UNIVERSITAT DE BARCELONA

Una aInnovative CAR T-cell based immunotherapy for the treatment of Relapsed/Refractory Acute Leukemias

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INNOVATIVE CAR T-CELL BASED IMMUNOTHERAPIES FOR THE TREATMENT OF RELAPSED/REFRACTORY ACUTE LEUKEMIAS

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INNOVATIVE CAR T-CELL BASED IMMUNOTHERAPIES FOR THE TREATMENT OF RELAPSED/REFRACTORY ACUTE LEUKEMIAS

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CERTIFY THAT

- The doctoral thesis entitled “Innovative CAR T-cell based immunotherapy for the treatment of Relapsed/Refractory Acute Leukemias” submitted for the award of Doctor in Biomedicine to the University of Barcelona is a record of authentic and original research conducted by Matteo Libero Baroni under their supervision and guidance.
- The two articles derived from this dissertation were published in 2020 and 2019 in “Journal for Immunotherapy of Cancer (Impact factor 8.278) and Blood (Impact Factor 16.6) respectively
- That the doctoral student has fully led the work included in this thesis doctoral. He has designed and discussed with his mentors the experiments to be carried out to respond to the objectives. On this basis he has carried out the experiments and has analyzed and interpreted the data. Finally, it has organized the results in the final figures of the works.
- No part of any paper published has been previously submitted for the award of any PhD degree

Pablo Menéndez Bujan

Clara Bueno Uroz

Barcelona, May 2020

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Sooner or later this moment would have come. As for many, if not for all, reaching this point marked the realization of a life goal, an endeavor, a won battle…call it whatever. I am thus no different than any other, I have experienced the same initial excitement, almost driven by a higher meaning, I have faced the same struggle to start, I have dealt with the same hopelessness, I have stared at the void and the void stared back at me…. But I made it, as many others made it before me and as many others will make it after me. These past 4 years have been among the most characterizing years of my life: the constant mental challenges I have been subjected to, like many others before and after me, have radically changed the way I view and perceive reality. “Question yourself before questioning others”, “Who said what and when”, “Let the idea sinks in…you’ll answer tomorrow”. These are the mantras I always repeat to myself when dealing with life circumstances, when I read the news, when I meet somebody new, with different background and origin. I am so grateful to have had the opportunity to work on such thrilling, thought-provoking topics, it has given me the chance to improve many aspects of my personality, which go well beyond the technical and scientific skill one gains through a PhD in Biomedicine. I am and I will benefit from them virtually in ANY situation I will be faced to. I thank the circumstances for having me mistreated and forced to confront myself with situations that were only up to me to be resolved. In retrospective, difficulties become wise counselors, adversaries become friends, headaches transform into novel insights and perspectives, uncertainty subside and serendipity takes place. I am a regular guy, comfortably sitting within a Gauss curve, however this experience has given me the chance to explore parts of me I didn’t know existed and forced me to improve my attitude and mental skills, thus

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-Matteo Libero Baroni-

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LIST OF ABBREVIATIONS

A

ADC: Antibody Drug Conjugate

ADCC: Antibody Dependent cytotoxicity

ALL: Acute Lymphoid Leukemia

AML: Acute Myeloid Leukemia

APC: Antigen Presenting Cell

AR: Adverse Risk

B

B-ALL: B-cell Acute Lymphoid Leukemia

β_c: Beta Common

BiTE: Bispecific T-cell Engager

BM: Bone Marrow

C

CAR: Chimeric Antigen Receptor

CBF: Core Binding Factor

CDC: Complement Mediated Cytotoxicity

CHIP: Clonal Hematopoiesis of Indeterminate Potential

CIK: Cytokine Induced Killer

CLL: Chronic Lymphoid Leukemia

CLP: Common Lymphoid Progenitor

CML: Chronic Myeloid Leukemia

CMP: Common Myeloid Progenitor

Co T-ALL: Cortical T-cell Acute Lymphoblastic Leukemia

CR: Complete Remission

D

DC: Dendritic Cells

dS DNA: Double Stranded DNA

E

E: Erythroid CFU

EFS: Event Free Survival

EGIL: European Group for the Immunological Characterization of Leukemias

ELN: European Leukemia Network

F

FL: Fetal Liver

FLT3: Fms Like Tyrosine Kinase 3

FR: High Risk

G

G: Granulocytic CFU

GM: Granulo-Monocytic CFU

GEMM: Granulocytic, Erythroid, Myelo-Monocytic CFU

GMP: Granulocyte/Monocyte Progenitor

Go: Gemtuzumab Ozogomicyn

H

HLA: Human Leukocyte Antigen

HPC: Hematopoietic Progenitor Cell

HSC: Hematopoietic Stem Cell

HSCT: allogeneic Hematopoietic Stem Cell Transplantation

I

ICAD: Inhibitor Casapse Activated DNAse

Ig: Immunoglobulin

IL: Interleukin

IM: Insertional Mutagenesis

IR: Intermediate Risk

ITAM: Immunoreceptor Tyrosine-based Activation Motif

ITBM: Intra Bone Marrow

ITD: Internal Tandem Repeat

IV: Intra Vein

L

LC: Langherans Cell

LTR: Long Terminal Repeat

LV: Lentiviral Vector

M

M: Monocytic CFU

MEP: Megakariocytic/Erythroid Progentiror

MHC: Major Histocompatibility complex

MLL: Mixed Lineage Leukemia

MoAb: Monoclonal Antibody

MRD: Minimal Residual Disease

MTX: Methotreaxate

N

NMP1: Nucleophosmin-1

O

OS: Overall survival

OTOT: On Target Off Tumor Toxicity

P

PIC: Pre-Integration Complex

PB: Peripheral Blood

R

RCL: Replication Competent Lentivirus

RUNX: Runt-Related Gene

RV: Retroviral Vector

S

scFv: Single Chain Variable Fragment

SIN: Self Inactivating Lentiviral Vector

sS DNA: Single Stranded DNA

T

TAA: Tumor Associated Antigen

TCM: T-Central memory

TCR: T-Cell Receptor

T-EFF: T-Effector

Tfh: T-Follicular Helper

Th: T-Helper

T-SCM: T-Stem Cell Memory

Tn: T-cell Naïve

Tm: T-cell memory

TRAIL: TNF-related Apoptosis Inducing Ligand

Treg: T-Regulatory

V

Vh: Heavy Chain

Vl: Light chian

1. HEMATOPOIESIS

1.1 The hematopoietic system.

Hematopoiesis, an ancient Greek derived word which stands for “Blood Making”, is the physiological multistep process ensuring proper blood system homeostasis through a finely tuned balance between cell proliferation, differentiation and cell death. Perturbations of the hematopoietic system’s homeostasis can eventually lead to different physiological and pathological conditions including leukemia.

1.2 The hematopoietic stem cells.

Hematopoietic stem cells (HSCs) are multipotent immature stem cells in charge of maintaining hematopoietic system’s homeostasis by constant renewal and replenishment of blood cell (Srikanth *et al.* 2015). HSCs show the remarkable property of sustaining the entire hematopoietic system, and such capacity resides in their ability to maintain a state of quiescence and self-renewal. The altered metabolism of quiescent HCSs contribute to their survival for long time periods in the hypoxic bone marrow (BM) environment (Srikanth *et al.* 2015). The quiescent stem cell pool comprises a small number of non-dividing or slowly dividing long-term repopulating cells that maintain stemness and prevent premature stem cell exhaustion through symmetric cell division. Some quiescent cells divide asymmetrically to generate short-term repopulating cells that maintain rapid cycling, self-renewal capacity (Morrison *et al.* 2006) and they tend to be located in the subendosteal zone, while dividing HSCs are closer to the central vascular niche, which promotes differentiation and mobilization of mature blood cells to the circulation (Ramaiah *et al*.2013).

1.3 The hematopoietic differentiation

According to the standard view of hematopoiesis, as presented in figure 1, HSCs gradually become lineage restricted through a multiplicity of divisions (Ramaiah *et al.* 2013). The model follows a highly hierarchical fashion, starting with a universal progenitor common for all lineages. Further differentiation gives rise to Common Myeloid progenitors (CMPs) and to Common Lymphoid progenitors (CLPs), which will sustain to the entire myeloid and lymphoid lineages respectively. The common myeloid progenitor can be additionally subdivided into a Megakaryocytic/Erythroid progenitor (MEP) originating megakaryocites erythrocytes and platelets, and a Granulocyte/Monocyte progenitor (GMP) giving rise to monocytes/macrophages, granulocytes, and mast cells (Ramaiah *et al.* 2013). Conversely, the Common lymphoid progenitor, will form B and T lymphocytes, as well as the Natural Killer (NK) cells (Birbrair *et al.* 2016).

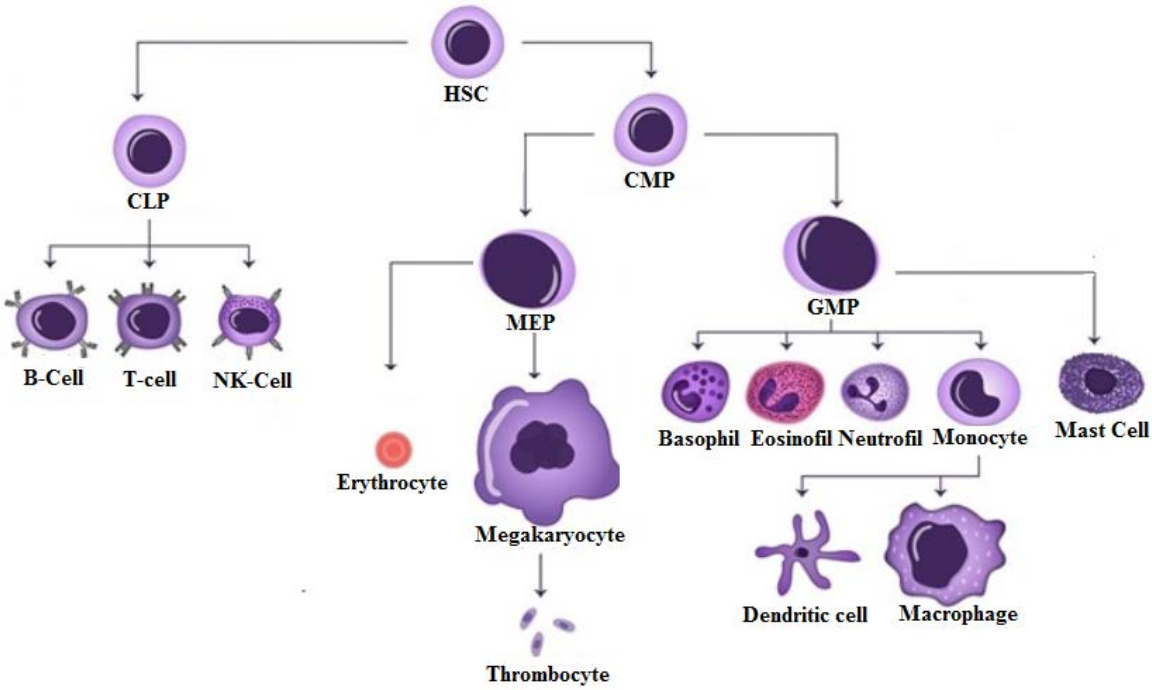


Fig.1) Hierarchical organization of the hematopoietic system. Diagram showing the development of different blood cells from HSC to mature cells. The process follows a highly hierarchical fashion, encompassing multiple steps of differentiation and maturation**. CLP:** Common Lymphoid Progenitor, **CMP**: Common Myeloid Progenitor, **GMP**: Granulocyte/Monocyte Progenitor, **MEP**: Megakaryocytic/Erythroid Progenitor (Adapted from Häggström, 2014).

2. LEUKEMIA

Leukemia comprises a wide group of blood cancers, usually originating in the BM, which ultimately give rise to an abnormal production and proliferation of aberrant blood cells (Vardiman *et al.* 2009). As shown in figure 2, leukemia is commonly characterized by the development of a clone of mutated hematopoietic progenitor cells that interferes or disrupts the normal functioning of the hematopoietic system (Vardiman *et al.* 2009). A developmentally stalled progenitor cell will then expand clonally with subsequent hematopoietic displacement, where various environmental and genetic factors cooperate to hamper the mechanisms controlling cell growth, proliferation and survival. Worldwide, in 2018 alone, over 437,000 new cases of leukemia were reported, accounting for approximately 2,4% of all new cancer diagnosis, and in the same year more than 309,000 Leukemia related deaths were reported, accounting for roughly 4,5 % of all cancer related deaths (Bray *et al.* 2018).

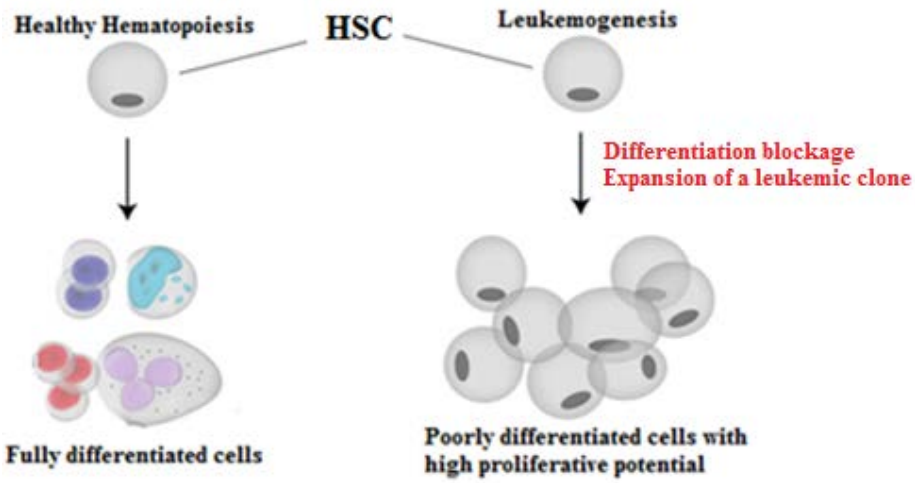


Fig.2) Schematics of the Leukemogenesis process. During leukemia initiation, the BM produces a large amount of abnormal, immature blood cells which display aberrant functions and a high degree of proliferation. The large number of fast dividing cells in the BM hampers the production of functional immune cells (a phenomenon known as hematopoietic displacement) which lead to a wide variety of symptoms. Adapted from National Institute of Health (NIH), 2019.

2.1 Classification

Leukemia entails a wide array of clinical and pathological features and in general terms this group of cancers is subdivided into two main categories: Acute and Chronic. Acute leukemia is defined by the rapid expansion of undifferentiated leukocytes and the overcrowding that results from such cell bulk, hinders the BM capacity to produce healthy blood cells. Urgent action is mandatory in acute leukemia because of the rapid formation and accumulation of the malignant cells, which then extravagate into the bloodstream and spread to other organs, leading to severe multi-organ dysfunction (Juliusson *et al.* 2016).

On the other hand, chronic leukemia is characterized by the excessive accumulation of relatively mature, however still abnormal, blood cells. Generally progression takes several months to years, and cancerous cells are produced at a higher rate than the normal counterpart, resulting in many abnormal white blood cells and consequent dysfunction of the hematopoietic system as well as other affected organs (Savage *et al.* 1997). Additional classifications are made based on the lineage affected by the disease: under this perspective it is possible to characterize Lymphoid and Myeloid neoplasia. As presented in figure 3, by using the aforementioned categorization, leukemia can be divided into four major subgroups: Acute Lymphoblastic Leukemia (ALL), which can be either B- or T-lineage, Chronic Lymphoblastic Leukemia (CLL) which can also encompass both B- and T-cell lineage, Acute Myeloid leukemia (AML) and Chronic Myeloid Leukemia (CML).

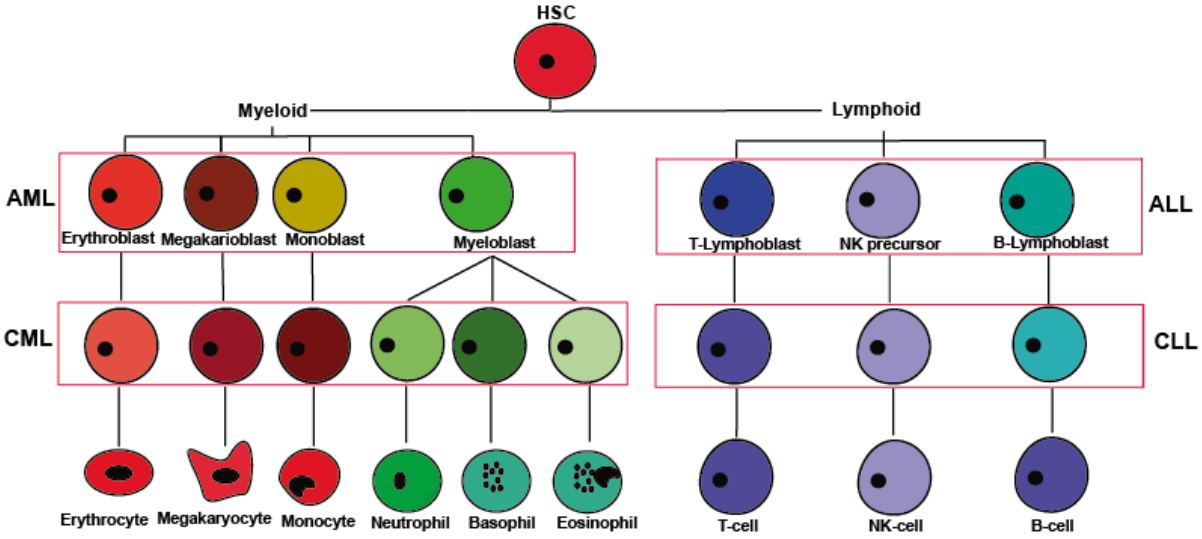


Fig.3) Difference between Acute and Chronic Leukemia. The four major classifications of leukemias depending on which developmental stage the normal maturation of blood cells stops, and which lineage is affected. Acute Leukemia sees the oncogenic transformation at an earlier stage compared to Chronic Leukemia. **AML,** Acute Myeloid Leukemia, **ALL**, Acute Lymphoid Leukemia, **CML**, Chronic Myeloid Leukemia, **CLL**, Chronic Lymphoid Leukemia

2.2 Acute Myeloid Leukemia

AML is the most common form of acute leukemia in the adult population, comprising the 80% of newly diagnosed cases in this group, while ALL is more common in young or pediatric population (Yamammoto *et al.* 2008, Thein et al. 2013). The occurrence of AML increases with age, ranging from approximately 1.3 cases per 100 000 individuals younger than 65 years old, to 12.2 cases per 100 000 individuals in those over 65 years. Although medicine has witnessed a substantial improvement in the treatment for younger patients, prognosis in the elderly remains dismal (Shah *et al.* 2013)

2.2.1 Pathophysiology of AML

AML can arise in patients with a hidden hematological disorder, or as a consequence of prior chemotherapy, for example, exposure to topoisomerases II, alkylating drugs or radiations (Sill *et al.* 2011). However, in the vast majority of cases, it appears as a *de novo* entity in otherwise healthy individuals. The pathogenesis of AML involves the clonal uncontrolled proliferation of a hematopoietic myeloid progenitor stalled in

differentiation, a phenomenon caused by mutations and/or chromosomal translocations that have been shown alter the normal maturation processes of myeloid progenitor cells (De Kouchovsky *et al.* 2016). Alternatively, AML can arise from what is known as clonal hematopoiesis of indeterminate potential (CHIP) (Sperling *et al.* 2017). It is a common aging-related phenomenon in which HSCs or other early blood cell progenitors contribute to the formation of a genetically distinct subpopulation of blood cell. As the definition implies, this subpopulation in the blood is characterized by a shared unique mutation in the cells' genetic material and it is thought that this subpopulation is "clonally" derived from a single originating cell and hence comprises genetic "clones" of the founder cell (Steensma *et al.* 2015). In any case, the development of AML is thought to be a multistep process that requires the cooperation of at least two classes of mutations to overt the full establishment of the disease. Current classification entails Class I mutations that activate signal transduction pathways and confer a proliferation advantage on hematopoietic cells, and class II mutations, that affect transcription factors and primarily cause impairment to hematopoietic differentiation and cell identity (Frolhing *et al.* 2005, Kelly *et al.* 2002).

2.2.2 Class I: Signaling and Kinase pathway mutations

Mutations affecting signaling cascade pathways are found in approximately two-thirds of AML cases, thus making up the most common mutational subset in AML (Papaemmanuil *et al.* 2016). A prominent example is represented by Fms-like tyrosine kinase-3 (FLT3) which is a receptor tyrosine kinase expressed by immature hematopoietic cells and plays vital roles for the normal development of HSCs and the immune system. The ligand for FLT3 is expressed by BM stromal cells and other cells and acts synergistically with other growth factors to stimulate proliferation of stem cells, progenitor cells, dendritic cells, and natural killer cells (Gilliland *et. al* 2002). FLT3 Internal Tandem Duplications (ITD)

and/or activating kinase domain point mutations in the *FLT3* gene are present in nearly one-third of patients with AML and are commonly associated with unfavorable clinical outcome (Badar *et al.* 2015).

FLT3 mutations lead to constitutive activation of downstream signaling through the RAS/RAF/MEK/MAPK and (PI3K)/AKT pro-survival pathways, resulting in uncontrolled cell proliferation.

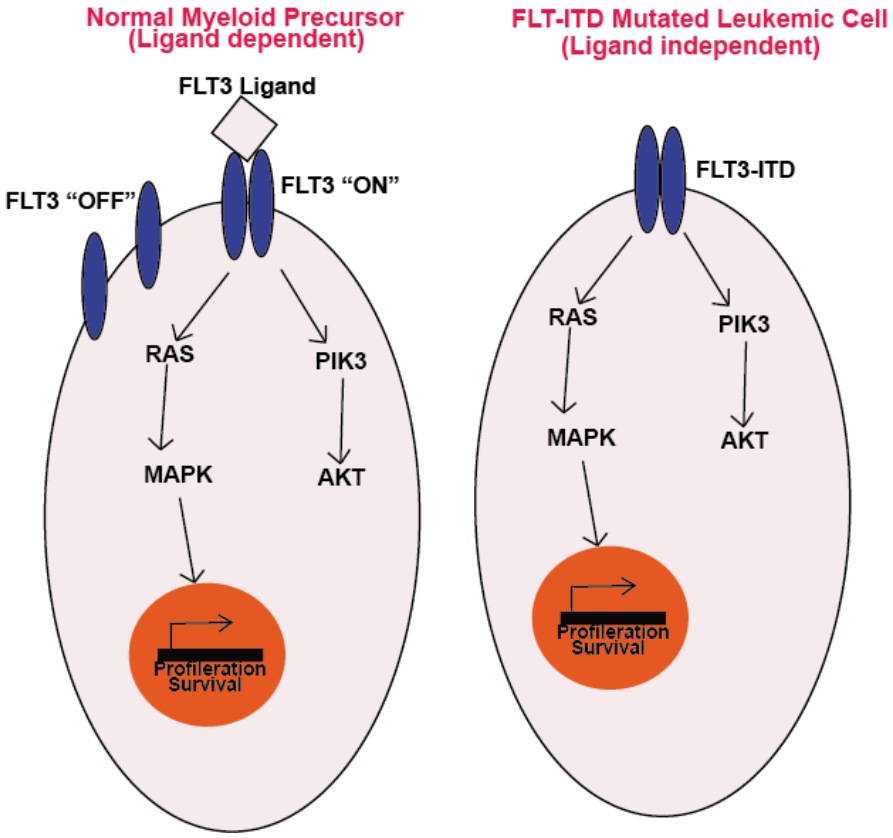


Fig.4) FLT3-ITD cells have a survival advantage over FLT3 *wt.* FLT3 signaling starts with FLT3 ligand binding to FLT3 protein. Dimerization causes activation of intracellular signaling cascades leading to cell proliferation and survival. FLT3-ITD causes a constitutive dimerization of FLT3 molecules which in turn causes a permanent activation of downstream signaling pathways

FLT3-ITD mutations are more frequent within younger adult patients. They are associated with normal karyotype (NK), high blood cell and elevated blast percentage, and increased risk of relapse leading to decreased overall survival (OS) (Ravandi *et al.* 2010).

Together with FLT3 mutations, alterations in the *NMP1* gene are considered among the most frequent acquired molecular abnormalities in AML (Verhaak *et al.* 2005) and both represent key candidates for diagnostic and outcome prediction (Diaz de la Guardia *et al.* 2020). NPM1 is essentially localized in the nucleolus and is believed to serve as a molecular chaperone of proteins, mediating the transport of ribosomal peptides across the nuclear membrane (Boher *et al.* 1989). Alterations of *NPM1* can result in the cytoplasmic dislocation of NPM1. The high frequency of *NPM1* mutations in AML with normal karyotypes, and the observation that cytoplasmic NPM1 cannot exert its normal functions as binding partner and transporter protein, lead to the notion that *NPM1* mutation may be an early event in leukemic initiation (Verhaak *et al.* 2005).

Another key player in this class of mutation, is represented by the family of *RAS* oncogenes, which is mutated in approximately 10% to 15% of AML cases (Renneville *et al.* 2008). *RAS* oncogenes encode a family of guanine nucleotide-binding proteins that regulate signal transduction upon binding to a variety of membrane receptors, including KIT and FLT3, thus playing important roles in cell proliferation, differentiation and apoptosis (Renneville *et al.* 2008). These patients might present activating mutations in *NRAS*, *KRAS*, *PTPN11*, and *NF1*, leading to aberrant proliferative signaling through the RAS/RAF/MEK kinase pathway, and the acquisition or clonal emergence of *RAS* mutations, similarly to *FLT3*, leads towards a poorer outcome compared to other AML subtypes lacking this type of mutation (Badar *et al.* 2015).

Another protagonist in the class I mutation landscape is the tyrosine-protein kinase KIT. It is a cytokine receptor belonging to the type III RTK family, and it can be found on the surface of HSCs as well as other cell types where it functions as activating kinase, which upon binding to its ligand Stem Cell Factor (SFC), phosphorylates and activates signal transduction molecules that propagate proliferative signals in the cell (Blume-Jensen *et*

al. 1991). Gain-of-function mutations can cause ligand-independent activation of KIT, causing downstream signaling pathway to become activated, ultimately leading to cell proliferation and increased survival (Kitayama *et al.* 1996).

2.2.3 Class II: recurring chromosomal aberrations and fusion transcripts

Class II mutations comprise recurring chromosomal aberrations such as t(6;9), t(8;21), inv(16), and t(15;17) which generate fusion transcripts of DEK-NUP214, RUNX1/ETO, CBFβ/MYH11 and PML/RARα respectively, as well as also mutations in transcription factors such as RUNX1, C/EBPα and MLL (Baer *et al.* 1998, Takahashi *et al.* 2011). RUNX1 belongs to the Runt-related (*RUNX*) gene family, it is required for definitive hematopoiesis and it is necessary for the differentiation of myeloid progenitor cells to granulocytes (Ito 2004). *RUNX* genes codify for the *α* subunits (polyomavirus enhancer-binding protein 2 (PEBP2)*α*/core-binding factor (CBF)*α*) of the Runt domain transcription factors, which act as developmental regulators, and bind the *β* subunits (PEBP2*β*/CBF*β*) to form the heterodimeric transcription factor CBF, which in turn will drive cell cycle progression (Sood *et al.* 2017). C/EBPα is codified by the *CEBPA* gene, and it belongs to the CCAAT/enhancer-binding protein family involved in the balance between cell proliferation and terminal differentiation. In hematopoiesis, *CEBPA* plays a critical role in early stages of myeloid differentiation and it is particularly expressed in myelomonocytic cells (Renneville *et al.* 2008). CEBPA acts in a variety of ways, most importantly by down regulation of c-MYC expression allowing differentiation by synergistic action with other key genes involved in myeloid development, including CBF (Radomska *et al.* 1998, Johansen *et al.* 2001, Smith *et al.* 1996).

The presence of 11q23 rearrangements in the Mixed Lineage Leukemia (*MLL)* gene results in the juxtaposition of the amino-terminus of the histone methyltransferase MLL

with a variety of different fusion partners that destroy normal histone H3K4 methyltransferase function of MLL and replace it by heterologous functions provided by the presence of the fusion partner (Slany *et al.* 2009). The chimeric proteins arising from the MLL fusion with its cognate partners, are transcriptional regulators that take over the transcriptional control of targets that would be otherwise controlled solely by MLL. Some of these proteins are themselves chromatin modifiers that introduce histone acetylation whereas other fusion partners can recruit histone methyltransferases (Dou *et al.* 2005). In particular, histone H3 specific methylation at lysine 79 catalyzed by DOT1L has been identified as a hallmark of chromatin activated by MLL fusion proteins (Steger *et al.* 2008). Interestingly, several frequent MLL fusion partners seem to coordinate DOT1L activity with a protein complex that stimulates the elongation phase of transcription (Peterlin *et al.* 2006). Interestingly, in addition to the 11q23 rearrangements associated with leukemia that develops *de novo*, translocations sporadically arises after cytotoxic treatment for a primary neoplasm. The t(9;l l)(p22;q23) and the t(11;19) are among the most common 11q23 rearrangements observed in therapy-related AML (t-AML) (Albain *et al.* 1990, Cowell *et al.* 2012). These patients develop leukemia, with often dismal prognosis, after a relatively short latent period, most often after treatment with drugs that target DNA-topoisomerase II (Super *et al.* 1993)

2.2.4 AML risk stratification

Outcome in patients with AML ranges from death within a few months from beginning of the treatment to complete eradication of the disease. A wide array of variables have been associated to such large variability in clinical outcome. Age, percentage of blasts, , comorbidities, molecular and genetic features are amongst the most common parameters dictating potential response to treatment and whether patients will display resistance to therapy (Estey *et al.* 2018). Genetic analysis of both classical cytogenetics and mutational

status of various genes, has helped the establishment of guidelines to assess the likelihood of patients to display refractoriness. As presented in table 1, such guidelines has led to the stratification of patients into three main groups: Adverse Risk (AR), Intermediate Risk (IR) and Favorable Risk (FR)

RISK	GENETIC ABNORMALITY
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD ^{low} Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD^{high} Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214* t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)* −5 or del(5q); −7; −17/abn(17p) Wild-type *NPM1* and *FLT3*-ITD^{high} Mutated *RUNX1* Mutated *ASXL1* Mutated *TP53*

Table 1) European LeukemiaNet (ELN) classification according to genetics (Donher *et al.* 2017)

2.2.5 Current therapy for AML

Current therapy in AML did not experience substantial changes in recent years. Initial assessments evaluate whether a patient is considered a suited candidate for “intensive induction chemotherapy” which is based on cycles of 3 days of an anthracycline and 7 days of cytarabine (commonly referred to as “7+3” regimens). Induction therapy is followed by a consolidation therapy, where current approaches include the deployment of further chemotherapeutic cycles with the goal of consolidating the patient into the

remission phase. Consolidation regimens often combine administration of single-agent cytarabine at high doses and multiagent chemotherapy (Donher *et al.* 2017). Complete Remission (CR) is achieved in 60% to 80% of younger adults and in 40% to 60% of older adults (60 years or above) (Donher *et al.* 2010, 2015, 2017).

Together with standard chemotherapeutic agents, the use of targeted therapies has started taking root in current standard-of-care for AML patients. Targeted therapy in AML can be divided into 3 groups. First, mutation-targeted agents act on oncogenic receptors of recurrent AML-associated mutations, examples of such agents include FLT3 inhibitors (figure 5). Second, targeted agents that disrupt key metabolic or cell signaling pathways without directly damaging DNA or its repair.

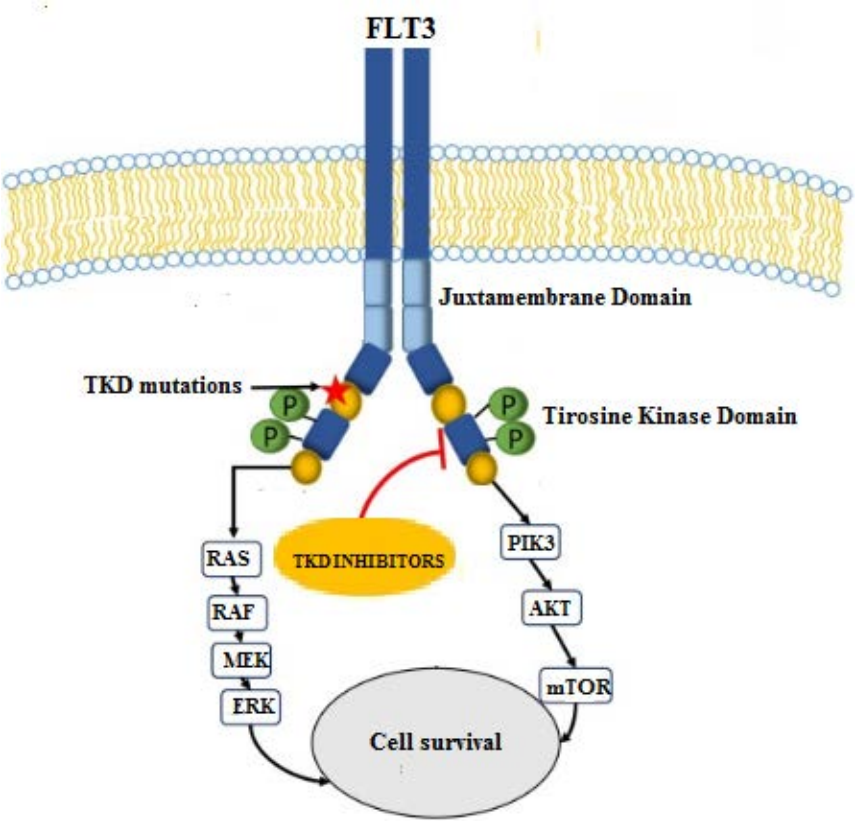


Fig.5) FLT3 Kinase Inhibitors' mechanism of action. Selective targeting of the TKD domain, which is often constitutively activated in AML, lead to suppression of downstream signaling cascades causing uncontrolled cell proliferation and survival

A final group consists in the targeted delivery of cytotoxic agents, such as Antibody Drug Conjugates (ADCs) (Perl *et al.* 2017). Among the first category of agents the initial drugs

tested were FLT3 Tyrosine Kinase Inhibitors (TKIs), which included “Midostaurin” and “Sorafenib”. These compounds showed a good *in vitro* inhibition of FLT3, but rather poor kinase selectivity, often leading to drug resistance (Grunwald *et al.* 2013). Soon thereafter, a second generation of FLT3 inhibitors emerged with stronger potency, more pronounced kinase inhibition properties, and improved pharmacokinetic parameters. In phase 1 testing, “Quizartinib”, fully abolished FLT3 signaling *in vivo* (Cortes *et al.* 2013). However, resistance to FLT3 inhibition might also occur due to concomitant hyper-activating mutation of downstream signaling or anti apoptotic pathways (Kornblau *et al*. 1999). An example of the latter is given by the anti-apoptotic BCL2 protein. A hallmark of many types of cancer is in fact the deregulation of apoptosis, and several hematologic malignancies, including AML, present the overexpression of BCL2 proteins which ultimately lead to increase survival and drug resistance. Early attempts to target BCL2 therapeutically focused on reducing BCL2 expression via antisense RNA (Dias *et al*.2002) and by designing mimetics of the activation domain of BCL2, which showed the property of blocking the anti-apoptotic functions of BLC2 (Ni Chonghale *et al.* 2008). Important advancements in the selective BCL2 targeting have been granted by the novel FDA-approved agent Venetoclax (Roberts *et al.* 2017),whose antitumoral property resides in the capacity of binding and inhibiting BCL2, thereby slowing or halting disease progression (Roberts *et al.* 2017). Another class of therapeutic agents that have recently gained great popularity and success, is the Antibody Drug Conjugates (ADC). The strategy behind the development of these drugs, was to combine the antigen specificity of monoclonal antibodies (MoAbs), with the cytotoxic properties of standard chemotherapeutics. Therefore, ADCs entails the delivery of traditional cytotoxic agents (and/or radioisotopes) to leukemic cells, thus increasing efficacy with a concomitant reduction of toxicity. The first FDA-approved ADC for cancer therapy was the CD33-

redirected Gemtuzumab-Ozogamicin (GO). Immunophenotyping of AML by flow cytometry has allowed an unprecedented number of surface antigens to be characterized for their frequency and stability across AML specimens. Among these, CD33 has emerged as a potential candidate for AML-targeted therapy since it is expressed on leukemic blasts of the majority of patients (Larson *et al.* 2005). Early attempts to target CD33 demonstrated that upon engagement with the target, antibodies were rapidly internalized and degraded, thus limiting their clinical efficacy. However, as depicted in figure 6, this feature could be exploited to create a novel drug-antibody conjugate which entailed the fusion of cytotoxic compounds to MoAbs. These engineered molecules are stable in the neutral environment of the blood stream, but subjected to lysis (with subsequent release of the carried toxin) once internalized and exposed to the highly acidic environment of the lysosome (Perl *et al.* 2017).

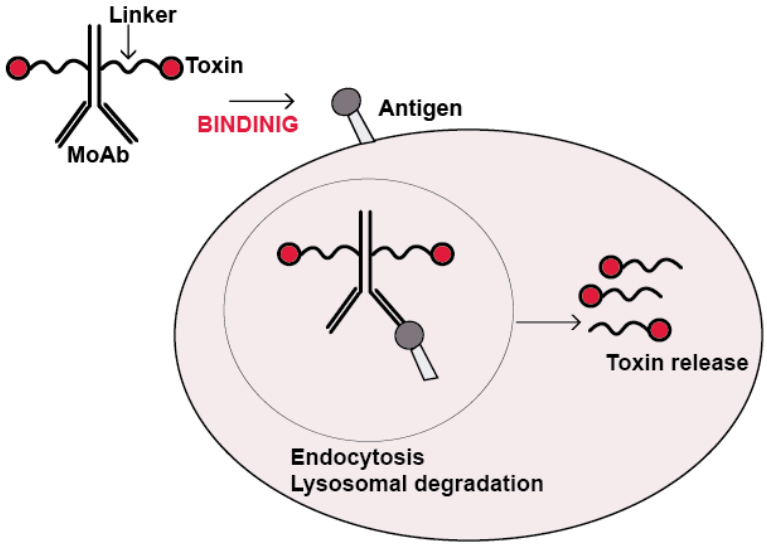


Fig.6) ADC mechanism of action. MoAb can be engineered to be linked to a toxic agent via a chemical linker. Upon antigen engagement the ADC-target is internalized via endocytosis and subjected to lysosomal degradation, which in turn causes the disruption of the complex antibody-immunotoxin thus promoting the release of the drug into the target cell.

A final, and perhaps the most decisive, approach to eradicate leukemic cells, is represented by the allogeneic Hematopoietic Stem Cell Transplantation (HSCT). Such

strategy took hold as a consequence to recurrent relapses observed after extensive cycles of chemotherapy. The agents used to treat cancers act primarily on highly proliferating cells. However, leukemia initiating cells (LICs), are quiescent and therefore show poor sensibility to such therapy (Copelan *et al.* 2006). Thus, although chemotherapy can greatly reduce tumor burden, the leukemic stem cells are spared, allowing the cancer to recur. Nonetheless, such cells may be eliminated by immunologically active donor cells (Copelan *et al.* 2006). As donor cells are infused into the patient, they will engraft into the BM and they will initiate the hematopoietic reconstitution. The newly formed donor-derived T-cells, as well as the T-cells co-transplanted, will react against the host cells including the remaining leukemic stem cells, thus initiating a process that is known as *Graft versus Leukemia* (Kolb 2008).In summary, the therapeutic rationale to treat AML follows the schematic overview presented in figure 7. As an example the PETHEMA protocol (programa español para el tratamiento de las hemopatias malignas), shows the different treatment phases and evaluations that patients undergo to.

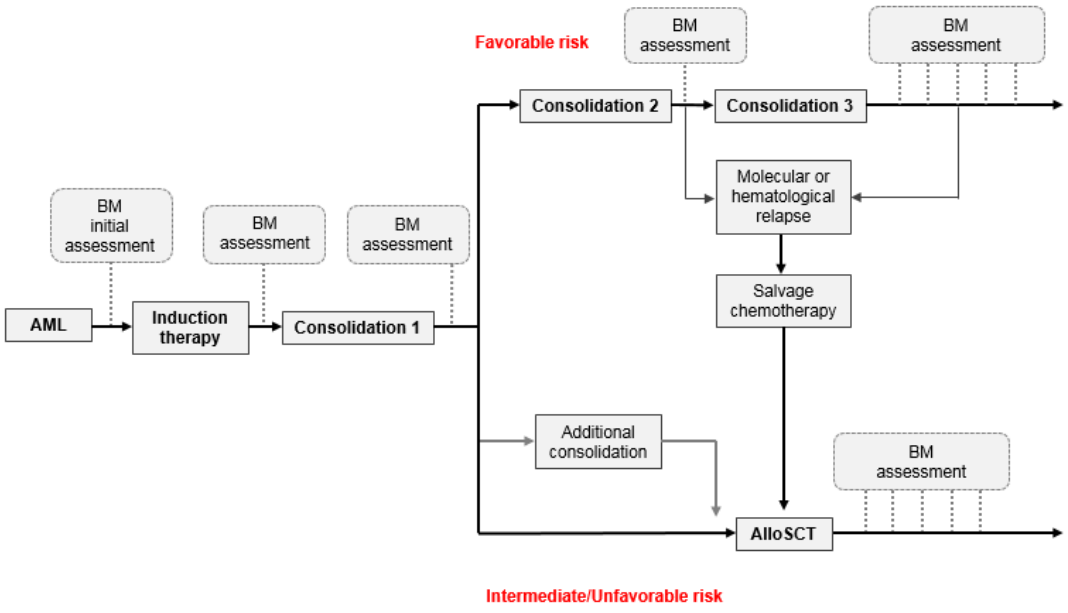


Fig 7) Schematic view of the AML adapted PETHEMA protocol. The Pethema protocol is a risk-adapted protocol eligible for fit patients up to 70 years diagnosed with *de novo* AML, excluding patients with relapsed/refractory AML

2.3 T-Cell Acute Lymphoblastic Leukemia

T-Cell Acute Lymphoblastic Leukemia (T-ALL) is a type of acute lymphoblastic leukemia (ALL), a cancer of the lymphocyte-forming cells called lymphoblasts. T-ALL blasts are developmentally stalled early during T-cell differentiation, before T-cell Receptor (TCR) rearrangements are in place. It represents approximately 15% of all newly diagnosed ALL cases and it displays unique clinical and biological features (Raetz *et al.* 2016). Clinical outcomes for T-ALL were historically worse than those for B lymphoblastic leukemia (B-ALL) cases (Ferrando *et al.* 2002). However, thanks to recent advances in chemo- and targeted cell therapy, five-year event-free survival (EFS) rates have improved and now are approximately 85% according to some studies (Vrooman *et al.* 2015, Vora *et al.* 2013, Möricke *et al*. 2016). However, a definitive cure is not readily available without a cost, as intensive (often toxic) therapy is required. Lastly, disease recurrence after treatment is very difficult to treat, and very few new drugs have been successfully implemented for patients with therapy-resistant disease (Raetz *et al.* 2016).

2.3.1 Pathophysiology of T-ALL

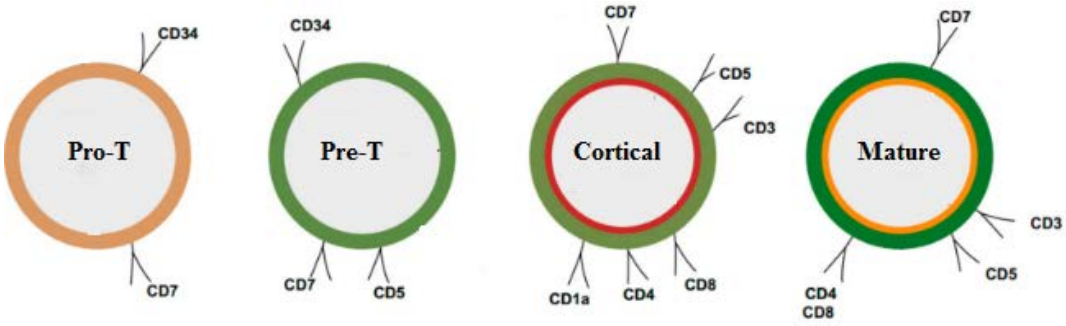
T cell acute lymphoblastic leukemia (T-ALL) arises from the malignant transformation of hematopoietic progenitors primed toward T cell development, as consequence of a multistep oncogenic transformation involving constitutive activation of various signaling and genetic alterations in transcription factors, signaling oncogenes, and tumor suppressors genes (Van Vlieberghe *et al.* 2012). The spectrum of genetic abnormalities in T-ALL is heterogeneous and diverse (Raetz *et al.* 2016), however, despite this significant variety, the majority of lesions can be classified into 2 categories: chromosomal translocations that come with distinct gene-expression patterns and mutations/deletions that affect signaling cascades and/or the cell cycle. Roughly 50% of blasts of patients with T-ALL have been associated with chromosomal translocations that

can be loosely divided into 2 subgroups (Belver *et al.* 2016). One category of translocation comprises rearrangements of proto-oncogenes to the T-cell receptor (TCR), causing the overexpression of the proto-oncogene. These genes include *TLX1* (*HOX11*), *MEF2C*, *HOXA*, *LMO1*, *LMO2*, *TAL1*, and *TAL1* (Van Vlierberghe *et al.* 2012). A second group includes rearrangements of two transcription factor genes, resulting in the abnormal fusion of transcription factors. These include *PCIALM-MLLT10*, *STIL-TAL1*, *TLX3-BCL11B*, and *NUP214-ABL1*, which affect ~8%, ~20%, ~15%, and less than 5% of patients, respectively (Kraszewska *et al.* 2012). Finally, ~5% to 10% of patients with T-ALL have *MLL* gene rearrangements. None of these fusions has been shown to predict outcome consistently and independently from end-of-consolidation minimal residual disease (MRD) (Giraldi *et al.* 2017).

2.3.2 Classification of T-ALL

To date, different clinically relevant biological groups of T-ALL have been established, and these are associated with unique gene expression profiles and with immunophenotypes that reflect thymocyte developmental arrest at different stages of maturation (Ferrando *et al.* 2002). T-ALL is characterized by a proliferation of lymphoblasts that might display a wide array of cytological features. The current designations L1 and L2 have no clinical significance but are helpful for describing such variance. L1-type blasts are small blasts with a high nucleus/cytoplasm ratio and small nucleoli. L2 blasts are larger with prominent nucleoli and more abundant cytoplasm. The diagnosis of T-ALL relies on immunophenotypic demonstration of T-cell lineage, with the most specific T-cell marker being CD3, which is found intra-cytoplasmatically in all T-ALL (Kappelmayer *et al.* 2000, Ferrando *et al.* 2002). Application of standard cytogenetic analysis have helped outlining the risk stratification, prognosis and treatment (You *et al.* 2015). The most immature surface marker of T-cell lineage is CD7, although

most specific marker is CD3 (Ginaldi *et al.* 1996). In an attempt to frame T-ALL in the context of normal T-cell development, the European Group for the Immunological Characterization of Leukemias (EGIL) attempted classifying the disease into four major groups: pro-T ($CD7^+$), pre-T ($CD2^+$ and/or $CD5^+$ and/or $CD8^+$), cortical T ($CD1a^+$), and mature T (surface $CD3^+$, $CD1a^-$) (You *et al.*2015).



Subtype	CD3	CD7	CD1a	CD4	CD8	CD5
Pro-T	+	+	-	-	-	+
Pre-T	+	+	-	-	-	+
Cortical	+	+	+	+	+	-/+
Mature T	+	+	-	-/+	-/+	+

Table 2) Immunologic classification of T-ALL (Litzow *et al.* 2015)

Besides the aforementioned categorization, an additional distinct pathological entity has been recently identified: early T-cell progenitor acute lymphoblastic leukemia (ETP-ALL) (Jain *et al.* 2016). Genetic studies have shed new light on the biology of ETP T-ALLs, a distinct disease entity associated with poor prognosis and defined by a characteristic immunophenotype and a gene expression signature indicating an early arrest in T-cell development. ETP blasts are freshly migrated from the bone BM to the thymus, in which they retain a certain level of multilineage pluripotency (Bell *et al.* 2008). By gene expression profiling, ETP cells share to some extent similarities with hematopoietic stem cells and myeloid progenitor cells (Coustan-Smith *et al.* 2009). In

terms of phonotypical characterization ETP is typically defined as $CD1a^{-}$, $CD8^{-}$, $CD5^{-/(dim)}$, and positive for at least one or more stem cell or myeloid antigens (Coustan-Smith *et al.* 2009).

2.3.3 Current therapy for T-ALL

The management of T-ALL has evolved from the use of standard lymphoma regimens to the use of ALL regimens, incorporating induction, consolidation and maintenance with high-dose chemotherapy and intrathecal therapy (Litzow *et al.* 2015, Raetz *et al.*2016). The most relevant predicting factor of outcome is the minimal residual disease (MRD) at end-of-consolidation, and further treatment for MRD positive patient, largely depend on the current risk classification in which they belong. The risk assessment include the repartition of patients in three major groups: standard (< 5% blasts in BM at Day 29 post induction), intermediate (5-25% blasts in BM at Day 29 post induction) and high risk (>25% blasts in BM at Day 29 post induction) (Raetz *et al.* 2016). Considering the poor prognosis for recurrent disease, the scientific field has witnessed major attempts to optimize the use of conventional therapeutic agents such as: dexamethasone, asparaginase, methotrexate (MTX) and intrathecal chemotherapy (Raetz *et al.* 2016). As a last resort for high risk patients, it has been raised the question whether they could benefit from an allogeneic HSCT with a reduced-intensity conditioning regimen (Hamilton *et al.* 2017). Allogeneic HSCT is a potentially curative option for patients with T-cell ALL, but relapse rates post allogeneic HSCT is higher compared to B-cell ALL and still remain a major cause of death (Hamilton *et al.* 2017).

Despite the refinement and optimization of therapeutic approaches, when considering the extensive variety of cytological and genetic features characterizing different T-ALL subgroups, there is a wide spectrum of clinical outcomes that can be expected. For instance, ETP-ALL is associated with a substantially worse prognosis in children and

young adults compared with other T-ALL subtypes and despite an overall CR rate of 90% to 95%, approximately one-third of patients relapse, and the five-year OS rate for adults is approximately 50% to 55% (Hoelzer *et al.*2009). On the other side of the spectrum, it has been shown that cortical and mature T-ALL patients had a higher complete remission (CR) rate than patients with more immature T-ALL phenotype, implying a beneficial effect of later developmental arrest on CR achievement and OS rate (Marks *et al.* 2009). As for AML however, current clinical practice entails the use of a standardized course of action aiming to induce, consolidate and maintain patients into CR. The PHETEMA T-ALL adapted protocol is presented in figure 8.

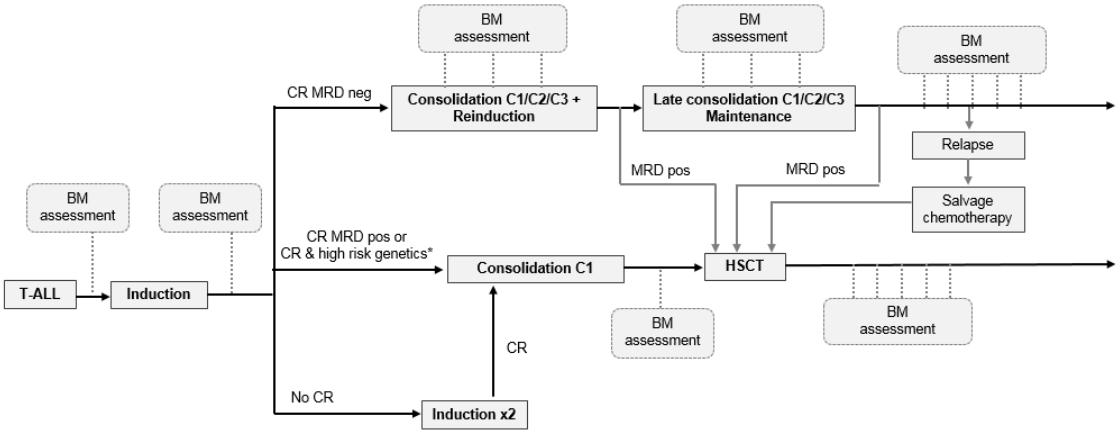


Fig 8) Schematic view of the T-ALL adapted PETHEMA protocol. The protocol follows consequential stages based on BM assessment, induction, consolidation, maintenance and based on patient's eligibility to HSCT

3. CANCER IMMUNOTHERAPY

3.1 Historical perspective on Cancer immunotherapy

At the beginning of the 20th century, it was proposed the existence of an immunological surveillance against tumor development (Ehrlich 1909), although it was not until the 70s when Burnet proposed a theory which would explain at the molecular and cellular level, the mechanisms giving rise to the proposed immuno-surveillance (Burnet 1970, Burnet 1971). Subsequently, it has been demonstrated that antitumor immunity could be transferred through the T lymphocytes among syngeneic mice (Whitney *et al.* 1975). In humans, it has been found that the presence of T lymphocytes (Haanen *et al.* 2006) or NK cells (Ishigami *et al.* 2000) infiltrated in different types of tumors lead to greater survival rate and, on the other hand, the incidence of cancer mortality in immunosuppressed patients is higher compared to patients who never received such treatments. Similarly, patients who underwent kidney transplantation and sub sequentially received immunosuppressive drugs, were in fact 10-30 times more likely to suffer from kidney cancer (Birkeland *et al* 1995). Taken together, these data corroborate the hypothesis that the immune system plays a vital role in regulating cancer onset and progression.

3.2 Current trends in cancer immunotherapy

Cancer immunotherapy is nowadays growing into an effective strategy among different therapeutic options and over the past years it has unraveled its potential against several types of cancers (Zhang *et al.* 2018). It relies onto the immune system's ability to induce sustained anti-tumor responses, thus blocking cancer progression (Mellman *et al.* 2011). In the last decade, cancer immunotherapy has witnessed a tremendous development, employing an ever growing array of therapeutic approaches such as utilization of cancer vaccinations, oncolytic viruses, immune checkpoint blockade therapy, TCR engineered

lymphocytes, Bispecific T-cell Engagers (BITEs) and Chimeric Antigen Receptor (CAR) T-cell therapy (Voena *et al.* 2016).

3.3 The role of T-cells in immunity

T cells are a type of lymphocyte which originate from BM-derived multipotent lymphoid progenitors (MLP) that enter the T cell pathway as they immigrate to the thymus. The most primitive cells in the thymus are the early thymocyte progenitors (ETP), which retain all lymphoid and myeloid potential, although transiently, as they rapidly differentiate into specific T-lineages with subsequent selection and export to the periphery. Developmentally, T cells comprise different subsets, including naive T cells (Tn), which have the capacity to respond to new antigens, memory T cells (Tm), which derive from previous antigen activation and maintain long-term immunity, and regulatory T (Treg) cells, that keep immune responses in check (Alberts *et al.* 2002). An immune response starts when naive T cells bind to a given antigen and to co-stimulatory ligands presented by dendritic cells (DCs), resulting in interleukin 2 (IL-2) production, proliferation, and differentiation to T effector cells (Teff) that then migrate to diverse sites to promote the removal of pathogens through the production of effector cytokines and cytotoxic mediators. Activated effector cells have a short life span, although a proportion survive as memory T cells (Saule *et al*. 2006). Memory T-cell subsets can participate in maintaining long-term immunity and recall protective responses. The role of T cells in immunity has to be framed taking into account the different phases across lifetime. Earlier in life, a large proportion of T cells are Tn freshly matured from the thymus, although Treg cells are also well represented (Kumar *et al.* 2018). It is within this stage, that the greatest number of new antigens are encountered and T cells have key protective functions in repelling pathogens, while Treg cells are critical for developing immune-tolerance to innocuous and ubiquitous antigens as long-term reserves of Tm are

established. Tm are generated from exposure to antigens and begin to accumulate during childhood. The level of Tm accumulation reaches a maximum in early adulthood and is maintained over several decades (Kumar *et al.* 2018). The changes occurring in T cell repertoire's composition, from naive to memory after childhood, and the relative stability of immunity over decades of adulthood, reinforce that T cells have different roles in children than in adults. Adults, tend to encounter fewer new antigens, so the role of T cells shifts from actively fighting incoming pathogens (and associated diseases) to maintaining homeostasis and regulation when an individual repeatedly and chronically encounters the same antigens spectrum (Goronzy *et al.* 2017).

3.4 Subsets of T-cells

T cells are grouped into a series of subsets based on their function as well as the associated gene or protein expression patterns. Current classification includes a multitude of T-cells subtypes, each of which was historically believed to serve a strictly defined set of tasks. Nowadays it is know that such strict compartmentalization might be misleading since there is not a clear consensus both at the semantic (nomenclature) and phenotypic level, regarding the functional characterization of these subsets (Mahnke *et al.* 2013). However, current knowledge establishes two major subgroups: "$CD4^+$ helper" and "$CD8^+$ cytotoxic" T-cells.

3.4.1 $CD4^+$ helper T-cells

$CD4^+$ cells differentiate into multiple subsets: T helper (Th) 1, Th2, Th9, Th17, Th22, regulatory T-cells (Treg), and follicular helper T cells (Tfh), which are characterized by different cytokine profiles (Cubas *et al.* 2015) All $CD4^+$ Th subsets arise from naive $CD4^+$ T cells through the action of different cytokines: Th1 by IL-12 and IFN-γ , Th-2 by IL-4,Th-9 by IL-4 and TGF-β , Th-17 by IL-1,IL-6, IL-23 and TGF-β meanwhile Th-22 by IL-6 and TNF. Conversely, each Th subset releases specific cytokines that can have

either pro- or anti-inflammatory functions, survival or protective functions. On the other hand, Treg production is influenced are by IL-2 and TGF-beta and Tfh by IL-6 and IL-21(Golubovskaya *et al.* 2016). A graphical depiction of different $CD4^+$ subsets formation is presented in figure 9.

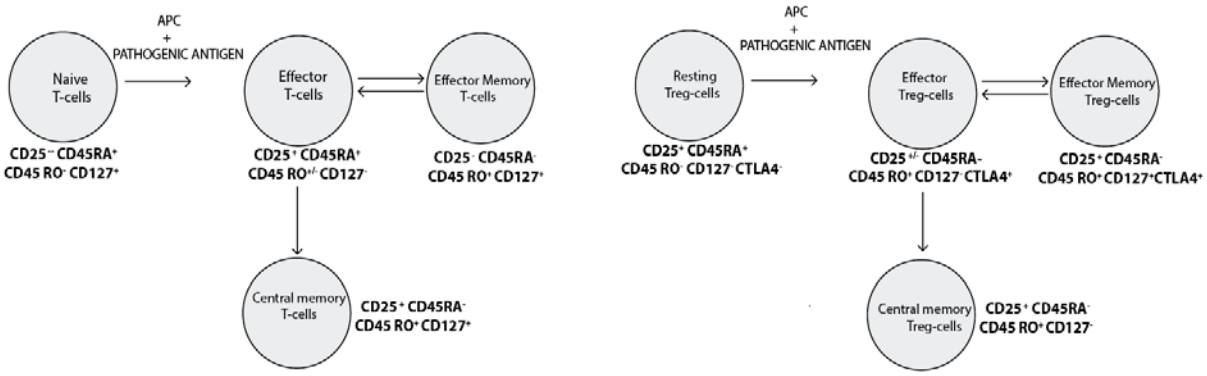


Figure 9) The differentiation of $CD4^+$ subsets. Graphical depiction of the maturation process leading to the formation of different $CD4^+$ subsets. Markers of each cell type for a given differentiation step are presented in bold

3.4.2 $CD8^+$ cytotoxic T-cells

Same as for the $CD4^+$ subsets, $CD8^+$ T cells undergo a differentiation process which sees multiple steps of maturation, till full effector phenotype is established. Naive T cells differentiate into stem cell memory cells (T-scm), T Central Memory cells (Tcm) T effector memory cells (Tem) and T effector cells (Teff). The different $CD8^+$-related markers upon cell differentiation, L-Selectin, CD45RO, CD45RA and CCR-7, are expressed in a sequential fashion as presented in figure 10. Effector functions increase upon $CD8^+$ T cell differentiation, while memory function decrease (Golubovskaya *et al.* 2016).

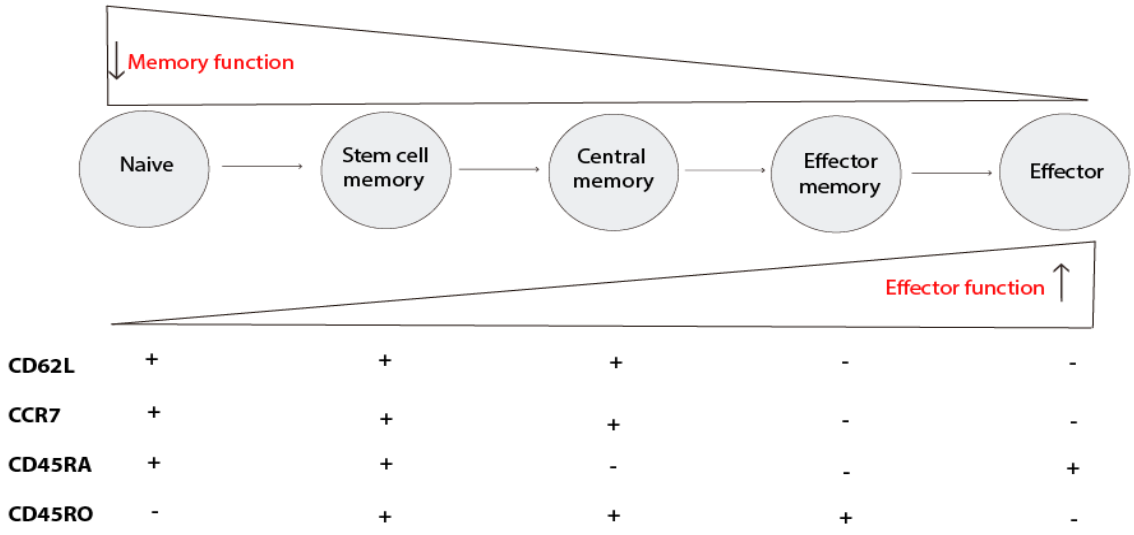


Fig.10) Differentiation of CD8$^+$ subsets. Graphical depiction of the maturation process leading to the formation of different CD8$^+$ subsets. Markers of each cell type for a given differentiation step are presented in bold

3.5 Mechanisms of T-cell activation

Upon completion of the maturation process, T-cells leave the thymus, and start circulating throughout peripheral blood (PB) until they recognize foreign antigens on the surface of specialized Antigen Presenting Cells (APCs). The activation process is a multistep mechanism which primarily involve the engagement of a T-cell Receptor (TCR) to a Major Histocompatibility Complex (MHC) on the APC. The TCR is a disulfide-linked membrane-anchored heterodimeric polypeptide consisting of the highly variable alpha (α) and beta (β) chains expressed as part of a complex with the invariant CD3 chain molecules. T cells expressing this receptor are denominated αβ T cells, although a minority of T cells express an alternative form of this receptor, formed by variable gamma (γ) and delta (δ) chains, referred as γδ T cells (Janeway *et al* 2001). Once the TCR, on both CD4$^+$ memory T cells and CD8$^+$ cytotoxic T cells, binds to the antigen held in place by the MHC complex, it triggers initial activation of T cells. The CD4 and CD8 molecules bind to the MHC molecule too, thus acting as stabilizing agent for the whole T-cell/APC

complex. This initial binding, the so called “first signal” (figure 11), sets the response in motion and normally takes place in the secondary lymphoid organs. However, in addition to TCR binding to antigen-loaded MHC, both helper T cells and cytotoxic T cells require a number of secondary signals to become activated and respond to the threat (Buckle *et al* 2018). In all T-cells, the first of these is provided by CD28, a molecule found on T cell membrane that binds to one of two molecules on the APC, B7.1 (CD80) or B7.2 (CD86) thus promoting T-cell proliferation (Peach *et al* 1995).

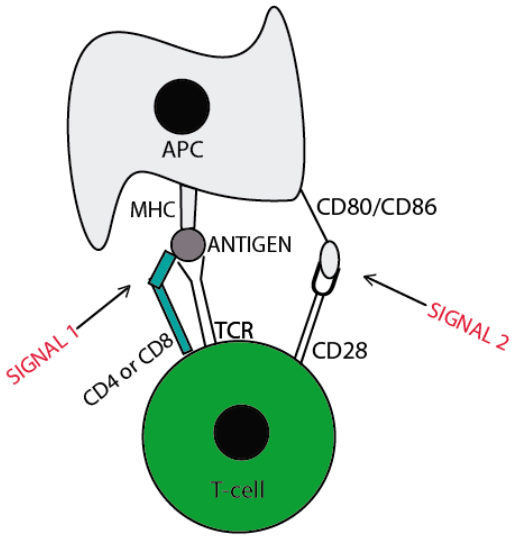


Fig.11) Basic mechanisms of T-cell activation. The “signal 1” for T cell activation, occurs following the recognition of MHC-peptide complex on an antigen-presenting cell (APC) by the T cell receptor (TCR), CD4/CD8 on a T cell. The “signal 2” for T cell activation is provided by binding of B7 molecules (CD80/CD86) on the APC to CD28 on the T cells. Following this interaction, T cells are activated and perform various effector functions.

3.6 T-cells effector functions

The antigen-specific TCR controls the delivery of effector signals in four ways: i) it creates a stable binding of effector cells to their selected targets by forming a tight junction, ii) it narrows the space in which effector molecules can be located, iii) it focuses their delivery at the site of contact by inducing a re-orientation of the secretory apparatus of the T-cell and iv) it triggers the synthesis and release of specialized molecules in the extracellular space (Janeway *et al.* 2001). All these mechanisms contribute to the selective

action of effector molecules towards the target antigen. In this way, T-cell activity is highly selective for those target cells that display the antigen, although the effector molecules themselves are not antigen-specific (Janeway *et al.* 2001). The effector compounds produced by armed effector T cells fall into two main categories: cytotoxins, which are stored in specialized lytic granules and released by cytotoxic CD8 T cells, and cytokines, which are synthesized *de novo* by all effector T cells (Janeway *et al.* 2001). The cytotoxins are the main effectors of cytotoxic T cells and, their release must be tightly regulated as they are not antigen-specific. By contrast, cytokines and membrane-associated proteins act by binding to specific receptors on the target cell. Cytokines and membrane-associated proteins are the principal mediators of $CD4^+$ T-cell effector functions, which are therefore directed towards specialized cells that express specific receptors for these molecules (Sallusto *et al.* 2004). $CD8^+$ T-cells recognize pathogen-derived peptides in a MHC class I dependent manner, and upon antigen engagement, they release most notably perforin and granzymes. Perforin is a glycoprotein responsible for pore formation in cell membranes of target cells as it is able to polymerize and form a channel across the target's surface (Lichtenhel *et al.* 1988). It does so by selective binding cells through membrane phospholipids, and the resulting pores disrupt membrane's integrity allowing free influx and efflux of ions and polypeptides, altering cell homeostasis and leading to tonic shock (Law *et al.* 2010). On the other hand, Granzymes, a family of serine proteases, act synergistically with perforin. Upon penetration within the target cell (whose integrity has been compromised by perforin), Granzymes (most notably Granzyme B) can cleave, and therefore activate, several procaspases and can also directly cleave downstream caspase substrates, including the inhibitor of caspase-activated DNase (ICAD), thus contributing in a major way to DNA fragmentation within the target cell (Trapani *et al.* 2001). A different mechanism by which $CD8^+$ T-cells induce

target cell death is related to the surface expression of a membrane-bound effector molecule termed Fas-ligand. In this context the $CD8^+$ T-cells mediated cytotoxicity, arise from the interplay between the Fas ligand expressed on T-cells and the Fas receptor expressed on target cells (Janeway *et al.* 2001). Trimerization of the Fas receptor by Fas ligand results in activation of caspase 8 that is responsible for triggering downstream caspases and cell death (Waring *et al.* 1999). Contrary to their $CD8^+$ counterpart, $CD4^+$ T-cells exert their effector functions mainly through secreting cytokines and chemokines that activate and/or recruit target cells. Proliferating $CD4^+$ T cells develop their effector functions to serve as a defense typically against intracellular bacteria and protozoa. They are triggered by the polarizing cytokine IL-12 and their effector cytokines are IFN-γ and IL-2. The main responders to helper T-cells action cells are macrophages, $CD8^+$ T cells and IgG B cells (Zhu *et al.* 2008). Through the production of IFN-γ secreted by $CD4^+$ T cells macrophages can receive the instruction to phagocytize and digest intracellular bacteria and protozoa. Helper T-cells can also lead to a humoral immune response, typically against extracellular parasites including helminths. Autocrine IL-2 secretion lead to the production of effector cytokines such as IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25. Within the $CD4^+$ T-cells mediated immune response, the main effector cells are by far represented by eosinophils, basophils, and mast cells as well as B cells (Wan *et al.* 2014).

4. CAR T-cells

Chimeric antigen receptor (CAR) T-cell therapy entails the genetic manipulation of healthy T-cells to express a membrane spanning chimeric protein with predefined specificity for a given tumor-associated antigen(s) (Kosti *et al.* 2018). They combine the antigen-binding property of MoAbs with the lytic capacity and self-renewal of T cells and display numerous advantages over unmanipulated T cells (Ramos *et al.* 2011). CAR-T cells are designed to engage and kill tumor cells irrespectively of the MHC recognition, in a way that target cell binding is unaffected by some of the major mechanisms by which tumors avoid MHC-restricted T-cell recognition, such as down-regulation of human leukocyte antigen (HLA) class I molecules or their defective processing (Maher *et al.* 2012). The general CAR architecture, as shown in figure 12 comprises three main modules, a binding domain, a hinge/trans-membrane domain and a signaling endodomain (s) (Dotti *et al.* 2014).

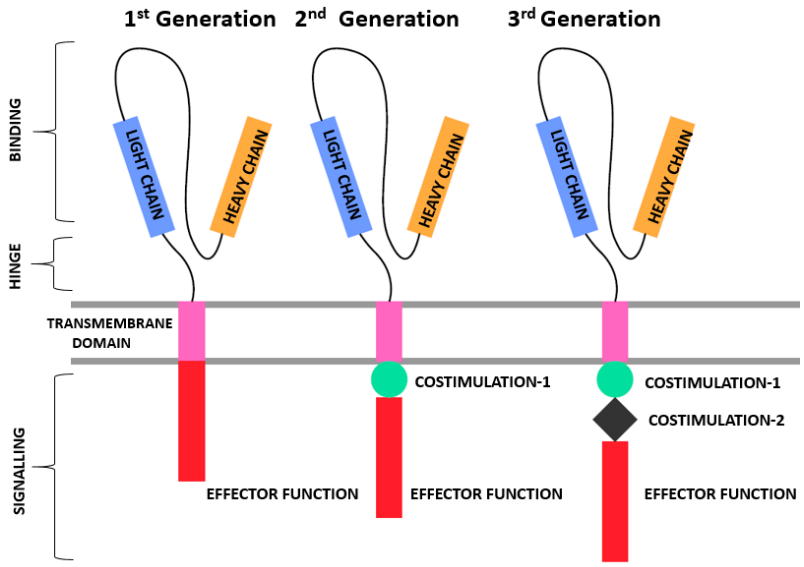


Fig. 12) Architecture of different CAR T-cells generations. CARs have the potential for suface redirection of activated T-cells. They are composed of the variable regions derived from a monoclonal antibody linked together by a serine-glycine peptide linker sequence. A molecular spacer or hinge domain facilitates outward projection of the binding domain away from the cellular surface while intracellular co-stimulation signaling domains mediate intracellular signal propagation to compensate for the lack of TCR and MHC co-stimulation, thus leading to T-cells effector functions.

4.1 The ectodomain

The ectodomain (binding domain) of a CAR, almost universally consist in a single chain variable fragment (scFv) of a MoAb, generated to target a tumor associated antigen (TAA) (Chmieleweski *et al.* 2004). Such scFvs, as shown in figure 13, consist in a fusion protein made of the variable regions of the heavy (V_H) and light chains (V_L) of immunoglobulins, inter-connected through a short linker peptide of 10 to 25 amino acids (Huston *et al.* 1988). The linker is generally rich in glycine to confer structural flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the V_H with the C-terminus of the V_L, or *vice versa* (Petersson *et al.* 2006). This polypeptide retains the binding specificity of the original immunoglobulin, despite the removal of the constant regions and the introduction of the linker (Petersson *et al.* 2006).

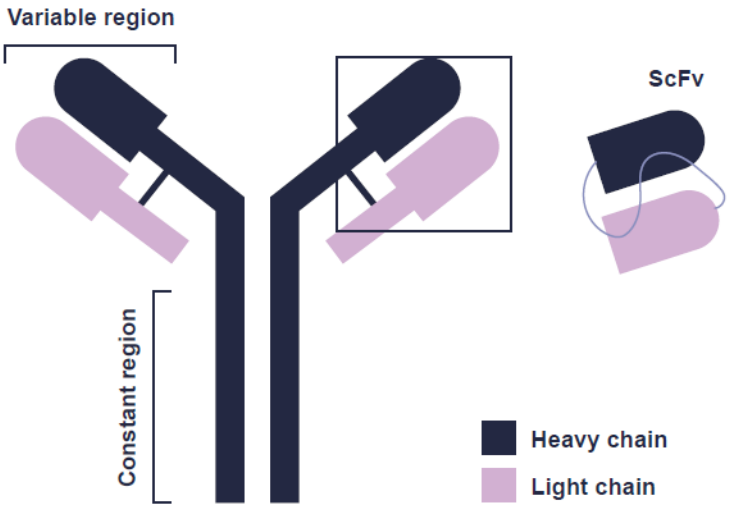


Fig.13) Representation of an antibody and scFv. MoAbs laid down the foundation for the development of CAR T-cells technology. scFvs are fusion protein constituted by the variable region of the Heavy and Light chain, united by a flexible linker which promote effective binding towards the designated target molecule

4.2 The hinge region

The ectodomain is followed by a hinge region, whose role is to augment the scFv flexibility, thereby relieving the spatial constraints between tumor antigens and CARs, while facilitating synapse formation between the CAR T cells and target cells (Qin *et al.* 2017). To the present date hinge regions most commonly derive from IgG subclasses (such as IgG1 and IgG4), IgD and CD8 domains (Qin *et al*.2017), and different lengths and amino acidic composition of such domain have been shown to profoundly affect CAR mediated cytotoxicity, as they primarily influence the binding capacity and the association of different CAR molecules on the surface (Alabanza *et. al* 2017, Hudecek *et al.* 2013).

4.3 The transmembrane domain

The transmembrane region represents a bridge between the hinge region and the endodomain of a CAR. Type I proteins such as CD3ζ, CD28, and CD8 have been historically used as transmembrane domains in CAR constructs (Dwivedi *et al.* 2018). In earlier times it was believed that the transmembrane domain does not have much impact on CAR T cell efficacy except anchoring CAR molecule to the membrane, however it is nowadays well established that they play an important role in CAR T-cell effector functions and persistence (Alabanza *et al.* 2017, Shirasu *et al.* 2012, Savoldo *et al.* 2011).

4.4 The endodomain

The endodomain represents the final step of the signaling propagation cascade started outside the T-cell when the anti scFv (or equivalent ectodomains) engaged with the target antigen. Historically, during the development of first generation CARs, the endodomain solely consisted in the CD3ζ subunit, which plays an important role in coupling antigen recognition to several intracellular signal-transduction pathways**.** In the context of CAR T-cells, upon target/anti scFv engagement, a phosphorylation cascade of immunoreceptor

tyrosine-based activation motif (ITAMs) present in CD3ζ intracellular domain, is initiated leading to activation and priming of CAR T cells (Dotti *et al.* 2014). However, more sophisticated intracellular modules have been created over time, and the CD3ζ subunit has been coupled with one or more co-stimulatory domains, serving the function of increasing the durability and potency of the cytotoxic signal. When the CD3ζ is paired with one co-stimulatory module, the CAR construct is defined as being "second generation" (Savoldo *et al.* 2011), and most notably, CARs containing either CD28 or 4-1BB costimulatory domains have been the most widely used to date, as both of them have granted impressive responses in clinical trials (Kochenderfer *et al.* 2012, Porter *et al.* 2012). Several studies suggest that the CD28 intracellular domain stimulates greater CAR T cell functionality, whereas the 4-1BB intracellular domain promotes greater CAR T cell persistence (Savoldo *et al.* 2011, Dotti *et al.* 2014, Guedan *et al.* 2018). Recent advancements in the field of CAR T-cells, have led to the development of more elaborated intracellular domains with the aim of granting higher T-cell proliferation and "in-patient" persistence. Such multi-modular design, sees the incorporation of two co-stimulatory domains cloned in frame (i.e CD28 followed by 4-1BB) (Zhong *et al.* 2010, Guedan *et al.* 2018), and they take the name of "third generation CAR". The combination of CD28 and 4-1BB signals are functionally additive when combined within a single CAR and that the improved T-cell activation is at least partially dependent on the activation of the Akt pathway (Morgan *et al.* 2018), which is independently and synergistically recruited by CD28 and 4-1BB. The incorporation of both modules, is thus superior to second generation CARs when it comes to promote *in vitro* and *in vivo* T-cell persistence and cytotoxicity (Zhong *et al.* 2010). In addition to second and third generation CAR T cells, a fourth generation of constructs have been generated that incorporate a third stimulatory signal. Colloquially known as "TRUCK" T cells, fourth generation CAR T cells are

defined as CAR T cells armed with immune stimulatory cytokines (Chmielewski *et al.* 2015) that improve CAR T cell expansion and persistence while rendering them less susceptible to the immunosuppressive tumor environment. Additionally, transgenic cytokine expression can potentially trigger bystander T cells to eliminate antigen-negative cancer cells at the target site (Petersen *et al.* 2019).

5. GENETIC ENGINEERING INTO T-CELLS

T-cell genetic modification strategies usually rely on integrating vectors to ensure effective sustained transgene expression by cellular progeny. Although a wide array of genetic modification strategies are currently available, the present work will focus exclusively on the two integrating vector strategies employed in clinical practices: retroviral and lentiviral vectors.

5.1 Retroviral vectors

The function of a retroviral expression vector (RV) (schematically represented in figure 14) is to ensure permanent integration of a replication deficient provirus, carrying the (trans)gene to be delivered, into the chromosomal DNA of the target cell. Three indispensable events have to take place to achieve successful transduction: viral penetration into target cells, reverse-transcription and proviral integration (Baum *et al.* 2006). Viral entry occurs via two possible mechanisms: receptor-based internalization or fusion on the viral envelope together with the plasmatic membrane. After entry, the retroviral RNA-based genome needs to be modified and carried inside the nucleus. The reverse transcriptase (*pol* gene), provided by the vector, will turn the ssRNA genome into dsDNA (Everson *et al*. 2016). Such transformation is accompanied by the establishment of a pre-integration complex (PIC) which combines the recently formed dsDNA along with the proteins of both eukaryotic and viral origin that will mediate its transfer to the host nucleus. Together with ssRNA to dsDNA conversion, the genes *gag* and *env* (provided by the vector) will mediated the formation of the structural components of the virus (a phenomenon common to both retroviral and lentiviral systems). However, as the PIC of retroviruses are relatively unstable, they are unable to cross the intact nuclear membrane of non-dividing cells (Goff *et al.* 2007). Such feature represent a key limiting

factor for the applicability of retrovirus-based gene delivery, thus reducing the spectrum of cells that can be infected (Goff *et al.* 2007).

Fig.14) Schematics of a retroviral vector. 5' long terminal repeat (LTR) which, in the DNA form found in the provirus acts as a transcriptional promoter, and in the RNA (genomic) form contains sequences important for reverse transcription of the genome. **Moloney Murine Leukemia Virus psi (MMLVψ)** sequence which directs packaging of the genomic RNA into the virion. **Transgene** represents the gene (CAR) to be inserted into the host genome. **3' LTR** which, in the DNA form (in the provirus) acts as a polyadenylation signal, and in the RNA (genomic) form contains sequences important for the reverse transcription process

5.2 Lentiviral vectors

Contrary to RVs, lentiviruses such as HIV-1, which constitute the vast majority of lentiviral vectors (LVs) currently in use, present a highly stable PIC, allowing for transport across an intact nuclear membrane (Beutler *et al*. 2001). Hence, the capability to transduce non-dividing cells attracted interest into the application of LVs as an alternative to retroviral mediated strategies.

Considering that the HIV-1 lentiviral genome is considerably more complex than retroviral genomes, the field of gene therapy has witnessed a constant improvements of LVs with the aim to increase biosafety and reliability from these gene transfer vehicles. Novel tools, such as second and third generation LVs have become the golden standard of gene delivery, offering the highest possible safety profile (Gandara *et al.* 2018). As a matter of fact, current third generation LVs solely requires 3 of the original 9 HIV genes, and significant deletions to the viral long LTR domains allowed the establishment of self-inactivating (SIN) vectors which are LTR independent and rely for transgene expression on exogenous promoters cloned upstream the gene of interest (figure 15) (Zufferey *et al*.1998). Furthermore, current standards of the field, see the separation of key components for the viral replication machinery (*gag*, *pol* and *env*) on three different plasmids, meaning that three separate recombination events would have to occur in order

to create replication competent lentivirus (RCL). On the other hand, it must be remarked that increased biosafety is obtained at the expenses of diminishing the viral titer calculated as infection units/ul (UI/ul). The present work, like many others (Sanchez *et al.* 2019, Castella *et. al* 2019) employ LVs pseudotyped with VSVg envelope protein (env), which is compatible with second or third LV systems and simultaneously permits ultracentrifugation, thus offering the best tradeoff between safety and high viral titers (Gandara *et al*. 2018)

Fig.15) Schematics of a SIN Lentiviral vector. 5'Truncated Long Terminal Repeat (LTR) which present inactivating deletions that down-regulate the enhancer/promoter in the long-terminal repeat. **Human Immunodeficency Virus psi (HIVψ)** sequence which directs packaging of the genomic RNA into the virion. **Human Elongation Factor α-promoter (EF-1α promoter)** which replaces the LTR to drive the expression of the gene to be inserted. **Transgene** represents the gene (CAR) to be inserted into the host genome. **3'Truncated Long Terminal Repeat (LTR)** shows deletions that renders it unable to contribute to the transcription of the Transgene, thus leaving its expression entirely dependent on the internal promoter.

5.3 Risks associated with T-cells engineering

Concomitantly with the desired anti-tumor effect, altering and manipulating normal T-cells and their naturally occurring immune responses, might lead towards unpredictable and undesirable toxicities (Tey *et al.* 2014). Concerns for patients' safety, regarding the deployment of T-cell therapies, have been identified: i) on-target off-tumour toxicity (OTOT), aberrant deletion of non-tumorous tissue, ii) insertional mutagenesis, disruption of healthy cellular homeostasis resulting from the introduction of genetic material, iii) immunological toxicities associated with *ex-vivo* T-cell manipulation (Yee *et al.* 2000, Schlimgen *et al.* 2016, Graham *et al.* 2018)

5.3.1 On target off tumor toxicities

CAR T-cell therapy needs the presence of a designated target molecule to enable recognition of cancerous cells. These TAAs) can often be found on healthy tissues, as the

cancer lesions may arise from normal cells (Sun *et al*. 2018). Thus, apart from rare cases such as viral proteins or characteristic mutations, expression of most tumor antigens is not exclusive to the bulk of cancerous cells, implying that targeted immunotherapy may also result in OTOT (Sun *et al*. 2018). In some fortunate cases, secondary damage resulting from therapeutic elimination of abnormal tissue may be readily overcome such as with anti CD19 CAR T-cells, where depletion of the healthy B-cell compartment can be replenished with gamma globulin replacement therapy (Maher *et al.* 2012).

5.3.2 Insertional mutagenesis

Any DNA alteration is by definition mutagenic, however not all mutations have the same impact on the overall homeostasis of a cell. Therefore, the potential dangers resulting from many genetic modifications can only be inferred. Stable incorporation of viral vectors, may induce mutagenic toxicity via a wide array of mechanisms. Since the viral LTR can serve as promoter, as well as, enhancer sequence, the most threatening risk from LTR-driven gene (over)expression is the possibility of altering endogenous gene expression (Weber *et al.* 2007). Interaction among transcription factors and the integrated viral LTR can result in activation of adjacent cellular proto-oncogenes. Although this phenomenon is most evident through excessive cellular proliferation, generally associated with oncogenic hits, negative impacts such as altered transcriptional reading frame might debilitate cellular functions without displaying a clear detectable phenotype (Goncalves *et al.* 2017). Conversely, direct vector insertion within coding sequences may cause deletion or abnormal truncation of cellular transcripts (Coffin *et al.* 1997). Further, viral enhancer regions have been shown to promote disruption of endogenous gene expression of coding regions situated hundreds of kilobases downstream the viral integration sites (Hargrove *et al.* 2008). Lastly, vector integration may cause genomic disruption through

impacts upon genetic regulatory domains causing altered splicing patterns or polyadenylation (Dudley 2003).

5.3.3 Immunological toxicities associated with *ex-vivo* T-cell manipulation

Under the safety stand point, a concluding remark should be made when addressing safety and efficacy of CAR-T cell therapy. Although the CAR T-cells manufacturing process has refined and optimized its standards to ensure the highest safety and reproducibility, the non-physiological stimulation that T-cells are subjected to, or signal leakage from (second generation onward) CAR-bearing vectors, might in principle give rise to unwanted cellular proliferation even in the absence of CAR T-cells/target engagement. Moreover, the possible occurrence of insertional mutagenesis, combined with potential selective advantage driven by additional intracellular co-stimulatory domain(s) could put at risk patients of uncontrolled lymphoproliferation (Zhang *et al.* 2017, Albert *et.al* 2019).

5.3.4 Safety Switches to reduce toxicity in *ex vivo* T-cell engineering

Safety switches, also known in the field as suicide genes (if considered at the DNA level), in broad terms comprise a native or engineered protein, which is normally absent in unmodified target cells. The expression of such artificially introduced peptide, allow selective termination of modified cells after being exposed to a therapeutic compound generally being an antibody or a small, otherwise inert, molecule. The presence of a suicide gene as a mean to increase safety in CAR T-cell therapies have been explored in case of immunological toxicities, OTOT or cytokine release syndrome (CRS) (Philip *et al.* 2014, Tasian *et al.*2017, Diaconu *et al.* 2017). In principle, an adequate suicide system should have the following characteristics: i) have no intrinsic toxicity, ii) don't elicit immune responses that would otherwise reject it, iii) be specifically inducible with a well-tolerated compound (not self-activation) and iv) it should not induce cellular resistance (i.e cells becoming resistant the action of the suicide drug).

5.3.4.1 Catalytic vs Non-Catalytic safety switches

Suicide strategies can be loosely based on two catagories: Catalytic and Non-catalytic. Catalytic suicide system encode for a protein which have the capacity to elicit intrinsic cellular pathways that would eventually lead to cell elimination. The Non-catalytic counterpart, rely on the presence of extracellular machineries to mediate deletion. Examples of the latter include: complement mediated cytotoxicity (CDC) or antibody dependent cytotoxicity (ADCC).

5.3.4.2 Inducible Capsase 9

The iC9 system utilizes a synthetic fusion gene construct comprising a mutated human caspase9 activation domain fused to a synthetic FKBP12 binding domain via a serine-glycine (Ser-Gly_{x4}) linker (Diaconu *et al.* 2017). Apoptosis machinery initiation is mediated through the administration of AP1903, a lipid-permeable, non-toxic analogue of the clinically approved dimerising agent FK506 (Straathof *et al.* 2005). iC9 represents a catalytic suicide gene strategy, where cellular deletion is dependent upon apoptosis mediated via endogenous cellular machinery. In a 2011 clinical trial (Di Stasi *et al.* 2011), the efficacy of iC9 in the contest of elimination allogeneic T-cell in case GVDH developed. It was demonstrated that single dose of AP1903 resolved of GvHD symptoms in all patients treated, with 90% of transgenic cells deleted within 30 minutes following therapeutic administration.

5.3.4.3 Transgenic CD20

CD20 historically seemed to constitute the best option when it came to choose a suicide system, offering clinical grade selection using Miltenyi magnetic sorting beads as well as depletion mediated by Rituximab (RTX), a clinically approved anti CD20 MoAb. This strategy represent a hybrid between the catalytic and non-catalytic approaches mentioned before. Extensive work (van Meerten *et al.* 2006, Tasian *et al.* 2017) has been conducted

on CD20 as a potential termination strategy for T-cell therapy. RTX is a MoAb that recognizes the human CD20 molecule and is joined to a human IgG1 constant region. This IgG1 region would be responsible for the activation of the CDC and the recruitment of effector cells (ADCC) to kill the RTX-bound cells. Such feature would then, according to the criteria established in the point 5.3.4.1 of this work, qualify CD20 as non-catalytic. However, it has also been demonstrated that RTX can induce direct apoptosis, growth arrest of CD20-positive cells as a result of the activation of CD20 apoptotic pathway (Smith *et al.* 2003). CD20 constitutively associates with lipid rafts in which src-family, which are in an inactive form. When anti CD20 MoAbs cross-link with CD20 antigen, they induce a conformational change in the membrane lipid rafts which in turn activate src-familiy kinases. When activate, they start downstream signaling resulting in the initiation of an apoptotic response (Deans *et al.* 2002), hence placing CD20 depletion system in the catalytic group

6. IMMUNOTHERAPY IN ACUTE LEUKEMIAS

Immunotherapy for Leukemia has created substantial interest in the hemato-oncology because of its non-overlapping toxicity with chemotherapy and radiation therapy (Mahoney *et al.* 1981, Leung *et al*. 2009). Moreover, our immune cells display an immense diversity of effector mechanisms, involving Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL), complement, Perforin, Granzyme, pro-inflamatory cytokines, myeloperoxidase, superoxide, and nitric oxide, that can be used to tackle the growth of leukemic cells (Leung *et al*. 2009). Anyhow, to ensure proper recognition and elimination of cancer cells by immune cells (such as T-cells or NK cells), distinct tumor antigens must be displayed coupled with the appropriate MHC. Considering the multifaceted nature of an anti-oncogenic immune response, there are countless mechanisms through which cancerous cells can evade the immune system recognition (Khong *et al.* 2002, Zitvogel *et al.* 2006). Major mechanisms include: natural selection of less immunogenic malignant cells during oncogenesis, mainly driven by antigen loss or by downregulation of MHC expression, or conversely, the expression of co-stimulator molecules can be tuned-down (Leung *et al*. 2009).

6.1 Immunotherapy with Monoclonal Antibodies

To date, an ever increasing number of MoAbs are pre-clinically validated and approved for the treatment of a wide variety of hematological malignancies. (Moccia *et al.* 2008). Some of the leading improvements in designing monoclonal antibodies consist in: i) humanization and reduced immunogenicity, ii) better pharmacokinetics, iii) increased ADCC (figure 16), and iv) improved capacity to elicit the activation of complement CDC (Figure 16) (Leung *et al.*2009). A summary of MoAbs with their specific characteristics are listed in table 3.

NAME	ANTIGEN/DISEASE	CHARACTCERISTICS
Rituximab	CD20/B-ALL	Chimeric Human/Murine MoAb
Cetuximab	EFGR/Colorectal Cancer	Chimeric Human/Murine MoAb
Talacotuzumab	CD123/AML	Humanized with Fc ennginered to increase affitity for NK cells
Gemtuzumab Ozogamicine	CD33/AML	Antibody-Drug conjugate
Blinatumumab	CD19/B-ALL	Bite with dual affinity for CD19 and CD3
Brentuximab vedotin	CD30/Hodgkin	Antibody-Drug conjugate
Mogamuluizumab	CCR4	Human MoAb

Table 3) Examples of MoAbs. List commonly used MoAb and their specific characteristics

Other key advances are represented by the ability to genetically engineer the antigen binding, the constant and the variable regions, to enhance antibody survival, as well as the Fc region to increase binding to FC-γ RIII (Leung *et al.*2009). Arming the MoAb with toxins, or engineered bi-specificity have shown great enhancement in patients remissions and survivals (table 3) (Larson *et al.* 2005, Chichili *et al.* 2015, Kantarjian *et al.* 2017). Combinations of these approaches may increase the potency and the specificity while reducing toxicity.

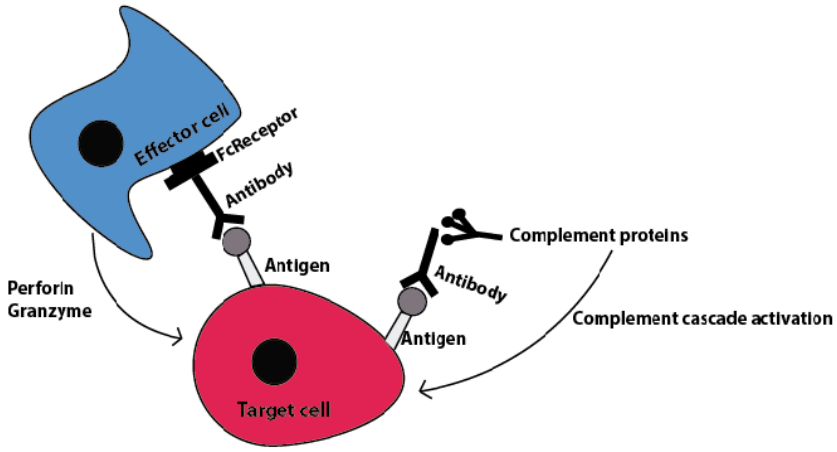


Fig.16) Schematics of CDC and ADCC mediated apoptosis. After antibody-antigen engagement two possible scenarios lead to targeted cell death. ADCC involves the engagement of an effector cells, which then releases Perforin or Granzyme, while in the CDC the antibody can bind the complement proteins leading to complement cascade activation and elimination of the target cell.

6.2 Immunotherapy with Bispecific T-cell engagers (BiTEs)

Bi-specific T-cell engagers (BiTEs) are a class of artificial MoAbs that are investigated for the use as anti-cancer drugs. They direct a host's immune system, more specifically the T cells' cytotoxic activity, against cancer cells. BiTEs are fusion proteins consisting

of two scFvs of different MoAbs. As shown in figure 17, one of the scFvs binds to T cells via the CD3 receptor, and the other to a tumor cell via a tumor specific surface antigen. As other bi-specific antibodies, but contrary to common MoAbs, BiTEs form a tight link between T cells and tumor cells (Nagorsen *et al.* 2009). This causes patient's T cells to exert autologous cytotoxic activity on tumor cells by producing proteins like perforin and granzymes, independently of the presence of MHC I or co-stimulatory molecules. These proteins enter tumor cells triggering cell's apoptosis thus mimicking the physiological responses during T cell attacks.

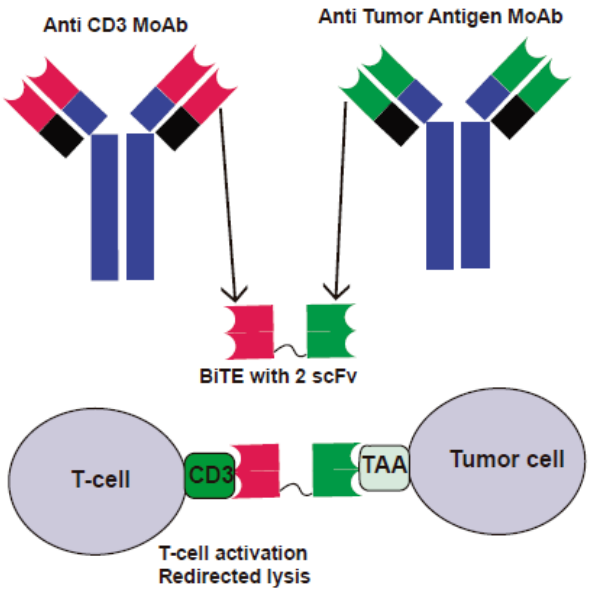


Fig 17) BiTE architercture and function. BiTEs comprise two distinct scFv united via a flexible linker. One scFv recognizes CD3, while the other one binds to a Tumor Associated Antigen (TAA). Upon binding to both cells, T-cells are activated and their cytotoxic function are redirected towards the

6.3 Immunotherapy with CAR T-cells

CAR T-cells have revolutionized the (pre) clinical landscape in respect of the patients' treatment with no further therapeutic options. The remarkable feats of effectiveness hold many promises for current and future therapeutic approaches in both "liquid" and "solid" tumors (Kosti *et al.* 2018, Morgan *et al.* 2018, Sun *et al.* 2018). As they combine the

specificity of MoAb with the increased persistence of effector cells (autologous as well as allogeneic) (Benjamin *et al.* 2019), they grant unprecedented response rates in very aggressive and/or resistant form of cancers (table 4). Worth to be mentioned that other source of effector cells are currently under investigation, such as NK cells, cytokine-induced killer (CIK) cells or Vδ1 γδ T-cells (DOTs) (Rotolo *et al.*2019), yet, for the scope of this work, only T-cells will be taken into consideration. CAR T-cells redirected against relapsed/chemo-resistant form of B-Cell ALL emerged as a potential therapeutic tool in the early 2010's (Hay *et al.* 2017), where CD19 was used as a therapeutic target in primarily, but not exclusively, pediatric B-cell ALL. The selective targeting of CD19 in refractory/relapsed patients showed impressive response rates, reaching up to 85% ((Kochenderfer *et al.* 2012, Brentjien *et al.* 2013, Alabanza *et al.* 2017)

INSTITUTE	VECTOR	ICD	DISEASE	LD CHEMOTHERAPY	CART DOSE	RESPONSE
CHOP	Lentivrus	4-1BB	Pediatric ALL; n = 53	Investigator's choice	1.0 × 10^6 to 17. × 10^6 CAR^+ T cells/kg	CR: 50/53 12 mo RFS: 45% 12 mo OS: 78%
NCI	γ-retrovirus	CD28	Pediatric ALL; n = 20	Cy 900 mg/m^2 ×1 + Flu 25 mg/m^2 ×3 d	1 × 10^6 (N = 16) vs 3 × 10^6 (N = 4) CAR^+ T cells/kg	CR: 14/20 LFS: 79% at 4.8 mo OS: 52% at 7.8 mo (all)
NCI	γ-retrovirus	CD28	Adult CLL B-NHL; n = 8	Cy 60 mg/kg ×2 d + Flu 25 mg/m^2 ×5 d	0.3-3.0×CAR^+ T cells/kg	ORR: 6/8 (CLL, 3/4; FL, 2/3); CR, n = 1 (CLL) and PR, n = 5
NCI	γ-retrovirus	CD28	Adult B-NHL; n = 15	Cy 60 mg/kg ×1-2 d + Flu 25 mg/m^2 ×5 d	1 × 10^6 to 5 × 10^6 CAR^+ T cells/kg	CR: 4/7 (refractory DLBCL), 4/6 (indolent B-NHL)
MSKCC	γ-retrovirus	CD28	Adult ALL; n = 46	Cy or Cy + Flu	1 × 10^6 vs 3 × 10^6 CAR^+ T cells/kg	CR: 37/45 6 mo OS: 65% (all)
FHCRC	Lentivirus	4-1BB	Adult ALL; n = 29	Cy or Cy 60 mg/kg ×1 + Flu 25 mg/m^2 ×3 d	2×10^5- 2×10^7 CAR^+ T cells/kg; 1:1 $CD4^+$:$CD8^+$	CR: 10/12 (Cy only) and 14/14 (Cy + fludarabine)
FHCRC	Lentivirus	4-1BB	Adult B-NHL; n = 28 Adult CLL; n = 6	Cy 60 mg/kg ×1 ± etoposide or Cy 60 mg/kg ×1 + Flu 25 mg/m^2 ×3 d	2 × 10^5, 2 × 10^6, and 2 × 10^7 CAR^+ T cells/kg 1:1 $CD4^+$:$CD8^+$	ORR: 8/14 Median PFS: 7 mo Median OS: 29 mo
UPenn	Lentivirus	4-1BB	Adult CLL; n = 14	Investigator's choice	0.14 × 10^8 to 11 × 10^8 CAR^+ T cells	ORR: 9/23 (CR, n = 5, PR, n = 4)
UPenn	Lentivirus	4-1BB	Adult CLL; n = 26	Investigator's choice	5 × 10^7 vs 5 × 10^8 CAR^+ T cells	ORR: 15/22 PFS: 62% at 11.7 mo
UPenn	Lentivirus	4-1BB	Adult B-NHL; n = 24†	Investigator's choice	3.08-8.87 *10^6 CAR^+ cells/kg	CR: 8/9 (all MRD− in all)

Table 4) Summary of anti CD19 CAR T-cell clinical trial. CHOP, Children's Hospital of Philadelphia; **CR**, complete response; **Cy**, cyclophosphamide; **DLBCL**, diffuse large B-cell lymphoma; **FHCR**C, Fred Hutchinson Cancer Research Center; **FL**, follicular lymphoma; **Flu**, fludarabine; **L**D, lymphodepleting, , **ICD**, Intracellular domain **LFS**, leukemia-free survival; **MCL**, mantle cell lymphoma; **MRD−**, minimal residual disease negative; **MSKCC**, Memorial Sloan Kettering Cancer Center; **NCI**, National Cancer Institute; **ORR**, objective response rate (in evaluable patients); **OS**, overall survival**; PFS**, progression-free survival; **PR**, partial response; **RFS**, relapse-free survival; **UPenn**, University of Pennsylvania. (Park *et al.* 2016)

The impressive clinical success of anti CD19 CAR T-cells, did not rely exclusively on the ability to strongly promote remission in $CD19^+$ leukemia. The biology and tissue distribution of CD19, as well as the existence of effective therapies (immunoglobulin replacement) to counteract CAR T-cell induced B-cell aplasia, made this surface molecule the ideal target to selectively redirect engineered T-cells (Hay *et al.* 2017). First, CD19 compared to other B-cell lineage antigens such as CD22, did not display internalization when bound to anti CD19 MoAb (Du *et al.* 2008), which implies that it would be less prone to immune evasion. Second, CD19 is exclusively expressed on B-cells, making the resulting "on target off tumor toxicity" less of an issue compared to other targetable antigens, whose expression is found throughout the body and in a variety of different tissues. Third, the prolonged (and perhaps desired) bystander aplasia of healthy B-cells, can be safely managed with monthly intravenous immunoglobulin administration to minimize infectious complications (Doan *et al.* 2018). Contrary to B-lineage malignancies, where B-cells specific antigens (CD19, CD20 and CD22) can be targeted in a relatively safe manner, the successful implementation of CAR T-cell for other hematological malignancies, is lagging behind at both pre-clinical and clinical level (Tasian *et al.*2018).

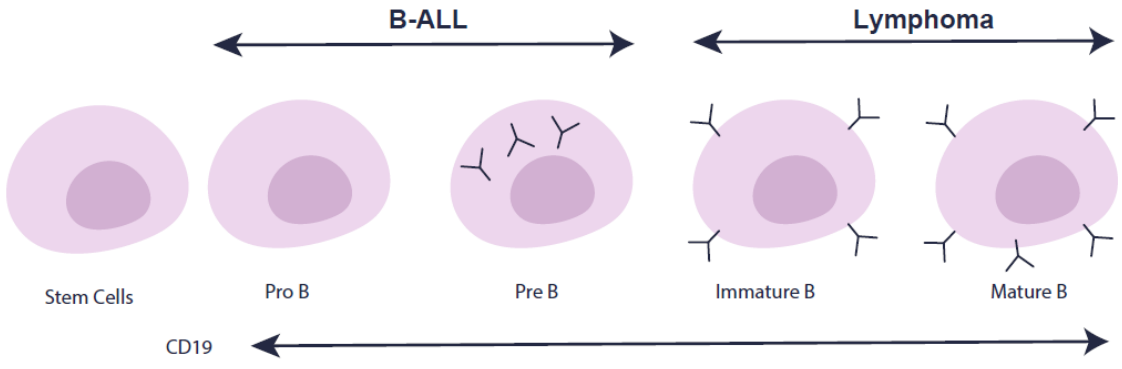


Fig.18) CD19 throughout B-cell development. Graphical representation of the major developmental stages of B-cell maturation. Depending on which stage the oncogenic process takes place and whether the tumor is in the BM or in extramedular hemopoietic sites such as lymph nodes, we can distinguish between B-ALL and Lymphoma. In all cases positivity for CD19 represent the hallmark for the majority of the B-lineage malignancies. Importantly, the stem cell compartment is $CD19^-$

6.3.1 CAR T-cells in myeloid malignancies

In the case of AML, the field is witnessing an alarming shortage of safe targetable molecules. Regarding AML, the optimal surface molecule characteristics would include its restriction to malignant cells, without concomitant expression on HSCs or normal tissues. Ideally, the antigen(s) should be critical to cancer initiation and/or maintenance (thus expressed on both stem cell compartment and bulk disease). Furthermore, an ideal target should be increasingly expressed a relapse or advanced disease stages, since CAR T-cells don't represent a first line of treatment. Taking into account the lack of AML-exclusive surface molecules, the majority of preclinical and clinical CAR T-cells studies have rather tried to identify a therapeutic window to redirect T-cells towards myeloid antigens overexpressed on AML blasts that are also present at variable levels on normal tissues, predominantly on hematopoietic stem cells (Tasian *et al.* 2018). While antecedent MoAb-based approaches redirected towards AML have laid down wishful hopes that targeting the same antigens with CARTs would be similarly well tolerated, it is of paramount importance to strengthen the fact that CARTs are living effector cells, which are in most cases, far more potent and provide longer lasting effects than their MoAb counterparts (Tasian *et al.* 2018). CAR T-cell persistence is undeniably an issue of an immense relevance, as it is required to eradicate leukemia, on the other hand however, it might come at the cost of myeloablation which poses patients at great risks.

6.3.1.1 Targetable antigens in AML

In the previous years, various preclinical works have demonstrated encouraging *in vitro* and *in vivo* effectiveness of CAR T cells engineered to target various myeloid antigens in human AML cells, those included but were not limited to including Lewis-Y (Peinert *et al*.2010) CD33 (Dutour *et al.* 2012, Pizzitola et *al.* 2014) CD123 (Mardiros *et al.* 2013, Gill *et al.* 2014, Tasian *et al.* 2017) CD44v6 (Casucci et al. 2013) ,the FLT3

receptor (Jetani et al.2018), CD38 (Drent *et al.* 2016) and C-kit (Arai *et al*. 2018). A summary of the current clinical trials in AML is represented in table 5.

INSTITUTE	ANTIGEN	IDENTIFIER	VECTOR/ICD	LD CHEMOTHERAPY	CART DOSE
PMCC	**Lewis-Y**	NCT03851146	Lenti/CD28	Flu (25 mg/m2) Cy (300mg/m2)	1×10^8 to 5×10^9 CAR^+ T cells/kg
MDA	**CD33**	NCT03126864	Lenti/NS	Flu (25 mg/m2) Cy (900mg/m2)	$>1.5x10^5$/kg CAR^+ T cells $\leq 4.5 \times 10^5$/kg
UPENN	**CD33**	NCT03971799	NS/NS	Flu (25 mg/m2) Cy (900mg/m2)	$3x10^5$/kg CAR^+ T cells
FENGTAI	**CD123**	NCT03114670	Lenti/4-1BB	Flu (NS) Cy (NS)	NS
COH	**CD123**	NCT02159495	Lenti/CD28	Flu (25 mg/m2) Cy (900mg/m2)	NS
UPENN	**CD123**	NCT03766126	Lenti/4-1BB	Flu (NS) Cy (NS)	$1x10^5$/kg to 5×10^5/kg CAR^+ T cells
IRCSS	**CD44v6**	NCT04097301	NS/NS	Flu (NS) Cy (NS)	$1x10^6$/kg to 2×10^6/kg CAR^+ T cells
COH/MDA	**FLT3**	NCT03904069	NS/NS	Flu (NS) Cy (NS)	NS
ZHUJANG	**C-kit**	NCT03473457	NS/NS	Flu (NS) Cy (NS)	NS

Table 5) Summary of ongoing AML CAR T-cell clinical trials. COH, City of Hope; **Cy**, cyclophosphamide; **Flu**, fludarabine; **ICD**, Intracellular domain: **IRCCS**, Istituto Di Ricovero e Cura a Carattere Scientifico; **LD**, lymph depleting, **Lenti**, Lentiviral; **MDA**, MD Anderson; **PMCC**, Peter Mac Callum Cancer Center; **UPENN**, University of Pennsylvania

6.3.2 CAR T-cells in T-cell malignancies

When it comes to translating CAR T-cells therapy to T-ALL, the field faces different challenges compared to AML, which are similarly causing important delays in the full applicability of CARTs to T-cell leukemia. Harnessing the potency of CAR T-cells effector functions to kill malignant T cells, while sparing normal T cells, revealed to be a very complex endeavor. Major obstacles lie within: the biological aspect of CAR T-cells/T-cells interaction and the technical hurdles faced while generating CAR T-cells. First, redirecting T-cells against T-cells in fact would unfold a major issue, namely T-cell aplasia (Fleischer *et al.* 2019, Sanchez *et al.* 2019, Mamonkin *et al.* 2015). Such phenomenon would likely cause a profound (transient or permanent) immunodeficiency,

which ultimately would increase comorbidity and mortality. Second, reprogramming "healthy" T-cells to target a T-cell specific antigen would induce CAR T-cells fratricide, thus decreasing *in vivo* CAR T-cell persistence. Third, the process of CAR T cells generation requires the harvesting and isolation of normal T cells from patients for CAR-modification. Because normal and tumoral T-cells usually share the same immunophenotype, it is likely to harvest blast cells for CAR transduction. An additional flaw in the separation process would lead to malignant cells contamination, thus a single circulating T-cell blast that would undergo CAR transduction, could express the scFv on its surface, which can result in masking the antigen leading to antigen-positive relapse (Ruella *et al.* 2018, Fleischer *et al.* 2019).

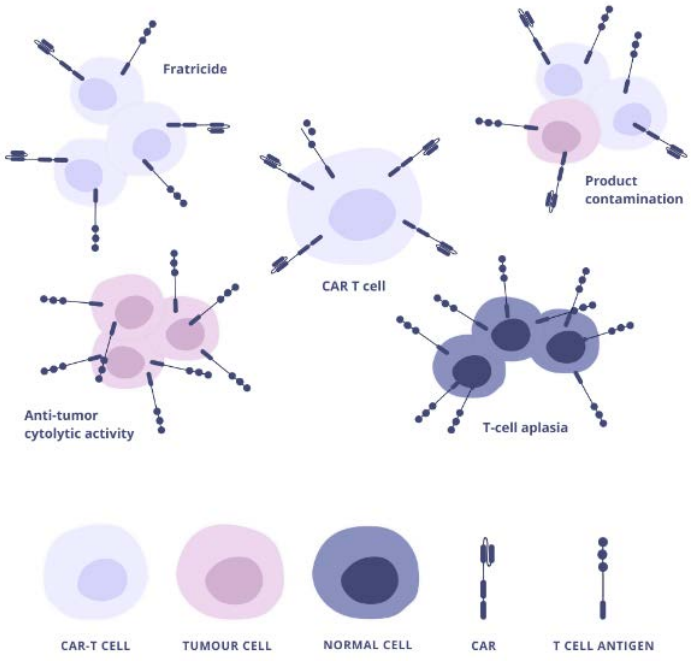


Fig.19) Potential outcomes for CAR therapy in T-ALL. Anti-tumor cytolitic activity. CAR T cells target tumor cells as intended, reducing tumor burden. **Fratricide.** Without further engineering, the CAR-modified T cells are likely to express the targeted antigen as well, resulting in fratricide. **T-cell aplasia**. CAR T cells would also target healthy T cells, resulting in unintended T cell aplasia. **Product contamination.** CAR T cell manufacturing implies the separation of normal T cells from malignant T cells for CAR-transduction. A single tumor cell contaminating the apheresis can result in masking of the antigen, causing antigen-positive relapse.

6.3.2.1 Targetable antigens in T-ALL

Current antigens in the T-ALL landscape have shown a limited exploitability due to the reasons discussed in the chapter 6.3. However major pre-clinical works have laid down the basis to bring some candidate antigens forward to clinical testing. The most important are CD5 (Mamonkin *et al.* 2015) CD7 (Gomes Silva *et al.* 2017), CD37 (Scarfo *et al.* 2018) and CD1a (Sanchez Martinez *et al.* 2019). A summary of the most recent clinical trial in the field of T-cell malignancies is enclosed in table 6.

INSTITUTE	ANTIGEN	IDENTIFIER	VECTOR/ICD	LD CHEMOTHERAPY	CART DOSE
HMH	**CD5**	NCT03081910	Retro/CD28	Flu (NS) Cy (NS)	1×10^7 to 5×10^8 CAR^+ T cells/kg
HMH	**CD7**	NCT03690011	Retro/CD28	Flu (30 mg/m2) Cy (500mg/m2)	1×10^7 to 5×10^8 CAR^+ T cells/kg
SBU	**CD4**	NCT03829540	Lenti/CD28-41BB	NS	4×10^6 CAR^+ T cells/kg
MGH	**CD37**	NCT04136275	NS/NS	NS/NS	NS

Table 6) Summary of ongoing T-ALL CAR T-cell clinical trials Cy, cyclophosphamide; **Flu**, fludarabine; **HMH**, Huston Methodist Hospital; **ICD**, Intracellular domain:; **LD**, lymph depleting**, Lenti**, Lentiviral; **MDA**, MD Anderson; **NS**, not specified; **Retro**, Retroviral; **SBU**, Stony Brooke University

7. CD123 AS A TARGET FOR AML

7.1 CD123 structure and function

The interleukin-3 receptor (CD123) is a molecule that belongs to a subfamily of membrane receptors, known as the Beta Common (β_c) family of cytokines, because all members of this family share the common signaling subunit β_c. CD123 is a heterodimer, composed by IL-3 specific α subunit and a shared β_c subunit. CD123 is a glycoprotein of 360 amino-acids, composed by an extracellular domain of 287 residues, involving an Ig-like domain, two FnIII domains, a trans-membrane domain of 30 amino-acids and by an intracellular domain of 53 residues (Woodcock *et al.* 1996). CD123 is found on several types of cells that propagate the signal of IL-3, a soluble cytokine playing vital roles for the immune system homeostasis, controlling normal and malignant hemopoiesis, native as well as adaptive immunity, and inflammatory response (Testa *el al.* 2014). Its cognate cytokine, IL3, mainly produced by activated T-lymphocytes, modulates the function and regulates the production of hematopoietic and immune cells. It shows in fact, the remarkable property of stimulating the development of a wide-range of hematopoietic cells from bone marrow, including basophils, neutrophils, eosinophils, macrophages, erythroid cells, megakaryocytes, and dendritic cells (Rothenberg *et al.* 1988).

7.2 CD123 expression on hematopoietic stem cells

Various groups have attempted to explore CD123 expression in stem cells and multiple subpopulations of hematopoietic progenitor cells. In this perspective, CD123 expression have been throughout the years assessed in different sources of hematopoietic cells, such as: cord blood (CB), BM, PB, and fetal liver (FL) (Sato *et al.* 1993, Wognum *et al.* 1996, Testa *et al.* 1996, Manz *et al.* 2002). Accordingly, various pieces of evidence were established regarding the function of CD123 in hematopoietic development and maintenance. It was initially suggested (Wognum *et al.* 1996) that early primate HSCs

identified as $CD34^+/HLA\text{-}DR^{low}$ cells express low levels of CD123, while $CD34^+$ cells with negative or high CD123 expression were committed erythroid and myeloid progenitors, respectively. Later works (Huang *et al.* 1999) have defined three subsets of $CD34^+$ cells in accordance with surface levels of CD123. $CD34^+CD123^+$ cells were myeloid and B-lymphoid progenitors, whereas the erythroid progenitors were mainly contained in the $CD34^+CD123^-$ subset. Conversely $CD34^+CD123^{low}$ cell subset contained a heterogeneous population of early progenitor cells. More recently (Manz *et al.* 2002), $CD34^+CD38^+$ cells were subdivided according to the positivity for CD123 and CD45RA and it was shown that: $CD123^{low}CD45RA^+$ cells mainly contained granulo-monocytic progenitors (GMP), $CD123^-CD45RA^-$ cells mainly contained erythroid and megakaryocytic progenitors (MEP), $CD123^{low}CD45RA^-$ cells give rise to both GMPs and MEPs and contained the progenitors of both populations. Lastly, CD123 involvement during hemopoietic development was further corroborated by the fact that this receptor was found on the majority of $CD34^+$ hemopoietic progenitors and its expression is rapidly lost during erythroid and megakaryocytic differentiation, moderately decreased during monocyte development and retained in the granulocytic lineage (Testa *et al.* 2014).

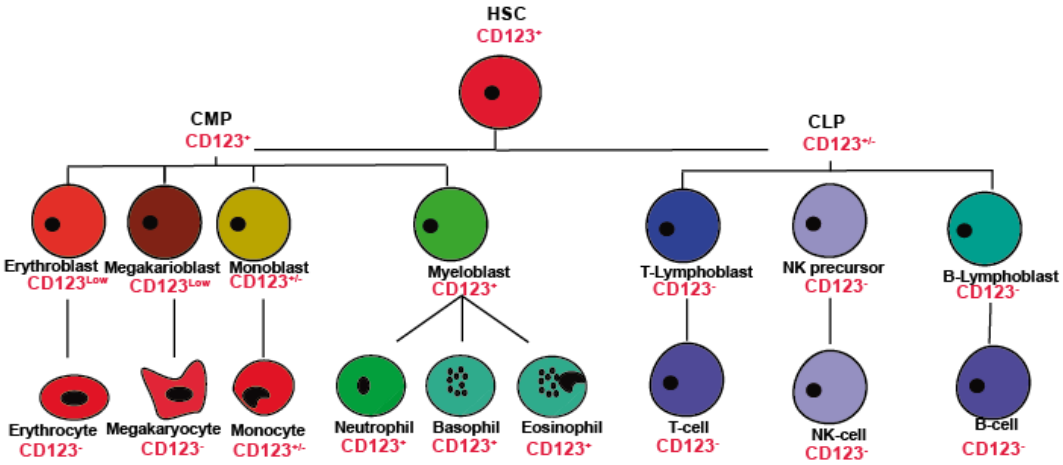


Fig.20) CD123 expression across hematopoiesis. Schematic representation of the expression of CD123 antigen from the HSC to the most mature blood cells.

7.3 CD123 expression in AML

Initial studies in AMLs have outlined a substantial overexpression of CD123 on bulk and $CD34^{+}CD38^{-}$ AML cells (Jordan *et al.* 2000). To assess the functional role of $CD34^{+}CD38^{-}CD123^{+}$ blasts, these cells were isolated from AML samples and injected into immunodeficient mice and they were able to initiate and maintain the leukemic process, thus acting as Leukemia Initiating Cells (LIC) (Jordan *et al.*2000). Further evidence supports CD123 targeting as a feasible therapeutic approach for AML. First, major phenotypic (immature, granulocytic and monocytic) and cytogenetic (FLT3- and NPM1-mutated) AML subgroups express CD123 (Bras *et al.*2019, Testa *et al.* 2014, Testa *et al*. 2019) Second, the presence of $CD34^{+}CD38^{-}CD123^{+}$ cells in AML at presentation is associated to lower disease-free and overall survival and failure to achieve complete remission (Zaharan *et al.* 2018). Third, CD123 expression enhance AML cell proliferation and induces down-regulation of CXCR4, which is the receptor of stromal-derived growth factor-1 (SDF-1) and plays an essential role in the regulation of HSC homing and migration. Thus, it was hypothesized that the CD123 overexpression, through CXCR4 downregulation, may induce the egress of BM AML leukemic stem cells (LSCs) into the circulation (Wittver *et al.* 2017). In fact, numerous preclinical studies have strengthen the potential of CD123 as a target for AML. Seminal work have explored the implementation of neutralizing MoAbs redirected towards CD123 (Nievergall *et al.* 2014, Leet *et al.* 2015), demonstrating their therapeutic potential as CD123-redirected MoAbs reduced AML LSC homing, engraftment, and self-renewal ability and improved the survival of xenografted NOD/SCID mice CD123 (Nievergall *et al.* 2014).

8. CD1a AS A TARGET FOR T-ALL

8.1 CD1a structure and function

CD1a belongs to the CD1 family of trans-membrane glycoproteins, whose overall structure closely resemble the major histocompatibility complex (MHC) proteins. Collectively they are involved in presenting lipid antigens to T cells, but their precise function yet to be established (Van Haarts *et al.* 1996, Ito *et al.* 1999). Upon recognition of the CD1 ligand complex by the T-cell receptor, CD1-dependent T cells are activated in a variety of immunological contexts. Loss-of-function studies revealed that CD1-deficient mice may be more susceptible to some viruses, bacteria, and protozoa (Smiley *et al.* 2005). In terms of bio-distribution, CD1a is essentially confined to cortical thymocytes and Langerhans cells (LC), the latter being the first line innate immune response in the skin, and it's virtually undetectable in human tissues (Van Haarts *et al.* 1996, Ito *et al.* 1999).

8.2 CD1a expression in healthy cells

CD1a is among the sole proteins that characterize the first described Dendritic Cells (DC) subset, Langherans Cells (LC). *In vivo* there is substantial heterogeneity in CD1a expression among DC, even among CD1a expressing DC in the skin. First biopsies from various patients, revealed an even wider distribution of $CD1a^+$ DC that are now known to be widely located in sites such as lung, tonsil, gastrointestinal and genital tracts (vanHaarst *et al.* 1996, Prakash *et al.* 2004) There are other tissues, such as peripheral blood and epidermis, in which the co-existence of both myeloid derived $CD1a^+$ (Langherans Cells) and $CD1a^-$ (Interstitial Dendritic Cells) have been described (figure 21) (Ito *et al.* 1999).

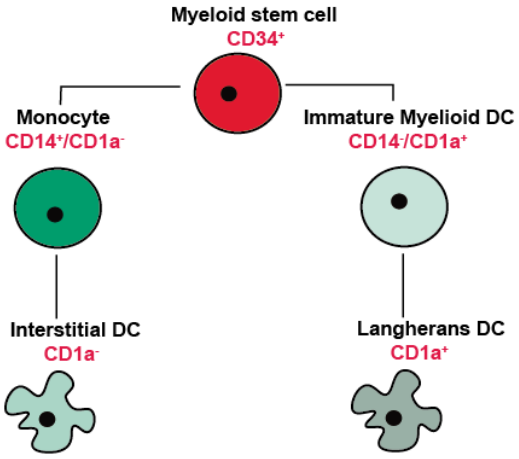


Fig. 21) Developmental stages of CD1a+ and CD1a- Dendritic cells. CD1a expression is confined to a subtype of Dendritic cells (DC) of myeloid origin, the Langherans DC, as opposed to the Interstitial DC which show no expression of CD1a antigen

Importantly however, as shown in figure 22, CD1a is transiently detected in cortical thymocytes (Galy *et al.* 1993), but it is absent in mature T cells in all extrathymic tissues. Noteworthy, $CD34^+$ HSPCs also lack CD1a expression in multiple hematopoietic location throughout various developmental stages (Bechan *et al.* 2012, Sanchez Martinez *et al.* 2019). T-cell maturation in fact is initiated within the thymus by a first colonizing $CD34^{high}CD7^-CD1a^-$ primitive HSPCs, whose fate is to differentiate in response to the thymic microenvironment into $CD34^{high}CD7^+CD1a^-$ early T-cell progenitors (Martin-Gayo *et al.* 2017).

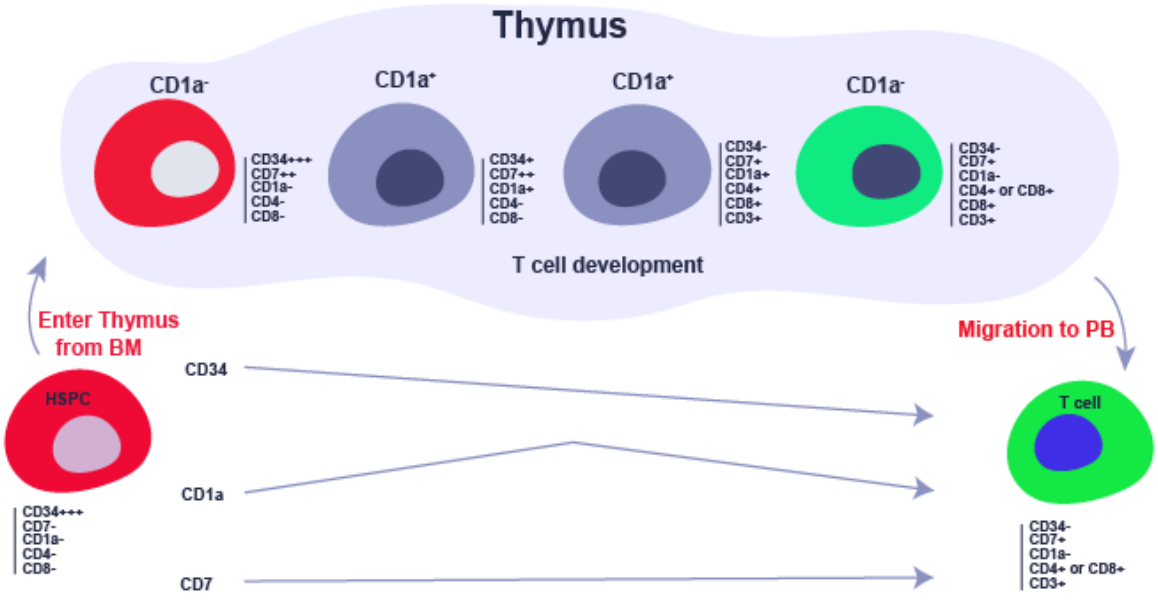


Fig.22) CD1a expression throughout T-cell development. T-cell development starts in the thymus by a first colonizing $CD34^{high}CD7^-CD1a^-$ primitive HSPC As thymic differentiation progressed, tymocytes gradually lost CD34, maintained CD7 and CD1a expression transiently emerged at intermediate stages for being lost before T-cell maturation

8.3 CD1a expression in T-ALL

T-ALL comprise a wide variety of diseases characterized by a distinct phenotype that indicates at which stage the developmental arrest took place. The group showing displaying the most immature phenotype is ETP-ALL, being $CD1a^{-}$,$CD5^{-}$,$CD8^{-}$ and $CD7^{+}$ (Jain *et al.* 2016). Later arrests during T-lymphocytes maturation give raise to pro-T ($CD7^{+}$), pre-T ($CD2^{+}$ and/or $CD5^{+}$ and/or $CD8^{+}$), cortical T ($CD1a^{+}$), and mature T (surface $CD3^{+}$, $CD1a^{-}$, $CD7^{+}$) (Litzow *et al* .2015). CD1a expression in T cell leukemias is only restricted to cortical T-ALL, a major subset of T-ALL accounting for ~ 35–40% of all T-ALL cases (Niehues *et al.* 1999, van Grotel *et al.* 2008) and, neither early T cells progenitors or mature T-cells, nor $CD34^{+}$ hematopoietic progenitors seem to express CD1a, making it a potential fratricide-resistant target, and limiting the risk of myeloablation (caused by loss $CD34^{+}$ cells).

GENERAL AIMS

The main goal of this thesis was to explore novel therapeutic approaches for relapsed or chemo-refractory Acute Leukemia. Within the past two decades, a wide number of targeted immunotherapies have been implemented in the clinical frame work for the treatment of myeloid and lymphoid malignancies. Engineered T-cells redirected towards B-cell lymphoid antigens have shown remarkable success rate. However T-cells redirected against T- or myeloid-specific antigens are lagging behind due to the lack of tumor specific surface molecules, and the potential toxicity as myeloablation and/or immunodeficiency.

We have focused our investigation into two main diseases: AML and cortical T-ALL

CD123 target for AML

There is an evident shortage of targetable antigens to safely implement CAR T-cell therapy into the AML landscape, as practically all of them are expressed at various degrees on different healthy cell types across myeloid differentiation, most notably on HSC. At the present date there is a controversy on whether targeting CD123 qualify as safe or not, as multiple reports put forward contradicting conclusions. We therefore sought to investigate the efficacy and safety of novel CD123 redirected T-cells using both *in vitro* and *in vivo* AML primary cells/PDX and humanized models of human hematopoiesis

CD1a target for coT-ALL

The lack of a blast-specific pan-T cell antigen has hindered the clinical implementation of successful CAR T-cell-based approaches redirected towards T-ALL. However CD1a has been suggested as a potential target with limited toxicity in a minority of cases of T-ALL, specifically coT-ALL. We therefore aimed to investigate the feasibility of CD1a as a target for R/R coT-ALL.

SPECIFIC AIMS

CD123-redirected CAR in AML

1. Characterize CD123 expression in a large cohort of AML patients
2. Characterize a novel CD123-redirected CAR
3. Functionally test *in vitro* and *in vivo* models for AML both 4-1BB based and CD28 based CD123 redirected T-cells
4. Characterize *in vitro* and *in vivo* the potential myeloablative toxicity of anti CD123 CAR T-cells due to on target off tumor on normal $CD34^+$ HSCs

CD1a-redicrected CAR in coT-ALL

1. Characterize CD1a expression in a large cohort of co T-ALL patients
2. Characterize a novel CD1a-redirected CAR
3. Functionally test *in vitro* and *in vivo* models for T-ALL L 4-1BB based CD1a redirected T-cells

RESULTS

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4-1BB-based and CD28-based CD123-redirected T-cells ablate human normal hematopoiesis *in vivo*

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ABSTRACT

Background: Acute Myeloid Leukemia (AML) is a hematopoietic malignancy which is biologically, phenotypically and genetically very heterogeneous. Outcome of AML patients remains dismal, highlighting the need for improved, less-toxic therapies. Chimeric antigen receptor T-cell (CARTs) immunotherapies for refractory or relapse (R/R) AML patients are challenging because the absence of a universal pan-AML target antigen, and the shared expression of target antigens with normal hematopoietic stem/progenitor cells (HSPCs), which may lead to life-threating on-target/off-tumor cytotoxicity. CD33- and CD123-redirected CARTs for AML are in advanced pre-clinical and clinical development and they exhibit robust anti-leukemic activity. However, preclinical and clinical controversy exists on whether such CARTs are myeloablative.

Methods: We set out to comparatively characterize *in vitro* and *in vivo* the efficacy and safety of 41BB- and CD28-based CARCD123. We analyzed 97 diagnostic and relapse AML primary samples to investigate whether CD123 is a suitable immunotherapeutic target, and we used several xenograft models and *in vitro* assays to assess the myeloablative potential of our second generation CD123 CARTs.

Results: Here, we show that CD123 represents a *bona fide* target for AML and show that both 41BB- and CD28-based CD123 CARTs are very efficient in eliminating both AML cell lines and primary cells *in vitro* and *in vivo*. However, both 41BB- and CD28-based CD123 CARTs ablate normal human hematopoiesis and prevent the establishment of *de novo* hematopoietic reconstitution by targeting both immature and myeloid HSPCs.

Conclusions: This study calls for caution when clinically implementing CD123 CARTs, encouraging its preferential use as a bridge to allo-HSCT in R/R AML patients.

BACKGROUND

Acute myeloid leukemia (AML) is a biologically, phenotypically and genetically very heterogeneous malignant disease which results from the uncontrolled accumulation of differentiation-defective hematopoietic stem/progenitor cells (HSPCs) or immature myeloid cells [1, 2]. AML is one of the most common hematopoietic malignances, and its incidence increases with age [3, 4]. Intensive chemotherapy combos based on nucleoside analogs plus anthracyclines remain the standard front-line treatment of AML [5], followed by allogeneic HSPC transplant (allo-HSCT), based on patient's eligibility, to consolidate complete remission (CR) and prevent relapse [6]. However, with the exception of a few molecular subgroups (the "so-called" low-risk AMLs), relapses are common after consolidation therapy and/or allo-HSCT. Chemotherapy-related toxicity, refractoriness, and failure to eradicate leukemia-initiating cells (LICs) are the major mechanisms underlying AML progression and relapse [7-10]. Unfortunately, improved AML treatments have only experienced minor developments over the last four decades, and current 5-year event-free survival (EFS) remains ~20% in adults and <70% in children [11, 12], highlighting the desperate need for safer and more efficient therapeutics.

Immunotherapy has generated unprecedented expectations in cancer treatment. In AML, both CD33- and CD123-specific antibody-drug conjugates have been used for combination therapy with standard chemotherapy with improved EFS [13, 14], and bi-specific T-cell engagers (BiTE) for CD33 and CD123 are being clinically assayed [15, 16]. Adoptive cellular immunotherapy based on the engineering of human T-cells with chimeric antigen receptors (CARTs) redirected against cell surface tumor antigens have shown robust clinical responses in patients with B-cell malignances thanks to the high efficacy, specificity and persistence of CARTs [17-19]. However, the clinical

implementation of AML-specific and safe CARTs for refractory or relapse (R/R) AML patients is still awaiting. Strategies targeting AML using CARTs have proven more challenging than in B-lineage malignancies because two-fold: i) the lack of a universal pan-AML target antigen due to the large disease heterogeneity which hampers clinical implementation, since a wide range of CARs would be needed to cover the different leukemic phenotypes, and ii) the shared expression of immature target antigens between normal HSPCs and myeloid blasts, which compromises safety due to potential on-target/off-tumor cytotoxicity against HSPCs leading to fatal aplasia [20, 21].

Adoptive immunotherapy for AML is in advanced pre-clinical and clinical development using CD33- and CD123-redirected CARs (CD123 CARTs), and they exhibit robust anti-leukemic activity *in vitro* and *in vivo* [22-25]. However, controversy exists on whether CD123- and CD33-directed CARTs are myeloablative. Some groups raised safety concerns leading to the development of complex target antigen knock-out in HSPC or T-cell suicide strategies to circumvent such a toxicity [23, 26]. In contrast, other groups showed a safety profile with limited on-target/off-tumor toxicity of such CARs [22, 27-31]. Here, we set out to characterize *in vitro* and *in vivo* the efficacy and the safety of 41BB- and CD28-costimulated CARCD123, based on a clinically relevant scFv from the CSL362 monoclonal antibody (MoAb). Analysis of a large cohort of diagnostic and relapse AML primary samples revealed that CD123 is a suitable target for AML, and CD123 CARTs were very efficient *in vitro* and *in vivo* in eliminating both AML cell lines and primary cells, regardless the co-stimulation motif. However, clonogenic assays and several xenograft models revealed that both 41BB- and CD28-costimulated CD123 CARTs strongly ablate normal human hematopoiesis by targeting both HSCs and

myeloid progenitors. This study calls for caution when clinically implementing CD123 CARTs and highlights its preferential use as a bridge to allo-HSCT in R/R AML patients.

MATERIAL AND METHODS

CAR design and vectors, lentiviral production and T-cells transduction

The anti-CD123 scFV derived from the clinically tested CSL362 MoAb was generated and cloned into the pCCL lentiviral-based second-generation CAR backbone containing a human CD8 transmembrane (TM) domain, a human co-stimulatory domain (either 41BB or CD28), CD3z endodomain, and a T2A-GFP cassette. The pCCL vector expressing green fluorescence protein (GFP) alone (Mock vector) was used as a control. CAR-expressing viral particles pseudotyped with VSV-G were generated using HEK 293T cells with a standard polyethylenimine transfection protocol. For each production, plasmid transfection was carried out using a 3:1 PEI to DNA ratio using 16 μg transfer vector, 16 μg of pSPAX2, and 8 μg VSV-G per plate and viral particles were concentrated by ultracentrifugation as previously described [32]. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy volunteers by Ficoll-Hypaque gradient centrifugation. Buffy coats were obtained from the Barcelona Blood and Tissue Bank (BST) upon IRB approval (HCB/2018/0030). T-cells were activated by plate-bound OKT3 and anti CD28 antibodies (BD Biosciences) for 2 days in the presence of interleukin-7 (IL-7) and IL-15 (10 ng/mL, Mitenyi Biotec) [33, 34]. Surface expression of CAR123 was traced by fluorecence-activated cell sorting (FACS). CAR detection was confirmed by GFP expression and by using an AffiniPure $F(ab')_2$ Fragment Goat Anti Human IgG (H+L) (Jackson ImmunoResearch). Activation and subsetting of lentivirally-transduced T-cells was confirmed by surface staining with CD25/CD69 (data no shown) and CD3/CD4/CD8, respectively.

Immunophenotyping of healthy HSPCs, primary AML samples and cell lines

Diagnostic immunophenotyping data for the most commonly expressed antigens in AML (CD123, CD33, CD13, CD34, CD15, c-kit, and CD66) was obtained for 97 patients diagnosed at local hospitals: *Germans Trias i Pujol* (Barcelona, Spain), Hospital Clínico (Madrid, Spain), Hôpital Armand Trousseau (Paris, France) and *Santa Creu i San Pau* (Barcelona, Spain). CD123 expression was also compared in diagnostic-relapsed paired samples (n=68 patients) and in paired bulk leukemia-leukemia stem cells (LSC) (n= 37 patients) [35]. Cell lines were stained with CD123-APC, CD33-BV-421, CD14-PerCP-Cy5.5 and CD19-APC. The expression of CD123 and CD33 antigens was prospectively compared in CD34+ HSPCs derived from healthy cord blood (CB, n=22), mobilized peripheral blood (PB, n=10) and diagnostic primary AML samples (n=24). For HSPC subsetting, CD34+ cells were stained with CD34-PE or CD34-PE-Cy7, CD133-PE, CD19-FITC, CD90-APC, CD13-PE-Cy7 and CD71-APC-Cy7, which allow for the identification and quantification of immature HSCs (CD34++CD133+CD90+), myeloid progenitors (CD34+CD13++CD71/low), erythroid progenitors (CD34+CD71++CD13low), and B-cell progenitors (CD34+CD19+CD71-CD13-). Isotype-matched, non-reactive fluorochrome-conjugated MoAbs were always used as a fluorescence reference. All antibodies were purchased from Beckton Dickinson. Cells were incubated with MoAbs (30min at 4°C in the darkness), then washed in PBS and analyzed in a FACSCanto-II flow cytometer equipped with FACSDiva software (Becton Dickinson) [36-38]. Determination of antigen density for CD33 and CD123 was performed using BDQuantibrite-PE (Becton Dickinson) according to manufacturer's instructions.

In vitro cytotoxicity assays and cytokine release determination

The cell lines THP-1, MOLM-13 and 697 were purchased from DSMZ (Germany) and expanded according to DSMZ recommendations. Primary AMLs and healthy CD34+ cells were obtained from the aforementioned hospitals and the BST (Barcelona Blood Bank), respectively, (IRB approval: HCB/2018/0030). Target cells were incubated with CAR123 or MOCK T-cells at different Effector:Target (E:T) ratios for the indicated time periods. CART-mediated cytotoxicity was determined by analyzing the residual alive (7-AAD-) target cells at each time point and E:T ratio. For absolute cell counting, Trucount absolute count beads (Becton Dickinson) were used. Furthermore, FACS-sorted CD3+ mature T-cells from BM of CD123+ AML patients were activated, transduced with CD123 CAR and tested against their autologous-matched CD123+ AML blasts. $1x10^5$ target cells were used for all cytotoxicity assays unless stated otherwise. **Table 1** shows the clinical-biological features of the CD123+ AML samples used for *in vitro* experiments. The production of the pro-inflammatory cytokines IL-2, TNFα and IFNγ was assessed by ELISA (Human ELISA SET, BD Biosciences) using *in vitro* supernatants harvested at 16h post T-cells exposure, and sera collected from mice 10 days after CART infusion.

Colony forming-units (CFU) assays

CB-derived CD34+ cells were exposed for 24 hours to either CD123 CARTs or MOCK T-cells (E:T 1:1), and then plated ($2x10^3$) onto serum-free methylcellulose H4435 (Stem Cell Technologies). CFUs were then counted and scored after 12-14 days following standard procedures.

In vivo xenograft models for AML, HSPCs and CARTs

8- to 12-week-old nonobese diabetic (NOD)-Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (Jackson Laboratory) were bred and housed under pathogen-free conditions in the animal facility of the Barcelona Biomedical Research Park (PRBB). In experiments addressing CAR123 efficacy, mice were intravenously transplanted with $0.25x10^6$ Luc-mCherry-expressing patient-derived xenograft AML cells (PDX-579)[39] 5 days before intravenous (*iv*) infusion of $3x10^6$ of either 41-BB- or CD28-CD123 CARTs derived from healthy PBMCs. Tumor burden was monitored at the indicated time points by bioluminescence (BLI) using the Xenogen IVIS 50 Imaging System (Perkin Elmer) [32]. In experiments addressing the myeloablative effect of CAR123, CD34+ HSPCs ($0.1x10^6$) were intra-BM transplanted in sublethally irradiated (2Gy) NSG mice, followed by *iv* infusion of $3x10^6$ of 41-BB-CD123, CD28-CD123 CARTs or MOCK T-cells either 1 day or 6 weeks after CD34+ transplantation. BM and PB were FACS-analyzed for human chimerism at sacrifice. Cells were stained with anti-HLA.ABC-PE and CD45-BV450. Engrafted mice were assessed for multilineage engraftment using anti-CD123-APC for myeloid cells, anti-CD19-BV421 for lymphoid cells, and anti-CD34-PE.Cy7 for immature cells. Human absolute engraftment in PB and BM was quantified using BD Trucount tubes according to manufacturer's instructions.

Statistical analysis

For comparison of CD123 expression between paired "diagnostic-relapse" and "bulk leukemic cells-LSC", the Mann-Whitney U test was used. For differences in antigen density and engraftment among groups a One-way ANOVA test was used. For the remaining comparisons, the Student's t-test was used. All *p-values* were considered statistically significant when <0.05(*).

RESULTS

CD123 represents a *bona fide* immunotarget for AML

We first analyzed by FACS the expression levels of the most common diagnostic myeloid markers in a cohort of 97 AML patients at presentation. We found that CD123 was the most common and homogeneously expressed antigen (86.4%±26.8 of AML blasts) followed by CD33 (77.4%±32.1) **(Fig 1A)**. Important, in 82% of the AML patients analyzed >80% of the blasts were CD123+, while only 66% of the patients showed positivity for CD33 in >80% of the blasts **(Fig 1A)**. A target antigen for immunotherapy in AML should ideally be absent in HSPCs. CD123 and CD33 are both partially expressed in healthy CD34+ HSPCs [22, 23], so we next quantified the density (molecules/cell) of both antigens in fresh primary AML blasts (n=24), healthy CB-derived (n=22) and healthy mobilized PB-derived CD34+ HSPCs (n=10). Of note, 67% (16/24) of AML patients displayed levels of CD123 significantly higher than those found in both CB- and PB-derived CD34+ HSPCs, while only 41% (10/24) of AML patients displayed levels of CD33 that segregate them from CB- and PB-derived CD34+ HSPCs (**Fig 1B**). This suggests that CD123 represents, *a priori*, a less myeloablative target than CD33. Of note, analysis of paired diagnostic-relapse AML samples revealed that CD123 expression is maintained at relapse, and in AML-LSC (identified as CD34+CD38-) [35] **(Fig 1C)**, reinforcing CD123 as a *bona fide* immunotarget for R/R AML.

41BB- and CD28-based CD123 CARTs efficiently eliminate AML primary cells *in vitro* and *in vivo*

We next designed second-generation 41BB-based and CD28-based CD123CARs coupled in-frame with GFP through a T2A sequence **(Fig 1D, Fig S1A)**. The expression of both 41BB- and CD28-CD123CAR in T-cells was confirmed through co-detection of scFv and

GFP (**Fig 1E, Fig S1B)** and did not affect the CD4:CD8 ratio **(Fig 1F)**. Importantly, activated (CD69+CD25+) T-cells continuously expanded ~50-fold over a 10-day period, similar to Mock T-cells **(Fig 1G)**, demonstrating that redirecting T-cells against CD123 does not hamper T-cells expansion.

We then tested the functionality of our 41BB- and CD28-CD123CARs *in vitro* and *in vivo* **(Fig 2, Fig S1, S2)**. *In vitro*, both 41BB- **(Fig 2A,B)** and CD28- **(Fig S1C)** CD123 CARTs, but not MOCK T-cells, specifically eliminated the CD123+ AML cell lines THP1 and MOLM13 in a E:T ratio-dependent manner **(Fig S2)**, while sparing the CD123- B-ALL cell line 697. In fact, CD123+ AML cells barely survived exposure to CD123 CARTs in a 48h absolute number assay at a 1:1 E:T ratio **(Fig 2B, S1C)**. We then examined in an autologous setting whether CD3+ T-cells deriving from AML patients can be isolated, modified to express CD123CAR, expanded and used as cytotoxic effector cells **(Fig 2C)**. Patient-derived CD123 CARTs were successfully generated from MACS-sorted CD3+ T-cells (>95% purity) and specifically eliminated autologous patient-matched CD123+ AML blasts **(Fig 2D)**. Important, both CD123 CARTs produced high levels of the proinflammatory cytokines IL-2, TNFα, and IFN-γ on co-culture with both AML cell lines **(Fig 2E, S1D)** and primary blasts **(Fig 2F)**, confirming their robust cytotoxicity.

We next compared the cytotoxic activity of 41BB- and CD28-CD123 CARTs *in vivo* using Luc-expressing CD123+ AML xenograft (**Fig 2G)**. NSG mice were transplanted with 0.25×10^6 Luc-expressing AML PDX cells five days prior to *iv* infusion of 3×10^6 41-BB- or CD28-CD123 CARTs, and leukemia establishment was followed-up weekly by BLI until disease signs were evident **(Fig 2H)**. While control mice increasingly showed

aggressive disease and disseminated leukemia, CD123 CARTs-treated mice showed extensive disease control across the experiment, regardless the co-stimulation domain used **(Fig 2H,I)**. Of note, T-cells persisted in PB and BM at sacrifice although at higher levels in 41BB-CD123 CART-treated mice, in line with the reported longer persistence/effector function of 41BB-stimulated CARTs **(Fig 2J)** [40]. Similarly, both CD123 CARTs, especially 41BB-stimulated CD123 CARTs produced high levels of the IFN-γ *in vivo* **(Fig 2K)**. Collectively, both 41BB- and CD28-CD123 CARTs have similarly potent and specific antileukemic activity against AML cells *in vitro* and *in vivo*.

On-target/off-tumor targeting of immature HSPCs and myeloid progenitors render both 41BB- and CD28-CD123 CARTs severely myeloablative *in vitro* and *in vivo*

There is controversy on whether CD123-redirected T-cells are myeloablative. To prospectively assess the potential myelotoxicity of CD123 CARTs, we first addressed *in vitro* whether exposure to CD123 CARTs hampers the viability and clonogenic capacity of CD34+ HSPCs **(Fig 3A)**. As compared to MOCK T-cells, both CD123 CARTs induced a massive reduction in CD34+ cell counts in a 72h (E:T ratio 2:1) assay **(Fig 3B,C)**. Similarly, CD34+ HSPCs pre-exposed to either CD123 CART (E:T ratio 1:1) for only 24h showed 50%-80% reduction in their clonogenic capacity **(Fig 3D)**.

We next assessed the myeloablative potential of both 41BB- and CD28-CD123 CARTs *in vivo* using xenograft models of human hematopoietic reconstitution. In an initial set of experiments, sublethally irradiated NSG mice were reconstituted with $0.1x10^6$ CD34+ HSPCs, and 6 weeks later, when human multilineage engraftment was established mice received $3x10^6$ 41-BB-, CD28-CD123 CARTs or MOCK T-cells **(Fig 3E)**. Human engraftment was biweekly analyzed in PB **(Fig 3F)** and BM **(Fig 3G)** over 6 weeks.

MOCK T-cells-treated mice consistently showed increased myeloid- (HLA-ABC+CD45+CD123+CD33+), B-lymphoid- (HLA-ABC+CD45+CD123-CD19+) and immature (HLA-ABC+CD45+CD34+) hematopoietic engraftment than that observed the day of CARTs infusion. In contrast, both 41BB- and CD28-CD123 CARTs-treated mice showed an impaired multilineage engraftment in both PB and BM **(Fig 3F,G)**. However, in this xenograft model of existing hematopoiesis CD28-based CD123 CARTs proved less myeloablative than 41BB-CD123 CARTs.

Next, we assessed the capacity of both CD123 CARTs in preventing *de novo* establishment of normal hematopoiesis by transplanting sublethally irradiated NSG mice with CD34+ HSPC and either 41-BB-, CD28-CD123 CARTs or MOCK T-cells one day after **(Fig 3H)**. Long-term multilineage human engraftment was found in both PB and BM in MOCK T-cells treated mice; however, human hematopoiesis was barely reconstituted in both 41BB- and CD28-CAR123 CARTs-treated mice **(Fig 3I,J)**, indicating that both 41BB- and CD28-CD123 CARTs prevent healthy hematopoietic reconstitution.

Finally, to further characterize the myeloablative effects of CD123 CARTs, we exposed total CD34+ HSPCs to either CD123 CARTs or MOCK T-cells for 48h at 1:1 E:T ratio, and quantified afterwards whether the myeloablative effects were CD34+ subset-specific **(Fig 3A, 4A,B)**. We found a significant loss of both immature/early HSPCs (CD34++CD133+CD90+) and myeloid progenitors (CD34+CD13+CD71low), while B-cell progenitors (CD34+CD19+CD13-CD71-) and erythroid progenitors (CD34+CD71+CD13low) were unaffected by CAR123 CART exposure **(Fig 4A, B)**. Of note, CD123 CART-mediated cytotoxicity correlated well with the expression levels of CD123 in the different CD34+ subsets **(Fig 4C)**. Collectively, our results suggest that

CD123 CARTs ablate human hematopoiesis by targeting both early/immature HSPCs and myeloid progenitors.

DISCUSSION

AML is a very heterogeneous stem cell malignant disease characterized by the progressive acquisition of (epi)genetic alterations resulting in a clonal rapid expansion of differentiation-defective HSPC in BM and PB [1]. Unfortunately, the prognosis of AML remains unfavorable, especially in patients >60 years old, due to common relapses, disease refractoriness and treatment-related toxicities [41]. Unfortunately, improved AML treatments have only experienced minor developments over the last four decades, reinforcing the high-demand for new therapeutics with improved efficacy and reduced toxicity [20, 23]. In this context, the undisputable clinical improvements of cancer immunotherapy have not gone unnoticed in AML and undoubtedly represents the great hope of the next decade in the treatment of AML. In fact, immunotherapeutic targeting in AML is already well-advanced in clinical trials using MoAb, antibody-drug conjugates, BiTEs, dual-affinity retargeting (DART) and CAR T-cell therapies against CD33 and CD123[13-16, 20, 22, 29] . However, clinical progress and regulatory approval of such immunotherapies have been hampered by the challenge to find a specific and safe targetable surface antigen [41, 42].

CD33 and CD123 are the most extensively explored antigens for AML and blastic plasmocytoid dendritic cells neoplasm immunotherapy [42, 43]. In this study, we aimed to better characterize the suitability of CD33 and CD123 in a large cohort of diagnostic and relapse AML primary samples. We show that CD123 represents a *bona fide* target for AML with a potentially safer profile than CD33. Not only is the most common and

homogeneously expressed antigen in AML, but its expression is also fully retained at relapse and in AML-LSC. This lack of antigen plasticity, a phenomenon widely observed during the progression and relapse of acute leukemias [44], further strengthens the potential of CD123 as immunotarget for AML. More importantly, a target antigen for immunotherapy in AML should ideally spare HSPCs. Previous studies about the expression of CD123 in CD34+ HSPCs have provided conflicting results based on the source of CD34+ cells and the MoAb used [20, 22, 29]. Here, we demonstrate that CD123 is expressed in both CB- and PB-derived CD34+ HSPCs; however, in contrast to CD33 which discriminate more poorly AML from either CB- or PB-derived CD34+ HSPCs, two thirds of AML patients express CD123 at levels significantly higher than those in healthy CD34+ cells, suggesting CD123 as a safer target than CD33 for AML.

Extensive evidence supports CD123 targeting as a therapeutic approach for AML. First, major phenotypic (immature, granulocytic and monocytic) and cytogenetic (FLT3- and NPM1-mutated) AML subgroups express CD123 [35, 45, 46]. Second, CD123+ AML cells are capable to initiate leukemogenesis when transplanted in immunodeficient mice, thus marking AML-LSC [47-49]. Third, the presence of CD34+CD38-CD123+ cells in AML at presentation is associated to lower disease-free and overall survival and failure to achieve complete remission [50, 51]. Four, CD123 expression enhance AML cell proliferation and induces downregulation of CXCR4 favoring the egress of BM AML-LSCs into the circulation [52]. Based on this background, we prompted to characterize and compare *in vitro* and *in vivo* the efficacy and safety profile of the second generation 4-1BB-based and CD28-based CARCD123s derived from the clinically tested CSL362 humanized MoAb [14, 53]. Regardless the co-stimulation motif, CD123 CARTs were very efficient *in vitro* and *in vivo* in eliminating both AML cell lines and primary cells,

even at a relatively low E:T ratios. Importantly however, CD123 CARTs ablated existing normal human hematopoiesis and prevented the establishment of *de novo* hematopoietic reconstitution, by directly targeting both myeloid progenitors and early/immature HSPCs, with subsequent functional consequences in all downstream normal hematopoietic progenitors, rendering severe impairment of multi-lineage hematopoiesis in BM and PB. This study adds information to the existing controversy about the myeloablative potential of CD123 CARTs. Despite several reports showing a limited cytotoxic effect on CD34+ HSPCs [22, 25, 29], our data supports the work by Gill and coworkers who reported a myeloablative *in vivo* potential of 4-1BB-based CD123 CARTs on CD34+ HSPCs.

The myeloablative effects here observed were not limited to the 41-BB-based CD123 CARTs but were similarly observed with the CD28-costimulated CAR123 CARTs. Previous studies used different sources of CD34+ cells, different vector designs, and distinct CD123 scFvs. Therefore, current conflicting data may be attributed to distinct vector architectures, CAR binding affinity, target density, source of healthy CD34+ cells, or even experimental designs [31]. The robust myeloablative effects reported in this study calls for caution when clinically implementing CD123 CARTs. Unfortunately, however, immunotherapies for AML different to CARTs such as DARTs or CD123-directed MoAb resulted in limited clinical efficacy unable to control the disease in the medium-long-term [14, 54, 55]. A potentially safer clinical approach to circumvent myeloablation would be the use of potent CD123 CARTs to achieve complete remission followed by allo-HSCT as a rescue therapy. Finally, alternative sources of effector cells, such as NK cells, cytokine-induced killer (CIK) cells or Vδ1 γδ T-cells (DOTs) are being explored in order to better control the *in vivo* persistence of CD123 CAR bearing cells [56, 57].

LIST OF ABBREVIATIONS: AML: Acute myeloid leukemia, B-ALL: B-cell acute lymphoblastic leukemia, BM: Bone marrow, CB: Cord blood, CIK: Cytokine induced killer, DOT: Delta-One-T-cells. EFS: Event free survival, HSPC: Hematopoietic stem cell progenitor, HSCT: Hematopoietic stem cell transplant, iv: Intra-vein, LIC: Leukemia-initiating cell, LSC: Leukemic stem cells, MoAb: Monoclonal antibody, NK: Natural killer, PB: Peripheral blood, PBCMs: Peripheral blood mononuclear cells, R/R: Refractory or relapsed, scFv: Single chain variable fragment.

DECLARATIONS

Ethics approval and consent to participate: This study was IRB-approved by the Barcelona Clinic Hospital Ethics Committee (HCB/2017/1056). All *in vivo* procedures were approved by the Animal Care Committee of The Barcelona Biomedical Research Park (HRH-17-0029-P1).

Consent for publication: not applicable

Data and material availability: the datasets and materials generated in this study are available from the corresponding author on reasonable request.

Competing interests: the authors have no competing interests

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Authors' contribution: ML-B, designed experiments, analyzed/interpreted the data, and wrote the manuscript. DS-M, F.G-A, H.R-H, M.C, S.R.Z, T.V-H, J.C, and R.D.dlG, performed experiments. EA, SV, JFN, HL, AEB, VHJvdH, JJ, PMa, AB, JE, AL, BV, IJ and MS provided clinical samples and biological data. CB, designed the experiments, interpreted the data, and financially supported the work. PM, conceived the study, designed experiments, wrote the manuscript, and financially supported the work.

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LEGENDS TO FIGURES

Figure 1. Expression of CD123 in AML and design, detection and expansion CD123 CARTs. (A) Immunophenotyping of the indicated diagnostic myeloid markers in a cohort of 97 AML patients at presentation. Each dot denotes an individual patient. Red circles identify patients with >80% of blasts positive for the indicated marker. **(B)** Comparative antigen density (measured as antigen molecules/cell) for CD123 and CD33 in primary AML samples (n=24), CB-derived (n=22) and PB-derived (n=10) CD34+ cells from healthy donors. AML blasts were identified as $7AAD^{-}CD3^{-}CD45^{+/low}CD123^{+}CD33^{+}$. One way-Anova *p<0.05, **P<0,01, ***P<0,001. **(C)** Comparison of CD123 expression in 68 paired diagnostic-relapse AML samples (left panel) and in bulk tumor *versus* AML-LSC (n=37, right panel) [33]. **(D)** Scheme of the CD123 CAR structure. **(E)** CAR detection in primary T-cells using an *anti-human IgG F(ab')2 antibody* and GFP. **(F)** Successful CAR123 transduction and detection in CD4+ and CD8+ T-cells (n=3). **(G)** Robust expansion of activated T-cells transduced with either MOCK (black line) or CAR123 (red line) (n=3).

Figure 2. 41BB-CD123 CARTs specifically target and eliminate CD123+ AML cells *in vitro* and *in vivo*. (A) Surface expression of CD123 (red) in THP-1, MOLM-13 and 697 cell lines. **(B)** Absolute counts of alive residual target cells measured by FACS in 48h cytotoxicity assays at 1:1 E:T ratio (n=3). Data are presented as mean±SEM. n.s=non-significant, *p<0.05, **p<0.01, ***p<0.001. **(C)** Graphical cartoon of the experimental design for autologous cytotoxic assays. Normal CD3+ T-cells were FACS-purified from the PB of AML patients (n=3), infected with CD123 CAR, expanded, and exposed to autologous total PBMCs (1:1 E:T). Residual CD123+ blasts were quantified 48h post 41BB-CD123 CART exposure. **(D)** *Left,* representative FACS analysis of the cytotoxicity

assay. T-cells are shown in black and CD123+ blasts in blue. *Right,* absolute counts of alive AML blasts in 48h cytotoxicity assays at 1:1 E:T ratio (n=3). **(E,F)** ELISA showing robust secretion of pro-inflammatory cytokines by 41BB-CD123 CARTs after exposure to CD123+ cell lines **(E)** and AML primary blasts **(F)** for 16h at 1:2 E:T ratio (n=3). **(G)** Experimental design to assess *in vivo* the efficacy of both 41BB- and CD28-based CD123 CAR. NSG mice were *iv* injected with 2.5×10^5 Luc-expressing xenograft AML cells (PDX-579) followed 5 days after by a single *iv* injection of 3×10^6 CD123 CARTs (either 41BB or CD28) generated from healthy PBMCs. Tumor burden was monitored every 7-10 days by BLI using IVIS imaging. **(H)** IVIS imaging of tumor burden monitored by BLI at the indicated time points. **(I)** *Left,* total radiance quantification (p/sec/cm^2/sr) at the indicated time points for both 41BB-CD123 CARTs, CD28-CD123 CARTs and untreated mice. *p<0.05. *Right,* absolute counts of residual AML cells in PB and BM at endpoint. **(J)** T-cell persistence in PB and BM at endpoint. **(K)** *In vivo* quantification by ELISA of INF-γ in PB sera collected in the acute phase (10 days post CARTs infusion). *p<0.05.

Figure 3. Both 41BB- and CD28-CD123 CARTs eliminate healthy CD34+ HSPCs *in vitro and in vivo*. A) Experimental scheme for *in vitro* assessment of CAR123 cytotoxicity on healthy CD34+ cells. **B)** Representative FACS showing the residual CD34+ HSPCs (red) after exposure to CAR123 CARTs or MOCK T-cells for 72h at E.T of 2:1. **C)** Absolute quantification of remaining alive CD34+ cells after exposure to either 41BB- or CD28-CD123 CARTs (72h, E:T 2:1). **D)** Clonogenic assays performed with residual alive CD34+ HSPCs after 24h co-incubation with either 41BB-CD123 CARTs, CD28-CD123 CARTs or MOCK T-cells (E:T=1:1) (n=3 donors). GEMM, Granulocytic, Erythroid, Myelo-Monocytic CFUs; GM, Granulo-Monocytic CFU; G, Granulocytic

CFU; M, Monocytic CFU; E, Erythroid CFU. **E)** Schematic representation of the *in vivo* experimental plan. CD34+ cells were intra-BM transplanted into NSG mice, and six weeks later the level of human engraftment was assessed by FACS analysis in PB and BM. Mice then received $3x10^6$ of either CD123 CARTs (41BB or CD28) or MOCK T-cells. PB bleedings were performed bi-weekly and PB/BM were analyzed at sacrifice (6 weeks after CART infusion). **F,G)** Analysis of murine PB **(F)** and BM **(G)** multi-lineage reconstitution (CD19+ B-lymphoid, CD123+ myeloid and CD34+ immature) at the indicated weeks post CARTs infusion. Final engraftment (POST) of myeloid, B-lymphoid and immature HSPCs is presented as fold change in comparison to pre-CARTs/MOCK infusion (PRE). **H)** Schematic representation of the *in vivo* experimental plan. CD34+ cells were intra-BM transplanted into NSG mice, followed, the day after, by infusion of either $3x10^6$ CD123 CARTs (41BB or CD28) or MOCK T-cells. Mice were sacrificed six weeks after and PB/BM were analyzed. **I,J)** Analysis of murine PB **(I)** and BM **(J)** multi-lineage reconstitution (CD19+ B-lymphoid, CD123+ myeloid and CD34+ immature) six weeks after CARTs infusion. *p<0.05, **p<0.01, ***p<0.001.

Figure 4. CAR123-mediated cytotoxicity is CD34 subset specific. A) Flow cytometry characterization of different subsets of $CD34^+$ HSPCs post CD123 CART exposure. *Left*, identification of CD34+ HSPCs and CD123 CARTs. *Middle-right*, identification of CD90+CD133+ early-immature CD34+ HSPCs (turquoise dots), CD13-CD71-CD19+ B-lymphoid CD34+ HSPCs (green dots), CD13++CD71dim myeloid CD34+ HSPCs (grey dots) and CD13lowCD71++ erythroid CD34+ HSPCs (black dots). **B)** Absolute quantification by FACS of the different CD34+ subsets (as identified in A) upon exposure to CD123 CARTs or MOCK T-cells (48h,1:1 E:T) (n=3). **C)** Mean fluorescence intensity (MFI) levels of CD123 in the different CD34+ cell subsets (n=3). *p<0.05

Figure 1

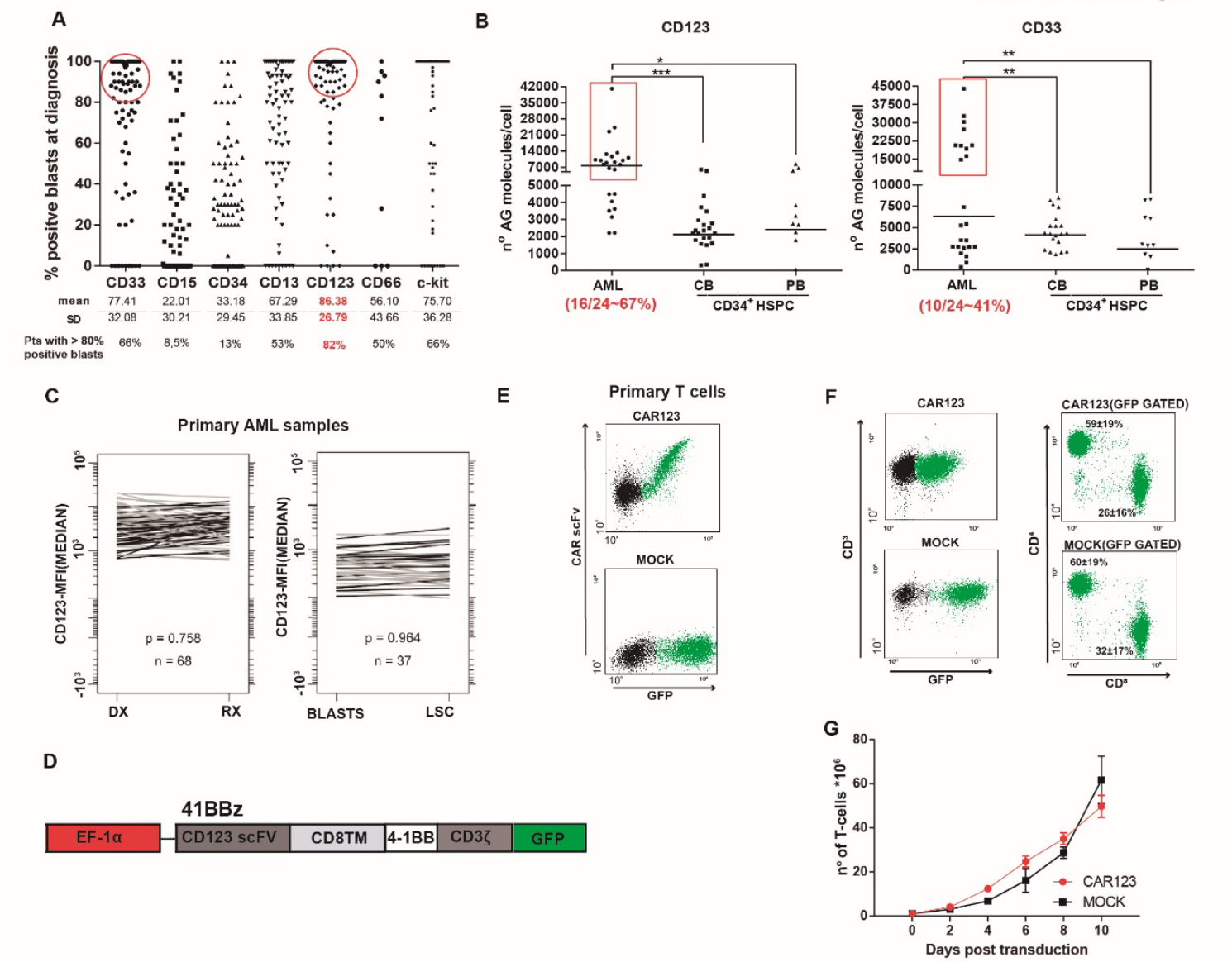

A
% positive blasts at diagnosis
CD33 CD15 CD34 CD13 CD123 CD66 c-kit
mean 77.41 22.01 33.18 67.29 86.38 56.10 75.70
SD 32.08 30.21 29.45 33.85 26.79 43.66 36.28
Pts with > 80% positive blasts 66% 8,5% 13% 53% 82% 50% 66%
B
CD123
CD33
n° AG molecules/cell
AML
CB
PB
(16/24~67%)
(10/24~41%)
CD34+ HSPC
C
Primary AML samples
CD123-MFI(MEDIAN)
p = 0.758
n = 68
p = 0.964
n = 37
DX
RX
BLASTS
LSC
D
41BBz
EF-1α
CD123 scFV
CD8TM
4-1BB
CD3ζ
GFP
E
Primary T cells
CAR123
MOCK
CAR scFv
GFP
F
CAR123
MOCK
CAR123(GFP GATED)
MOCK(GFP GATED)
59±19%
26±16%
60±19%
32±17%
CD3
CD4
CD8
G
n° of T-cells *10^6
CAR123
MOCK
Days post transduction

Figure 2

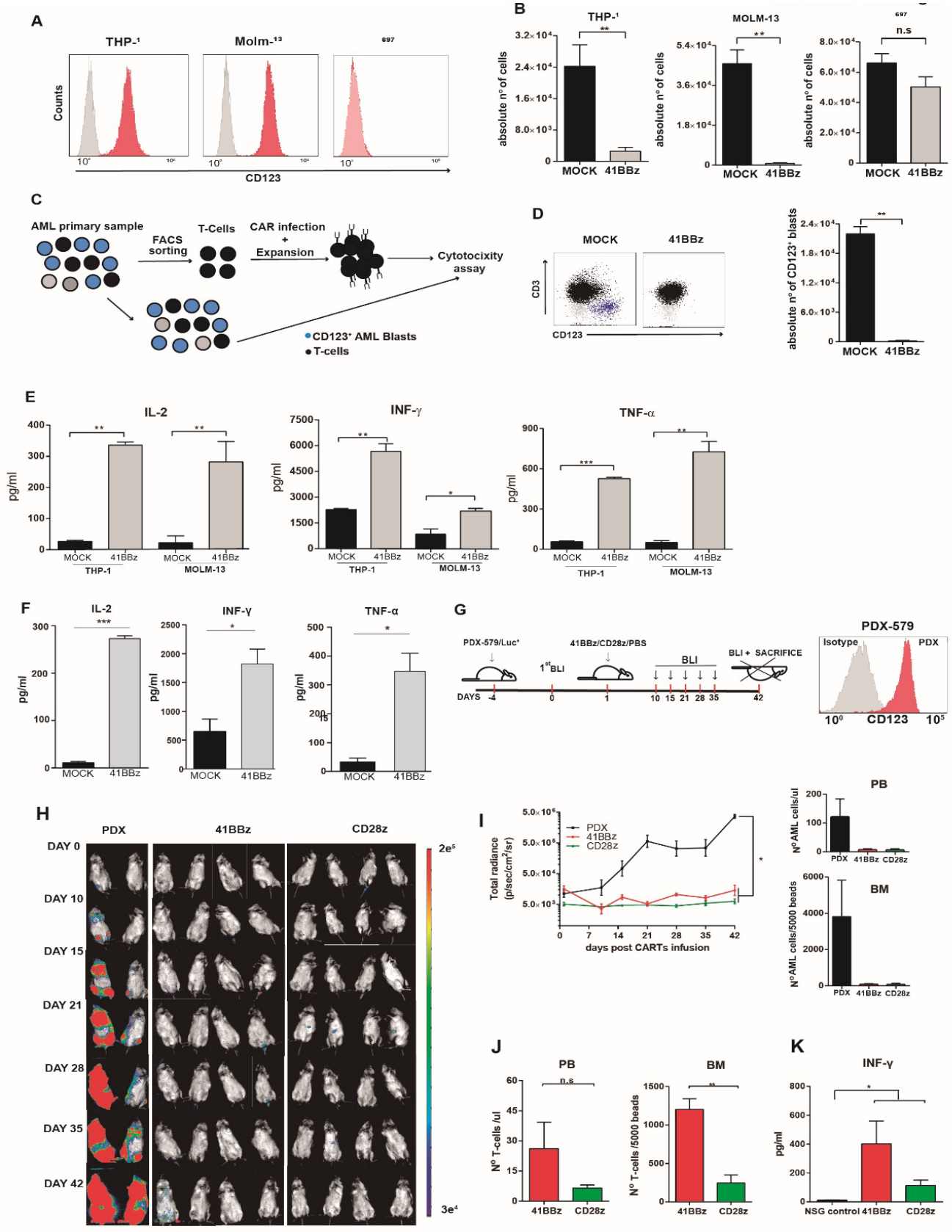

A
THP-1
Molm-13
697
Counts
CD123
B
THP-1
MOLM-13
697
absolute n° of cells
MOCK
41BBz
n.s
C
AML primary sample
FACS sorting
T-Cells
CAR infection + Expansion
Cytotoxicity assay
CD123+ AML Blasts
T-cells
D
MOCK
41BBz
CD3
CD123
absolute n° of CD123+ blasts
E
IL-2
INF-γ
TNF-α
pg/ml
THP-1
MOLM-13
F
IL-2
INF-γ
TNF-α
G
PDX-579/Luc+
41BBz/CD28z/PBS
1st BLI
BLI
BLI + SACRIFICE
DAYS
PDX-579
Isotype
PDX
CD123
H
PDX
41BBz
CD28z
DAY 0
DAY 10
DAY 15
DAY 21
DAY 28
DAY 35
DAY 42
2e5
3e4
I
PDX
41BBz
CD28z
Total radiance (p/sec/cm²/sr)
days post CARTs infusion
PB
BM
N°AML cells/ul
N°AML cells/5000 beads
J
PB
BM
N° T-cells /ul
N° T-cells /5000 beads
K
INF-γ
pg/ml
NSG control
41BBz
CD28z

Figure 3

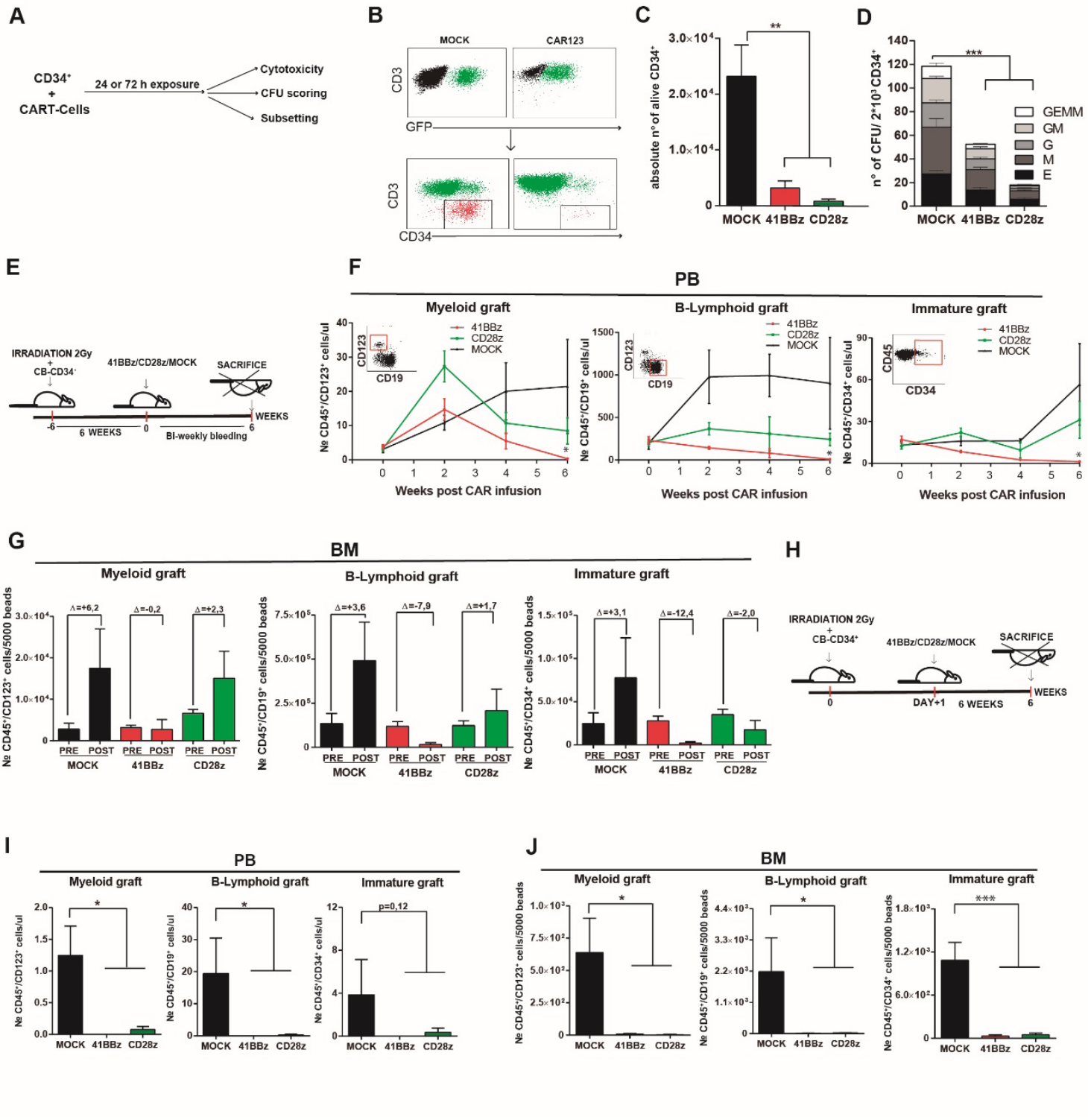

A
CD34+ + CART-Cells
24 or 72 h exposure
Cytotoxicity
CFU scoring
Subsetting
B
MOCK
CAR123
CD3
GFP
CD34
C
absolute n° of alive CD34+
MOCK 41BBz CD28z
D
n° of CFU/ 2*10³ CD34+
GEMM
GM
G
M
E
MOCK 41BBz CD28z
E
IRRADIATION 2Gy + CB-CD34+
41BBz/CD28z/MOCK
SACRIFICE
WEEKS
6 WEEKS
Bl-weekly bleeding
F
PB
Myeloid graft
B-Lymphoid graft
Immature graft
41BBz
CD28z
MOCK
№ CD45+/CD123+ cells/ul
№ CD45+/CD19+ cells/ul
№ CD45+/CD34+ cells/ul
Weeks post CAR infusion
G
BM
Myeloid graft
B-Lymphoid graft
Immature graft
№ CD45+/CD123+ cells/5000 beads
№ CD45+/CD19+ cells/5000 beads
№ CD45+/CD34+ cells/5000 beads
PRE POST
MOCK 41BBz CD28z
H
IRRADIATION 2Gy + CB-CD34+
41BBz/CD28z/MOCK
SACRIFICE
WEEKS
DAY+1
6 WEEKS
I
PB
Myeloid graft
B-Lymphoid graft
Immature graft
p=0,12
MOCK 41BBz CD28z
J
BM
Myeloid graft
B-Lymphoid graft
Immature graft
MOCK 41BBz CD28z

Figure 4

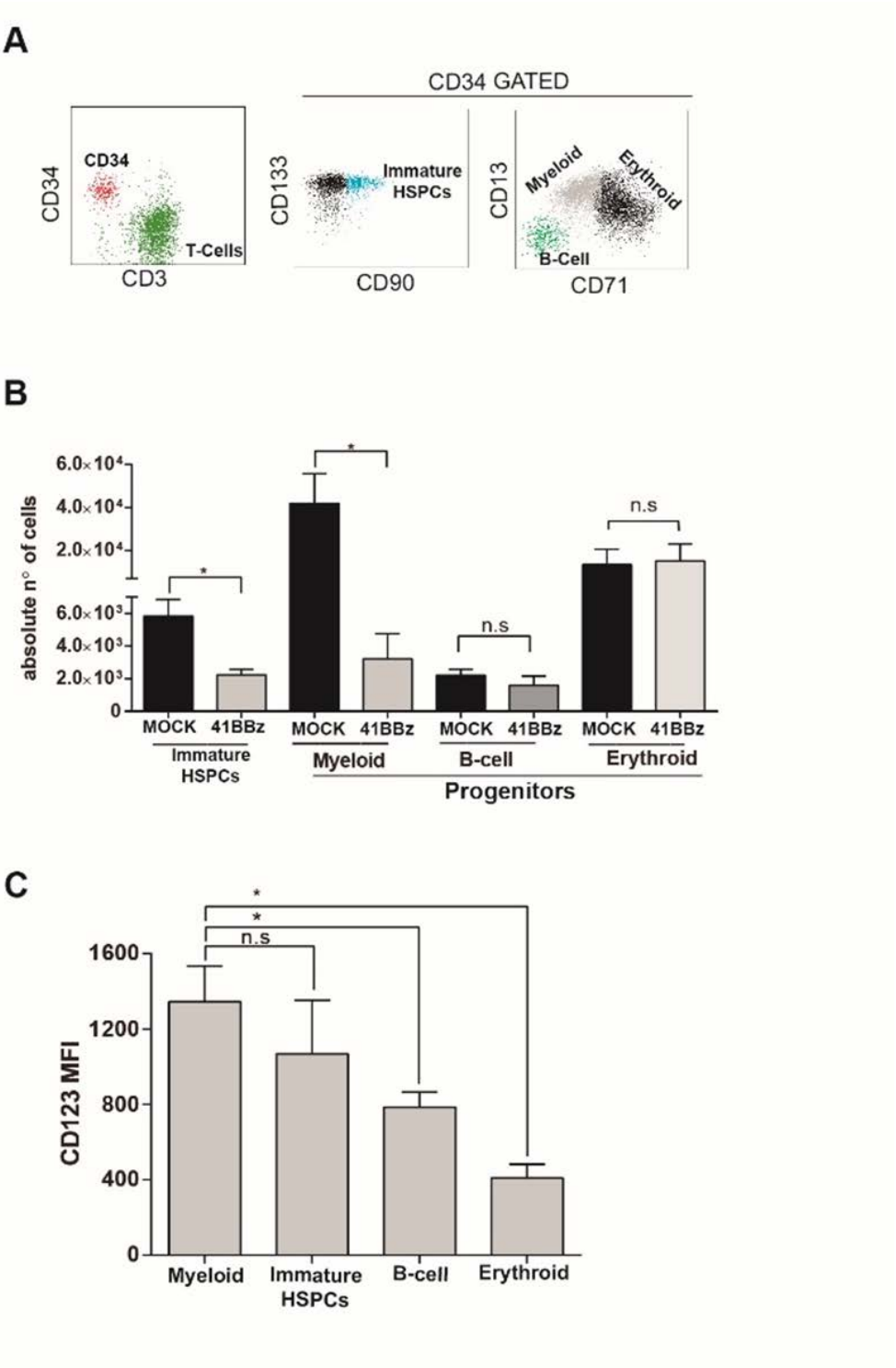

A
CD34 GATED
CD34
CD3
CD34
T-Cells
CD133
CD90
Immature HSPCs
CD13
CD71
Myeloid
Erythroid
B-Cell
B
absolute n° of cells
6.0×10⁴
4.0×10⁴
2.0×10⁴
6.0×10³
4.0×10³
2.0×10³
0
*
*
n.s
n.s
MOCK
41BBz
Immature HSPCs
Myeloid
B-cell
Erythroid
Progenitors
C
CD123 MFI
1600
1200
800
400
0
*
*
n.s
Myeloid
Immature HSPCs
B-cell
Erythroid

Table 1. Biological and cytogenetic-molecular characteristics of blasts from diagnostic AML

Patient ID	Diagnostic	Cytogenetics	Molecular	Age (y)	Gender	Blasts (%)	CD123 (%)	USE
14085	AML	46,XY	NPM1+, FLT3-ITD	42	M	87	80	AG density
14176	AML-M1	46,XX	Normal	44	F	83	75	AG density ELISA
14093	AML-M4	46,XX	$NPM1^{MUT}$, FLT3-ITD	52	F	81	86	AG density
14184	AML-M1	46,XX, t(8;21)	AML1-ETO	14	F	90	84	AG density
14268	AML	46,XY, inv(16)	$NPM1^{MUT}$, FLT3-ITD	69	M	95	92	AG density Autologous
14269	AML-M5	47,XX,+8	Normal	64	F	92	90	AG density ELISA
14266	AML	46,XX,t(8;21)	AML1-ETO, FLT3-ITD+	48	F	90	92	AG density ELISA
14123	AML	46,XY,t(3;3)	Normal	28	M	42	77	AG density
14185	AML	46,XY,inv(16)	Normal	8	M	77	72	AG density
14143	AML-M1	46,XY	Normal	43	M	90	90	AG density
14156	AML	46,XY,11q23	MLL-AF6	1	M	88	100	AG density
14144	AML	46,XY,del(7)(q22)	Normal	61	M	88	98	AG density
14141	AML-M5	46,XX, t(8;21)	AML1-ETO	39	F	83	82	AG density
14091	AML-M4	47,XX,+8	Normal	61	F	95	80	AG density
14272	AML	46,XX	$NPM1^{MUT}$, FLT3-ITD	44	F	73	88	Autologous
14274	AML	46,XX, t(8;21)	AML1-ETO	13	F	85	75	Autologous
ABT3974	AML	46,XY, +9, inv(16)(p13;q22), der(17)t(11;17)(q13;q25)	CEBPA, FLT3-TKD, WT1	37	M	91	87	AG density
ABT5270	AML	46,XY	NPM1, DNMT3A, IDH1	55	M	74	77	AG density
ABT4435	AML	46,XX	IDH2, DNMT3A	70	F	24	96	AG density
ABT8326	sAML	44-45X-,Y, der(3),del(7)(q22),der(8),add(12)(p13),-18,add(21)(q28)	TET2, CALR	61	M	6	56	AG density
ABT7693	AML	46,XY	NPM1, IDH1, PTPN11	45	M	80	99	AG density
ABT4470	AML	47,XX	NPM1, IDH1, BCOR	72	F	71	76	AG density
ABT5718	AML	46,XX	$CEBPA^{bi}$, DNMT3A, TET2	52	F	34	65	AG density
ABT8597	AML	NA	NPM1, IDH1, NRAS	69	F	71	100	AG density
ABT3906	AML	NA	NPM1, $FLT3\text{-}ITD^{HIGH}$, IDH1	40	M	93	77	AG density
ABT4685	tAML	46,XX	$CEBPA^{bi}$, TET2, WT1	67	F	49	93	AG density

Abbreviations: sAML= Secondary AML, tAML = therapy related AML, M=Male, F=Female, AG=Antigen,y=years

LEGENDS TO SUPPLEMENTARY FIGURES

Figure S1. CD28-costimulated CD123 CARTs exert potent *in vitro* effector functions against AML cells. A) Scheme of the CD28-costimulated CD123 CAR structure. **B)** CD28-CAR123 detection in primary T-cells using an *anti-human IgG F(ab')2 antibody* and GFP. **C)** Absolute counts of alive residual target cells measured by FACS in 48h cytotoxicity assays at 1:1 E:T ratio (n=3). Data are presented as mean±SEM. n.s,non-significant, *p<0.05, **p<0.01. **D)** ELISA showing robust secretion of pro-inflammatory cytokines by CD28-costimulated CD123 CARTs after exposure to CD123+ AML cells for 16h at 1:2 E:T ratio (n=3).

Figure S2. CAR123-mediated cytotoxicity is E:T ratio dependent. A) Relative quantification by FACS of the remaining alive target cells in a 16h cytotoxicity assay at the indicated E:T ratios. CAR-mediated cytotoxicity was normalized against MOCK T-cells (n=3) *p<0.05, **p<0.01. ***p<0.001 **B)** Representative FACS plot showing the gating strategy to identify remaining target cells (green dots) 16h post CAR/MOCK T-cells exposure.

Figure Supplementary 1

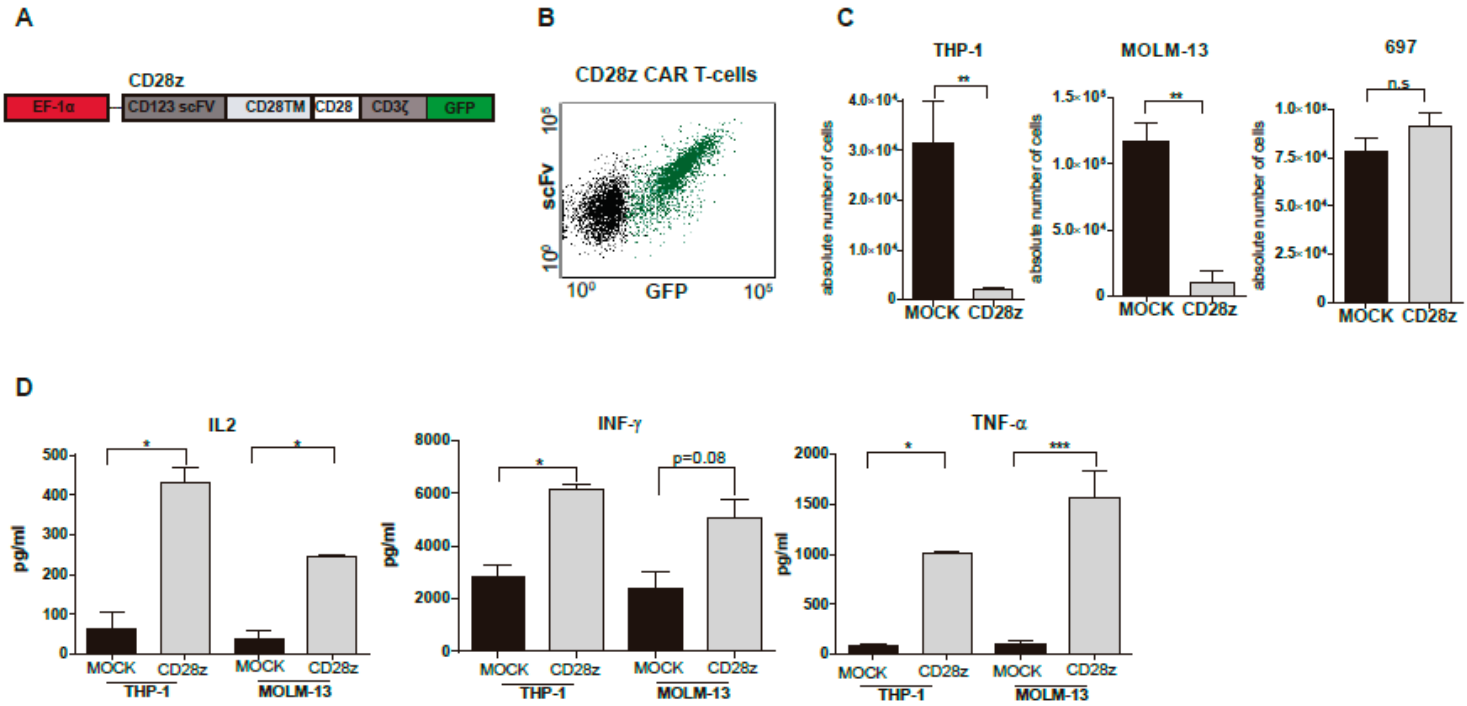
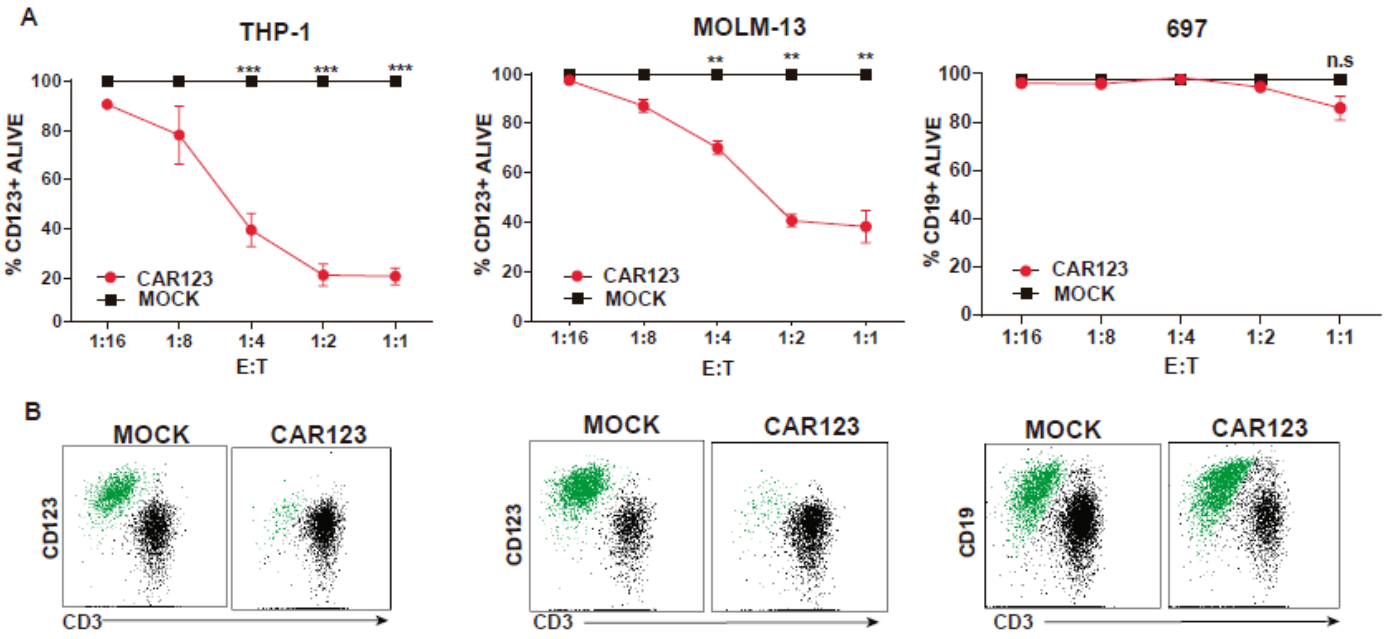


Figure Supplementary 2



PAPER 2: Published in Blood 2019; 133(21):2291-2304.

Fratricide-resistant CD1a-specific CAR T-cells for the treatment of cortical T-cell acute lymphoblastic leukemia

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Running title: CD1a-directed CARTs for coT-ALL

ABSTRACT

Relapsed/refractory T-cell acute lymphoblastic leukemia (R/R T-ALL) has a dismal outcome, and no effective targeted immunotherapies for T-ALL exist. The extension of chimeric antigen receptor T-cells (CARTs) to T-ALL remains challenging because the shared expression of target antigens between CARTs and T-ALL blasts leads to CARTs fratricide. CD1a is exclusively expressed in cortical T-ALLs (coT-ALL), a major subset of T-ALL, and retained at relapse. Here, we report that the expression of CD1a is mainly restricted to developing cortical thymocytes and neither CD34+ progenitors nor T-cells express CD1a during ontogeny, confining the risk of on-target/off-tumor toxicity. We thus developed and pre-clinically validated a CD1a-specific CAR with robust and specific cytotoxicity *in vitro* and antileukemic activity *in vivo* in xenograft models of coT-ALL, using both cell lines and coT-ALL patient-derived primary blasts. CD1a-CARTs are fratricide-resistant, persist long-term *in vivo* retaining antileukemic activity in re-challenge experiments, and respond to viral antigens. Our data supports the therapeutic and safe use of fratricide-resistant CD1a-CARTs for R/R coT-ALL.

INTRODUCTION

T-cell lineage acute lymphoblastic leukemia (T-ALL) is a malignant disorder resulting from leukemic transformation of thymic T-cell precursors[58]. T-ALL is phenotypically and genetically heterogeneous, and is commonly associated with genetic alterations/mutations in transcription factors involved in hematopoietic stem/progenitor cell (HSPC) homeostasis and in master regulators of T-cell development[59]. T-ALL comprises 10–15% and 20–25% of all acute leukemias diagnosed in children and adults, respectively[60, 61] with a median diagnostic age of 9 years[62-64]. Intensive chemotherapy regimens have led to the improved

survival of patients with T-ALL; however, the event-free (EFS) and overall (OS) survival remains <70%, and relapsed/refractory (R/R) T-ALL has a particularly poor outcome. There are currently no potential curative options beyond hematopoietic cell transplantation and conventional chemotherapy, which is linked to large trade-offs in toxicities[61, 65], reinforcing the need for novel targeted therapies.

Immunotherapy has generated unprecedented expectations in cancer treatment and relies on the immune system as a powerful weapon against cancer. In recent years, adoptive cellular immunotherapy based on chimeric antigen receptors (CARs) has shown great potential. CAR therapy redirects genetically modified T-cells to specifically recognize and eliminate specific antigen-expressing tumor cells in a major histocompatibility complex-independent fashion[66, 67]. The success of CAR T-cells (CARTs) re-directed against CD19 or CD22 is now indisputable for B-cell malignancies (mainly B-ALL)[68-71]. Strategies targeting T-cell malignancies using CARTs remain, however, challenging because of the shared expression of target antigens between CARTs and T-lineage tumoral cells. In this regard, CARTs against pan T-cell antigens have two major drawbacks: i) CARTs self-targeting/fratricide and, ii) T-cell aplasia, leading to life-threating immunodeficiency[33, 72, 73].

Recent elegant pre-clinical studies demonstrated that T-cells transduced with either CD7, CD3, CD5 or TCR CARs, the most expressed pan-T-cell antigens, efficiently eliminate T-ALL blasts *in vitro* and are able to control the disease *in vivo*[33, 34, 72-75], leading very recently to pioneering phase I clinical trials with CAR T-cells for T-ALL (NCT03690011, NCT03590574). Yet, innovative approaches, such as

CRISPR/Cas9 genome editing or protein expression blockers, seem needed for disruption of the target antigen in T-cells prior to CAR transduction, to avoid extensive self-antigen driven fratricide[33, 72-74].

Gene expression profiling and multicolour immunophenotyping classify T-ALLs into distinct subgroups that mostly reflect a particular stage of differentiation arrest[76]. Cortical T-ALL (coT-ALL) is a major subgroup of T-ALL characterized by the surface expression of CD1a, consistent with a developmental arrest at the cortical stage[77-79]. There are four CD1 isoforms (CD1a, CD1b, CD1c and CD1d) in humans while only the CD1d isoform is expressed in the mouse[80]. Upon recognition of the CD1 ligand complex by the T-cell receptor, CD1-dependent T-cells are activated in a variety of immunological contexts[80]. Loss-of-function studies revealed that CD1-deficient mice may be more susceptible to some viruses, bacteria and protozoa [81, 82]. Unfortunately, the role of CD1 isoforms in human infection remains elusive. CD1a is a lipid-presenting molecule whose expression is essentially restricted to coT-ALL and Langerhans Cell Histiocytosis (LCH), and practically absent in human tissues with the exception of developing cortical thymocytes and LC[83, 84]. Here, we tested the feasibility of targeting CD1a+ coT-ALL using CD1a CARTs. We report that CD1a-specific CARTs show robust cytotoxicity against CD1a+ coT-ALL cell lines and primary coT-ALL cells, both *in vitro* and *in vivo*. CD1a CARTs are fratricide-resistant and remain functional *in vivo* after 13 weeks, as demonstrated by leukemia re-challenge experiments. Fratricide-resistant CD1a CARTs thus represent a safe and innovative adoptive immunotherapy for coT-ALL, and potentially for other CD1a+ tumors such as LCH.

METHODS

CD1a-specific scFv generation and CAR design

The CD1a-specific single-chain variable fragment (scFv) derived from the NA1/34.HLK clone of CD1a-specific antibody was obtained using commercial synthesis (Sigma-Aldrich) with the mouse IgG Library Primer Set (Progen), and was cloned into a pCCL lentiviral-based second-generation CAR backbone containing a human CD8 transmembrane (TM) domain, human 4-1BB and CD3z endodomains, and a T2A-GFP cassette. Identical lentiviral vectors expressing either GFP alone (mock) or CD22 CAR backbone were used as controls.

CAR-expressing lentiviral production, T-cell transduction, activation and expansion

CAR-expressing viral particles pseudotyped with VSV-G were generated in 293T cells using standard polyethylenimine transfection protocols, and were concentrated by ultracentrifugation as described elsewhere[85]. Viral titers were consistently in the range of 10^8 TU/mL. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy volunteers by Ficoll-Hypaque gradient centrifugation. Buffy coats were obtained from the Barcelona Blood and Tissue Bank (BST) upon IRB-approval (HCB/2018/0030). T-cells were activated by plate-bound anti-CD3 (OKT3) and anti-CD28 antibodies (BDBiosciences) for 2 days and were then transduced with CAR-expressing lentivirus (MOI=10) in the presence of interleukin-7 (IL-7) and IL-15 (10 ng/mL, Mitenyi Biotec)[33, 34]. The cell surface expression of CD1aCAR was traced by fluorescence-activated cell sorting (FACS) expression of GFP and using an AffiniPure $F(ab')_2$ Fragment Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch). Proper

activation of CAR-transduced T-cells was demonstrated by staining for CD25 and CD69 after 2-day expansion.

Immunophenotyping of healthy CD34+ progenitors, T-cells and primary T-ALL samples

The expression of CD1a antigen in CD34+ stem/progenitor cells (HSPCs), CD34+CD7+ thymic T-cell progenitors and CD3+ T-cells was prospectively analyzed in fresh human thymus, fetal liver and bone marrow (BM), cord blood and adult BM and peripheral blood (PB) (n=3). Fetal tissue was collected as previously described[86, 87] from developing embryos aborted at 18–22 weeks of pregnancy, obtained from the MRC/Wellcome Trust Human Developmental Biology Resource upon informed consent and approval by our local ethics committee (CMRB-CEIC-26/2013). Neonatal and adult tissues were obtained from the BST upon IRB approval (HCB/2018/0030). Primary T-ALL samples and diagnostic immunophenotyping data (n=38) were obtained from the Spanish hospitals *Sant Joan de Déu*, *Germans Trias i Pujol*, *Santa Creu i San Pau* (Barcelona), *Niño Jesús* (Madrid), and *Virgen de la Arrixaca* (Murcia). **Table S1** shows the main clinical-biological features of the CD1a++ coT-ALL cases. For immunophenotyping of T-ALL primary samples, the following fluorochrome-conjugated monoclonal antibodies (MoAb) were used: anti-CD2-PE, CD7-FITC/PE, CD13-PerCP-Cy5.5, CD34-APC, CD3-PE, CD5-FITC, CD4-BV-421, CD8-APC-Cy7, CD45-AmCyan, CD1a-BV-421/APC/PE, CD33-APC and CD123-APC (BDBiosciencies). Isotype-matched, non-reactive fluorochrome-conjugated MoAb were always used as a fluorescence reference. Briefly, 5×10^5 PBMCs were incubated with erythrocyte-lysing solution (BDBiosciencies) for 10min and then stained with MoAb (20min at 4°C in the dark). Stained cells were washed in phosphate buffered saline and FACS-analyzed on a FACSCanto-II cytometer equipped

with FACSDiva software (BDBiosciencies)[36-38]. CD1a antigen density was determined using BDQuantibrite-PE, as described elsewhere[88].

In vitro cytotoxicity assays and cytokine release determination

Cell lines Jurkat, MOLT4 and NALM6 were purchased from DSMZ (Germany) and expanded according to DSMZ recommendations. Luciferase (Luc)/GFP-expressing cells were stably generated by retroviral transduction and FACS purification of GFP+ cells[89]. Target cells (cell lines and primary T-ALL blasts) were labeled with 3 μM eFluor670 (eBioscience) and incubated with CD1a, CD22 or mock CARTs at different Effector:Target (E:T) ratios for the indicated time periods. CART-mediated cytotoxicity was determined by analyzing the residual alive (7-AAD-) eFluor670+ target cells at each time point and E:T ratio. Absolute cell counts were determined using Trucount absolute count beads (BDBiosciences). Additionally, FACS-sorted CD3+CD1a- mature T-cells from PB of coT-ALL patients at presentation were activated, transduced with CD1a CAR and tested against their eFluor670-labeled autologous-matched CD1a+ coT-ALL blasts. Leukemic blasts were never FACS-sorted in primary samples. The production of the pro-inflammatory cytokines IL-2, TNFα and IFNγ was measured by ELISA (BDBiosciences) in supernatants harvested after 16h.

In vivo Jurkat and T-ALL patient-derived xenograft (PDX) models

Six- to 12-week-old nonobese diabetic (NOD)-Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (Jackson Laboratory) were bred and housed under pathogen-free conditions in the animal facility of the Barcelona Biomedical Research Park (PRBB). Mice were irradiated (2Gy) and intravenously (i.v.) transplanted with 3×10^6 Luc-GFP-expressing Jurkat cells or with 1×10^6 primary cortical CD1a+ T-ALL blasts (primary and primograft-expanded)[90]. Between $1.5–5\times10^6$ CD1a or mock CARTs were i.v. infused 3 days later.

When Luc-Jurkat cells were used, tumor burden was followed by bioluminescence (BLI) using the Xenogen IVIS 50 Imaging System (Perkin Elmer). To measure luminescence, mice received 150 mg/kg of D-luciferin intraperitoneally, and tumor burden was monitored at the indicated time points. Living Image software (Perkin Elmer) was used to visualize and calculate total luminescence. Tumor burden of primary coT-ALL samples was followed-up by biweekly bleeding and FACS analysis. Mice were sacrificed when mock CARTs-treated animals were leukemic, and tumor burden (hHLA-$ABC^+hCD45^+hCD3^{-/low}hCD1a^+$ graft) and effector T persistence (hHLA-$ABC^+hCD45^+hCD3^+hCD1a^-GFP^+$) was analysed in BM, PB and spleen by FACS. In re-challenge experiments, leukemia-free animals that had received an infusion of CD1a CARTs 5-7 weeks before were re-infused with either 1.5×10^6 Luc-Jurkat cells or 1×10^6 CD1a+ coT-ALL primary cells, and disease reappearance was followed-up by BLI and FACS. All procedures were performed in compliance with the institutional animal care committee of the PRBB (DAAM7393).

Enzyme-linked immunospot assay (ELISpot)

ELISpot plates (Millipore) were coated with anti-human IFNγ antibody (1-D1K, Mabtech) and kept overnight at 4°C. Plates were then washed six times with PBS containing 1% fetal calf serum and then cells from three independent donors were plated at $5–10\times10^5$ cells/well and cultured in triplicate for 20h at 37°C and 5% CO_2. We measured IFNγ-secreting cells in response to CEF at 1 μg/mL, a peptide pool of T-cell epitopes of Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Flu and to staphylococcal enterotoxin B (SEB) at 1 μg/mL as a positive control. Plates were then revealed with biotinylated anti-human IFNγ, streptavidin-alkaline phosphatase (Mabtech), as previously described[91, 92]. The frequency of IFNγ-secreting cells was

quantified using ImmunoCapture and ImmunoSpot software to calculate the number of IFNγ Spot Forming Units per 10^5 (SFU).

Statistical analysis

Data from at least three individual donors are shown in all figures, and experimental duplicates were always performed. At least five animals were used in each *in vivo* condition. All p-values were calculated by unpaired two-tailed Student´s t-test using Prism software (GraphPad). OS of mice was determined using a Mantle-Cox test. A p-value <0.05 was considered statistically significant.

RESULTS

CD1a specifically marks coT-ALL blasts

The shared expression of target antigens between CARTs and T-lineage blasts has limited immunotherapy approaches in T-ALL due to CARTs fratricide and potential life-threating T-cell aplasia. CD1a antigen is expressed in coT-ALLs, a major subset of T-ALLs (**Fig1A,B,S1**), and retained at relapse (**Fig1C**). In line with previous studies[93-95], in our cohort of T-ALL patients (n=38), 75% (n=29) are phenotypically defined as CD1a+ coT-ALL; however, in only 50% (19/38) of our T-ALL patients the expression of CD1a is homogeneous (**Fig1A,B,S1B**). Importantly, CD1a is completely absent in T-cells in all extra-thymic tissues (**Fig1D**)[83], and steady-state CD34+ HSPCs also lack CD1a expression in multiple hematopoietic sites across ontogeny (**Fig1D**). T-cell development is initiated within the thymus by a first colonizing $CD34^{high}$CD7-CD1a- primitive HSPC with lympho-myeloid potential, which then differentiate in response to the thymic microenvironment into $CD34^{high}$CD7+CD1a- early T-cell progenitors (ETP)[96]. As thymic differentiation progresses, ETPs maintain CD7 expression and gradually lose

CD34 expression, whereas CD1a expression emerges and is transiently confined to cortical thymocytes[97] (**Fig1E**). Within the CD34+ thymic HSPC population, ~50% is represented by pre-cortical CD34^{high}CD7+CD1a- ETPs (**Fig1F**, blue cells), allowing us to hypothesize that CD1a may be a feasible and safe immunotherapeutic target for R/R coT-ALL[60, 93, 98, 99].

CD1a-redirected T-cells (CD1a CARTs) expand without T-cell fratricide

We designed a second-generation CD1a CAR consisting of anti-CD1a scFv, a CD8 TM spacer, and intracellular signaling domains from 4-1BB and CD3z coupled in-frame with GFP through a T2A sequence (**Fig2A**). The expression of the CD1a CAR was easily detected through co-expression of both scFv and GFP in 293T cells (**Fig2B**) and in primary CD4+ and CD8+ T-cell subsets (**Fig2C**). Importantly, activated (CD69+CD25+) CD1a CARTs (**Fig2D**) continuously expanded 200-fold over a 12-day period, similar to MOCK T-cells (**Fig2E**), demonstrating that redirecting CARTs against CD1a antigen does not induce T-cell fratricide.

CD1a CARTs specifically eradicate T-ALL cell lines and primary blasts *in vitro*

An initial analysis of CD1a density in cell surface confirmed high expression level of the target antigen specifically in coT-ALL primary cells, primografts and cell lines (**Fig2F,G**), further validating CD1a as an immunotarget. Consequently, CD1a CARTs were first tested *in vitro* using the CD1a+ T-ALL cell lines Jurkat and MOLT4, and the B-ALL cell line NALM6 as a negative control (**Fig2F**). Compared with control CARTs (either MOCK T-cells or CD22 CARTs), CD1a CARTs specifically eliminated CD1a+ T-ALL cells in a manner dependent on the E:T ratio. A relatively low E:T ratio of 2:1 or 4:1 induced 50%-90% specific cell lysis in 16h-assays (**Fig2H,S2**). Importantly, CD1a+

T-ALL cells barely survived exposure to CD1a CARTs in a 72h absolute number assay at a 1:1 E:T ratio (**Fig2I,J**). CD1a CARTs produced high levels of the pro-inflammatory cytokines IL-2, TNFα and IFNγ on co-culture with CD1a+ cell lines, confirming their cytotoxicity (**Fig2K**).

To further address their ability to eliminate primary tumors, CD1a CARTs were co-cultured with primary coT-ALL samples (either freshly harvested or PDX-derived), with a proportion of CD1a+ blasts ranging between 80% and 99% (**Fig3A**). Compared with MOCK T-cells, CD1a CARTs specifically eliminated primary CD1a+ coT-ALL cells in 48h cytotoxicity assays at 4:1 E:T ratio (**Fig3B,C**). BM normal hematopoietic cells (CD1a-) as well as CD1a- T-ALL blasts were not lysed by CD1a CARTs, further confirming the specificity of the CD1a CAR (**Fig3C,S5**). High-levels of IFNγ and TNFα were also secreted on co-culture with CD1a+ primary T-ALL cells (**Fig3D**).Collectively, CD1a CARTs have a potent and specific anti-leukemic activity against coT-ALL cell lines and primary blasts *in vitro*.

CD1a CARTs demonstrate potent anti-leukemia activity *in vivo*

We next evaluated the activity of CD1a CARTs *in vivo* using both Luc-expressing Jurkat T-ALL cells (**Fig4, S3**) and a primary coT-ALL xenograft model[90] (**Fig5**). NSG mice were transplanted with 3×10^6 Luc-expressing Jurkat cells three days prior to i.v. infusion of either 2×10^6 or 5×10^6 CD1a (or MOCK) CARTs, and leukemia establishment was followed-up weekly by BLI (**Fig4A,S3**). In contrast to the mice receiving MOCK T-cells, which showed massive tumor burden by BLI, those mice receiving CD1a CARTs were practically leukemia-free by day 25 (**Fig4B,C,S3**). The control of leukemia progression was CD1a CART cell dose-dependent (**FigS3B,C**). Flow cytometry analysis of tumor burden in PB confirmed the BLI data (**Fig4D**). Importantly, FACS analysis revealed T-

cell persistence in all hematopoietic tissues analyzed (**Fig4E**); however, we found a significantly increased biodistribution of CD1a-directed effector T-cells in BM and spleen, as compared with the biodistribution of MOCK T-cells (**Fig4E**), indicative of an active control of disseminated leukemia by CD1a CARTs.

In a clinically more relevant PDX model of coT-ALL, NSG mice were first transplanted with 1×10^6 primary CD1a+ T-ALL blasts followed three days later by infusion of 1×10^6 CD1a (or MOCK) CARTs, and leukemia engraftment was then followed-up bi-weekly by bleeding and endpoint BM analysis (**Fig5A**). Engraftment of CD1a+ coT-ALL cells gradually increased over time both in BM (**Fig5B**, 50%±13% and 55%±11% on week 6 and 9, respectively) and PB (**Fig5C,** 4.4%±2% and 18%±6% on week 6 and 9, respectively) in MOCK T-cells-treated PDXs, and associated with a significantly lower 9-week OS (42% *vs* 100%, p=0.01;**Fig5D**). In contrast, CD1a CARTs fully abolished T-ALL growth/engraftment even 9 weeks after CARTs infusion (0.36% and 0% T-ALL blasts in BM and PB, respectively), and, importantly, they persisted in PB and BM overtime (**Fig5E**).

***In vivo* persistent CD1a CARTs are functional in re-challenge assays**

Because the persistence of CARTs in hematopoietic tissues is a major biological parameter for their clinical success, we next assessed whether CD1a CARTs persisting after 40–50 days remained functional and efficient in controlling T-ALL progression. To do this, T-ALL-transplanted mice in which the leukemia was cleaned on treatment with CD1a CARTs were re-challenged with either Luc-Jurkat cells (**Fig6A-D**) or primary T-ALLs from primografts (**Fig6E-G**). Opposite to controls in which the secondary leukemias rapidly (as soon as 2 weeks after) and massively engrafted, T-ALL engraftment

was barely detectable by either BLI or FACS in the Jurkat (**Fig6A-C**) or primograft model after 6 weeks (**Fig6F**). Importantly, FACS analysis confirmed persisting effector T-cells in PB (**Fig6D,G**), BM and spleen (**Fig6D**) of re-challenged animals, further supporting the functional effect CD1a CARTs in controlling disease progression in re-challenge assays.

Patient-derived CD1a CARTs specifically target autologous CD1a+ blasts and retain antiviral activity

The proper choice of the target antigen and avoiding T-cell fratricide are crucial for the success of CARTs in T-ALL. Accordingly, we examined whether PB-derived mature CD3+CD1a- T-cells from patients with coT-ALL can be isolated and genetically modified to express CD1a CAR (**Fig7**). For this, CD3+CD1a- T-cells from patients were isolated (>95% purity, data not shown), activated with CD3/CD28 and lentivirally transduced (31–70% transduction) with CD1a CAR or MOCK. Next, we investigated the cytolytic capacity of CD1a CARTs derived from primary T-ALLs against active T-ALL patient-matched PBMCs (**Fig7A**). Total PBMCs were used as targets because it allows to assess both the autologous cytotoxicity potential and the degree of fratricide. Within eFluor670-labelled target PBMCs, the great majority (~75%) are CD1a+ blasts and ~15% are CD3+CD1a- mature T-cells (**Fig7B**). As compared with MOCK T-cells, the CD1a CARTs showed a massive and specific cytolytic capacity against autologous CD1a+ blasts but not against CD1a- mature T-cells (**Fig7C**), further demonstrating that CD1a CARTs are fratricide-resistant.

To further assess the potential thymic toxicity of CD1a CARTs, we next used human normal fetal thymus-derived CD7+ thymocytes as target cells. Only the CD1a+ cortical

thymocytes (red) were eliminated by the CD1a CARTs, whereas developmentally earlier and later CD1a- (blue) thymic T-lineage populations (CD7+CD34+ and CD7+CD34-) were not targeted (**FigS4**), limiting the on-target/off-tumor effects to a developmentally transient thymic population of cortical thymocytes. We finally sought to determine whether CD1a CARTs can protect, by themselves, the host by targeting the most common pathogens causing viremia in immunosuppressed patients. To do this, we tested the reactivity of CD1a CARTs to CMV, EBV and Flu antigens (CEF) and quantified the SCFs by INFγ ELISpot. Both MOCK T-cells and CD1a CARTs responded very similarly to stimulation with viral peptides, suggesting that CD1a CARTs retain antiviral activity (**Fig7D**).

DISCUSSION

T-ALL is an aggressive hematological cancer with poor clinical outcome both in children and adults, for which there is currently no targeted therapy[98, 99]. Salvage chemotherapy regimens induce remissions in only 20–50% of R/R cases, and allogeneic HSPC transplantation is largely associated with toxicities[61]. Despite intensive multi-agent chemotherapy protocols, 5-year survival remains ~50%[61, 93], reinforcing the need for novel targeted therapies. Along this line, the only targeted therapies previously used for eradication of malignant T-cells, with suboptimal clinical outcome, are the ricin A chain toxin-conjugated MoAbs anti-CD5 and anti-CD7[100, 101].

Adoptive cellular immunotherapy based on CARs holds great promise in cancer-targeted treatment. T-cells can be modified to specifically recognize and eliminate tumor cells through the expression of CARs, which redirect genetically modified T-cells to specific antigen-expressing tumor cells in a major histocompatibility complex-independent

manner[66, 67]. However, broadening the scope of CARTs to treat T-ALL and T-cell lymphomas has proven challenging because of the shared expression of target antigens between CARTs and T-lineage tumoral cells[33, 34, 72-75]. Accordingly, two major stumbling blocks need to be overcome for the use of CARTs for T-ALL and T-cell lymphoma: first, CARTs self-targeting/fratricide occurs when CARTs recognize pan T-cell antigens; and second, CARTs targeting pan T-cell antigens will induce T-cell aplasia, leading to a life-threating immunodeficiency[33, 34, 72-74].

Here, we hypothesized that the choice of the antigen against which we wish to re-direct T-cells would represent a major advance to solve the problems associated with the shared expression of T-cell markers between normal and malignant T-cells. CD1a is a lipid-presenting molecule whose expression is basically restricted to coT-ALL, retained at relapse, and is practically absent in human tissues with the exception of cortical thymocytes, skin LC and some circulating myeloid dendritic cells during development[83, 84, 102, 103]. Given this, we opted for the CD1a antigen as a feasible and safe target for CAR immunotherapy in R/R coT-ALL, the most common subtype of T-ALL[76, 83, 104].

We developed and functionally characterized CD1a-specific CARTs, which displayed robust cytotoxicity against T-ALL cell lines and primary CD1a+ coT-ALL cells both *in vitro* and *in vivo* in xenograft models. The CD1a CARTs continuously expanded 200-fold, similar to MOCK T-cells, demonstrating that redirecting CARTs against CD1a antigen does not induce T-cell fratricide. Also, the use of CD1a CARTs for coT-ALL bypasses the need for sophisticated genome editing-based disruption of target antigens in T-cells prior to CAR transduction as a strategy to avoid self-antigen-driven fratricide[33,

72-74]. We further demonstrated that in steady-state hematopoiesis, CD1a is exclusively expressed in a subset of CD34+CD7+ cortical thymic T-progenitors, whereas earlier $CD34^{high}CD7^{high}$ T-progenitors lack CD1a. In addition, neither normal CD34+ HSPCs nor mature T-cells from multiple tissues express CD1a during ontogeny, thereby minimizing the risk of on-target/off-tumor toxicity. Indeed, when human fetal thymus-derived CD7+ thymocytes were exposed to CD1a CARTs, only the CD1a+ cortical thymocytes were eliminated by the CD1a CARTs, while developmentally earlier and later thymic T-lineage populations (CD34+ and CD34-) were not targeted, limiting the on-target/off-tumor effects to a developmentally transient thymic population of cortical thymocytes and further confirming the fratricide resistant nature of CD1a CARTs.

Regarding safety, we do not expect irreversible toxicities or immunodeficiency attributed to CD1a CARTs for the following reasons: i) CD1a+ thymocytes represent a transient and thymus-restricted population, eventually regenerated by “non-targetable” upstream CD34+CD7+CD1a- T-cell progenitors physiologically/constantly maturing into functional T-cells; ii) CD1a CARTs themselves respond normally to viral antigens and therefore are likely to be protective against pathogens; iii) the clinical use of specific antibodies against CD5 or CD7[100] did not reveal severe or irreversible toxicities; iv) postnatal thymectomy does not lead to immunodeficiency in humans[105, 106], likely because thymic emigrants generated early in life persist for decades[105], suggesting that potential transient elimination of thymic progenitors by CD1a CARTs in pediatric patients would not compromise the complete anti-viral T-cell repertoire in adult life. Nonetheless, whether infants could eventually develop a premature immunosenescence later in life merits caution, as this was reported for infants thymectomized before 1 year of age[107-111]. Fortunately, however, T-ALL is extremely infrequent in infants. Safer

ultimate strategies would include i) the implementation of an inducible molecular switch to control potential toxicities linked to CARTs[112-114] and/or ii) the use of CD1a CARTs as a therapy bridge before curative allogeneic HSPC transplantation.

Beyond the hematopoietic system, CD1a expression in humans is restricted to LCs in the skin, which constitute a very rare subset of dendritic cells in the epidermis[115]. The immunological homeostasis of LCs is vital to avoid inflammatory skin diseases such as dermatitis and psoriasis[115]. The *in vivo* role of CD1a has long remained a challenge because CD1a is not expressed in mice. However, a recent seminal paper based on mice with transgenic expression of CD1a[116] showed that CD1a drives the pathogenesis of poison-induced dermatitis and psoriasis[115]. Importantly, they also demonstrated that treatment with CD1a blocking antibodies alleviated skin inflammation with no co-morbidity or side-effects. This highlights that CD1a is a potential therapeutic target in inflammatory skin diseases but also supports CD1a as a safe target for R/R coT-ALL. A phase I clinical trial is planned in our institution to confirm the safety of CD1a-directed CARTs in R/R coT-ALL. Unfortunately, CD1a-directed CAR is not a therapeutic choice for the aggressive ETP T-ALL which is commonly CD1a-.

Last but not least, it remains to be determined whether autologous mature T-cells can be recovered from the PB of T-ALL patients, modified to express the CAR of interest, and retain cytolytic activity against the tumoral cells expressing the target antigen. Along this line, flow cytometric analysis of PB from patients with active T-ALL revealed the presence of mature CD3+CD1a- T-cells in all the patients. These PB-derived T-cells were efficiently harvested from coT-ALL patients, modified to express CD1aCAR and exhibited a potent and specific cytolytic activity against autologous CD1a+ T-ALL blasts,

reinforcing the notion that CD1a CARTs are fratricide-resistant. Furthermore, as a note of safety, the genome-wide mutational landscape of both pediatric and adult T-ALL[117] has revealed 106 somatic oncogenic drivers present in diagnostic samples but absent in remission mononuclear cells and T-cells, suggesting that mature T-cells would be a relatively safe source of autologous T-cells for CAR transduction. We propose CD1a CART immunotherapy once tumor burden has been extensively reduced with standard therapies. Alternatively, donor T-cells would represent an ideal effector source in coT-ALL patients who have previously underwent allogeneic HSPC transplantation. Finally, universal, “off-the-shelf” allogeneic-suitable T-cells are the short-term “holy-grail” for leukemia immunotherapies since T-cell lymphopenia or T-cell dysfunction occurs in many multi-treated refractory/relapse patients suffering from hematological malignances. As such, preliminary studies have already demonstrated the potential of using universal allo-tolerant "off-the-shelf" CARTs generated by genomic editing-mediated deletion of receptors such as CD3, TCR or β2-microglobulin, which are essential for antigen recognition and immune function in a MHC-dependent context[72, 74]. We provide a preclinical evidence for the therapeutic and safe use of fratricide-resistant CD1a CARTs for R/R coT-ALL.

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Author contributions:

DS-M conceived the study, designed and performed experiments, analyzed data and wrote the paper. MB, FG-A, HR-H, M.T, JJ, TV-F, OB-L, JG-P, BU, JCa performed experiments and analyzed data. MC, JLF, MR-O, S.G-G, PF, JCo, MLT provided primary T-ALL samples, primografts and FACS data. CB performed experiments and financially support the study.

PM conceived the study, designed experiments, wrote the manuscript and financially supported the study.

Declaration of Interests: The authors have nothing to disclose.

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Figure 1

Figure 2

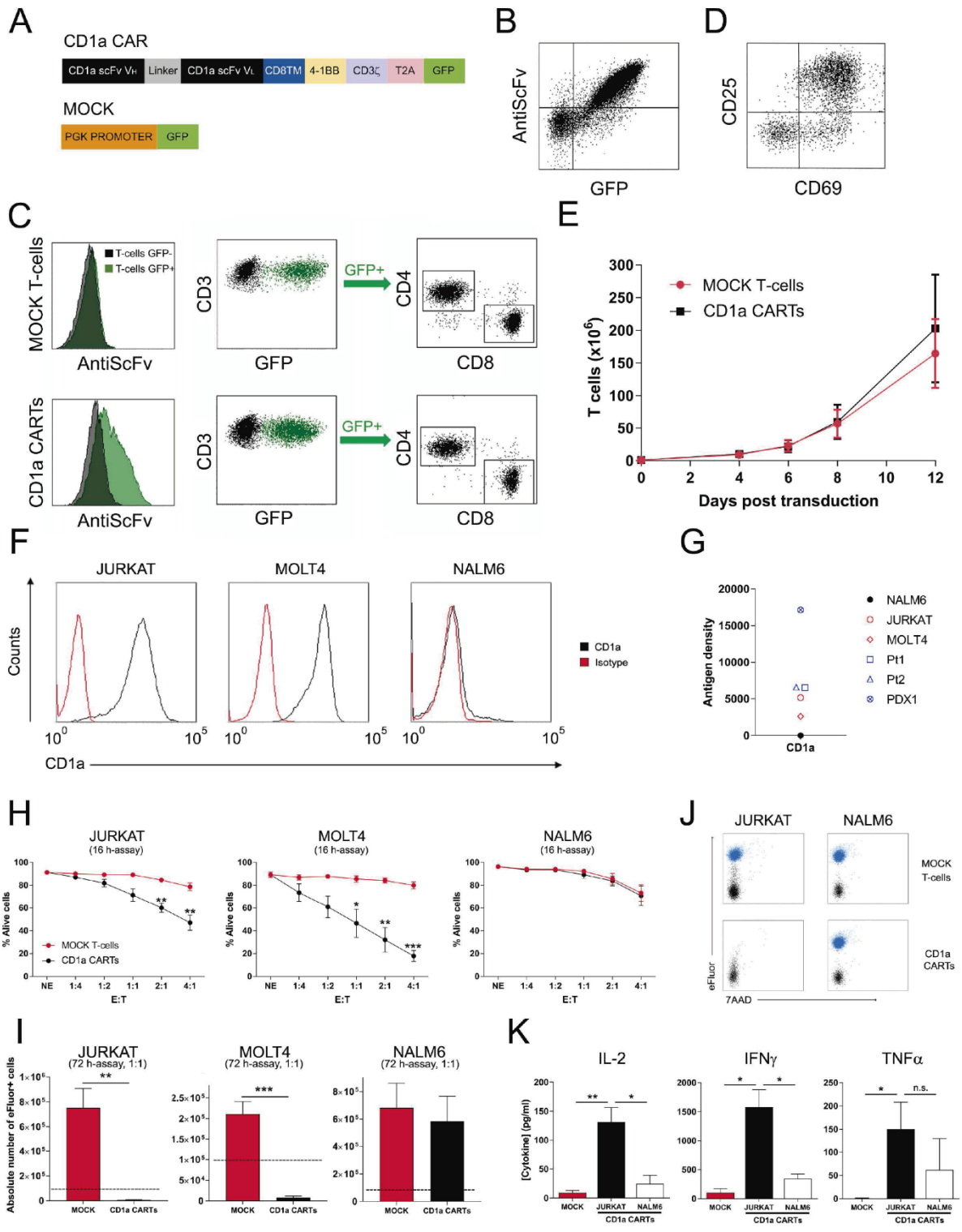

A
CD1a CAR
CD1a scFv VH
Linker
CD1a scFv VL
CD8TM
4-1BB
CD3ζ
T2A
GFP
MOCK
PGK PROMOTER
GFP
B
AntiScFv
GFP
D
CD25
CD69
C
MOCK T-cells
T-cells GFP-
T-cells GFP+
AntiScFv
CD3
GFP
GFP+
CD4
CD8
CD1a CARTs
E
MOCK T-cells
CD1a CARTs
T cells (x10^6)
Days post transduction
F
JURKAT
MOLT4
NALM6
Counts
CD1a
Isotype
G
Antigen density
NALM6
JURKAT
MOLT4
Pt1
Pt2
PDX1
H
JURKAT
(16 h-assay)
MOLT4
(16 h-assay)
NALM6
(16 h-assay)
% Alive cells
MOCK T-cells
CD1a CARTs
E:T
J
JURKAT
NALM6
MOCK T-cells
CD1a CARTs
eFluor
7AAD
I
JURKAT
(72 h-assay, 1:1)
MOLT4
(72 h-assay, 1:1)
NALM6
(72 h-assay, 1:1)
Absolute number of eFluor+ cells
MOCK
CD1a CARTs
K
IL-2
IFNγ
TNFα
[Cytokine] (pg/ml)
n.s.

Figure 3

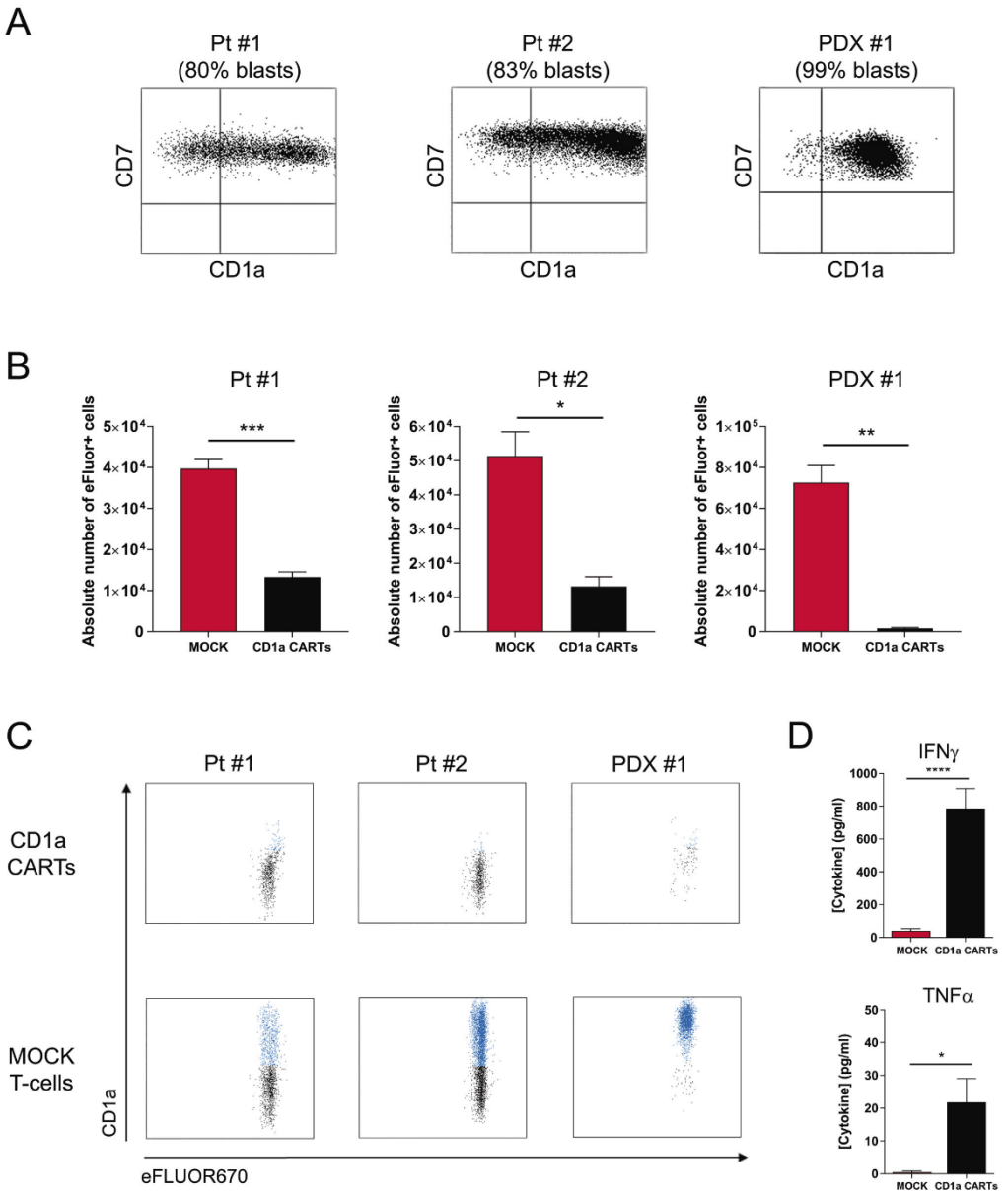

A
Pt #1
(80% blasts)
Pt #2
(83% blasts)
PDX #1
(99% blasts)
CD7
CD1a
B
Pt #1
Pt #2
PDX #1
Absolute number of eFluor+ cells
MOCK
CD1a CARTs
C
Pt #1
Pt #2
PDX #1
CD1a CARTs
MOCK T-cells
CD1a
eFLUOR670
D
IFNγ
TNFα
[Cytokine] (pg/ml)
MOCK
CD1a CARTs

Figure 4

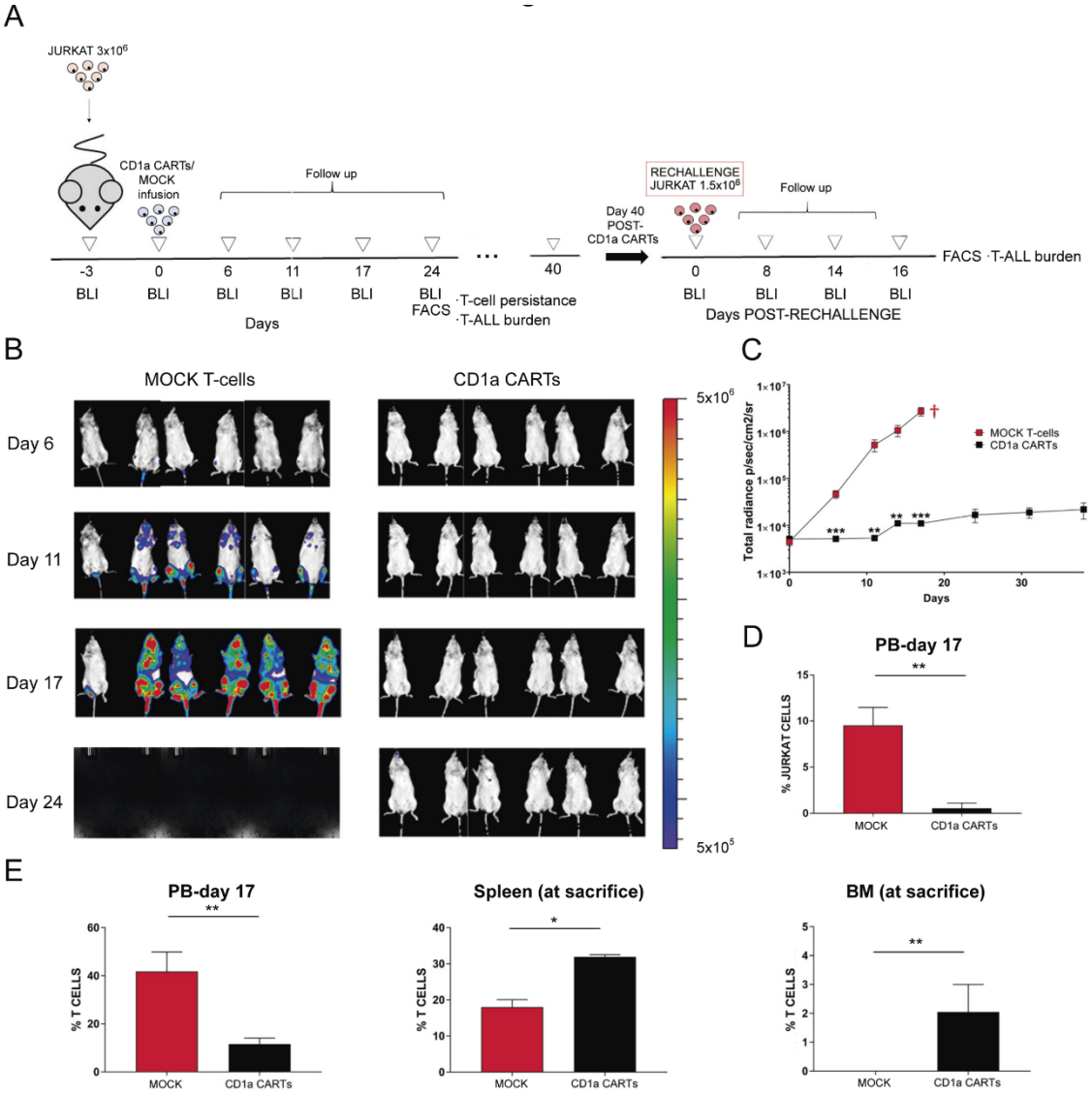

A
JURKAT 3x10^6
CD1a CARTs/ MOCK infusion
Follow up
RECHALLENGE JURKAT 1.5x10^6
Day 40 POST- CD1a CARTs
Follow up
FACS ·T-ALL burden
-3 BLI
0 BLI
6 BLI
11 BLI
17 BLI
24 BLI FACS
40
·T-cell persistance
·T-ALL burden
Days
0 BLI
8 BLI
14 BLI
16 BLI
Days POST-RECHALLENGE
B
MOCK T-cells
CD1a CARTs
Day 6
Day 11
Day 17
Day 24
5x10^6
5x10^5
C
Total radiance p/sec/cm2/sr
MOCK T-cells
CD1a CARTs
Days
D
PB-day 17
% JURKAT CELLS
MOCK
CD1a CARTs
E
PB-day 17
% T CELLS
MOCK
CD1a CARTs
Spleen (at sacrifice)
% T CELLS
MOCK
CD1a CARTs
BM (at sacrifice)
% T CELLS
MOCK
CD1a CARTs

Figure 5

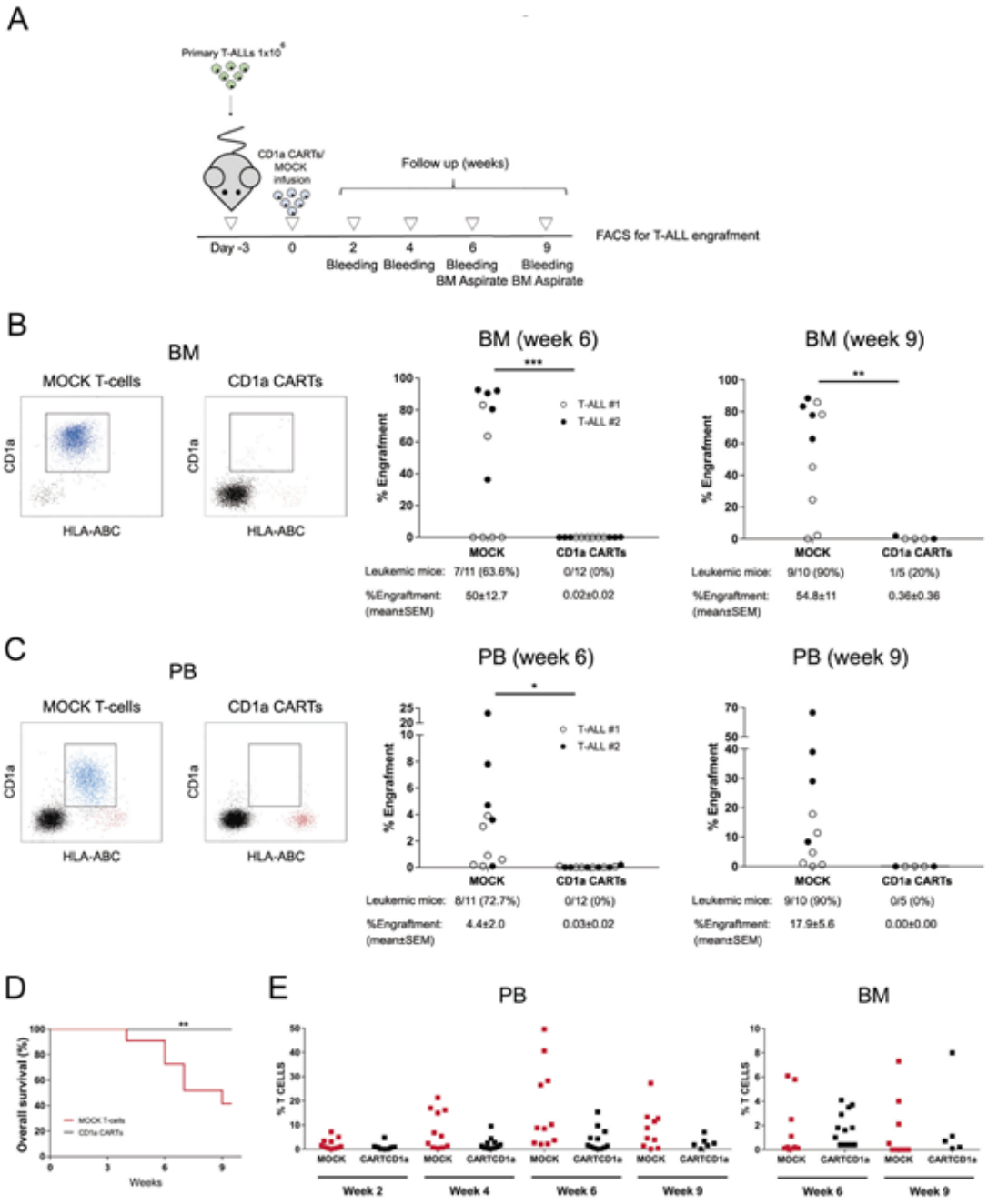

A
Primary T-ALLs 1x10^6
CD1a CARTs/ MOCK infusion
Follow up (weeks)
Day -3
0
2
4
6
9
Bleeding
Bleeding
Bleeding
BM Aspirate
Bleeding
BM Aspirate
FACS for T-ALL engrafment
B
BM
MOCK T-cells
CD1a CARTs
CD1a
HLA-ABC
BM (week 6)
BM (week 9)
% Engraftment
T-ALL #1
T-ALL #2
MOCK
CD1a CARTs
Leukemic mice: 7/11 (63.6%) 0/12 (0%)
%Engraftment: (mean±SEM) 50±12.7 0.02±0.02
Leukemic mice: 9/10 (90%) 1/5 (20%)
%Engraftment: (mean±SEM) 54.8±11 0.36±0.36
C
PB
PB (week 6)
PB (week 9)
Leukemic mice: 8/11 (72.7%) 0/12 (0%)
%Engraftment: (mean±SEM) 4.4±2.0 0.03±0.02
Leukemic mice: 9/10 (90%) 0/5 (0%)
%Engraftment: (mean±SEM) 17.9±5.6 0.00±0.00
D
Overall survival (%)
Weeks
MOCK T-cells
CD1a CARTs
E
PB
BM
% T CELLS
MOCK
CARTCD1a
Week 2
Week 4
Week 6
Week 9

Figure 6

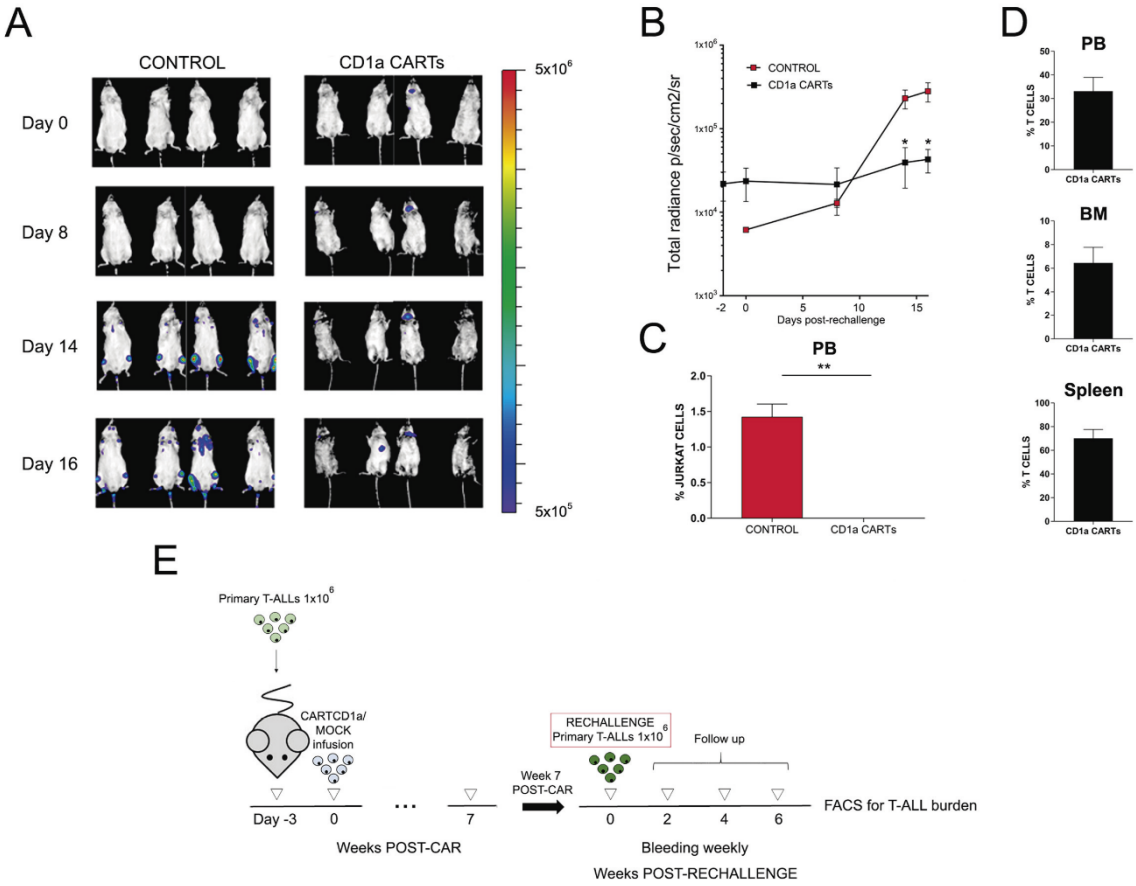
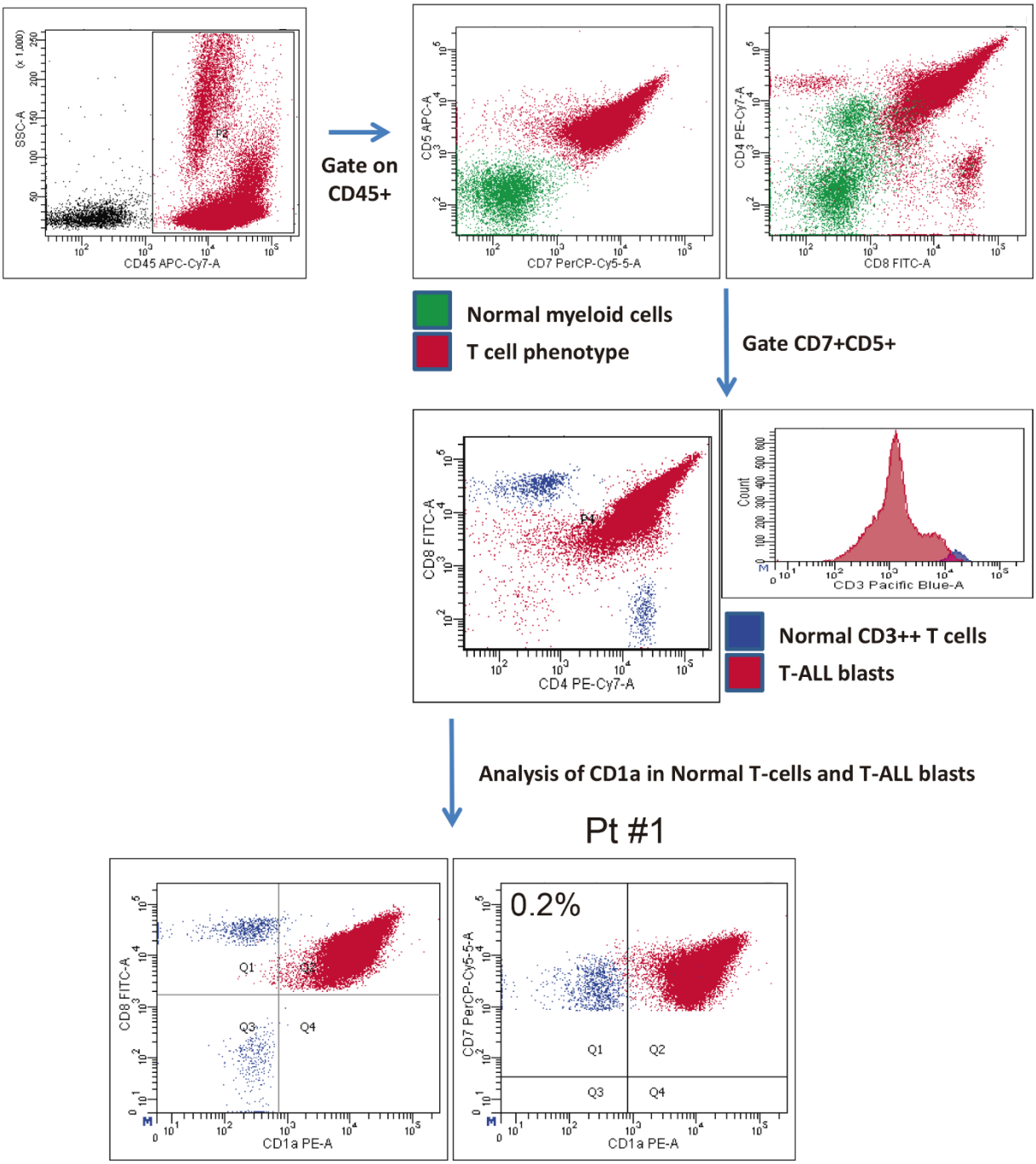

A
CONTROL
CD1a CARTs
Day 0
Day 8
Day 14
Day 16
5x10^6
5x10^5
B
CONTROL
CD1a CARTs
Total radiance p/sec/cm2/sr
Days post-rechallenge
C
PB
% JURKAT CELLS
CONTROL
CD1a CARTs
D
PB
BM
Spleen
% T CELLS
CD1a CARTs
E
Primary T-ALLs 1x10^6
CARTCD1a/
MOCK
infusion
RECHALLENGE
Primary T-ALLs 1x10^6
Follow up
Week 7
POST-CAR
Day -3
0
7
0
2
4
6
FACS for T-ALL burden
Weeks POST-CAR
Bleeding weekly
Weeks POST-RECHALLENGE
F
PB
(week 6 post-rechallenge)
BM
(week 6 post-rechallenge)
% Engrafment
CONTROL
CD1a CARTs
G
PB
% T CELLS
WEEK2
WEEK4
WEEK6

Figure Supplementary 1

A



B

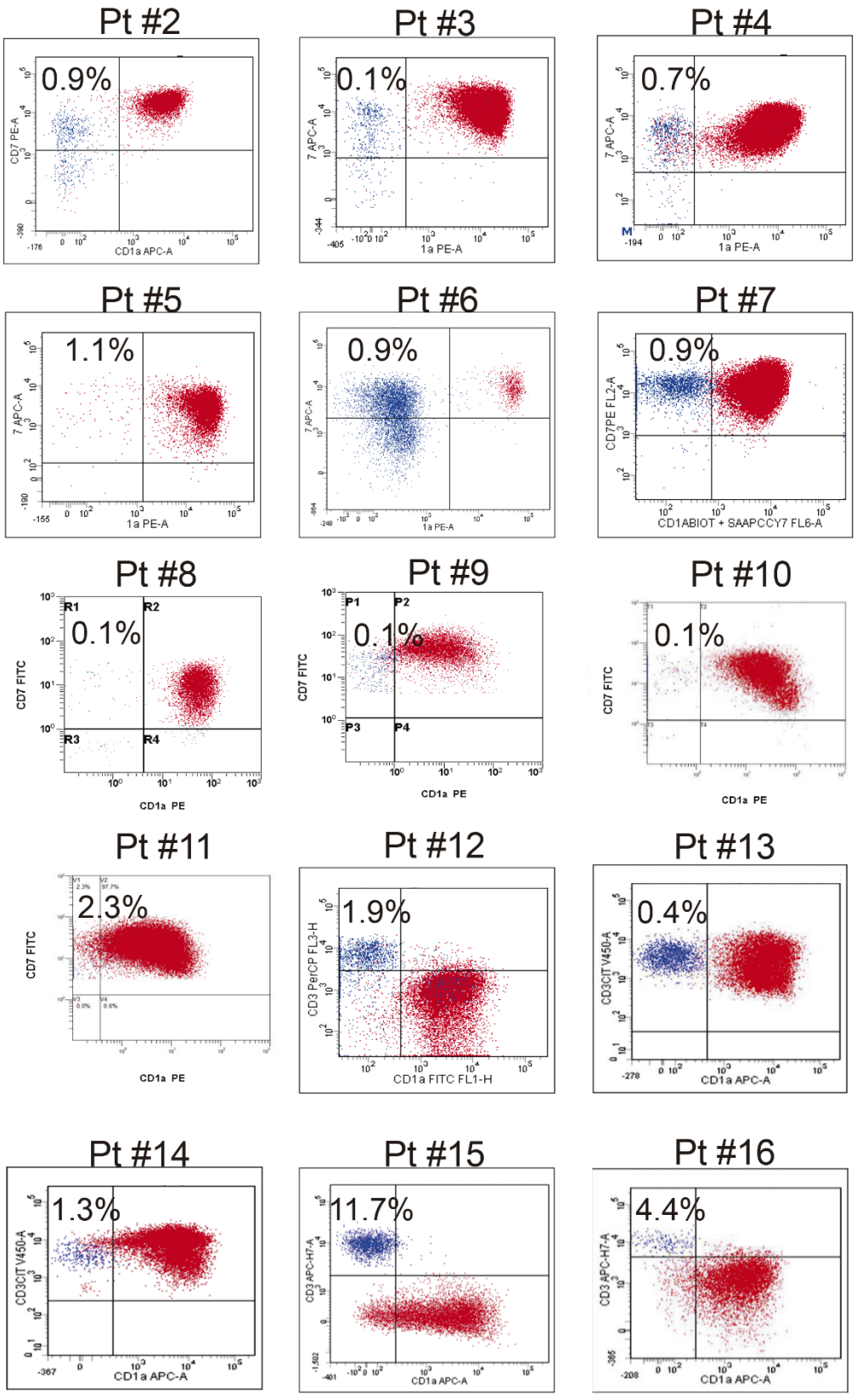

Pt #2
0.9%
CD7 PE-A
CD1a APC-A
Pt #3
0.1%
7 APC-A
1a PE-A
Pt #4
0.7%
7 APC-A
1a PE-A
Pt #5
1.1%
7 APC-A
1a PE-A
Pt #6
0.9%
7 APC-A
1a PE-A
Pt #7
0.9%
CD7PE FL2-A
CD1ABIOT + SAAPCCY7 FL6-A
Pt #8
0.1%
R1
R2
R3
R4
CD7 FITC
CD1a PE
Pt #9
0.1%
P1
P2
P3
P4
CD7 FITC
CD1a PE
Pt #10
0.1%
CD7 FITC
CD1a PE
Pt #11
2.3%
CD7 FITC
CD1a PE
Pt #12
1.9%
CD3 PerCP FL3-H
CD1a FITC FL1-H
Pt #13
0.4%
CD3CIT V450-A
CD1a APC-A
Pt #14
1.3%
CD3CIT V450-A
CD1a APC-A
Pt #15
11.7%
CD3 APC-H7-A
CD1a APC-A
Pt #16
4.4%
CD3 APC-H7-A
CD1a APC-A

Figure S1. Immunophenotype for each individual CD1a++ coT-ALL patient presented in this study. (A) Gating strategy distinguishing mature normal T-cells (CD3++CD1a- either CD4+ or CD8+, blue cells) and coT-ALLs blasts (CD7+CD1a+, red cells). Note that coT-ALL blasts commonly have aberrant expression for CD3 and/or CD4/CD8). **(B)** CD7/CD3 *vs* CD1a FACS dot plots for n=16 available CD1a++ coT-ALL patients showing the percentage of mature normal T-cells (blue) and coT-ALLs blasts (red).

DISCUSSION

This thesis has been developed with the goal of exploring innovative CAR T-cell based treatments for R/R AL, more specifically AML and coT-ALL. CAR T-cells are emerging as a promising therapeutic tool for a wide variety of hematological malignancies, however pitfalls and drawbacks are quickly emerging. The lack of leukemia specific antigens and on target off tumor toxicity (among others) are the main obstacles impeding the successful clinical implementation of CARTs in AML and T-ALL, as it would constitute a potential source of myeloablation in the former, and immunosuppression in latter.

In the first study, we aimed to resolve a controversy currently pervading the field of CARTs in AML: Does the selective targeting of CD123 induce myeloablation? To address this issue we used various *in vitro* and *in vivo* approaches. We first prospectively analyze the expression of CD123 in a wide cohort of primary AML samples and compare it with healthy HSCs. Second, we established two distinct *in vivo* humanized xenograft models aiming to characterize the effects of CAR123 over i) existing human hematopoiesis or ii) the establishment of de novo human hematopoiesis. Third, we compared two distinct co-stimulation motifs (4-1BB and CD28) to set out whether the observed effects were specific for the construct used rather than the antigen to be targeted. In the second study we explored the feasibility of redirecting engineered T-cells towards CD1a antigen, a surface molecule which marks approximately 40% of co-T-ALL cases. The first key point to address was weather CD1a represent a *bona fide* target for coT-ALL. As CD1a was never tested before as a potential target for CAR T-cells, it was deemed as mandatory to revise CD1a expression across T-cell development and address its expression in CD34+ HSCs and mature T-cells across ontogeny in order to infer its potential as immunotherapeutic target for R/R coT-ALL. Second, we set out several *in vivo* models, using both $CD1a^{+}$ cell lines and primary samples, to characterize anti CD1a

CAR T-cells effector functions. Very importantly, rechallange in vivo experiments were implemented to ensure CD1a CAR T-cells efficiency and persistance.

STUDY I

4-1BB-based and CD28-based CD123-redirected T-cells ablate human normal hematopoiesis *in vivo.*

To date intensive chemotherapy combos based on nucleoside analogs plus anthracyclines remain the standard front-line treatment of AML, followed by allogeneic HSCT, based on patient's eligibility, to consolidate CR and prevent relapse (Thol *et al.* 2015). However, resistance to standard treatment, relapse and chemotherapy-related toxicities are still important sources of mortality for AML patients, and this problem encompasses both juvenile and adult populations, showing the highest death toll in patients over 65 years of age (Tasian *et al.* 2017).

Implementing new targeted treatments, which might sustain leukemia remission and reduce toxicity, is thus a major endeavor to be taken. TAAs-redirected CAR T-cell immunotherapies have induced remarkable responses in patients with R/R B-ALL, and similar strategies have been under clinical investigation in adults with R/R AML, nonetheless, potential on target off tumor toxicity of AML CAR T-cell immunotherapies is a major hurdle to overcome. First among all, the abolishment of normal myelopoiesis is hindering broader implementation of such strategies. Thoughtfully choosing an optimal targetable surface molecule, understanding its biology within healthy and tumoral tissues, represents a key step forward in the establishment of CAR T-cell for AML as a robust anti-tumoral approach. CD123 has emerged as one of the most promising targets for CARTs therapy (Tasian *et al.* 2018, Mardiros *et al.* 2014, Gill *et al.* 2014) as it displays multiple features that render it a good surface molecule for targeted therapies. In the first place, major phenotypic (immature, granulocytic and monocytic) and cytogenetic (FLT3- and NPM1-mutated) AML subgroups express CD123 (Testa *et al.* 2014, 2019). Second, $CD123^{+}$ AML cells are capable to initiate leukemogenesis when transplanted in immune-suppressed mice, therefore marking AML-LSC compartment, and this may obviate the

need to target selectively the AML stem cell (Al-Mawali *et al.* 2015, Angelini *et al.* 2015). Third, the presence of $CD34^{+}CD38^{-}CD123^{+}$ cells in AML at presentation is associated to lower disease-free survival (DFS) and OS and failure to achieve complete remission (Aria *et al.* 2019). Fourth, CD123 expression enhance AML cell proliferation and induces down-regulation of CXCR4 favoring the egress of BM AML-LSCs into the circulation (Wittwer *et al.* 2017). Importantly however, recent studies support the evaluation of CD123 expression as a detection marker for MRD. MRD can be defined as the persistence after therapy of a small mass of tumor cells, not detectable by conventional histopathological analysis (bone marrow morphology), and the presence of MRD after therapy is a negative prognostic marker of increased incidence of relapse and mortality in AML patients (Ravandi *et al.* 2018). MRD can be assessed through the detection of leukemia-specific molecular transcript or flow cytometric analysis of BM cells using a panel of antigens aberrantly expressed in AML blasts, including CD123 (Ravandi *et al.* 2018).

As AML blasts evolve by either acquiring drug resistance, by losing mutations associated with sensitivity to the treatment or by outgrowth of a sub-clone following eradication of the major clone during the first line of treatment, the leukemic population at relapse may arise from either clonal or sub-clonal cell populations (Ivey *et al.* 2016, Varandi *et al.* 2018, Testa *et al.* 2019). This implies that, either through linear or branching evolution, leukemic cells undergo a process of adaptation to survive to new environment (Eppert *et al.* 2011). This means that the processes of dynamic evolution relies on the consistent clonal heterogeneity of AMLs and on the extreme difficulty to tackle the leukemic process through the targeting of single genetic abnormalities. Contrary to the molecular markers involved in the dynamics of clonal evolution, CD123 is equally expressed in AML bulk and LICs at initial diagnosis and relapse and therefore its expression is not related to

leukemic clonal evolution (Bras et al. 2019, Haubner *et al.* 2019) The independency of CD123 expression from AML clonal evolution strongly supports CD123 as a potential therapeutic target of AMLs at various disease stages: diagnosis, MRD and relapse (Testa *et al.* 2019).Taken together these observation highlight the fact that CD123 is a particularly attractive target due to the importance of IL-3 signaling in both normal and malignant hematopoiesis and it has become evident that CD123 is a *bona fide* target for CAR T-cell immunotherapies.

However, in respect to the safety of selectively redirecting T-cells towards CD123, the field has been pervaded by somewhat contradicting results. Seminal pre-clinical studies using MoAb and DARTs (Al-Hussain *et al.* 2016, Bonifant *et al.* 2016, Lee *et al.* 2015, He *et al.* 2015) did not report substantial long term impairments on normal hematopoiesis following CD123 redirected therapies, a phenomenon likely attributable to the short persistence of such compounds into the human body or the inability to fully abolish $CD123^{dim}$ cells (Cohen *et al.* 2005). Conversely, when targeting CD123 via autologous CAR T-cells, substantial discrepancies over safety emerged: as a matter of fact, there are numerous studies, at both pre- and clinical level, reporting opposite findings (Bole-Richard *et al*. 2020, Gill *et al.* 2014, Luo *et al.* 2015, Mardiros *et al.* 2013 Pizzitola *et al.* 2014, Tasian *et al.* 2017). On one instance CAR T-cells redirected towards CD123 demonstrate robust anitleukemic properties without inflicting significant damages to normal hematopoiesis, therefore aligning with previous seminal works with MoAb and DARTs, while on the other side, strong myelotoxic effects are reported. A wide range of variables could be taken into account to provide a tentative explanation for such marked discrepancies. Experimental design, source of effector and target cells, scFv binding properties and CAR co-stimulatory modules are the major (but not the unique)

determinants that can retrospectively guide scientists to unravel the controversy surrounding the safety of CARTs against CD123.

Based on this background, we prompted to characterize and compare *in vitro* and *in vivo* the efficacy and safety profile of two second generation 4-1BB-based and CD28-based anti CD123 CARTs derived from the clinically tested CSL362 humanized MoAb (Lee *et al.* 2015, He *et al.* 2015). These novel CARs, regardless the co-stimulation motif, proved to be very efficient *in vitro* and *in vivo* in eliminating both AML cell lines and primary cells, even at a relatively low E:T ratios. Importantly however, CD123 CAR T-cells ablated existing normal human hematopoiesis and prevented the establishment of *de novo* hematopoietic reconstitution, by directly targeting both myeloid progenitors and early/immature HSPCs. This lead to subsequent functional consequences in all downstream normal hematopoietic progenitors, rendering severe impairment of multi-lineage hematopoiesis in BM and PB. The robust myeloablative effects reported in this study calls for caution when clinically implementing CD123 CAR T-cells and it raises the question whether the use of anti CD123 CAR T-cells should be implemented exclusively as a bridge to allogeneic HSTC. Additionally it pushes to explore potentially safer approaches to circumvent myeloablation such as using alternative sources of effector cells, like NK cells, cytokine-induced killer (CIK) cells or Vδ1 γδ T-cells (DOTs) (Correia *et al.* 2011, Rotolo *et al.* 2019). These cells in fact would entail strong and acute antileukemic responses coupled with a shorter persistence that could allow a better control *in vivo* of the CD123 CAR bearing cells.

STUDY II

Fratricide-resistant CD1a-specific CAR T cells for the treatment of cortical T-cell acute lymphoblastic leukemia

T-ALL is an aggressive hematological cancer resulting from leukemic transformation of thymic T-cell precursors. Even if intensive chemotherapy regimens have led to the improved survival of patients, it shows unfavorable clinical outcome, both in juvenile and adult populations. The 5 year EFS and OS remains <70%, and R/R T-ALL has a particularly poor prognosis (Marks *et al.* 2009, Litzow *et al.* 2015). There are currently no potential curative options beyond hematopoietic cell transplantation and conventional chemotherapy, which comes at cost of large trade-offs in toxicity (Ferrando *et al.* 2002, Belver *et al.* 2016, Litzow *et al.* 2015) This lack of therapeutic options for R/R T-ALL poses extensive demands in order to find alternative therapies which could potentially offer a safer profile than standard treatments. In recent years the T-ALL landscape has witnessed only marginal improvements when it comes to implementing targeted immunotherapeutic regimens, as the majority of exploitable antigens are shared between malignant and healthy T-cells. As a matter of fact, the only targeted therapies previously used for eradication of malignant T cells, with suboptimal clinical outcome, are the ricin A chain toxin–conjugated MoAbs anti-CD5 and anti-CD7 (Lemaistre *et al.* 1991, Frankel *et al.* 1997). Such compounds, in fact, granted only short-lasting partial responses in a fraction of the treated patients.

At the same time of the emergence of CAR T-cells for B-ALL, it became quickly evident that broadening the scope of CARTs to treat T-ALL and T-cell lymphomas would have been a challenging endeavor not only because of the shared expression of target antigens between healthy T-cells and T-lineage tumoral cells. Both scenarios would in fact lead to either T-cell aplasia or to a premature depletion of CAR T-cells, thus failing to achieve any significant long lasting response.

Current strategies to overcome CAR T-cell fratricide include the targeted disruption of the surface antigen gene using CRISPR/Cas9 prior to CAR expression (Gomes- Silva *et al.* 2017), or by antigen down-regulation using a protein expression blocker (PEBL) motif, which entails the coupling of an intracellular retention domain to the anti-scFv (Kamia *et al.* 2018, Scarfó *et al.* 2019).

We hypothesized that the choice of the antigen against which we wish to re-direct T cells would represent a major advance to solving the problems associated with the shared expression of T-cell markers between normal and malignant T cells. CD1a is a lipid-presenting molecule whose expression is basically restricted to coT-ALL, retained at relapse, and is practically absent in human tissues with the exception of cortical thymocytes, skin LC, and some circulating myeloid dendritic cells during development (Cernadas *et al.* 2009, Bechan *et al.* 2012, Carrera Silva *et al.* 2017). Given this scenario, we opted for the CD1a antigen as a feasible and safe target for CAR immunotherapy in R/R coT-ALL, the most common subtype of T-ALL. Applicability of CD1a targeting however could also be extended to other $CD1a^+$ tumors such $CD1a^+$ Peripheral Lymphoblastic Lymphoma (PLL) and Langherans Cell Hystiocytosis (LCH) (Cortellazzo *et al.* 2011, Powell *et al.* 2017)

With this background, we developed and functionally characterized CD1a-specific CARTs, which displayed robust cytotoxicity against T-ALL cell lines and primary $CD1a^+$ coT-ALL cells, both in vitro and in vivo in xenograft models. We demonstrated that activated CD1a CARTs continuously expanded 200-fold over a 12-day period, similar to MOCK T cells, proving that that redirecting CARTs against CD1a antigen did not induce T-cell fratricide. When exposed to $CD1a^+$ cells, being them cell lines or primary samples, CAR T-cells exerted strong and specific cytotoxic effects in a ratio and time dependent manner, further corroborating the specificity of this CAR construct, and

these effects were promptly followed by specific secretion of high-levels of IL-2, IFN-γ and TNFα. For assessing the efficacy of our anti CD1a CARTs *in vivo*, evaluated their activity and persistence using both Luc-expressing Jurkat T-ALL cells and primary patients coT-ALL xenograft cells. Irrespective of the model used, CD1a CARTs as compared to control T-cells, demonstrated to have potent antileukemic activity that was retained over long period of time (up to 13 weeks) as demonstrated by multiple experiments in which CAR treated mice, re-challenged with a second dose of target cells, showed the remarkable capacity to contain and reduce the tumor burden.. Also, the use of CD1a CARTs for coT-ALL bypasses the need for sophisticated genome editing–based disruption of target antigens in T cells before CAR transduction as a strategy to avoid self-antigen–driven fratricide (Gomes-Silva *et al.* 2017, Rasayia *et al.* 2018,).

We further showed that in steady-state hematopoiesis, CD1a is exclusively expressed in a subset of $CD34^{+}CD7^{+}$ cortical thymic T-progenitors, whereas earlier $CD34^{high}CD7^{high}$ T-progenitors lack CD1a. In addition, neither normal $CD34^{+}$ HSPCs nor mature T cells from multiple tissues express CD1a during ontogeny, thereby minimizing the risk of on-target/off-tumor toxicity. Indeed, when human fetal thymus-derived $CD7^{+}$ thymocytes were exposed to CD1a CART-cells, only the $CD1a^{+}$ cortical thymocytes were eliminated by the CD1a CARTs. Developmentally earlier and later thymic T-lineage populations ($CD34^{+}$ and $CD34^{-}$) were not targeted, limiting the on-target/off-tumor effects to a developmentally transient thymic population of cortical thymocytes and further confirming the fratricide-resistant nature of CD1a CAR T-cells. Regarding safety, we do not expect irreversible toxicities or immunodeficiency attributed to CD1a CARTs for the following reasons: i) $CD1a^{+}$ thymocytes represent a transient and thymus-restricted population, eventually regenerated by “nontargetable” upstream $CD34^{+}CD7^{+}CD1a^{-}$ T-cell progenitors physiologically/constantly maturing into

functional T cells; ii) CD1a CARTs themselves respond normally to viral antigens and therefore are likely to be protective against pathogens iii) postnatal thymectomy does not lead to immunodeficiency in humans likely because thymic emigrants generated early in life persist for decades (Haynes *et al.* 1999) suggesting that potential transient elimination of thymic progenitors by CD1a CARTs in pediatric patients would not compromise the complete antiviral T-cell repertoire in adult life. Nonetheless, whether infants could eventually develop a premature immunosenescence later in life merits caution, as this finding was reported for infants thymectomized before 1 year of age (van Der Broek et al. 2016). Fortunately, however, T-ALL is extremely infrequent in infants. Safer ultimate strategies would include the implementation of an inducible molecular switch to control potential toxicities linked to CAR T-cells and/or the use of CD1a CAR T-cells as a therapy bridge before curative allogeneic HSPC transplantation. lternatively, donor T cells would represent an ideal effector source in patients with coT-ALL who previously underwent allogeneic HSPC transplantation. Alternatively, universal "off-the-shelf" allogeneic-suitable T cells would represent the short-term "panacea" for leukemia immunotherapies because T-cell lymphopenia or T-cell dysfunction are often associated in many multitreated R/R patients. As such, preliminary studies have already shown the potential of using universal allo-tolerant off-the-shelf CARTs generated by genomic editing–mediated deletion of receptors such as CD3, T-cell receptor, or β_2-microglobulin, which are essential for antigen recognition and immune function (Cooper *et al*. 2018, Rasaiyhaa *et al*. 2019)

Taken together these results demonstrated the feasibility of CD1a redirected targeting and strengthens the fratricide-resistant nature of the resulting anti CD1a CAR T-cells.

CONCLUDING REMARKS.

Resistance to standard therapy and relapses are observed in several cases of both myeloid and lymphoid cell malignancies. Such phenomenon poses unquestionable limits to the utility of current therapeutic approaches, and remains a critical endeavor for the clinical management of patients with advanced cancers. To counteract resistance and to prevent relapse, the deployment of a wide portfolio of agents was put into action, providing at times only marginal benefits with large trade off in toxicity and tolerability. With the advent of immunotherapy huge improvements in patients' management and overall survival was achieved, particularly in the field of CAR T-cells. Engineering T-cells to redirect them against a tumor antigen have granted impressive responses in the treatment of B-cell malignancies. On the other hand, the clinical implementation of CARTs for other blood cancers is lagging behind. Major reasons lay within the biology, expression and localization of the antigen to be targeted, as it can play vital roles in cell regulation and development in healthy tissues. The present work have provided extensive contribution to the field of CAR T-cells in two different manners: i) by elucidating with robust *in vitro* and *in vivo* comparative experiments whether CD123 selective targeting qualifies as myeloablative or not ii) by validating CD1a as a feasible therapeutic antigen for T-ALL and by creating a novel anti CD1a CAR, which showed robust antileukemic activity while preserving safety towards healthy T-cells and T-cells precursors. The development of innovative therapeutic tools that permit to tackle refractory-relapsed cancer with otherwise no further therapeutic options, opens the doors for important advances that will enable society to win the battle against blood cancers.

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