. Relative gene expression in whole blood samples from patients with CML ($n = 5$) at diagnosis, PBMCs from healthy donors (HD; $n = 3$), CD14$^+$-sorted monocytes from healthy donors, IL1RAP variant 1 or 5 cDNA-transfected K562 cells, or other cell lines (lymphoma, ALL; $n = 4$) is provided to compare to calibrator KU812 cell line.

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; $n = 4$; Supplementary Fig. S2B).

Western blotting of subcellular fractions demonstrated that IL1RAP CAR is associated with CD3ζ signaling (signal at 55 kDa compared with the expected endogenous CD3ζ signal at 16 kDa; Fig. 2C). Additional analysis using serial dilution of biotinylated IL1RAP protein (from 20 ng to 2.4 pg/mL) and FACS analysis detected IL1RAP 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 (Supplementary Fig. S3). Moreover, the addition of equivalent amounts of sIL1RAP (up to 500 pg/mL) to E:T coculture did not affect the cytotoxicity of IL1RAP CAR T cells, but a higher amount (from 125 ng/mL to 10 μg/mL) of cold recombinant IL1RAP protein led to significant inhibition of effector cytotoxicity (Supplementary Fig. S4A). K562v5 cell culture SN containing secreted sIL1RAP did not affect the CAR T-cell cytotoxicity (Supplementary Fig. S4B). Overall, these results confirmed that CAR is present at the cell surface and that soluble ILRAP does not affect IL1RAP/IL1RAP CAR T-cell binding.

IL1RAP–CAR T 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 IL1RAP 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 (Supplementary Fig. S5A; Supplementary Table S2). Interestingly, the microvascular HMEC-1 endothelial cell line is not recognized by our #A3C3 IL1RAP mAb (Supplementary Fig. S5B), whereas

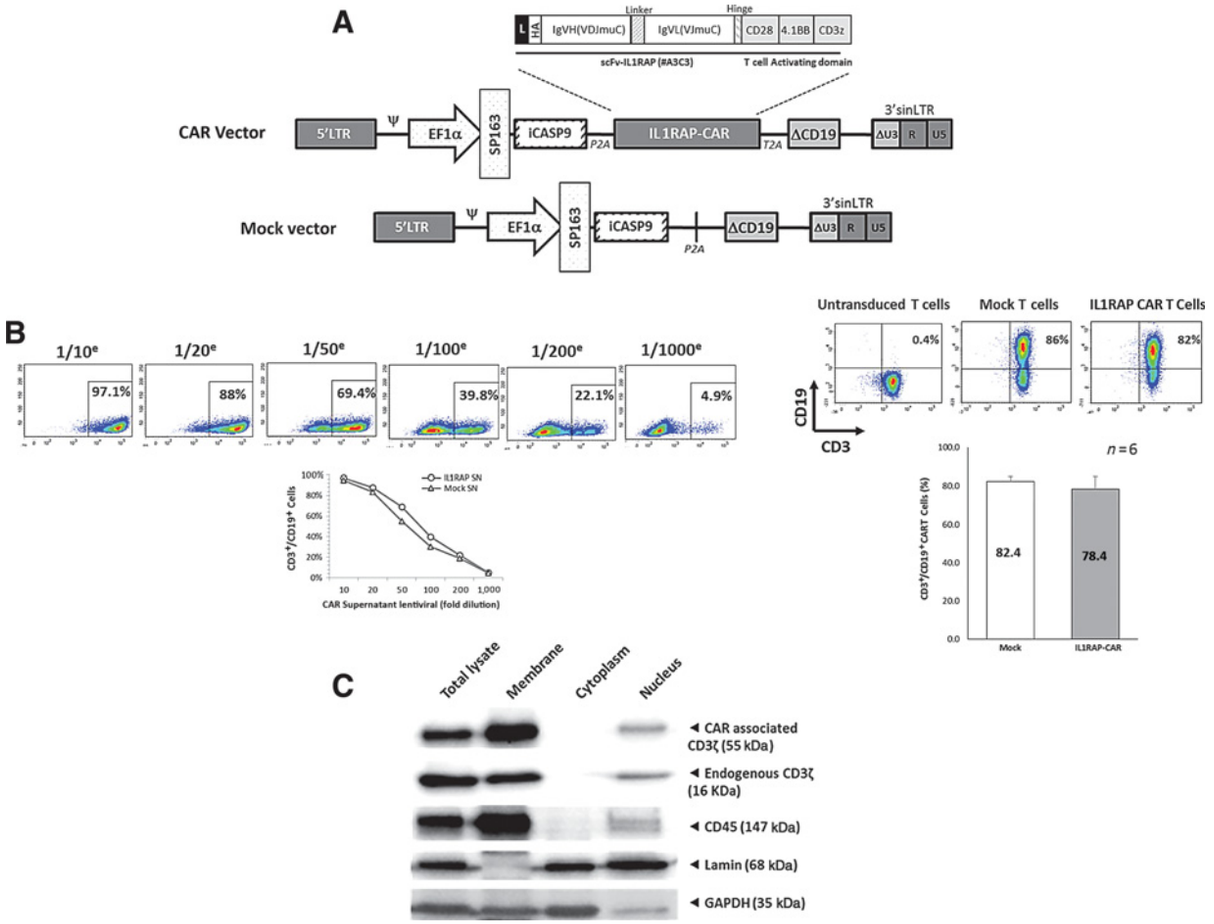

Figure 2.

Generation of IL1RAP CAR–expressing gene-modified T cells. **A,** Schematic overview of the IL1RAP CAR lentiviral vector. The construct carries three different parts: the suicide safety cassette iCASP9 (50), the IL1RAP 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,** Top, lentiviral transduction efficiency of donor T cells measured by flow cytometry. 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 IL1RAP CAR T cells (bottom left). Results are presented as mean ± SD of 6 independent transductions of 6 different donor PBMCs (bottom right) using 100X concentrated SN. **C,** Western blot analysis of IL1RAP 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.

the R&D IL1RAP 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 (Fig. 3A and B) or normal cord blood (Fig. 3B and C), we noted weak staining (RFI < 2) of the monocyte subpopulation in PB (2/5) and BM (3/5) from healthy donors (Fig. 3A). Thus, we studied *in vitro* sensitivity of monocytes by coculturing PBMCs with autologous CAR T cells at various E:T ratios. At ratio of 1:1, only some of the monocytes are targeted, leaving 41.45% of monocytes alive (Fig. 3D; Supplementary Table S3), whereas lymphocytes, granulocytes, and the K562 IL1RAP–negative cell lines are not affected (Fig. 3D), even at superior E:T ratios. Interestingly, at this E:T ratio, 94.77% of leukemic cells are killed (Fig. 3E). These results were confirmed *in vivo*, in an hCD34-engrafted murine model (hu-NOG; Supplementary Fig. S6A) receiving functional IL1RAP CAR T cells (Supplementary Fig. S6B), in which we demonstrated that, although monocytes decreased on day 15 (41% ± 25%, $n = 3$, P = n.s), other human immunocompetent cells derived from $hCD34^+$ cells are not affected by CAR T cells (Supplementary Fig. S6C). HSC culture assay after *in vitro* coculture of healthy $CD34^+$ cord blood HSCs with autologous CAR T cells ($n = 3$) confirmed that HSCs are not affected (Supplementary Fig. S7). These results agree with IL1RAP CAR T-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 nmol/L) exposure. First, using optical microscopy, 293T cell culture transduced by IL1RAP CAR was sensitive to the CID (Supplementary Fig. S8A, top). Cytometric analysis showed that, in a mixed population of $CD19^+$ and $CD19^-$ IL1RAP CAR T cells, only the $CD19^-$ $CD3^+$ cells persisted after

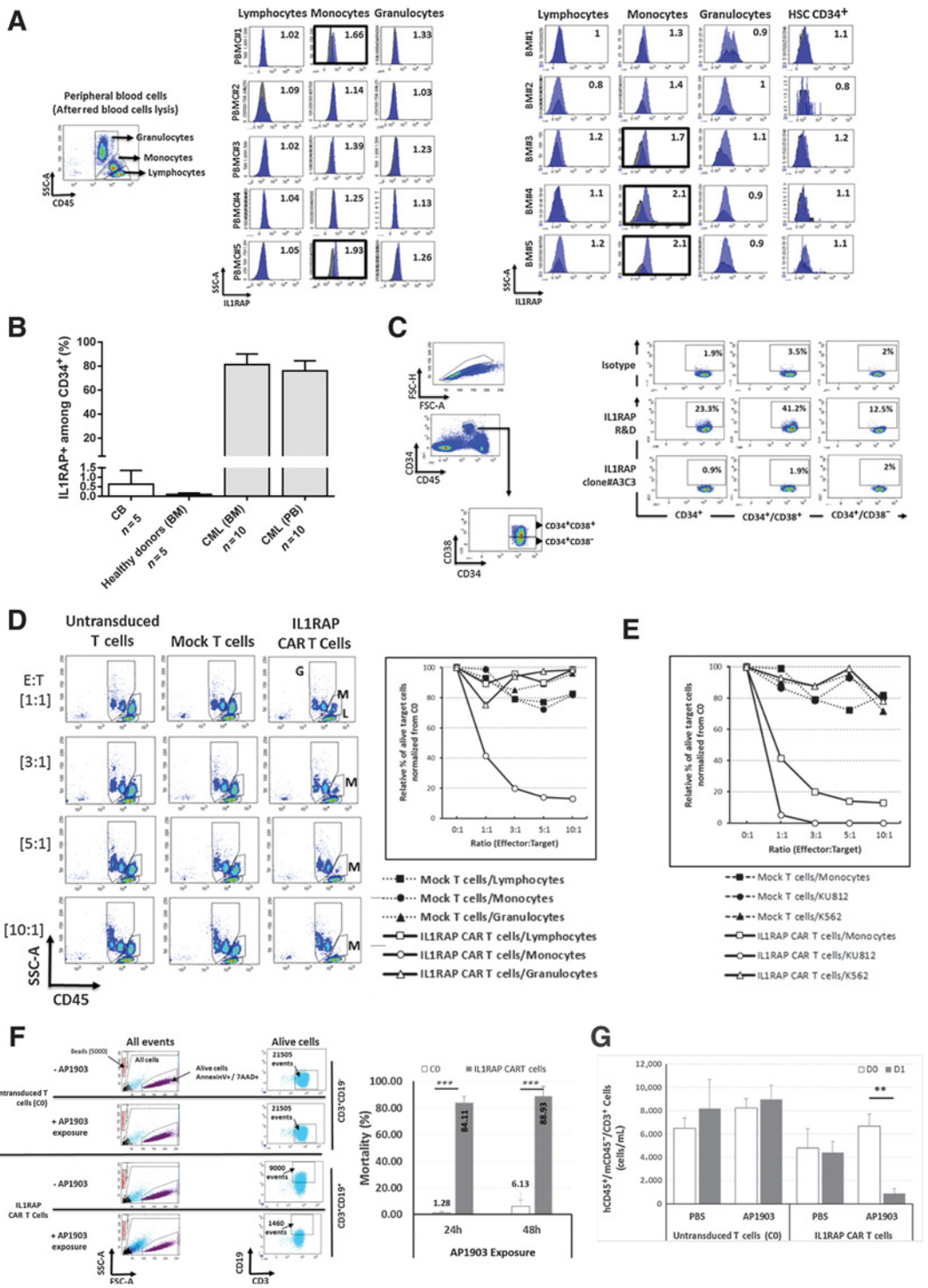

Figure 3.

Effect of IL1RAP CAR T cells on healthy hematopoietic cells and efficiency of the safety suicide gene iCASP9 cassette. **A,** IL1RAP cell-surface expression on PB (left) or BM (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,** IL1RAP-positive cells among CD34$^+$ cells in cord blood (CB; $n = 5$) or BM from healthy donors ($n = 5$) compared with CD34$^+$ cells from the BM ($n = 10$) or PB ($n = 10$) from patients with CML. **C,** Representative IL1RAP staining of whole human cord blood cells. IL1RAP 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 E:T ratios of autologous nontransduced T cells, Mock, or IL1RAP CAR T cells. Right, relative percentage (*Continued on the following page.*)

24 hours of CID exposure (Supplementary Fig. S8A, bottom). More precisely, in a quantitative assay of apoptosis, 84.11% and 88.93% of IL1RAP CAR T cells were eliminated after 24 or 48 hours of CID exposure, respectively, compared with nontransduced T cells (C0; 1.28% and 6.13% at 24 or 48 hours, respectively; $P < 0.001$, $n = 3$; Fig. 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 IL1RAP CAR T 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 (Fig. 3G; Supplementary Fig. S8B).

IL1RAP-dependent proliferation and cytokine secretion of IL1RAP CAR–expressing T cells

To analyze the proliferative and functional properties of IL1RAP CAR T cells, in addition to the KU812 cell line naturally expressing IL1RAP, we generated a deficient MHC class I cell line, K562, expressing either the membrane (isoform 1) or soluble (isoform 3) forms of IL1RAP translated from variant 1 (v1) or 5 (v5) transcripts, respectively (Supplementary Fig. S9A–S9C). Interestingly, we showed that IL1RAP expression was higher in transfected K562-v1 cells than KU812 cells (RFI = 10.57 vs. 33.46; Supplementary Fig. S9D).

The proliferative capability of IL1RAP CAR T cells triggered by IL1RAP target-expressing cells was determined by cocultured (E:T = 1:1) CFSE-stained (C0), Mock, or IL1RAP CAR T cells in the presence of K562, K562-v1, -v5, or KU812 cells. Effector IL1RAP CAR T cells divided significantly only in response to the presence of cell-surface IL1RAP-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%; Fig. 4A; $P < 0.001$, $n = 4$).

IL1RAP CAR T $CD8^+$ or $CD8^-$ cells, but not C0 or Mock cells (E:T ratio of 1:5), produced IFNγ and exclusively against IL1RAP-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 (Fig. 4B).

Finally, coculturing of target cells with C0, Mock, or CAR T cells (E:T = 1:1) showed that only cell-surface IL1RAP-expressing K562-v1 and KU812 cells could trigger cytokine secretion with robust IFNγ and IL2 secretion, moderate TNFα, and low IL4, IL6, and IL10, but not IL17 secretion, indicating a specific Th1 profile (Fig. 4C; Supplementary Fig. S10).

IL1RAP-dependent CAR cytotoxicity and lysis of IL1RAP-expressing tumor target cells

The CD107a&b degranulation assay applied to IL1RAP CAR T cells cocultured at an E:T ratio of 1:5 against $IL1RAP^+$ (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 IL1RAP-specific T cells ($P < 0.001$, $n = 4$; Fig. 5A). IL1RAP-dependent cytolytic potency of IL1RAP CAR–expressing T cells *in vitro* was determined using fluorescent (eFluor) and 7-AAD staining to discriminate CAR T cells and living cells, respectively. As expected, coculture at an E:T ratio of 1:1 and compared with C0 or Mock T cells revealed significant lytic activity characterized by the disappearance of cells in the $7\text{-AAD}^-/\text{eFluor}^-$ gate between $IL1RAP^+$ (K562-v1 and KU812) target cells and $IL1RAP^-$ (K562, K562-v5) target cells ($P < 0.001$, $n = 4$; Fig. 5B).

Xenograft murine model

In a tumor xenograft murine model (Fig. 6A), following tumor engraftment (D4), IL1RAP CAR T cells (E:T = 1:1) were allowed to target K562-v1 $IL1RAP^+/Luc^+$ (i.p., Fig. 6B) or CML KU812/Luc^+ (i.v., Fig. 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 CAR T-cell–treated group. Notably, tumors continued to grow in the absence of CAR T cells in surviving mice in the untreated and Mock-T–treated groups (Fig. 6B and C). In a second animal experiment, mice ($n = 6$/group) were grafted with bulk tumor cells (97.3% K562-v1 $IL1RAP^+/GFP^+/Luc^+$) containing residual (2.7%) IL1RAP-/GFP-/Luc^+ cells (Supplementary Fig. S11A). After the first objective response (D7, in 5/6 mice), and despite a second CAR T-cell injection, we noted the recurrence of Luc^+ cells (D24; Supplementary Fig. S11B). Flow cytometric analysis of human ($hCD45^+$) tumor cells sorted revealed that cells are $IL1RAP^-$ (GFP^-) cells originated from initial bulk injection (Supplementary Fig. S11C).

In vitro cytotoxicity against primary IL1RAP-expressing cells from patients with CML

From a primary TKI-resistant CML patient (always with BCR-ABL(IS) ratio > 10%) to five lines with four TKIs (Fig. 7A) treatment for a period of 4 years, we were able to produce CAR T cells with a transduction efficiency of 95.5% (Fig. 7A). IL1RAP CAR T cells exhibited dose-dependent cytotoxic activity against $IL1RAP^+$ KU812 cells with 95% efficiency at an E:T ratio of 3:1 compared with an alloreactive cytotoxicity of 18% and 21% for C0 or Mock-T cells, respectively (Fig. 7B). Coculture of autologous IL1RAP CAR T cells against CML patient PBMCs exhibited specific lysis (76.65% ± 9.2% for IL1RAP CAR T cells compared with 4.16% ± 4.3% and 2.78% ± 1.72% for C0 or Mock-T cells, respectively) of $IL1RAP^+/CD34^+$ cells after 24 hours (Fig. 7C).

(*Continued.*) of alive cells among lymphocytes (square), monocytes (circle), and granulocytes (triangle), normalized to nontransduced autologous T cells (C0) cocultured 24 hours with autologous Mock-T cells (dashed line) or IL1RAP CAR T cells (solid line). **E,** Comparison of IL1RAP 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 IL1RAP CAR T cells (white, solid line). Percentages were calculated using absolute cell number determined using Trucount tubes (cytometry acquisition of 5,000 fluorescent beads). **F,** Left, gating strategy and analysis for absolute count of CID AP1903-induced cell death. Nontransduced (C0) or IL1RAP CAR T cells were exposed to medium alone or medium + CID (20 nmol/L, 24 hours). The quantification was performed after acquiring 5,000 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 at 24 or 48 hours C0 or IL1RAP CAR (gated on $CD3^+/CD19^+$) T-cell CID exposure. Right, mean from three independent experiments. **, $P < 0.001$. See also Supplementary Data. **G,** Absolute quantification of IL1RAP CAR T cells injected in a tumor (CML KU812, i.v.) xenograft NSG model 24 hours 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.

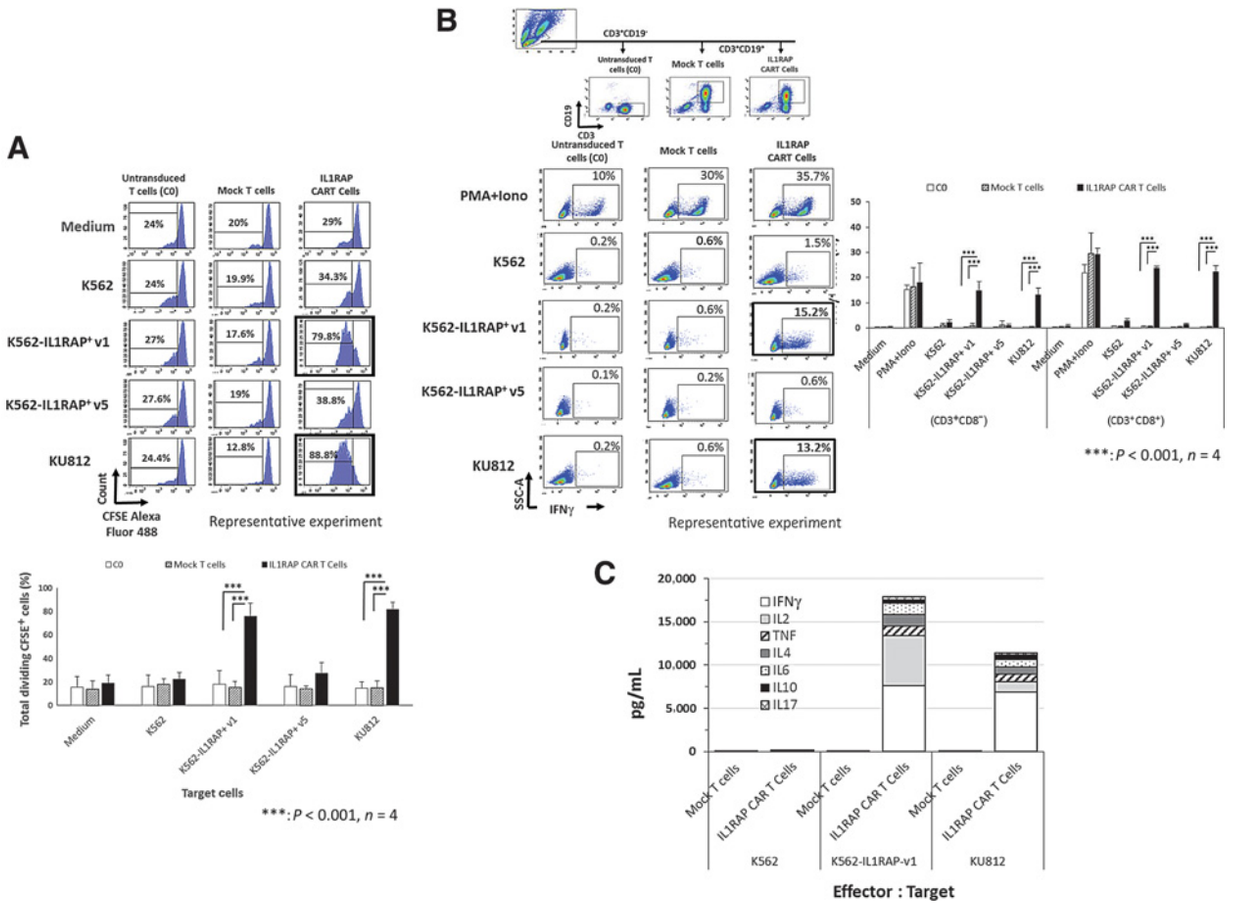

Figure 4.

IL1RAP CAR T cells proliferate in coculture with IL1RAP-expressing target cells secreting and releasing IFNγ and proinflammatory cytokines. **A,** Top, gating strategy for flow cytometry CFSE dilution analysis. Nontransduced (C0) and Mock or IL1RAP CAR-transduced T cells were cultured in medium alone, in contact with IL1RAP$^-$, cell surface (K562-v1 and KU812), or soluble (K562-v5) IL1RAP$^+$-expressing target cells at an E:T ratio of 1:1. Effectors were labeled with 0.5 μmol/L CFSE. After 48 or 96 hours of coculture without IL2 supplementation, measurement of CFSE dye dilution allowed assessment of 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 IL1RAP CAR T cells were cocultured for at least 16 hours at an E:T of 1:5 in the presence of target cells expressing or not expressing IL1RAP 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 secrete IFNγ, IL2, TNF, IL4, IL6, IL10, and IL17 cytokines within the culture medium. See also Supplementary Data.

Moreover, autologous IL1-RAP CAR T cells produced (transduction efficiency: 85.33% ± 8.8%) from patients with CML ($n = 3$) under long-term treatment, including TKIs, or free of treatment (Supplementary Table S4), and directed against their respective initial long-term cryopreserved (>20 years) peripheral blood stem cell autograft, killing the CD34$^+$/IL1RAP$^+$ cells with an efficiency of 79.78% ± 10.7% (Fig. 7D; Supplementary Fig. 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 CAR T 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, IL1RAP, 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.

IL1RAP 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 IL1RAP is overexpressed in AML and high-risk MDS and correlates, in this case, with poor clinical outcome (37). Anti-IL1RAP antibody has been demonstrated to kill primary cells from patients with AML *in vitro* (25) and in a xenograft

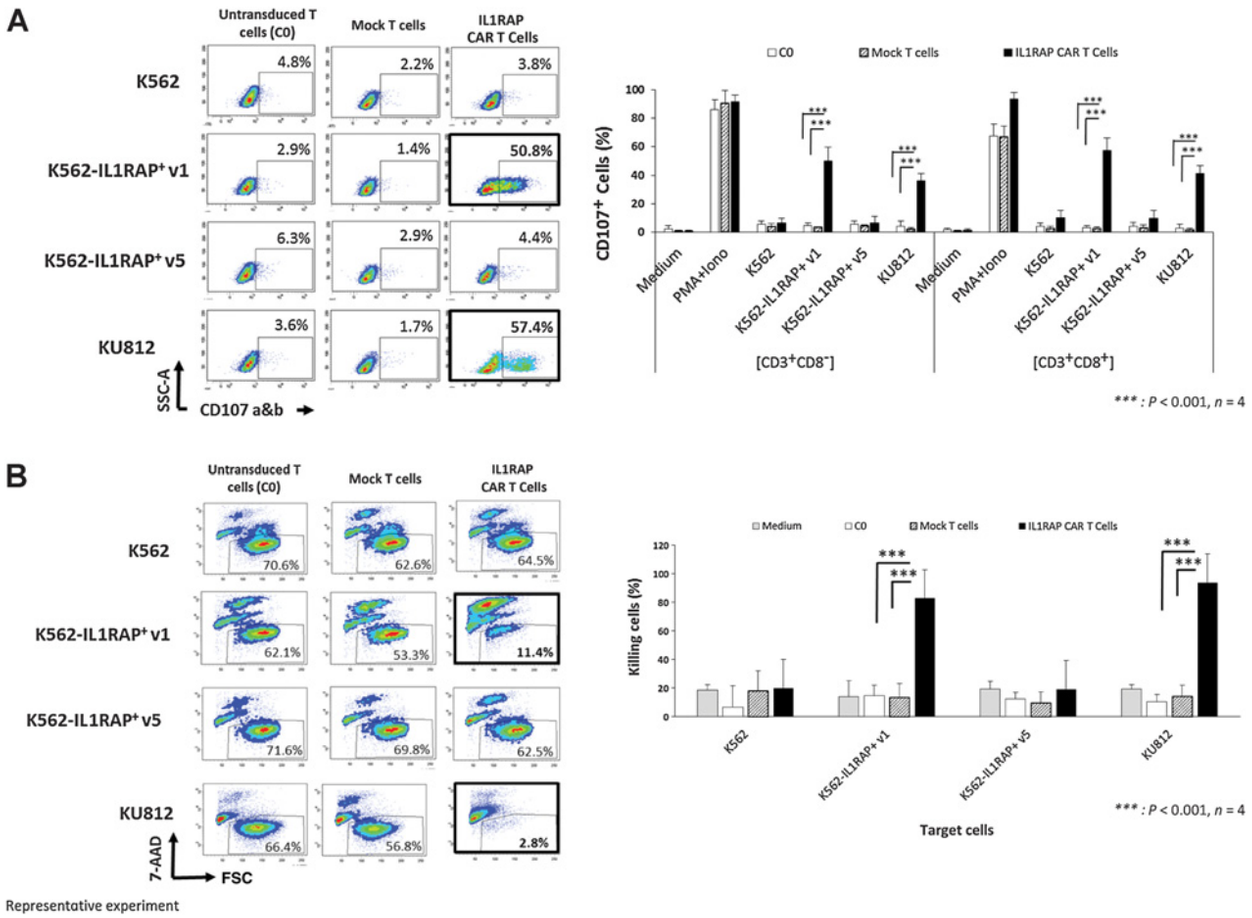

Figure 5.

Lysis function analysis of effector cells. **A,** Left, CD107a&b degranulation assay. Transduced (Mock or IL1RAP CAR) and nontransduced T cells were cocultured at an E:T ratio of 1:5 for 6 hours with target cells expressing or not expressing IL1RAP. After 5 hours, $CD3^+/CD19^+/CD8^+$ 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 IL1RAP CAR T cells at lysing cell-surface IL1RAP-expressing cells. Effectors, labeled with e-Fluor, were cultured at an E:T of 1:5 in the presence/absence of $IL1RAP^+$ target cells. Right, percentage of total killed target cells. Mean ± SD of four independent experiments. ***, $P < 0.001$. See also Supplementary Data.

murine model (31). Thus, IL1RAP is an additional target for new cell immunotherapy in myeloid malignancies in addition to CD123, CD33, or CD44v6 targeting.

Despite the impressive success of CAR T-cell immunotherapies in relapsed/refractory acute lymphoid leukemia, non-Hodgkin's lymphoma, chronic lymphoid leukemia, or multiple myeloma treatment (38), this approach is frequently associated with toxicity, such as cytokine release syndrome (CRS), 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 CAR T cells target HSCs expressing cell-surface antigens. We have shown that IL1RAP 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* coculture of autologous IL1RAP CAR T 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, IL1 secreted by monocyte/macrophages was recently reported to be involved in CRS and neurotoxicity after CAR T-cell infusion (40). Thus, partial targeting of this population, as shown in our IL1RAP CAR T-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 IL1RAP targeting may be associated with limited side effects. This is in favor of continuing investigations of IL1RAP CAR T-cell targeting, especially toxicity studies, at the preclinical level, until the first use in humans.

Use of an anti-IL6 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 CAR T-cell depletion possible if complications occur. To limit potential adverse events, other tools should be

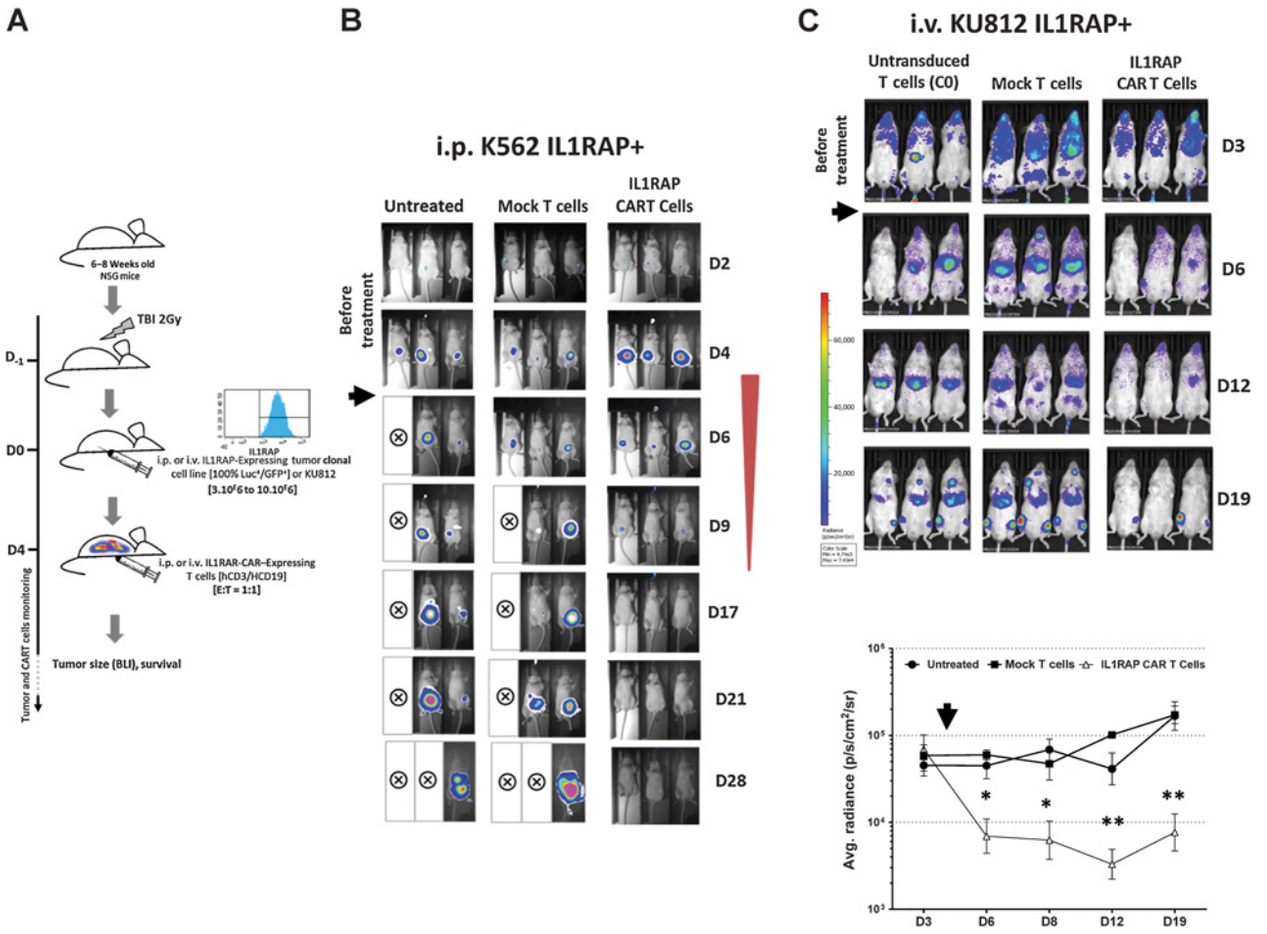

Figure 6.

NSG murine xenograft tumor model experiment. **A,** Mice were sublethally irradiated at a dose of 2 Gy ($n = 3$/group) 24 hours before tumor transplantation. The 5 × 10E6 clonal cells of K562-v1, an IL1RAP^{+}-, luciferase^{+}-, and GFP-positive cell line (K562-v1$^{IL1RAP+/GFP+/Luc+}$), or CML KU812^{Luc+} cell line were respectively transplanted via i.p. or i.v. injection into 6- to 8-week-old NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice. At an E:T ratio of 1:1, Mock-T or IL1RAP 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,** Bioluminescent imaging analysis of mice of different groups from days 2 to 28. (x), dead mice; ⊗, euthanized mice; →, the time of gene-modified T-cell (IL1RAP CAR or Mock-T cells) injection. See also Supplementary Data. **C,** Top, bioluminescent imaging analysis of mice of different groups from days 3 to 19. →, the time of gene-modified T-cell (IL1RAP CAR- or Mock-T cells) injection. Bottom, radiance of the *in vivo* bioluminescent signal (radiance p/s/cm^2/sr) harvested using the IVIS Illumina III (Perkin Elmer). ↓, time of IL1RAP CAR T-cell injection. *, $P < 0.05$; **, $P < 0.01$.

explored, such as new designs of viral constructs, integrating our IL1RAP 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 patients with CML are considered clinically cured. CML disease is highly susceptible to the allogenic immune system, as indicated by allo-SCT (42) and 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 CAR T-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 CAR T-cell manufacturing failures arising from the use of T cells from heavily treated patients (44). Especially in CML, the use of these donor CAR T cells will enhance the antileukemic effects by combining the alloreactivity and specific cytotoxicity of IL1RAP 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 CRS, cerebral nervous system toxicity, off-target toxicity, or tumor lysis syndrome (46).

IL1RAP ADCC eliminates CML stem cells (26, 32) and primary AML cells (31) through IL1/IL1RAP signaling blockade via proliferation and expansion of the suppression mechanism of cells responsive to IL1. The same work highlights this lysis mechanism by IL1RAP 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

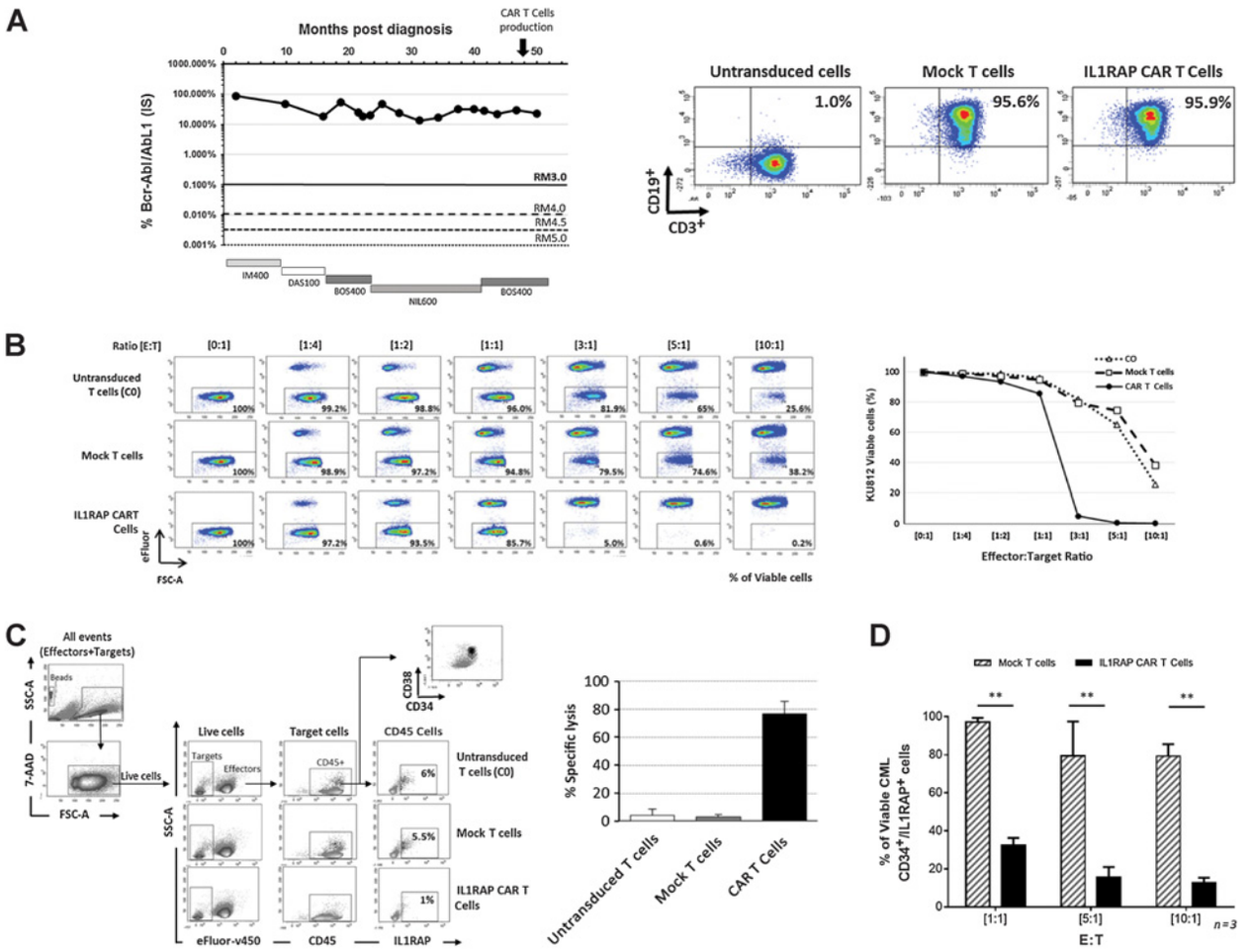

Figure 7.

In vitro toxicity against primary IL1-RAP$^+$ circulating cells from a patient with CML. **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 patient with CML. **B,** Left, dot plot of cytometry of effector cells, C0, Mock-T, or CAR T cells, labeled with eFluor and cocultured 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 IL1RAP CAR T cells against IL1RAP-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 IL1RAP CAR or Mock-T cells against their respective CML autografts at various (E:T) ratios. Aggregate results of three independent experiments (three different patients with CML). The percentage of remaining viable CD34$^+$/IL1RAP$^+$ cells calculated from control cells (C0) is provided. **, $P < 0.01$.

(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 CAR T cells, to achieve a cure. Here, we provided a new therapeutic approach with direct recognition of the IL1RAP cell-surface protein and without the need for accessory immunocompetent NK cells.

In addition to the transmembrane form of IL1RAP, alternative splicing results in an mRNA encoding a secreted and soluble (sIL1RAP) form characterized by the extracellular domain of the protein. The soluble form may interfere with membrane IL1RAP-expressing tumor cells by saturating the CAR. In healthy people, circulating levels of soluble IL1RAP are detected at a mean concentration of 200 pg/mL (49). We demonstrated *in vitro* that a higher concentration (2,400 pg/mL) of IL1RAP recombinant protein did not result in staining of IL1RAP CAR–expressing T cells (Supplementary Fig. S3). Moreover, both recombinant IL1RAP protein < 125 ng/mL (Supplementary Fig. S4A) and the culture SN of K562-v5, secreting sIL1RAP (Supplementary Fig. S4B), did not affect CAR T cytotoxicity against IL1RAP-expressing targets. Overall, these findings suggest that sIL1RAP would not interfere with IL1RAP tumor cell recognition, though an accurate serum sIL1RAP concentration needs to be identified in a cohort of CML or AML patients for precise quantification of expression.

This preclinical work demonstrates for the first time the whole production and validation process of CAR T cells directed against IL1RAP-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 CAR T 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 CAR T cells has also been demonstrated in patients with CML, even if the CML model is a limited application field in the TKI area, and would be beneficial to TKI nonresponders or TKI-intolerant CML patients and young or accelerated/blastic phase CML patients, who are all candidates for allograft. Because AML leukemic cells express IL1RAP (37), this promising CAR T-cell immunotherapy approach may be applied in clinically critical acute hematologic disease. In this context (CML), use of IL1RAP CAR T allogenic cells would constitute a bridge toward 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 IL1RAP an interesting tumor-associated antigen for immunotherapy cell targeting using CAR T cells. In-depth studies are required to determine and/or reduce potential toxicities and side effects before phase I clinical trials.

Disclosure of Potential Conflicts of Interest

C. Faure is a consultant/advisory board member for Abbvie. C. Ferrand reports receiving commercial research grant from Novartis and is a consultant/advisory board member at BMS, Novartis, and Incyte. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank DiacloneSA for IL1RAP monoclonal antibody production; Drs. Desbrosse and Berceanu for recruiting CML patients; Francis Bonnefoy and Emmanuel Salvado for xenograft murine models; the Immunology Medical Lab (LBMG) for assistance with the CFU assay; and Sarah Odrion for reading the article.

This study is supported by Ligue contre le cancer—CCIR-GE 2014, Programme de Recherche Translationnelle INCa (PRTK N°PRT-K-275), Association *Nausicaa combat sa Leucémie* (nonprofit association 1901; Gift 2017-03), SFGMTC/Association Capucine Grant 2013-AO2, the MiMedi project funded by BPI France (no. DOS0060162/00), and the EU through the European Regional Development Fund of the Region BFC (no. FC0013440).

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Received May 25, 2018; revised October 9, 2018; accepted November 29, 2018; published first December 4, 2018.

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