



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

Rim Trad

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**THESE DE DOCTORAT DE L'ETABLISSEMENT UNIVERSITE BOURGOGNE FRANCHE-COMTE**  
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Par

Mlle **TRAD Rim**

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

**Cellules T réceptrices d'antigènes chimériques ciblant IL-1 RAP : une nouvelle immunothérapie cellulaire prometteuse pour traiter la leucémie myéloïde aiguë**

Thèse présentée et soutenue à « l'UFR Santé-Université de Franche-Comté », le « 05 Novembre 2021 »

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## List of abbreviations

### A

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AML: Acute myeloid leukemia  
ANLL: Acute non-lymphoblastic leukemia  
AL : acute leukemia  
ASCT: Allogenic stem cell transplantation  
ASLX1: Additional Sex Combs Like 1  
ADCT: Adoptive Cell Transfer  
AP-1: Activator protein-1  
ADCC: Antibody-dependent cell cytotoxicity  
AICD: Activation Induced Cell Death  
APC: antigen-presenting cell  
AAV: Adeno-Associated Virus  
Ad: Adenovirus  
ASH: American Society of Hematology  
ATG: anti-thymocyte globulin  
ATP: adenosine triphosphate  
A2AR: adenosine receptor

### B

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BET: Bromodomain and extra-terminal protein  
BCL2: B-Cell Lymphoma 2  
BiTE: Bispecific T-cell Engager  
BiKE: Bispecific Killer Engagers  
BCMA: B-cell maturation antigen  
BissCAR: bispecific CAR

### C

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CTLs: cytotoxic T lymphocytes  
CAR: Chimeric Antigen Receptor  
CiTE: Checkpoint inhibitor T-cell Engager  
CTLA-4: Cytotoxic T Lymphocyte Associated protein 4

CD: cluster of differentiation

CIK: Cytokines Induced Killer

CML: Chronic Myeloid Leukemia

CRISPR/Cas9: Clustered Regularly Interspaced  
Short Palindromic Repeats/Cas9Cas9: CRISPR  
associated protein 9

CLL: chronic lymphocytic leukaemia

CRS: cytokine release syndrome

CRES: Cytokine Release Encephalopathy  
Syndrome

CSF: cerebrospinal fluid

CDC: complement-dependent cytotoxicity

CLL-1: C-type Lectine-Like-1

cCAR: compound CAR

CEA: carcinoembryonic antigen

### D

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DOT1L: Disruptor of telomeric silencing 1-like

DART: Dual-Affinity Re-targeting antibodies

DC: dendritic cell

DLBCL: diffuse large B-cell lymphoma

DNMT: DNA methyl transferase

### E

---

EZH2: Enhancer of Zeste Homolog 2

EMA: European Medicines Agency

EGFR: epidermal growth factor receptor

### F

---

FLT3: Fms-Like tyrosine kinase 3

FLT3-ITD/TKD: FLT3-Internal Tandem

Duplication/Tyrosine kinase domain

Fv: Foamy virus

FDA: Food and Drug Administration Agency

FITC: Fluorescein isothiocyanate

---

## G

GMTLs: genetically modified T lymphocytes

GO: GemtuzumAb ozogamicin

GMP: Good Manufacturing Process

GMCSF: Granulocyte-macrophage colony  
stimulating factor

GVHD: graft-versus-host disease

GSV: Ganciclovir

GVL: graft-versus-leukemia

GITR: glucocorticoid induced tumor necrosis factor  
receptor related protein

---

## H

HSC : Hematopoietic stem cell

HPC : hematopoietic progenitor cell

HMA: Hypomethylating agents

HIV: human immunodeficiency virus

HSV-tk: herpes simplex virus thymidine kinase

HLA: human leukocyte antigen

---

## I

IDH1/2: Isocitrate dehydrogenase 1/2

ICD: Intermediate cytarabine dose

IS: Immune System

IL-1RAP: Interleukin-1 Receptor Accessory Protein

IL-1: interleukin-1

IFN $\gamma$ : interferon gamma

ICOS: Inducible T-cell COStimulator

ICANS: immune effector cell-associated  
neurotoxicity syndrome

iCasp9: inducible caspase 9

iCAR: inhibitory CAR

IDO: Indoleamine 2,3 dioxygenase

---

## K

KIRs: killer inhibitory receptors

---

## L

LDS1: Lysine specific demethylase1

LDC: Low Doses Cytarabine

LAG3: Lymphocyte Activation Gene  
3 protein

LSC: Leukemia Stem Cell

LDL: Low-density lipoprotein

LXR: liver-X nuclear hormone  
receptor

---

## M

MDS : Myelodysplastic syndrome

MDM2: murine double minute 2

mAbs: Monoclonal antibodies

MM: multiple myeloma

MHC: Major Histocompatibility  
Complex

MCL: mantle cell lymphoma

MAS: macrophage activation  
syndrome

MRD: measurable residual disease

MDSCs: myeloid-derived suppressor  
cells

MSC: mesenchymal stromal cells

---

## N

NPM1: Nucleolar phosphoprotein

B23 or Numatrin



NK: Natural killer

NF-Kappa-B: Nuclear factor-kappa B

NFAT: nuclear factor of the activated T-cell

NHL: non-Hodgkin's lymphoma

NAD: nicotinamide adenine dinucleotide

## **P**

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PD-1/PDL-1: Programmed Death-1/Programmed Death Ligand-1

PR-1: Protein-1

PDX: patient derived xenograft

## **R**

---

RT: reverse transcriptase

REV: Regulator of Expression of Virion

ROS: reactive oxygen species

## **S**

---

ScFV: single cell fragment variable

SB: Sleeping Beauty

synNotch: synthetic Notch

sCAR: Switchable CAR

## **T**

---

TET2: Ten-Eleven Translocation 2

TKI: Tyrosine Kinase Inhibitor

TP53: tumor protein 53

tgTCR: transgenic T-cell receptor

TIM3: T-cell Immunoglobulin and Mucin domain containing protein 3

TILs: Tumor Infiltrating Lymphocytes

TRiKE: Trispecific Killer Engager

TIR: Toll interleukin receptor

TLs: T Lymphocytes

TRUCKs: T-cells redirected for universal cytokine-mediated killing

TNF $\alpha$ : Tumor Necrosis Factor alpha

Tcm: central memory T-cells

Tem: effector memory T-cells

Treg: regulatory T lymphocyte

TALEN: nucleoside transcription activator-like effector nuclease

TAT: Trans-Activator of Transcription

TLS: tumor lysis syndrome

TAA: Tumor-Associated Antigens

Tan-CAR: Tandem-CAR

TF: transcription factor

TRE3G: tet response element 3G

TIGIT: T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain

## **V**

---

VH: Heavy chain

VL: Light chain

VSV-G: Vesicular Stomatitis Virus Glycoprotein

## **W**

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WT1: Wilm's Tumor gene 1

## **Z**

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ZFNs: Zinc fingers

## Abstract

Acute myeloid leukemia (AML) remains a very difficult disease to cure due to the persistence of leukemic stem cells (LSCs), a subpopulation of AML cells with self-renewal, and chemorefractory capacity. AML LSCs are the origin of refractory/relapsed (R/R) disease in 80% of AML patients not receiving allogeneic hematopoietic stem cell transplantation (allo-HSCT). Targeted therapies combined with chemotherapy and bone marrow transplantation have improved the prognosis. Immunotherapy [monoclonal / bispecific antibodies, checkpoint inhibitors, Chimeric Antigen Receptor Lymphocytes (CAR T-cells)] offers great hope for improvement in treatment. However, treatment of R/R AML is still a substantial challenge and is associated with poor prognosis and low chance for cure, especially for elderly patients. Hence the necessity of new alternatives with robust anti-leukemic activity while avoiding T-cell cytotoxicity against healthy tissues for treating AML patients. Interleukin-1 Receptor Accessory Protein (IL-1RAP) has been identified as being involved in the oncogenic pathway of AML, but also as a potential target for cytotoxic blast elimination. We have previously established the proof of concept in-vitro and in-vivo that a third-generation CART-cell targeting IL-1RAP was able to eliminate LSCs in Chronic Myeloid Leukemia (CML). In this study, we showed that the IL-1RAP protein is overexpressed on the surface of LSCs in all subtypes of AML and confirmed it as an interesting and promising target in AML compared to the most common potential AML targets. We hypothesized that third-generation IL-1RAP CART-cells could eliminate AML LSCs. We first demonstrated that IL-1RAP CART-cells could be produced from T-cells of AML patients at the time of diagnosis but also at relapse. Characterization of IL-1RAP CART-cells showed expression of checkpoint markers at the end of the production process. Interestingly, we showed, in-vitro and in-vivo, the effectiveness of IL-1RAP CART-cells against AML cell lines expressing different levels of IL-1RAP and the cytotoxicity of autologous CART-cells against primary cells from AML patients at diagnosis or at relapse. In patient-derived AML xenograft (PDX) models, we confirmed that IL-1RAP CART-cells are able to circulate in peripheral blood and to migrate in the bone marrow and spleen and are cytotoxic against primary AML cells.

**Keywords:** Acute myeloid leukemia (AML); Hematopoietic stem cells (HSCs); Leukemic stem cells (LSCs); Interleukin-1 receptor accessory protein (IL-1RAP); Chimeric antigen receptor (CAR); T lymphocytes (TLs); relapse.

## Résumé

La leucémie aiguë myéloïde (LAM) reste une maladie très difficile à guérir en raison de la persistance des cellules souches leucémiques (CSLs), une sous-population de cellules de la LAM avec un auto-renouvellement et une capacité chimioréfractaire. Les CSLs de LAM sont à l'origine d'une maladie réfractaire/récidive (R/R) chez 80 % des patients atteints de LAM ne recevant pas d'allogreffe de cellules souches hématopoïétiques (allo-CSH). Les thérapies ciblées associées à la chimiothérapie et à la greffe de moelle osseuse ont amélioré le pronostic. L'immunothérapie [anticorps monoclonaux / bispécifiques, inhibiteurs de points de contrôle, lymphocytes à récepteurs chimériques d'antigènes (cellules CAR T)] offre un grand espoir d'amélioration du traitement. Cependant, le traitement de la R/R LAM reste un substantiel défi et est associé à un mauvais pronostic et à de faibles chances de guérison, en particulier chez les patients âgés. D'où la nécessité de nouvelles alternatives avec une activité anti-leucémique robuste tout en évitant la cytotoxicité des lymphocytes T contre les tissus sains pour le traitement des patients atteints de LAM. La protéine accessoire du récepteur de l'interleukine-1 (IL-1RAP) a été identifiée comme étant impliquée dans la voie oncogène de la LAM, mais aussi comme une cible potentielle pour l'élimination cytotoxique des blastes. Nous avons précédemment établi la preuve de concept in-vitro et in-vivo qu'un CART-cell de troisième génération ciblant IL-1RAP était capable d'éliminer les LSCs dans la leucémie myéloïde chronique (LMC). Dans cette étude, nous avons montré que la protéine IL-1RAP est surexprimée à la surface des LSCs dans tous les sous-types de LAM et l'avons confirmée comme une cible intéressante et prometteuse dans la LAM par rapport aux cibles potentielles les plus courantes de LAM. Nous avons émis l'hypothèse que les IL-1RAP CART-cells de troisième génération pourraient éliminer les LSCs de la LAM. Nous avons d'abord montré que les IL-1RAP CART-cells pouvaient être produites à partir de cellules T de patients atteints de LAM au moment du diagnostic mais aussi lors de la rechute. La caractérisation des IL-1RAP CART-cells a montré l'expression de marqueurs de point de contrôle à la fin du processus de production. Fait intéressant, nous avons montré, in-vitro et in-vivo, l'efficacité des IL-1RAP CART-cells contre des lignées cellulaires LAM exprimant différents niveaux d'IL-1RAP et la cytotoxicité des IL-1RAP CART-cells autologues contre des cellules primaires de patients atteints de LAM au moment du diagnostic ou à la rechute. Dans les modèles de xénogreffe de LAM dérivées de patients, nous avons confirmé que les IL-1RAP CART-cells sont capables de circuler dans le sang périphérique et de migrer dans la moelle osseuse et la rate et sont cytotoxiques contre les cellules primaires de la LAM.

**Mots clés :** Leucémie aiguë myéloïde (LAM) ; Cellules souches hématopoïétiques (CSHs) ; Cellules souches leucémiques (CSLs); protéine accessoire du récepteur de l'interleukine-1 (IL-1RAP); récepteur d'antigène chimérique (CAR); Lymphocytes T (LT); rechute.

## Introduction

### Chapter 1: Acute Myeloid Leukemia (AML)

#### I. AML physiopathology

Acute Myeloid Leukemia (AML) is a set of clonal and malignant proliferations resulting in the accumulation in the bone marrow (BM), blood and possibly other organs, of myeloid stem blood cells, that have totally, or partially lost their ability to differentiate themselves (Döhner, Weisdorf, and Bloomfield 2015) (Figure 1). It is a rapidly growing malignant cancer. Several names can be given to AML: acute myeloid leukemia, acute myelocytic leukemia, acute myelogenous leukemia, acute granular leukemia and acute non-lymphoblastic leukemia (ANLL). AML is the most common type of leukemia in adults, it constitutes 80% of acute leukemia (AL) in adults (median age at diagnosis of 65 years) with a prevalence of 3-5 cases per 100,000 inhabitants (De Kouchkovsky and Abdul-Hay 2016). It is less common in children with fewer etiologic studies exist (Puumala et al. 2013). Myelodysplastic-myeloproliferative neoplasms can develop into acute myelogenous leukemia. In fact, Myelodysplastic Syndromes (MDS) originate from abnormal hematopoietic stem cells (HSCs) which proliferate and differentiate into abnormal hematopoietic progenitor cells (HPCs) which can transform into AML (Cazzola 2020).

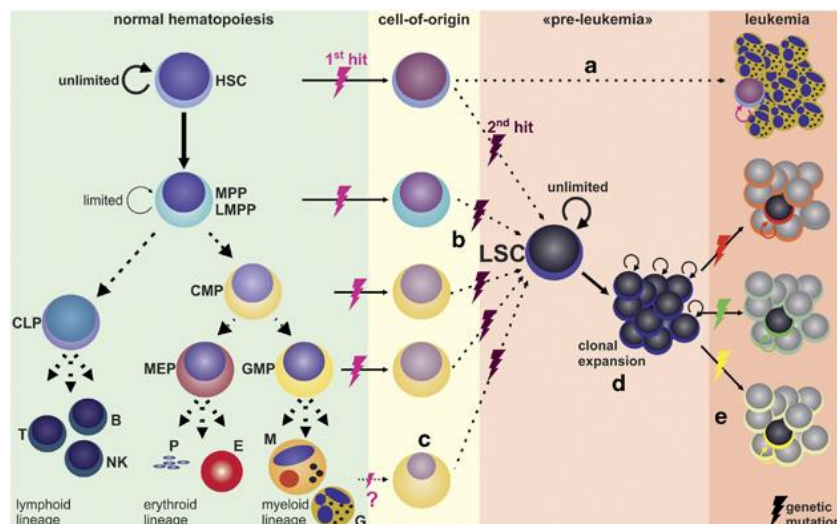


Figure 1: Acute myeloid leukemia hematopoiesis. MPPs: multipotent progenitors, LMPPs: lineage-restricted progenitors, CLPs, CMPs: common lymphoid and myeloid progenitors, GMP: granulocyte-macrophage progenitor, MEP: megakaryocyte-erythrocyte progenitor (Riether, Schürch, and Ochsenbein 2015).

## A. Genetic and Cytogenetic abnormalities

The leukemic phenotype is given to normal HSCs or to a progenitor engaged in differentiation by at least two mutational events in different stages of the hematopoietic hierarchy, which makes AML a genetically heterogeneous and oligo clonal disease. A 2-hit model is defined necessary for the development of de novo AML:

- Class 1 genetic damage characterized by a constitutive activation of surface receptors for proliferative pathways: RAS (GTPase of the family of monomeric G proteins), tyrosine kinase receptors such as Fms-Like tyrosine kinase 3 (FLT3) or c-KIT, which activate the hematopoiesis. These mutations are found in 50% of AML cases and result in a worse prognosis.
- Class 2 genetic damage inducing hyper-expression of Homeobox (HOX) genes with homeoboxes allowing them to bind strongly to DNA and triggering a cascade activation of other genes as fusion genes blocking normal myeloid differentiation such as those induced by t-translocation (8;21) or inversion (inv16).

Added to this model, class 3 mutations in genes involved in epigenetic regulation like chromosome 5 and 7 abnormalities such as hyper-methylation of tumor suppressor genes inducing their inhibition with downstream effects on both cellular differentiation and proliferation. This type of mutations is seen in up to 40% of AML cases (De Kouchkovsky and Abdul-Hay 2016).

This therefore results in a variable mutational profile between the different stages of the disease, with clonal selection at relapse (Ding et al. 2012a) (Figure 2). An initiating "driver" mutation within a progenitor such as, for example, Nucleolar Phosphoprotein B23 or Numatrin (NPM1), Isocitrate Dehydrogenase 1 (IDH1), DNA Methyl Transferase 3A (DNMT3A) and others, already containing mutations "passenger" will confer anomalies of self-renewal, proliferation and differentiation in AML (Cancer Genome Atlas Research Network et al. 2013) and in the MDS (Papaemmanuil et al. 2013). Other cooperative "driver" mutations can occur (FLT3-ITD: Internal Tandem Duplication of the FLT3 gene) thus forming a founder clone containing several "driver" and "passenger" mutations. Thus, AML results from anomalies of a variable number of genes (translocations, mutations) present from the time of diagnosis, within different subclones.

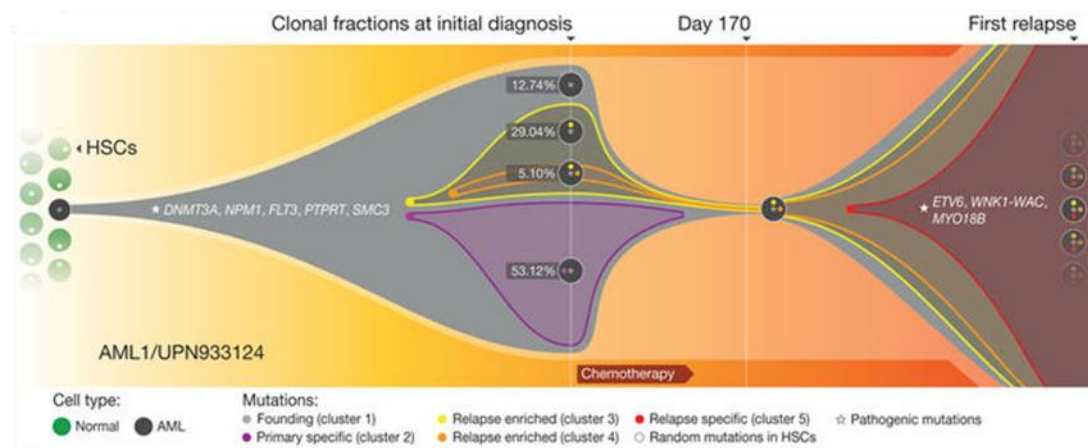


Figure 2: Clonal evolution from diagnosis to relapse, after accumulation of new mutations (Ding et al. 2012b).

Mutations can occur in many genes involved in different signaling pathways (Grove and Vassiliou 2014) (Table 1), making it difficult to prioritize the weight of each in the course of the disease. The World Health Organization (WHO) proposes a 2016 AML classification based on molecular criteria and some work has identified genomic subgroups (fusion genes or mutated genes) of AML with different prognosis allowing orienting therapeutic management (Papaemmanuil et al. 2016). Selection of a dominant clone and/or additional mutations may be caused by inadequate treatments resulting in treatment resistance and play an important role in relapses. Recently, distinct leukemia associated mutations expression modeled AML pathogenesis. The cooperation of three types of mutations induce the AML phenotype; Type A, type B and type C mutations for the expression of AML associated fusion genes, constitutively activated kinases by fusion or mutation and clonal hematopoiesis and preleukemic state respectively (Fisher et al. 2019). AML is further classified into three prognostic risk groups: favorable, intermediate, and adverse. These are based on both cytogenetics and relatively recent recognition of molecular diseases subsets (Pelcovits and Niroula, n.d.).

Table 1: Recurrent gene mutations in AML (Roussel et al. 2020).

TABLE 1   Eight functional categories of genes mutations in acute myeloid leukemia (AML).			
Functional categories		Genes involved	Frequency
Signaling genes		FLT3, KRAS, NRAS, and KIT mutations	59%
Epigenetic homeostasis genes	Chromatin-modifying genes	ASXL1 and EZH2 mutations, MLL fusions	30%
	Methylation-related genes	DNMT3A, TET2, IDH1, and IDH2 mutations	44%
Nucleophosmin gene		NPM1 mutations	27%
Spliceosome-complex genes		SRSF2, SF3B1, U2AF1, and ZRSR2 mutations	14%
Cohesin-complex genes		RAD21, STAG1, STAG2, SMC1A, SMC3 mutations	13%
Myeloid transcription factors		RUNX1, CEBPA, GATA2 mutations	22%
		RUNX1-RUNX1T1, PML-RARA, MYH11-CBFB fusions	18%
Tumor suppressive genes		WT1, TP53 and PHF6 mutations (PTEN and DMM2 deregulations)	16%

## B. Diagnostic

The clinical signs are the consequence of the proliferation of blasts in the bone marrow and their spread in the blood. From genetic studies, it has become clear that the evolution of human AML is a multi-step process.

The myelogram is essential for the diagnosis and characterization of blasts (Auer body, myeloperoxidase positive staining, myeloid markers as cluster of differentiation (CD) CD34, CD13, CD33) (Döhner et al. 2017). The number of blasts must be greater than 20% of the total cells in the BM or circulating cells in the Peripheral Blood (PB) (Narayanan and Weinberg 2020), except for hemopathies with translocations inv16, t (8;21) and t (15;17) or an extramedullary tissue infiltrate. Certain cases require urgent management, in particular hyperleukocytosis (> 50G/L), severe hemorrhagic syndromes, metabolic disorders (lysis syndrome, renal failure).

Symptoms related to cytopenias in the blood are more or less marked: Anemic syndrome, Infectious syndrome, Hemorrhagic syndrome.

The tumor syndrome is inconstant: Lymphadenopathy is rare; splenomegaly is encountered in 15 - 20% of cases, leading to acute myeloid leukemia.

Gingival hyperplasia and skin localizations (leukemids) are more common for AL with a monocyte component, sometimes neurological locations (as in ALL) (De Kouchkovsky and Abdul-Hay 2016).

## II. History of AML treatment

Treatment of AML is generally divided into 2 phases: an induction phase and a consolidation phase. The first is intended to stabilize the patient's condition by reducing the tumor mass in the blood and the number of blasts in the BM, until complete remission. The second phase is to prevent relapse, after the patient has recovered from the induction phase; this phase can go as far as transplant strategies.

### A. Conventional chemotherapy

The induction phase in AML, generally consists of high doses of cytotoxic chemotherapy with cytarabine and an anthracycline (Daunorubicin), type "7 + 3" which means 7 days of continuous infusion of cytarabine with the addition of Daunorubin daily for the first 3 days (Huguët and Récher 2012) (Pelcovits and Niroula, n.d.). Although this option allows a reduction in tumor mass, it is nevertheless associated with high toxicity such as aplasia, infection, hemorrhages, inflammation, etc.,

and a high rate of relapse (Dombret and Gardin 2016), with selection of a clone, by selection pressure. In order to reduce toxicity, the use of CPX-351, allowing the delivery of Cytarabine and Daunorubicin by the liposomal route may be an alternative leading to better overall survival and tolerance and better post-allogeneic survival (Feldman et al. 2011) (Shlush and Mitchell 2015) (Figure 3). In addition, the toxicity of the two treatments, with CPX-351 and that with 7 + 3, is almost the same (Kansal et al. 2017).

In the consolidation phase, in order to eradicate the residual disease to prevent relapse, an allogeneic hematopoietic Stem Cell Transplantation (ASCT) is employed or a treatment offering cycles of chemotherapy with a single drug (cytarabine) (Pelcovits and Niroula, n.d.). Given that, Intermediate Cytarabine Dose (ICD) consolidation is recommended for younger and older patients who are not undergoing ASCT (Döhner et al. 2017). The addition of a third drug, fludarabine or clofarabine (a purine analog) to the 7+3 regimen induced a better response rate in AML (Estey 2018).

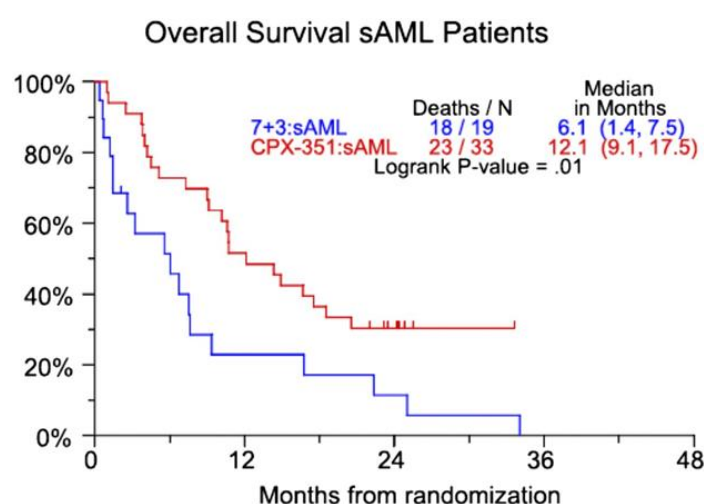


Figure 3: Percentage of AML patients' survival treated with CPX-351 (red line) versus those treated with Cytarabine and Daunorubicin (7 + 3) (blue line) (Zeidner and Karp 2014).

## B. Non-targeted therapy

Within mutated genes identified in AML are those involved in the epigenetic regulatory mechanisms of gene expression, including genes involved in DNA methylation (DNMT2), Wilm's Tumor gene 1 (WT1), Ten-Eleven Translocation 2 (TET2)) or histone regulation (Additional Sex Combs Like 1 (ASXL1), Enhancer of Zeste Homolog 2 (EZH2)) and KMT2A translocation. DNA methyl transferase inhibitors like Hypomethylating agents (HMA) using azacytidine, decitabine, guadecitabin (SGI-110, a novel HMA)



and many others are known to have significant clinical activity in the treatment of AML (Dombret et al. 2015). New agents, like OTX015, a Bromodomain and extra-terminal (BET) protein inhibitor, pinometostat, a Disruptor of telomeric silencing 1-like (DOT1L) inhibitor and iadademstat (ORY-1001), a Lysine specific demethylase 1 (LSD1) showed a clinically significant activity in relapse and refractory AML (Cerrano and Itzykson 2021).

### C. Targeted therapy

Another therapeutic alternative is targeted therapy, which is less toxic than traditional chemotherapy (Kayser and Levis 2018) (Figure 4). Targeted therapies are used as monotherapy or combined with non-targeted therapy or conventional chemotherapy.

Tyrosine Kinase Inhibitors (TKIs), targeting a tyrosine kinase receptor FLT3 mutations (FLT3-ITD/FLT3-TKD), are divided into 2 groups: a first generation multi-kinase inhibitors (such as Midostaurin (RATIFY assay (Stone et al. 2017), Sorafenib, Lestaurtinib), and next generation inhibitors (including Gilteritinib (ADMIRAL assay (Perl et al. 2019), Quizartinib, Crenolitinib). These inhibitors may be a treatment option for AML either used alone or in combination with intensive chemotherapy (Antar et al. 2020). Gilteritinib is the only FLT3 inhibitor for the treatment of relapsed or refractory AML with mutations of FLT3-ITD or FLT3-TKD (Bertoli, Fourrier, and Puisset 2021).

IDH1 and IDH2 are soluble enzymes expressed ubiquitously in the cytoplasm and mitochondria respectively. They are involved in the Krebs cycle by catalyzing an oxidative decarboxylation reaction to produce  $\alpha$ -Ketoglutarate ( $\alpha$ -KG). Mutations in IDH genes, identified in AML by whole genome sequencing, cause DNA and histone hypermethylation leading to blocked cellular differentiation, proliferation suppression and induce early leukemogenesis, by production of an abnormal metabolite, 2-hydroxyglutarate (2HG) which is an  $\alpha$ -KG antagonist. Enasidenib and Ivosidenib are oral small-molecules inhibitors that have been developed for mutant IDH2 and IDH1 respectively (Stein et al. 2019) (Roboz et al. 2020) and available under temporary authorization of use for relapse or refractory AML with IDH1 and IDH2 mutations (Bertoli, Fourrier, and Puisset 2021).

Among agents targeting the non-mutated targets, we note the antagonist inhibitors of B-Cell Lymphoma 2 (BCL2) such as Venetoclax or antisenses (Moore et al. 2006), which target the pathway of apoptosis. Venetoclax was first used as a single agent in AML relapse. For elderly patients, who are not eligible for standard induction chemotherapy, Venetoclax is used in combination with HMA (DiNardo et al. 2020), with Low Doses Cytarabine (LDC) (A. H. Wei et al. 2019) or with other targeted

therapies (H. Liu 2021). The association azacytidine-venetoclax (Phase III VIALE-A assay) (Bertoli, Fourrier, and Puisset 2021) is a first line oral treatment for relapse or refractory AML patients ineligible for intensive chemotherapy (Bertoli, Fourrier, and Puisset 2021).

The tumor protein 53 (TP53) mutation has been associated with a poor prognosis in both AML and MDS (Short, Rytting, and Cortes 2018) (Hunter and Sallman 2020). Despite of being “undruggable”, many studies have been done to target TP53 mutation. 10-days of decitabine was used as a first line therapy for older AML patients. A novel, first-in-class small molecule APR-246 (Eprexetapopt) alone or combined with azacytidine induce apoptosis in TP53 mutated cancer cells in MDS and AML. In addition, inhibitors targeting the interaction between TP53 and murine double minute 2 (MDM2) like idasanutlin (a second-generation-nutlin molecule) binding MDM2 and leading to decrease TP53 transcriptional activity. Idasanutlin was also used with cytarabine and venetoclax (H. Liu 2021).

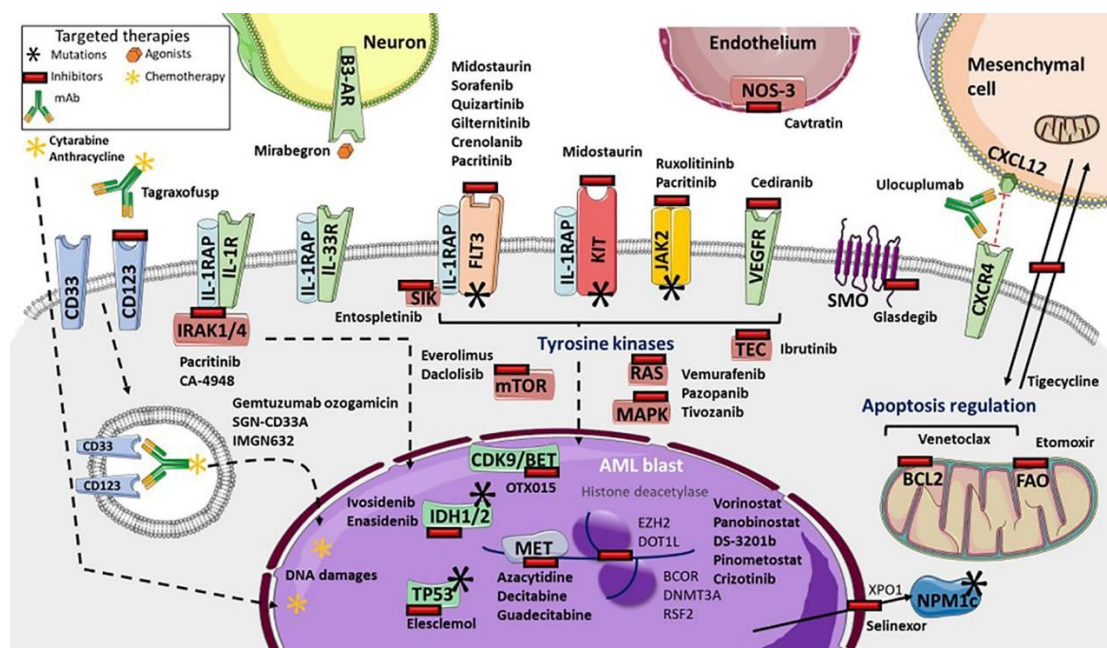


Figure 4: Targeted therapies available and in development in AML (Roussel et al. 2020).

#### D. Immunotherapy

The progression of AML results from the escape of leukemia cells to the Immune System (IS) (Marcus et al. 2014). These cells are able to turn off the expression of stress molecules on their surface or block receptors that serve as recognition with the IS. Treatment of AML with immunotherapy approaches involves reversing the strategies implemented by the leukemia cell. This treatment includes either the

use of agents allowing elimination of the tumor cell (coupled antibodies), or reactivation and recruitment of immunocompetent cells (vaccines, cytotoxic T lymphocytes (CTLs)). Plus, by injecting genetically modified T lymphocytes (GMTLs) to redirect them against the tumor such as Chimeric Antigen Receptor (CAR) T-cells and transgenic T-cell receptor (tgTCR) (Yang et al. 2017a) (Figure 5).

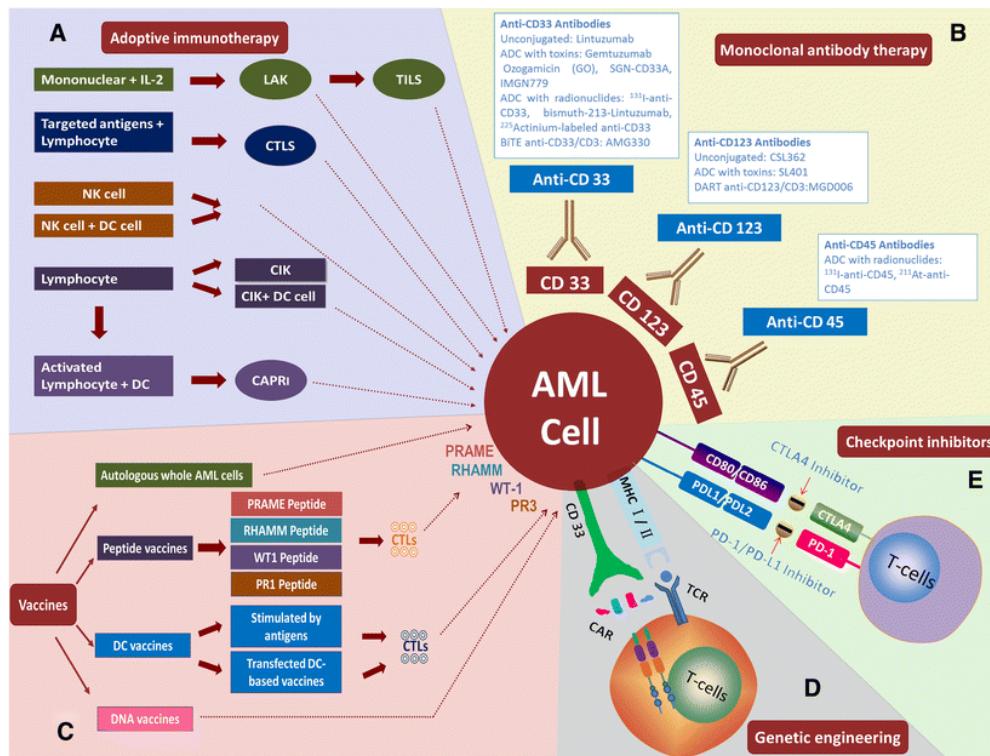


Figure 5: Immunotherapeutic approaches in AML (Yang et al. 2017b).

## 1. Passive immunotherapy

Different membrane antigens (such as CD33, CD123, CD47, CD64...) are expressed on the surface of leukemia cells allowing their identification, and especially their targeting with antibodies in order to be able to deliver cytotoxic drugs in a specific manner. On the other hand, targeting AML microenvironment is still under investigation.

Monoclonal antibodies (mAbs) are used alone or with a conjugated drug, targeting several leukemia cells' membrane antigens (Morsink and Walter 2019a). Only few clinical trials have shown a satisfactory response in AML (Shang and Zhou 2019a) (Morsink and Walter 2019b). CD33-targeting with an anti-CD33 mAb coupled to a toxin (GemtuzumAb ozogamicin (GO)) is the most advanced strategy actually used but anti-CD33 mAb could not provide the results expected in monotherapy

(Petersdorf et al. 2013). Anti-CD123 mAb (talacotuzumab) showed toxicity issues but when coupled to IMG632 with or without adding venetoclax, it showed better responses. Indeed, the CD123 antagonist, the diphtheria toxin IL-3 fusion protein (SL-401), indicates potent activity against AML blasts (Mani et al. 2018).

The progress in immunotherapy has permitted to develop synthetic antibodies with several antigens targeting, such as bispecific antibodies whose double affinity at a time redirect T effector cells against tumor cells. Bispecific T cell Engager (BiTE) antibodies: anti-CD3/CD33 (AMG330), anti-CD3/CD119, anti-CD3/CD123 (XmAb14045); Dual-Affinity Re-targeting (DART) antibodies: anti-CD3/CD123 (flotetuzumab) (Shang and Zhou 2019b); Checkpoint inhibitor T cells Engager (CiTE): anti-CD3/CD33 with blocking PD-1 extracellular domain (Shang and Zhou 2019a).

Finally, antibodies blocking the checkpoint molecules of the IS: Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4), Programmed Death-1/Programmed Death Ligand-1 (PD-1/PDL-1), T-cell Immunoglobulin and Mucin domain-containing protein 3 (TIM3), Lymphocyte Activation Gene 3 protein (LAG3), CD47 pathway, CXCR4 chemokine receptor, are, as in many cancers, targets explored in preclinical or clinical settings, without yet evidence of efficacy in AML (H. Liu 2021).

## 2. Active immunotherapy

Stimulation of the IS by tumor antigen, with autologous AML cells, peptides, DNA or dendritic cells (DC) have been used in the treatment of AML. Administration of WT1 peptides, overexpressed in 90% of AML and MDS, as vaccines or as a first-in-human trial of TCR-gene transduced T-cell (TCRT-cell) transfer in patients with refractory AML (WT1-specific TCRT-cells) (Tawara et al. 2017) has shown clinical improvement (phase 1 and 2) and control of residual disease (Maslak et al. 2018) (Tsuboi et al. 2012). The infusion of ex-vivo-derived CTLs following stimulation by Protein-1 (PR-1) (expressed on the surface of AML cells), tested in a phase 1 and 2 clinical trial of patients with AML, resulted in positive responses in 24% of these patients (Qazilbash et al. 2017). Finally, DC loaded with WT1 (by WT1 messenger RNA electroporation) shows in a phase 1 trial an effective strategy in the treatment of AML relapse by stimulating the toxicity of specific LT-CD8 + cells (Van Tendeloo et al. 2010) (Anguille et al. 2017).

### 3. Adoptive immunotherapy

In 2011, Hanahan and Weinberg (Hanahan and Weinberg 2011) described immunomodulating properties within tumors characteristics. Some tumors are infiltrated by effector cells for innate immunity and adaptive immunity with a good prognosis, so Adoptive Cell Transfer therapies (ADCT) can be a promising immunotherapy in AML.

Allogeneic BM transplantation, a first and benchmark cell immunotherapy, is indicated in first complete remission in AML (Koreth et al. 2009). Even though allogeneic transplantation is associated with high procedural toxicity (TRM: Transplant-Related Mortality), it remains the only curative option in patients at unfavorable risk (Kassim and Savani 2017).

Autologous Tumor Infiltrating Lymphocytes (TILs) cultured in ex-vivo, in the presence of IL-2, were the first adoptive cellular immunotherapies. TILs are not suitable for hematologic tumors, because of the difficulties in obtaining these cells, although recent work has shown the possibility of obtaining them from the microenvironment of the BM (Borrello and Noonan 2016). TILs from AML patients' BM are expandable ex-vivo and possess anti-tumor activity (Teo et al. 2019) (L. Wei et al. 2019).

AML blast cells escape the vigilance of Natural killer (NK) cells, through the overexpression of specific ligands for immunoglobulin-like killer inhibitory receptors (KIRs) expressed on the surface of NK cells. The use of NK cells may therefore be an interesting avenue in the treatment of AML (Carlsten and Järås 2019). Clinical studies have shown the feasibility of using NK cells from a healthy donor stimulated ex-vivo by IL-2, which aims to amplify the activating signal and inhibit alloreactivity with human leukocyte antigen (HLA) molecules from the patient's tumor cells (Koehl et al. 2004). In addition, Bispecific Killer Engagers (BiKE) (anti-CD16/CD33) and Trispecific Killer Engager (TRiKE) (anti-CD16/IL-15/CD33, anti-CD33/CD123/CD16 is under investigation (Braciak et al. 2018)). NK cells activated by different cytokines for Cytokines Induced Killer cells (CIK) are made more cytotoxic and more proliferative than those activated with IL-2 (Linn et al. 2009). Immunotherapy using Cytokine-induced memory-like NK cells demonstrates the ability to fight AML leukemia cells in a first-in-human phase 1 clinical trial (Romee et al. 2016).

## E. Genetically modified T Lymphocytes

Cancer immunotherapy is based on the use of immune effector cells cytotoxicity against leukemia cells. The potential of T-cells to eradicate tumors has inspired new immunotherapy strategies and has led to the development of GMTL to express a specific receptor of malignant cells (Turtle et al. 2012).

As ADCT, autologous T-cells can be redirected to leukemia cells of an AML patient by two recently used methods based on the genetic modification of these T-cells; either by generating T-cells with a tgTCR specific to one tumor antigen, or by generating T-cells expressing a CAR against a tumor antigen (Figure 6).

Several tgTCRT-cells targeting a tumor antigen are used in AML with potent anti-tumor efficacy. However, tgTCRT-cells therapy may be associated with off-target toxicities induced by mispairing between the endogenous and the introduced TCR chains and it is limited to HLA restriction and the small number of suitable targets (Bonte et al. 2020).

In AML, classically, blasts escape the IS by down-regulating HLA molecules, resulting in an altered TCR/HLA/tumor antigen interaction. Therefore, immunotherapy using T-cells free of HLA-recognized (CART-cells) will be more preferable.

CART-cells therapy is a novel ADCT therapy with promising results in BCL and multiple myeloma (MM) using CD19 CAR, CD22 CAR -T-cells (Park et al. 2018). CART-cells targeting various antigens (CD33, CD123, FLT3, CLL-1, etc.) are under clinical investigations for AML treatment. These assays showed potent efficacy but also toxicities on healthy hematopoietic cells (Shang and Zhou 2019a). The ideal antigenic target in AML has not yet been identified.

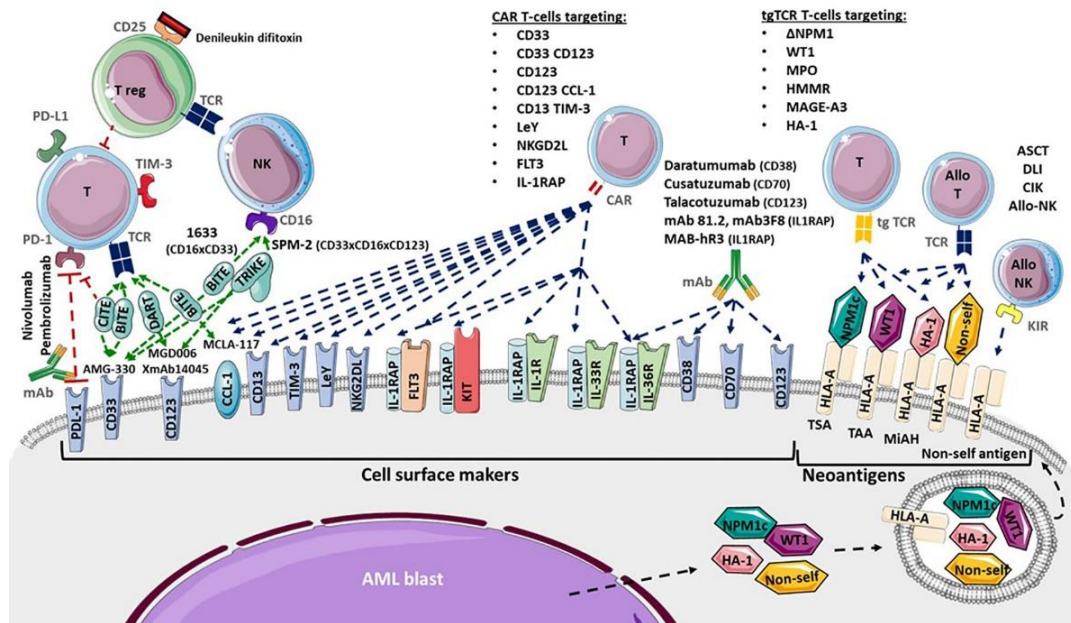


Figure 6: Immunotherapies using genetically modified immune cells in AML (Roussel et al. 2020).

### III. Relapse of AML patients and need for new therapeutic alternatives

#### A. Resistance to treatments

Despite the progress made in the treatment of AML over the past 10 years, survival has not been significantly improved especially in elderly patients, mainly due to relapses. Resistance to treatments occurs due to acquisition and/or enrichment of clones in the AML tumor cell with activation of the signaling pathways such as FLT3 (FLT3-ITD mutation) or RAS or bi-allelic mutations affecting the function of TP53. Moreover, combination of venetoclax and azacitidin may select monocytic disease in AML, which will lead to treatment resistance and relapse (Pei et al. 2020).

The strong interactions of AML blasts with the IS suggest avenues for improving treatments, thanks to immunotherapy approaches, particularly in maintaining remission during the maintenance phase (Table 2). Currently, various research groups are trying to identify appropriate targets to develop alternative immunotherapies to treat patients refractory or relapsing from conventional treatments or existing immunotherapies.



Several studies aimed to target the Leukemia Stem Cell (LSC), which is responsible of the maintenance and the propagation of the AML phenotype after treatments, and prevent relapse by targeting various markers expressed on the LSC's surface (Gentles et al. 2010).

The Interleukin-1 Receptor Accessory Protein (IL-1RAP) has been identified for its expression on LSC (Tasian, Bornhäuser, and Rutella 2018), thus as a potential target to destroy AML leukemia cells. This approach will make it possible to target AML blasts expressing the IL-1RAP protein, in order to provide an alternative treatment to CART-Cells approaches conventionally targeting CD33 or CD123.

*Table 2: Mechanisms of AML LSC resistance against immunotherapies and some possible solutions (Valent et al. 2020).*



Type of resistance and mechanism	Strategies proposed to overcome resistance
Intrinsic stem cell resistance	Antibody-based therapies; HSCT; CAR
LSC dormancy	Antibody-mediated targeting of dormant LSC; activating LSC with cytokines to induce cell cycling (cytokine-priming)—and combination with chemotherapy or TKI <sup>a</sup> ; CAR; HSCT
Expression of efflux-transporters like MDR1	MDR1-targeting drugs; HSCT; TKI <sup>a</sup> ; CAR antibody-based therapies; HSCT; TKI <sup>a</sup> ; CAR
Lack of drug transporters (lack of drug uptake)	Antibody-based therapies; HSCT; TKI <sup>a</sup> ; CAR
Niche-mediated resistance	Niche cell-targeting drugs, drug combinations, antibody-based therapies; HSCT; CAR; TKI <sup>a</sup>
Osteoblastic LSC niche	Osteoblast cell-targeting drugs and/or BET/MYC-targeting drugs <sup>b</sup>
Vascular LSC niche	Endothelial cell-targeting (anti-angiogenic) drugs
LSC-retention in niche	Mobilizing drugs (plerixafor); HSCT; CAR; TKI <sup>a</sup>
LSC-hypermobilization (eg, CD26-mediated)	Mobilization blocker (eg, gliptins or CD26 Ab); antibody-based therapies; HSCT; CAR; TKI <sup>a</sup>
Acquired resistance of LSC mutations in subclones	Drug combinations; antibody-based therapies; HSCT; CAR; TKI <sup>a</sup>
Immune checkpoint-induced resistance of LSC	Checkpoint-targeting antibodies Checkpoint-targeting CAR cells or BiTE BET/MYC-targeting drugs <sup>b</sup> ; antibody-based therapies; HSCT; CAR; TKI <sup>a</sup>
Loss of cell surface targets	Mixtures of antibodies; HSCT; CAR directed against 2 or more surface targets; drug combinations + HSCT or CAR
General immunosuppression	Repeated T/NK cell infusion
Blocked immune cells	Bi-specific antibodies against LSC and immune effector cells; HSCT; CAR; TKI <sup>a</sup>
Loss of CAR-T cells or CAR-NK cells	Repeated infusions of CAR cells; cytokine-induced expansion of CAR cells; drug combinations; HSCT; TKI <sup>a</sup>
Development of blocking antibodies against CARs	Use of single domain scFvs humanize the scFvs; HSCT; TKI <sup>a</sup>

## B. IL-1 Receptor Accessory Protein (IL-1RAP)

### 1. Definition of IL-1RAP

IL-1RAP (also called IL-1RAcP, IL-1R3 or C3orf13) is a protein encoded by chromosome 3q28. It belongs to the interleukin-1 (IL-1) family of proteins. It is a protein expressed on the surface of cells and forms a complex with the receptor for IL-1 $\alpha$ , IL-1 $\beta$ , and IL-33 and it is essential for their signaling. There are five known forms of alternative splicing for IL-1RAP mRNA. This protein exists in two forms, a membrane form (variant 1, 3, and 4) and a secreted soluble form (variant 2 and 5). The membrane form of IL-1RAP induces an intracellular signal after binding to IL-1. While the soluble form has a neutralizing effect of IL-1 (D. E. Smith et al. 2003). IL-1 is a pro-inflammatory cytokine in response to infection, stress or tissue damage via the (Nuclear factor-kappa B) NF-Kappa-B pathway to infection (Figure 7).

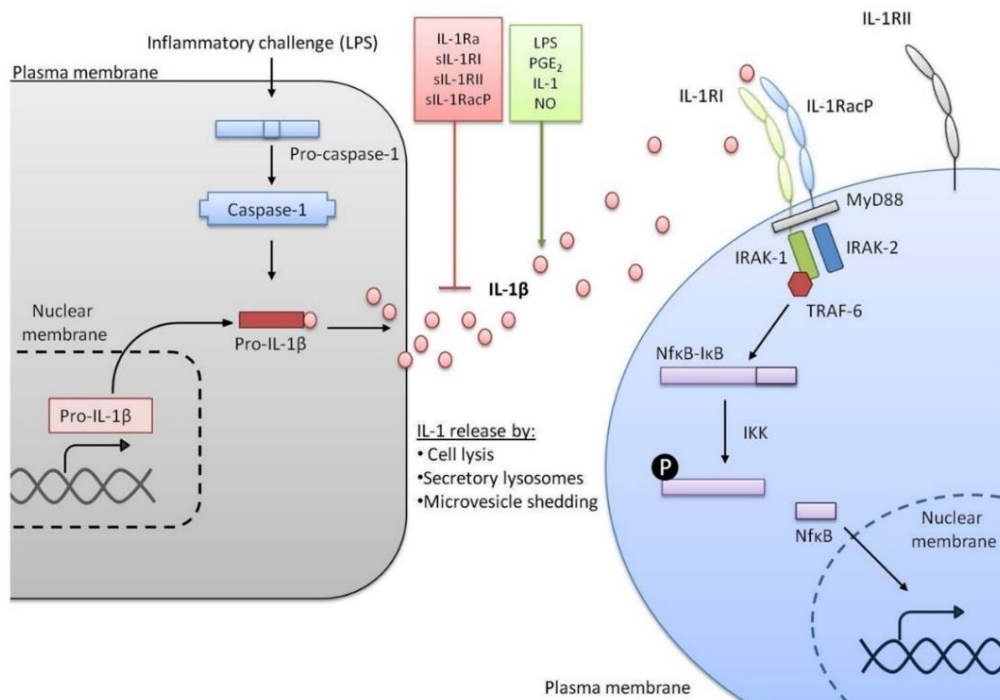


Figure 7: IL-1 production and signaling. IL-1Ra: Interleukin-1 antagonist, sIL-1RI: Soluble IL-1 receptor I, sIL-1RII: Soluble IL-1 receptor II, sIL-1RacP: Accessory protein to the IL-1 receptor (Murray, Parry-Jones, and Allan 2015).

## 2. Function of IL-1RAP

Cytokines of the IL-1 family are secreted very early in the immune response by DC, monocytes and macrophages. IL-1 secretion is stimulated by recognition of viral, parasitic or bacterial antigens by innate immunity receptors. Cytokines that are members of the IL-1 family (IL-1 $\alpha$ , IL-1 $\beta$ ) are generally pro-inflammatory, which means that they induce an increase in the permeability of the capillaries at the site of cytokine secretion, thus causing an amplification of the migration of leukocytes to infected tissues. IL-1 can be stored as a precursor (Pro-IL-1 $\beta$ ) which is then hydrolyzed to IL-1 $\beta$  by the IL-1 converting enzyme or caspase 1.

The binding of IL-1 $\beta$  to its type 1 receptor, IL-1RI (IL-1 Receptor I) (Figure 7) induces a conformational modification of the toll interleukin receptor (TIR) domain, which allows the binding of the Myeloid Differentiation primary response 88 (MyD88) adapter molecule via its TIR domain. MyD88 recruits one or more IL-1 Receptor Activated Kinases (IRAK kinases) forming an activating complex at the receptor level which in turn induces the activation of the transcription factor NF- $\kappa$ B and the Activator protein-1 (AP-1).

NF- $\kappa$ B and AP-1 are involved in the induction of different signaling pathways such as proliferation, differentiation and secretion of cell growth factors. In contrast, the binding of IL-1 $\beta$  to its type 2

receptor IL-1RII, does not induce signaling because IL-1RII lacks the intracellular TIR fragment. IL-1Ry $\alpha$  (IL-1 receptor antagonist) has an effect that opposes the effects of IL-1 $\beta$  by competing with IL-1RI. In addition, the soluble forms of IL-1RI (sIL-1RI), IL-1RII (sIL-1RII), and IL-1RAP (sIL-1RAP) have an inhibitory effect. They can bind to IL-1 $\beta$  and therefore prevent its binding to its IL-1RI membrane receptor.

A transcriptomic analysis, compares the populations of Leukemic Hematopoietic Stem Cells (LHSC) (AML with monosomy 7), primitive -LT (Lin-/CD34+/CD38-/CD90+ : long-term LHSCs), less primitive-ST (Lin-/CD34+/ CD38-/CD90- : short-term LHSC) and more differentiated GMP (Lin-/CD34+/CD38+/CD123+/CD45RA+ : Granulocytes/Macrophages progenitors) respectively with the same populations of healthy subjects. Among a list of 11 genes, common to the three types of populations, an overexpression of the IL-1RAP protein emerges. Thus, the IL-1RAP gene expression allows discriminating normal HSC and LSC.

IL-1RAP overexpression is observed in Chronic Myeloid Leukemia (CML) correlated with the mutation of BCR-ABL gene, in ALL with Philadelphia chromosome, and in MDS and AML stem and progenitors cells (with normal karyotype or AML-7/7q- (monosomie 7 or deletion of 7q) but not on healthy hematopoietic cells (Barreyro et al. 2012) (Askmyr et al. 2013).

In an inflammatory microenvironment such as in AML pathogenesis, IL-1RAP has an oncogenic effect through two tyrosine kinase receptors pathways FLT3 and c-kit (Mitchell et al. 2018) which promotes the proliferation of leukemic cells and drives HSC clonal evolution (Pietras et al. 2016). IL-1RAP signaling axis plays an important role in enhancing inflammation in the leukemic niche via p38 MAPK and NF- $\kappa$ B signaling pathways (De Boer et al. 2020) (Figure 8). In addition, IL-1RAP has a crucial role in the regulation of tumor microenvironment-related inflammatory factors in solid tumors ((Lv et al. 2021).

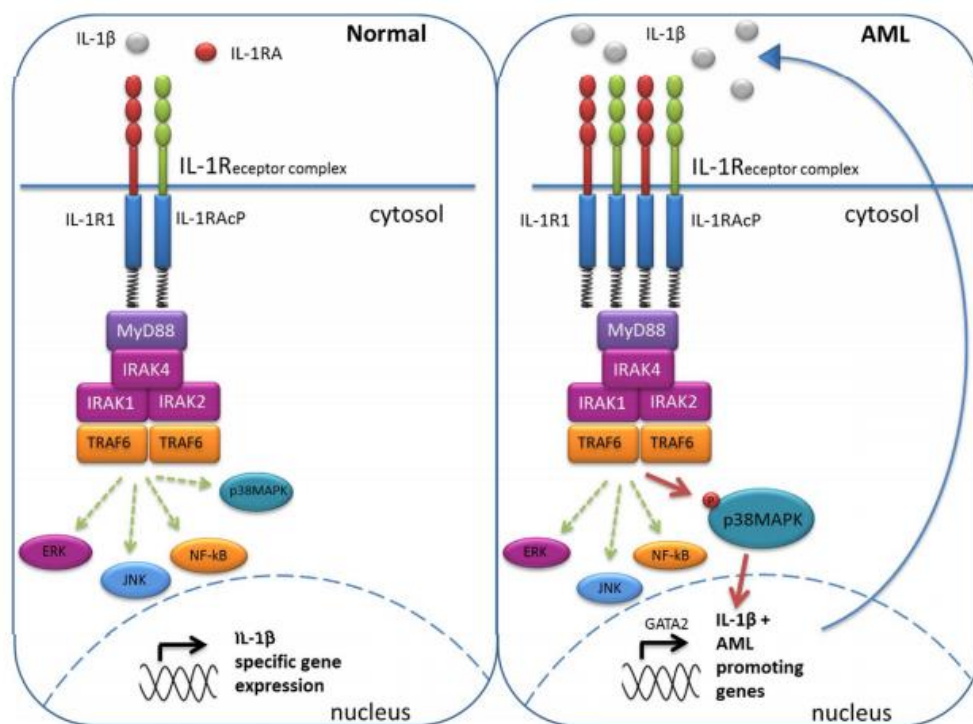


Figure 8: IL-1RAP signaling pathway promotes AML cell survival (Binder, Luciano, and Horejs-Hoeck 2018).

### 3. Targeting of IL-1RAP

Based on these findings, IL-1RAP appears to be an interest and efficient target for cancers therapies. Anti-IL-1RAP have been developed to enable the killing of tumor cells, via an Antibody-dependent cell-mediated cytotoxicity (ADCC).

In solid tumors, IL-1RAP is targeted by synthetic mAbs (P. Zheng et al. 2018), showing safety profile in a clinical study (CAN04 (nidanilimab) targeted IL-1RAP, 2019) and overcoming cancer metastasis in an aggressive childhood sarcoma (H.-F. Zhang et al. 2021).

Anti-IL-1RAP mAbs (mAb81.2 and mAb3F8) and IL-1 signaling blockade induced potent cytotoxicity in AML and CML cells (Askmyr et al. 2013) (Ågerstam et al. 2016). In a murine AML xenograft model, blocking the IL-1 receptor by an IL-1RAP antibody and the ADCC effect led to suppression of blasts proliferation (H et al. 2015).

Blocking IL-1RAP using the human IL-1RAP Ab, MAB-hR3, attenuate most of the functions of IL-1 family leading to an anti-inflammatory activity (Højen et al. 2019), and might reduce inflammation in the BM niche (De Boer et al. 2020).

IL-1RAP was also targeted with CART-cells in CML. IL-1RAP CART-cells efficiently eliminate quiescent tumor HSCs, which fall outside the spectrum of action of tyrosine kinase inhibitors in-vitro and in-vivo in a xenograft murine model (Warda et al. 2019a).

Recently, a novel therapy was developed in targeting IL-1RAP in AML using a bio-reducible lipidoid-encapsulated CRISPR associated protein 9 (Cas9)/single guide IL-1RAP RNA ribonucleoprotein (Ho et al. 2021). This strategy provides an effective attenuation of AML LSC growth.

In addition, bispecific Abs have been developed in order to target IL-1RAP and Thomsen–Friedenreich in CML to increase the specificity towards LSC by using additional biomarkers (Eldesouki et al. 2021).

## **Chapter 2: 1. Cellular immunotherapy in AML using CART-cells**

### **I. Principle of CAR development**

The growing understanding of the natural T Lymphocytes (TL)-TCR construction and function, has allowed developing the CAR receptor. Five generations of the CAR receptor were constructed over the years (Figure 9).

The CAR development history was first begun in 1989 when Gross's team built a chimeric TCR (cTCR) by replacing the V $\alpha$  and V $\beta$  extracellular variable domains of the TCR chains with their homologous immunoglobulin Heavy chain (VH) and Light chain (VL) (C $\alpha$ VH + C $\beta$ VL) or C $\alpha$ VL + C $\beta$ VH). TLs expressing this cTCR were activated against target cells independently from the Major Histocompatibility Complex (MHC) (Gross, Waks, and Eshhar 1989).

This experiment validated that the cytoplasmic domain of the TCR, CD3 $\zeta$  (zeta), can reproduce many of the signals of a TCR (van der Stegen, Hamieh, and Sadelain 2015). In light of these advances, in 1993 was constructed a first functional CAR receptor composed of the single cell fragment variable (ScFv) of a mAb linked to the intracytoplasmic sequence of CD3 $\zeta$  (Eshhar et al. 1993). This new artificial receptor is known as the first generation CAR.

However, in order to enhance cytotoxicity by cytokine production and naïve TLs activation, some costimulatory molecules were added to the first generation of CAR construct. The addition of the costimulatory element CD28 was described in 1998, producing a second generation of CAR. The second generation CARs are more efficient in inducing cytokine production (for example IL-2) and CART-cell proliferation compared to the first generation CAR, which has been proved in several preclinical studies (Haynes et al. 2002).

The third generation CAR contains two costimulatory domains, resulting in a more potent persistence and other CART-cell functions in treated patients (Jinjuan Wang et al. 2007).

For further improve the killing function of CART-cells against tumors, a fourth generation of CAR, also named T-cells redirected for universal cytokine-mediated killing (TRUCKs), has been developed based on the second generation. It can induce cytokine production for example IL-12 through the nuclear factor of the activated T-cell (NFAT) which can direct the TL to express transgenic products (Chmielewski and Abken 2015). Recently, a novel fifth generation of CAR was developed containing intracellular domains of cytokine receptors, such as IL-2R $\beta$  chain fragment, to enhance anti-tumor effects (Kagoya et al. 2018).

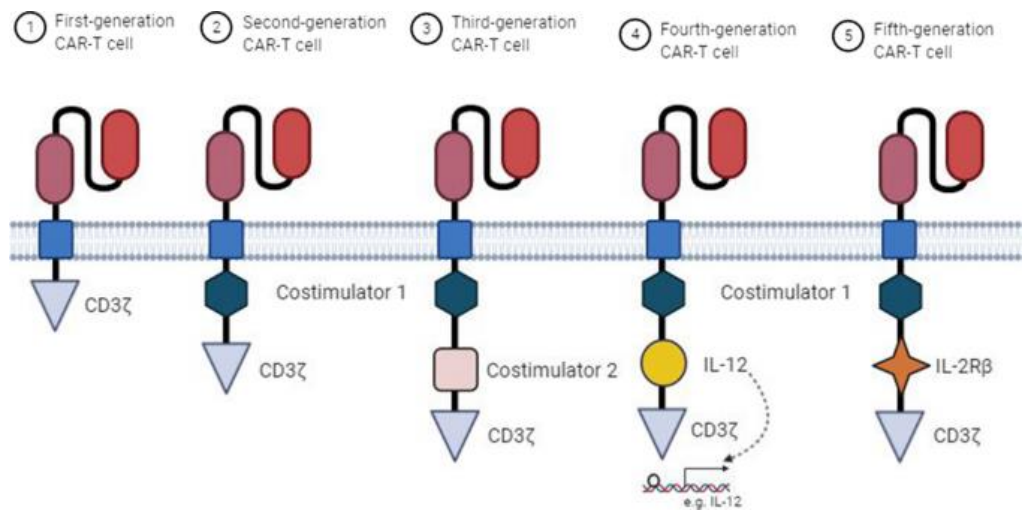


Figure 9: Different generations of the CAR receptor (Jin et al. 2021).

## II. CAR structure

The CAR is made up of three domains: an extracellular domain, a transmembrane domain and an intracellular domain, which may contain different co-stimulation endo-domains depending on the type of the CAR (Guedan et al. 2019) (Figure 10).

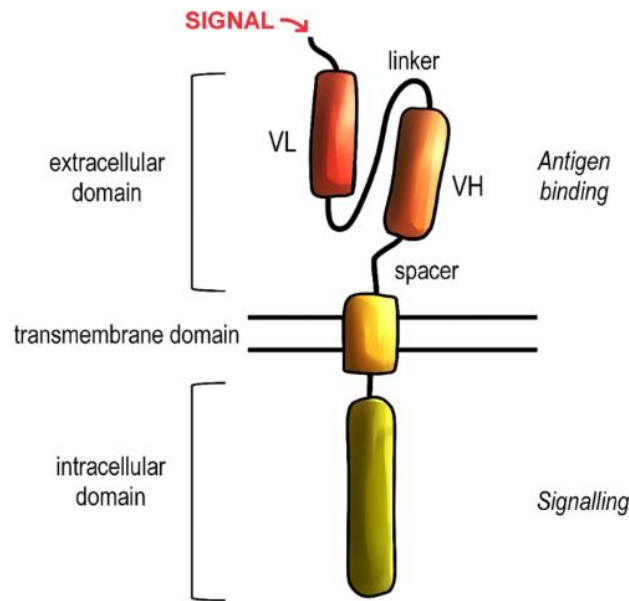


Figure 10: Schematic representation of the different domains of the CAR (Skorka et al. 2020).

#### A. Extracellular and stimulation domain

The extracellular region (ectodomain) of the CAR, which forms the specific antigen-binding site, is a ScFv derived from a mAb specific for a target antigen. ScFv is a fusion protein composed of a VL chain attached to a VH chain of a fragment (Fab) of a monoclonal immunoglobulin (Ig) via oligo-peptides called "Linker". The "flexible GS Linker" was used in order to improve the folding and the stability of the construction of the ScFv fragment. The "linker's" structure is formed of a peptide sequence (GGGGS) repeated 3 times, allows a correct orientation of the VH and VL domains and does not interfere with the folding of the protein domains. The length of this "Linker" was adjusted as a function of the distance between the C-terminus of the VH domain and the N-terminus of the VL domain in its natural orientation (3.5 nm) to ensure, at the same time, a good affinity and a better CAR function (Xiaoying Chen, Zaro, and Shen 2013) (Figure 11). The ScFv can be carefully designed and manipulated in order to influence specificity and differential targeting of tumors versus normal tissues.

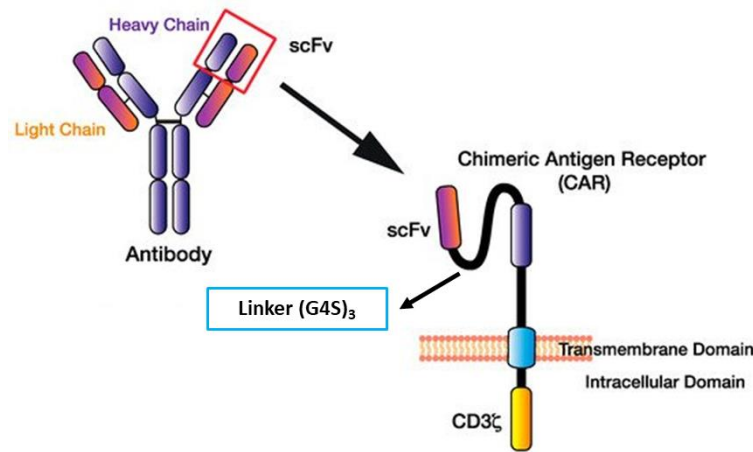


Figure 11: CAR ScFV structure. ScFV: single chain variable fragment (Hughes-Parry, Cross, and Jenkins 2020).

## B. Hinge region

The “Hinge” or “spacer” part of the CAR construct links the extracellular part to the intracellular part of this receptor. It plays an important role in activating the CART-cell. The role of this region is to provide flexibility to the ScFv, whose "hinge" length plays a role in enhancing the expression of ScFv at the TL membrane (Guest et al. 2005). These properties have been described as modulating effector cell/target cell interactions, thus affecting the strength of the activation signal of the CART-cell. The hinge region can be of different types such as an extracellular fragment derived from CD28, TCR $\beta$  chain, CD8 $\alpha$ , or NKG2D or an Ig-like domain with the Fc regions of an IgG antibody (IgG1 hinge CH2-CH3) due to the stability of the protein domain (Lipowska-Bhalla et al. 2012). The position of the target epitope regarding to the target cell surface determine the need of an extracellular spacer domain (A. A. Hombach et al. 2007). Studies have shown that the hinge region has a role in the activation and secretion of cytokines by CART-cells and regulates the CAR signaling threshold (Fujiwara et al. 2020).

## C. Transmembrane domain

The transmembrane domain (TM) connects the ectodomain to the endodomain and serves as the anchor to the cell membrane. Few studies have been done on the transmembrane domain of the CAR. An earlier study had used a CD3 $\zeta$  transmembrane domain in the CAR construct and subsequently showed that this domain is important for stability of membrane expression of the CAR (Romeo, Amiot, and Seed 1992). In 2010, Bridgeman et al. have shown that the biochemical interactions that occur



between the wild-type CD3 $\zeta$  transmembrane domain and other components of the endogenous TCR/CD3 complex are important for the optimal activity of the CD3 $\zeta$  CAR (Bridgeman et al. 2010). Various transmembrane regions have also been employed in CARs including those derived from CD28, CD3, CD8, CD4, or Fc $\epsilon$ R1 $\gamma$ . Using CD19 CAR T-cells with TNFRS19 TM showed clinical promoting results (Caimi et al. 2019). The TM can regulate the amount of CAR signaling via control of CAR expression level. Similar to the hinge domain, changing the length of the TM can affect the CAR T-cell proliferation and further clinical studies are needed to prove the advantages of the TM for decreasing tonic signals and increasing CAR T-cell persistence (Fujiwara et al. 2020).

#### D. Intracellular and signaling domain

The CAR construct has a functional cytoplasmic region that provides downstream signaling and directs the immune responses of this receptor. The different generations of the CAR differ in the number and properties of the intracellular signaling domains (endodomains) (Pehlivan, Duncan, and Lee 2018) (Figure 12). The first generation CAR has only a single signaling domain, the CD3 $\zeta$  signaling domain of a natural TCR which provides the activating signal (Lee and Kim 2019). The addition of different signaling domains in association with the different constructions are defined in a certain order for a full activation of the CAR T-cell. In most constructions, the CAR costimulatory domain is located directly between the transmembrane domain and the CD3 $\zeta$ . Being in close proximity to the membrane, this domain can easily interact with its signaling molecules located in this region of the cell (Kalaitzidou et al. 2015). Different co-stimulatory molecules are used in the other CAR constructs.

The CD28 which provides the signal 2 in the activation of T cells, was used to increase the proliferation of CAR T-cells and allow greater secretion of cytokines such as IL-2, IL-10, interferon gamma (IFN $\gamma$ ) and Tumor Necrosis Factor alpha (TNF $\alpha$ ) in comparison with the first generation CAR lacking a co-stimulatory domain (A. Hombach et al. 2001). In clinical trials, CD19-CD28 CAR T-cells were effective in eradicating B lymphomas (Davila et al. 2014).

The 4-1BB co-stimulatory domain also called CD137, which is a part of the TNF receptor (TNFR) family was used to increase cytokines secretion, cell survival and resistance to Activation Induced Cell Death (AICD). In addition, CAR T-cells expressing 4-1BB were shown to decrease tonic signaling and cell exhaustion greater than CAR T-cells with CD28 (Long et al. 2015), and promotes the formation of central memory T-cells (T<sub>cm</sub>) versus the formation of effector memory T-cells (T<sub>em</sub>) with CD28 (Kawalekar et al. 2016). CD19-4-1BB CAR T-cells also showed clinical outcomes in refractory B cell ALL (Locke et al. 2019).

Additional preclinical studies discovered more molecules, which can be used for co-stimulation of the CAR in combination with CD28 and 4-1BB.

The Inducible T-cell COStimulator (ICOS) molecule, also called CD278, is a member of the CD28 superfamily. This co-stimulator plays a promoter role in the differentiation of TL-CD4<sup>+</sup> Helper 1 (Th1) and Helper 2 (Th2) subsets and their effector functions in cytokines production (IL-10). CART-cells with the two intracellular signaling domains ICOS and 4-1BB demonstrate increased efficacy in solid tumor models regarding the use of only 4-1BB (Guedan et al. 2018).

Combined 4-1BB and OX40 (CD134), two agonist of the TNFR costimulatory receptors, has been shown to generate very high effector T-cells with longer survival, more differentiation and production of greater quantity of cytokines compared to T-cells stimulated with only 4-1BB (Konstorum et al. 2019). In addition, OX40 stimulates the secretion of pro-inflammatory cytokines such as IL-4, IL-6 and IFN $\gamma$  by CART-Cells, thus inhibiting the suppressive activity of regulatory TL (Treg), and can induce differentiation of CART-cells to a memory phenotype allowing the escape of AICD (Redmond, Ruby, and Weinberg 2009).

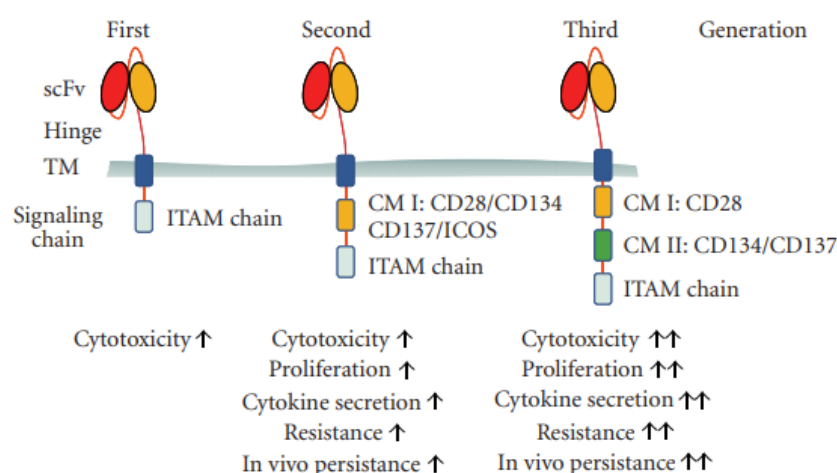


Figure 12: A brief of the different CAR co-stimulatory molecules and their functions. ITAM: Immunoreceptor tyrosine-based activation motif (CD3 $\zeta$ ), CM: Co-stimulatory Molecule (Cartellieri et al. 2010).

Activation of TL via the TCR requires its interaction with MHC molecules forming an immunological synapse and activating the Zap70 molecule by phosphorylation, which provides signal 1, and which occurs by exclusion of the inhibitory receptor CD45 due to its large ectodomain regarding the small special distance (15nm) between the TL and the antigen-presenting cell (APC). Signal 2 is then provided by the binding of the co-stimulatory molecule CD28, expressed by TL, to its ligand CD80/CD86 (B7) on the target cell resulting in activation of the PI3K transduction pathway. While for a CART-cell, activation by the CAR signal, following its interaction with the specific tumor-antigen, is sufficient to deliver both

signals 1 and 2, resulting in T-cell activation and triggering of an immune response. The special distance between the CART-cell and the target tumor cell is not yet known. Indeed, the activation of the BiTE needs the presence of two independent signals. Signal 1 is delivered upon BiTE ligation with target tumor cell through secreting bispecific antibodies, and signal 2 is delivered by CD28/B7 (Y. Wang et al. 2017) (Figure 13 A-B-C).

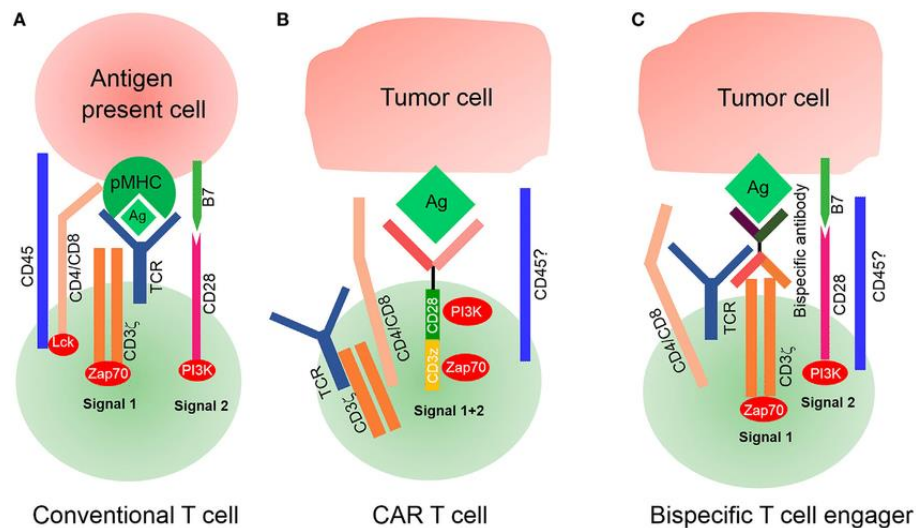


Figure 13: Signaling mechanisms of conventional TCR and CAR receptor (Y. Wang et al. 2017).

### III. CAR vector transfer

In gene therapy, the transgenesis is the act of transfer a nucleic acid (DNA or RNA) into a host cell, which is foreign to it. Gene vectors mediate the delivery of the nucleic acid because of its negative charge and considerable larger size. Different delivery systems like Viral, transposon, Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9) and electroporation systems are frequently used to transfer the CAR transgene into immune effector cells (T-cells, NK cells) (Figure 14).

Viral vectors have been used as modified virus in more than two-thirds of clinical trials for their advantage of high gene transfection. Y-retrovirus, lentivirus, Adeno-Associated Virus (AAV), Adenovirus (Ad) and Foamy virus (FV), remains the reference to this day, they provide a stable and efficient tool to deliver the genetic material to different cell types and tissues, for either transient or persistent expression (Piscopo et al. 2018) (Tumaini et al. 2013).

Recently, new technologies using non-viral systems have been developed in order to decrease the cost, the production time and the risks associated with virus vectors such as possible random insertions or DNA damage. Sleeping Beauty (SB) and PiggyBac transposon systems showed clinical anti-tumor

efficiency and CART-cells persistence with producing a higher ratio of Tcm (Clauss et al. 2018) (Bishop et al. 2019). SB-modified CART-cells also demonstrate potent outcomes in-vitro and in-vivo in chemoresistant AML patient derived xenograft (PDX) (Rotiroti et al. 2020).

Among the evolution of the manufacturing technology, gene editing methods such as Zinc finger (ZFNs) and nucleoside transcription activator-like effector nucleases (TALENs) were developed and used in the CAR therapy (Qasim et al. 2017). The breakthrough in the genetic “editing” was the CRISPR/Cas9 technology. Using a short RNA guide (gRNA) to direct any desired region in the genome, it provides a powerful tool to enhance the ability of engineered T-cells to fight cancer cells and decrease the immunogenicity. PD-1 disrupted CART-cells in solid tumors using CRISPR/Cas9 system showed preclinical tumor-killing efficacy (Hu et al. 2019) (H. Zhu et al. 2020). In AML, CRISPR/Cas9 system was used recently to target the IL-1RAP protein providing an effective strategy to improve AML therapy (Ho et al. 2021). In addition, this system is actually in use to enhance CART-cells efficacy by deletion of immunosuppressive factors (Giuffrida et al. 2021).

The electroporation of CAR mRNA in NK and T-cells was described as efficient enough, with low electroporation-related apoptosis. It demonstrated successful clinical anti-tumor effects in solid tumors (Beatty et al. 2014). Despite the short lifetime and transiency of its expression, CAR mRNA degradation over time allows a complete removal of the CAR from the patient without the need for suicide genes (Angel and Yanik 2010). This system is being investigated in early clinical trials. However, some limits are also described using this system such as the long ex-vivo culture time to generate therapeutic doses of GMTs and the severe cell damage following the electroporation.

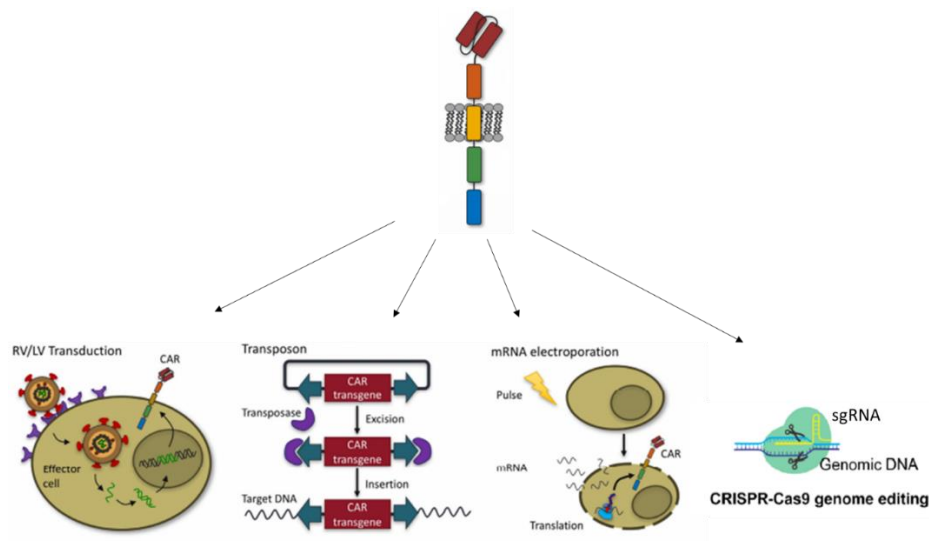


Figure 14: Different mechanisms of engineering immune effector cells with the CAR transgene (Oldham and Medin 2017).

## Lentivirus

Lentivirus is a member of Retroviridae family like  $\gamma$ -retroviral. The most commonly used lentiviral vectors are based on the human immunodeficiency virus (HIV) (Figure 15). The nucleic acid of these viruses integrates into transcribed rather than regulatory genes of the host cell, thus allowing prolonged expression, of longer duration compared to that obtained using retroviruses. Lentiviruses are capable of infecting resting cells with great efficiency. Indeed, the presence of cell divisions facilitates the penetration of nucleic acid through the nuclear pores.

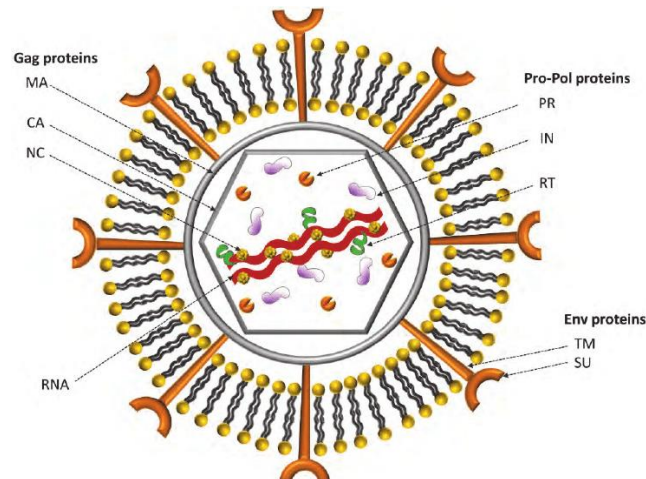


Figure 15: Structure of a lentiviral virus (Rodrigues, M., and Coroadinh 2011).

Essential genes gag, pol and env are removed from the viral backbone and they are provided in helper plasmids, for viral production. The CAR transgene is introduced in place of eliminated genes in the viral backbone. Cell lines can be then transfected with the CAR transgene vector plus the helper plasmids, in order to generate cell lines stably producing viruses containing the CAR transgene (Durand and Cimorelli 2011) (Figure 16).

- The psPAX2 plasmid: containing the sequence encoding the GAG gene (Group specific antigen) which will allow the translation of the structural polyprotein of the virus, and that encoding the POL gene (polymerase) which will code for reverse transcriptase (RT retrotranscriptase ). This plasmid also has the Regulator of Expression of Virion (REV) protein and Trans-Activator of Transcription (TAT) genes. The REV protein allows the nucleo-cytoplasmic transport of viral mRNAs that are not completely spliced (encoding the structural proteins). The TAT protein, on the other hand, acts as a transcription factor.

- The pMDG plasmid: will code for the envelope of the virus. It encodes the Vesicular Stomatitis Virus Glycoprotein (VSV-G) gene. This envelope allows the virus to bind to the Low-density lipoprotein (LDL) receptors of the cell.

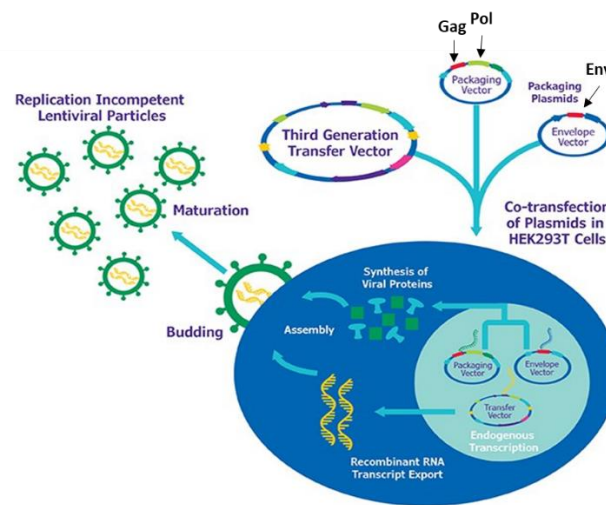


Figure 16: Production of lentiviral virus containing the CAR transgene (Durand and Cimorelli 2011).

#### IV. Clinical trials using CART-cells

##### A. Strategy of CART-cells production for patients' treatment

The production process of CART-cells begins with a leukopheresis. This procedure consist in separate TLs from the PB of the patient. Patient's TLs are then isolated and activated using magnetic beads coated with an Ab specific for CD3/CD28 with high doses of IL-2 cytokine for increasing TLs proliferation. In addition, more methods have been developed to isolate TLs like using CD4/CD8 magnetic beads (Turtle et al. 2016) or to induce TLs differentiation in certain phenotypes to reduce toxicity (Ramos et al. 2016). Following TLs activation and proliferation, they are transduced, in most clinical trials by lentiviral vectors, in order to express the CAR on their surface. GMTLs (CART-cells) are expanded in-vitro in an adequate medium containing cytokines (Harrison et al. 2019). Quality controls are done to validate the process for the efficiency and the safety of CART therapy. After having the needed volume that can be applied to the patient, autologous CART-cells are then reinjected to the patient as therapeutic agent after 48-96 hours from completing the lymphodepletion chemotherapy (Turtle et al. 2012) (Figure 17). The entire CART-cells process administration lasts about 3 weeks.

Patients receive CART-cells through intravenous infusion as standard mechanism in 1 to 2 weeks. However, the type of the cancer determine the type of injection. Thus, CART-cells can be injected through intra-tumor (You et al. 2016), intracranial (Brown et al. 2015), intraperitoneal (Koneru et al. 2015), or in the hepatic artery (Katz et al. 2015).

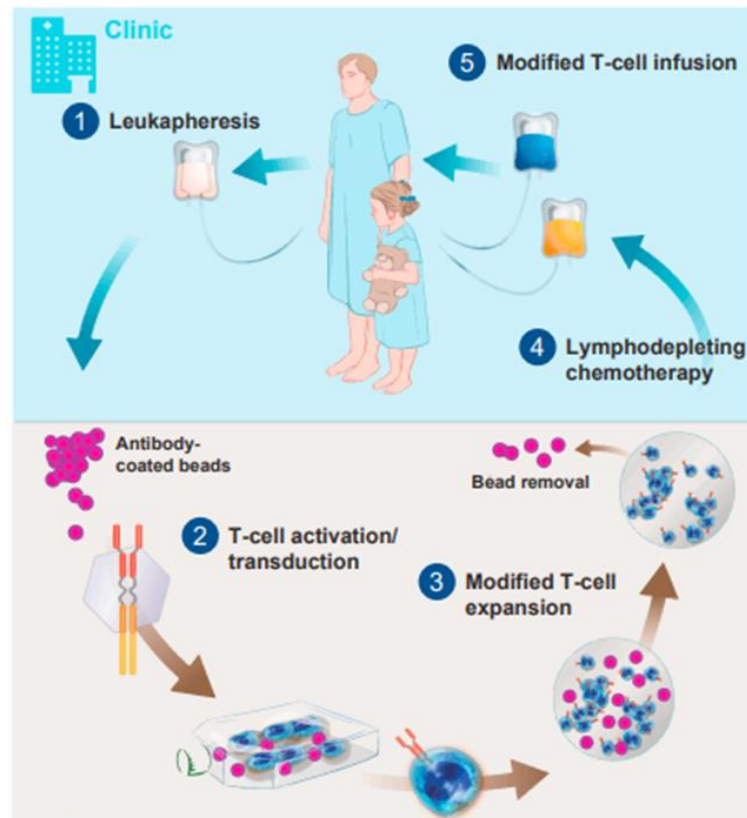


Figure 17: Strategy of CART-cells production for clinical use (Tyagarajan, Spencer, and Smith 2020).

Patient conditioning based on cyclophosphamide (CY) and fludarabine or bendamustine, is needed for obtaining better responses than using CART-cells without prior conditioning, for example treatment of non-Hodgkin's lymphoma (NHL) with second generation anti-CD19 CART-cells (Davila and Sadelain 2016).

The excessive need of CART-cells in quantities suitable for clinical applications, led to develop a closed automated Good Manufacturing Process (GMP) grade system allowing reproducible and rapid production of CART-cells. An example of this is the "Octane Cocoon™" cell culture and tissue engineering system (Octane), which is a patient-scale cell therapy platform as a central core of a series of cell and tissue therapy production systems with the possibility of running different manufacturing processes in parallel. Another example is the CliniMACS Prodigy® (Figure 18) (Miltenyi Biotec). In fact,



automated CliniMACS Prodigy was used for many CART-cells productions allowing activation, high transduction efficiencies, amplification and harvesting of CART-cells (Köhl et al. 2018). It have been used for production of CD19 CART-cells (Mock et al. 2016), NKG2D CAR memory T-cells (Fernández et al. 2019), CD19 and dual-targeted CD20/CD19 CART-cells (F. Zhu et al. 2018), CD20 CART-cells (Aleksandrova et al. 2019).



*Figure 18: A close automated manufacturing system for CART-cells production in GMP-like grade (Miltenyi Biotec).*

#### B. Clinical trials of CART-cells in hematological malignancies

Patients with hematological malignancies are faced with the possibility of disease relapse after the implementation of conventional chemo-immunotherapy. Therapy using autologous CART-cells remains one of the most advanced and promising form of ADCT for treating hematological diseases including ALL, chronic lymphocytic leukaemia (CLL), diffuse large B-cell lymphoma (DLBCL), NHL and MM (June et al. 2018) (Rohaani, Wilgenhof, and Haanen 2019) (Table 3).

The first clinical application of CART-cells was the administration of CD19 CART-cells to patients with CLL in 2011 (Porter et al. 2011) and to children and young adults with relapsed and refractory ALL in 2012 (Grupp et al. 2013). A 7-year-old Emily Whitehead achieved a real and successful progress in



CART-cells therapy (Rosenbaum 2017). Novel therapies are being investigated in CLL treatment with CD20 CART-cells (Hosing et al. 2013). CD20 CART-cells have demonstrated potential antitumor activity for treatment of indolent NHL and mantle cell lymphoma (MCL) (Till et al. 2008).

Thereby, two products of CART-cells were licensed by the US Food and Drug Administration Agency (FDA) in 2017 and the European Medicines Agency (EMA) in 2018. Tisagenlecleucel (Novartis' Kymriah) (Y. Liu et al. 2017) and Axicabtagene ciloleuce (Kite/Gilead's Yescarta) (Viardot et al. 2019). They are autologous CD19 CART-cells used respectively for patients with B-cell ALL who do not respond to treatment or have relapsed (Jacoby et al. 2018) based on the phase II clinical results (ELIANA study), and for relapsed or refractory (R/R) DLBCL based on the phase I/II clinical results (ZUMA study). Other than CD19, CD20 and CD22 antigens highly expressed by DBLCL cells could become potential targets for CART therapy.

CART-cells targeting the B-cell maturation antigen (BCMA) have been used in multiple clinical trials for MM treatment (ABECMA, idecabtagene vicleucel (ide-cel) (H. Huang, Wu, and Hu 2020). Different other therapeutic targets for CART-cells were used in MM, CD138 CART-cells showed potent results in phase I/II clinical trials (Guo et al. 2016). Several clinical trials also have focused on using CD19 CART-cells for MM treatment (Garfall et al. 2018), for R/R MCL (TECARTUS, brexucabtagene autoleucel) and for R/R large B-cell lymphoma (LBCL) (BREYANZI, lisocabtagene maraleucel).

The promoting progress in CAR technology allowed its use in other hematological malignancies and solid tumors. Even the use of dual CART-cells, CAR-NK cells or CAR NKT cells.

The American Society of Hematology (ASH) meeting in 2019 presented new therapeutic agents for treatment of NHL using lisocabtagene maraleucel (liso-cel; JCAR017) in R/R LBCL and ZUMA-2 (Gilead/Kite's KTE-X19) in R/R MCL. Liso-cel differs from Yescarta and Kymriah, as it consists of a specified ratio of CD4+/CD8+ T-cells.

The number of clinical trials using CAR-NK cells is limited. Recently, the administration of CD19 CAR-NK cells to relapsed or refractory patients with NHL and CLL showed a response to treatment without the development of major toxic effects (E. Liu et al. 2020). However, most of CAR-NK clinical trials are still in early planning or recruiting. Similarly, clinical trials using autologous CAR NKT cells for the treatment of R/R metastatic neuroblastoma are ongoing with promising results.

Table 3: Selected clinical trials using CART-cells for hematological malignancies (Skorka et al. 2020).

Study	Phase	Condition or disease included in data analysis	CART generation	Costimulatory domain	Target molecule	Outcome (n/n infused)
NCT01593696	I	ALL	2nd	CD28	CD19	CR: 70% (14/20)
NCT01044069	I	ALL	2nd	CD28	CD19	CR: 83% (44/53)
NCT02028455 (PLAT-02)	I/II	ALL	2nd	4-1BB	CD19	CR: 93% (40/43)
NCT02772198	I/II	ALL	2nd	CD28	CD19	CR: 90% (18/20)
NCT02435849 (ELIANA)	II	ALL	2nd	4-1BB	CD19	CR: 81% (61/75); FDA approval for ALL
NCT01029366	I	CLL	2nd	4-1BB	CD19	OR: 57% (8/14; 4 CR and 4 PR)
NCT01416974	I	CLL	2nd	CD28	CD19	OR: 38% (3/8; 2 CR and 1 PR)
NCT01865617	I/II	CLL	2nd	4-1BB	CD19	OR: 74% (14/19; 4 CR and 10 PR)
NCT03331198 (TRANSCEND CLL 004)	I/II	CLL	2nd	4-1BB	CD19	OR: 87% (13/15; 7 CR and 8 PR)
NCT00924326	I	DLBCL	2nd	CD28	CD19	OR: 68% (13/19; 9 CR and 4 PR)
NCT02631044 (TRANSCEND NHL 001)	I	DLBCL	2nd	4-1BB	CD19	OR: 68% (89/131; 64 CR and 25 PR)
NCT02348216 (ZUMA-1)	I/II	DLBCL, PMBCL, tFL	2nd	CD28	CD19	OR: 83% (84/101; 59 CR and 25 PR); FDA approval for DLBCL
NCT02445248 (JULIET)	II	DLBCL	2nd	4-1BB	CD19	OR: 52% (48/93; 37 CR and 11 PR); FDA approval for DLBCL
NCT02215967	I	MM	2nd	CD28	BCMA	OR: 81% (13/16; 2 sCR, 8 VGPR and 3 PR)
NCT02658929	I	MM	2nd	4-1BB	BCMA	OR: 85% (28/33; 15 CR and 13 PR)
NCT01886976	I/II	MM	2nd	4-1BB	CD138	OR: 80% (4/5; 3 SD)

## V. Limitations and challenges of CART-cell therapy

Therapy using CART-cells has produced impressive anti-tumor responses to treat malignancies or solid tumors, but some limitations exist and limit the use of this therapy.

The first factor is the high cost and the availability of the treatment. So far, CART-cells are not universal and it is not possible to be produced in massive quantities like other existing immunotherapy-based approaches. Moreover, generating autologous CART-cells require advanced and expansive technologies like viral vectors or gene editing tools in addition to the overall equipment to prevent infections in patients receiving CART-cells (Hettle et al. 2017). Therefore, the development of effective and persistent universal “off-the-shelf” allogenic CART-cells products could reduce manufacturing costs. This strategy consists on inactivate the endogenous TCR or the genes encoding the associated HLA molecules of CART-cells, in order to prevent the potential allogenic reactivity, by the TALEN or CRIPR/Cas9 technologies (Poirot et al. 2015).

The risk of developing a resistance to CART therapy is also an important limiting factor. In fact, the insufficiency of expansion or limited persistence of CART-cells, the loss or the down-regulation of tumor-antigen in relapsed disease, all these factors can induce resistance to the treatment (N. N. Shah and Fry 2019). Accordingly, administering combinations of CARs against multiple targets will overcome relapse owing to the antigen-negative tumor cells (Orlando et al. 2018) or combining CART therapy with blocking of inhibitory Abs such as anti-PD-1 mAb (Chong et al. 2017).

The healthy status of patients undergoing CART-cells therapy is also required. Before T-cells administration, the lymphodepletion chemotherapy can expose vulnerable patients to infections (S. D. Smith et al. 2019).

## **VI. Toxicities of CART therapy**

Although CART-cells therapy has revolutionized the treatment of cancers, it is still associated with several toxicities and safety issues. CART-cells application presents frequent toxic side effects with significant risks (Table 4) (Miliotou and Papadopoulou 2018). It will be simpler to classify these toxicities into five categories: on-target on-tumor, on-target off-tumor, off-target, neurotoxicity, and other toxicities (Figure 19) (Sun et al. 2018).

*On-Target On-Tumor Toxicity (a):* The most common toxicity specific to the administration of CART-cells is the on-target on tumor toxicity. It is characterized by an excessive release of cytokines or tumor cell necrosis. The excessive cytokine release may result in cytokine release syndrome (CRS), which can vary from mild moderate, to severe potentially fatal forms. CRS is triggered often during the first week after CART-cells infusion, as a first rapid immune reaction of activation and proliferation of injected CART-cells leading to a massive secretion of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, IL-10 and IFN- $\gamma$  (Hay et al. 2017). In addition, the rapid devastation of a large quantity of tumor cells can develop a tumor lysis syndrome (TLS) (Howard, Jones, and Pui 2011).

In rare cases, CRS can progress into other immune-related toxicities such as prolonged cytopenias, macrophage activation syndrome (MAS) (A. R. Shah et al. 2016), Cytokine Release Encephalopathy Syndrome (CRES) (Sattva S. Neelapu et al. 2017).

*On-Target Off-Tumor Toxicity (b):* The most detrimental toxicity is when injected CART-cells attack normal tissues that have the shared expression of the targeted tumor-antigen. This on-target off-tumor toxicity of CART-cells on nonpathogenic tissues expressing low levels of the same antigen expressed on tumor cells may be fatal in many cases (C. H. Lamers et al. 2013) (A. Hombach, Hombach,

and Abken 2010). Thus, the selection of target antigen, which is strictly specific to the tumor or to generate CART-cells with lower affinity for the targeted antigen and less recognition of normal cells expressing low levels of this antigen (Song et al. 2015), in addition, to infuse lower doses of CART-cells to patients could potentially overcome this toxicity (Ahmed et al. 2015).

*Off-Target Toxicity (c):* The off-target toxicity occurs when infused CART-cells attacks another antigen than the one they target or activate themselves independently from the antigen recognition due sometimes to their artificial synthetic construct. For example, anti-HER2/neu CART-cells (trastuzumab) carrying the IgG1-derived CH2CH3 domain in the spacer extracellular domain may interact with the Fc receptor expressed on innate immune cells such as macrophages and NK cells, and resulting of an antigen-independent activation (A. Hombach, Hombach, and Abken 2010). Likely, to date, this toxicity cross-reactive antigen has not been evident in CART-cells clinical trials. However, it was reported a fatal off target toxicity with tgTCR T-cells (Kötter, Andresen, and Krüger 2014).

*Neurotoxicity (d):* In most cases, CRS is associated with immune effector cell-associated neurotoxicity syndrome (ICANS) in patients receiving CD19 CART-cells therapy. Neurotoxicity can appear in the days to weeks post transfusion in patients with different types of cancers (Rubin et al. 2019). In most patients with neurotoxicity, CART-cells have been found in cerebrospinal fluid (CSF) without reporting a clear expression of CD19 in the affected brain areas. Suggestion that this infiltration may be caused by hyperthermia and IL-6, released during CRS (Prudent and Breitbart 2017).

*Other toxicities (e-f):* Some other toxicities are also triggered by CART-cells infusion, such as (1) Immunosuppression: caused by the lymphodepleting and nonmyeloablative regimen before the infusion. It comes along with anemia, coagulopathy, and neutropenic sepsis (Dudley et al. 2008). (2) Immunogenicity: due to immune reaction against the ScFv, which derives from a mouse mAb, leading to sever anaphylaxis (C. H. J. Lamers et al. 2011) (Gorovits and Koren 2019). (3) Genotoxicity: may results from the integrating viral vectors used for the engineered T-cells which may pose a potential risk of oncogenic insertional mutagenesis including modifications of normal gene expression and function (Hacein-Bey-Abina et al. 2008).

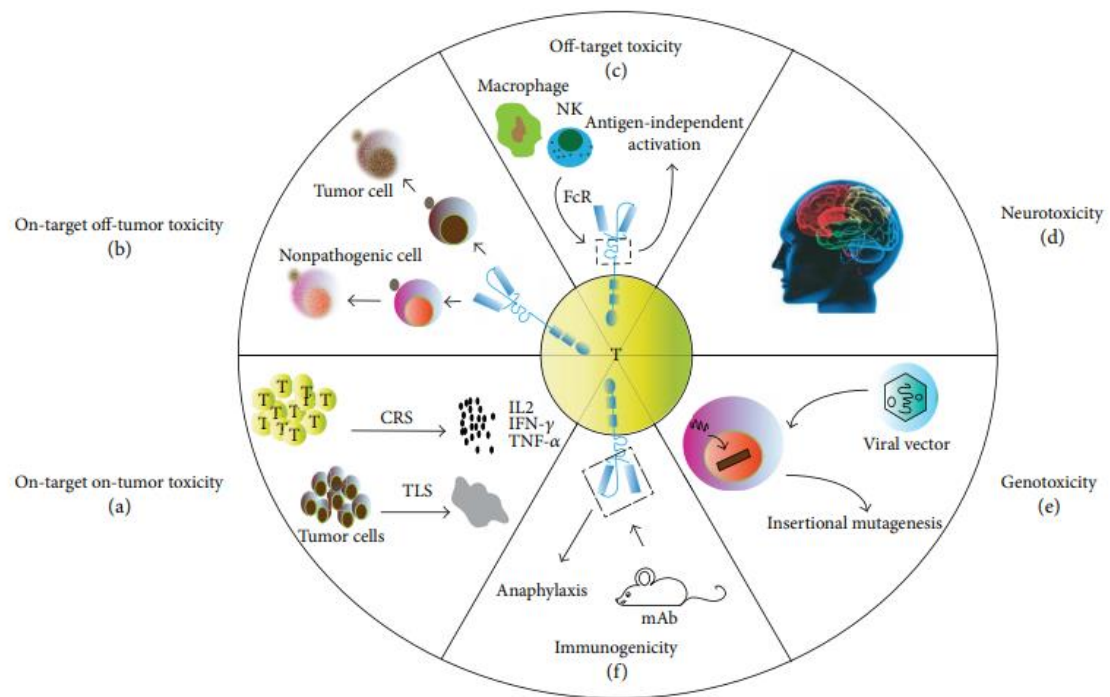


Figure 19: CART-cells related toxicities (Sun et al. 2018).

Table 4: Toxicities associated with CART-cells treatment in hematological and solid tumors (Miliotou and Papadopoulou 2018).

Type of Toxicity	Caused by:	Observed During:
"On-target on-tumor"	<ul style="list-style-type: none"> <li>Rapid destruction of a large tumor mass</li> <li>Massive release of tumor cell components into the circulation</li> </ul>	<ul style="list-style-type: none"> <li>Leukemia treatment</li> <li>Treatment of solid cancer (low toxicity)</li> </ul>
"On-target off-tumor"	<ul style="list-style-type: none"> <li>Engagement of cognate antigen on healthy tissues</li> </ul>	<ul style="list-style-type: none"> <li>Treatment of solid tumors</li> <li>Treatment of leukemia/lymphoma (low toxicity)</li> </ul>
"Off-target off-tumor"	<ul style="list-style-type: none"> <li>Inflammatory reaction beyond the targeted tumor tissue</li> </ul>	<ul style="list-style-type: none"> <li>Independently of the CAR specificity and the malignant disease</li> </ul>
Cytokine Release Syndrome	<ul style="list-style-type: none"> <li>Release of supra-physiological serum levels of proinflammatory cytokines by CAR T-cells (IFN-<math>\gamma</math>, IL-6, TNF-<math>\alpha</math>)</li> </ul>	<ul style="list-style-type: none"> <li>Treatment of hematologic malignancies</li> </ul>
Neurotoxicity	<ul style="list-style-type: none"> <li>Diffuse encephalopathy, mainly due to systemic cytokines' trafficking to the CSF</li> </ul>	<ul style="list-style-type: none"> <li>Independently of the CAR specificity and the malignant disease</li> </ul>

#### Approaches to manage CRS and ICANS-related toxicities in clinical trials:

Nowadays, there are different ways in clinical usage to manage CRS and ICANS-related toxicities. The aim of the overcoming strategy is to inhibit CART-cells persistence and suppress T-cell function or induce apoptosis.

Given that IL-6 is a major cytokine contributing in CRS development, IL-6 receptor (IL-6R) is targeted by Tocilizumab (an FDA approved mAb against IL-6R) (Khadka et al. 2019) and Siltuximab (anti-IL-6 chimeric mAb) used especially in tocilizumab and steroid refractory cases.

High doses of corticosteroid drugs such as dexamethasone are used for patients who do not respond to tocilizumab as a second line treatment. But it is the first line treatment for ICANS because of its capability to cross the blood-brain barrier (S. S. Neelapu et al. 2017).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by CART-cells and stimulates myeloid cells suggesting its implication in CRS development. Targeting the GM-CSF with lenzilumab or Abs or gene editing systems have a potential to reduce severe adverse effects of CRS and ICANS while preserving an antitumor activity of CART-cells (Sachdeva et al. 2019).

Blocking the IL-1R with Anakinra (an IL-1 antagonist) showed in recent clinical study promising results regarding the use of anakinra to mitigate CART-associated toxicities in large BCL (Strati et al. 2020).

In addition, some tyrosine kinase inhibitors targeting signaling pathways (such as JAK Inhibitors, Ibrutinib, Dasatinib) are also in use to alleviate CART-cells-related toxicities in different malignancies. Noted that clinical trials have begun testing the usage of Defibrotide in preventing CART-associated ICANS with no published data yet (Zahid, Siegler, and Kenderian 2020).

The “fourth-generation” CART-cells with inducible release of IL-12 activate innate immune cells to target and eliminate tumor cells not recognized by CART-cells (Chmielewski and Abken 2015). Using less differentiated T-cells (Y. Xu et al. 2014) or NKT-cells (Heczey et al. 2014) for CART-cells or CAR NKT-cells therapy showed more powerful antitumor effect in preclinical models with low toxicities.

## **VII. Strategies to overcome CART therapy related toxicities**

It remains very important to find a balance between tumor elimination and unexpected and undesirable toxicities. Accordingly, innovative strategies have been performed to offer imperious opportunities.

### **A. Enhance the safety of CART therapy**

As well the traditional cancer drugs (such as small molecules and antibodies) wield as passive targeting, T-cell-based therapeutic agents can actively home to disease sites, and trigger a strong immune response along with their capability of self-renewal, amplification and differentiation into different

effectors subsets. Therefore, feedback control systems and safety modules have been investigated to modulate the activity of CARs.

*Suicide switch systems:* Considering the CAR persistence-related toxicities, a mechanism for rapid ablation through suicide switch was included in the CAR construct (Figure 20) (Sun et al. 2018):

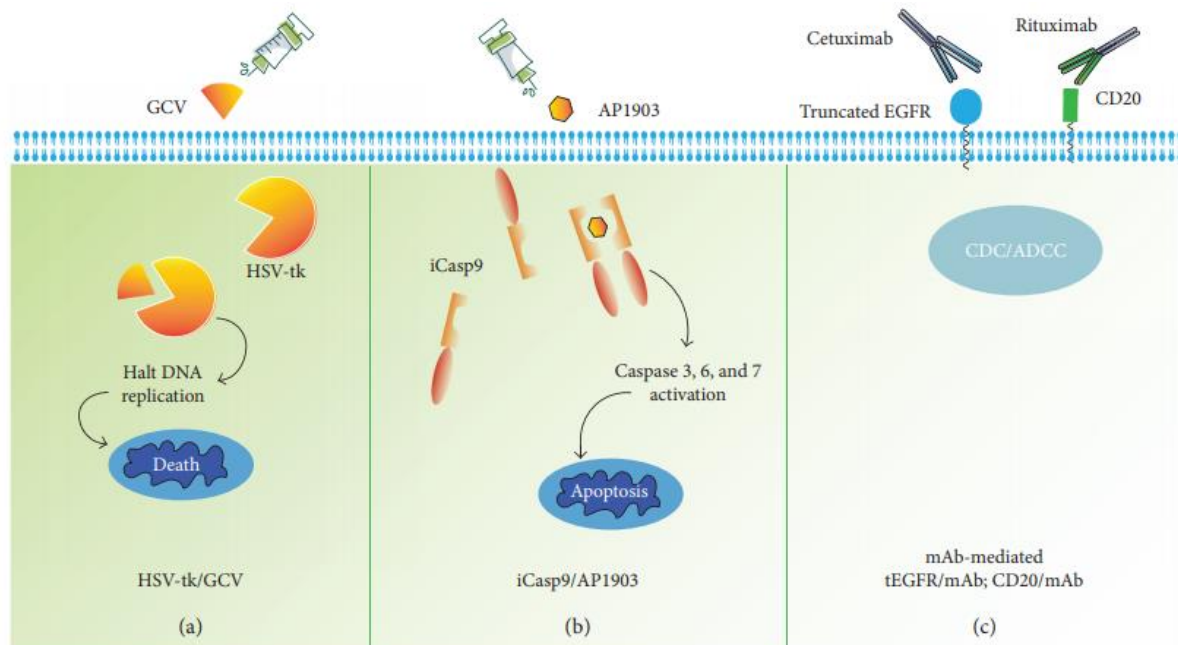


Figure 20: overcoming toxicities rest on the suicide gene co-expression in CART-cells. GCV: ganciclovir, HSV-tk: herpes simplex virus thymidine kinase, iCasp9: inducible caspase 9, EGFR: epidermal growth factor receptor, tEGFR: truncated EGFR, CDC: complement-dependent cytotoxicity, ADCC: antibody-dependent cell-mediated cytotoxicity (Sun et al. 2018).

*iCasp9/AP1903 (a):* Through the addition of a small-molecule drug that dimerizes and activates the inducible caspase 9 (iCasp9) expressed by CART-cells, it induces CART-cells elimination by programmed cell death (apoptosis) (Diaconu et al. 2017). The efficacy and safety of this system have been first demonstrated in allogeneic HSCT with reversed graft-versus-host disease (GVHD) leading to the elimination of 90% of T-cells expressing iCasp9 within 30 minutes (Di Stasi et al. 2011) (Zhou et al. 2015).

*HSV-tk/GCV (b):* Another suicide gene, the herpes simplex virus thymidine kinase (HSV-TK), expressed on CART-cells converts the prodrug Ganciclovir (GCV) into a cytotoxic molecule resulting in T-cells apoptosis (Jones et al. 2014). For example, this system was used for control of allogeneic graft-versus-leukemia (GvL) in patients with relapsed or developed Epstein-Barr virus-induced lymphoma after T-cell-depleted bone marrow transplantation (BMT) (Bonini et al. 1997). Nevertheless, this system is

limited by the immunogenicity engendered by viral enzymes and the long time (several days) to achieve full elimination (Chalmers et al. 2001).

*mAb-mediated tEGFR/mAb (c)*: Alternative safety switches built on targeting a specific surface antigen expressed by CART-cells such as CD20 and truncated epidermal growth factor receptor (EGFR). These epitope tags are recognized by FDA-approved mAb such as cetuximab (Paszkievicz et al. 2016) and rituximab (Tasian et al. 2017a) respectively, allowing CART-cells apoptosis through complement-dependent cytotoxicity (CDC) and ADCC.

## B. Optimize the efficiency of CART therapy

Suicide switches are effective at stopping the toxicity of CART-cells, however they induce an irreversible ending of the therapeutic treatment. Otherwise, non-cytotoxic reversible systems may be practical for controlling unfavorable toxicity without limiting the therapeutic effects.

*1- Targeting Two Tumor-Associated Antigens (TAA)*: Consisting on CART-cells whose activation can be guide through combinatorial antigen-targeting activation with separated signals, such as Dual CAR, Tandem-CAR (Tan-CAR), inhibitory CAR (iCAR) and synthetic Notch (synNotch) (Figure 21) (Sun et al. 2018).



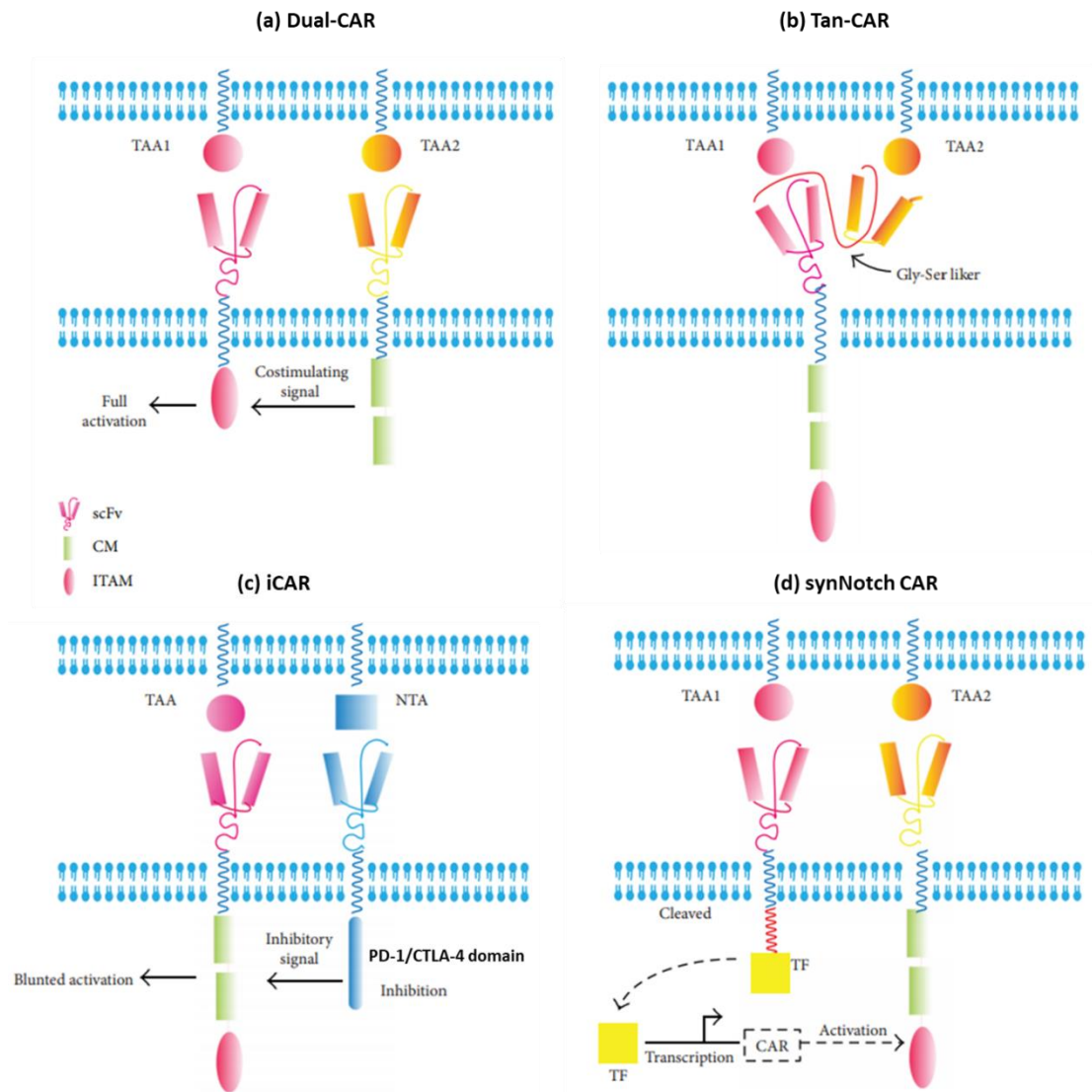


Figure 21: Targeted activation strategies to overcome toxicities. ScFV: single-chain variable fragment of antibody, CM: costimulatory molecule, ITAM: immune-receptor tyrosine-based activation motif, TAA: tumor-associated antigen, NTA: normal tissue antigen, CAR: chimeric antigen receptor, iCAR: inhibitory CAR, TanCAR: tandem CAR, synNotch CAR: synthetic notch CAR, TF: transcription factor (Sun et al. 2018).

**Dual CAR (a):** A dual CAR is a bispecific CAR generated by modifying T-cells by “splitting” the activation signal and the endo-costimulatory signal in two different CAR constructs to express two CARs targeting different TAA to guarantee that they will be activated only against tumor cells (Kloss et al. 2013).

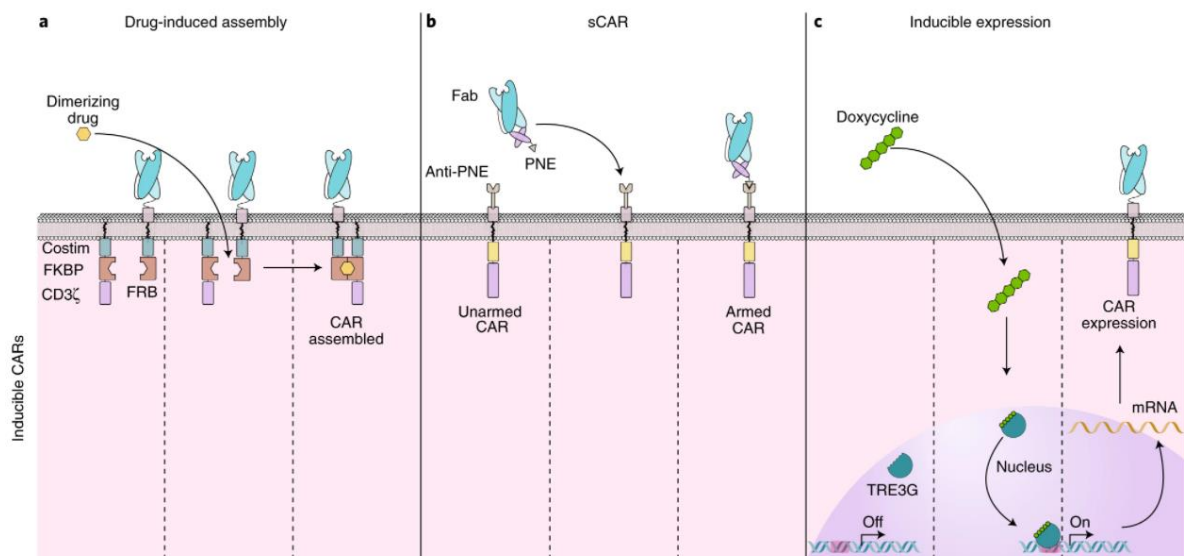
**Tan-CAR (b):** Another bispecific CAR, the Tandem CAR, which unlike the dual CAR is a single CAR specific for two antigens due to the expression of two tandemly arranged ScFVs combined to the same signaling domain (Grada et al. 2013).

**inhibitory CARs (iCARs) (c):** The iCAR construct include an ScFv specific to an antigen expressed on normal tissues. This ScFv is coupled to the intracellular signaling domain of an inhibitory receptor such

as a checkpoint molecule (PD-1 or CTLA-4) (Ok and Young 2017). Consequently, when iCAR binds its antigen found on normal cells, it results of the inhibition of CART-cell function. This self-regulation switch allows to reduce undesirable side effects and to target exclusively tumor cells (Fedorov, Themeli, and Sadelain 2013).

*synthetic Notch (synNotch) (d)*: The synNotch is a novel dual-receptor AND-gate CAR. It consists of an ScFv targeting a specific antigen, a Notch core, and an artificial transcription factor (TF). Upon TAA binding by the notch receptor, an orthogonal TF is cleaved from the cytoplasmic domain of this receptor and allow the transcription of the CAR receptor that will bind to a second antigen. Hence, only in the presence of the two antigens on tumor cells that the synNotch is activated and not affecting normal cells which express one of these antigens (Roybal et al. 2016).

**2- Switchable CAR (sCAR)**: Promising strategies have been evolved in order to enhance the specificity of CART-cells and reduce their toxicities. The activation of CART-cells via an “on-off” switch system included in CAR design enable the precise control and regulation of the location, duration, and intensity of therapeutic activities (Figure 22) (Labanieh, Majzner, and Mackall 2018).



**Figure 22: Switchable CAR strategies to overcome toxicities.** FKBP: FK506 binding protein, FRB: FKBP12-rapamycin binding domain, TRE3G: tet response element 3G (Labanieh, Majzner, and Mackall 2018).

**On-Switch CAR (a)**: The on-switch CAR consists in separation the extracellular antigen-binding (ScFv) domain and the intracellular signaling domain of the conventional CAR into two distinct parts and only the adding of a heterodimerized molecule can reassemble the CAR receptor and activate it (Wu et al. 2015). Another way for a split-CAR strategy is the “transient” CART-cells that are directly dimerized at

the hinge domain with the addition of a small molecule (Juillerat et al. 2016). These two procedures transform conventional CART-cells into smart CART-cells more safer, more precise and more efficient (E. Zhang and Xu 2017).

*Switch CAR using recombinant Abs (b):* Furthermore, another type of switchable CAR was created using a system of two antibody fragments (Fab) as bifunctional molecules. In this system, a tumor antigen-specific Fab molecule engrafted with the neoepitope peptide (PNE) or FITC (Fluorescein isothiocyanate) that binds to the CAR receptor containing the antigen-specific scFv anti-PNE or anti-FITC (M. S. Kim et al. 2015).

*Inducible CAR (c):* The CAR expression can be induced by the addition of a small molecule such as doxycycline or tetracycline. This molecule induces a conformational change in tet response element 3G (TRE3G) that enables the transcription of CAR mRNA and the CAR expression (Sakemura et al. 2016).

## **Chapter 2: 2. CART-cells immunotherapy in AML**

### **I. Therapeutic approaches in AML using CART-cells and clinical trials**

AML cancer is globally a rare disease affecting more commonly older adults and among men compared to women. The rate of new cases of AML was 4.3 per 100,000 men and women per year based on 2014–2018 cases, age-adjusted, with a 5-year related survival around 29.5%.

#### **A. The ongoing use of CART-cells therapy in AML**

In AML application, CART-cell therapy is at its dawn, with a limited number of reported clinical trials. In 2013, Ritchie et al. published the first reported phase 1 clinical trial using CART-cells in AML. They used a second generation CD28- $\zeta$  CAR directed against the Lewis Y (LeY) antigen (NCT01716364). Although only limited efficacy was observed, this study was very important, as it has given a clear demonstration of CART-cell biological activity in AML patients in the absence of observed hematopoietic toxicities (Ritchie et al. 2013). Nowadays, there are more than twenty CART-cell clinical trials applying patients with AML registered with the [clinicaltrials.gov](https://clinicaltrials.gov) (Table 5), substantially targeting CD33, CD123, C-type Lectine-Like-1 (CLL-1/CLEC12A), NKG2DL, FLT3 (CD135), CD38 or CD44v6 (Fiorenza and Turtle 2021). Whereas more AML antigens are evaluated in preclinical for being targeted

with CART-cells including: c-kit (CD117), B7-H3 (CD276), folate receptor (FR)- $\beta$ , IL-1RAP, CD13 and WT1 (Przespolewski and Griffiths 2020) (Shang and Zhou 2019c).

*Table 5: CART-cell therapy applications in AML (Mardiana and Gill 2020).*

Disease	Interventions	Identifier ID	Phase
AML	CD123/CLL1 CAR T cells	<a href="#">NCT03631576</a>	II/III
	CLL-1, CD33 and/or CD123 CAR T cells	<a href="#">NCT04010877</a>	I/II
	CD123 CAR T cells	<a href="#">NCT03796390</a>	I
	CD123 CAR T cells	<a href="#">NCT03585517</a>	I
	Muc1/CLL1/CD33/CD38/CD56/CD123 CAR T cells	<a href="#">NCT03222674</a>	I/II
	CD38/CD33/CD56/CD123/CD117 /CD133/CD34/Muc1 CAR T cells	<a href="#">NCT03473457</a>	N/A
	CD123 CAR T cells expressing EGFRt		I
	CD44v6 CAR T cells	<a href="#">NCT04097301</a>	I/II
	CD33 CAR T cells	<a href="#">NCT03971799</a>	I/II
	Universal CD123 CAR T cells	<a href="#">NCT03190278</a>	I
	CD123 CAR T cells	<a href="#">NCT04014881</a>	I
	CD123 CAR T cells	<a href="#">NCT03556982</a>	I/II
	CD123 CAR T cells expressing EGFRt	<a href="#">NCT02159495</a>	I
	CD123 CAR T cells	<a href="#">NCT03766126</a>	I
AML/MDS	CD33/CD38/CD56/CD117/CD123/CD34 /Muc1 CAR T cells + Eps8 or WT1 peptide specific dendritic cell	<a href="#">NCT03291444</a>	I
	NKG2D CAR T cells	<a href="#">NCT03018405</a>	I/II
AML/MDS/MPN	CD123-CD33 cCAR T cells	<a href="#">NCT04156256</a>	I
	CLL1-CD33 cCAR T cells	<a href="#">NCT03795779</a>	I

CD33 and CD123 are interesting target antigens with many CAR constructions improvement over time. CD33 and CD123 are ubiquitously expressed on AML blasts, nonetheless, they also are expressed on healthy HSPCs (Ehninger et al. 2014). In preclinical studies, CART-cells targeted CD33 (Dutour et al. 2012) (W. Zheng et al. 2018) and CD123 (Mardiros et al. 2013) (Arcangeli et al. 2017) (Tasian et al. 2017b).

CLL-1 is considering as an attractive target for CART-cells due to its high expression in AML and its absence in healthy HSPCs, apart from its expression on monocytes and on some early hematopoietic cells. Additionally, CLL-1 rare expression is reported on non-hematological cells (Ma et al. 2019). CLL-

1 CART-cells generated by Zhou, is a CAR of 3<sup>rd</sup> generation with two co-stimulatory domains CD28 and 4-1BB coupled to the CD3- $\zeta$  chain. CLL-1 CART-cells had efficient anti-tumor activity against AML cells, with no cytotoxicity toward healthy HSCs in preclinical studies (Jinghua Wang et al. 2018).

A phase 1 clinical trial of autologous second generation CART-cells targeting CD33 (CD33 CART-cells) reported a partial response in a 41-year-old male with relapsed and refractory AML (NCT01864902). This patient showed a remarkable transient reduction of AML blasts in the bone marrow until he relapsed with a disease progression that occurred at 9 weeks after CART-cells treatment. A significant CRS was observed (Q. Wang et al. 2015). In order to reduce CD33 toxicity on healthy HSCs, the genetic inactivation of CD33 gene in these cells prior to transplantation allowed to avoid on-tumor off-target toxicities (M. Y. Kim et al. 2018).

Recently completed a phase 1 clinical trial of CD123 CART-cells produced via mRNA electroporation (NCT02623582) with the intention of preventing long-term persistence of CART-cells thus avoiding the risk of severe toxicities (Cummins et al. 2017b). While no measurable anti-tumor responses were reported in R/R AML patients, CRS demonstrated CART-cells bioactivity. A transient detection of CART-cells was noted with no adverse effects regarding healthy tissues expressing CD123. This favorable safety profile encouraged the development of a lentiviral CD123 CART-cells clinical trial. A phase 1 trial (NCT02159495, NCT03766126) of R/R AML patients, treated with second generation autologous CD123 CART-cells, experienced complete remission with no treatment-related cytopenias (Budde et al. 2017). The modification of anti-CD123 ScFv affinity with VH and VL chains of different mAbs can decrease the BM toxicity of AML mice (Arcangeli et al. 2017). Moreover, the combination of CD123 CART-cells with ASCT offer a promising strategy for treating R/R AML patients (Testa, Pelosi, and Castelli 2019).

Up to 2021, an estimate of 65 AML patients have been treated with CART-cell therapy. Only a quarter of whom have achieved complete remission. The majority of the CD33 CART-cell therapies resulted in partial responses, with no responses observed in all 31 patients treated with NKG2DL CART-cells for three studies (Fiorenza and Turtle 2021). In a phase I study (NCT03018405, NCT02203825), seven of eight R/R AML patients treated with NKG2DL CART-cells (CYAD-01), without prior preconditioning chemotherapy, showed promising responses (Sallman et al. 2018). Promising and interesting results have been reported by 3 patients who reached complete remission within one month after being infused with CLL1 CART-cells (Bu et al. 2020).

In order to overcome the lack of tumor-specific antigen resulting in AML heterogeneity and to prevent toxicity due to the common antigens between leukemic blasts and normal tissues, novel targeting strategies are being investigated using dual-targeting CART-cells. A transcriptomic and surfaceome

proteomic analysis profiled human AML cells and normal hematopoietic cells, reported unique pairings of antigens detected on AML blasts but not co-expressed on normal cells. Consequently, given that one antigen is expressed on normal hematopoietic and non-hematopoietic tissues, dual-targeting CART-cells will induce less toxicity toward normal cells (Perna et al. 2017).

In a first in human phase 1 study (NCT03795779), Liu et al. evaluated compound CAR (cCAR) T-cells targeting two AML antigens, CD33 and CLL-1. It was reported that the majority of CD33 positive AML cells are also positive for CLL-1 expression.

The dual CD33/CLL-1 CART-cell construct consists of two individually complete and functional CAR receptors on the surface of a T-cell with a CD52 safety switch. Two R/R AML patients treated with multiple lines of chemotherapy were the unique responders that have been reported from this trial so far, with a measurable residual disease (MRD)-negative remission so they were able to proceed to allo-HSCT (F. Liu et al. 2018).

In preclinical studies, a compound CD33/CD123 CART-cells demonstrate anti-leukemic activity and prolonged animal survival in AML cell line xenograft models *compared to a single antigen-redirected* CART-cells (Petrov et al. 2018). Furthermore, compound CD123/CLL-1 CART-cells may be useful in AML targeting with limiting healthy HSCs toxicities (Shang and Zhou 2019b).

Another novel preclinical targeting strategy, recently published developed a bispecific and split CAR (BissCAR) targeting CD13 and TIM3. CD13 is expressed in healthy HSCs but highly overexpressed in AML cells, while TIM3 is only expressed in AML cells. Accordingly, this BissCART-cell was capable to effectively eradicate patient-derived AML cells (He et al. 2020). Thus, BissCART therapy might be a hopeful approach for developing an effective CART-cell therapy for AML.

## **II. CART therapy limitations in AML**

### **A. Lack of an AML-specific target antigen**

The main challenge limiting the application of CART-cells in AML is lack of suitable target antigen. AML cells express various cell surface myeloid antigens mainly CD33 and CD123 that are often co-expressed on normal HSPCs and their myeloid and/or lymphoid progenitors (Cummins and Gill 2019). This fundamental biological barrier of CART-cells use remain a primary problem in AML treatment resulting in an intolerable myeloablation and major toxicities as observed with CD33 CART- and CD123 CART-

cells. Despite the high anti-tumor efficacy in preclinical studies, there was several undesired toxicities on normal cells.

An optimal AML target should be mightily expressed on the surface of the AML blasts in particularly on LSCs in most AML cases and with no or lower expression on normal hematopoietic and non-hematopoietic tissues.

The antigenic heterogeneity of AML give some potential targets for CART-cells therapy, each with advantages and disadvantages.

#### Classification of AML target antigens:

*Leukemia-specific antigens:* These antigens resulting from aberrant proteins encoded by leukemia mutations are usually expressed intracellularly (for example, NUP98 fusion proteins, mutated FLT-3 and NPM1, IDH-1) and exclusively in malignant clones so they might be ideal targets. To date, no clinical trials evaluating these leukemia-specific neoantigens.

*Lineage-restricted antigens:* These antigens are restricted to the myeloid lineage and usually expressed on the surface of myeloid cells. The majority of current clinical trials of antibodies or CART-cells in AML patients target this type of antigens, such as CD33, CD123, CLL-1/CLEC12A, IL-1RAP, CD135/FLT-3).

*Leukemia-associated antigens:* Leukemia-associated antigens are overexpressed on AML cells comparable to healthy tissues. Usually these antigens are not lineage specific which make their expression on healthy hematopoietic cells less likely. Nevertheless, they may be found on non-hematopoietic tissues, causing on-target off-tumor toxicities. For instance, WT1 and PRAME are under early-phase clinical trials evaluation in AML patients (Lichtenegger et al. 2020).

#### B. Persistence of CART-cells

Since CART-cells target highly expressed non-leukemia specific antigens including CD33 and CD123 resulting in the AML-BM failure, limiting the long-term CART-cell persistence in-vivo could offer an important opportunity to prevent toxicities. The considerable strategy of a “safety switch” system that consists on incorporating a suicide gene into T-cells allowing the elimination of these cells in-vivo has been evaluated in various preclinical and clinical studies. A suicide gene that has been employed for a long time in CART-cell treatment is the HSV-tk. Upon administration of the prodrug Ganciclovir, this suicide gene is able to transform this prodrug into a toxic compound that stop the DNA replication.

Hence, providing a selective depletion of T-cells expressing the HSV-tk gene (Bordignon et al. 1995). However, the immunogenicity of the viral enzyme associated with the HSV-tk/Ganciclovir system and the long latency to activation and effects manifestation, are considerable limits for its use, as the management of toxicity require immediate termination.

A more safe suicide system utilized is the iCasp9/AP1903 system. In this system, the intracellular domain of a pro-apoptotic protein the caspase 9 is fused to the extracellular binding domain of the FK506 drug protein resulting in the co-expression of iCasp9 in CART-cells. Following the administration of a synthetic molecule drug called AP1903, a dimerization of the fusion proteins occurs allowing a rapid ablation of CART-cells (Gargett and Brown 2014) (Figure 20 (b)). The iCasp9 suicide system was clinically evaluated in haploidentical stem cell transplantation background (Di Stasi et al. 2011). Then, it was explored by Hoyos et al. in a preclinical situation using CART-cell therapy (Hoyos et al. 2010). Thereafter, the iCasp9 suicide system has been integrated in the CAR vector construct of many clinical trials (such as *clinicaltrials.gov identifier: 02992210, 01822652*). So far, the AP1903 administration has never been needed thus its efficacy in CART-cells setting is not proved yet. Rapamycin caspase 9 (rapaCDasp9) is also a safe suicide system, based on the fusion of both FRB and FKBP12 with the catalytic domain of caspase 9, used for CD19 CART-cells elimination in preclinical studies (Stavrou et al. 2018).

Another alternative for a safety switch is to use  $\Delta$ EGFR/cetuximab or  $\Delta$ CD20/rituximab systems (Figure 20 (c)). These two systems are based on engineering CART-cells to co-express a truncated ( $\Delta$ ) well-characterized cell surface antigen against a clinically approved mAb (such as  $\Delta$ EGFR targetable by cetuximab, and  $\Delta$ CD20 targetable by rituximab). Through antibody-based elimination, these systems are able to eradicate transferred CART-cells by ADCC or CDC (H. Li and Zhao 2017).

Some approaches use non-specific drugs to eliminate infused CART-cells as well as endogenous T-cells principally anti-thymocyte globulin (ATG), or the anti-CD52 mAb (alemtuzumab) (Ali et al. 2017).

In another hand, in order to limit CART-cells persistence, some other different strategies do not require administration of exogenous antibodies such as using the mRNA electroporation for incorporation of the CAR transgene into T-cells, by which the degradation of the mRNA inherently limit CART-cells function (Cummins et al. 2017b) (Figure 23).



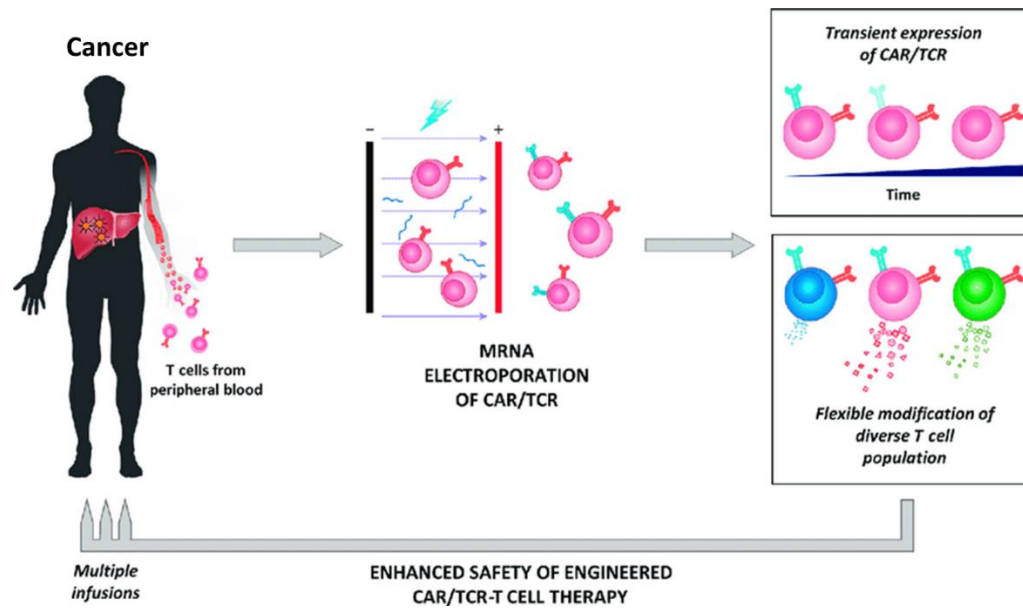


Figure 23: mRNA electroporation to engineer CAR/TCR T-cells (Bertoletti and Tan 2020).

Moreover, the presence and the type of the co-stimulatory domain in the CAR construct likely affect CART-cells persistence in-vivo. It has been demonstrated that incorporation of mainly the intracellular co-stimulatory molecules CD28 and 4-1BB in the CAR receptor enhance the persistence of CART-cells (Zhao et al. 2015). However, it has been never exhibited the effect of CD28 on CART-cells persistence in the context of AML and its impact on limiting the duration of CART-cell-induced myeloablation.

Notably, the ideal duration of CART-cells persistence is predicted to be at least 3–6 months, and it has been revealed that the durable clinical response of CD19 CART-cells in BCL seems to be correlated with CART-cells expansion and persistence, and limiting this persistence appears to be likely related with high risks of relapse (Finney et al. 2019).

### C. Challenges in CART-cells production

Following the use of CART-cells in patients with active AML, a notable problematic was encountered. Some recent clinical trials using CD123 CART-cells for the treatment of R/R AML patients, faced serious manufacturing problems limiting the treatment (Cummins et al. 2017a) (Budde et al. 2017). The possible cause of these difficulties may be related to the methods used in CART-cells production. For example, some studies used mRNA electroporation to transfer the CAR transgene, whereas some others used lentiviral transduction.

Another potential issue in the generation of CART-cells is the inability of AML patients' T-cells to express the CAR transgene due to the treatment background of these patients. In fact, almost all AML patients that are candidates for CART-cell therapy have previously undergone heavy and intense treatments that will possibly hinder obtaining T-cells of good quality for CART-cell manufacture. Thus, a careful selection of AML patients may be in early treatment will potentially address this issue (Döhner et al. 2017).

Currently, several studies are evaluating the use of allogeneic T-cells from healthy donors as an alternative source for CART-cells production, though it is likely associated with risks of GvHD and possibly a rejection of the infused CART-cells (Graham et al. 2018). The iCasp9/AP1903 suicide safety switch system can be used for treating GvHD in allografts through a potential elimination of the majority of transferred CART-cells with improvement of its activity and enhancement of the sensitivity of CART-cells to AP1903 by treating them with 5-azacytidin (Bôle-Richard et al. 2016).

#### D. Epitope masking

An important reason of relapse after treatment is the loss of the expression of the membrane target antigen. One of the causes of this loss is an epitope masking of the cell surface antigen. It was reported a relapse of a patient with BCL after 9 months of CD19 CART-cells (CTL019) infusion. This relapse was due to the persistence of CD19 negative leukemic cells that expressed the anti-CD19 CAR. In fact, during the viral transduction of autologous T-cells with the CAR vector, the CAR transgene was unintentionally introduced into a single leukemic B-cell. Consequently, the CAR receptor bound in cis to the CD19 epitope on the surface of leukemic cells. Thus, this autorecognition of the CD19 target antigen by the tumor-expressed CAR masked the recognition of CD19 by CD19 CART-cells giving out to a resistance of transduced B-cells to CTL019 (Ruella et al. 2018a).

Various strategies exists to overcome antigen escape and resistance to CART-cells immunotherapy that lead to the relapse. Notably, enhancing CART-cell production (using IL-7 and IL-15 rather than IL-2), using dual CAR or tan-CAR targeting more than one surface epitope, using a universal CAR, combining the CART-cell therapy with checkpoint inhibitor antibodies. Anti-CAR CART-cell therapy was also recently proposed to eradicate transduced leukemic B-cells (Ruella et al. 2020). However, this antidote cannot be applicable to CART-cells directed against other targets. Therefore, identifying more strategies is needed. The use of a suicide gene for a safety switch may be a universal and interesting safe approach to limit adverse events of CART-cell therapy namely accidental tumor cell transduction. Recently, the iCasp9/AP1903 suicide system safety switch was evaluated in AML in order to eliminate transduced AML cell lines and primary cells (expressing IL-1RAP) with autologous IL-1RAP CART-cells

for triggering an epitope masking (Figure 24) (Warda et al. 2021). The elimination of >85% of tumor cells IL-1RAP+/CAR+ in a 24 hours of exposure to AP1903 (Rimiducid) encourage the use of this approach in a phase I trial.

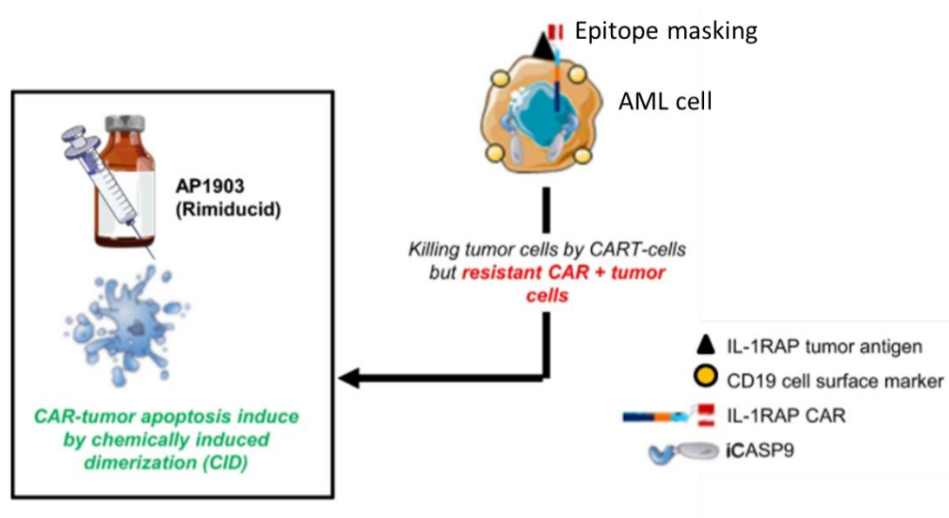


Figure 24: iCasp9 safety switch system mechanism for an epitope masking model in autologous AML tumor cells (Warda et al. 2021).

#### E. Immunosuppression worn by AML toward CART-cells therapy

While AML has long been identified being immune-responsive, featured by the GVL effects following allo-HSCT, it is also considered as an immunosuppressive or immune-evasive disease. A wide range of AML immunosuppressive mechanisms have been described based on in-vitro and preclinical studies (Figure 25) (Mardiana and Gill 2020). Identifying the immunosuppressive pathways responsible for AML evasion and relapse remains very crucial to develop appropriate efficient therapies.



can hamper the proliferation and the differentiation of T-cells. Besides, another function of IDO enzyme is to stimulate Treg conversion and increase their immunosuppressive activity. It was reported an increased level of kynurenine in AML patients associated with a reduced overall survival. Clinical trials are currently ongoing for IDO inhibitors in AML (clinicaltrials.gov identifier: 02835729) and other types of malignancies (Soliman et al. 2016). Indeed, IDO inhibition was shown to upgrade CART-cells efficacy in preclinical studies (Ninomiya et al. 2015) (Q. Huang et al. 2018).

#### *(B) Enhance immunosuppressive cells' activity*

One of the immunosuppressive manifestations of AML cells is that they can recruit and enhance the expansion and the activity of immunosuppressive immune cells such as Tregs and myeloid-derived suppressor cells (MDSCs) (Epperly, Gottschalk, and Velasquez 2020). AML cells as well as Tregs express ectonucleotidases (CD39 and CD73) enzymes that function is to breakdown the adenosine triphosphate (ATP) or nicotinamide adenine dinucleotide (NAD<sup>+</sup>) producing large number of adenosine. Extracellular adenosine aggregation enhance Tregs activity and alter the effector function of T-cells (Dulphy et al. 2014). MDSCs are also found in high levels in patients with AML. This expansion was shown to be raised by both primary AML cells and AML cell lines in a contact-dependent manner through the MUC-1 oncoprotein (Pyzer et al. 2017).

In the context of CART-cell therapy, many potential approaches were investigated in order to address the issue of the inhibitory function of abundant immunosuppressive cells against CART-cells. As such, blocking the adenosine immunosuppression activity by an antagonists targeting the adenosine receptor (A2AR) administered in combination with CART-cell therapy (Beavis et al. 2017) or by the deletion of this receptor in the CAR construct using CRISPR/Cas9 system (Giuffrida et al. 2021). In the aim to target MDSCs immunosuppression, some clinical trials have been initiated in multiple malignancies using small molecules to reduce the recruitment of MDSCs, for example chemokine inhibitors or the liver-X nuclear hormone receptor (LXR) agonist (Fleming et al. 2018). Furthermore, the in-vivo depletion of MDSCs increased CART-cells anti-tumor effects (Di et al. 2019). Several strategies also have been evaluated to enhance CART-cells expansion and persistence including IL-8 or IL-15 transgene expression, addition of different cytokines (IL-7, IL-15, and IL-21) and many others. However, these strategies are not yet investigated in AML.

### *(C) Upregulation of Inhibitory mechanisms*

Immune checkpoints such as CTLA-4 and PD-1 have long been studying in a wide range of cancers. Clinical trials have evaluated different mAbs in blocking these immune checkpoints signaling pathways. Several mAbs essentially  $\alpha$ -PD-1,  $\alpha$ -PD-L1, and  $\alpha$ -CTLA-4 have been FDA approved for certain cancers treatment with the Nobel Prize in physiology and medicine to Tasuku Honjo and James Allison in 2019 for solid cancers (Mardiana, Solomon, et al. 2019).

In AML, it has been demonstrated an enhancement of the expansion and the activity of autologous T-cells against AML cells following the blockage of CTLA-4 (Zhong et al. 2006). Moreover, a phase I trial in AML patients relapsing after an allo-HSCT, blocking CTLA-4 with ipilimumab had an anti-tumor effect with a complete remission (Davids et al. 2016).

Another checkpoint axis is PD-1/PDL-1, PDL-2. PDL-1 expression on AML primary cells is upregulated in an inflammatory microenvironment through different stimuli such as IFN $\gamma$  and TLR ligands resulting in an immunosuppression and a disease progression with poor prognosis (Xiangli Chen et al. 2008). Furthermore, an expression of PD-1 was described on T-cells of relapsed AML patients.

So far, the effect of clinical trials blocking checkpoint pathways in AML as a single agents are very limited. Therefore, new strategies are ongoing combining the blockage of checkpoints with other types of therapy such as azacitidine, a hypomethylating agent which upregulate the expression of PD-1 and PD-L1 (clinicaltrials.gov identifier: 02775903, 03092674) (Daver et al. 2019).

T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT), TIM-3, and glucocorticoid induced tumor necrosis factor receptor related protein (GITR) are all also attractive checkpoints pathways to be investigated in AML (Kong et al. 2016).

Among the mechanisms used by AML cells and in particularly monocytes, is that they can directly suppress T-cells anti-tumor responses *(D)* by producing large quantities of reactive oxygen species (ROS), thereafter inducing the apoptosis of T-cells (Aurelius et al. 2012). Furthermore, *(E)* AML blasts can downregulate the expression of their MHC class I and class II, thus reducing the antigen presentation leading to immune evasion and relapse after transplantation (Christopher et al. 2018).

### **III. Targeting the AML tumor microenvironment with CART-Cells**

As represented before, the microenvironment could play a substantial role in the regulation of the functions and toxicities of CART-cells. The deregulation of several costimulatory ligands by the microenvironment in addition to the immunosuppressive and hypoxic nature of AML-BM niche, they may presumably hamper CART-cell functionality. To date, only one study using CD44v6 CART-cells has assessed the role of the BM-mesenchymal stromal cells (MSC) in CART-cell resistance in AML and further investigation is required (Casucci et al. 2013).

In aim to hamper the tumor-associated immunosuppressive environment, different CAR constructs have been evolved such as: the fourth-generation CAR that have been engineered to produce immune-stimulatory cytokines (IL-12) (Charrot and Hallam 2019), to release PD-1-blocking ScFv (Sarwish Rafiq et al. 2018) with preclinical anti-tumor efficacy, or to enhance endogenous anti-tumor immune response by constitutively express CD40L (Curran et al. 2015). Additionally, a switch CAR with the extracellular domain of an inhibitory T-cell receptor (like PD-1) coupled to an intracellular costimulatory signal have been described (X. Liu et al. 2016). Furthermore, many other strategies are also used (Figure 26).

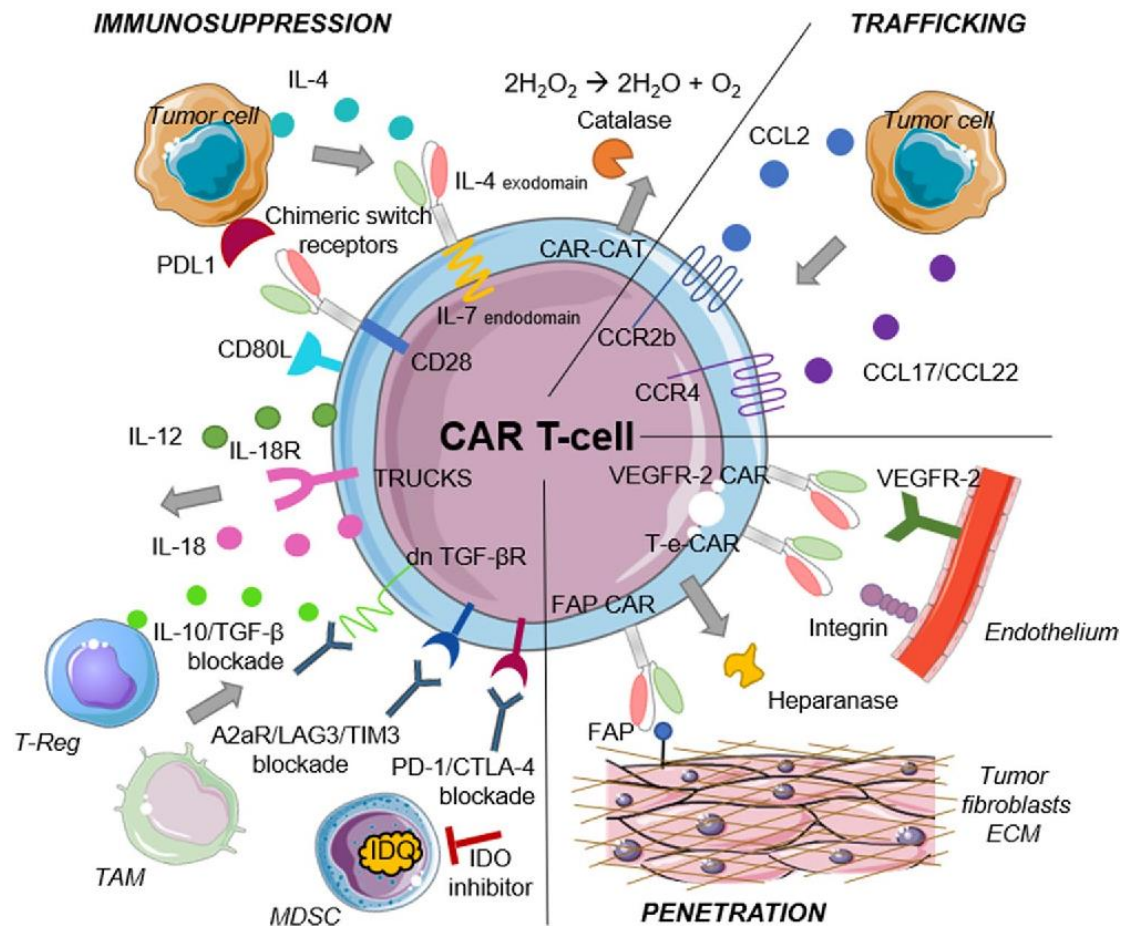


Figure 26: Overcoming the immunosuppression of AML microenvironment by adoptive cell transfer of CART-cells. TAM: Tumor-associated macrophages, MDSC: myeloid-derived suppressor cells, VEGFR: vascular endothelial growth factor receptor, FAP: fibroblast activating protein, ECM: extracellular matrix (Gowrishankar, Birtwistle, and Micklethwaite 2018).

Furthermore, c-kit CART-cells expressing a trafficking receptor (CXCR4) allowed to enhance the traffic to the BM and the expansion of CART-cells resulted in significant depletion of the BM c-kit<sup>+</sup> cells (Arai et al. 2018).

Besides, the hypoxic environment of AML-BM niche have been demonstrated to promote central memory phenotype in CART-cells (X. Wang et al. 2016), which is advantageous for their functionality. However, at the same time hypoxia can decrease CART-cells proliferation and hinder their effector memory differentiation and functionality (Rodriguez-Garcia et al. 2020). In another hand, hypoxic environment can likely induce alteration of the surface phenotype of AML cells (Sironi et al. 2015) leading to antigen escape in CART-cell therapy. Consequently, using hypoxia-sensitive CART-cells which can specifically be activated in hypoxic sites (such as the AML-BM microenvironment) can prevent undesirable off-site toxicities (Juillerat et al. 2017). This approach could be credibly assessed to



eradicate residual LSC persisting in the hypoxic BM niche after chemotherapy and responsible for the relapse. More investigations are required to better understand the effect of the AML-BM niche on CART-cells functions for developing greater and more efficient therapeutic targets.

#### **IV. Approaches to overcome CART-cells resistance in AML and to manage toxicities**

##### **A. Genome editing using CRISPR/Cas9:**

Toward the prevention of undesired prolonged myeloablation effect and for a long-term CART-cells persistence, a novel strategy has been evaluated based on editing out the CAR target antigen from a donor allograft. This technique consist on the deletion of the CAR target antigen for example CD33 in the HSPCs of the donor using CRISPR/Cas9 technology (Figure 27) (C. Liu et al. 2017) and then transplant these CD33<sup>-/-</sup> HSPCs into the patient before administrating CD33 CART-cells manufactured from the same donor. Thereby, CD33<sup>-/-</sup> allograft HSPCs will trigger a normal hematopoiesis always with continued persistence of CART-cells. In fact, a group of researchers has demonstrated by CRISPR/Cas9 genome editing that autologous CD33 CART-cells (from the donor) did not affect CD33<sup>-/-</sup> HSPCs in-vitro and in AML murine xenograft and resulting in an efficient elimination of leukemia without myelotoxicity. They have also exhibit that CD33-deficient HSPCs have consistently kept their hematopoietic and immunological functions. Accordingly, genetically engineering the donor derived HSPCs represent an innovative approach to create an artificial AML-specific antigen immunotherapy with avoiding on-target, off-tumor toxicity. Indeed, a clinical trial implying the use of allogeneic CD33<sup>-/-</sup> HSCT prior to CART-cell infusion is currently being investigated at the University of Pennsylvania for patients with R/R AML (M. Y. Kim et al. 2018).

Besides CD33, CD123 is also a potential AML target antigen that may be edited using a similar approach. However, since CD123 plays an important role in hematopoiesis as it is the alpha-chain IL-3R, the complete deletion of CD123 in hematopoietic cells will results in an abnormal hematopoietic system (Testa, Pelosi, and Frankel 2014). Therefore, two alternative approaches could be potentially used: the deletion of the epitope on the CD123 receptor that can be recognized by the CART-cells, as well as the knockdown of the CD123 expression in donor-derived HSPCs instead of a complete knockout of its expression. Herewith, the expression of CD123 on hematopoietic allograft cells will be minimized to a level below the CART-cells activation threshold, so CART-cells will not be activated against normal HSPCs while preserving a normal hematopoiesis with CD123 presence. So far, this approach is under investigation.

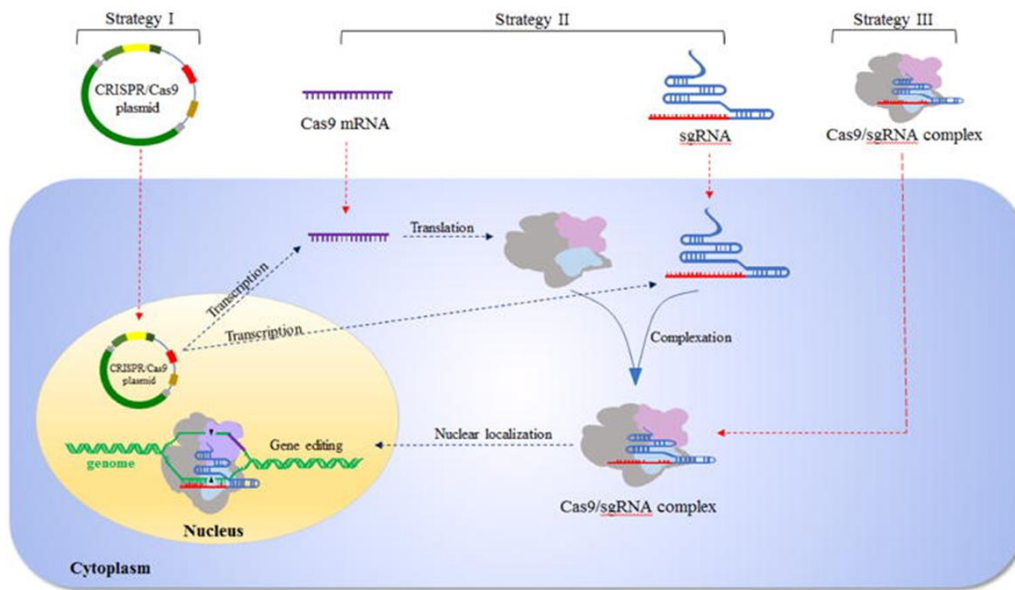


Figure 27: Different strategies of gene editing using CRISPR/Cas9 system (C. Liu et al. 2017).

## B. Identify new AML specific neoantigens

The major obstacle in cellular therapy is the lack of a specific tumor target antigen facilitating tumor eradication whilst sparing normal cells. To date, most of CART-cells target antigens overexpressed on tumor cells are also expressed at lower levels on normal cells. Lewis Y antigen, carcinoembryonic antigen (CEA) and GD2 antigen are considered as efficient and safe targets for CART-cells in sight of their highly restricted expression on healthy tissues make them distinguishable from malignant tissues (Mardiana, Lai, et al. 2019). Unfortunately, this type of antigens with differential expression does not exists for most myeloid diseases such as AML. This problem led to various researches to find a real AML specific target antigen. To this aim, studies have been based on discovering antigens produced specifically by the disease or disease-associated mutations. These neoantigens would be the perfect targets for CART-cells as they must be expressed only by tumor cells and not by healthy cells (Wirth and Kühnel 2017). As a result of studies developed by the Cancer Genome Atlas Research Network to discover the mutational profile of de novo AML, a number of certain mutations leading up to the development of the AML phenotype have been identified (Cancer Genome Atlas Research Network et al. 2013) (Papaemmanuil et al. 2016). Although a few number of genome mutations have been found in patients with AML (Alexandrov et al. 2013). Some neoantigens have been described including mutation in the metabolic enzymes IDH1 and IDH2 (Goswami and Hourigan 2017) present in nearly 20% of de novo AML cases, mutation in NPM1 gene present in up to 60% of AML patients (Greiner et al. 2012). These mutant epitopes have been shown to be immunogenic following their immune

recognition which make them attractive targets for T-cell therapy. However, resulted mutant proteins are expressed intracellularly and consequently not reachable by the CAR.

Another potential source of neoantigens is the dysregulated splicing. Actually, an aberrant splicing of genes can lead to alternative isoforms of a protein that are discernable from their wild type counterpart. According to the investigations of the genome-wide splicing abnormalities in AML, it have been found that about a third of genes expressed in AML undergo differential RNA splicing. This may crate different variants and thus potential neoantigens (Adamia, Haibe-Kains, et al. 2014). A splice variant for FLT3 and another for NOTCH2 have been found in 50-73% of AML cases respectively with no presence in healthy donors (Adamia, Bar-Natan, et al. 2014). Additionally, the CD44v6 variant which arise from the CD44 was also described as an AML-specific isoform present in more than 60% of AML patients and not present in healthy donors. Among AML associated mutations, CD44v6 is expressed on the cell surface therefore unlike other mutations, it will be accessible to the CAR. This was demonstrated against AML primary cells where CD44v6 CART-cells had an efficient anti-tumor responses while no affecting normal HSPCs (Casucci et al. 2013).

To cross the incapability of targeting intracellular mutations with CART-cells, a recent innovative study has demonstrated the possibility of generating a CAR that recognizes an intracellular antigen. In this study they generated a TCR-mimic CAR targeting the peptide-MHC complex, it is in fact specific to the intracellular onco-protein WT1 that was presented on the cell surface by the MHC (S. Rafiq et al. 2017).

Indeed, a TCR targeting WT1 was clinically used in AML with an effective reduction of relapse risk following HSCT (Chapuis et al. 2019). Nevertheless, to date no clinical tests reported using CART-cells against WT1.

### C. Strategies to mitigate GvHD risks:

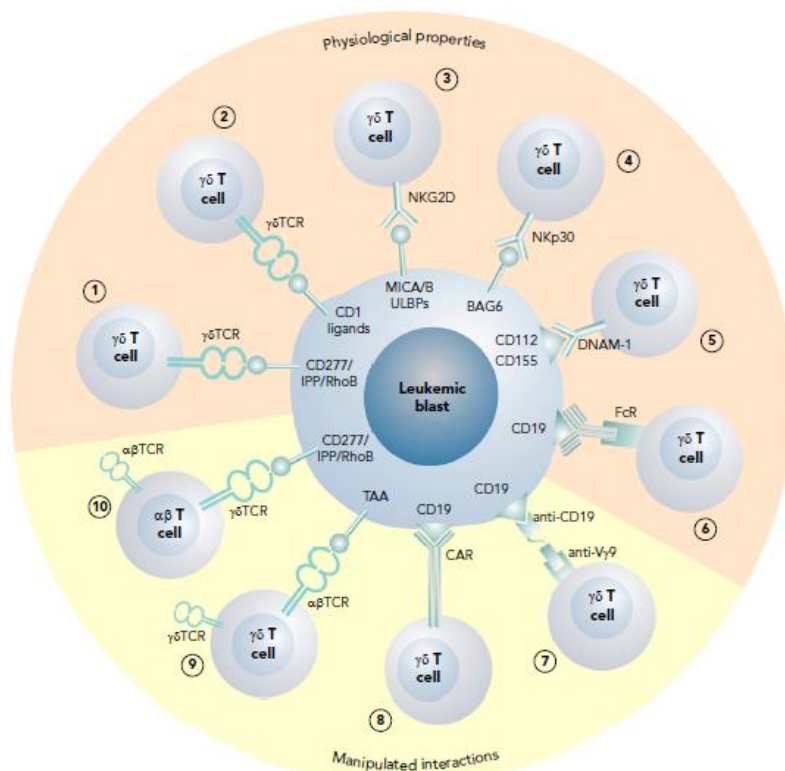
#### *Universal CART-cells*

Allogenic CART-cells therapy is unfortunately associated with high risks of GvHD and rejection of transferred T-cells. Consequently, in an attempt to alleviate GvHD, some solutions have been evaluated in different preclinical studies such as producing universal allogenic CART-cells by a genetic disruption of the endogenous TCR from the CART-cells (Torikai et al. 2012). This strategy has resulted in successful clinical outcomes with R/R B-ALL children treated with allogeneic TCR-deficient CD19 CART-cells (Qasim et al. 2017). Another study with promising results is with as an off-switch FLT3 CART-cells (Sommer et al. 2020). Further clinical trials using universal CART-cells are ongoing

(clinicaltrials.gov identifier: 03166878, 03229876). However, the risk of GvHD is not completely abolished due to the presence of T-cells with even very small percentage of TCR, which the need to an optimization of TCR deletion during CART-cells production. A phase I clinical trial is currently under investigation for the applicability of a universal CD123 CART-cells in AML (NCT03190278).

#### *Non alloreactive cells: $\gamma\delta$ T-cells*

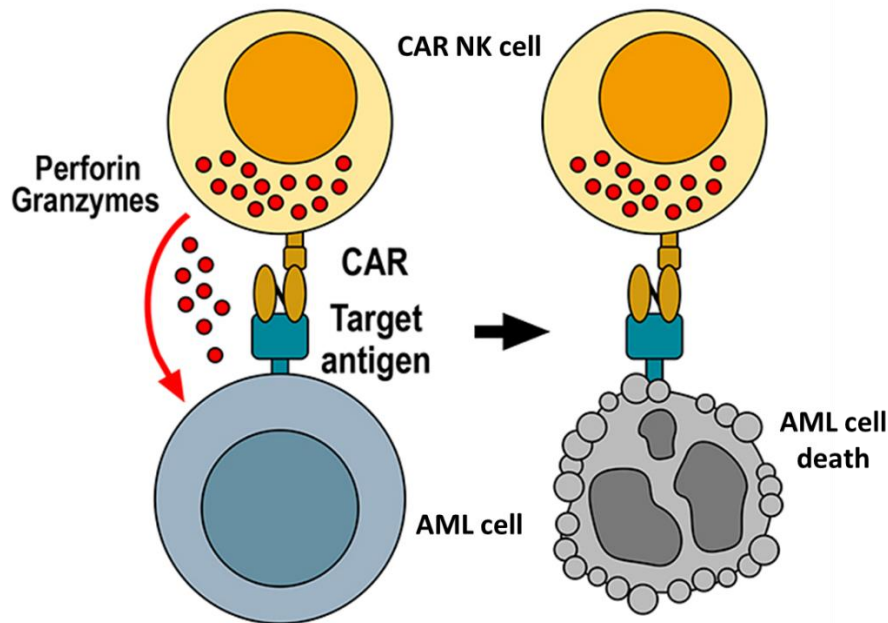
In another hand, the use of a specific T-cells subset seems to play an important role in reducing the risk of GvHD. So far, most of the CART-cells used in the clinic are fabricated from unselected T-cells, primarily  $\alpha\beta$  T-cells. The selection of a T-cell subset that is less likely to induce GvHD for the CART-cells production, appear to be an attractive option. For example,  $\gamma\delta$  T-cells differently from  $\alpha\beta$  T-cells are not alloreactive and do not induce GvHD (Figure 28) (Handgretinger and Schilbach 2018). It was reported in 2004 a first use of  $\gamma\delta$  CART-cells with a CAR of first generation targeted toward GD2 antigen resulting in a reduction of antigen-specific tumor reactivity (Rischer et al. 2004). Besides, it was demonstrated that  $\gamma\delta$  cells in GD2-directed CART-cells could be easily mass produced for clinical use despite their small percentage (5%) in circulating T-cells and associated with high cytotoxicity (Capsomidis et al. 2018). To date, no clinical use of CAR  $\gamma\delta$  cells has been reported.



*Figure 28: Anti-leukemic cytotoxic mechanisms of  $\gamma\delta$  T-cells applications (Handgretinger and Schilbach 2018).*

### *Other effector cells: NKT and NK cells*

Alternatively, it is possible to use other effector cells to mitigate GvHD risks such as CIK-cells, NKT-cells and NK-cells for the CAR engineering and the AML treatment (Figure 29) (Burger et al. 2019).



*Figure 29: CAR NK cells anti-tumor function in AML (Burger et al. 2019).*

As published in preclinical studies, using CD123 CAR CIK-cells and CD33 CAR CIK-cells in-vivo promoted a very efficient reduction of AML leukemia burden with limited toxicity on normal HSCs with only CD123 CAR CIK-cells (Tettamanti et al. 2013) (Pizzitola et al. 2014) (Rotiroti et al. 2020). Currently, CAR NK-cells are being investigated in phase I/II clinical trials, targeting different antigens mainly CD19 but also others including CD7, CD33, MUC-I, and Her2 (Kloess, Kretschmer, et al. 2019). CD33 CAR NK-92 cells was evaluated in a phase 1 trial (Tang et al. 2018) and recently engineered CD33 targeted CAR NK-cells, using CRISPR/Cas9 gene editing and AAV-mediated gene delivery, were able to kill CD33+ AML cells in-vitro (Naeimi Kararoudi et al. 2020). CD123-specific CAR cord blood NK-cells eliminate CD123+ AML cells which support their potential use in the clinic for patients with relapsed AML (Kerbaui et al. 2017). However, CAR NK therapy was associated with a poor CAR NK-cells persistence, thus in order to enhance CAR persistence it was recently reported an in-vitro activity of affinity optimized CD38 CAR NK-cells in AML. In another hand, the expression of an NKG2D CAR on NK-cells naturally expressing the NKG2D receptor enhances NK-cell activity with a poor risk to a downregulation encountered with endogenous NKG2D in AML (Gurney and O'Dwyer 2021). A phase I clinical trial of

NKG2D CAR NK-cells from a haploidentical donor in MDS and AML was recently opened ([NCT04623944](https://clinicaltrials.gov/ct2/show/study/NCT04623944)). Moreover, in order to solve the problem of the insufficient dose of CAR NK-cells in-vivo, a phase I/II dose escalation study of CAR NK-cells has begun in patients with B-cell malignancies (clinicaltrials.gov identifier: 03056339). Thus, CAR NK therapy may be a potential therapy for ‘off-the-shelf’ application with a safer adverse effect profile describe an attractive approach to AML immunotherapy (Kloess, Oberschmidt, et al. 2019).

#### D. Combine CART-cells treatment with checkpoints inhibitors

Several preclinical studies have tested the combination of CART-cell therapy with checkpoint inhibitors. Antibodies blocking checkpoints are either administered with CART-cells (John et al. 2013), or genetically engineered with the CAR vector of the CART-cells that so they can synthesized themselves the checkpoint blocking antibodies (S. Li et al. 2017) (Figure 30).

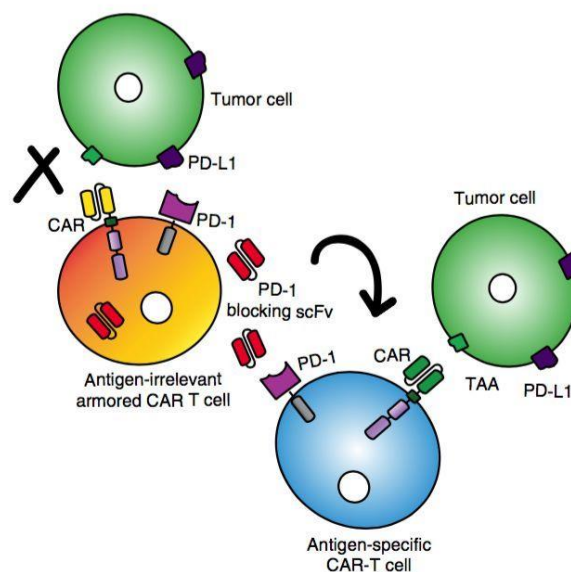


Figure 30: CART-cells secrete ScFv anti-PD-1 as combined immunotherapy (Sarwish Rafiq et al. 2018).

Promising results of preclinical data encouraged the translation of these combined strategies to the clinic. Clinical trials are currently ongoing for the evaluation of the safety and efficacy of these strategies in lymphomas and solid tumors. However, as noted previously, the effect of checkpoint inhibition in AML is limited owing to the low genomic mutations in AML, subsequently the few target antigens that can be targeted by CART-cells.

## Main objectives of this work

Given the toxicities and the high relapses that are associated with the current treatments in AML, a need of a safe and curative alternative treatment strategy for AML patients is crucial. Following our previous work using CART-cells targeted the IL-1RAP protein in CML that showed promising results, we made the proof of concept that IL-1RAP CART-cells are highly functional and cytotoxic against leukemic blasts that express IL-1RAP at their surface. Therefore, the objective of this project was to develop a new therapeutic strategy in AML using adoptive CART-cell immunotherapy targeting an antigen specifically expressed by AML LSCs who are the origin of the relapse. With published data that demonstrated an expression of IL-1RAP on the surface of AML cells, the aim of this thesis is to evaluate the IL-1RAP molecular and cellular expression in AML primary cells and AML cell lines using our own anti-IL-1RAP monoclonal antibody (mAb). Then we validate the functionality of IL-1RAP CART-cells, that we produce using a CAR of 3<sup>rd</sup> generation specific for IL-1RAP, against AML primary cells (diagnosis or R/R) and AML cell lines *in-vitro* and *in-vivo* in xenograft murine models and PDX models. We also characterize the immunophenotyping profile and the exhaustion status (checkpoints expression) of IL-1RAP CART-cells produced from AML patients (diagnosis or R/R).

## Results (Submitted article)

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

**Scientific category:** Myeloid neoplasia

**Title page:** AML as a candidate for IL-1RAP CAR T cell immunotherapy

**Key points:** Identification of a novel antigen expressed by AML cells (IL-1RAP) allowed the development of an alternative AML treatment: CAR-T cells targeting IL-1RAP.

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## Abstract

Acute myeloid leukemia (AML) remains a very difficult disease to cure due to the persistence of leukemic stem cells (LSCs), which are resistant to different lines of chemotherapy and are the basis of refractory/relapsed (R/R) disease in 80% of AML patients not receiving allogeneic transplantation. In this study, we showed that the IL-1RAP protein is overexpressed on the cell surface of LSCs in all subtypes of AML and confirmed it as an interesting and promising target in AML compared to the most common potential AML targets. After establishing the proof of concept for the efficacy of CAR T cells targeting IL-1RAP in chronic myeloid leukemia (CML), we hypothesized that third-generation IL-1RAP CAR T cells could eliminate AML LSCs. We first demonstrated that IL-1RAP CAR T cells can be produced from AML T cells at the time of diagnosis but also at relapse. The characterization of IL-1RAP CAR T cells showed an expression of checkpoint markers at the end of the production. In vitro and in vivo, we showed the effectiveness of IL-1RAP CAR T cells against AML cell lines expressing different levels of IL-1RAP and the cytotoxicity of autologous IL-1RAP CAR T cells against primary cells from AML patients at diagnosis or at relapse. In patient-derived relapsed AML xenograft models, we confirmed that IL-1RAP CAR T cells are able to circulate in peripheral blood and to migrate in the bone marrow and spleen and are cytotoxic against primary AML cells.

## INTRODUCTION

Despite several successive improvement in treatment, acute myeloid leukemia (AML), a hematological malignancy of leukemic stem cells (1), remains difficult to treat and to cure (2).

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

Immunotherapy targeting leukemia-specific AML antigens has been explored to improve the outcome of AML patients, such as conjugated monoclonal antibodies (CD33-GO, gemtuzumab ozogamicin, and antibodies targeting CD44, CD123 or CD47) (4), bispecific T cell engager (BiTE) antibodies (targeting CD3/CD33 or CD3/CD123), and immune checkpoint inhibitors (targeting PD-1/PDL-1, anti-TIM-3, and anti-CTLA4) (5).

Since SCT is not suitable for every AML patient and based on the success and subsequent approval of cellular gene therapy CD19-targeted immunotherapies (6), these technologies, particularly chimeric antigen receptor (CAR) T-cells, are being translated into robust anti-AML therapies for refractory/relapsed (R/R) patients (7). Several AML cell surface targets have been explored, like CD33, CD123, CD44v6, CLL-1, and B7H6 (8); however, they have potential toxicities due to their frequent expression on healthy HSCs or progenitors (9) and can lead to ablation of all myeloid progeny, thus requiring investigation of other targets.

IL-1 receptor accessory protein (IL-1RAP) forms a complex with the IL-1 $\alpha$ , IL-1 $\beta$ , and IL-33 receptors on the surface of hematopoietic cells. The IL-1RAP protein has been shown to be overexpressed on the surface of leukemic stem cells (LSCs) of AML, myelodysplastic syndrome (MDS) and chronic myeloid leukemia (CML) but not on normal HSCs (10). It is a proinflammatory protein that has an oncogenic effect in AML via the FLT3 and C-kit pathways (11) that promotes leukemic proliferation over normal hematopoiesis (12) and has been shown to be related to some solid tumors (13).

Currently, IL-1RAP is targeted only by monoclonal antibodies (14-16), which are still under clinical evaluation (ClinicalTrials.gov: NCT03267316 and NCT04452214) in solid tumors (17). The goals of such antibodies are to block the interaction with the IL-1 receptor, impair tumor progression and enhance

antibody-dependent cellular cytotoxicity (ADCC) by recruiting natural killer (NK) cells. In both cases, the antibodies did not generate a persistent memory effect compared with CAR T cells.

Based on our previous work demonstrating the proof of concept that 3<sup>rd</sup> generation IL-1RAP CAR T cells (18, 19) are an interesting and robust immunotherapy approach in CML that does not affect healthy HSCs, in this work, we evaluated this innovative cell immunotherapy in AML to demonstrate its feasibility for a future first phase I clinical trial.

## Methods

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

### **Transcriptomic and RNAseq *in-silico* analysis**

Gene expression profiling (GEP) was performed using Human Genome U133 Plus 2.0 arrays (Affymetrix/ThermoFisher, Santa Clara, CA), as previously described (20). A total of 244 samples were subjected to transcriptomic analysis, among which 74 AML samples included different French-American-British subtypes: 28 AML0, 11 AML1, 17 AML2 including t(8;21), 12 AML4 including t(inv16), 6 AML5, 60 T-ALL, 24 B-ALL and 13 blastic plasmacytoid dendritic cell neoplasm (BPDCN) samples. Gene expression intensity values were log-transformed and normalized using the robust multiarray average (RMA) algorithm with DNA-Chip Analyzer DCHIP software (21)[1]. Gene expression data from 32 peripheral blood samples from healthy donors available in the Gene Expression Omnibus (22) database were included.

Raw RNA-seq data of sorted normal bone marrow and peripheral blood mononuclear cells (n=49 samples from 9 healthy donors) and leukemic bone marrow cells (n=32 samples from 12 AML patients) were retrieved from the NCBI Gene Expression Omnibus (GEO) portal under the accession number GSE74246 (23). Statistical analysis was performed using R software v 3.6.0 (see supplemental methods).

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

The human AML tumor cell lines Molm-13 (ACC-554), HL-60 (CCL-240), EOL-1 (ACC-386), HEL (ACC-11), Mono-Mac-6 (ACC-124), THP-1 (TIB-202) and Ma9RAS (MLL/AF9), the CML cell lines KU812 (CRL-2099) and K562 (CCL-243), and the human embryonic kidney epithelial cell line 239T (CRL-3216) were obtained from ATCC and stored in a master cell bank. Cells derived from working cell banks were used for the present study. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient density centrifugation using Ficoll-Paque (Velizy-Villacoublay, France) from anonymous blood samples collected from healthy donors at a French blood center (Besançon, France). AML primary cell collection from AML patients was performed at the time of diagnosis (Cohort French Innovative Leukemia Organization (FILO) Cell Biobank; AML collection (N° BB-0033-00073, declaration 2009-944 and authorization AC 201261739)) and at follow-up and relapse (AML-CAR Collection, N° CPP2019-03-022a/2018-A03300-55, harvested from Besançon and Dijon clinical centers). Patients and donors provided written informed consent, and the study was conducted in accordance with ethical guidelines

(Declaration of Helsinki) and approved by the local ethics committee of the CPP-Sud-Ouest et Outre-Mer IV (France).

### **Determination of IL-1RAP mRNA expression, western blotting, and determination of the number of IL-1RAP antigenic sites**

Relative IL-1RAP mRNA expression was determined by RT-QRT-PCR (real-time quantitative reverse transcription-polymerase chain reaction) using the Hs\_00895050\_m1 TaqMan qPCR gene expression assay (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) targeting the mRNA variant codon for the intracellular domain of the cell surface protein. IL-1RAP protein expression analysis was performed by western blotting on AML cell lines ( $5.10^6$  cells) using our own primary antibody targeting IL1RAP (#A3C3 mAb; BL-43, Diaclone, France; (diluted 1,000x) and an antibody targeting  $\beta$ -actin (clone AC15, #A5441, Sigma-Aldrich, St Louis, MO, USA) (diluted 1,000x) as an internal loading control.

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

### **Production of lentiviral construct supernatant and genetically modified CART-cells**

The CAR construct and the self-inactivating lentiviral vector have been previously described (19). Briefly, the extracellular receptor domain, derived from the IL-1RAP monoclonal antibody (mAb, #A3C3), is linked to a third-generation intracellular T-cell activating domain (CD28/4-1BB/CD3 $\zeta$ ). The vector (Supplemental Figure S1A) also carries a safety switch consisting of a suicide gene (inducible caspase 9 (iCASP9) and a truncated CD19 gene ( $\Delta$ CD19) encoding the cell surface marker). Lentiviral (LV) supernatant was harvested from tritransfected 293T cells (pMDG, pPAX2 and transgene plasmids) (Supplemental Figure S1B). Cells taken from healthy donor or AML patient samples, after initial T cell selection and activation using anti-CD3/CD28 magnetic beads and interleukin-2 (IL-2), were transduced with either IL-1RAP CAR or mock (lacking the CAR sequence) LV supernatant. After 6 days, genetically modified CD3 $^{+}$ /CD19 $^{+}$  T cells were evaluated by flow cytometry and expanded for 9 days.

### **Flow cytometry immunophenotyping**

Patient primary AML cell immunophenotyping was performed using a panel of mAbs targeting human CD45, CD34 (stem cells), CD38 (progenitor cells), CD33 (AML blasts), CD14 (monocytes), and

CD123 (AML blasts), including our own murine FITC-labeled IL-1RAP mAb (#A3C3, clone BL-43, from which the CAR was derived) and/or #H6E11 (clone BR-58; Diaclone, Besançon, France). Transduced T cells were stained using both anti-CD3 and anti-CD19 antibodies. T lymphocytes from AML patients were stained using an immunophenotyping panel containing mAbs targeting CD3, CD8, CCR7 and CD95 and a checkpoint inhibitor panel containing mAbs targeting PD-1, LAG-3 and TIM-3. Stained cells were collected by a CANTO II cytometer (BD Biosciences, Le Pont-de-Claix, France) or an LSR Fortessa flow cytometer and analyzed using DIVA software (BD Biosciences, Le Pont-de-Claix, France). Suitable matched isotype controls were used for analysis. The relative fluorescence intensity ratio (RFI) was calculated by dividing the mean fluorescence intensity (MFI) of IL-1RAP staining by that of the isotype control mAb. Cells were considered positive for IL-1RAP expression at an RFI > 1. The mAbs used for phenotyping, intracellular staining, cytometry and other cytometry reagents are described in Supplemental Table S1.

#### **In vitro cellular analysis: cell activation, IFN $\gamma$ intracellular expression and cytotoxicity analysis**

IL-1RAP CAR T cells were cocultured with target tumor cells (AML cell lines or primary AML blasts) overnight at an effector:target (E:T) ratio of 1:5 for the detection of IFN- $\gamma$  intracellular expression. An IL-1RAP CAR T cell proliferation assay was performed after 3 days of coculture (E:T ratio= 1:3) and assessed by flow cytometry after V450 e-Fluor staining. The cytotoxicity of IL-1RAP CAR T cells against AML leukemic cells was assessed after incubation for 24 h at different E:T ratios by flow cytometry using trucount Absolute Counting tubes (ref: 340334, BD biosciences, Le Pont-de-Claix, France). Cell death was evaluated by 7-amino actinomycin D (7-AAD+) labeling. Effector cells were distinguished from target cells with a previously described V450 e-Fluor labeling method. Gating on CD3+/CD19+ cells and on IL-1RAP+ cells allowed discrimination of CAR T cells from IL-1RAP+ tumor AML cells. In the case of allogeneic mismatch, alloreactivity was taken into account by subtracting the cytotoxicity of untransduced T cells (C0) or mock-treated T (MockT) cells at the respective E:T ratio. C0 and MockT cells were used as controls.

#### **Xenograft murine models and patient-derived xenograft murine model**

Six- to 8-week-old NSG mice (Jackson Laboratory, Sacramento, CA, USA) were sublethally irradiated (250 cGy) on day 4 prior to tumor injection. One day later, each mouse was injected intravenously with  $1.10^6$  HL-60 (IL-1RAP<sup>Low</sup>), Molm-13 (IL-1RAP<sup>Int</sup>) or Mono-Mac-6 (IL-1RAP<sup>High</sup>) luciferase-expressing AML cells or primary cells from AML patients (taken at diagnosis or relapse). Following AML cell engraftment (day 0), mice were treated intravenously with MockT or IL-1RAP CAR T cells ( $10.10^6$  cells) and assessed

for leukemia progression on days 3, 5, 10, 14, 17 and 21 by in vivo bioluminescent imaging or determination of the percentage of human CD45+/IL-1RAP+ cells in mouse blood for the PDX model.

In the PDX model, at D32 and D71, NSGS mice (triple transgenic NSG-SGM3 mice expressing human IL-3, GM-CSF and SCF, Jackson Laboratory, Sacramento, CA, USA) were sacrificed, and bone marrow or spleen cells were collected and identified by FCM staining (hCD45+/CD3-/CD34+). Monitoring of CAR T cells was performed by digital PCR as previously described (Haderbach et al). CART-cells Effector/Memory phenotype was assessed by the expression of CD95/CCR7 among CD45RO+/CD45RA- or CD45RO-/CD45RA+ cells. Mouse experiments were approved by the local ethical committees (CELEAG and protocol 11007R, Veterinary Services for Animal Health & Protection).

### **Statistical analysis and graph constructions**

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



## Results

### **Compared to the large number of AML cell surface-targeted markers, IL-1RAP is a promising marker expressed at various levels in primary AML blasts**

In silico analysis of IL-1RAP expression was performed with a public dataset of 81 samples of sorted bone marrow and peripheral blood cells from 9 healthy donors and 12 high-risk AML patients (23). First, IL-1RAP mRNA expression was compared to that of the other current AML cell surface targets in ongoing CAR T cell studies (24). Differential gene expression analysis between AML cells (LSCs or blasts) and normal hematopoietic progenitor and stem cells (HPSCs) revealed significant overexpression of IL-1RAP in LSCs and blasts (fold changes of 21.7 and 15, respectively), with a significant false discovery rate (FDR)-adjusted p-value ( $\sim 1.10 \times 10^{-20}$ ). The IL-1RAP marker was highly discriminatory compared to common AML cell surface targets, such as TIM3, CLEC12A (CLL-1), IL3RA (CD123), MICA/MICB (NKG2DL), CD44, CD33 and FLT3 (Figure 1A).

As reported in Figure 1B, comparison of IL-1RAP expression between total normal PB/BM and AML cells clearly showed a higher level in blasts and LSCs. Importantly, IL-1RAP is not expressed in B, T (CD4+ or CD8+) or NK cells but is known to be expressed in monocyte PB subpopulations. In addition, all BM progenitor subpopulations expressed IL-1RAP. Using TCGA LAML cohort (n=139), we found that a high IL-1RAP expression was associated with a poor disease-free survival (DFS) and overall survival (OS). This association stood significant in a multivariate Cox model using age and ELN2017 group as confounding variables (HR 1.60 for high versus low IL1-RAP expression level for both DFS and OS, p-value=0.037 and 0.014 respectively) (Supplemental Figure S2). Finally, Affymetrix transcriptional profiling of the IL-1RAP mRNA encoding the membrane expressed isoform (mRNA variant 1) showed that it was overexpressed in all AML FAB subtypes compared to the soluble IL-1RAP isoform (variant 5) (Figure 1C).

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

By RT-qPCR, we detected mRNA in different AML cell lines (n=6) (Supplemental Figure S3A). IL1-RAP protein was detected by western blotting in different AML cell lines (Figure 2A). Cell surface expression at various levels was identified in 7 AML cell lines ( $2 < \text{RFI} < 4.3$ ) (Figure 2B) as well as in primary AML blasts and LSCs ( $1.8 < \text{RFI} < 3.6$ , n=30), while it was consistent on monocytes from AML patients ( $1.6 < \text{RFI} < 2.3$ , n=30) (Figure 2C). No expression of IL-1RAP was detected on normal hematopoietic cells and normal monocytes. Clinical information of AML patients are provided in supplemental table 2. Absolute quantification of the number of IL-1RAP antigen sites confirmed the different levels of expression in different antigen cell lines (n=7) (Supplemental Figure S3B) as well as in primary AML blasts (n=11) (Figure 2D). The absolute number of IL-1RAP antigen sites was not statistically different according to ELN group. To model IL-1RAP cell surface expression for further analysis, we classified 3

AML cell lines and defined them as "low (HL-60)", "intermediate (Molm-13)" and "high (Mono-Mac-6)" IL-1RAP expressers.

### **Efficient generation of IL-1RAP CAR T cells from T cells taken from AML patients at either diagnosis or relapse with memory profiles and checkpoint inhibitor expression**

Using our lentiviral vector (19), we were able to efficiently transduce T cells from healthy donors with either the mock ( $75.29\% \pm 22.7$ ) or IL-1RAP CAR ( $74.06\% \pm 16.48$ ) T cell supernatant (n=24). More interestingly, T cells from AML patients taken either at diagnosis (D) or at the time of relapse (R/R) could be transduced ( $90.4\% \pm 6.58$  and  $88.1\% \pm 9.28$ , respectively, for mock or IL-1RAP CAR T cell supernatant, n=12) without noticeable differences from T cells from healthy donors (Figure 3A). We then identified the ratio of CD4<sup>+</sup> and CD8<sup>+</sup> cells before CAR T cell production (D0) and at the end of the process (D10) using either IL-2 or IL-7/15 as cytokines in the culture medium. As reported in Figure 3B, similar to IL-1RAP CAR T cells from healthy donors, IL-1RAP CAR T cells produced from AML patients conserved the CD4<sup>+</sup>/CD8<sup>+</sup> ratio during the culture. Ultimately, the IL-1RAP CAR T cells (either CD4<sup>+</sup> or CD8<sup>+</sup>) produced from T cells taken from AML patients (D or R/R) showed the same profile of phenotype that was observed with T cells taken from healthy donors with a conservation of this phenotype during culture with either IL-2 or IL-7/15 (Figure 3C). A slight tendency of acquisition a naïve/memory phenotype by the end of the production process was observed with CD8<sup>+</sup> CAR T cells.

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

The HL-60 (IL-1RAP<sup>Low</sup>), Molm-13 (IL-1RAP<sup>Int</sup>) and Mono-Mac-6 (IL-1RAP<sup>High</sup>) IL-1RAP<sup>+</sup> AML cell lines were used for *in vitro* and *in vivo* functional analysis. For the proliferation test, IL-1RAP CAR T cells were cocultured for 3 days with the 3 AML cell lines at a 1:3 Effector:Target (E:T) ratio. We demonstrated that IL-1RAP CAR T cells proliferated at  $71.5 \pm 18.09\%$ ,  $81.58 \pm 6.4\%$ , and  $80.3 \pm 15.3\%$  when cultured with the low, int and high IL-1RAP cell surface expression cell lines (n=4), respectively, independent of the IL-1RAP expression level, and these results were different than those achieved with culture in the absence of targets or with coculture of targets with untransduced T cells (C0) or MockT cells (Figure 4A). Similarly, in the presence of IL-1RAP-positive targets, CD4<sup>+</sup> [ $27.23 \pm 7.5\%$  (low),  $41.63 \pm 12.2\%$  (int), and  $58.25 \pm 12.9\%$  (high)] or CD8<sup>+</sup> [ $26.8 \pm 6.7\%$  (low),  $38.73 \pm 3.7\%$  (int), and  $71.75 \pm 17.2\%$  (high)] cells were activated and expressed IFN $\gamma$ . Interestingly, the level of IL-1RAP cell surface expression affected the levels of IFN $\gamma$  expression (Figure 4B). Finally, IL-1RAP CAR T cell cytotoxicity was evaluated

at different E:T ratios against the 3 AML cell lines and the negative IL-1RAP cell line K562. After coculture, in contrast to MockT cells or IL-1RAP CAR T cells that were unable to kill IL-1RAP-negative K562 cells, IL-1RAP CAR T cells were able to statistically eliminate CML KU812 IL-1RAP+ cells ( $99 \pm 0.1\%$ ) and all AML cell lines at different E:T ratios ( $95.3 \pm 1.52\%$  (Low);  $99 \pm 0.1\%$  (Int);  $99.3 \pm 0.57\%$  (High); a high and significant cytotoxic effect was observed at a low ratio E:T = 10:1, n=3) (figure 5A). Interestingly, specificity was confirmed by coculturing IL-1RAP CAR T cells with different healthy peri(solid)tumor tissues, thus in an inflammatory context (Supplemental Figure S4).

### **IL-1RAP CAR T cells clearly decrease the cell numbers of leukemia-derived AML tumor cell lines in vitro and decrease the tumor burden in an in vivo xenograft murine model**

To test the efficacy of IL-1RAP CAR T cells in vivo in murine xenograft models, we generated 3 luciferase-positive AML cell lines to be injected intravenously into immunodeficient NSG mice. After AML tumor engraftment, IL-1RAP CAR T cell-treated mice showed a rapid decrease in tumor burden in peripheral blood, whereas AML tumors (formed from cells with low, intermediate or high IL-1RAP cell surface expression) progressed to very high tumor burdens or until mouse death (day 21) in the untreated group and group treated with untransduced T cells (Figure 5B). The luminescence measurement confirmed a significant decrease at day 21 (average radiance  $>10^5$  p/s/cm<sup>2</sup>/sr versus average radiance  $<10^3$  p/s/cm<sup>2</sup> for untreated or T cell-treated mice and IL-1RAP CAR T cell-infused mice,  $P < 0.001$ , n=3 mice/group). Taken together, these results show that IL-1RAP CAR T cells efficiently controlled AML cell line growth in vivo.

### **IL-1RAP CAR T cells generated from healthy donors or AML patients (at diagnosis or relapse) are efficient against primary AML cells (taken from patients at diagnosis or relapse)**

We then produced IL-1RAP CAR T cells from PBMCs of healthy donors or harvested from AML patients either at the time of diagnosis or at the time of relapse. We evaluated the ability of allogeneic (from a donor or an AML patient) or autologous (from an AML patient) CAR T cells to kill primary AML blasts (taken at diagnosis or relapse) after 24 h of coculture at different E:T ratios (0:1, 1:2, 1:1, 3:1, 5:1 and 10:1). As reported in Figure 6, we confirmed that IL-1RAP CAR T cells prepared from healthy donor T cells were able to effectively kill primary AML blasts extracted at diagnosis or relapse at as low as a 1:1 E:T ratio. Moreover, IL-1RAP CAR T cells produced from T cells taken from AML patients at diagnosis or after relapse were also cytotoxic against primary AML blasts in allogeneic and autologous settings also at a low E:T ratio of 1:1. Finally, and more interestingly, autologous IL-1RAP CAR T cells produced from PBMCs taken from a relapsed AML patient with FLT3+NPM1+CD33+ disease (following several

lines of treatment: chemotherapy, midostaurin, CD33-GO, sorafenib and quizartinib) eliminated primary AML blasts taken at relapse at as low as a 1:2 E:T ratio. Hence, CART-cells obtained from HDs or newly diagnosed or even R/R AML patients had the same cytotoxic effects in eliminating AML target cells in an autologous or allogenic settings.

### **R/R primary AML blasts are targeted in the bone marrow and successfully eliminated by IL-1RAP CAR T cells in an in vivo AML patient-derived xenograft murine model**

The cytotoxicity of healthy donor IL-1RAP CAR T cells against R/R patient primary AML cells in vitro was next confirmed in an AML PDX murine model (Figure 7A). After engraftment of fully refractory AML primary blasts and human T cell treatment (control or IL-1RAP CAR T cells), the mice were sacrificed when blasts invade untreated mice. From two independent experiments, we showed that human IL-1RAP CART-cells migrate specifically to the spleen and the BM (where the AML blasts home), compare with C0 T cells, which the majority remains in the PB in mice (Figure 7B). We clearly confirmed by dPCR quantification of the CAR transgene at the day of sacrifice (day 32), that not only T cells but also IL-1RAP CAR T cells migrated to the BM and the spleen with a higher copy number in the bone marrow than in all other organs ( $p < 0.0001$ ,  $n = 3$ ) (Figure 7C, left). Additionally, we carried out a biodistribution of IL-1RAP CART-cells in AML PDX mice by dPCR of different types of organs at day 71 (Figure 7C, right). The results showed a distribution of IL-1RAP CART-cells in a high level in the spleen of mice (to kill AML blasts) and in lower levels in other types of organs with no visible toxicities detected. We performed flow cytometry analysis of AML blasts (Supplemental Figure S5) and statistically showed that the number of AML primary blasts remained higher in the BM of untreated mice than in the BM of mice treated with either C0 ( $p < 0.05$ ) or IL-1RAP CAR T cells ( $p < 0.001$ ). Interestingly, R/R AML blasts were more effectively eliminated by IL-1RAP CAR T cells than by C0 T cells ( $p < 0.01$ ). The difference between untransduced T cells and C0 T cells reflects their alloreactivity (Figure 7D). Finally, either IL-1RAP CAR or C0 T cells in the spleen and the BM, had a more effector than a memory phenotype (Figure 7E) and had an exhausted status, as evidenced by expression of 3 different checkpoints, PD-1, LAG-3 and TIM-3 (Figure 7F).

In another AML PDX murine model, in which primary AML blasts harvested at diagnosis from a patient in the favorable ELN group (NPM1+ and FLT3wt) were engrafted 3 months before treatment, we

showed a higher overall survival (OS) of PDX mice treated with IL-1RAP CAR T cells (n=5) than of untreated animals (n=2) or C0 T cell-treated (n=5) (240 days versus 98 and 126 days for untreated mice and C0 T cell-treated mice, respectively; n=5 mice per group;  $P < 0.01$ ) (Figure 7G). Importantly, no visible toxicities were observed on dead mice treated with IL-1RAP CART-cells.

## Discussion

AML remains one of the most malignant hematological diseases and has a poor outcome, especially for high-risk patients, despite improvements in hematopoietic stem cell transplantation and several other treatments. Indeed, though approximately 40%-45% of young patients and 10%-20% of elderly patients can be cured, the cure rate for R/R AML patients is lower than 10%, suggesting the necessity of new alternatives for treating patients (25). Immunotherapy using CAR T cells, particularly CD19 CAR T cells, which have shown remarkable results in ALL and lymphoma (26) as well as in MM (27), is an interesting strategy to explore in AML. Several cell surface AML antigen targets, mainly CD123, CD33, CLL1, FLT3, and CD44v6, are under investigation at preclinical stages; however, none are truly perfect targets due to their hematopoietic toxicity (for example, they can induce profound myeloablation) (28). Thus, there is a need to continue investigating new AML markers targetable by CAR T cells.

In this work, we investigated IL-1RAP, a novel and unexplored AML cell surface marker expressed by leukemia cells but not healthy hematopoietic stem cells, progenitors, or T and B lymphocytes, though it is expressed by circulating monocytes (19). IL-1RAP, the accessory protein of the IL-1 receptor, plays a crucial role in immunity, inflammation and cancer. It is involved in a tumor microenvironment that favors leukemia proliferation (12, 13), potentiates multiple oncogenic signaling pathways and oncogenic pathways and contributes to AML oncogenesis (11, 29). The need for IL-1RAP for tumorigenesis and its overexpression in cancer make it an interesting target for avoiding further selection and escape because of antigen loss after CAR T cell immunotherapy, as occurs with CD19 (30) or BCMA (31) CAR T cell immunotherapies. Michaud et al. demonstrated that soluble IL-1RAP is present at a low level in healthy people, and Warda et al. showed that high concentrations of soluble IL-1RAP did not result in staining of IL1RAP CAR-expressing T cells and did not affect CAR T cytotoxicity against IL1RAP+ target cells.

Currently, only a monoclonal antibody targeting IL-1RAP (nidanilimab) is under investigation in phase I/II clinical trials (NCT03267316 and NCT04452214, [www.clinicaltrials.gov](http://www.clinicaltrials.gov)) in pancreatic and triple-negative breast cancer. This antibody is used as a blocking agent in association with pembrolizumab or to enhance chemotherapy (15) and has shown a manageable safety profile (22). Regarding toxicity, in addition to our previous work (19), where we showed absence of targeting of the healthy hematopoietic stem cells, we give here another argument in favor of IL-1RAP targeting by showing absence of degranulation of IL-1RAP CAR T cell against healthy peritumoral tissues from different solid tumors, thus in an inflammatory context.

We demonstrated that IL-1RAP mRNA is expressed in all AML FAB subtypes and that the IL-RAP protein is expressed at different levels on the cell surface of AML blasts independent of ELN

classification. Interestingly, we showed in 30 AML patient cohorts that most primary IL-1RAP<sup>+</sup> blasts coexpressed CD123, making this target suitable for bispecific CAR T cell targeting, which will increase specificity via “and/or” CAR activation signaling and presumably without increasing toxicity (32).

Following our previous work in CML demonstrating a proof of concept for targeting IL-1RAP (19), we here showed in a preclinical study that this new alternative treatment has efficacy in AML using an innovative approach: targeting the cell surface marker IL-1RAP, which is expressed by both leukemic stem cells and primary AML blasts. The fact that the cytotoxicity and efficacy of IL-1RAP CAR T cells were the same against both cells with low IL-1RAP and cells with high IL-1RAP expression (primary AML cells and AML cell lines) suggests their efficacy.

Interestingly, while chemotherapy and other targeted therapies are recognized as early-line treatments currently, it is important to demonstrate that T cells from R/R AML patients can be used for CAR T cell generation. Indeed, nonresponder AML patients are eligible for targeted therapies following chemotherapy, and some of these patients receive FLT3 tyrosine kinase inhibitor drugs (33) that may interact with intrinsic tyrosine kinases (34) involved in CAR transduction signals, such as Fyn, ZAP-70, and Lck(35). We were able to transduce T cells taken from AML patients at diagnosis and also at relapse with high efficiency, and we demonstrated that autologous CAR T cells are effective in vitro against primary R/R AML blasts. Moreover, the presence of a suicide gene within our lentiviral construct will protect against adverse events in cases of unexpected leukemic cell transduction, as previously shown (18). This mechanism will also help in the case of persistent adverse events affecting monocytes by functioning as a safety switch in order to eliminate CAR T cells and allow a mature myeloid cell recovery. The functionality of the safety switch system has been evaluated in killing persistent IL-1RAP CAR T cells (18).

Characterization of the IL-1RAP CAR T cells at the end of the production process (supplemental Figure S6) and when in PDX mice, showed that they acquire the expression of exhaustion markers such as PD1, TIM3 and LAG3. Interestingly, the expression of these markers did not affect the function of IL-1RAP CART-cells in killing AML blasts. Further studies are needed in order to evaluate the expression of these markers' ligands (such as PDL1) on the surface of AML cells. Importantly, these results allow using inhibitors of these markers in combination with the CART-cells in case of unqualified patient's T lymphocytes (because of early treatment lines), their activity can be increased by inhibiting the exhaustion markers (36). Importantly, the persistence of IL-1RAP CART-cells in the bone marrow and the spleen of PDX mice was confirmed by dPCR for approximately 2 months after treatment with no visible side effects on treated mice. This persistence may be due to the co-stimulation molecule 4-1BB present in the CAR construct.

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

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## Disclosure of Conflicts of Interest

- CF: Consulting: Novartis, BMS, Incyte, and Daiichi. Research grants: Novartis, Daiichi, Bellicum Pharmaceutical
- MD and CF: co-founders of CanCell Therapeutics SAS, Besançon, France
- SD: Founder of ErVaccine
- VA: ErVaccine consultant

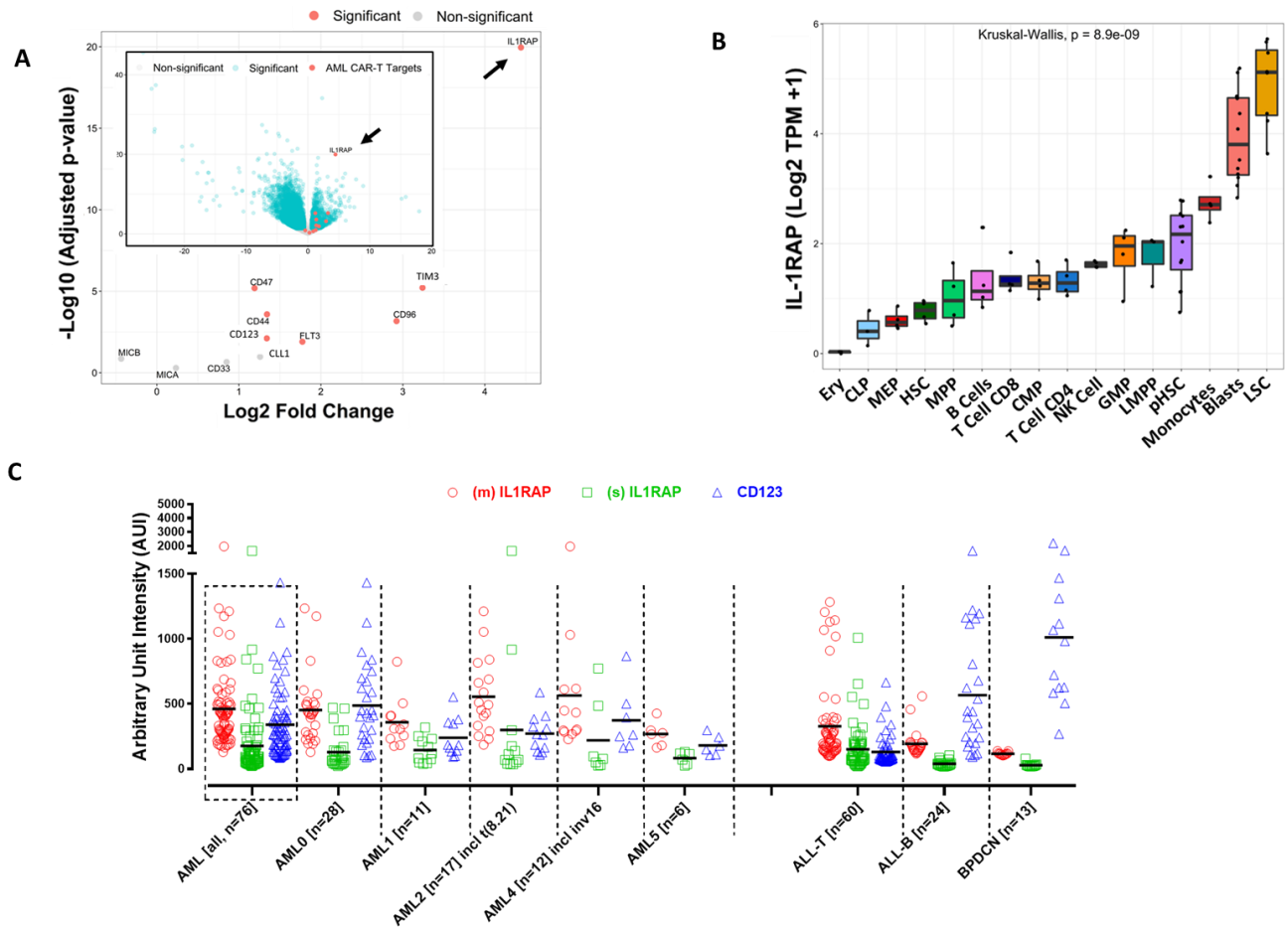
## Author's contributions

- **Conceptualization and study design:** RT, WW, MD, CF
- **Methodology, experiments and data analysis:** RT, WW, ES, MNdR, LB, RH, CN, SB, FR, MD, CF
- **Original draft and figure construction:** RT, WW, MNdR, CN, VA, ES, RH, MD CF
- **Clinical advice and patient collection:** AB, XR, YD, ED, CF
- **Writing, review and/or revision of the manuscript:** VA, SD, WW, RT, MD, CF
- **Overall supervision and research funding:** WW, MD, CF

All authors reviewed the manuscript and contributed to improving the paper.

## Figures and legends

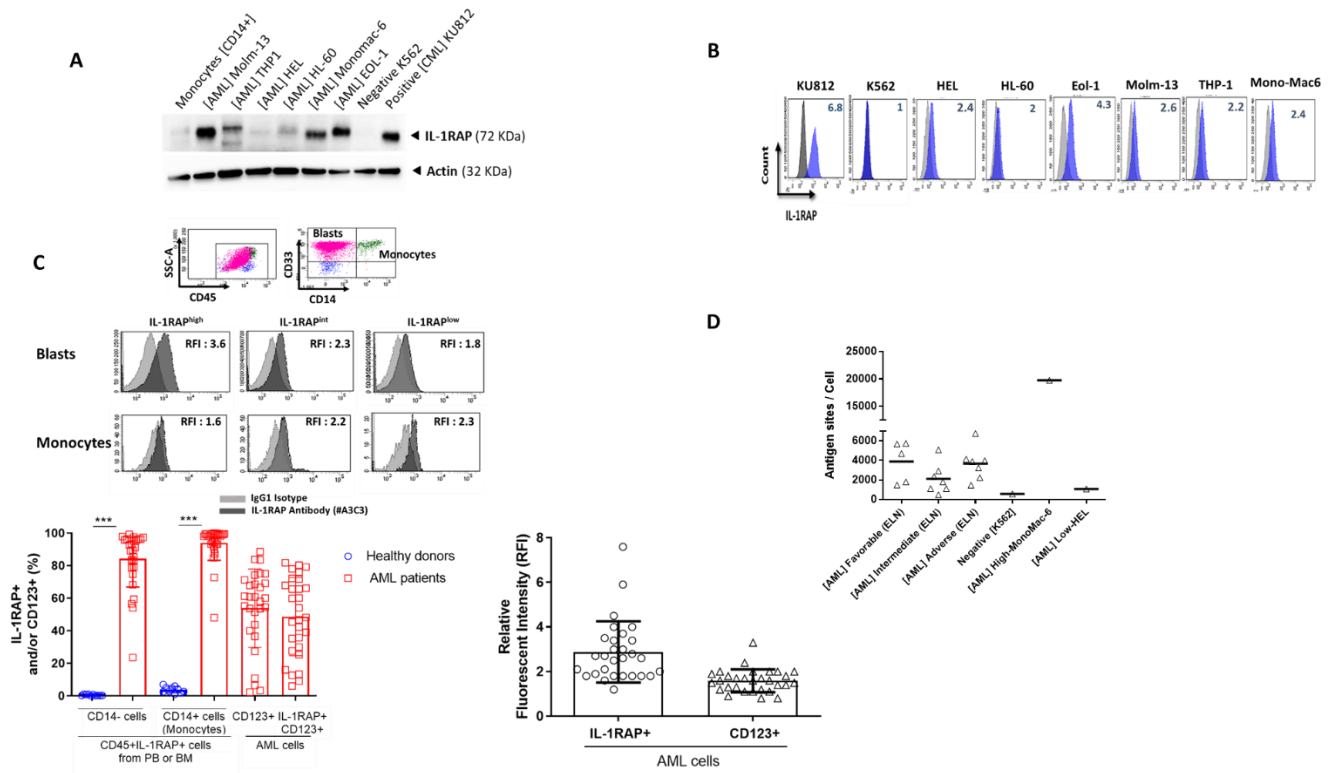
Figure 1



**Figure 1: In silico analysis of IL-1RAP mRNA expression in an AML public database and Affymetrix gene expression profiling**

**A/** Volcano plot showing differentially expressed genes in AML leukemic stem cells compared to normal hematopoietic stem cells. Light green or red dots represent significant AML markers. The fold change is presented on the x-axis (expressed as log2), and the p-value (expressed as the  $-\log_{10}$  adjusted p-value) is presented on the y-axis. Genes with log2 fold change  $> |1|$  and adjusted p-value  $< 0.05$  and with log2 mean expression  $> -5$  were tagged as significant. **B/** IL-1RAP gene expression (expressed as the log2 TPM+1 value) averaged over the replicates of each sample across AML blasts and leukemic stem cells compared to normal peripheral blood or bone marrow cells. Ery: erythroid progenitor, CLP: common lymphoid progenitor, MEP: megakaryocyte/erythrocyte progenitor, HSC: hematopoietic stem cell, MPP: multipotent progenitor, CMP: common myeloid progenitor, NK: natural killer, GMP: granulocyte/macrophage progenitor, LMPP: lymphoid-primed multipotent progenitor, pHSC: hematopoietic stem cell progenitor, LSC: leukemic stem cell. **C/** Transcriptome analysis of the IL-1RAP mRNA transcripts encoding the cell surface (m) and soluble (s) IL-1RAP isoforms in AML patients classified according to FAB classification. CD123 mRNA expression is given for comparison. ALL: acute lymphoid leukemia. BPDON: blastic plasmacytoid dendritic cell neoplasm.

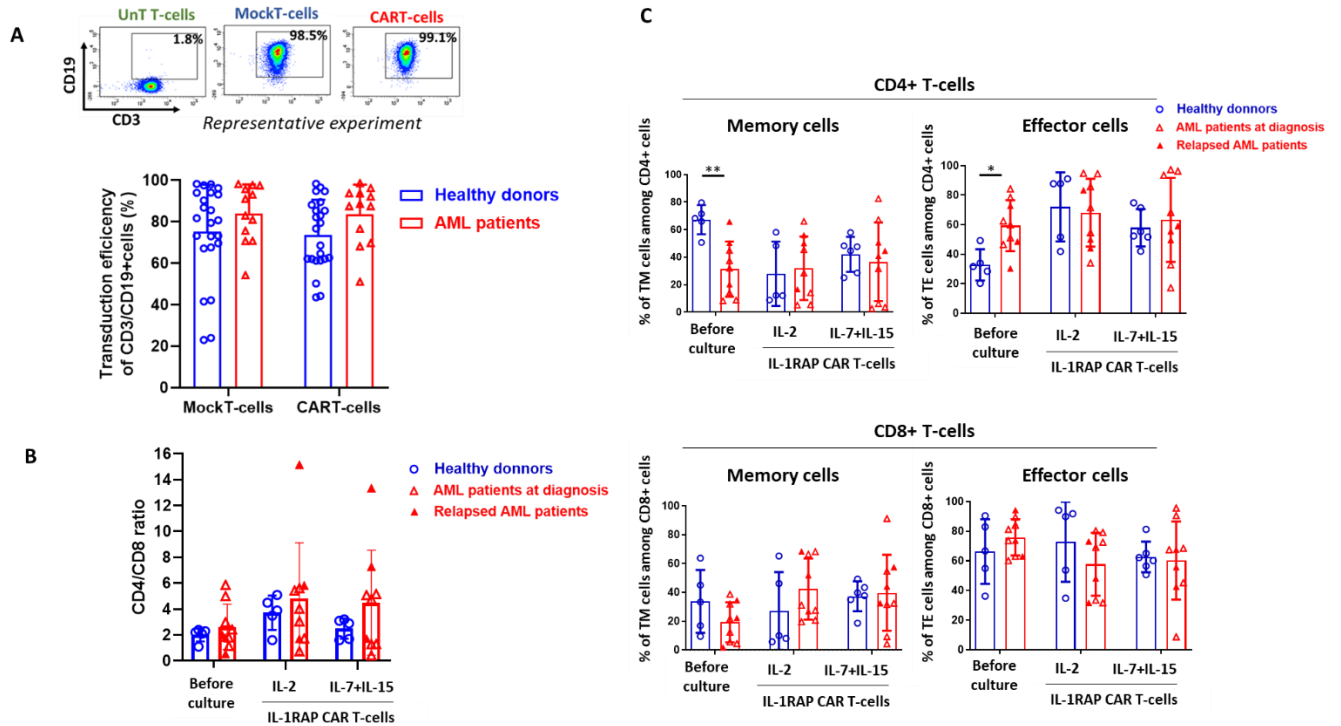
**Figure 2**



**Figure 2: Expression of the IL-1RAP protein in AML blasts.**

**A/** Western blot analysis of IL-1RAP in different AML cell lines and monocyte subpopulations derived from sorted primary CD14+ cells. The membrane was hybridized with the #A3C3 IL-1RAP mAb. The membrane isoform of IL-1RAP was detected at the expected size (72 kDa). Protein load was assessed by actin detection at 43 kDa. The K562 and KU812 cell lines served as negative and positive controls, respectively. **B/** Flow cytometry detection of IL-1RAP cell surface expression (blue histogram) compared to that of the IgG-1 isotype (gray histogram). The calculated RFI is provided. **C/** Upper: representative gating strategies for primary AML blasts (CD45+/CD14-/CD33+ or CD33-) and monocytes (CD45+/CD14+/CD33+). Representative histograms showing the IL-1RAP cell surface expression levels (dark gray) on primary blasts (CD14-) and monocytes (CD14+) compared to the IgG-1 isotype levels (light gray). The RFI was calculated by comparing the IL-1RAP and isotype signals. Bottom left: percentages of IL-1RAP+ cells in AML (red rectangle) blasts and AML LSCs as well as CD14+ monocytes in AML patient samples compared to healthy (blue rectangle) hematopoietic cells (CD14-) and normal monocytes. The percentages of CD123+ and CD123+/IL-1RAP+ cells are also provided. Bottom right: IL-1RAP and CD123 RFIs in CD45+/CD14- AML primary blasts (n=29). **D/** Quantification of the absolute number of IL-1RAP antigen sites on the surface of cells according to ELN classification using the #A3C3 IL-1RAP monoclonal antibody. K562 cells served as a negative control. The Mono-Mac-6 and HEL AML cell lines show high and low IL-1RAP expression, respectively.

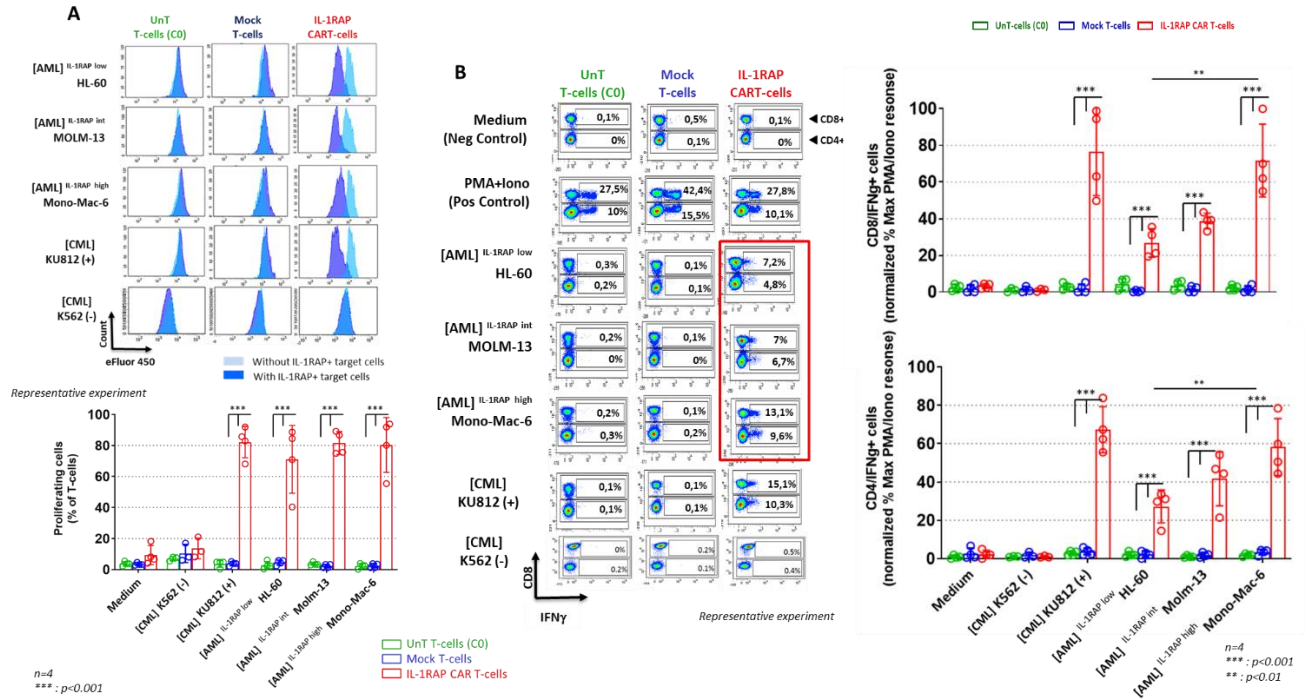
**Figure 3**



**Figure 3: Production and characterization of IL-1RAP CAR T cells taken from healthy donors or AML patients at diagnosis or relapse**

**A/** Generation of mock or IL-1RAP CAR gene-modified T cells using 30X concentrated supernatant. Upper: representative gating strategy after staining with anti-CD19 and anti-CD3 mAbs. Lower: genetically modified T cells obtained from healthy donors (n=24, blue rectangle) and from AML patients at diagnosis or relapse (n=12, red rectangle), all from different independent experiments. The results are presented as the mean  $\pm$  standard deviation (SD). \*\*\*\*:  $p < 0.0001$ . **B/** Identifying the CD4+/CD8+ ratio of IL-1RAP CART-cells produced from healthy donors (circles, blue lines) and from samples from AML patients at diagnosis (empty triangles, red lines) or at time of relapse (filled triangles, red lines) and cultured in medium with IL-2 or IL-7/IL-15 cytokines. **C/** Effector (CD95+/CCR7-)/memory (CD95+/CCR7+) phenotyping of CAR T cells (CD4+ or CD8+) generated from T cells taken from healthy donors (circles, blue lines) or from AML patients at diagnosis (empty triangles, red lines) or at relapse (filled triangles, red lines) and cultured in medium with IL-2 or IL-7/IL-15 (\*:  $p < 0.0074$ , \*\*:  $p < 0.0118$ ).

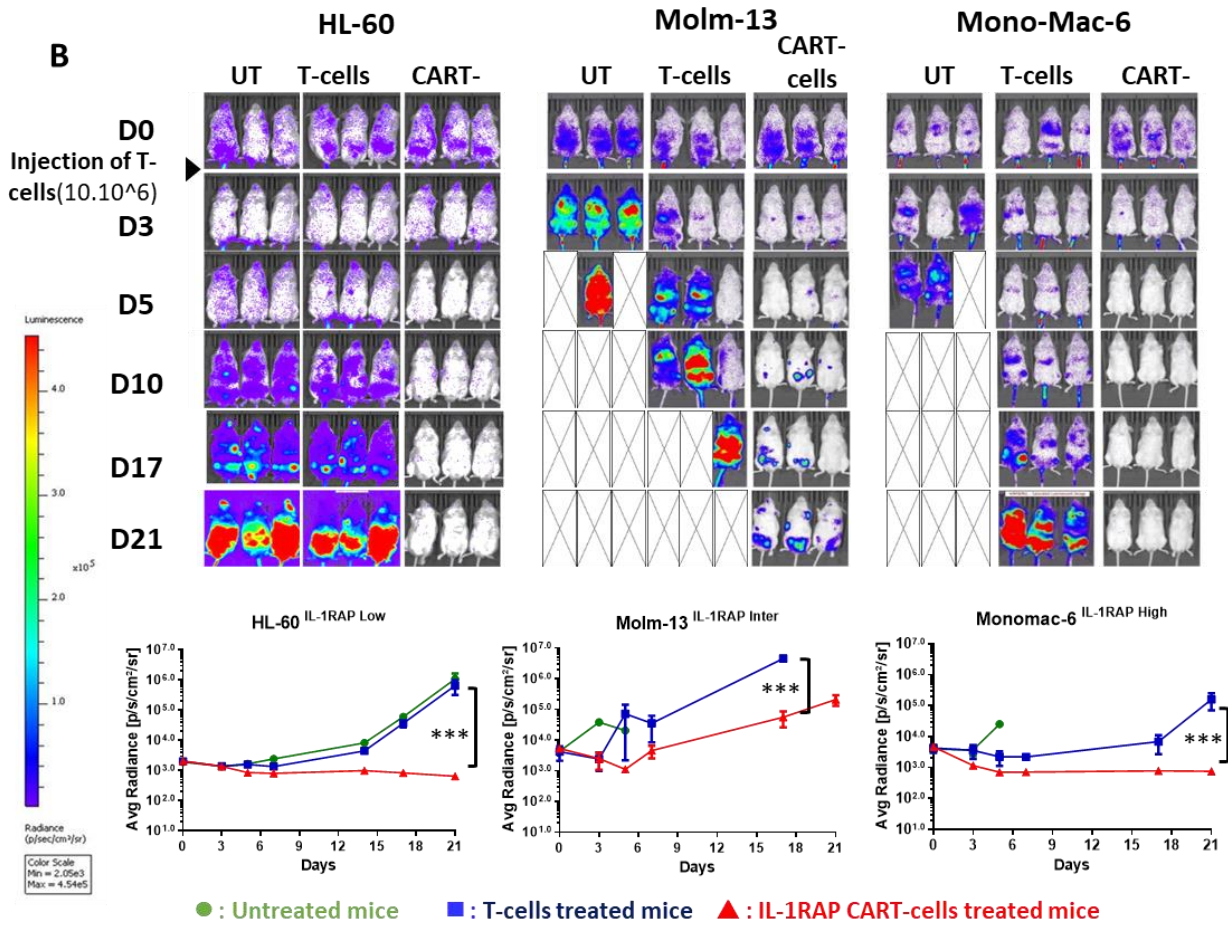
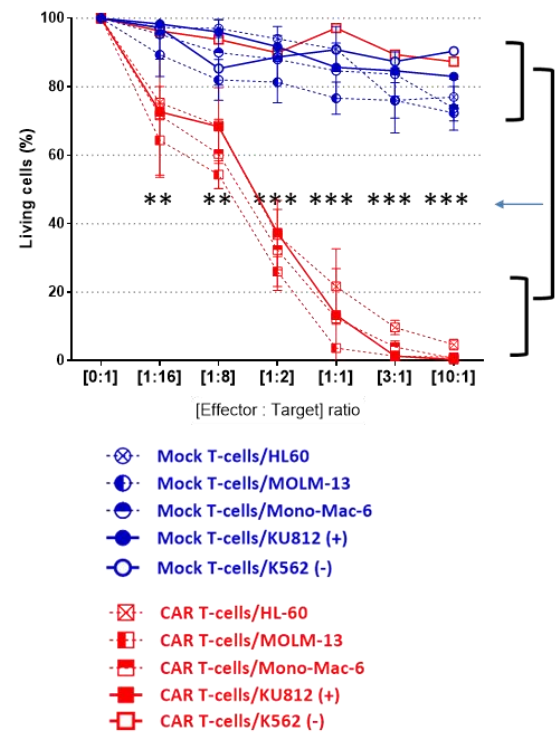
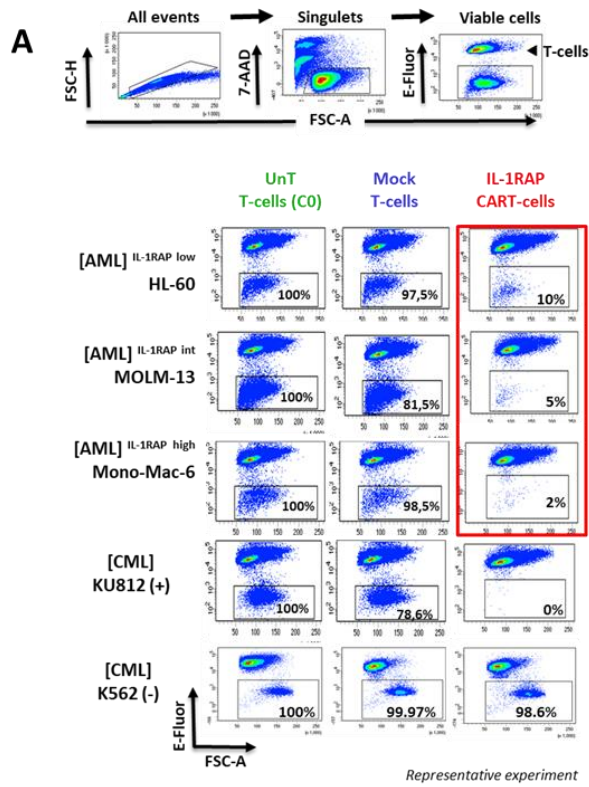
**Figure 4**



**Figure 4: In vitro proliferation assay and determination of the IFN $\gamma$  expression of IL-1RAP CAR T cells following coculture with AML cell lines**

**A/** Untransduced effector T (UnT) cells and T cells transduced with either mock or the IL-1RAP CAR were labeled with eFluor 450 diluted at 1:1,000 and cocultured (72 h at an E:T ratio of 3) or not with 3 AML cell lines with different levels (low, intermediate and high) of IL-1RAP cell surface expression. The K562 and KU812 cell line were used as targets and as negative and positive IL-1RAP expressers, respectively. Dilution of the eFluor level enabled estimation of effector cell division and thus proliferation after contact with target cells (dark blue histogram), which was compared to the division and proliferation of cells cultured without target cells (light blue histogram). Upper: representative experiment scheme. Lower: percentage of proliferative IL-1RAP CAR T cells for n=4 independent experiments (below). Mean  $\pm$  SD of three independent experiments. \*\*\*: p<0.001. **B/** Left: intracellular IFN $\gamma$  analysis by flow cytometry of CD3+/CD19+/CD8+ or CD3+/CD19+/CD8- subpopulations of UnT cells, mock T cells and IL-1RAP CAR T cells cocultured overnight with AML cell lines with various levels of IL-1RAP cell surface expression at an E:T ratio of 1:5. Cultures with medium alone and with PMA/ionomycin were used as negative and positive controls, respectively. The K562 and KU812 cell lines were used as targets and as negative and positive IL-1RAP expressers, respectively. Right: percentage of intracellular IFN- $\gamma$  (relative to PMA/ionomycin) expressed by stimulated CD8+ and CD4+ (CD8-) IL-1RAP CAR T cells in response to the IL-1RAP antigen (lower). Mean  $\pm$  SD of n=3 independent experiments. \*\*\*: p<0.001, \*\*: p<0.01.

**Figure 5**





**Figure 5: IL-1RAP CAR T cells are cytotoxic against AML cell lines in vitro and in vivo in an AML xenograft NSG mouse model.**

**A/** Left: Untransduced (C0) T cells, MockT cells and IL-1RAP CAR T cells generated via genetic modification of healthy donor PBMCs were cultured at different E:T ratios for 24 h, with target AML cell lines expressing different levels of IL-1RAP. The K562 and KU812 cell lines were used as targets and as negative and positive IL-1RAP expressers, respectively. Viable cells were gated based on 7-AAD labeling via flow cytometry, and T cells were distinguished from tumor cells by eFluor labeling. The percentage of remaining tumor cells within the eFluor negative gate is provided. Right: percentages of living cells within the tumor cell population after coculture at different E:T ratios with MockT cells (blue circle, dotted line) or IL-1RAP CAR T cells (red square, dotted line). The results are from n=3 independent experiments. Solid lines (blue or red) represent coculture with IL-1RAP+ or IL-1RAP- targets. \*\*: p<0.01; \*\*\*: p<0.001. **B/** Six- to 8-week-old NSG mice were irradiated at a dose of 250 cGy (n=3/group) before tail vein (i.v.) injection of  $1.10^6$  HL-60, Molm-13 or Mono-Mac-6 luciferase+/IL-RAP+ cells (which have low, intermediate and high expression of IL-RAP, respectively). After tumor engraftment (D0),  $10.10^6$  C0 T cells or IL-1RAP CAR T cells were i.v. injected. Mice engrafted with AML tumor cell lines but not treated (UT) or treated with T cells were used as controls. BLI was performed until the tumor load was too high or the mice died (X) in the control groups. The radiance of the *in vivo* bioluminescent signal (radiance p/s/cm<sup>2</sup>/sr) collected using the IVIS Illumina III (Perkin Elmer) is shown. (►) indicates the time of IL-1RAP CAR T cell injection. \*\*: p<0.01; \*\*\*: p<0.001.

Figure 6

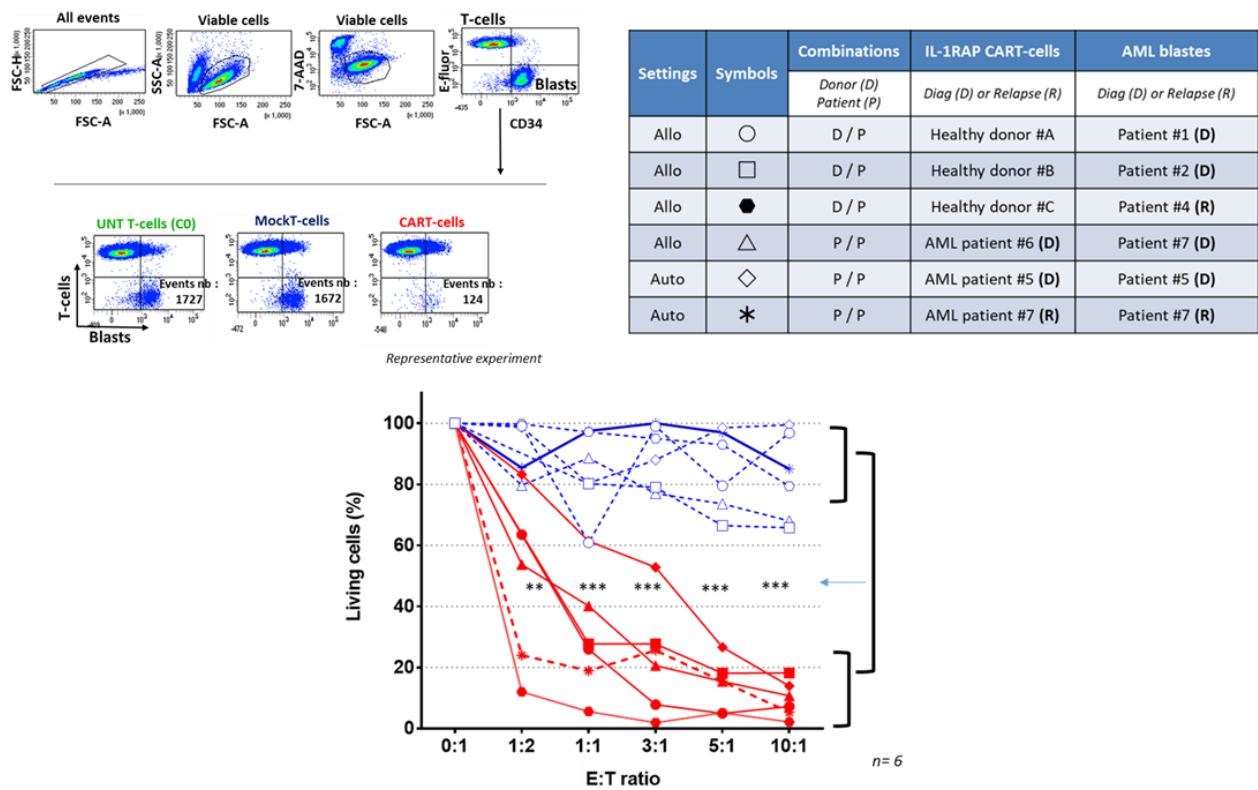
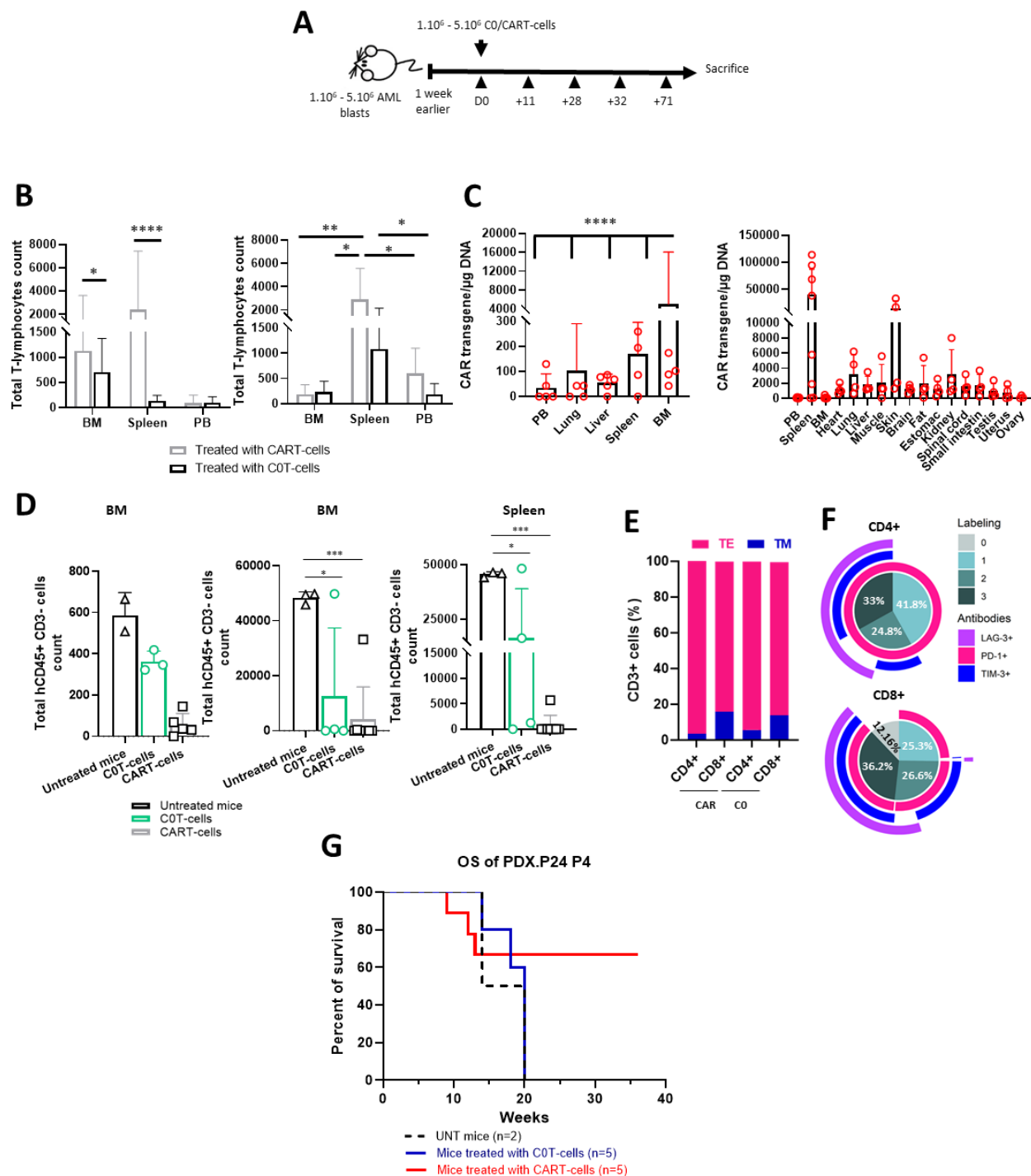


Figure 6: IL-1RAP CAR T cells are cytotoxic against AML primary cells taken from patients at diagnosis or at relapse in-vitro and in-vivo in an AML patient-derived xenograft (PDX) NSGS mouse model.

Gating strategy for discrimination of effector T cells (cells stained with eFluor) from primary AML blasts (human CD34+ cells). Primary AML blasts taken from patients at diagnosis or relapse were cocultured at different E:T ratios with effector T cells [untransduced (UnT and C0) or transduced with mock or the CAR lentiviral vector] harvested from healthy donors or AML patients at diagnosis or relapse. Alloreactivity was accounted for by subtracting the UnT (C0) cell coculture value for all cytotoxicity tests. The absolute number of events within the AML tumor cell gate is provided. The percentages of viable CD34+/IL-1RAP+ cells after coculture with MockT cells (blue line) or IL-1RAP CAR T cells (red line) for n=6 independent donor:patient combinations are also reported. Solid lines represent coculture of effector T cells (mock or IL-1RAP CAR T cells) generated from PBMCs taken from an AML patient at the time of relapse with the same patient's own R/R primary AML blasts. Percentage of living cells was calculated from absolute cell count using truccount tubes. \*\*: p<0.01; \*\*\*: p<0.001.

### Figure 7



**Figure 7: IL-1RAP CAR T cells are cytotoxic against AML primary cells taken from a R/R patient in-vivo in an AML patient-derived xenograft (PDX) NSGS mouse model.**

**A/** NSGS mice were injected with  $1.10^6$  -  $5.10^6$  AML blasts taken from a relapsed patient treated with multiple therapies. One week after human AML blasts first appeared in the PB, the mice were treated with  $1.10^6$  -  $5.10^6$  CAR T cells or C0 T cells with an untreated group. Mice were sacrificed 32-71 days after treatment, and leukemic cells and human T cells (untransduced or CAR-transduced T cells) levels in PB or harvested organs were monitored. In figures 7B-C-D: left graphs represent experiment 1 and right graphs represent experiment 2. **B/** Total T lymphocyte counts within the BM, spleen and PB assessed by flow cytometry. **C/** IL-1RAP CAR transgene copies per  $\mu\text{g}$  of DNA quantified by digital PCR (the PCR targeted the 4.1BB/CD3z junction) in different types of organs (PCR was performed in duplicate for  $n=4$  mice) (\*:  $p<0.05$ , \*\*:  $p<0.01$ , \*\*\*\*:  $p<0.0001$ ). Statistical significance was calculated with the Mental-Cox (log rank) test,  $P<0.01$ . **D/** Total human blast count (mCD45-/hCD45+/CD3-) in the BM and the spleen of untreated mice (black bar, triangle,  $n=3$ ), mice treated with control untransduced C0 T cells (green bar, circle,  $n=4$ ) and mice treated with IL-1RAP CAR T cells (solid gray bar, square,  $n=8$ ) quantified by flow cytometry (\*:  $p<0.05$ , \*\*\*:  $p<0.01$ , \*\*\*\*:  $p<0.001$ ). **E/** Percentage of effector (TE, pink bars) and memory (TM, blue bars) CAR T cells (gated on CD3+/CD19+ and CD4+ or CD8+) harvested from the spleen at the time of sacrifice of mice with xenografted tumors from a R/R AML patient ( $n=6$ ) treated with either C0 or IL-1RAP CAR T cells. **F/** Number of checkpoint markers (PD-1, LAG-3, and TIM-3) expressed on CD3+/CAR+ cells (CD4+ or CD8+) in the spleen of six PDX mice (with tumors derived from a R/R AML patient) after sacrifice. **G/** Kaplan-Meier survival curve representing the survival of untreated mice or mice treated with C0 or CAR T cells (week 0). Patient leukemic cells were engrafted 3 months before. Effector cells were infused when AML blast cells started to be detectable in PB. Statistical significance was calculated with the Mental-Cox (log rank) test,  $P<0.01$ .

## Supplemental

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

*Trad et al*

## Supplemental material and Methods

### Transcriptomic and RNAseq *in silico* analyses

Gene expression profiling (GEP) was performed using Human Genome U133 Plus 2.0 arrays (Affymetrix/ThermoFisher, Santa Clara, CA). Gene expression data available in the Gene Expression Omnibus (22) database were included for 32 peripheral blood samples from healthy donors (GSE48060: GSM1167102-21 and GSM1167123; GSE54992: GSM1327541,42,44,46,49,50, GSE20489: GSM514766,71,76,81,86) and 26 bone marrow samples from healthy donors (GSE3526: GSM80576, 577, 602-604; GSE18674: GSM463920; GSE32725: GSM813068-71; GSE33075: GSM818813-19,23,24; GSE41130: GSM1008985-91).

### RNAseq dataset *in silico* analysis

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

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

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

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

All statistical analyses were performed using R software v 3.6.0.

### **Lentiviral vector construction**

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

We designed a CAR plasmid (pSDY-iC9-IL-1RAPCAR-dCD19) using a self-inactivating lentiviral backbone carrying the scFv of mAb #A3C3, an iCASP9 safety cassette and a cell surface-expressed marker ( $\Delta$ CD19) for monitoring and potential cell selection (Figure S1A). The three transgenes were all separated by peptide cleavage sequences (P2A and T2A) and under the control of the elongation factor 1 alpha (EF1) promoter in addition to the enhancer sequence SP163. The mock plasmid carried the same construct but lacking the gene sequence encoding the specific scFv of IL-1RAP.

### **Determination of IL-1RAP mRNA expression and western blotting**

IL-1RAP mRNA was extracted from AML cell lines and primary cells from AML patients ( $5 \times 10^6$  cells lysed in RLT buffer) following the manufacturer's protocols (RNAeasy Mini Kit, Qiagen, Cat n°: 74106). The relative IL-1RAP mRNA expression was determined by RT-qPCR using the Hs\_00895050\_m1 TaqMan qPCR gene expression assay (Thermo Fisher Scientific) targeting the mRNA variant codon for the intracellular domain of the cell surface protein. PCR was performed using a CFX96 TM Real Time System (C1000TM Thermal Cycler, BioRad) according to the following program: enzymatic activation at 95°C within 20 sec-3 min (1 cycle), denaturation at 95°C within 1-3 sec (40 cycles), and pairing/elongation/acquisition at 60°C for 15 sec (40 cycles). The results were analyzed using BioRad CFX Manager Software.

For western blotting, AML cell lines ( $5 \times 10^6$  cells) were sonicated and suspended in RIPA buffer supplemented with a protease inhibitor cocktail. Proteins were transferred to membranes and probed overnight with primary antibodies targeting IL-1RAP: #A3C3 mAb (clone A3C3, 8423A3C3, Diaclone)

(diluted 1:3e3) and anti- $\beta$ -actin antibody (clone AC15, #A5441, Sigma-Aldrich, St Louis, MO, USA) (diluted 1:10e3) as an internal loading control. For immunodetection, an HRP-anti-mouse IgG was added (#515-035-062, Jackson ImmunoResearch, USA). Detection was performed by chemiluminescence using a camera and Bio-1D software (Vilber-Lourmat).

### **Functional tests in vitro**

Effector CO (untransduced), mock and IL-1RAP CAR T cells ( $1.10^6$  cells/mL) were labeled with Cell Proliferation Dye e-Fluor-V450 (ref: 65-0842-85, eBioscience) following the manufacturer's protocol. Labeled cells were cultured in a 96-well plate at an appropriate E:T ratio with target cells at 37°C for the needed time. The final volume/well was of 200  $\mu$ L. After coculture, cells were labeled with 7-AAD (Cat N°: 51- 68981E, BD) and anti-CD3-PE, anti-CD19-APC (for evaluating T-cells) and anti-IL-1RAP (for evaluating target cells) antibodies.

A CD107a-PE degranulation assay (Clone H4A3, BD Biosciences) and an intracellular IFN- $\gamma$ -PE expression assay (BD Cytofix/Cytoperm™ Plus Kit, ref: 555028) were performed according to the manufacturers' protocols.

### **Xenograft murine models and the patient-derived xenograft murine model**

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

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



## Supplemental Table

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

Fluorochrome	Antibody	Provider	Reference
FITC	Mouse anti-human IL-1RAP (BR-58)	Diaclone	DSP- FF190327
FITC	Mouse IgG-1 isotype control (B-Z1)	Diaclone	857.071.010
FITC	Mouse anti-human IL-1RAP (BL-43)	Diaclone	8423A3C3
BV421	Mouse anti-human CD34	BD Biosciences	562577
APC	Mouse anti-human CD38	BD Biosciences	345807
V500	Mouse anti-human CD45	BD Biosciences	560777
APC-H7	Mouse anti-human CD14	BD Biosciences	641394
PerCP-Cy5.5	Mouse anti-human CD33	BD Biosciences	333146
Vio-Blue	Mouse anti-human CD3	Miltenyi Biotec	130-094-363
APC	Mouse anti-human CD19 (LT19)	Miltenyi Biotec	130-113-165
APC	Mouse anti-human CD34 (581 RUO)	BD Biosciences	555824
PE	Mouse anti-human IFN- $\gamma$ (27 RUO)	BD Biosciences	559327
FITC	Mouse anti-human CD8 (B-Z31)	Diaclone	854.961.010
PE-Cy7	Mouse anti-human CD123	Biolegend	306010
PE-Cy7	Mouse IgG-1 isotype control	Biolegend	400-126
PE	Mouse anti-human CD3 (B-B11)	Diaclone	954.012.010
Pacific-Blue	Mouse anti-human CD3	BD Biosciences	558117
PerCP-Cy5.5	Mouse anti-human CD45	BD Biosciences	317428
BV510	Mouse anti-human PD-1	BD Biosciences	563076
BV510	Mouse IgG-1 isotype control	BD Biosciences	562946
FITC	Mouse anti-human LAG-3	Biolegend	369308
FITC	Mouse IgG-1 isotype control (MOPC-1)	Biolegend	400110
APC-H7	Mouse anti-human TIM-3	Biolegend	345026
APC-H7	Mouse IgG-1 isotype control	Biolegend	400128
BV650	Rat anti-mouse CD45	BD Biosciences	563410
BV500	Mouse anti-human CD45	BD Biosciences	560777
BV421	Mouse anti-human CD3 (UCHT1)	BD Biosciences	562426
BV510	Mouse anti-human CD8 (SK1)	BD Biosciences	563919

PE	Mouse anti-human CD95 (DX2)	BD Biosciences	555674
PE	Mouse IgG-1 isotype control (MOPC-21)	BD Biosciences	555749
PECy7	Mouse anti-human CD45RA (HI100)	Sony	RT2120630
PerCP-Cy5.5	Mouse anti-human CD45RO (UCHL1)	Sony	RT2121110
FITC	Mouse anti-human CCR7 (150503)	R&D Systems	FAB197F-100
FITC	Mouse IgG-2a isotype control (20102)	R&D Systems	IC003F
PE	Streptavidin	BD Biosciences	5150605051

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

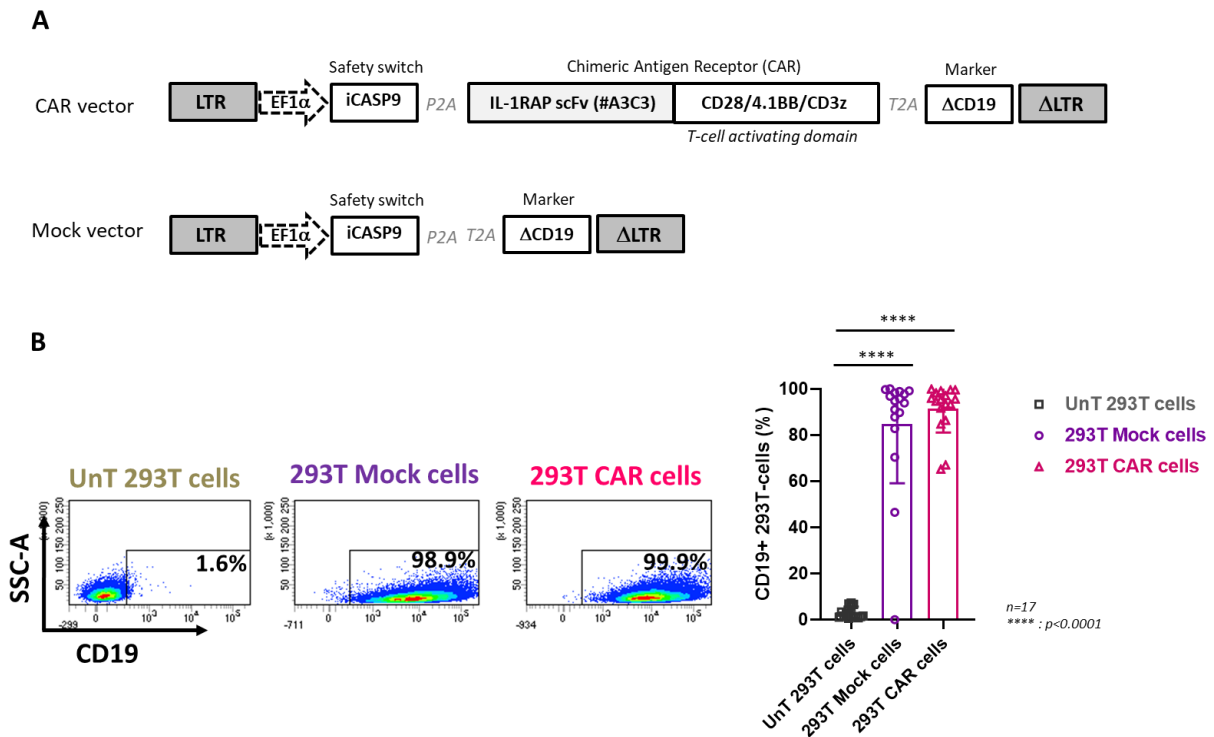
**Supplemental Table 2 : Clinical, cytogenetic and molecular information of AML patients from the Filo cohort.**

AML patients	Sexe	% Blastosis	Groupe	Karyotype	NPM1	FLT3
1	Female	21	Favorable	Normal	Mutated	Wild type
2	Female	53	Favorable	Normal	Mutated	Wild type
3	Male	42	Favorable	Normal	Mutated	Wild type
4	Female	29	Favorable	Normal	Mutated	Wild type
5	Male	25	Favorable	Normal	Mutated	Wild type
6	Male	80	Favorable	Normal	Mutated	Wild type
7	Male	43	Favorable	Normal	Mutated	Wild type
8	Female	30	Favorable	Normal	Mutated	Wild type
9	Female	54	Favorable	Normal	Mutated	Wild type
10	Male	80	Favorable	Normal	Mutated	Wild type
11	Female	63	Intermediate	Normal	Mutated	Mutated
12	Male	81	Intermediate	Normal	Wild type	Mutated
13	Male	53	Intermediate	Normal	Wild type	Wild type
14	Male	39	Intermediate	Normal	Mutated	Mutated

15	Male	95	Intermediate	Normal	Mutated	Mutated
16	Male	99	Intermediate	Normal	Wild type	Wild type
17	Female	84	Intermediate	Normal	Wild type	Mutated
18	Male	84	Intermediate	Normal	Wild type	Mutated
19	Female	99	Intermediate	Normal	Mutated	Mutated
20	Male	22	Intermediate	Normal	Wild type	Wild type
21	Female	53	Adverse			
22	Male	93	Adverse			
23	Female	82	Adverse			
24	Female	72	Adverse			
25	Male	70	Adverse			
26	Male	75	Adverse			
27	Female	65	Adverse			
28	Male	34	Adverse			
29	Female	82	Adverse			
30	Male	53	Adverse			

## Supplemental Figures

Figure S1



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

Figure S2

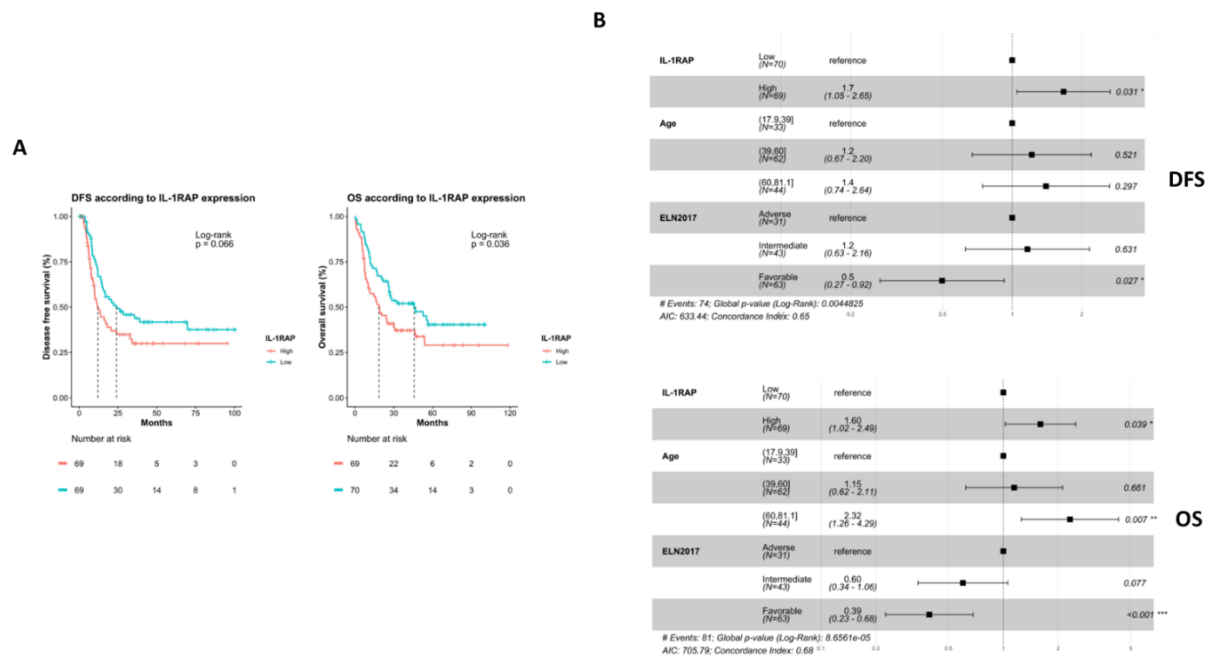
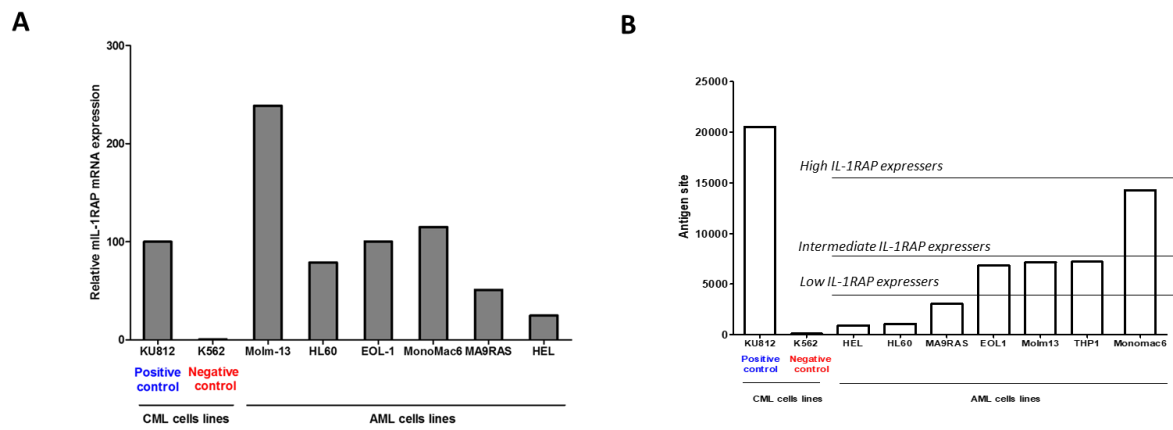
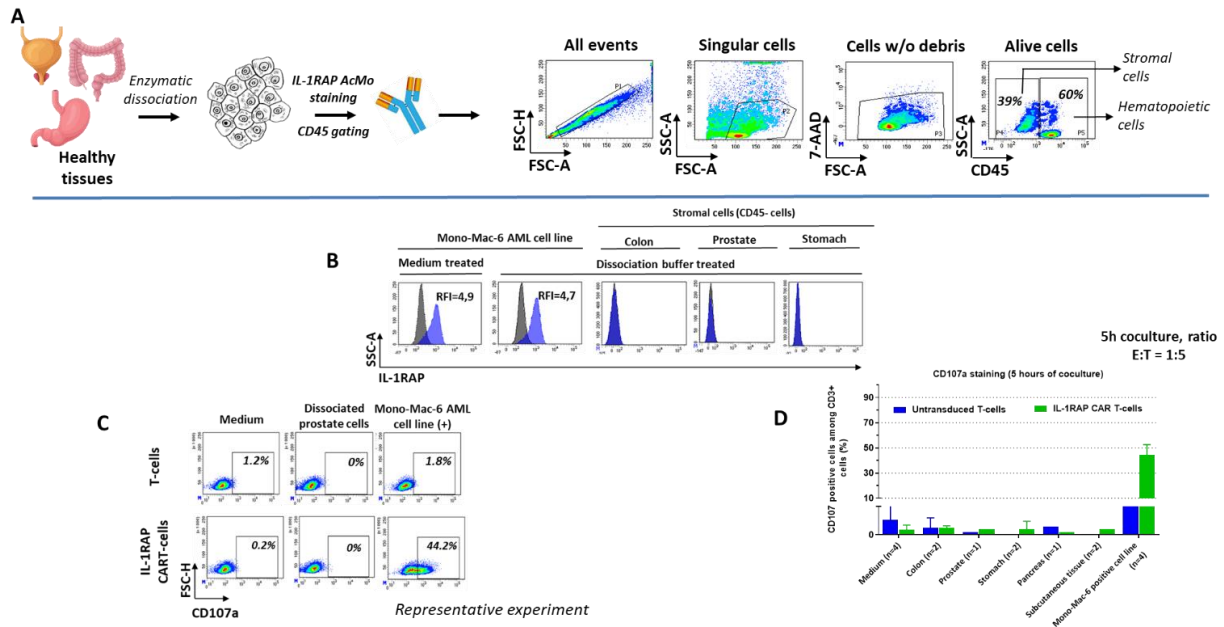


Figure S3



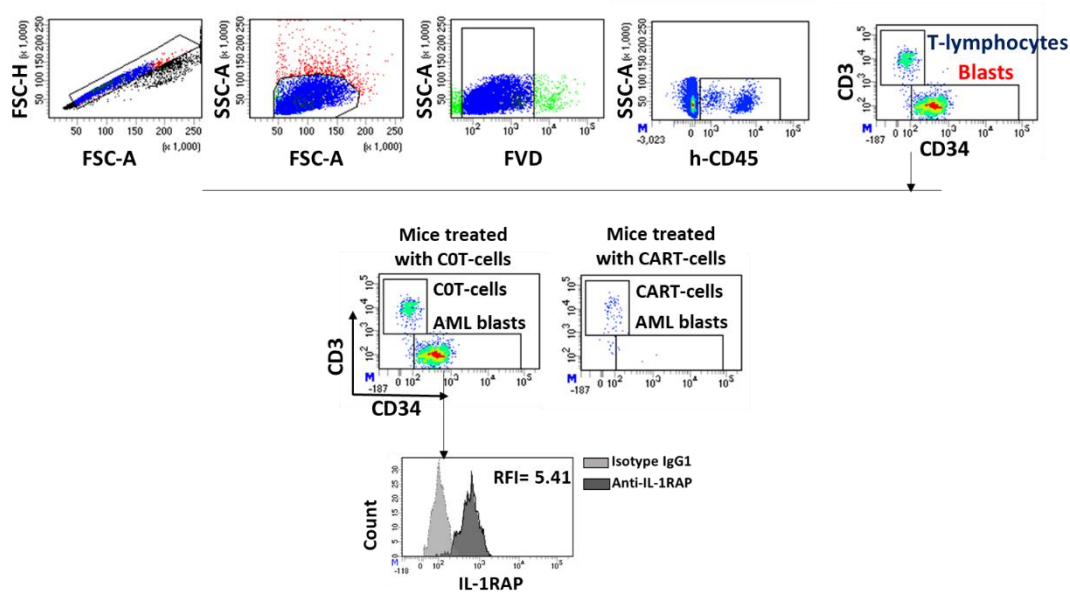
**Figure S3: A/** Quantification of IL-1RAP mRNA by RT-qPCR in AML cell lines. RT-qPCR was performed on AML cell lines. **B/** Determination of the absolute number of IL-1RAP antigenic sites expressed on the cell surface of AML cell lines. The KU812 and K562 CML cell lines were used as positive and negative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

**Figure S4**



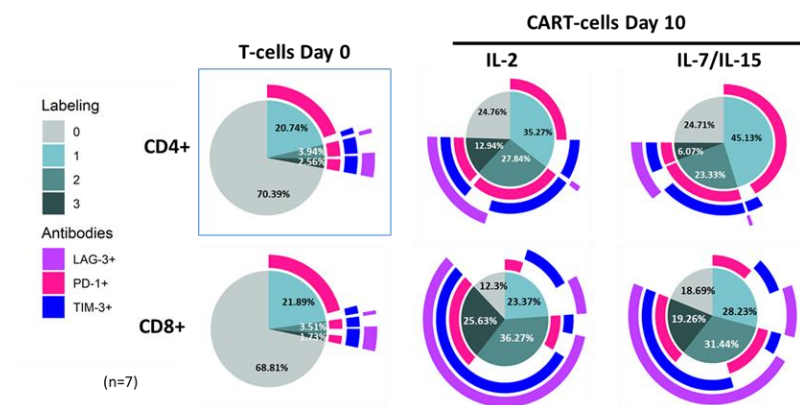
**Figure S4: Coculture of IL-1RAP CAR T cells with healthy tissues** harvested close or at the periphery of different solid tumors. Selection's section is made by an experimented cytopathologist. **A/** Briefly, healthy tissues (1 to 3 mm<sup>3</sup> pieces) were enzymatically dissociated in DTTD (BD Tissue and Tumor Dissociation; BD Biosciences) either 2 or 16 hours, then stained with CD45 monoclonal antibody in order to discriminate stromal cells (CD45 negative). **B/** IL-1RAP staining of healthy tissues. Mono-Mac-6 DTTD treated or not, are used as control staining. **C/** Representative experiment of CD107 staining of IL-1RAP CAR T cells after co culture (5h, E:T ratio = 5) with dissociated prostate healthy tissue and AML IL-1RAP+ cell line **D/** CD107 staining among IL-1RAP CAR T Cells (CD3+/CD19+)

**Figure S5**



**Figure S5: Gating strategy for human AML blasts from murine bone marrow samples of AML PDX mouse models.** Human AML primary cells with hCD45+/CD34+/IL-1RAP+ labeling were detected by flow cytometry. T cells were gated as CD3+. An IgG-1 isotype (light gray peak) was used as a control for IL-1RAP staining.

**Figure S6**



**Figure S6 : Evaluation of the checkpoint inhibitor expression on the surface of CAR T cells (CD4+ or CD8+ T cell subpopulations) produced from samples from AML patients (n=7).** The expression of PD-1, TIM-3 and LAG-3 was measured by flow cytometry prior to production (day 0) and at the end of the CAR T cell production (day 10) using either IL-2 or a combination of IL-7/IL-15. The percentage of cells expressing 0, 1, 2 or 3 checkpoint inhibitors (PD-1, LAG-3, and TIM-3) among CD3+CAR+ cells (CD4+/CD8+) is provided.



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## Discussion

AML remains a rare but lethal malignancy with a poor prognosis, especially for high-risk patients. AML treatment is always in progress with novel approaches that have emerged since 2017. Despite the progress made over the last ten years in the treatment of AML and improvements in allogeneic HSCT, and given that AML is an aggressive disease with a clonal evolution, it is in most cases related to a fatal relapse after treatment. This relapse owing to the resistance and the persistence of leukemia cells, specifically LSCs, which is a subpopulation of AML cells with self-renewal, and chemorefractory capacity.

Actually, the relapse after conventional chemotherapy, targeted therapy or immunotherapy remains a major problem in AML patients and a crucial cause of death. Hence the necessity of new alternatives for treating AML patients (Thol and Heuser 2021). In the field of CART-cells immunotherapies, the stellar success of autologous CD19 CART-cells in different types of B-cell malignancies such as in ALL and lymphoma (Frigault and Maus 2020) as well as in multiple myeloma (MM) (Munshi et al. 2021) have yielded significant efforts to translate this triumph to myeloid malignancies such as AML. Almost all antigens expressed on the surface of LSC, are targeted with at least one therapeutic pathway (targeted therapies, immunotherapies). A surface protein, the IL-1 receptor accessory protein (IL-1RAP) has been defined as a selective marker of LSCs while the other antigens are also expressed by normal HSCs (Askmyr et al. 2013). The pro-inflammatory protein IL-1RAP plays a crucial role in immunity, inflammation and cancer (De Boer et al. 2020) (Lv et al. 2021).

In this work, we investigated IL-1RAP, as a novel and unexplored potential target in AML. We have reproduced what has already been demonstrated that IL-1RAP is overexpressed by AML leukemia cells specifically LSCs and not by healthy HSPCs, or T and B lymphocytes, though it is expressed by circulating monocytes (Askmyr et al. 2013). We demonstrated that IL-1RAP mRNA is expressed in LSCs and blasts in all AML FAB subtypes and was discriminatory compared to common AML cell surface targets. As published data, we showed that the IL-1RAP protein is expressed at three different levels “Low”, “Intermediate” and “High” on the cell surface of AML blasts (Askmyr et al. 2013) and independently of ELN classification.

Interestingly, we showed in a cohort of 30 AML patients that most primary IL-1RAP<sup>+</sup> blasts co-expressed CD123, making this target suitable for bispecific CART-cell targeting, which will increase specificity via “and/or” CAR activation signaling and presumably without increasing toxicity (Jain and Davila 2018).

In our preceded work in CML, we established the proof of concept for targeting IL-1RAP with CART-cells (Warda et al. 2019b). We demonstrated that IL-1RAP CART-cells efficiently eliminate CML resistant cells after therapy with tyrosine kinase inhibitors (Warda et al. 2019b). Furthermore, we showed the absence of toxicities toward healthy HSCs that were not targeted by IL-1RAP CART-cells (Warda et al. 2019b). Here, we show in a preclinical study that this new alternative treatment has efficacy in AML. We targeted the cell surface biomarker IL-1RAP that is expressed on leukemic stem cells and primary AML blasts using CART-cells specific for IL-1RAP. This innovative approach showed efficiency and high cytotoxicity against both cells AML cell lines and primary AML cells either with low expression of IL-1RAP or with high IL-1RAP expression. We were able to produce IL-1RAP CART-cells of 3<sup>d</sup> generation from healthy donors and from AML patients at diagnosis or at relapse with high efficiency. We demonstrated that allogeneic and interestingly autologous IL-1RAP CART-cells are effective in-vitro against primary R/R AML blasts and LSCs and allogenic IL-1RAP CART-cells were able to eradicate AML blasts and LSCs from R/R AML patients in the bone marrow and spleen of PDX mice in-vivo. Therefore, CART-cells offer a strong treatment option apart from chemotherapy and targeted therapies, which are recognized as early-line treatments.

Moreover, the characterization of T-cells taken from diagnostic AML patients and from R/R AML patients showed an acquisition of at least one checkpoint marker such as PD-1, TIM-3 and LAG-3 at the end of the production process in-vitro as in-vivo in PDX mice. Further studies are needed to evaluate the surface expression of the ligands of these markers such as PDL-1 on AML cells for a possible combination of IL-1RAP CART-cells therapy with checkpoints inhibitors (Jimbu et al. 2021).

In addition, these T-cells showed a conservation of the phenotype profile during in-vitro culture with a slight tendency of acquisition a naïve/memory phenotype by CD8<sup>+</sup> CART-cells at the end of the production process (Schnorfeil et al. 2015) (Williams et al. 2019). No impact of the cytokine type added in the culture medium (IL-2 or IL-7/IL-15). In PDX mice, an acquisition of an effector phenotype was observed after the sacrifice.

Importantly, the persistence of IL-1RAP CART-cells in the bone marrow and the spleen of PDX mice was confirmed by dPCR for approximately 2 months after treatment. This persistence may be due to the co-stimulation molecule 4-1BB present in the CAR construct.

From a clinical point of view, in order to circumvent the problem of inducing a graft-versus-host disease, following the transfusion of allogeneic TL, we incorporated within our lentiviral construct an iCASP9/AP1903 suicide system. This safety switch system will protect patients who received CART-cells against adverse events such as off-target toxicities, neurological toxicities, unexpected leukemic cell transduction, as previously shown (Ruella et al. 2018b) or when persistent adverse events affect

monocytes and many other toxicities in order to eliminate CART-cells and allow a mature myeloid cell recovery. In addition, this system could be facilitate the use of allogeneic IL-1RAP CART-cells in AML.

We are also working on a clinical transfer of IL-1RAP CART-cells treatment to a phase I/IIa trial on R/R AML patients with a GMP like-grade Prodigy system.

## Conclusion

Our synthetic mAb is specific for IL-1RAP protein expressed on the surface of AML cell lines and primary cells and not expressed on normal HSPC except monocytes. With a high lentiviral transduction, IL-1RAP CART-cells were produced.

Subsequently, the functionality of IL-1RAP CART-cells was evaluated on different AML cell lines expressing the IL-1RAP protein at different levels (Low, intermediate and high, respectively). Despite this difference in IL-1RAP expression, the stimulation and activation of IL-1RAP CART-cells was similar after co-culture with the target lines, regard to the level of cell proliferation, the expression of IFN $\gamma$  and induction of target mortality in-vitro, and in-vivo with xenograft murine model.

Additionally, IL-1RAP CART-cells were highly cytotoxic in-vitro and in-vivo with PDX murine model against AML primary cells from AML patients at diagnosis or specially when relapsed following chemotherapy or targeted therapy treatments.

Accordingly, this work clearly confirms the potential of IL-1RAP as a target in AML and the strong anti-leukemic effects of strategies targeting this marker both in-vitro and in-vivo, laying the foundation for a promising future first-in-human clinical trial in R/R AML. IL-1RAP CART-cells have not yet been evaluated in humans, and doing so will help determine the optimal IL-1RAP CART-cells dose and evaluate the efficacy and safety of this approach.

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## **Annexes: published articles**

### **Annexe 1: CML Hematopoietic Stem Cells Expressing IL-1RAP Can Be Targeted by Chimeric Antigen Receptor-Engineered T Cells, Cancer Research 2019**

Walid Warda, Fabrice Larosa, Mathieu Neto Da Rocha, [Rim Trad](#), Eric Deconinck, Ziad Fajloun, Cyril Faure, Denis Caillot, Marius Moldovan, Severine Valmary-Degano, Sabeha Biichle, Etienne Daguindau, Francine Garnache-Ottou, Sebastien Tabruyn, Olivier Adotevi, Marina Deschamps, and Christophe Ferrand.

### **Annexe 2: IL-1RAP, un candidat pour l'immunothérapie par CAR T-cells, Médecine/Sciences, 2019**

Mathieu Neto da Rocha, [Rim Trad](#), Walid Warda, Rafik Haderbache, Lucie Bouquet, Clémentine Nicod, Marina Deschamps, Christophe Ferrand.

### **Annexe 3: Acute Myeloid Leukemia: From Biology to Clinical Practices Through Development and Pre-Clinical Therapeutics, Frontiers in Oncology 2020**

Xavier Roussel, Etienne Daguindau, Ana Berceanu, Yohan Desbrosses, Walid Warda, Mathieu Neto da Rocha, [Rim Trad](#), Eric Deconinck, Marina Deschamps and Christophe Ferrand.

### **Annexe 4: Overcoming target epitope masking resistance that can occur on low-antigen-expresser AML blasts after IL-1RAP chimeric antigen receptor T cell therapy using the inducible caspase 9 suicide gene safety switch, Cancer gene therapy 2021**

Walid Warda, Mathieu Neto Da Rocha, [Rim Trad](#), Rafik Haderbache, Yahya Salma, Lucie Bouquet, Xavier Roussel, Clémentine Nicod, Marina Deschamps & Christophe Ferrand.

### **Annexe 5: Droplet digital PCR allows vector copy number assessment and monitoring of experimental CAR T cells in murine xenograft models or approved CD19 CAR T cell-treated patients, Journal of Translational Medicine, 2021**

Rafik Haderbache, Walid Warda, Eric Hervouet, Mathieu Neto da Rocha, [Rim Trad](#), Vincent Allain, Clementine Nicod, Catherine Thieblemeont, Nicolas Boissel, Pauline Varlet, Ibrahim Yakoub Agha, Lucie Bouquet, Melanie Guiot, Fabienne Venet, Pierre Sujobert, Xavier Roussel, Paul-Oliver Rouzaire, Denis Caillot, Olivier Casasnovas, Jean Christophe Bories, Emmanuel Bachy, Sophie Caillat-Zucman, Marina Deschamps, Christophe Ferrand.

### **Annexe 6: Coated recombinant target protein helps explore IL-1RAP CAR T-cell functionality in vitro, submitted article (Cytotherapy, 2021)**

Mathieu Neto Da Rocha, Melanie Guiot, Clementine Nicod, [Rim Trad](#), Lucie Bouquet, Rafik Haderbache, Walid Warda, Pierre-Emmanuel Baurand, Chloe Jouanneau, Philippe Dulieu, Marina Deschamps and Christophe Ferrand.