PhD Program in Translational and Molecular Medicine

DIMET

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University of Milano-Bicocca
School of Medicine and Surgery

Characterization of Chimeric Antigen Receptors (CARs) as a potential tool for the treatment of Acute Myeloid Leukemia (AML).

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Chapter 1

General introduction

1. Hematopoiesis and acute myeloid leukemia (AML)

Hematopoiesis is the process by which the different types of blood cells are formed. The site where hematopoiesis occurs changes during embryonic development, having postnatally bone Marrow (BM) as the major site. Mature blood cells have a finite lifespan and are continuously reconstituted by hematopoietic stem cells (HSCs). HSCs are rare, mostly quiescent, multipotent and long-term self-renewing cells located at the top of the hematopoietic hierarchy. Through asymmetric cell divisions, HSCs give rise to both identical stem cells and intermediate progenitors that, progressively, lose their multi-lineage and self-renewing potency and differentiate toward specific mature blood cells (Riether, Schürch, & Ochsenbein, 2015).

Alterations in this dynamic and finely tuned developmental program result in hematological diseases including leukemia. Depending on the lineage affected, leukemias can be distinguished between myeloid and lymphoid, which in turn can be acute or chronic according to their progression.

Acute myeloid leukemia (AML) occurs in the myeloid lineage and consists in the accumulation of abnormal cells, known as blasts, which fail to correctly differentiate and interfere with the production of normal blood cells. According to the “Cancer Stem Cell hypothesis”, AML can be viewed as an aberrant hematopoietic process (Dominique Bonnet, 2005) initiated and sustained by rare leukemic stem cells (LSCs) which share many characteristics with normal HSCs, including quiescence, pluripotency and self-renewal. AML was indeed one of the

first disease for which the existence of a small population of cancer stem cells has been proven through xenotransplantation functional assays (D Bonnet & Dick, 1997) (Ng et al., 2016). The phenotypic and molecular heterogeneity of LSCs (Sarry et al., 2011) (David C Taussig et al., 2008) (Goardon et al., 2011) has suggested the hypothesis that LSCs could arise not only from mutations at the HSCs level but even from mutations occurring in more committed progenitors, allowing to reacquire, ectopically, the unique self-renewal properties of stem cells and interfering with their subsequent ability to correctly differentiate (U. Testa, 2011), figure 1.

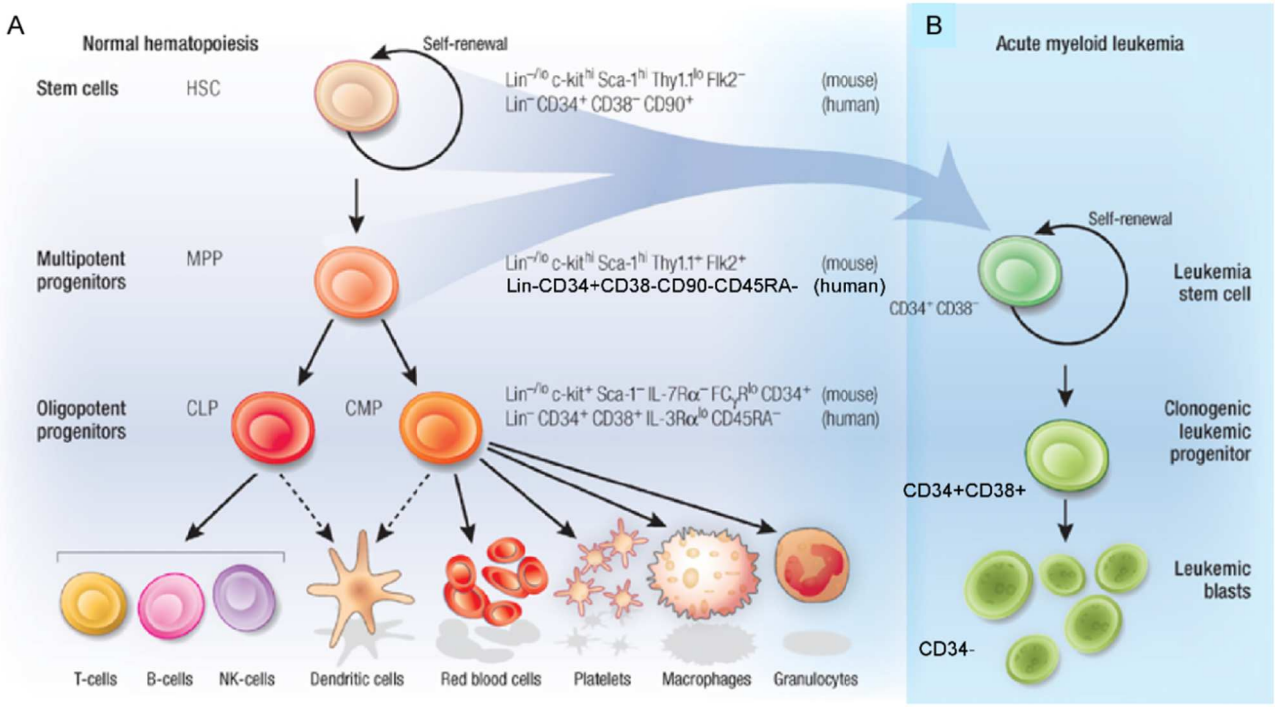

Figure 1. Schematic model of normal hematopoiesis and acute myeloid leukemia
Adapted from Majeti Lab web page.

1.2 Epidemiology and incidence of AML

AML is the most common form of leukemia in adults, most frequently diagnosed among people aged 65-74, with a higher incidence in men than women (NIH National Cancer Institute Surveillance, Epidemiology and End Results Program: Acute Myeloid Leukemia, available from http://seer.cancer.gov/statfacts/html/amyl.html). In contrast, AML is less common in pediatric age, accounting for approximately 20% of childhood leukemias (Whelan & Alva, 2017).

1.3 Pathogenesis and risk factors

Excluding acute promyelocytic leukemia (APL), generally AML is the result of multiple genetic lesions. Studies in mice (Kitamura et al., 2014) led to the development of a "two-hit" model of leukemogenesis according to which at least two kinds of mutations are required: class I mutations, involving genes in the kinase signaling pathways and promoting cell survival and proliferation or inhibiting apoptosis (*RAS*, *FLT3*, *JAK2* activating mutations, *P53* inactivating mutations), and class II mutations affecting transcription factors or cofactors which alter the hematopoietic differentiation (t(15;17)/*PML-RARα*, inv(16)/*CBFß-MYH11* and t(8;21)/*RUNX1-RUNX1T1* fusions, *MLL*/*PTD*, *CEBPα* and *AML1*/*RUNX1* mutations) (Kitamura et al., 2014). The hypothesis is that leukemia develops after class I mutations conferring a proliferative advantage to hematopoietic cells already blocked in differentiation by class II mutations. In addition to these, further mutations involving genes related to epigenetic regulation have been

detected (*IDH1*, *IDH2*, *TET2*, *DNMT3A*, *ASXL1*, *EZH2*) (Chen, Odenike, & Rowley, 2010).

Even if it is not known what triggers AML genetic lesions, a number of different risk factors have been associated to AML development, including genetic and environmental factors, lifestyle, drugs, and antecedent blood disorder. Some of these are summarized in Table 1.

Generally accepted risk factors	**Suggestive of increased risk**	**Suggestive of decreased risk**	**Limited evidence**
Down syndrome	Older maternal age	Long term breastfeeding	Paternal exposure to benzene
Fanconi anemia	Increasing birth order		Paternal smoking
Familial monosomy 7	Prior fetal loss		Maternal exposure to benzene
Ataxia telangectasia	Maternal alcohol assumption		Maternal use of antibiotics
Shwachman-Diamond syndrome	Maternal exposure to pesticides		Maternal dietary consumption of DNA topoisomerase II inhibitors
Bloom syndrome	High birth weight		
Ionizing radiation *in utero*	Low birth weight		

Table 1: Summary of Risk factors for Childhood AML. Adapted from Puumala *et al.*, Pediatr Blood Cancer 2013 (S.E., J.A., & R., 2013)

1.4 Signs and symptoms

Clinical manifestations of AML are the result of blast proliferation and their consequent accumulation in the BM, peripheral blood (PB) and eventually in other organs. Blasts accumulation in the BM interfere with normal hematopoiesis leading to pancytopenia which is responsible for anemia, leukopenia, thrombocytopenia. Leukemia spreading in various tissues is responsible for organomegaly, bone pain and a variety of other symptoms such as headaches, vomiting and loss of balance when the infiltrate reaches the nervous system.

1.5 Diagnosis

The starting point for AML diagnosis is the assessment of at least 20% of blasts in the BM or in the PB by means of morphologic examination. Additionally, cytochemical, immunophenotypic, molecular and cytogenetic analyses are performed to confirm the diagnosis and identify the AML subtype. Cytochemistry allows to distinguish between myeloid (myeloperoxidase (MPO) positive) and monoblastic differentiation (MPO negative and nonspecific esterase (NSE) positive). Nowadays, more than cytochemistry, immunophenotyping, molecular and cytogenetic analyses are crucial for AML characterization, especially for more rare subtypes of AML (acute leukemia with minimal differentiation, acute megakaryoblastic leukemia, acute leukemias of ambiguous lineage) (Hartmut Dohner et al., 2010).

Approximately 55% of adult and 70-80% of childhood AML (Mrózek, Heerema, & Bloomfield, 2004) (Creutzig et al., 2012) have numerical and structural chromosomal abnormalities (e.g., translocations, inversions or deletions) and the presence of any of these cytogenetic abnormalities: t(15;17), t(8;21), inv(16), or t(16;16), lead to AML diagnosis without any regard of the blast threshold ((Arber et al., 2016).

In addition to diagnosis, cytogenetic aberrations have traditionally been considered the most important prognostic tool for defining prognosis and risk stratification of AML patients.

Novel insights in risk-stratification and prognosis, as well as in the development of targeted therapies, have been provided by molecular analysis which led to the discovery of nucleotide-level mutations in a great number of genes having particular impact for sub-classifying cytogenetically normal AML (CN-AML).

Regarding immunophenotyping, cell-surface and cytoplasmic markers are combined to CD45 for recognizing the AML blast population within the context of normal residual hematopoiesis (Béné et al., 2011). Indeed, leukemic blasts exhibit a decreased CD45 expression compared to normal hematopoietic cells. Below are listed the immunophenotype panels following the European LeukemiaNet (ELN) Work Package recommendations (Béné et al., 2011).

- Precursor markers: CD34, CD117, CD33, CD13, HLA-DR;
- Granulocytic markers: CD65, cytoplasmic MPO;
- Monocytic markers: CD14, CD36, CD64;
- Megakaryocytic markers: CD41, CD61;

- Erythroid markers: CD235a, CD36.
- Mixed phenotype acute leukemia (MPAL, including leukemias expressing antigens of more than one lineage): B-lineage (CD19, cytoplasmic CD79a, cCD22, CD10), T-lineage (surface or cytoplasmic CD3), myeloid lineage (MPO, CD11c, CD14, CD64).

1.6 Classification

AML diagnosis is followed by its classification. The French–American–British (FAB) system (Bennett et al., 1985) represents the first attempt of classification and defines eight AML subtypes (M0 through M7) based on the morphological and cyto-chemical characteristics of the leukemic cells and their relative stage of differentiation.

Current AML classification, which incorporates genetic information with morphology, immunophenotype and clinical presentation, was firstly proposed by the World Health Organization (WHO) in 2001, then revised in 2008 and later in 2016. According to a multidisciplinary approach this classification recognizes different disease entities having an impact on prognosis and on the selection of specific treatment (table 2: FAB classification and table 3: WHO classification).

FAB Subtype	Frequency	Description
M0	2-3%	Non-differentiated - immature blasts with minimal myeloid differentiation
M1	20%	Myeloblastic without maturation – immature blast cells without signs of myeloid differentiation
M2	30-40%	Myeloblastic with granulocytic maturation
M3	5-10%	Promyelocytic (APL)
M4	15%	Myelomonocytic (M4eo = with bone marrow eosinophilia)
M5	10%	Monocytic (M5a = monocytic leukemia without maturation; M5b = monocytic leukemia with partial maturation)
M6	5%	Erythroleukemia
M7	1%	Megakaryoblastic

Table 2: FAB classification. Adapted from Bennet et *al.*, Annals of internal medicine 1985 (Bennett et al., 1985)

Acute myeloid leukemia and related neoplasms
AML with recurrent genetic abnormalities
AML with t(8:21)(q22;q22); RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
APL with PML-RARA
AML with t(9;11)(p21.3;q23.3); MLLT3-KMT2A
ML with t(6;9)(p23;q34.1); DEK-NUP214
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); RBM15-MKL1
AML with BCR-ABL1 (provisional entity)
AML with mutated NPM1
AML with biallelic mutations of CEBPA
AML with mutated RUNX1 (provisional entity)
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML not otherwise specified (NOS)
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
ML associated with Down syndrome
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemias

Table 3: WHO classification. Adapted from Dohner et *al.* , Blood 2017 (Döhner et al., 2017)

1.7 Prognostic Factors

AML prognosis takes into account risk-defining parameters that provide estimates of clinical outcomes. These include pre-treatment factors, patient-related and disease-related, but also post-treatment factors, including markers evaluated at distinct time points during treatment and follow up which allow patient stratification refinement and treatment adaptation.

- Patient-related factors:

  Patient age at diagnosis is the most important patient-related factor in AML and increasing age is associated with poor outcome (Appelbaum et al., 2006). This is due to AML adverse features, indeed AML in older patients is often secondary to antecedent myelodysplastic syndrome (MDS) or cytotoxic treatment, it has frequently unfavorable cytogenetics and commonly exhibits the expression of multidrug resistance proteins (Leith et al., 1999). Moreover, older patients are less able to tolerate intensive chemotherapy also because of comorbidities and poor performance status. However, even taking all these factors into account, patient age remains a strong independent prognostic factor (Hartmut Dohner et al., 2010). This can be the result of a greater genetic instability and clonal diversity of AML in elderly due to accumulation of non-clonal mutations in HSCs with aging (Appelbaum et al., 2006).

- Disease-related factors:

  Genetic aberrations represent the most significant disease-related prognostic factors for complete remission (CR) and overall survival (OS) (Byrd et al., 2002) (Schlenk & Döhner, 2013). Accordingly, the 2017 ELN classification combine information from both cytogenetics and molecular aberrations, mainly *NPM1*, *FLT3*, and *CEBPA* genes, to redefine the three risk groups, favorable, intermediate and unfavorable, conventionally defined by cytogenetic alone (Döhner et al., 2017).

  The favorable group includes patients with either inv16 (*MYH11-CBFB*), t(8;21) (*RUNX1-RUNX1T1*), mutant *NPM1* without *FLT3* internal tandem duplications (ITD) or with low *FLT3*-ITD allelic ratio (<0.5), or biallelic mutations of *CEBPA*. The adverse group consists of patients with either monosomy 7 (-7), monosomy 5 (-5), deletion of the long arm (q) of chromosome 5 (5q-), abnormalities of 17p, 3q, 11q (except t(9;11)), t(6;9) (*DEK/NUP214*), t(9;22) (*BCR-ABL1*), wild type *NPM1* and high *FLT3*-ITD allelic ratio, mutated *TP53*, *RUNX1*, *ASXL1* (the two latter if not occurring in favorable risk AML) or patients with complex karyotype (three or more unrelated chromosome abnormalities not including translocations or inversions) or with a monosomal karyotype.

  The intermediate group comprises patients with a normal karyotype with mutated *NPM1* and high *FLT3*-ITD allelic ratio, wild type *NPM1* without *FLT3*-ITD or with low *FLT3*-ITD allelic

ratio, patients with t(9;11) (*MLLT3-KMT2A*), or patients with cytogenetic abnormalities not included in the previous categories.

LSC frequency offers additional prognostic information.

A high percentage of LSC enriched CD34+/CD38−/CD45−/low cells at diagnosis correlates with high minimal residual disease (MRD) after chemotherapy and poor clinical outcome (Anna Van Rhenen et al., 2005) (Terwijn et al., 2014).

Furthermore, a high degree of correlation between HSCs and LSCs signature, underlying stemness properties, is predictive of lower survival (Eppert et al., 2011).

The CD34+/CD38+ and CD34- compartments have been also shown to contain leukemia initiating cells. However, these fractions seem to lack clinical relevance, at least in patients with CD34+/CD38− cells (Costello et al., 2000) (Terwijn et al., 2014).

Finally, hyperleukocytosis (white blood cell count (WBC)> 200.000/µl) at diagnosis is associated with poor response to induction therapy (Vardiman, 2010).

- <u>Response to therapy</u>:

  Alongside pre-treatment prognostic factors, measurement of treatment response to induction therapy is recognized as an independent prognostic factor, improving risk stratification and allowing a subsequent risk adapted management of the disease. Historically, response to induction therapy has been assessed by morphological examination of BM aspirates at

defined time points. However, the limit of detection of the morphologic analysis is not sufficient in identifying submicroscopic levels of disease which have been shown to harbor a comparable and eventually superior prognostic relevance to that associated with morphological persisting leukemia (Araki et al., 2015) (Inaba et al., 2012). MRD is defined as any measurable disease or detectable leukemia above a certain threshold, depending on the methodology applied. The AML heterogeneity as well as the differences in the molecular landscape between pre-leukemic and leukemic clones, makes MRD assessment more difficult. However, multiparametric flow cytometry (MFC) and molecular techniques, including real-time quantitative PCR (RT-qPCR), digital PCR and next-generation sequencing (NGS)-based technologies, have shown promise. MRD monitoring by PCR relies on the detection of leukemia specific target, such as: fusion genes (e.g., RUNX1-RUNX1T1, CBFB-MYH11, PML-RARA); mutations, (for example in NPM1); over expression of genes such as WT1, collectively covering ≈ 60% of AML cases.

MRD monitoring by MFC relies instead on the principle that the antigen expression patterns on the leukemic blasts differ from that seen on normal cells. Accordingly, two related approaches are applied. The first one is based on the identification of “leukemia associated immunophenotypes” (LAIPs) defined as the presence of a combination of antigens and/or flow cytometric physical abnormalities that are absent or very

infrequent in normal BM. Since LAIPs were often unstable between diagnosis and relapse, the alternative approach "different than normal" consists in identifying myeloblasts by their deviation from normal patterns. In this way > 90% of patients can be monitored by MFC. Many international groups recently published that in AML MRD detection by PCR or MFC is feasible and rapid also enabling a better treatment response assessment, thus facilitating timely and tailored intervention, even though it is not yet widely used in clinical decision making; major reasons for this include the lack of large prospective studies, the lack of standardization among different laboratories, immunophenotypic instability, and lack of thresholds and time-points identifications during follow-up (Rizzari, Cazzaniga, Coliva, De Angelis, & Conter, 2011). Some groups such as the ELN are trying to fill this gap in order to provide guidelines for the use of MRD in clinical practice (Schuurhuis et al., 2018).

1.8 Treatment of AML

Despite the progresses in understanding the pathophysiology and molecular heterogeneity of the disease, AML treatment has remained significantly unchanged and uniform in the past four decades relying mainly on refinements of doses and schedules of standard cytotoxic chemotherapy or progresses in hematopoietic stem cell transplantation (HSCT) procedures. With these improvements, although CR rates of ≥80% may be reached, the long term survival

remains dismal, with a 5-year OS ranging from 40%-50% for patients younger than 60 years to 10%-20% for elderly patients (Hartmut Dohner et al., 2010) (Döhner, Weisdorf, & Bloomfield, 2015). However, a new era for AML treatment is about to begin. Indeed, in 2017-2018 five new drugs have been approved by the US Food and Drug Administration (FDA):

-Midostaurin (Rydapt), a multi-tyrosine kinase inhibitor active against FLT3-mutations, for the treatment of FLT3-mutant AML in combination with cytarabine and daunorubicin induction and cytarabine consolidation.

-Enasidenib (Idhifa), an IDH2 inhibitor, for adults with relapsed or refractory AML with IDH2 mutation.

-Ivosidenib (Tibsovo), a small-molecule inhibitor of mutant IDH1, for adult patients with relapsed or refractory AML with IDH1 mutation.

-CPX-351 (Vyxeos), a liposomal formulation of cytarabine and daunorubicin with better pharmacokinetic properties which lead to increased leukemia blast exposure, for patients with secondary AML, therapy-related AML (t-AML) or AML with MDS-related changes (AML-MRC). The recommendation for approval was based on findings from a phase III study comparing CPX-351 with the standard induction therapy ("7+3"). The median OS was 9.56 months (95% CI, 6.60-11.86) with CPX-351 versus 5.95 months (95% CI, 4.99-7.75) with "7+3", representing a 31% reduction in the risk of death (HR, 0.69; P = .005) (Jeffrey E. Lancet, Geoffrey L. Uy, Jorge E. Cortes, Laura F. Newell, Tara L. Lin, 2016).

-Gemtuzumab ozogamicin (GO) (Mylotarg), an anti-CD33 antibody drug conjugate delivering a DNA-damaging calicheamicin derivative, for newly diagnosed CD33-positive AML in adults and for relapsed/refractory CD33-positive AML in adults and pediatric patients who are at least two years of age (Omar Abdel-Wahab, 2018). The latter has been already approved in 2000 and then withdrawn in 2010 because of failure to confirm efficacy data and for safety concerns. Since then, an Individual Patient Data (IPD) Meta-Analysis from 5 Cooperative Group and the ALFA-0701 French study reported a beneficial effect of GO in terms of better survival in adults with favorable- and intermediate-risk cytogenetics but not adverse-risk AML randomized to receive GO along with intensive induction chemotherapy. As a result, GO has been re-evaluated by regulatory agencies (Castaigne et al., 2012). The current approval is for a lower recommended dose and fractionated schedule than the previous one. Furthermore, several promising targeted agents are under evaluation bringing new hope to AML treatment.

Conventional treatment of AML is generally divided into two phases: induction of remission and post-remission therapy for consolidation.

-INDUCTION THERAPY:

Induction therapy aims to achieve complete morphologic remission. The standard induction therapy, referred as “7+3” regimen, consists in a 7 day continuous infusion of cytarabine concomitantly to an anthracycline drug on days 1 to 3 (Saultz & Garzon, 2016). The induction phase usually consists of one or two cycles and higher CR rates are observed in younger adults compared to elderly patients (60-

80% versus 40-60%) (Hartmut Dohner et al., 2010) (H Dohner et al., 2015). Reasons for the poorer CR rates in aged AML patients rely on more frequent resistance to conventional chemotherapy, due to inherent adverse features of AML biology and antecedent hematologic disorders as well as on poor tolerance to intensive chemotherapy which results in higher treatment related morbidity and mortality. Alternatively, for this patient category, hypomethylating agents (HMA), such as azacytidine and decitabine, offer a less intensive option for disease control even in absence of CR (Fenaux et al., 2010; Ferrara & Musto, 2011).

Recent randomized studies have exploited the use in younger patients of higher doses of cytarabine (HiDAC) plus a nucleoside analogue (fludarabine, cladribine or claforabine). Although currently there is no conclusive evidence to recommend this approach over the standard "7+3"regimen, the results are quite promising (Burnett et al., 2013).

-<u>POST-REMISSION THERAPY</u>:

After achieving apparent CR, post-remission therapy is necessary to eradicate MRD and to prevent disease relapse. Usually, two main strategies are adopted: intensive chemotherapy (consolidation), usually consisting in repetitive cycles of HiDAC, and high dose therapy followed by autologous or allogeneic HSCT (allo-HSCT). The therapy choice depends on patient age, comorbidities and risk of relapse (Shipley & Butera, 2009). Allo-HSCT offers the most effective curable option, but remains associated with higher treatment-related morbidity and mortality (TRM), especially in elderly patients, thus it represents the treatment of choice particularly in fit patients with

intermediate and high risk features while it is delayed for those with a lower relapse risk. For intermediate-risk patients autologous HSCT (auto-HSCT) is also considered.

The benefits of allo-HSCT are the result of both myeloablative preparatory regimen used and the immunotherapeutic anti-leukemic activity exerted by the donor cells (Hamilton & Copelan, 2012) (Horowitz et al., 1990).

Allo-HSCT consists in the infusion of stem cells derived from an HLA-matched healthy donor, ideally a sibling related donor (matched related donor, MRD) or a matched unrelated donor, MUD. Moreover, the use of mismatched/haploidentical donors or cord blood as stem cell source provides the opportunity to benefit of HSCT to nearly all patients lacking a HLA matched donor. However, initial results from the use of mismatched allografts led to graft-versus-host disease (GVHD) and infections exacerbating TRM (Ciurea et al., 2015; Passweg et al., 2017).

For this reason, T-cell depleted (TCD) stem cells offer the potential for GVHD prevention without the morbidity associated with immunosuppressive drugs (Pasquini et al., 2012). However, this strategy did not translate into improved OS because of opportunistic infections, due to a delayed immune reconstitution, and disease recurrence, being T cell the major mediators of the graft versus leukemia (GVL) effect (Ho & Soiffer, 2001). Thus, to accelerate immune reconstitution and to prevent relapse some strategies have been developed: donor lymphocyte infusion (DLI), which is however still associated to GVHD development, and depletion of donor alloreactive

T cells (Scarisbrick et al., 2015) (Oevermann et al., 2012). The latter strategy will theoretically result in significant GVHD reduction without affecting GVL and immune reconstitution.

-LSCs specific targeting:

It appears critical that improving AML cure rates is strictly dependent on eradication of LSCs thus, considering the lack of responsiveness of LSCs to conventional therapies, LSC-directed therapies are mandatory. However, the development of anti-LSC therapies is hampered by the substantial biological parallelisms between AML and normal hematopoiesis and thus between LSCs and HSCs, including quiescence, multipotency and self-renewal. Identifying agents that selectively target LSCs without harming HSCs currently represents a challenging aspect.

Recently, several studies have expanded the knowledge on LSCs biology and heterogeneity by exploiting advanced xenograft models and high-throughput genome sequencing and DNA methylation profiling. Thus, considering the inter- and intra-patient variability, LSCs can be only functionally defined through the ability to engraft in serial xenotransplantation assays. Earlier experiments by John Dick and colleagues identified LSCs exclusively within the CD34+/CD38- subpopulation (D Bonnet & Dick, 1997), a phenotype shared by the HSCs capable of reconstituting normal hematopoiesis in NOD/SCID mice (Bhatia, Wang, Kapp, Bonnet, & Dick, 1997). However, by using NOD/SCID/IL2Rγ-null (NSG) mice, which are more permissive than the original NOD/SCID mice, LSC activity has been found also in the more mature CD34+/CD38+ fraction (D. C. Taussig et al., 2010). In particular,

a more detailed analysis has revealed that, in the vast majority of CD34+ AML patients, LSCs resemble lymphoid-primed multipotential progenitors (LMPP) or granulocyte-monocyte progenitors (GMP) (Goardon et al., 2011).

Concerning AML with mutated NPM1, a molecular lesion usually associated with low CD34 expression, LSC activity has been detected in the CD34- fraction in half of the cases, and in both CD34- and CD34+ fractions in the others (D. C. Taussig et al., 2010).

Current strategies under evaluation for developing LSC-directed therapies consists in targeting conserved markers of LSC population which are also differentially expressed on LSCs compared to normal HSCs (i.e., deregulated molecular pathways and overexpressed surface antigens), thus offering a therapeutic window for minimizing the risk of toxicity. In this context, some LSC targets are LSC-related molecular pathways: NFκB signaling pathway, PI3K/AKT/mTOR pathway, BCL-2 pathway, which could be targeted with small molecule inhibitors (Guzman et al., 2001) (Fransecky, Mochmann, & Baldus, 2015; Lagadinou et al., 2013) and many overexpressed surface antigens such as CD33, CD123, CLL-1, CD44, CD47, CD96, TIM3, which could be targeted with immunotherapeutic strategies (Liqing Jin et al., 2009; R.B. Walter, Appelbaum, Estey, & Bernstein, 2012) (L Jin, Hope, Zhai, Smadja-Joffe, & Dick, 2006; Leong et al., 2017; Majeti et al., 2009) (Jan et al., 2011). One alternative is represented by the targeting of microenvironment/leukemia interactions, trying to overcome resistance to chemotherapy linked to BM protection. To this aim, the emerging therapeutic targets include chemokine receptors (CXCR4),

and adhesion molecules (VLA4 and CD44) (Hsieh et al., 2013; L Jin et al., 2006; Rashidi & Dipersio, 2016).

1.9 Immunotherapy and AML

The evidence of the GVL effect in patients receiving allo-HSCT advances the concept that leukemia immunogenicity could be exploited to develop novel therapeutic strategies aimed at enhancing the anti-leukemia immune response. Indeed, the immune system alone, even if potentially capable of recognizing and eliminating tumor cells, is not able to prevent cancer progression, as tumor cells employ various strategies to escape immune surveillance.

In particular, AML cells have been shown to evade immune control by upregulating ligands for inhibitory receptors, down-regulating costimulatory molecules, inducing a suppressive cellular microenvironment, inducing a metabolically unfavorable microenvironment and immunoediting.

AML cells can indeed upregulate molecules such as Gal-9, CD80 or CD86, CD200, PDL1 or PDL2 that bind the corresponding receptors TIM-3, CTLA4, CD200R, and PD-1 respectively, thus suppressing T cell functions (Coles et al., 2015, 2012; Kong et al., 2015; Yao & Chen, 2013; Zhou et al., 2011). Interestingly, both TIM-3 and GAL-9 are expressed on AML cells constituting an autocrine loop critical for development and maintenance of AML (Kikushige et al., 2015). AML cells also express CD47 molecule which prevents phagocytosis by binding to the macrophage inhibitory receptor SIRPα (Majeti et al., 2009). Conversely, a decreased expression of the activating receptor DNAM-1 on immune cells is induced (Sanchez-Correa et al., 2012).

Similar to solid tumors, AML is capable of creating an immunosuppressive and metabolically unfavorable microenvironment. AML cells are indeed able to “educate” stromal cells to secrete inhibitory cytokines. Additionally, the depletion of arginine by arginase II, ROS secretion and tryptophan starvation due to indoleamine 2,3-dioxygenase 1 (IDO) activity elicited by both AML cells and induced myeloid derived suppressor cells (MDSCs), concur in inducing T cell dysfunction and promote T-reg differentiation, thus fostering immune escape and leukemia progression (Fracchiolla, Fattizzo, & Cortelezzi, 2017).

Loss or down-regulation of HLA molecules represents an immune escape mechanism frequently described in solid tumors known as immunoediting (Garrido & Algarra, 2001). This mechanism has been found to be less common in leukemic cells at the diagnosis while more frequent at relapse, having implications on HSCT effectiveness (Masuda et al., 2007). As an example, in 2009, Vago et al described, in 5 out 17 patients relapsed after haploidentical transplantation and infusion of donor T cells, the loss of the mismatched HLA determinants, targeted by alloreactive donor T cells, due to the mechanism of acquired uniparental disomy of chromosome 6p (Vago et al., 2009).

In conclusion, all these interactions with the immune system provide the rational for developing different immunotherapeutic strategies to target leukemic blasts, trying to overcome AML intrinsic immunosuppression.

In particular, in the last decades both active and passive approaches have been investigated.

ACTIVE IMMUNOTHERAPY

Active immunotherapy aims at enhancing patient own immune response toward the tumor in non-specific or specific manners.

Non-specific strategies include the administration of exogenous cytokines to restore T cell and NK cell effector functions. For example, IL-2 administration has been widely investigated, however, discordant results have been reported, depending on which patient subgroup has been included and which other treatments have been combined with IL-2 (Baer et al., 2008; Brune et al., 2006; Lange et al., 2008; Maraninchi et al., 1998). Furthermore, severe toxicity has been reported following IL-2 treatment (Maraninchi et al., 1998).

The newest emerging dugs that boost the immune system in a non-specific way are represented by the immune checkpoint inhibitors (ICI). Immune checkpoints (PD-1, CTLA-4) are negative regulators of the immune system and, physiologically, are crucial for maintaining immune homeostasis and preventing autoimmunity. However, as mentioned above, cancer cells take advantage of these mechanisms to dampen anti-tumor immune responses, thus immune checkpoint blockade represents a promising approach to reinstate anti-tumor responses. In particular, successful results have been experienced in the treatment of solid tumors such as metastatic melanoma and metastatic non-small cell lung cancer (Lipson & Drake, 2011; Robert et al., 2015). Concerning AML, both anti-PD-1 and anti-CTLA-4 are

currently object of clinical investigation (Assi, Kantarjian, Ravandi, & Daver, 2018).

To elicit specific immune responses cancer vaccines have been developed. Various vaccination strategies have been explored, including immunization with leukemia peptides, inactivated leukemic cells (eventually modified to enhance T cell response), AML-derived dendritic cells (DCs) or DCs loaded with leukemia-associated antigens (LAA) (Smits, Berneman, & Van Tendeloo, 2009).

Of note, when pursuing active immunotherapy, a competent immune system is required, which is likely not the case for AML patients, due to both chemotherapy regimens and immunosuppression exerted by leukemic cells itself. Additionally, the majority of tumor associated antigens (TAA) are self-proteins toward which self T cells have developed tolerance by clonal deletion or anergy, and tumor specific T cells, if present, are low avidity T cells resulting in weak responses which are inadequate in definitively fighting tumors (De Visser, Schumacher, & Kruisbeek, 2003). Therefore, in parallel to this approach, passive immunotherapeutic strategies have been explored.

PASSIVE IMMUNOTHERAPY

Passive immunotherapy refers to the direct administration of immune system components, such as tumor specific antibodies or T cells.

- Antibody-based approaches

Monoclonal antibodies (mAbs) have successfully entered in the clinical practice for treating patients with hematological malignancies and solid tumors.

Targeted mAbs can attack tumor cells via different mechanisms including blocking particular signaling pathways, relevant to cancer cell growth and proliferation, by binding to the related receptor or ligand, induction of apoptosis or delivery of a drug or cytotoxic agents. Alternatively, tumor destruction can be mediated by recruiting immune effector cells or by complement activation, mechanisms known as antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC), respectively.

Concerning AML, multiple surface antigens have been object of investigation for the development of mAb-based therapies due to their overexpression on leukemic cells, such as CD33, CD123, CD44, CLL-1, CD96, CD47, CD32, and CD25 (Majeti, 2011).

-Adoptive cell therapy (ACT):

Adoptive cell therapy (ACT) consists in the administration to cancer patients of *ex-vivo* expanded patient's own (autologous) or donor derived (allogeneic) T cells with anti-tumor activity (June, 2007).

This approach has been firstly described in 1988 by Rosenberg and colleagues who found tumor infiltrating lymphocytes (TILs) in patients

with malignant melanoma (MM) (Rosenberg, Spiess, & Lafreniere, 1986). In 2002 a progress has been made by introducing an immunodepleting regimen prior to T cell transfer to favor clonal repopulation of patients with anti-tumor T cells (Dudley et al., 2002).

However, TILs with high avidity for the target antigens can be found only in some cancer patients, furthermore the isolation and expansion of antigen specific T cells is time and labor intensive.

Concerning AML, the therapeutic advantage for T-cell immunotherapy arises from the above mentioned GVL effect evidenced after HSCT. To amplify this effect, DLIs have been explored. However, in addition to the risk of developing GVHD, DLIs exhibited a limited efficacy in AML (Frey & Porter, 2008; Schmid et al., 2007).

Thus, various efforts have been made overtime to overcome these limitations, improving the efficacy of the ACT. For example alternative immune effectors have been investigated, such as EBV-specific cytotoxic T cells (CTLs), NK cells, γδ T cells, LAK and CIK cells (Rooney, Aguilar, Huls, Brenner, & Heslop, 2001) (Boyiadzis, Foon, & Herberman, 2006) (Braza & Klein, 2013) (Grimm, 1982), with the aim to identify an effector population being easily expandable *in vitro* and effective once *in vivo*. Furthermore T cells engineered with artificial T-cell receptors (TCRs) (Clay et al., 1999) and **Chimeric Antigen Receptors** (**CARs**) (Gross, Waks, & Eshhar, 1989) have been generated.

2. Cytokine-induced killer (CIK) cells

Cytokine-induced killer (CIK) cells represent a really suitable T cell source to be employed for cancer immunotherapy being easily expandable *ex vivo* via a straightforward and cost-effective protocol and having an intrinsic MHC-unrestricted cytotoxic activity towards both solid and hematological tumors (Lu & Negrin, 1994; Schmidt-Wolf, Negrin, Kiem, Blume, & Weissman, 1991) and a limited capacity of causing GVHD in allogeneic settings (Gao et al., 2017). Furthermore, they do not require either a previous target contact or the exogenous administration of IL-2 (which turned to be toxic at high dosages) to become fully activated once *in vivo* (Lu & Negrin, 1994; Schmidt-Wolf et al., 1993, 1991).

In particular, CIK cells are a heterogeneous polyclonal T cell population with NK-like phenotypic and functional features.

CIK cells were first described by Schmidt-Wolf in 1991 (Schmidt-Wolf et al., 1991) who then firstly explored CIK cells in the treatment of cancer patients in 1999 (Schmidt-Wolf et al., 1999).

Classically, CIK cells can be obtained from peripheral blood mononuclear cells (PBMCs) through the timed addition of interferon-γ (IFNγ) at day 0, an anti-CD3 mAb (OKT3) and IL-2 24 hours later and by repeated addition of IL-2 every three days. After a culture period of 21-28 days CIK cell expansion rates range from few up to thousand folds (Schmidt-Wolf et al., 1991).

At the end of the differentiation process the population is composed by more than 95% of CD3+ cells, of which the vast majority is

represented by T-CD8+ rather than T-CD4+ cells, and also enriched in T-CD3+/CD56+ cells (accounting for 40 to 80%), that display the major cytotoxic properties. There is also a small percentage of NK cells (CD3-CD56+). The addition of IL-2 and OKT3 after the prior administration of IFN-γ seems to be crucial for the induction of an elevated cytotoxic potential, operating the signals at different levels (Schmidt-Wolf et al., 1991). Indeed, the IFN-γ priming leads to the monocytes activation which results in IL-12 production as well as upregulation of CD2 that interacts with leukocyte function-associated antigen 3 (LFA-3) for optimal *in vitro* expansion of the human CD3+/CD56+ effector cells (Lopez, Waller, Lu, & Negrin, 2001), while mitogenic stimuli are subsequently provided by the administration of both IL-2 and OKT-3 (Ochoa, Gromo, Alter, Sondel, & Bach, 1987). To improve the expansion protocol and cytotoxic potential of CIK cells several strategies have been investigated, such as the addition to the culture of cytokines like IL-1, IL-7, IL-15 or thymoglobulin (Bonanno et al., 2010; Rettinger et al., 2012; Zoll et al., 1998), the transient stimulation with allogeneic irradiated feeders (Todorovic et al., 2012) or T-reg depletion from the starting cell population (Li et al., 2007).

While the CD3+/CD56+ compartment exhibits a limited proliferative potential, being more terminally differentiated, the CD3+/CD56- subset present within the CIK cell culture comprises early effector T cells with a greater proliferative aptitude (Yeh C. Linn et al., 2009) that continuously supply the CD3+/CD56+ fraction by acquiring CD56 expression upon activation (Yeh C. Linn et al., 2009; Sangiolo et al., 2008).

Mature CIK cells express some typical NK receptors, such as NKG2D, DNAM-1 and low levels of NKp30. Differently from NK, they do not express NKp44 and NKp46, iKIR, NKG2A and CD94. CIK cells present a variable αβ-TCR repertoire thus being different entities from NKT lymphocytes, characterized by invariant αβ-TCR recognizing glycolipids presented by CD1d molecules on antigen presenting cells (Girardi & Zajonc, 2012). CIK cell TCR is biologically active and capable to respond to proper MHC-dependent antigen stimulation. Thus, even if CIK cell cytotoxic activity largely relies on NKG2D receptor, TCR-mediated cytotoxicity is retained (Verneris, Karami, Baker, Jayaswal, & Negrin, 2004) (Pievani et al., 2011).

NKG2D is the major responsible of CIK cell MHC-unrestricted tumor killing activity by the recognition of MHC-class I-like molecules, MICA, MICB and members of the ULBP family, ULPB1-4 which are often overexpressed by both solid and hematological tumor cells (Yeh C. Linn et al., 2009) (Groh et al., 1999) (Salih et al., 2003) (Pende et al., 2002). Along with NKG2D, also NKp30, DNAM-1 and the LFA-1/ICAM-1 complex have been recently described as implicated in tumor recognition (Pievani et al., 2011; Schmidt-Wolf et al., 1993). Additionally, it has been reported that CD56 membrane molecule itself could be implicated in tumor recognition and killing through hemophilic interaction with CD56 expressing hematopoietic tumor cells (Valgardsdottir et al., 2014). The final killing mechanism is mainly granzyme and perforine dependent (Baker, Verneris, Ito, Shizuru, & Negrin, 2001).

Another important peculiar feature of CIK cells is their reduced alloreactivity across HLA barriers. This feature is very important, underlining the possibility of employing CIK cells also in an allogeneic clinical setting. Early preclinical studies of HSCT in murine models demonstrated that CIK cell infusion across major MHC barriers was associated to limited GVHD occurrence compared with conventional lymphocytes (Verneris et al., 2001).

The reduced incidence of GVHD after CIK cell administration is not yet fully understood, however it is thought to be dependent on CIK cells capacity to produce high amounts of IFN-γ that has been demonstrated to have a protective effect towards the GVHD. Indeed, experiments performed with a number of knock-out (KO) mice strains, such as FAS, FASL, perforin, IL-2 and IFN-γ defective, revealed that GVHD could be prevented by all mice, except for IFN-γ -/- KO, with a lethal GVHD caused by this specific KO condition (Baker et al., 2001). In particular, the principal requirement to avoid GVHD development has been demonstrated to rely in the IFN-γ production by donor CD8+ NKT cells, rather than by recipient cells, since the injection of wt CD8+ NKT cells in IFN-γ -/- recipients did not cause GVHD occurrence.

Another explanation for the reduced ability of causing GVHD by allogeneic CIK cells is to be found in their ability to eliminate the DCs of the host, which are known to be the most important component in initiating the GVHD reaction, due to the enhanced CIK cell killing activity mediated by IFN-γ, as demonstrated by Mase and colleagues (Mase et al., 2012). Moreover, CIK cells showed an aptitude of being less proliferative and more susceptible to apoptosis *in vivo*, in

association with a reduced pro-inflammatory cytokine production at the site of GVHD and a lower expression of homing molecules and chemokine receptors that usually allow T cells to enter GVHD target organs (Nishimura et al., 2008). Additionally, GVHD target tissues, such as small bowel epithelium, skin and liver have been shown to not up-regulate NKG2D ligands, after the infusion of CIK cells in an allogeneic marrow transplant model, further accounting for the reduced capacity of allogeneic CIK cells of causing GVHD (Nishimura et al., 2008).

The other advantage of CIK cells is their tumor-directed trafficking capacity likely due to the interaction of chemokines released from the tumor microenvironment with chemokine receptors (CKRs) on the CIK cell surface (Baker et al., 2001).

Finally, CIK cells can be generated successfully from healthy donors as well as from cancer patients (Alvarnas, Linn, Hope, & Negrin, 2001; Y. C. Linn & Hui, 2002). Alternatively, CIK cell large-scale expansion has been reported for cord blood (CB) and even from washouts of leftover mononuclear cells from CB unit bags (M. Introna et al., 2006).The first experience with CB-derived CIK cells in a limited number of cancer patients has confirmed some clinical effectiveness (Niu et al., 2011). The possibility of expanding CIK cells from CB offers several advantages, including the availability of high number of umbilical CB (UCB) units tested for HLA and infectious markers in cord blood banks; lower alloreactivity (less GVHD); low risk of viral transmission; no risk of donor withdrawal; potential to provide ‘off-the-shelf’ products (Balassa & Rocha, 2017).

In conclusion, all these features paved the way for the employment of CIK cells in clinical trials for the treatment of both solid and hematological malignancies (Martino Introna & Correnti, 2018). Among the different research groups who employed CIK cells for the treatment of relapsed hematological malignancies after HSCT, in 2007 our group participated with a pioneering phase I study, in which allogeneic CIK cells have been infused into 11 patients. The most important finding of this study has been the feasibility of the employment of CIK cells, which could be easily produced in a short time, demonstrating to be effective in obtaining several clinical responses, such as complete remission in 3 patients and the stabilization of the disease in 1 of them. Indeed, even if high doses of CIK cells were required (i.e. from 3 x 10^6/kg to 15 x 10^6/kg), no serious adverse events have been encountered and only 4 patients developed GVHD, but at most with a second level severity (M Introna et al., 2007). Based on the results of this phase I study, a phase IIA protocol was opened to evaluate the safety and efficacy of sequential administration of donor derived DLIs and CIK cells in patients with hematologic malignancies relapsing after related or unrelated allogeneic HSCT. A low incidence of GVHD has been observed and disease control has been achieved mostly in patients with low tumor burden (M. Introna et al., 2017).

In order to report autologous or allogeneic CIK cell-based clinical trials against several type of tumors and to collect and interpret the results obtained worldwide, an International Registry on CIK Cells (IRCC) has been created (Hontscha, Borck, Zhou, Messmer, & Schmidt-Wolf,

2011). According to the latest published report, 45 studies (24 phase I and 21 phase II) employing CIK cells in 22 different tumor conditions have been evaluated, for a total of 2729 treated patients. The analysis revealed a mean response rate of 39% and a significantly improved OS, with limited side effects and an improved quality of life. (Schmeel, Schmeel, Coch, & Schmidt-Wolf, 2015).

Overall, CIK cells emerged as a promising immune effector population to be adopted in oncology, both as immunotherapy adjuvant in order to prevent recurrence and as a potential effector population in combination with ICI (Poh & Linn, 2016) or genetically redirected with CARs (Marin et al., 2006).

3. Chimeric antigen receptors (CARs)

Chimeric antigen receptors (CARs) were firstly developed by Zelig Eshhar and collaborators, in the late 1980s (Gross et al., 1989) and have now reached bedside applications, demonstrating their therapeutic potential. Indeed, the unprecedented clinical responses achieved (Wohlfarth, Worel, & Hopfinger, 2018) led the FDA to grant, in August 2017, the first ever approval to the Novartis' CD19.CAR-T cell product “Kymriah” for relapsed/refractory pediatric Acute Lymphoblastic Leukemia (ALL) and subsequently to Gilead Sciences’ CD19.CAR-T cell product “Yescarta”. On June 2018, also the European Medicines Agency (EMA) gave its favourable opinion.

In its classical design, CAR consists of an antigen-binding domain, usually derived from a mAb in the form of single chain fragment variable (scFv), a spacer, a transmembrane domain, and one or more intracellular T cell triggering domains (figure 2).

Therefore, CARs offer the advantages of combining the antigen binding properties of antibodies with the typical T cell effector functions such as cytotoxicity, capability of migration and infiltration within the tumor sites, cytokine release with the subsequent recruitment of other immune components and an overall sustained anti-tumor immune response (Bachmann et al., 2010). The antibody-mediated CAR recognition avoid the need for MHC antigen presentation and permits to recognize any target for which an antibody is available, including carbohydrates and lipids. MHC independent target recognition allows the use of CARs in a broad

range of patients and enables to overcome the tumor's immune escape mechanism of MHC downregulation. In contrast to TCR, the CAR recognizes only antigens on the cell surface, however, it can acquire TCR-like specificity by using an antibody detecting a specific peptide in the context of MHC (Stewart-Jones et al., 2009).

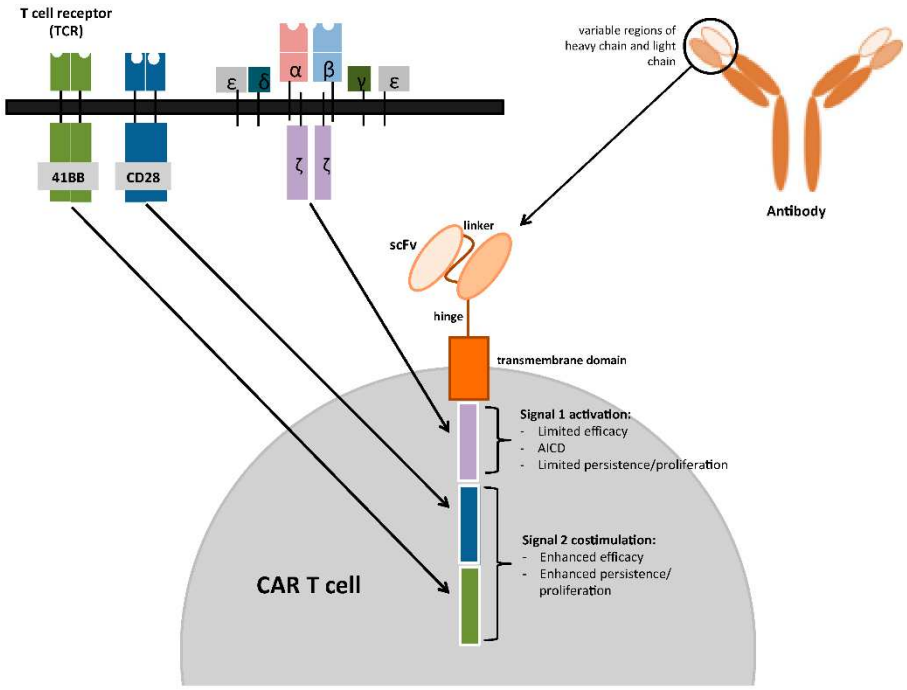

Figure 2. Schematic representation of a third generation CAR. Adapted from Klampatsa A et *al.,* Cancers 2017 (Klampatsa, Haas, Moon, & Albelda, 2017)

In order to optimize CAR-T cell performance in each particular tumor context, the CAR design has undergone to continuous developments over the last two decades. Though the early CAR prototype has been empirically designed, preclinical characterizations have highlighted that the efficiency of CAR-mediated T cell activation depends on various parameters including: CAR binding affinity, CAR and antigen densities, target antigen epitope accessibility, the achievement of an *"optimum of distance"* between the target antigen and the CAR, the type of costimulation and other variables implicated in the CAR-T cell functionality. Additionally, first clinical applications pointed out the need to control CAR-T cell safety profile, since "on target-off tumor" effects, uncontrolled secretion of pro-inflammatory cytokines or tumor lysis syndromes have occurred and have been fatal in certain occasions.

Thus, CAR design implementation will be fundamental for the development of CAR therapies appropriately matching efficacy with safety. To this aim, each modular component of a CAR can be arranged to optimize T-cell functions in a context dependent manner.

-<u>The antigen binding domain</u>:

As above stated, the most common extracellular binding regions exploited in the CAR construction are scFvs which comprise the variable light (VL) and heavy (VH) chains of a mAb fused together with a flexible linker. Recent studies have revealed that the scFv can impact the safety and/or efficacy of CAR T cells beyond just antigen-specificity. For example, the non-human origin of the scFv is

responsible for CAR immunogenicity, which can limit CAR T cell persistence (Jensen, 2010) as well as causing serious adverse events such as a reported case of fatal anaphylaxis in a patient infused with multiple doses of anti-mesothelin CAR-T cells (Maus et al., 2013). Therefore, scFv humanization is recommended to minimize CAR antigenic potential.

The affinity of the scFv for its target is another feature that has been shown to affect CAR-T cell function. One of the major concern associated with CAR-T cell therapy is the "on target-off tumor" effect which refers to antigen recognition by the CAR outside the tumor context due to the absence of real tumor specific antigens. In this sense, the CD19 molecule has proven to be an optimal antigen to be targeted by CAR-T cells. Indeed, the toxicity associated with B cell depletion has shown to be well tolerated, although immunoglobulin administration is required (Brentjens et al., 2011). In other circumstances, target expression on critical tissues, such as ERBB2 on respiratory epithelium, has resulted in death in at least one patient (Morgan et al., 2010). Lamers and colleagues also reported hepatic toxicity in clear cell renal cell carcinoma bearing patients treated with anti-carbonic anhydrase IX (CAIX) CAR-T cells, probably due to CAIX expression in the biliary tract (Lamers et al., 2013).

Concerning this aspect, investigators have recently demonstrated that scFv affinity and CAR density can be tuned to discriminate between tumor targets and normal tissues, taking advantage of the differential target antigen expression between tumor cells and the normal ones. It has been demonstrated that CAR-T cells owning low-affinity scFvs can

be more discriminative between tumor cell lines with high target antigen density and normal cells or tumor cell lines expressing low levels of target antigen. (Liu et al., 2015) (Caruso et al., 2015).

However, the use of a low antigen positive tumor cell line as representative of the healthy tissue limits the conclusion that by lowering scFv affinity there could be a benefit in terms of ameliorating the CAR therapeutic index. Indeed, this evidence raises concerns for a reduced effectiveness of low-affinity scFvs when challenged against low antigen positive tumor cells. Notably, not only the target antigen density but also the CAR density on the T cell has to be taken into account in identifying the ideal affinity range for a specific scFv-antigen pair considered.

Thus, it appears fundamental to deeply characterize CAR-T cell responses towards different target antigen densities to define CAR "lytic" and "activation" thresholds given by the number of antigens on the target cells surface required to produce a full cytotoxic activity and the subsequent activation and expansion of the CAR-T cells (K. Watanabe et al., 2015).

A rational affinity tuning of the CAR antigen-binding domain should allow the modulation of these thresholds in order to decrease CAR sensitivity toward low antigen, such as healthy tissues whose antigen expression is expected to be below the lytic threshold, thus favoring the recognition of overexpressing tumor cells.

For example, our *in vitro* characterization of wild type anti-CD123 CAR and its high and low affinity mutants confirmed CD123 as a safe target antigen for CAR-CIK cell approach. Furthermore, we found that i) early

cytotoxic activity is not affected by either CAR affinity or expression (within the tested range); ii) later effector functions are not affected by CAR affinity but rather by CAR density; iii) the mutant with 100-fold reduced affinity is just as effective as the high affinity ones while remaining safer or, at worst, equally safe (Arcangeli et al., 2017).

Similarly, in a different context, Hombach and colleagues showed that their anti-CD30 CAR-T cells did not attack CD30+ HSPCs while eliminating CD30+ lymphoma cells due to a subthreshold level of the targeted CD30 on HSPCs (Hombach et al., 2016). The issue of low antigen tumor cells could be bypassed by exploiting bi-specific CARs, that, while being more specific for the double antigen expressing-tumor cells, they ensure the tumor recognition even if one of the two target antigens is downmodulated. In the optimization of the CAR design, another aspect that has to be taken into account is the antigen-independent scFv clustering. This phenomenon has been associated to tonic CAR CD3ζ phosphorylation leading to early CAR-T cell exhaustion, thus lowering their proliferative and cytokine-producing capacities (Long et al., 2015). This issue can be addressed by improving scFv stability, particularly at the VH:VL interface to minimize scFv aggregation and reducing tonic signaling (Zhou L, Abel T, Schneider IC, Beghelli S, Labbal F, David M, 2017).

Alternatively to the scFvs, some groups have utilized receptor or ligand proteins for the CAR-targeting moiety. For example, binding peptide against vascular endothelial growth factor receptor (VEGF) (Niederman et al., 2002), anti-integrin α5β6 peptide (Pameijer et al., 2007), heregulin (Muniappan, Banapour, Lebkowski, & Talib, 2000),

interleukin (IL)-13 mutein (Kahlon et al., 2004), and the NKG2D receptor (Barber, Zhang, & Sentman, 2008; Zhang, Barber, & Sentman, 2007) have been introduced into CARs conferring anti-tumor activities to redirected T cells.

Different approaches for achieving multiple target specificities and programmability of CAR-T cells rely on universal switchable CARs, wherein the targeting moiety is a molecularly independent soluble tagged molecule (switch) with an epitope that is recognized by a 'universal' CAR. As an example, anti-FITC CAR-T cells have been exploited to recognize various tumors when bound by FITC-labeled antibodies (Tamada et al., 2012). This approach allows to control CAR T cell specificity (i.e., simultaneously targeting of multiple antigens or changing if necessary the target antigen) and to modulate T-cell activity by dosing the switch molecule. However, this strategy demands for T cell temporal and physical co-localization with both the tumor cell and the antigen binding intermediate.

-<u>The hinge and spacer domains</u>

The hinge and spacer domains connect the antigen-binding domain and the transmembrane region. Recent studies have demonstrated that, like the canonical TCR-MHC-peptide complex, the distance between a CAR-T cell and a tumor cell may be crucial in ensuring adequate immune synapse formation and thus functional activation.

Thus, CAR hinge and spacer length have significant impact on CAR-T cell effector functions and their architecture needs to be adjusted based on the specific target epitope. Indeed, the location and

accessibility of the target epitope are the principal constraining factors (Guest et al., 2005; James et al., 2008). Common hinge/spacer regions have been derived from CD8α or immunoglobulin (IgG1 or IgG4) constant domain (Fc).

However, it has been shown that inclusion of Fc domains can support binding of CARs with IgG Fc gamma receptors (FcγRs) on myeloid cells, thereby initiating an unintended "off-target" activation of both innate cells and T cells together with the initiation of activation induced cell death (AICD) through overstimulation of the T cell. (A Hombach, 2010; Hudecek et al., 2015; N. Watanabe et al., 2016). Thus, modifications of IgG-derived extracellular spacer domains have been performed to abrogate binding to Fc receptors (e.g. CH2 domain deletion within the IgG1 or IgG4 domains or introduction of specific mutations).

-<u>The transmembrane domain</u>:

The transmembrane domain lies between the hinge/spacer region and the signaling domains. It consists of hydrophobic alpha helix usually derived from CD3-ζ, CD4, CD8 or CD28 molecules. This region has been initially viewed as a non-functional structural element. Conversely, several studies have shown that the transmembrane domain has a significant influence on CAR surface expression and function. One early study (Romeo C, Amiot M, 1992) showed that CAR CD3ζ transmembrane domains dimerize with other CD3ζ proteins and dimerization disruption by point mutations has an impact on CAR-T cell activation leading to weaker cytolytic activity.

Zhang and colleagues found that the transmembrane region of their NKG2D CAR needs to be associated with an adaptor protein Dap10 for surface expression (Zhang et al., 2007). Similar to CD28, Dap10 has been shown to transduce a costimulatory signal via a phosphatidylinositol 3-kinase pathway (Raulet, 2003), thus ensuring a proper T cell activation.

Thus, it is possible to take advantage of transmembrane domains from already existing proteins to allow a CAR to associate with other beneficial co-receptors and T cell surface proteins.

-The intracellular signaling domain:

CAR cytoplasmic domain provides the downstream signal to trigger T cell activation. According to the incorporated signaling domains, CARs can be classified into three different generations. First generations CARs contained a primary activation signaling motif, utilizing either CD3ζ or FcRγ. The CD3-ζ has become the most widely used signaling domain and provides the required 'signal 1' for T cell activation. However, the anti-tumor activity of first generation CARs has been shown to be limited overtime because the 'signal 1' induces only transient cell division and suboptimal cytokine production, failing in promoting T cell persistence *in vivo* and sustained antitumor effect (Brocker, 1995). In principle, 'signal 2' could potentially be provided by the native co-receptors present in the CAR-T cells; however, tumor cells and tumor microenvironment often lack the expression of co-stimulatory ligands such as CD80 and CD86. Thus, to overcome the limitation of first generation CARs, second and third generation CARs

have been developed. These ‘generations’ incorporate one or two co-stimulatory molecules, respectively, which provide the ‘signal 2’ resulting in an efficient T cell activation.

CD28 was the first costimulatory domain exploited (Finney, Lawson, Bebbington, & Weir, 1998), and subsequently, other co-stimulatory molecules have been described, including ICOS (inducible costimulatory), OX40 (CD134), 4-1BB (CD137), CD27, DAP10 or 2B4 (CD244) (Maher, 2012) each of which endow CAR-T cells with different and peculiar functional properties.

Second-generation CARs incorporating CD28-CD3ζ or 4-1BB-CD3ζ sequences still remain the most frequent CAR formats used in clinical practice. Several studies suggest that CD28 stimulates CAR-T cell cytotoxic activity and functionality, whereas the 4-1BB promotes greater CAR-T cell persistence. Notably, we are now gathering the first clinical evidences coming from trials using different costimulatory domains, thus starting to collect data on the potential underlying mechanisms by which different transmembrane and intracellular domains influence T cell expansion, function, and persistence.

3.1 Improving the efficacy and safety of CAR-T cell therapy

The need to translate the CAR-T cell technology beyond B-cell malignancies, as well as to improve its safety and anti-tumor efficacy, is triggering investigators to devise innovative strategies for next-generation CAR design, some of which are summarized below (figure 3).

-TRUCK (T cells redirected for universal cytokine-mediated killing)

TRUCKs are CAR-redirected T cells which additionally release a transgenic product that accumulates in the tumor microenvironment. The accessory product is mostly a pro-inflammatory cytokine whose expression can be constitutive or inducible following CAR-T cell activation. This strategy is of particular interest for the targeting of solid tumors, since CAR-T cells fail to be as effective as liquid tumors for the inability to reach and survive in the microenvironment surrounding the neoplastic foci.

This approach is indeed aimed at recruiting, in a locally restricted fashion, other immune cells that can cooperate with CAR-T cell in fighting the tumors. In a specific development, TRUCKs were engineered to locally release IL-12 (Chmielewski, Kopecky, Hombach, & Abken, 2011), a pleiotropic cytokine that may induce potent antitumor responses by stimulating the immune system at multiple levels, which however causes severe toxicity following systemic administration. Chmielewski and colleagues demonstrated that such IL-12 TRUCK T cells exhibited improved efficacy towards solid tumors and could recruit and activate innate immune cells to mediate an antigen-independent antitumor reaction in the tumor lesion.

TRUCKs expressing catalase to protect T cells from oxidative stress (Ligtenberg et al., 2016) or heparanase to improve T cell penetration through the stroma (Caruana et al., 2015), are also being explored.

-Inducible suicide

To temporally control CAR-T cell activity, suicide genes have been introduced. Suicide genes encode for enzymes which convert non-toxic prodrug substrates into toxic drugs which ultimately results in the death of transduced T cells. Two main strategies are currently explored, the co-expression of the herpes simplex virus thymidine kinase (HSV-tk) which phosphorylates the guanosine analog gancyclovir into a toxic derivative, or an inducible caspase system that has been engineered to require a synthetic drug to dimerize, thereby initiating the caspase-9 apoptotic cascade (Bonini et al., 1997; Straathof et al., 2005).

-Switchable CARs

In order to fine control the timing, location, and the potency of T cell activition, “ON-switch” CARs have been designed. In this system, the antigen binding domain and the intracellular signaling moieties of a CAR have been separated and can assemble only in the presence of a heterodimerizing small molecule (Wu CY, Roybal KT, Puchner EM, Onuffer J, 2015).The previously described universal switchable CARs are another example.

-Dual CAR systems

Combinatorial antigen recognition

To minimize the “on-target-off-tumor” toxicity and improve tumor selectivity, some designs make use of a dual CAR system dissociating the activation signaling domain from the costimulatory signaling domain into two separate CARs with different antigen specificities.

Thus, ideally, only simultaneous engagement of both antigens initiates a robust CAR mediated antitumor response (Kloss, Condomines, Cartellieri, Bachmann, & Sadelain, 2013; Wilkie et al., 2012). However, to make this approach effective, a suboptimal CD3ζ primary signaling has to be pursued.

An alternative strategy is based on synthetic Notch (synNotch) receptors (Roybal et al., 2016). Target antigen recognition by the SynNotch receptor does not trigger T cell activation but leads to the cleavage of a transcriptional activator domain, which in turn enters the nucleus and drives the expression of a CAR with a distinct specificity. Thus, the engineered T cell is only activated when both the synNotch and the CAR are engaged on the target cell. However, one of the main limitations of this approach is that the induction of CAR expression should be fast enough to engage the tumor cell and time-limited to spare healthy tissues expressing the cognate antigen.

Bispecific CARs

Bispecific CARs (TanCARs) harboring two linked scFvs of different specificities have been designed to recognize two distinct antigens in order to offset antigen escape or to simultaneously target the tumor and its microenvironment (Grada et al., 2013). For example, to mitigate glioblastoma antigen escape and improve the antitumor activity of adoptively transferred T cells, Hegde and colleagues generated a HER2/ IL13Rα2 TanCAR while Byrd and colleagues described a TEM8/HER2 TanCAR targeting both $HER2^{+}$ tumors and their vasculature (TEM8: Tumor endothelium marker 8, a tumor endothelium-restricted antigen) (Hegde et al., 2016) (Tiara Byrd,

Kristen Fousek, Antonella Pignata, Amanda Wakefield, Brad St Croix, Bradley S Fletcher, Meenakshi Hegde, 2014).

-Inhibitory CARs (iCARs)

In this scenario, T cells co-express, together with a conventional CAR, an inhibitory CAR (iCAR) that recognizes an antigen expressed on normal tissues. The iCAR binding delivers a dominant inhibitory signal via a PD-1 or CTLA-4 endodomain. Thus, engagement of both target antigens on normal cells would suppress T cell activation, whereas recognition of only the activating ligand on tumor cells would result in T cell activation and effector functions (Fedorov, Themeli, & Sadelain, 2013). As proof of concept, this strategy has been exploited to modify T cells with a CD19.CAR and a prostate-specific membrane antigen (PSMA) iCAR containing PD-1 signaling domains. Transduced T cells were able to attack $CD19^{+}/PSMA^{-}$ cells but not $CD19^{+}/PSMA^{+}$ cells, both *in vitro* and *in vivo*, preventing off-target effects on $PSMA^{+}$ cells. (Fedorov et al., 2013).

-Switch CARs

In the attempt to overcome immune checkpoint pathways and suppression of anti-cancer T cell responses, T cells can be transduced with both a CAR and a chimeric switch receptor containing the extracellular domain of PD-1 fused to the transmembrane and cytoplasmic domain of the costimulatory molecule CD28 (PD-1-CD28 switch receptor) which allows to divert T cell negative costimulatory signals into positive ones. Liu and collaborators demonstrated the ability to augment CAR-T cell activity against different solid tumors

(mesothelioma and prostate cancer) by co-expressing a chimeric PD-1-CD28 switch-receptor (Liu et al., 2016).

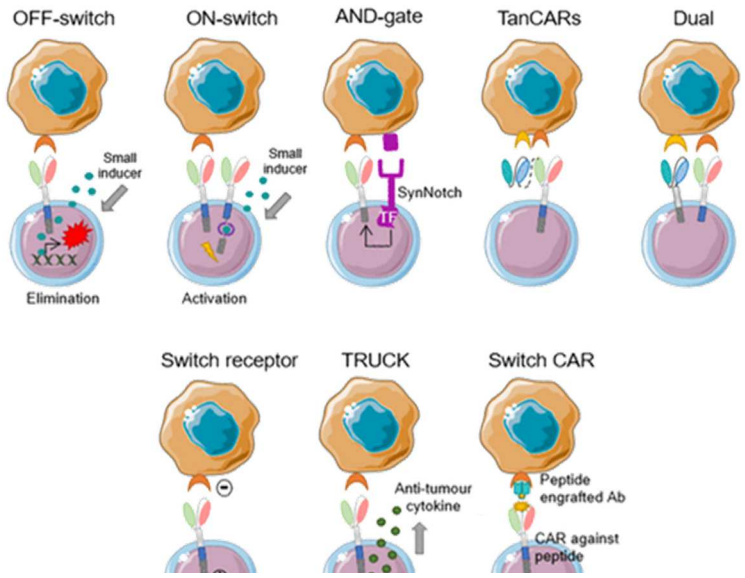

Figure 3. Schematic representation of next generation CARs. Adapted from Gowrishankar · Birtwistle, Mammalian Genome 2018 (Gowrishankar K , Birtwistle L, 2018)

4. Target antigen choice for AML immunotherapy

The choice of the target antigen is crucial for the success of any AML immunotherapeutic approach. Ideally, the target antigen expression should be restricted to AML cells, including LSCs, without expression on normal HSCs and other tissues and, preferably, it should play a critical role in AML pathogenesis. The AML target antigens described to date are "leukemia associated" (TAA) and thus also found on non-leukemic cells, especially those of hematopoietic origin, including progenitor and stem cell populations, raising concerns of myelotoxicity. Thus, current efforts are attempted to ameliorate the therapeutic index of AML targeted therapies in order to limit the off-target toxicity.

Among the well-characterized antigens, the CD123 and CD33 molecules have been largely explored in both preclinical and clinical studies.

4.1 CD123 immunotherapeutic target

Together with the CD33 molecule, the CD123 has emerged as a good therapeutic target in AML. CD123 is the α subunit of the human interleukin-3 receptor (IL-3R), it binds IL-3 with low affinity and together with the β subunit (βc or CD131, shared with IL-5 and GM-CSF) forms the functional heterodimeric high affinity IL-3R (Moretti et al., 2001). Thus, the α subunit provides specificity for its cytokine, while the βc subunit is involved in the formation of the high affinity receptor and in the signal transduction that occurs through three

principal pathways: JAK/STAT, MAPK and PI3-K (Martinez-Moczygemba & Huston, 2003) (figure 4).

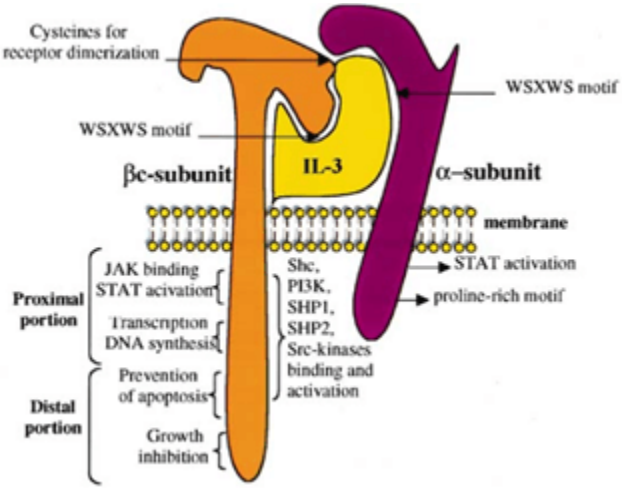

Figure 4. Structure and signaling pathways of CD123.
Adapted from *Creative Biolabs web page*

IL-3 is a pleiotropic cytokine, mainly produced by activated T lymphocytes, with a key role in hematopoiesis (Ihle, 1992). In addition, IL-3 acts also as a stimulator of endothelial cell proliferation (Brizzi et al., 1993). CD123 is present on a range of early and more mature hematopoietic progenitors and, within the committed cell populations, on both myeloid and lymphoid lineages (Huang et al., 1999). In particular, CD123 is expressed on the majority of CD34+ hematopoietic progenitor cells and while its expression is rapidly lost during erythroid and megakaryocytic differentiation, CD123 remains expressed on the granulocytic forms through mature neutrophils, as well as on monocytes and macrophages (Gabbianelli et al., 1996). The main characteristic that renders CD123 an attracting AML target

antigen is its differential pattern of expression between HSPC and AML cells, being overexpressed on AML blasts, CD34+ progenitors and more importantly on LSCs (Florian et al., 2006; Jordan et al., 2000; Ugo Testa, Torelli, et al., 2002; A. van Rhenen et al., 2007), and at the same time expressed at low levels by HSPC, both in terms of percentage of positive cells and MFI (Jordan et al., 2000). Moreover, recently, Ehninger and colleagues have evaluated the CD123 expression on AML blasts in a cohort of 298 patients founding that this TAA, like CD33, is expressed at high level on different AML FAB and particularly overexpressed in patients with unfavourable cytogenetics and molecular aberrations (Ehninger et al., 2014). CD123 is also expressed on other hematological malignancies, including blastic plasmacytoid dendritic cell neoplasm (BPDCN), plasma cell myeloma, Hodgkin lymphoma, mast cell disorders, myelodysplastic syndrome and ALL (Ugo Testa, Pelosi, & Frankel, 2014) (Li LJ, Tao JL, Fu R, Wang HQ, Jiang HJ, Yue LZ, Zhang W, Liu H, 2014) (Ruella et al., 2016).

CD123 expression in AML has been deeply investigated. Testa and colleagues have found that leukemic blasts overexpressing CD123 exhibit constitutive activation of STAT5, which results in a proliferative advantage and resistance to apoptosis (Ugo Testa, Riccioni, et al., 2002). Furthermore, CD123 overexpression has been associated with the number of leukemic blasts at the diagnosis and a negative prognosis (Ugo Testa, Riccioni, et al., 2002), representing a valuable marker for MRD assessment (Roug et al., 2014). Then, all these evidences make CD123 an interesting molecule to be targeted. To this aim, several different approaches have been investigated including the

use of its natural ligand, IL-3, or specifc mAbs. Concerning the first strategy, IL-3 has been conjugated with cytotoxic molecules. One of these compounds is DT_{388}IL-3, in which IL-3 is fused to the catalytic and translocation domains of the diphteria toxin (corresponding to the amino acid residues 1-388, DT_{388}) (A. E. Frankel et al., 2000). A variant was obtained by fusing the diphtheria toxin with a high affinity variant IL-3(K116W) resulting in the new fusion toxin DT_{388}IL3(K116W) (Hogge, Yalcintepe, Wong, Gerhard, & Frankel, 2006). Both products were able to induce pronounced cytotoxic effect both *in vitro* and *in vivo* directly correlated with the level of the receptor (Ugo Testa et al., 2005; Yalcintepe, Frankel, & Hogge, 2006). Following toxicology studies the toxin has been developed as a drug (SL-401) and tested in phase I clinical trials to evaluate the safety profile and its potential therapeutic impact in heavily pretreated AML patients (Frankel AE, Konopleva M, Hogge D, Rizzieri D, Brooks C, 2013). However, among 70 patients, only 2 durable complete responses and 5 partial responses were reported (Frankel AE, Konopleva M, Hogge D, Rizzieri D, Brooks C, 2013) (NCT00397579). Another clinical trial is currently recruiting participants for testing the DT388IL-3 as consolidation therapy in patients with adverse risk AML in first CR (NCT02270463). More encouraging results were instead obtained in the treatment of the BPCDN. Indeed, among 9 patients, 5 complete responses and 2 partial responses were reported (A. Frankel, Liu, Rizzieri, & Hogge, 2008). On November 2015, the EMA has granted orphan drug designation to SL-401 for the treatment of BPDCN.

The second CD123-targeting approach consists in the development of anti CD123 mAbs. In 1996, Sun and collaborators isolated a mAb, 7G3 clone, directed against the N-terminal domain of the IL-3Rα, demonstrating that it was capable of inhibiting IL-3-mediated proliferation of leukemic cell lines (Sun et al., 1996). The same effect was subsequently confirmed *in vivo* both on AML blasts and LSCs while marginally affecting HSCs (Liqing Jin et al., 2009). From 7G3 "humanization", affinity optimization and Fc engineering to improve binding to the FcR CD16, CSL362 antibody was developed (Busfield et al., 2014). Compared to CSL360, a non-Fc-engineered version, this antibody mediates ADCC of AML blasts, including LSCs, sparing normal pluripotent progenitors/stem cells. CSL362 synergizes with antileukemic drugs to inhibit the growth of primary AML cells grafted into NOD-SCID mice (Lee et al., 2015). Preclinical toxicology studies have been carried out in cynomolgus monkeys to evaluate the general CSL362 safety as well as the pharmacokinetic and pharmacodynamic properties. Thus, a phase I study using CSL362 was carried out in AML patients in CR but with high relapse risk (NCT0272145). At follow-up ≥ 6 months from first dose, 10 of 20 evaluable patients maintained CR. Of 6 evaluable patients who were MRD positive at baseline, 3 converted to negative on study. The treatment has shown to be safe and well tolerated (Smith et al., 2015).

Other approaches of CD123 targeting are based on the development and therapeutic use of bi- and tri- specific mAbs (CD123xCD3 bispecific scFv (Kuo, Wong, & Liu, 2012), CD123xCD16 bispecific scFv (Stein et al.,

2010) and CD123xCD33xCD16 trispecific scFv (Kügler et al., 2010) and T cell expressing CD123 CARs.

In particular, several phase I trials have been opened in various centers (City of Hope Comprehensive Cancer Center, University of Pennsylvania (Penn) Abramson Cancer Center, Weill Cornell Medicine, MDACC, Beijing) to exploit CD123 redirected CAR-T cells. At the UPenn, a pilot trial (NCT02623582) has been opened to study the feasibility, the safety and the potential myelotoxicity of a CAR-mediated CD123 targeting. To this aim, transiently modified mRNA CD123 CAR-autologous T cells have been exploited. Five adults with relapsed/refractory AML were treated with lymphodepleting chemotherapy followed by one to three doses (administered every 48–72 h) of CD123.CAR-T cells. It has been shown that less than 60% of the planned T cell doses have been successfully manufactured and all the treated patients had leukemia progression within a month of cell infusion. All patients experienced fever or CRS necessitating tocilizumab administration for toxicity management. No hematologic, neurologic, or vascular toxicity has been observed (Cummins et al., 2017). Due to the lack of anti-leukemia efficacy, the trial was terminated early. However, the limited “on target-off tumor” toxicity encouraged the development of a subsequent trial with stable CD123.CAR expression.

The City of Hope group also recently reported early results from its phase I trial (NCT02159495). Six adults with relapsed AML after HSCT were treated with 50×10^6 (dose level 1, DL1) or 100×10^6 (DL2) lentivirally transduced CD123CAR-CD28/CD3ζ autologous T cells

following lymphodepleting chemotherapy. Eligible patients were also treated with a second T cell infusion. One of two DL1 patients had transient morphologic remission and then responded to a second cell dose upon disease recurrence, ultimately achieving an MRD-level disease response. Two of 4 DL2 patients had CR and were able to undergo a second HSCT. The other 2 patients had partial responses. Grade 2 or less CRS were observed in most patients, and no dose-limiting toxicity, including myeloablation, has been observed to date. A patient with refractory BPDCN has also been treated and experienced a CR. This trial is currently enrolling patients (Budde et al., 2017).

4.2 CD33 immunotherapeutic target

CD33 is an attractive therapeutic target in AML due to its broad expression on leukemic blasts in the majority of AML patients and on LSCs in some patients (Ehninger et al., 2014; Roland B. Walter, Appelbaum, Estey, & Bernstein, 2012). CD33 is a member of the sialic acid-binding immunoglobulin (Ig)-like lectins (Siglecs). It is composed by two extracellular Ig-like domains (a V-set Ig-like domain and a C2-set Ig-like domain), a transmembrane region and a cytoplasmic tail with two conserved tyrosine-based inhibitory signaling motifs for recruitment and activation of the tyrosine phosphatases SHP-1 and SHP-2 or suppressor of cytokine signaling 3 (SOCS3) (figure 5). Indeed, CD33 has molecular features of an inhibitory receptor, however, its downstream signaling and physiological function are poorly understood. Some evidences suggest that CD33 and related Siglecs

with inhibitory signaling motifs negatively regulates inflammatory and immune responses through dampening tyrosine kinase-driven signaling pathways (MacAuley, Crocker, & Paulson, 2014).

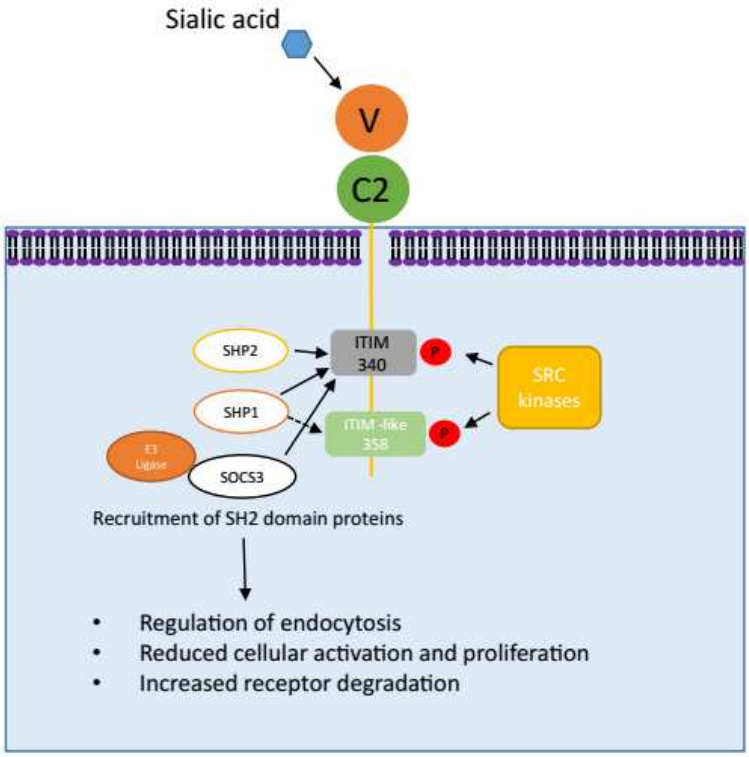

Figure 5. Structure and signaling pathways of CD33.
Adapted from Laing A.A. *et al* Experimental Hematology 2017(Laing, Harrison, Gibson, & Keeshan, 2017)

CD33 has endocytic properties that have been exploited for intracellular delivery of cytotoxic drugs by exploiting CD33-directed antibody-drug conjugates (ADC). Thus, the existence of CD33 splice variants is relevant for its therapeutic targeting. Indeed, a CD33 splice variant missing exon 2 results in loss of the V-set domain which is recognized by all currently clinically exploited CD33-directed

therapeutics (Hernández-Caselles et al., 2006). Also intracellular mutations in the ITIMs have been shown to impair the endocytic properties of the receptor and subsequent internalization of the antibody–CD33 complex (Linenberger, 2005).

CD33 expression on the myeloid lineage occurs at the early stages of myeloid differentiation, and is found on normal MPPs and unipotent myeloid progenitors, myeloid precursors, and maturing granulocytes and monocytes (Laszlo, Estey, & Walter, 2014). However, CD33 expression is also found on HSCs. Taussig et al., by performing xenotransplantation assays in immunodeficient mice, showed that CD34+CD38-CD33+ CB and healthy BM cells were capable of multilineage engraftment and self-renewal, indicating that this fraction contains HSCs (David C. Taussig et al., 2005). Importantly, Krupka et al. showed that CD33 was expressed on the CD34+CD38- BM cells from healthy donor but at significantly lower levels compared to CD34+CD38- cells from AML patients (Krupka et al., 2014). CD33 expression on leukemia blasts varies greatly across individual patients with regard to antigen density per cell and percentage of CD33+ cells but correlates with certain cytogenetic and molecular abnormalities. FLT3-ITD-, NPM1-, and CEBPA-mutated subgroups are consistently associated with high CD33 expression in both adult and childhood AML. Complex karyotypes and core-binding factor (CBF)-AML (t(8;21) and inv(16)) are associated with low CD33 expression in the bulk AML population (Ehninger et al., 2014; Khan et al., 2017; Krupka et al., 2014; Pollard et al., 2016).

Overall, considering the CD33 differential expression between leukemic cells and normal HSPCs (Ehninger et al., 2014), CD33 targeted immunotherapeutic approaches have been investigated. Gemtuzumab Ozogamicin (GO, Mylotarg®), an anti-CD33 mAb delivering a DNA-damaging calicheamicin derivative, was the first targeted treatment in AML to receive accelerated approval by the FDA in 2000 for adults older than 60 years with relapsed AML. This approval was based on early clinical studies of GO monotherapy in AML patients. In a phase I dose escalation study, GO has been administered to 40 patients with relapsed or refractory AML with doses ranging from 0.25–9 mg/m2. Twenty percent of patients achieved a reduction in morphological detectable AML with 12.5% achieving CR. The occurred adverse events were infusion-related fever and chills and mylosuppression. The recommended phase II dose was 9 mg/m2 given at days 1 and 14 (Sievers, 1999). Three open-label multicenter phase II studies started. Preliminary analysis of 142 patients (median age = 61 years) with CD33+ AML in first relapse who were enrolled in these three studies demonstrated an overall response rate of 30% (Pagano, Fianchi, Caira, Rutella, & Leone, 2007).

As part of the post-approval commitment, a randomized phase III Southwest Oncology Group (SWOG) S0106 study (NCT00085709) was designed to compare two different regimens of chemotherapy with or without a single dose of GO in adult patients less than 60 years with previously untreated de novo AML. Six hundred and thirty seven AML patients were randomized to receive daunorubicin and cytarabine (DA) plus GO (6 mg/m2 on day 4) versus DA alone. No clinical benefit

was observed in patients treated with GO. Remarkably, there was an increase in treatment-related mortality in the GO arm compared to the DA arm (5% vs 1% respectively, P = 0.0062) (Petersdorf et al., 2013). For these reasons, GO was voluntarily withdrawn from market in October 2010. Results from several subsequent large randomized clinical trials have shown that dose and sequence of GO administration are critical to avoid toxicity and achieve maximum benefit from addition of GO to chemotherapy. In the phase III randomized ALFA0701 study (NCT00927498), 278 untreated *de novo* AML patients were randomized to receive induction therapy with daunorubicin (60 mg/m2 for 3 days) and cytarabine (200 mg/m2 for 7 days) plus fractionated doses of GO (3 mg/m2 on days 1, 4, 7) versus DA alone. CR rates and treatment-mortality rates were similar between the two arms. However, the primary endpoint of the study which was 3-year event free survival (EFS) was significantly longer in patients in the GO arm compared to the control arm (15.6 vs 9.7 months respectively, HR = 0.66, P = 0.0026) (Castaigne et al., 2014). In the phase III GOELAMS AML 2006 IR study (NCT00860639), 238 adults with *de novo* AML were enrolled and randomized to receive either standard chemotherapy with or without GO (6 mg/m2). Similar to the ALFA0701 study, no differences in CR rates and treatment mortality rates have been observed between the two arms. However, the EFS was significantly longer in patients in the GO arm (53.7% vs 27%, P = 0.03) (Hills et al., 2014). Furthermore, findings from the UK MRC AML-15 and UK NCRI AML-16 trials, which evaluated the role of GO (3 mg/m2) with induction therapy among younger and older patients respectively,

showed that GO significantly improved the survival and reduced relapse risk with little increase in toxicity (Hills et al., 2014).

Finally, a meta-analysis of the ALFA-0701, S0106, GOELAMS AML2006IR, AML-15 and AML-16 studies which comprised of 3325 patients demonstrated that GO significantly reduced the risk of relapse and improved 5-year relapse free survival (OR = 0.87, P = 0.005) and overall survival (OR = 0.81, P = 0.0001). Additionally, it has been shown that lower doses (3 mg/m2) of GO were as effective as higher doses and had improved safety (Hills et al., 2014).

These results, have allowed recent breakthrough re-approval of GO for treating both de novo and relapsed/refractory CD33+ AML as a fractionated low dose of 3 mg/m2.

In addition to GO, several other CD33-directed ADC have been designed and are at different stages of clinical development. Vadastuximab talirine (SGN33A) is generated by conjugating a highly potent, synthetic DNA cross-linking pyrrolobenzodiazepine dimer (SGD-1882) to a humanized CD33 mAb (h2H12ec) via a protease-cleavable maleimidocaproyl-valine-alanine linker (Sutherland et al., 2013). Early clinical trials in relapsed AML have shown encouraging results. However, following a higher rate of mortality in a Phase III clinical trial, all SGN33A studies have been placed on hold. IMGN779 is another ADC consisting of a humanized CD33 antibody (Z4681A) conjugated via a cleavable disulfide linker (sulfo-SPDB) to a DNA crosslinking indolino-benzodiazepine dimer containing a monoimine moiety (DGN462) (Kovtun et al., 2018). Preliminary results from the first-in-human experience display a tolerable safety profile and anti-

leukemia activity (Cortes et al., 2017). ^{225}Ac-lintuzumab, is an anti-CD33 radioimmunoconjugate exploiting ^{225}Actinium radioisotope to generate a cytotoxic dose of α radiation killing AML blasts. Promising preliminary results, in terms of blast reduction and CR induction, have been reported from phase I trial (Jurcic et al., 2016) and recently also from a phase II trial (Finn et al., 2017).

CD33 targeting has also been explored in the context of adoptive T-cell therapy. AMG330 is a CD33/CD3 Bi-specific T-cell engager (BiTE), which simultaneously binds to both CD33 on the leukemic cells and CD3 on the effector T cells, which in turn are redirected and activated against the leukemic target. No preliminary data have been so far reported from the ongoing first-in human trial (NCT02520427) in adults aged ≥18 years.

Chimeric antigen receptor T cell (CART) therapy, using CD33 as a target, is being investigated as well. A clinical trial started in Beijing (NCT01864902) to assess the safety of second-generation autologous CD33.CAR CIK cells in patients with relapsed/refractory AML. A case report of a 41-year-old patient with refractory AML has been described. CD33.CAR-CIK cells were infused in a total of $1.12x10^9$ cells (38% CAR expression) without any conditioning chemotherapy regimen. He experienced a partial response with marked reduction in marrow blasts prior to leukemia progression at 9 weeks from CIK cell infusion. The adverse event documented has been a clinically significant CRS with fever and elevated inflammatory cytokine levels. Of note, no signs of sinusoidal obstruction syndrome/veno-occlusive disease (a potential “on target-off tumor” toxicity effect due to CD33

expression on hepatic Kupffer cells) have been observed (Q. S. Wang et al., 2015).

A first-in-man phase I trial started in Suzhou to explore the safety of escalating infusion doses of CD33.CAR redirected NK-92 irradiated cell line in the treatment of relapsed/refractory AML patients (NCT02944162), with the aim of provide an off-the-shelf CD33.CAR immunotherapy that could be advantageous and cost-effective. Despite the treatment has been well tolerated (only grade I CRS has been observed) this study did not demonstrate clinical efficacy (Tang et al., 2018). A trial exploiting CD33 CAR T-cells is also opened at the MD Anderson Cancer Center (MDACC) (NCT03126864) and another one specific for children, adolescents, and young adults with relapsed/refractory AML is in development at the National Cancer Institute and Children's Hospital of Philadelphia, sponsored by the Pediatric Blood and Marrow Transplant Consortium.

5. *Sleeping Beauty* (SB) non viral gene transfer technology

Transposons are mobile genetic elements naturally occurring in the genome that are able to 'jump' from a donor site to an acceptor site. This feature has been exploited to harness transposons for gene transfer. Since, to date, no active vertebrate transposons are available, most of the transposon systems used as gene vectors have been reconstructed from fixed transposon fossils. In particular, the *Sleeping Beauty* (SB) platform, currently representing the most widely used non viral gene delivery system, belongs to the Tc1/mariner class of transposons and has been derived from salmonid fish (Ivics, Hackett, Plasterk, & Izsvák, 1997). Like the other transposon systems, it consists of two components that can be physically separated in the form of bi-component vector systems: the transposon, containing the transgene of interest flanked by inverted repeat containing direct repeated sequences (IR/DR), and the transposase, which is responsible for the transposition. In particular, the SB transposase recognizes the IR/DR sequences and, by a "cut and paste" mechanism, mobilizes the SB transposon from one DNA site to another, which invariably contains a TA dinucleotide that is duplicated on both flanks of the transposon following integration. To improve the transposition rates, both the components of the system have been extensively engineered. Optimization of the original transposon (pT) at the level of the inverted terminal repeats (ITRs) resulted in the improved transposon versions pT2, pT3, pT2B and pT4 (Cui, Geurts, Liu, Kaufman, & Hackett, 2002; Izsvák et al., 2002; Y. Wang et al., 2017; Yant, Park, Huang, Mikkelsen,

& Kay, 2004). Starting from the original SB10 transposase, the mutagenized hyperactive versions include SB11 (3 fold higher than SB10), SB12 (4 fold higher than SB10), HSB1-HSB5 (up to 10 fold higher than SB10), HSB13-HSB17 (up to 17 fold higher than SB10), SB100X (100 fold higher than SB10) and SB150X (130 fold higher than SB10) (Baus, Liu, Heggestad, Sanz, & Fletcher, 2005; Geurts et al., 2003; Ivics et al., 1997; Mátés et al., 2009; Voigt et al., 2012; Yant et al., 2004; Zayed, Izsvák, Walisco, & Ivics, 2004).

Compared to viral vectors, the SB technology exhibits several advantageous features: close-to-random integration profile; greater capacity for genetic cargo; low immunogenicity; decreased production costs; increased biosafety (P. Hackett, Largaespada, Switzer, & Cooper, 2013).

Importantly, the implementation of an mRNA source for transient delivery of the SB transposase increases the biosafety of this approach by reducing the toxicity associated to DNA nucleofection and decreasing the risk of remobilization of the transgene due to the transient transposase expression (Wilber et al., 2007). Furthermore, a recent improvement consists in the employment of minicircle (MC) vectors as carriers of the SB transposon components. MC vectors are small and supercoiled molecules lacking any bacterial sequences and containing almost exclusively the transgene. It has been shown that the use of MC vectors improve the transposition efficiency and the transgene expression, while decreasing the toxicity associated with DNA delivery (Monjezi et al., 2017). This is likely due to their smaller sizes which facilitate both their cross through the cell membranes and

transposon/transposase complex formation (Izsvák, Ivics, & Plasterk, 2000). Additionally, the lack of antibiotic resistance genes is relevant in clinical applications, because excludes the potential for horizontal transfer to host bacteria and unintended integration into the host genome. Finally, further safety improvements can be achieved by the development of different systems of site-directed gene delivery into target selected loci to improve the safety profile of transposons (Kovač & Ivics, 2017).

Thanks to the preclinical optimization, the SB gene therapy approach is now also applicable in clinical practice. FDA has approved the first clinical trial using SB to generate T cells expressing CD19 CARs for B cell malignancies therapy (P. B. Hackett, Largaespada, & Cooper, 2010) and the first preliminary results have been establish the proof-of-concept for the feasibility and safety of using SB-modified CAR-T cells. As a follow-up to this study, a clinical trial (NCT02529813) is currently underway at MDACC infusing CAR-T cells in patients with refractory active ALL and lymphoma to evaluate refinements to the SB system. In USA there are currently ten ongoing clinical trials employing SB in the version SB11/pT2 to treat B-cell malignancies and metastatic breast cancer (Narayanavari & Izsvák, 2017). Also our group recently described the large-scale production and preclinical evaluation, in terms of both efficacy and toxicity, of donor-derived SB engineered CD19.CAR-CIK cells for a pilot Phase I/II trial that is currently ongoing to treat relapsed B-ALL (Magnani et al., 2018) (NCT03389035).

Scope of the thesis

The aim of the present PhD project was to characterize non-viral Sleeping-Beauty engineered cytokine-induced killer (CIK) cells with both anti- CD123 and CD33 CARs as a potential tool for the treatment of acute myeloid leukemia (AML).

The first chapter provides a general overview on AML pathological features as well as on current and promising therapeutic strategies, focusing in particular on CAR-T cell technology, providing the literature elements to understand the background scenario of the developed projects.

While the on-target off-tumor effect associated to the CD33 targeting is mostly limited to the hematological compartment, the CD123 targeting, due to the potential recognition of low CD123-positive endothelial tissue, demands a higher level of caution. Thus, in search for an optimization of this strategy in the second chapter we present our recent published work focused on dissecting the effect of several variables involved in the CAR design, known to modulate CAR-T cell functional profiles in a context-dependent manner, such as CAR binding affinity, CAR expression and target antigen density.

The third chapter includes our in preparation manuscript focused on the CD33 targeting in which we preclinically assessed *in vitro* and *in vivo* efficacy profiles of CD33.CAR-CIK cells by exploiting the novel combination of the Sleeping-Beauty system comprising the SB100X hyperactive transposase with the novel pT4 transposon.

Finally, the forth chapter reports the general conclusions of these studies, summarizing the main findings achieved, including a focus on future developments.

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Chapter 2

Balance of Anti-CD123 Chimeric Antigen Receptor Binding Affinity and Density for the Targeting of Acute Myeloid Leukemia

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ABSTRACT

Chimeric antigen receptor (CAR)-redirected T lymphocytes are a promising immunotherapeutic approach and object of pre-clinical evaluation for the treatment of acute myeloid leukemia (AML). We developed a CAR against CD123, overexpressed on AML blasts and leukemic stem cells. However, potential recognition of low CD123-positive healthy tissues, through the on-target, off-tumor effect, limits safe clinical employment of CAR-redirected T cells. Therefore, we evaluated the effect of context-dependent variables capable of modulating CAR T cell functional profiles, such as CAR binding affinity, CAR expression, and target antigen density. Computational structural biology tools allowed for the design of rational mutations in the anti-CD123 CAR antigen binding domain that altered CAR expression and CAR binding affinity without affecting the overall CAR design. We defined both lytic and activation antigen thresholds, with early cytotoxic activity unaffected by either CAR expression or CAR affinity tuning but later effector functions impaired by low CAR expression. Moreover, the anti-CD123 CAR safety profile was confirmed by lowering CAR binding affinity, corroborating CD123 is a good therapeutic target antigen. Overall, full dissection of these variables offers suitable anti-CD123 CAR design optimization for the treatment of AML.

INTRODUCTION

Adoptive cellular immunotherapy (ACT) employing T lymphocytes engineered with chimeric antigen receptors (CARs) has demonstrated impressive antitumor effects in relapsed or refractory patients affected by B cell neoplasms,[1–4] providing ACT with the possibility of being translated to other aggressive hematological malignancies, such as acute myeloid leukemia (AML), which is still associated with high relapse rates.[5] CARs are artificial molecules comprising an extracellular antigen binding domain, usually derived from a monoclonal antibody (mAb) in the form of a single-chain fragment variable (scFv) and an intracellular signaling moiety, such as the CD3-z complex, together with one or more co-stimulatory signaling modules.[6–8] CAR T cell technology is an extremely sensitive approach, because the minimum number of surface target molecules that can be recognized is markedly low compared to the mAb from which the same CAR has been derived due to avidity effects arising from the presence of multiple CARs on the same T cell.[9] Therefore, the target antigen choice for CAR-based therapies should be carefully evaluated, because drawbacks have emerged in the clinical translation of CARs. Serious side effects led in the worst cases to the death of the treated patients because of CAR-mediated recognition of low expressed tumor-associated antigens (TAAs) on normal tissues, a problem known as “on target, off tumor.”[10,11] Thus, strategies aimed at ameliorating the CAR-based approach should focus on an off-tumor target antigen expression at

very low levels or be limited to healthy tissues whose depletion could be well tolerated by the organism without causing major morbidity (i.e., CD19 targeting in B cell malignancies). In the context of AML, the disease heterogeneity and similarities between hematopoietic stem cells (HSCs) and leukemia stem cells (LSCs) make the identification of a good target antigen a challenging aspect. In this scenario, our attention has been focused on the interleukin-3 receptor alpha (IL-3RA; CD123), which is a poor-prognosis overexpressed marker on AML blasts and LSCs.[12] An anti-CD123 CAR previously generated by our group was able to eradicate AML blasts in vitro and in vivo, showing a safer profile toward normal hematopoietic stem or progenitor cells (HSPCs) compared to anti-CD33 CAR.[13,14] However, a moderate in vitro toxicity was observed toward CD123-low-expressing endothelium and monocytes, suggesting a higher caution level for future clinical translation to avoid potential on-target, off-tumor effects.

Although the exact structural and signaling features for an ideal CAR design are still undefined, it has been demonstrated in several tumor models that it is possible to affect the functional activity of engineered T cells by tuning the CAR-TAA binding properties.[15–18] However, further investigation of CAR binding affinity in a more context-dependent manner is warranted given the differences in each pathological situation in terms of percentage and density of antigen-positive cells, antigen localization, accessibility of the targeted epitope, and CAR design. In a model of anti-CD20 CAR, both a lytic and an activation

threshold have been defined as the number of cell surface antigens required to produce full cytotoxic activity and activation or expansion of CAR^+ T cells, respectively.[9] Therefore, we aimed to unravel how the interplay among CAR affinity, antigen density, and CAR expression could affect anti-CD123 CAR-redirected effector cell efficacy and safety profiles. We generated and tested anti-CD123 lower-affinity CAR mutants by single-residue substitution on the wild-type (WT) CAR scFv. In this way, the affinity should be the only variable in the system while the same epitope binding site and the overall features within the CAR structure are maintained. Mutation of these residues resulted in lower-affinity antibodies, with K_{off} (off-rate constant) identical to the original molecule but a slower K_{on} (on-rate constant). This is expected to decrease CAR sensitivity toward low antigen concentrations, as it does in off-tumor organs with low target antigen expression, thus favoring the recognition of overexpressing tumor cells. The CAR-redirected effector T cell population has been represented by cytokine-induced killer (CIK) cells, an ex vivo, easily expandable population that is greatly heterogeneous, displaying natural killer-like properties and showing powerful cytolytic activity against both solid and hematological tumors with no graft versus host disease in the allogeneic clinical setting.[18–20] We show that the CAR expression profile can strongly influence CAR-CIK cell effector functions, particularly their later properties, such as proliferation and cytokine production. In addition, our set of anti-CD123 CAR affinity mutants

(CAMs) allowed us to define in vitro antigen-specific lytic and activation functional thresholds, influenced by CAR binding kinetics.

RESULTS

Structure-Based Design of CAR Mutants with Lower Affinity for the CD123 Antigen

To explore the role of the sole CAR binding affinity, we used a combination of computational tools and structural analysis to design mutants of the anti-CD123 antibody that would (1) equally recognize the same epitope within the CD123 antigen and (2) have reduced binding affinity due to slower association rates. We first obtained an atomic model of the 7G3 antibody (previously used to design our anti-CD123 CAR)[13] and of its complex with the CD123 target antigen by computational simulations, validated by available experimental information regarding the antibody binding site.[21,22] Computational and visual analysis allowed us to identify single antibody residues in the antigen binding loops that, once mutated, could decrease, but not abrogate, the antibody binding. We then produced the mutated antibodies as scFv versions and measured their binding properties for the CD123 antigen with surface plasmon resonance (SPR). In addition to the WT antibody, four mutants named CAMs (CAR affinity mutants) were selected and incorporated in a CAR to engineer CIK cells for biological characterization. Two antibody mutants had an affinity approximately 10- and 100-fold weaker than the WT antibody (K_D: 3 x

10^{-9} M for WT, 3 x 10^{-7} M for CAM-L [CAM low affinity], and 3 x 10^{-8} M for CAM-M [CAM medium affinity]). Two further mutants with binding properties similar to the WT molecule were generated as controls (1 x 10^{-9} M for CAM-H1 [CAM high affinity mutant 1] and 2 x 10^{-9} M for CAM-H2 [CAM high affinity mutant 2]). All mutants had K_{off} rates similar to the WT molecule but different K_{on} rates (K_{on}: 1 x 10^5 $M^{-1}s^{-1}$ for WT, 8 x 10^2 $M^{-1}s^{-1}$ for CAM-L, and 4 x 10^4 $M^{-1}s^{-1}$ for CAM-M; K_{off}: 2 x 10^{-4} s^{-1} for WT, 2 x 10^{-4} s^{-1} for CAM-L, and 9 x10^{-4} s^{-1} for CAM-M) (Figure 1). The mutations did not alter the binding site and cross-competed with the WT (data not shown).

Reduced CAR Expression Impairs the Anti-CD123 CAR-CIK Cell's Later Efficacy Profile

The typical CIK cell phenotype was minimally affected by CAR engineering for all tested conditions (WT CAR and CAMs), being comparable to the unmanipulated NO DNA control (Figure S1). The percentage of CAR expression at the end of culture accounted for 61% ± 13%, for WT CAR, 62% ± 14% for CAM-H1, and 20% ± 6% for CAM-H2 (Figure 2A). We also observed a reduction of CAM-H2 mean fluorescence intensity (MFI) in comparison to the other constructs (Figure 2B). Cytotoxic activity, cytokine production, and proliferation ability of CAR-redirected CIK cells against two cell targets were evaluated: THP-1 (AML cell line, CD123-positive control) and MHH-CALL-4 (B-cell acute lymphoblastic leukemia [B-ALL] cell line, CD123-

negative control). We applied a variant to the canonical cytotoxic assay by performing a double-target challenge through co-culture of both target cell lines, together with CAR-redirected CIK cells. This allowed evaluation of their ability to specifically recognize the $CD123^{+}$ AML cells in the presence of a second, unintended target (negative control or low antigen-positive target cells). CAR-engineered CIK cell lytic activity against the MHH-CALL-4-negative control was comparable to NO DNA, indicating that the mutations introduced did not render them unspecifically active against a CD123-negative target. Furthermore, CAR-engineered CIK cells showed similar and higher killing ability against the THP-1-positive target, compared to NO DNA, despite the markedly reduced expression of CAM-H2 (Figure 2C). By contrast, upon stimulation with THP-1 target cells, the CAM-H2-CIK cell variant proliferated less than the others, with levels comparable to the NO DNA control (Figure 2D). This suggests that the low expression of CAM-H2, and not the affinity for its target, has a strong impact on later effector functions (Figure 2D). CAR-redirected CIK cells were also able to produce cytokines, such as interleukin-2 (IL-2) and interferon gamma (IFN-γ), in response to the THP-1 cell line, and not to MHH-CALL-4. However, CAM-H2-CIK cells stimulated with the THP-1 target produced statistically significant lower levels of IFN-γ and IL-2 in comparison to WT- and CAM-H1-redirected CIK cells (Figures 2E and 2F). These results suggest that, at comparable binding affinities (CAM-H1 and CAM-H2), an optimal CAR expression is required for later

effector functions, such as proliferation and cytokine production, in contrast to the more immediate cytotoxic activity.

CAR Expression Affects the Anti-CD123 CAR-CIK Cell's Later Efficacy Compared to CAR Affinity

To characterize the biological effects of the affinity tuning on CAR-CIK cell functional properties, we focused on CAM-L and CAM-M mutants with binding affinity approximately 100- and 10-fold weaker than the WT (K_D: 3 x 10^{-9} M for WT, 3 x 10^{-8} for CAM-M, and 3 x 10^{-7} for CAM-L). Both the genetic manipulation and the affinity tuning minimally affected the phenotype of engineered CIK cells, being comparable to the NO DNA control (Figure S2). WT CAR, CAM-H1, and CAM-L had similar expression profiles, whereas both CAR expression and MFI values appeared lower for CAM-M (CAM-M = 41% ± 7%; WT CAR = 70% ± 5%; CAM-H1 = 74% ± 5%; CAM-L = 62% ± 5%) (Figures 3A and 3B). The double-target challenge cytotoxic assay showed that CIK cells engineered with the lower-affinity CAM-M and CAM-L receptors maintained significant killing activity for highly $CD123^+$ cells in comparison to NO DNA. All tested CAM-CIK cells showed comparable killing capacity against primary AML cells and no activity toward the co-cultured healthy bone marrow cells (Figure 3C). Similarly, the highly CD123-positive THP-1 cells were lysed by the engineered CIK cells, whereas the co-cultured CD123-negative MHH-CALL-4 cells were not. The proliferation assay confirmed CAM-L- and CAM-M-redirected CIK

cell specificity and ability to respond to the high CD123+ target compared to the negative control. If CAM-L had a response comparable to WT- and CAM-H1-CIK cells, proliferation induced by CAM-M was lower. This highlights that CAR expression profile is more relevant than affinity in determining later effector functions (Figure 3D). CAM-L expression appeared sufficiently adequate to grant redirected CIK cells a proliferative advantage with respect to CAM-M and CAM-H2, even if its binding affinity is one and two orders of magnitude lower, respectively. The cytokine production profile after stimulation with CD123+ target cells further showed that CAM-M-CIK cells, like CAM-H2, produced significantly less IFN-γ and IL-2 than the other constructs (Figures 3E and 3F). Cytokine production was more abundant in the presence of CAM-L, strengthening the more prominent role of CAR expression over target affinity for later cellular functions. All these assays confirmed that CAM-L is equally effective in redirecting CIK cells as WT CAR and CAM-H1, despite having a 100-fold lower binding affinity for its target antigen. We also measured the killing of CD123-positive cells over time in a long-term cytotoxic assay for 1 week (Figure S3A). CAM-L-, WT CAR-, and CAM-H1-CIK cells were all found to be effective in killing THP-1 cells, while sparing $CD123^-$ negative MHH-CALL-4 cells, in comparison to NO DNA. Altogether, the preceding observations indicate that a 10^{-7} M binding affinity for the target antigen is sufficient to ensure significant and robust functional

responses in vitro against high CD123$^+$ leukemic conditions in both THP-1 and primary AML cells.

Lowering CAR Binding Affinity Confirms CD123 as a Safe Target Antigen for CAR-CIK Cell Approach

With the aim of studying the role played by CAR binding affinity in the recognition of cells with lower CD123 expression, we investigated the functional effects of CAM-L-CIK cells on cell lines with a progressive CD123 expression and density (Figure S4). We chose THP-1 (97% of CD123$^+$ cells, with 7,435 ± 1,986 CD123 molecules/cell) and KG-1 (81% of CD123$^+$ cells, with 4,550 ± 559 CD123 molecules/cell) AML cell lines as high CD123$^+$ target cells. The AML U937 cell line and healthy telomerase immortalized human microvascular endothelium (TIME) cells were representative of CD123 low-expressing cells, with a CD123 expression of 13% for U937 and 9% for TIME and comparable numbers of CD123 surface molecules (U937 = 1,603 ± 215; TIME = 1,688 ± 328). As before, we confirmed both the efficacy and the specificity of CAR-CIK cells when co-cultured with CD123+ THP-1 and CD123$^-$ MHH-CALL-4 (Figure 4A). Co-culture of the THP-1 cell line with low CD123- positive conditions showed preferential killing of THP-1 cells (Figures 4B and 4C), highlighting that the presence of a second target, low CD123$^+$, does not inhibit the killing of a first intended highly CD123$^+$ target. Although CAM-L-CIK cells were as effective as WT- and CAM-H1-CIK cells in killing highly CD123$^+$ cells, they showed a trend of lower killing

activity in comparison to the other constructs when challenged with low $CD123^+$ cells (Figure 4D). To characterize the safety profile of CAM-L-CIK cells against the TIME healthy tissue, we also performed a 72 hr long-term cytotoxicity assay (Figure S3B). Results supported the observation that CAM-L-CIK cells had cytotoxic effects against THP-1 cells, but not against the MHH-CALL-4-negative control or the TIME endothelial cells. This strengthens the idea that a mutant with 10^{-7} M affinity, 100-fold lower than the WT, is able to retain an optimal effector profile, carrying a safe profile against the healthy tissue. Furthermore, because the killing activity exerted by CAR-CIK cells against the low $CD123^+$ cells was higher than the negative control, but significantly lower compared to the high $CD123^+$ leukemic target, it appeared that the presence of approximately 1,600 CD123 molecules/cell is sufficient to induce the activation of the lytic cell machinery by CAR-redirected CIK cells, in line with the presence of a lytic threshold suggested by other literature.[9] By contrast, no proliferation activity was detected against the low $CD123^+$ cells (Figure 5A), while there was a strong and equal proliferative response obtained against the high $CD123^+$ targets for all CARs studied. According to these findings, ≈1,600 CD123 molecules are sufficient to trigger the lytic activity, but not to induce the proliferation of CAR-redirected CIK cells. Concerning the cytokine production, CAR-CIK cell sensitivity to the targets appeared to be clearly influenced by their CD123 positivity. A hierarchical cytokine production, from the highly

CD123^{+} THP-1 cells to the lower and lowest CD123 levels of KG-1 and U937 cells, was found. This was particularly evident in the case of IFN-γ and IL-2 production. Instead, a significant amount of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) was only produced in the presence of THP-1 cells (Figure 5). No activation was induced against the CD123-negative control and the healthy tissue by all CAR-CIK cells tested and for all cytokines analyzed. Moreover, with the aim of evaluating the response of anti-CD123 CAR-redirected CIK cells against the endothelium in a more physiological context, we set up a co-culture of Matrigel-embedded endothelial cells and CIK cells. Endothelial TIME cells are known to undergo tubule formation when cultured on Matrigel.[23] Thus, the impact on vessel formation induced by unmodified and CAR-redirected CIK cells was monitored after 4 hr co-culture. Branching points were counted to have a measure of the potential impairment on endothelial network stability and spreading. The same and limited impairment of vessel formation was observed, with no differences encountered between NO DNA and CAR-redirected CIK cells (Figures 6A and 6B). Therefore, in this model of endothelial vessel formation, anti-CD123 CAR CIK cells were not activated more than the unmanipulated counterpart, independently of CAR binding affinity.

Later Effector Functions, and Not Early Cytotoxic Activity, Are Proportional to CAR Downmodulation

After antigen engagement by T cells with canonical T cell receptors (TCRs), a serial triggering process leads to the internalization of surface TCR molecules until the activation threshold is overcome. This process is due to the low affinity of TCRs, which can be sequentially engaged and thus serially downmodulated. According to this model, the signaling strength is proportional to the rate of receptor internalization and thus to the potency of T cell activation.[24–28] Similarly, CAR+ T cell activation can be described as a specific case of TCR-antigen engagement in which serial CAR triggering is abrogated due to the high affinity of CARs compared to TCRs.[29] Therefore, to better understand the mechanisms at the basis of the lower CIK cell functional later responses encountered with both CAM-M and CAM-H2 receptors, we analyzed the CAR downmodulation in relation to both later cellular responses and lytic activity. The amount of CAR downmodulation was found to be independent of binding affinity for the target antigen. CAM-L, for instance, had the same amount of downmodulation as WT CAR and CAM-H1, despite a 100-fold lower binding affinity. By contrast, the lower-expressing CAMs, CAM-M and CAM-H2, had progressively lower amounts of downmodulation and lower IFN-γ production while having higher affinity than CAM-L (Figures 7A and 7B). By plotting the percentage of the killing activity detected against THP-1 cells as a function of CAR downmodulation, we found no apparent relation between the cytotoxic activity on highly CD123-positive THP-1 cell lines and the amount of CAR

downmodulation. The killing response of CAM-M- and CAM-H2-CIK cells was similar to that of the other CARs, even with a lower CAR downmodulation pattern (Figure 7C). The amount of CAR downmodulation was found to be related to CD123 antigen density (Figure 7D), accounting for a different strength of CAR^+ T cell activation and thus functional related responses (Figures 5 and 7D). Increasing numbers of CD123 molecules per cell lead to a larger amount of CAR downmodulation, resulting in escalating CAR^+ T cell activation. The results are comparable among WT CAR, CAM-H1, and CAM-L and thus independent of binding affinity, reflecting the hierarchical trend previously observed with cytokine production.

DISCUSSION

In search for a CAR-mediated targeting optimization, we investigated the effect of three of the main variables affecting CAR T cell activity: (1) CAR binding affinity for the target antigen, reported to modulate CAR T cell effector functions;[15–18] (2) CAR T cell expression levels; and (3) target antigen density. Anti-CD123 CAMs were rationally designed on the basis of computational and structural investigation of CD123 antigen-anti-CD123 antibody interaction to produce antibody mutants with slower association rate in comparison to the WT. Experimental SPR analysis confirmed that two anti-CD123 mutants had 100-fold (CAM-L) and 10-fold (CAM-M) lower affinity in comparison to the WT. Introduction of single amino acid substitutions in the antigen binding

loops allows evaluating the real effect of CAR affinity tuning without simply comparing antibodies targeting different binding epitopes. Altogether, computational simulations allowed rapid and inexpensive design of a limited set of mutants, bypassing the need to generate and screen large number of mutations in randomized sequence searches. This integrated approach connects the biochemical aspects of in silico selection of antibody mutants with the biological relevance given by their subsequent experimental validation.

We found that all CAMs were specific for $CD123^+$ AML cells, including AML primary samples. Unlike other published contexts,[15,16,18,30] even the weaker binders CAM-L and CAM-M showed enhanced redirected-CIK cell effector properties. However, CAM-H2 and CAM-M had lower CAR expression and MFI, which strongly affected the later functional cell properties, independently of CAR binding affinity. CAM-M-CIK cells showed lower proliferation and cytokine production than CAM-L-CIK cells, despite a 10-fold higher binding affinity. At the same time, the low CAM-M- and CAM-H2-CAR expression profile was not a limiting factor in determining a powerful cytotoxic activity against a highly $CD123^+$ leukemic target, similar to WT. These results are in sharp contrast with previous findings suggesting the presence of a functional balance between TAA densities and CAR expression profiles. Accordingly, a lower CAR density was equally able to induce both target cell lysis and production of pro-inflammatory cytokines against a high TAA^+ target cell.[31] In our model, a CAR expression ceiling of

≈40% CAR^+ cells, together with lower CAR MFI values, was observed. Below this threshold, no satisfactory cell activation was detected, even in the presence of high CAR affinity and high TAA density, leading to decreased later cellular effector functions. We then investigated the effect of CAR binding affinity and target antigen density on the efficacy and safety profiles of CAR-redirected CIK cells. No reduction of cytotoxic activity against a first intended target (THP-1), highly $CD123^+$, in the presence of a second unintended target (U937- AML or TIME-endothelium), low $CD123^+$, was detected. This is in contrast to what observed in an anti-CD20 CAR model described by James et al.[29] A value of approximately 1,600 CD123 molecules/cell (in U937 and TIME) was found sufficient to produce a detectable lytic activity, albeit significantly lower than the response to highly $CD123^+$ THP-1 cells. Watanabe et al. described this feature as the CAR lytic threshold.[9] The double-target challenge cytotoxic assay, which is to our knowledge the first attempt in challenging CAR T cells with multiple targets, allowed us to better assess this threshold. Moreover, CAM-L-CIK cells were as able to kill THP-1 cells as WT CAR- and CAM-H1-CIK cells, despite having a 100-fold lower binding affinity. Therefore, binding affinity above 10^{-7} M is sufficient to achieve a satisfactory CAR T cell lytic activation. In contrast, CAM-L showed a trend of inferior killing levels in comparison to CAM-H1- and WT CAR-redirected cells, against the low $CD123^+$ U937 and TIME cells, pointing out that 10^{-7} M binding affinity could offer a better safety profile. In a model of anti-ErbB2

targeting, Chmielewski et al. identified an affinity ceiling of 10^{-8} M, below which there is no additional improvement of receptor-mediated cellular activation.[16] However, differences in CAR affinities arose from the dissociation rate. In our case, by contrast, the lower affinity depends on the association rate. This could allow a long-lasting interaction between CAR and antigen, which might be important to induce potent CAR-CIK cell later effector functions. Furthermore, we found later T cell effector properties being affected by target antigen density. An antigen escalation seems to govern CAR-CIK cell-dependent cytokine production, particularly concerning IFN-γ and IL-2. In contrast, CAR-CIK cell proliferative capability was no longer sensitive to an antigen density above ≈5,000 antigen molecules/cell. We observed that the triggering of cytokine production and T cell proliferation depends on different antigen thresholds, whose definition is of fundamental importance to propose proper and specific target antigens in any CAR-based adoptive cell immunotherapy approach. Caruso et al. proposed the possibility of limiting the recognition of healthy tissues by lowering anti-epidermal growth factor receptor (EGFR) CAR binding affinity. However, similar later effector functions by low-affinity CARs were detected against the low $EGFR^{+}$ healthy tissue (≈15,000 molecules/cell) and the U87 tumor cell line (engineered with ≈30,000 antigens/cell). Thus, the lytic and activation thresholds might have been exceeded,[30] limiting the proper interpretation of a potential on-target, off-tumor effect.

Anti-CD123 CAR-CIK cells could recognize healthy targets, such as HSPCs, monocytes, and endothelium, reaching the antigen activation thresholds. In a previous publication, we employed colony assays and in vivo secondary transplantation experiments to demonstrate that anti-CD123 WT CAR was able to spare the different CD34 subpopulations at levels similar to the unmanipulated CIK cells and was safer than an anti-CD33 CAR.[13,14] In the present work, we performed cytotoxic assays against healthy bone marrow (BM) cells and observed that the CAM mutants had the same low toxicity as the previously characterized anti-CD123 WT CAR (Figure 3C), strongly suggesting that we are in a safe window in regards to the potential on-target, off-tumor effect against the normal hematopoietic compartment. Therefore, we investigated CAR affinity-tuned targeting of low $CD123^{+}$ endothelium by co-culturing differentiated endothelial cells and CIK cells on Matrigel. The antigen presentation within the fully differentiated endothelial network has been shown to be different from monolayer plating on plastic wells.[23] Same and limited impairment of vessel formation was found, with no differences between NO DNA and CAR-CIK cells, suggesting lower CD123 antigen detection in this more physiological context. The lack of homology of human and mouse CD123 molecules represents the major limitation for performing in vivo experiments on safety profile evaluation. Gene-editing approaches might deserve further consideration to generate a humanized mouse model to confirm the safety profile of our CD123

CAR mutants against the endothelial tissue and are under evaluation for future studies. TCR downmodulation was reported to be proportional to antigen-TCR binding strength and thus to T cell activation.[24–28] Similarly, CAR-antigen engagement can trigger CAR internalization at a specific rate, leading to reduced CAR availability on the engineered T cell surface.[29,30] We noted that CAR affinity has no impact on CAR downmodulation when CAR-CIK cells are challenged with highly antigen-positive THP-1 cells. The lytic activity is not related to CAR downmodulation, because equal killing activity was detected for mutants with different downmodulation levels. Instead, mutants with higher downmodulation appeared to have increased IFN-γ production, strengthening the idea that downmodulation is mainly responsible for later effector functions rather than early lytic activity.

Finally, there was a clear correlation between antigen density and amount of CAR downmodulation, suggesting that the number of CARs internalized (downmodulation) depends on the number of interactions with antigen molecules. According to this functional profile, there would be a threshold of CAR downmodulation able to trigger the cytotoxic activity of CAR T cells. Internalization of CARs above such threshold would represent accessory signaling events that can be required to sustain different and later effector functions.

In conclusion, low CAR expression levels resulted in impaired CAR-CIK cell activity, especially regarding later functions. This seems to arise from decreased CAR internalization or downmodulation levels upon

antigen binding, which has no impact on early lytic activity but leads to decreased cytokine production. As a consequence, when designing a new CAR within the specific tumor context, efforts should be directed toward the screening of optimal CAR expression profiles, and not exclusively toward the hunt for a high-affinity antibody-derived CAR. CIK cells redirected with the lowest-affinity mutant showed a trend of decreased killing of low $CD123^{+}$ targets and were as active as high-affinity CAR-CIK cells against highly antigen-positive cells. In conclusion, a combination of computational structural biology and cellular assays allowed us to characterize the role and interplay of CAR binding affinity and CAR expression in the efficacy and safety profiles of anti-CD123 CAR-redirected CIK cells toward AML cells and healthy tissues, expressing different target antigen levels. Overall, the pre-clinical identification of a proper balance between efficacy and safety of CAR therapy will help the improvement of the therapeutic index of such innovative treatments, particularly when dealing with highly aggressive malignancies not responsive to available standard treatments.

MATERIALS AND METHODS

Cell Lines and AML Primary Cells

THP-1, U937, KG-1 (AML cell lines), MHH-CALL-4 (B-ALL cell line), and TIME (ATCC CRL-4025, dermal micro-vascular endothelial cell line) have been obtained from the American Type Culture Collection

(ATCC). The THP-1, U937, and KG-1 cell lines were maintained in culture with Advanced RPMI medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 25 IU/mL of penicillin and 25 mg/mL of streptomycin (Lonza). The MHH-CALL-4 cell line was maintained in culture with 20% FBS Advanced RPMI complete medium. The TIME cell line was maintained in culture with Vascular Cell Basal Medium (ATCC), supplemented with the Microvascular Endothelial Cell Growth Kit-VEGF, containing several purified human recombinant (rh) growth factors (rh_VEGF [vascular endothelial growth factor], rh_EGF [epidermal growth factor], rh_FGF [fibroblast growth factor] basic, and rh IGF-1 [insulin growth factor 1]) and combined with 10 mM L-glutamine, 0.75 U/mL of heparin sulfate, 1 mg/mL of hydrocortisone hemisuccinate, 5% FBS, and 50 mg/mL of ascorbic acid (ATCC). Bone marrow and peripheral blood cells were collected from non-leukemic controls and children with AML at diagnosis. The Institutional Review Board approved this study, and informed consent was obtained from patients or their guardians.

Protein Modeling of Anti-CD123 and CD123

Structural analysis of anti-CD123 antibody in complex with its antigen was performed using a computational approach. CD123 experimental structure is available at PDB: 4JZJ, while anti-CD123 antibody was obtained using RosettaAntibody software. The templates are coded as follows (sequence identity is indicated in parentheses)—PDB: 1MVU

for the light-chain framework (96.77%), PDB: 1MJ8 for the heavy-chain framework (91%); PDB: 1MVU for L1 (100%) and for L3 (88.89%), PDB: 1Q9R for L2 (100%), PDB: 1EGJ for H1 (100%) and for H2 (82.35%), and PDB: 12E8 for H3 (same length, no identity). The best 10 of 2,100 antibody models were selected because of their RosettaAntibody algorithm score.

Computational Modeling of Anti-CD123-CD123 Complex

Complex models were predicted using Rosetta Docking 2.3, a computational docking software. The starting structures were visually oriented with the antibody (Ab) complementarity-determining region (CDR) loops facing the N-terminal part of the CD123 protein, because it is known that domain 1 of CD123 is fundamental for anti-CD123 binding,[32] and then separated by 25 Å. Docking runs of ten antibody models with its antigen were conducted as previously described.[33] Best models of each simulation, selected because of their energetic score and by visual analysis after discarding complexes with an interface far from the first 30 residues of N-terminal CD123, were then subjected to clustering and contact map analysis. This allowed us to select residues showing interaction in several models likely to be important in mediating binding.

Antigen, Antibody, and Variant Protein Production

The human CD123 domain 1+2 nucleotide sequence was cloned in frame into pET21a plasmid and expressed using the *E. coli* system

(Rosetta DE2 cells). Single-clone cells were grown in Luria-Bertani broth ampicillin+cyclohexylammonium salt+ (LB AMP+CHA+) media until 0.6 optical density (OD) and then induced with 1 mM isopropil-b-D-1-tiogalattopiranoside (IPTG) and harvested after 3 hr. CD123 domain 1+2 was found in the insoluble fraction, so the protein pellet was washed and solubilized using mild-denaturing buffer (100 mM Tris [pH 12.5], 2 M urea, 5 mM b-mercaptoethanol [B-ME]). Protein was loaded into an anion exchange column (HiPrep Q FF 16/10, GE Healthcare) pre-equilibrated with solubilization buffer and eluted with NaCl gradient, starting from 0 to 1M. Fractions containing CD123 1+2 protein were then loaded into size exclusion column (HiLoad 16/60 Superdex 75, GE Healthcare) pre-equilibrated with 50 mM Tris (pH 8.5), 150 mM NaCl, and 1% PEG3350. CD123 domain 1+2 elutes at 68 mL according to its monomeric molecular weight.

The anti-CD123 single-chain antibody nucleotide sequence was cloned in frame into pET21a plasmid and expressed using the *E. coli* system (Rosetta DE2 cells). Single-clone cells were grown in LB AMP+CHA+ media until 0.6 OD and then induced with 1 mM IPTG and harvested after 3 hr. Single-chain antibody was found in the insoluble fraction, so the protein pellet was washed and solubilized using denaturing buffer (50 mM MES [pH 6.5], 1 M NaCl, 6 M guanidinium-HCl). Protein was loaded into a HiTrap column (GE Healthcare) pre-equilibrated with solubilization buffer and eluted with same buffer plus 500 mM imidazole. Fractions containing antibody were refolded using direct

dilution into 20 mM NaP (pH 10), 150 mM NaCl, 200 mM arginine, and 1 mM Glut Red 0.1 Glut Ox. Protein was concentrated by centrifugation (2,000 rpm, 4°C) with 10 kDa Vivaspin (Sartorius) and loaded on a size exclusion column (HiLoad 16/60 Superdex 75, GE Healthcare) pre-equilibrated with 50 mM Tris (pH 9) and 50 mM NaCl. Anti-CD123 elutes at 64 mL according to its monomeric molecular weight.

Mutagenesis of Anti-CD123 Single-Chain Ab

Mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Specific mutated primers were synthesized by Microsynth and used to generate protein variants. Site-directed mutagenesis PCRs were performed according to the protocol kit and following the cycling parameters listed below. After the PCR reaction, the mixtures were treated with DpnI restriction enzyme at 37°C for 5 min to remove the parental (i.e., the nonmutated), supercoiled, double-stranded DNA (dsDNA). The DpnI-treated DNAs (mutated plasmids) were used to transform XL10-Gold Ultracompetent Cells; the colonies obtained from the transformations were used for DNA amplification and extraction with the QIAGEN Maxi or Mini Prep Kit. The sequence of each mutated plasmid was verified by DNA sequencing (Microsynth); the verified plasmids were used for protein expression and stored in aliquots at -80°C. Antibody variants

were tested for protein purification, showing no difference in yield and stability in comparison to WT.

PCR cycling parameters were as follows:

95°C 2 min
95°C 20 s
60°C 10 s
68°C 30 s/kb of plasmid length } x 18 cycles
68°C 5 min
4°C∞.

SPR

SPR experiments were performed to validate computational results. The scFvs were immobilized on the surface of a GLC chip (a thin alginate layer for amine coupling) at 500 nM in 10 mM NaOAc (pH 4.0). CD123 domain 1+2 was used as analyte (protein and running buffer: 20 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20). The injection of the antigen spanned a concentration range between 200–12.5 nM at flow rate of 70 mL/min. Data were fit using the Langmuir equation.

Transposons Plasmids

The WT anti-CD123/pTMNDU3 Sleeping Beauty (SB) transposon expresses the human third-generation anti-CD123-CD28-OX40-CD3z CAR under pTMNDU3 promoter. The construct has been derived as a SB expression plasmid, replacing the EGFP sequence from pT-MNDU3-EGFP with the scFv CD123 (7G3 clone) previously cloned in frame with

CH2CH3-CD28-OX40-z from SFG.aGD2 (provided by Dr. Martin Pule, University College of London). The DNA sequences of each anti-CD123 affinity mutant scFv were cloned in place of the anti-CD123 WT scFv. The plasmid pCMV-SB11 encodes for the SB11X transposase (from the University of Minnesota).

Generation of CIK Cells Genetically Modified for the Expression of the Anti-CD123 CARs

CIK cells were generated starting from peripheral blood mononuclear cells (PBMCs) from healthy subjects, obtained after centrifugation of fresh blood on a density gradient using Ficoll-Hypaque (Pharmacia). Once collected, PBMCs were resuspended in Amaxa Nucleofector solution, provided with the P3 Primary Cell 4D-Nuceofector X kit (Lonza), together with SB11X transposase and DNA plasmid encoding for one of the anti-CD123 CAR mutants, and were transfected using the 4D-Amaxa Nucleofector device (Lonza). Our controls included unmodified cells, which were nucleofected with no DNA, and EGFP episomal transfected cells (with 2 mg of Amaxa control plasmid) to assess the gene transfer efficiency. After nucleofection, the cells were transferred into a 6-well plate containing 4 mL of pre-warmed medium (advanced RPMI supplemented with 20% of heat-inactivated FBS and 1% of L-glutamine) and 1,000 U/mL of IFN-g (Dompè Biotec) were added to each well.[34] Twenty-four hours later, IL-2 (Chiron) and OKT-3 (Janssen-Cilag) were added at 300 U/mL and at 50 ng/mL, respectively.

Fresh medium and IL-2 were added twice a week, and cell concentration was maintained around 0.75 x 10^6 cells/mL. Cells were then cultured for 21 days.

Flow Cytometry

Immunostaining and flow cytometric analysis were performed with the following antibodies: allophycocyanin (APC)-anti-CD123 (Becton Dickinson [BD]), phycoerythrin (PE)-anti-CD123 (BD), v-500-anti-CD45 (BD), phycoerythrin-cyanine 7 (Pe-Cy7)-antiCD34 (BD), PE-anti-CD38 (BD), peridinin-chlorophyll-protein complex (PerCP)-anti-CD3, PE-anti-CD56 (BD), fluorescein isothiocyanate (FITC)-anti-CD8 (BD), PE-anti-CD4 (BD), PE-anti-CD62L (BD), FITC-anti-CD45RO (BD), Alexa Fluor 647-F(ab')2-anti-immunoglobulin G (IgG) (H+L) (anti-Fc), PE-anti-IL-2, FITC-antiIFN-γ, Pe-Cy7-anti-TNF-α, Horizon BV421-anti-IL-6, Horizon BV421-anti-Ki-67, FITC-anti-CD19 (BD), and PE-anti-CD144 (BD). Cell death and apoptosis were detected using the GFP-Certified Apoptosis/Necrosis detection kit (Enzo Life Sciences), according to the manufacturer's instructions. Cell membrane labeling was also performed using two lipophilic fluorescent dyes: FITC- and PE-Cell Tracker (Invitrogen).

To quantify the number of CD123 molecules on the surface of the target cell lines and primary AML samples, we used the QuantiBRITE PE fluorescence quantitation kit (BD), which allows the conversion of cell fluorescence intensity values into absolute numbers of receptors

per cell through the creation of a calibration curve.[35] Flow cytometry was performed on a FACSCanto II flow cytometer (BD), and data were analyzed using BD FACSDiva software v.6.1.3.

Short- and Long-Term Cytotoxicity Assays

To evaluate the killing ability of both unmodified and CAR-redirected CIK cells, short- and long-term cytotoxicity assays were performed. In the short-term cytotoxic assay assessed by means of the double-target challenge, CIK cells were co-cultured for 4 hr with the CD123-positive targets (THP-1, primary AML cells, U937, and TIME cell line), together with the CD123-negative control (MHHCALL-4 cell line), at an effector-target (E:T) ratio of 5:1. Target cells were previously labeled with FITC- and PE-Cell Trackers. At the end of the incubation, target cell killing was measured through apoptosis detection by flow cytometry, after annexin V and 7-amino-actinomycin D (7-AAD) (AnnV-7AAD) staining, gating in the Cell Tracker PE+ and FITC+ cells. The percentage of killed cells was calculated according to the following formula.

$$\frac{(\%\ \text{annexin V}^+\ \text{target cells} + \%\ \text{annexin V}^+\ 7\text{AAD}^+\ \text{target cells})\text{after co-culture with CIK cells}}{(\%\ \text{annexin V}^+\ \text{target cells} + \%\ \text{annexin V}^+\ 7\text{AAD}^+\ \text{target cells})\text{alone}}$$

Two long-term cytotoxicity assays were conducted at an E:T ratio of 1:5. The first was performed by co-culturing CIK cells with THP-1 and MHH-CALL-4 cell lines for 1 week, and the second was performed by co-culturing CIK cells with THP-1, MHH-CALL-4, and TIME cell lines for 72 hr. Target cell survival was then evaluated after surface staining with PerCP-anti-CD3 (BD) antibody, to detect CIK cells, and by

comprising the following antibodies: APC-anti-CD123 (BD), FITC-anti-CD19 (BD), and PE-anti-CD144 (BD), for THP-1, MHH-CALL-4, and TIME cell line detection, respectively. The percentage of target cell survival was calculated according to the following formula.

$$\left(\frac{\text{No. target cells after co-culture with effector T cells}}{\text{Total No. target cells alone}}\right) \times 100$$

Proliferation Assay

The proliferation ability of CAR-CIK cells was evaluated after co-culture with the various Cell Tracker-labeled targets, irradiated at 100 Gy γ-radiations at an E:T ratio of 1:1. After a 72 hr co-culture, the cells were collected, immunostained for intracellular Ki-67, and then analyzed by flow cytometry by performing detection of Cell Tracker negative-Ki-67^{+} cells.

Intracellular Cytokine Staining

CAR-CIK cell ability to produce cytokines was evaluated following a stimulation with the various target cell conditions at an E:T ratio of 1:3. After a 2 hr and 30 min co-culture, BD GolgiStop was added. The co-culture was then maintained for an additional period of 2 hr and 30 min, after which the cells were collected and stained for anti-CD3 and anti-Fc surface molecule detection. Finally, intracellular cytokine staining (ICS) for IL-2, IFN-γ, IL-6, and TNF-α was performed using the BD Cytofix/Cytoperm kit, according to the manufacturer's protocol. Specimens were then analyzed by flow cytometry.

Matrigel-Embedded Endothelium and CIK Cell Assay

250 mL of Matrigel (Corning) were plated on the surface of a 24-well plate and allowed to solidify by incubation at 37°C for 30 min. Subsequently, a number of 60,000 TIME cells and CIK cells (both unmodified and CAR redirected) were plated together, at an E:T ratio of 1:1, and left for additional 2 hr at 37°C. After this time interval, real-time monitoring of vessel formation was performed by microscope observation to assess the state of tubule formation of each sample, duplicated in every independent experiment. Four hours after plating, picture acquisition was performed by collecting nine pictures per well at 10x magnification. Branching point count through ImageJ software was then performed in each of the nine pictures per well to obtain a sufficient representation of the entire well surface.

CAR Downmodulation Quantitation

Following the ICS protocol previously described, after the CIK cell surface staining of CAR expression, we analyzed the CAR MFI values with no stimulation and in response to different targets. The CAR downmodulation percentage was calculated according to the following formula, as described by James et al.[29]

$$\%\ \text{CAR downmodulation} = 100 \times \left(\frac{1 - \text{stimulated MFI}}{\text{unstimulated MFI}}\right)$$

Statistical Analysis

Data were analyzed using GraphPad Prism 5 software (GraphPad). One-way ANOVA and two-way ANOVA statistical tests were performed for column statistics and grouped statistics, respectively, using no matching and repeated-measures criteria according to the type of dataset analyzed and presence or absence of matched values, as indicated in the figure legends. Reported values of the statistical analyses are the result of the evaluation of mean ± SEM. All p values are provided in the figure legends.

AUTHOR CONTRIBUTIONS

S.A., M.C.R., M.B., and S.T. designed the research, performed the experiments, analyzed the data, and wrote the paper. L.S. contributed in performing the experiments. C.F.M. offered technical support and optimized the non-viral gene transfer protocol for CIK cell CAR engineering. S.T. and L.V. designed and coordinated the research. E.B., A.B., and L.V. revised the data and performed a final revision of the paper.

CONFLICTS OF INTEREST

The authors have no potential conflict of interest.

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Research, CSL. The authors also thank Prof. Olivier Michielin and Dr. Mattia Pedotti for scientific discussions and Dr. Roberto Giovannoni and Dr. Stefania Galimberti for giving precious suggestions in planning the experiments and during paper writing. The authors thank the parent committees “Quelli che...con LUCA onlus,” “Comitato Maria Letizia Verga,” and “Stefano Verri” for their generous and constant support. This work was supported by grants from AIRC, Molecular Clinical Oncology 5 per mille 2010, “Innate immunity in cancer: Molecular targeting and cellular therapy” (grant 9962), and IG 2015, “Novel leukemia treatment by the use of chimeric antigen receptors (CARs)” (grant 17248); “Libera Le Ali” 2011 project, Fondazione Just Italia; SNF (grant 310030 166445); and Oncosuisse (grant KFS-3728-08-2015 to L.V. S.A. is a fellow of the University of Milano-Bicocca, Milan, Doctoral Program in Molecular and Translational Medicine (DIMET). Gratitude goes to “Quelli che...con LUCA onlus,” which generously supported S.T.’s fellowship and funded the project.

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FIGURES

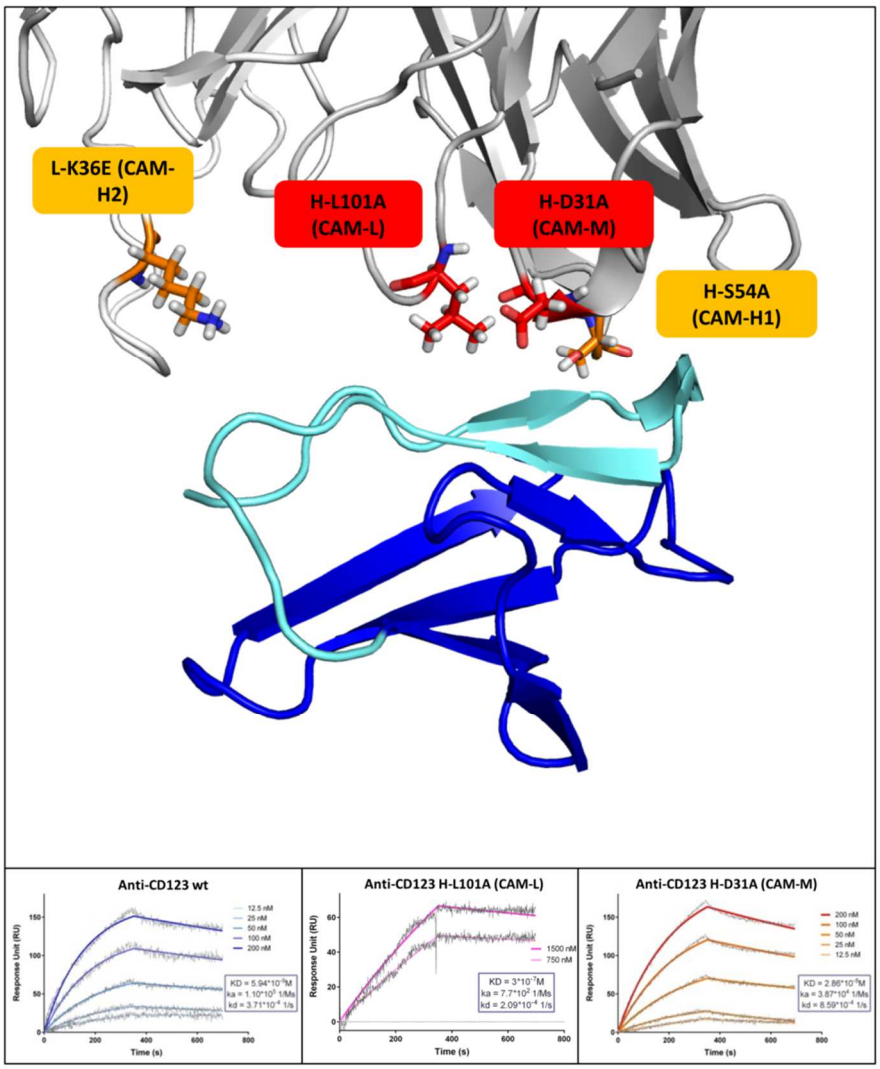


Figure 1. Three-Dimensional Model of the AntiCD123/CD123 Complex and SPR Binding Analysis

(Top) Cartoon visualization of CD123 domain 1 (blue, with the region important for anti-CD123 binding in cyan) and anti-CD123 antibody (heavy and light chain in dark and light gray, respectively). Single mutations, shown

as stick, were introduced in the antigen binding loops to affect CAR affinity. (Bottom) SPR sensogram showing the binding of immobilized WT single-chain antibody, CAM-L, and CAM-M to CD123. Raw data are shown in grey, whereas the fit used to calculate the binding properties is in color, with gradation indicating different concentrations. Association, dissociation, and binding affinity are shown.

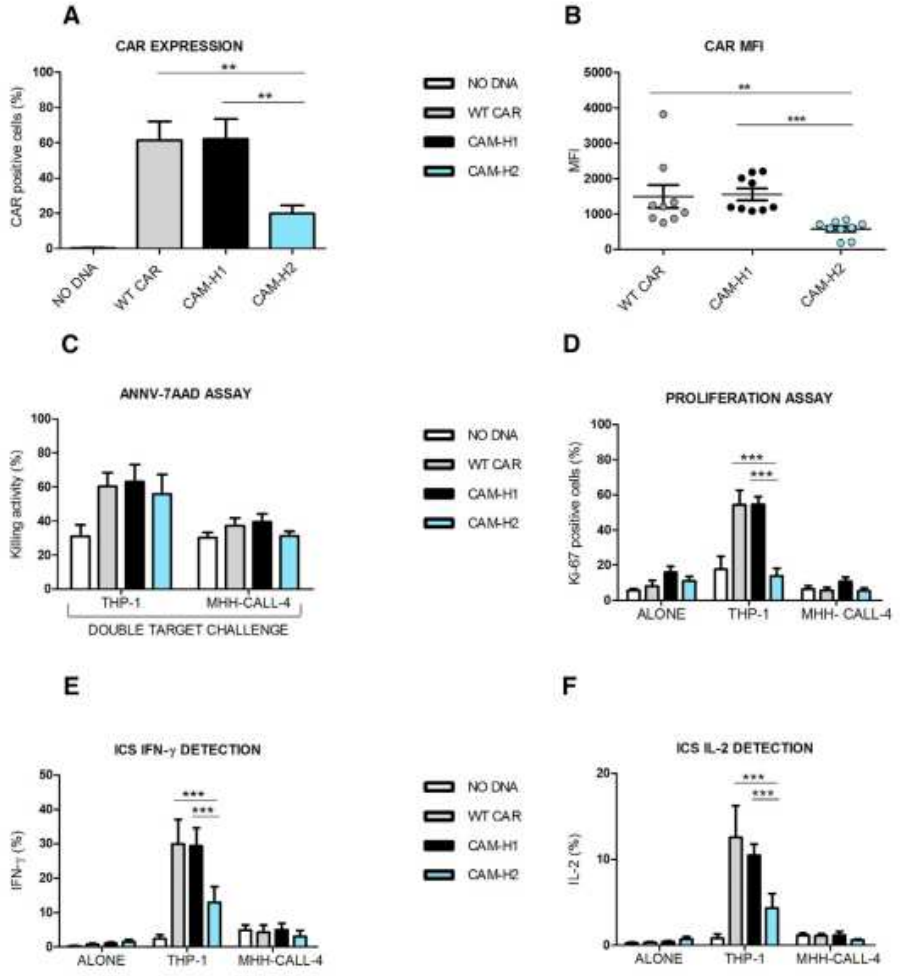


Figure 2. Later Effector Functions, and Not Early Cytotoxic Profile, Are Impaired in CAR-CIK Cells Displaying Identical Binding Affinity but Reduced Expression

(A and B) CAR expression (A) and MFI values (B) at day 21 of anti-CD123 CAR-CIK cells and NO DNA control. Data represented are the result of mean ± SEM. (A) n = 6 (**p < 0.01; one-way ANOVA, Bonferroni test); (B) n = 9 (**p < 0.01; ***p < 0.001; one-way ANOVA, Dunn's multiple comparison test). (C–F) Short-term AnnV-7AAD assay by means of the cytotoxic double-target challenge at an effector-target (E:T) ratio of 5:1 after a 4 hr co-culture with both THP-1 and MHH-CALL-4 cell lines (C). Long-term proliferation assay after

a 72 hr co-culture of the effector cells with THP-1 and MHH-CALL-4 cell lines (D). Intracellular cytokine staining of IFN-g (E) and IL-2 (F) after a 5 hr co-culture between effector cells and both THP-1 and MHH-CALL-4 targets. Data represented are the result of mean ± SEM. n = 4 (***p < 0.001; two-way ANOVA, Bonferroni test).

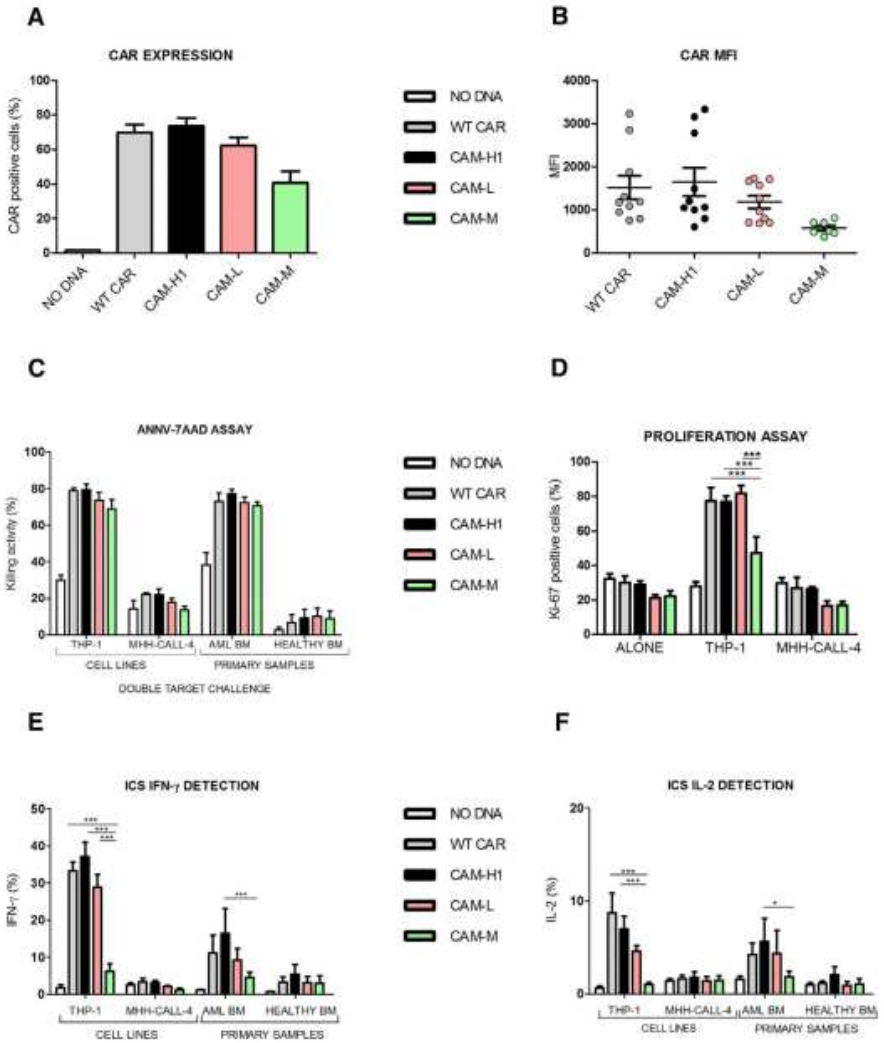


Figure 3. Reduced CAR Binding Affinity Does Not Affect CIK Cell Cytotoxic Functions in Response to Highly CD123⁺ Target Cells

(A and B) CAR expression (A) and MFI values (B) at day 21 of anti-CD123 CAR-CIK cells and NO DNA control. Data represented are the result of mean ± SEM. (A) WT CAR, CAM-H1, and CAM-L: n = 18; CAM-3: n = 7; (B) n = 7 (one-way ANOVA, Bonferroni test). (C–F) AnnV-7AAD assay through double-target challenge after 4 hr co-culture with both THP-1 and MHH-CALL-4 cell lines and primary AML and healthy bone marrow samples (C). E:T ratio 5:1, n = 4. Long-term proliferation assay after the co-culture of the effector cells with

THP-1 and MHH-CALL-4 cell lines for 72 hr (D). Intracellular cytokine staining of IFN-g (E) and IL-2 (F) after 5 hr co-culture between effector cells and THP-1, MHH-CALL-4 targets, primary AML, and healthy BM samples. Data represented are the result of mean ± SEM. WT CAR, CAM-H1, and CAM-L: n = 7; CAM-M: n = 4 (*$p < 0.05$, ***$p < 0.001$; two-way ANOVA, Bonferroni test).

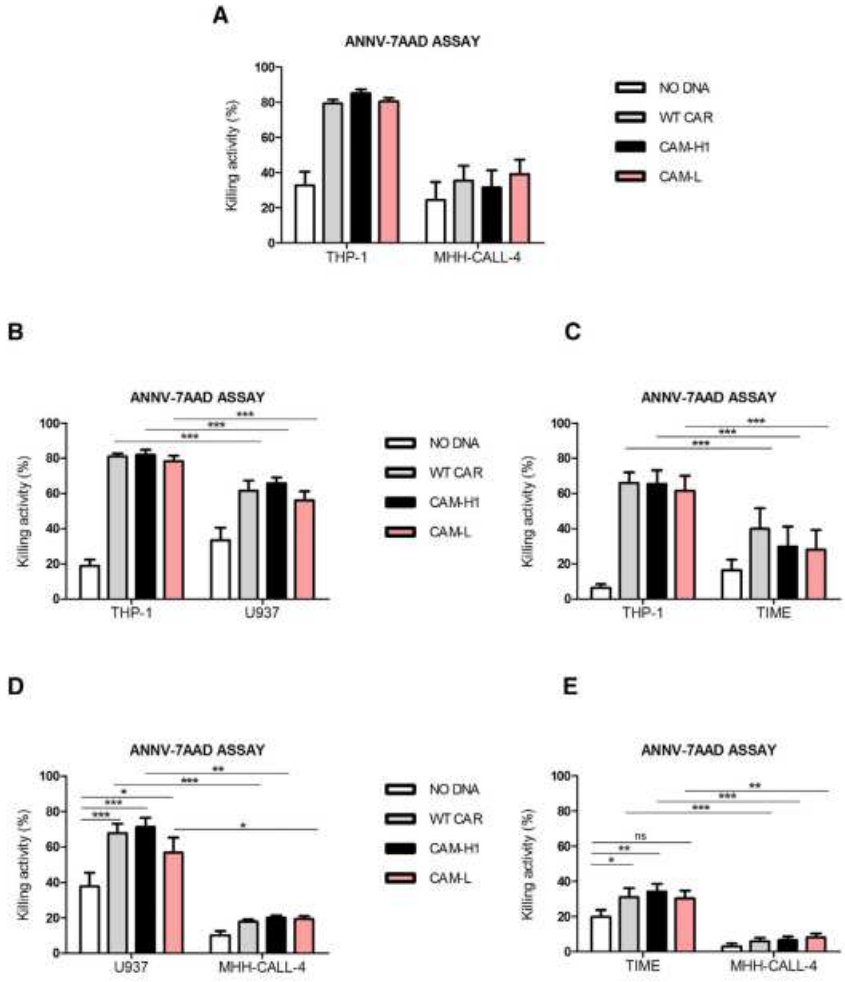


Figure 4. Short-Term Cytotoxic Double-Target Challenge
AnnV-7AAD assay, E:T ratio 5:1, (A) THP-1/MHH-CALL-4 (n = 5; CAM-H1: n = 4), (B) THP-1/U937 (n = 5; CAM-H1: n = 4), (C) THP-1/TIME (n = 6), (D) U937/MHH-CALL-4 (n = 5; CAM-H1: n = 4), and (E) TIME/MHH-CALL-4 (n = 6) double-target combinations. Data represented are the result of mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001; two-way ANOVA, Bonferroni test).

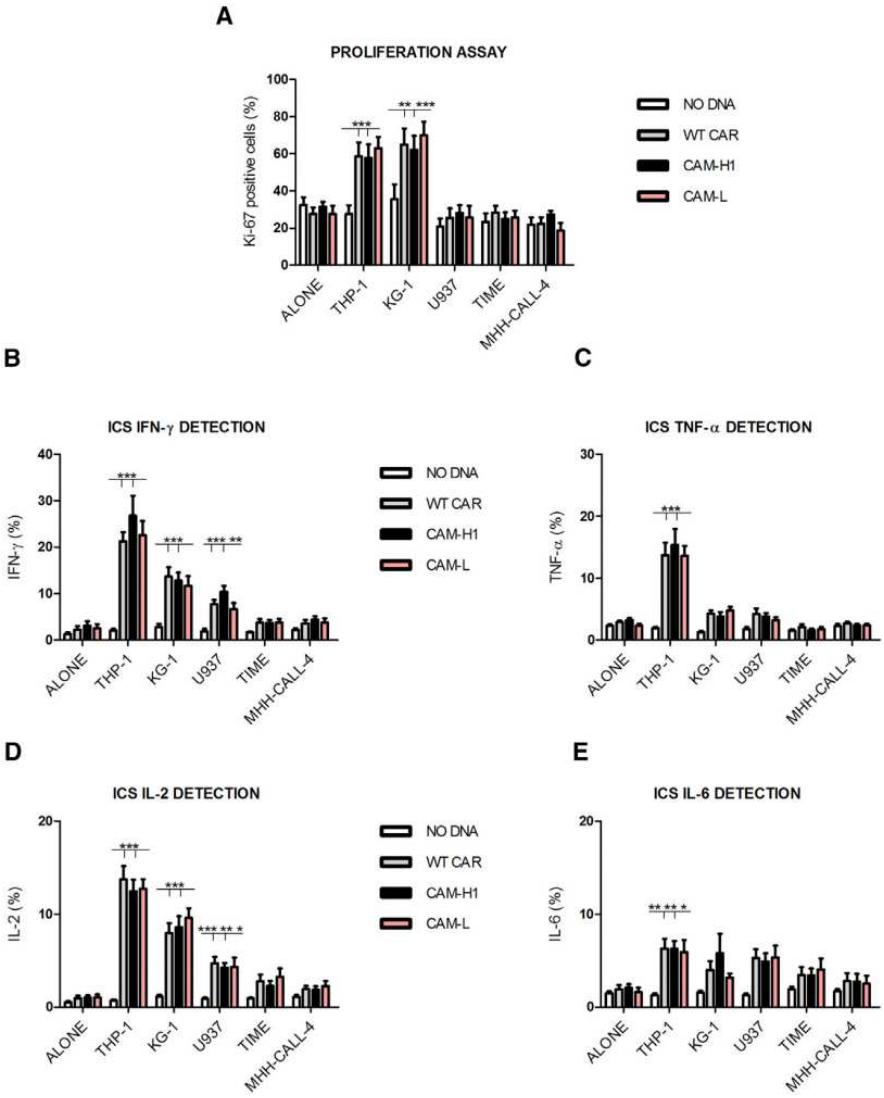


Figure 5. Target Antigen Density, and Not CAR Binding Affinity, Affects Later Effector Functions

(A) Long-term proliferation assay after the co-culture of the effector cells with the indicated cell lines for 72 hr. (B–E) Intracellular cytokine staining of IFN-γ (B), TNF-α (C), IL-2 (D), and IL-6 (E). Effector cells were co-cultured for 5 hr with THP-1, KG-1, U937, TIME, and MHH-CALL-4 targets. Data represented are the result of mean ± SEM. n = 12 (ALONE

condition); n = 9 (THP-1, U937, and TIME experimental cell conditions); n = 3 for all CARs tested against the KG-1 cell line (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; two-way ANOVA, Bonferroni test).

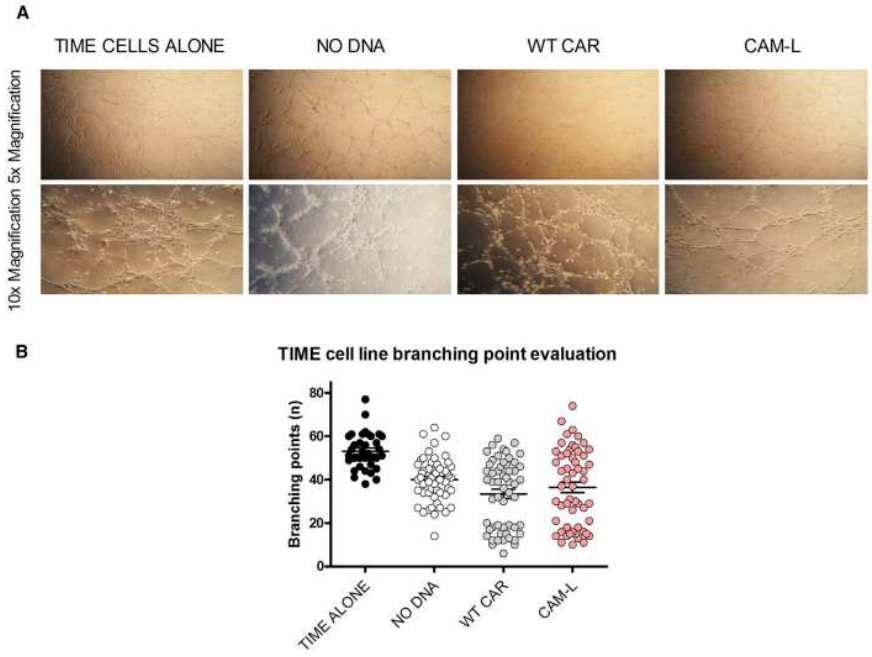


Figure 6. TIME Cell Vessels Are Not Hampered by CAR-CIK Cells
(A) Representative 5x and 10x magnifications of TIME cell vessels after a 4 hr culture on Matrigel (TIME cells alone) or with NO DNA and CAR-CIK cells (WT CAR and CAM-L), respectively. (B) Analysis of TIME cell branching point number, alone or after a 4 hr co-culture with unmanipulated and CAR-CIK cells, as a measure of TIME cell tissue integrity and vessel spreading. Data represented are the result of mean ± SEM. n = 3 (***$p < 0.001$; one-way ANOVA, Bonferroni test).

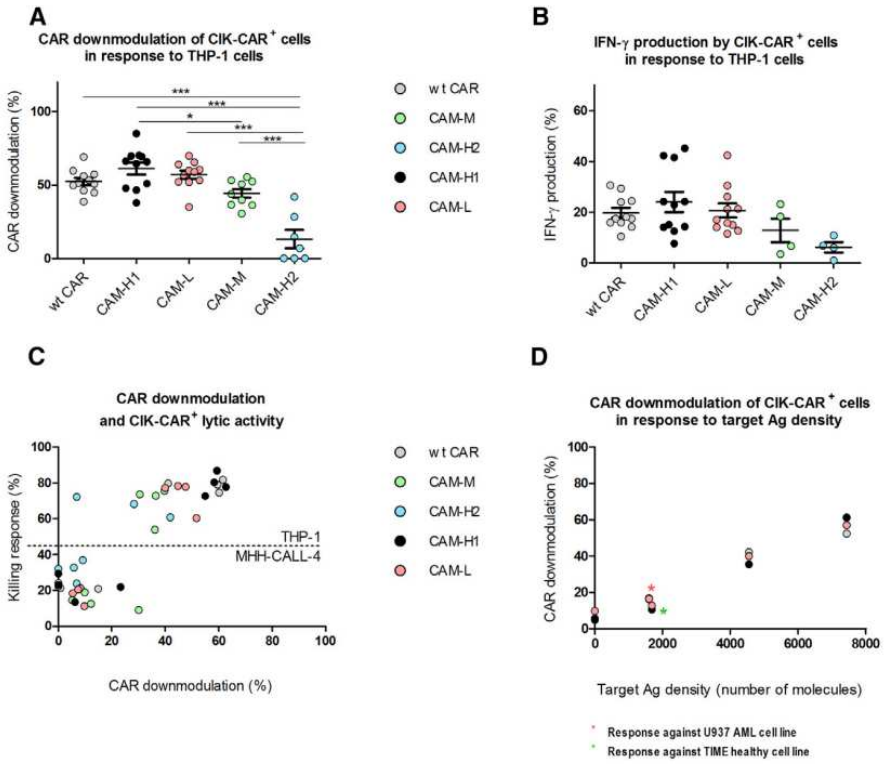


Figure 7. CAR Downmodulation in CAR-CIK Cells

(A and B) Percentage of CAR-CIK cell downmodulation (A) and percentage of IFN-γ production by CAR-CIK cells (B) in response to THP-1 cells. (A) WT CAR, CAM-H1, and CAM-L: n = 11; CAM-M: n = 9; CAM-H2: n = 7 (*p < 0.05, **p < 0.01, ***p < 0.001; one-way ANOVA, Bonferroni test). (B) WT CAR, CAM-H1, and CAM-L: n = 11; CAM-H2 and CAM-M: n = 4. (C) Plot of CAR downmodulation as a function of killing activity against both THP-1 and MHH-CALL-4 targets. (D) CAR-CIK cell downmodulation as a function of target antigen density. Data represented are the result of mean ± SEM, n = 11 for the stimulation with MHH-CALL-4, TIME, U937, and THP-1 cell lines; n = 7 for the stimulation with KG-1 cells.

SUPPLEMENTAL INFORMATION

Fig. S1

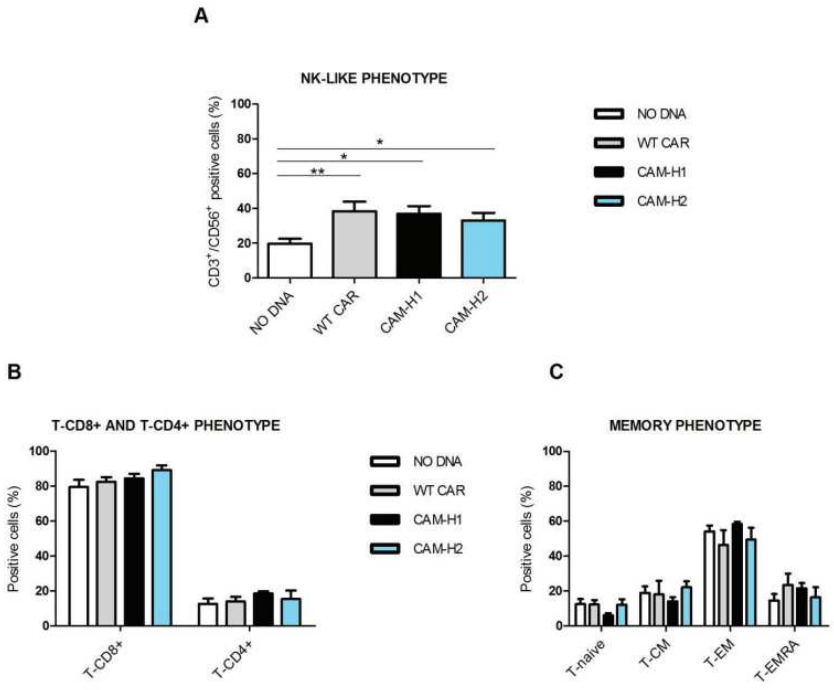


Fig. S2

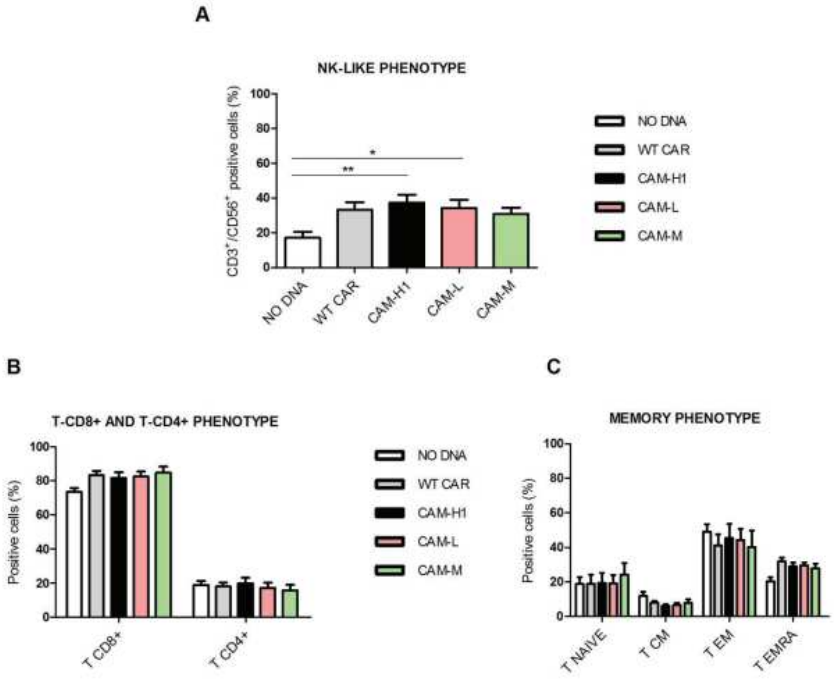

A
NK-LIKE PHENOTYPE
CD3+/CD56+ positive cells (%)
NO DNA
WT CAR
CAM-H1
CAM-L
CAM-M
B
T-CD8+ AND T-CD4+ PHENOTYPE
Positive cells (%)
T CD8+
T CD4+
C
MEMORY PHENOTYPE
T NAIVE
T CM
T EM
T EMRA

Fig. S3

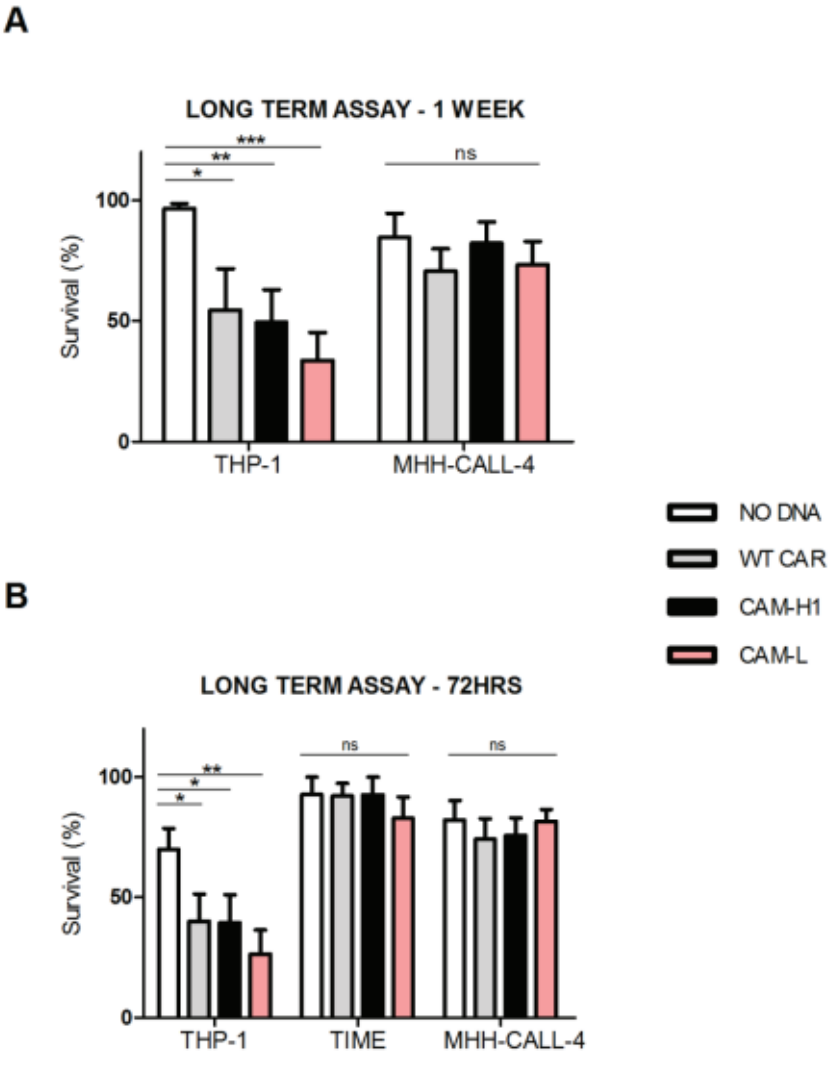
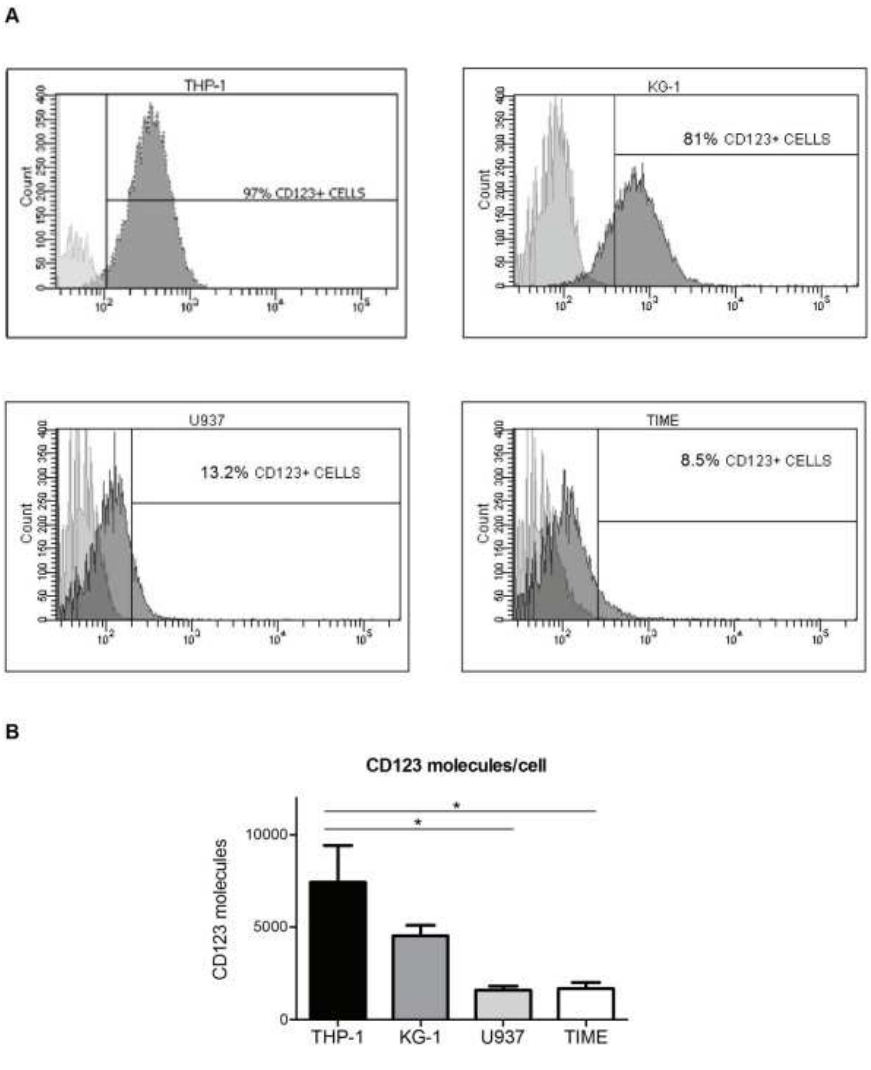

A
LONG TERM ASSAY - 1 WEEK
***
**
*
ns
100
50
0
Survival (%)
THP-1
MHH-CALL-4
NO DNA
WT CAR
CAM-H1
CAM-L
B
LONG TERM ASSAY - 72HRS
**
*
*
ns
ns
100
50
0
Survival (%)
THP-1
TIME
MHH-CALL-4

Fig. S4


A
THP-1
97% CD123+ CELLS
KG-1
81% CD123+ CELLS
U937
13.2% CD123+ CELLS
TIME
8.5% CD123+ CELLS
Count
B
CD123 molecules/cell
CD123 molecules
10000
5000
0
THP-1
KG-1
U937
TIME
*
*

Supplemental Data

Supplemental Figures 1 and 2. CAR-redirected CIK cell and NO DNA control phenotype characterization at day 21. In order to verify the impact of both the in silico selected mutations and the genetic manipulation on CIK cell phenotype, we performed $CD3^+/CD56^+$ and $CD3^+/CD8^+/CD4^+$ surface staining, together with a memory phenotype CD45RO/CD62L.

Supplemental Figure 3. Characterization of the efficacy and safety profiles of CAM-L-CIK cells over time, by means of long term cytotoxic assays. In order to corroborate the long term efficacy profile of CAM-L-CIK cells, we performed a long-term cytotoxic assay at an E:T ratio of 1:5, in the absence of exogenous IL-2 for 1 week (Supplementary Figure 3A). Furthermore, in order to characterize the safety profile over-time of CAM-L-CIK cells against the healthy tissue, represented by the TIME cell line, we performed a 72 hour long-term cytotoxicity assay at an E:T ratio of 1:5, in the absence of exogenous IL-2 (Supplementary Figure 3, B panel).

Supplemental Figure 4. Characterization of CD123 expression and CD123 molecules on different target cell lines.

Supplemental Figure legends

Fig. S1. CAR-redirected CIK cell and NO DNA control phenotype at the end of the differentiation period (Day21). (A) NK-like, (B) T-$CD8^+$ and T-$CD4^+$ and (C) memory phenotype at day 21 of NO DNA, wt CAR-, CAM-H1- and CAMH2-CIK cells. Data represented are the result of mean ± SEM, (A) n=6 (*p<0.05, **p<0,01, One way analysis of variance (ANOVA), Dunnet's test), (B and C) n=4 (two way analysis of variance (ANOVA), Bonferroni test).

Fig S2. CAR-redirected CIK cell and NO DNA control phenotype at the end of the differentiation period (Day21). (A) NK-like, (B) T-$CD8^+$ and T-$CD4^+$ and (C) memory phenotype at day 21 of NO DNA, wt CAR-, CAM-H1-, CAM-L and CAM-M-CIK cells. Data represented are the result of mean ± SEM, (A) NO DNA, wt CAR, CAM-H1, CAM-L: n=12; CAM-M: n=7 (*p<0.05, **p<0,01, One way analysis of variance (ANOVA), Dunnet's test), (B and C) n=7 (two way analysis of variance (ANOVA), Bonferroni test).

Fig. S3. CIK cells redirected with the low affinity CAM-L in vitro control the leukemic growth over time, while preserving its safety profile towards the healthy tissue. (A and B) Long-term cytotoxic assays performed with, wt CAR, CAM-H1 and CAM-L redirected CIK cells and NO DNA control for 1 week and

72 hours respectively, E:T ratio 1:5, in the absence of exogenous IL-2. Data represented are the result of mean ± SEM, n=7 (*p<0.05, **p<0.01, ***p<0.001; two way analysis of variance (ANOVA), Bonferroni test).

Fig. S4. CD123 expression in different target cell lines. (A) CD123 expression and (B) QuantiBrite quantification of CD123 molecules per cell in THP-1, KG-1, U937 and TIME cells. Data represented are the result of mean ± SEM, THP-1: n=6, KG-1: n=3, U937 and TIME cell line: n=5 (*p<0.05; one way analysis of variance (ANOVA), Bonferroni test).

Chapter 3

Preclinical assessment of CD33.CAR-CIK cells engineered by using a latest optimized version of the *Sleeping Beauty* transposon system in Acute Myeloid Leukemia

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ABSTRACT

Chimeric antigen receptor (CAR)-T cell therapy has been successfully clinically deployed in B-cell malignancies, paving the way for further development also in other tumor entities. In this context, Acute Myeloid Leukemia (AML) demands for such innovative targeted therapies, still representing one of the major unmet clinical need in the field of oncohematology. The CD33 molecule is so far one of the main validated target antigen in AML and, being broadly expressed on AML blasts, thus representing a suitable antigen to be targeted with CAR-T cells. Here, we describe the feasibility of engineering Cytokine-induced killer (CIK) cells with a CD33.CAR by using the latest optimized version of the non viral *Sleeping Beauty* transposon system "SB100X-pT4" which offers the advantage of improving CAR transposition in CIK cells compared to the previous optimized version "SB11-pT". SB-modified CD33.CAR-CIK cells exhibited significant anti-leukemia activity *in vitro* and *in vivo*, reducing AML development when administered as an "early treatment" approach and delaying AML progression in mice with established disease. Being the CAR-T cell therapy nowadays still considered as a second-line treatment, an additional xenograft chemotherapy model, mimicking human induction therapy, will be exploited to preclinically test the potential benefit of CAR-T cell therapy considering the real unmet clinical need, which is represented by the resistance to standard of care treatments.

INTRODUCTION

Starting from their first laboratory characterization by Zelig Eshhar and coworkers, in the late 1980s (Gross, Waks, & Eshhar, 1989), Chimeric Antigen Receptors (CARs) have now reached bedside applications, revolutionizing the field of cancer immunotherapy. Indeed, the unprecedented clinical responses achieved (Wohlfarth, Worel, & Hopfinger, 2018), led the US Food and Drug Administration (FDA) to grant, in August 2017, the first ever approval to the Novartis' CD19.CAR-T cell product “Kymriah™” for relapsed/refractory pediatric Acute Lymphoblastic Leukemia (ALL).

CARs are recombinant molecules joining an antigen binding domain, usually derived from a monoclonal antibody (mAb), in the form of single chain fragment variable (scFv), to the T cell receptor (TCR) CD3ζ and additional costimulatory domains, thus allowing to redirect T cell effector functions towards any surface antigen in a HLA independent fashion. These CAR features enable to broaden the number of patients who can benefit from adoptive immunotherapy and to overcome the tumor's immune escape mechanism of HLA downregulation.

Non-viral transposon-based gene transfer systems have recently been considered as alternative to viral vectors for CAR gene transfer. These systems are generally composed by two elements: the transposon, which contains the transgene of interest flanked by inverted terminal repeats (ITRs) and the transposase that, recognizing the ITRs, mediates the excision and integration of the transposon from the donor plasmid

to the target genome through a “cut and paste” mechanism. Among the different transposon system exploited, Sleeping-Beauty (SB) has been found to exhibit significantly higher activity in vertebrates compared to the other transposons tested (Fischer, Wienholds, & Plasterk, 2001) with a close-to-random genomic integration (P. Hackett, Largaespada, Switzer, & Cooper, 2013). Furthermore, the ease and reduced costs associated with manufacturing and quality control and the increased biosafety compared to viral vectors make SB vectors appealing for clinical application. Of note, the SB system has been already employed in clinic to genetically modify T cells with a CD19.CAR (Kebriaei et al., 2016). The transposition efficiency of the SB technology has undergone to a progressive improvement due to both the generation of hyperactive transposase mutants (SB10, SB11, SB100X) and the design of optimized transposon donor vector architecture (pT, pT2, pT3, pT4) (Kebriaei, Izsvák, Narayanavari, Singh, & Ivics, 2017).

Our group has pioneered the use of the SB platform in the version of SB11-pT to redirect Cytokine Induced Killer (CIK) cells with different CARs achieving, with a single stimulation step, a stable and efficient CAR expression while preserving phenotype, viability, and expansion of transduced CIK cells (Magnani et al., 2016).

Here, we exploited the novel molecular evolution of the SB technology, comprising the hyperactive SB100X transposase (Mátés et al., 2009) and the pT4 transposon (Y. Wang et al., 2017) (SB100X-pT4),

to further optimize the CIK cell engineering with a CD33.CAR for the treatment of Acute Myeloid Leukemia (AML).

Conventional AML therapeutic strategies fail in ensuring satisfactory long term overall survival, thus new treatment options are mandatory. Immunotherapy has emerged as an effective treatment for several cancer patients while representing a still challenging route of investigation in the AML setting, whose application is hampered by intrinsic disease characteristics, including the disease heterogeneity, the absence of leukemia-restricted target antigens and the resistance mechanisms towards the immune responses.

Among the lineage-specific antigens, the CD33 molecule has been extensively validated as an effective AML immunotherapeutic target in several clinical trials (Walter, Appelbaum, Estey, & Bernstein, 2012) being broadly expressed by leukemic blasts in the majority of AML patients (Ehninger et al., 2014) and present on Leukemic Stem Cells (LSCs) in some patients (Walter et al., 2012).

We therefore generated CIK cells genetically modified with the SB transposon system to express a CD33 specific CAR. We found that the SB100X-pT4 molecular evolution results in superior CD33.CAR expression on CIK cells than the earlier generation SB11-pT. Moreover, lowering the amount of DNA required for the transposition could help in limiting the unintended events of transposase integration or transgene remobilization, potentially improving the safety profile for

clinical translation and likely shortening the classical CIK cell culture period.

CD33.CAR expressing CIK cells exhibited a potent anti-leukemic activity *in vitro* and *in vivo*, significantly reducing AML development when administered as an "early treatment" approach and delaying the progression of established disease in mice. With the final aim of moving towards the clinical application, the potential benefit of CAR T-cell therapy for AML has to be characterized considering the real unmet clinical need, which is represented by the resistance to chemotherapeutic drugs. Since CAR-T cells are currently exploited as a second line treatment, the SB modified CD33.CAR-CIK cell preclinical activity will be evaluated also in combination with standard chemotherapy by exploiting an already established xenograft chemotherapy model (Wunderlich et al., 2013) which mimics human induction therapy in AML xenograft mouse models. This model paves the way for a more effective assessment of the potential benefit of innovative targeted therapies in combination with standard-of-care treatments. Our expectation is that such approach could help bridge the gap between experimental research and clinical trials, thus contributing in ameliorating future study design of CAR-T cell immunotherapy.

RESULTS

Transposition using SB100x/pT4 combination grants higher levels of CD33.CAR expression on CIK cells compared to SB11/pT system

According to our previously optimized protocol (Magnani et al., 2016), CIK cells were engineered with the third-generation CD33.CAR by using both the SB11/pT and the SB100X/pT4 combinations. To establish the optimal amounts of SB100X transposase- and CD33.CAR pT4 transposon-encoding plasmids to be used, different SB100X/pT4 ratios have been tested (Suppl Figure 1A). We found that 0.5 µg/nucleofection of transposase successfully integrated 15 µg/nucleofection of transposon resulting in CD33.CAR stably engineered CIK cells. Additionally, at the end of the differentiation process, at day 21, we found the novel SB100X/pT4 combination to result in higher levels of gene transfer than the previously optimized SB11/pT platform, in terms of both percentage and MFI of CAR^+ cells (63,5%, vs 43,5%; 2872 vs 1622; n=9 for SB100X/pT4, n=7 SB11/pT) (Figure 1A-B) and the CD33.CAR expression was similarly high in all the CIK cell subpopulations (data not shown). The typical CIK cell phenotype was minimally affected by the genetic modification as compared to the un-manipulated condition (NO DNA) (Suppl Figure 2A) and CD33.CAR CIK cells showed efficient expansion after 3 weeks of culture with an average of 43 fold increase (Suppl Figure 2B).

Sleeping-Beauty modified CD33.CAR-CIK cells exhibit potent *in vitro* anti-leukemia ability

CD33.CAR-CIK cells modified with SB11/pT and SB100X/pT4 combinations were evaluated for their ability to mount effective anti-leukemia responses *in vitro*. Despite differences in CAR expression, we found that both transpositions conferred a specific killing of $CD33^+$ AML target cell lines (THP-1 and MA9) as well as primary AML cells, as compared to the NO DNA control, whilst a basal killing capacity, equal to the NO DNA control, towards the $CD33^-$ cell line MHH-CALL-4 has been obtained (Figure 2A). Furthermore, CD33.CAR-CIK cells were able to retain a significant cytotoxic activity when re-challenged with the target cells following a previous stimulation, indicating their ability to respond to repetitive antigen exposure (Figure 2B). We also corroborated CD33.CAR-CIK cytotoxic potential by performing a long-term cytotoxic assay at an E:T ratio of 1:10 (Figure 2F) against THP-1 cells.

Intracellular staining for IFN-γ and IL-2 revealed a CD33.CAR-specific cytokine production after co-culture with $CD33^+$ target cells (Figure 2C-D). Furthermore, both sets of CD33.CAR-CIK cells extensively proliferate in a CD33.CAR-specific manner, as measured by Ki67 staining and CFSE dilution (Figue 2E-G-H). However, there was a trend of CD33.CAR CIK cells generated by SB100X/pT4 transposition in being more responsive to AML targets. This result is in line with our previous evidence that, above a certain threshold, CAR expression is not a

limiting factor in determining a powerful cytotoxic activity, at least in the presence of a high target antigen density, while low CAR expression impacts on later effector functions, such as decreased cytokine production and proliferation capacity (Arcangeli et al., 2017).

Sleeping-Beauty modified CD33.CAR-CIK cells are able to target AML *in vivo*

We sought to investigate the ability of CD33.CAR-CIK cells to control AML growth *in vivo* focusing on CD33.CAR-CIK cells generated by the new platform SB100X/pT4. To evaluate the effect of CD33.CAR-CIK cell immunotherapy particularly on Leukemia Initiating Cells (LICs), CD33.CAR-CIK cells were administered as an "early treatment" approach in secondary transplanted PDX, a setting which presumably reflects the typical LIC properties of self-renewal and leukemia reconstitution. Starting from day 5, mice received once-weekly, for 3 weeks, an intravenous infusion of 10^7 CD33.CAR-CIK cells. We observed a clear engraftment reduction in the treated cohort, nearly undetectable in 2 out 5 mice (Figure 3E), while a high leukemic burden has been detected in the untreated mice (up to 70% of engraftment in bone marrow (BM)) (Figure 3B). A significant leukemia reduction has been detected also in the peripheral blood (PB) and spleen of treated mice (Figure 3A-C), suggesting CD33.CAR-CIK cell potential of reducing AML dissemination in the periphery. Furthermore, the CD33 expression on residual AML cells were similar to the freshly injected

cells (data not shown), thus indicating that no resistance to CD33.CAR-mediated immunotherapy has occurred.

CD33.CAR-CIK cells could be detected in all the analyzed districts with a preferential homing in the spleens (Figure 3D) mostly dysplaying an effector memory and central memory phenotype (Suppl Figure 3).

To determine whether CD33.CAR-CIK cells are also effective in treating established AML, leukemic cells were transplanted in secondary recipient mice, which were treated when leukemia engraftment was clearly manifested in the BM (Figure 4A). Even in this situation, a significant decrease of the disease in the treated cohort was found, confirming the efficiency of the CAR approach (Figure 4B-D). CIK cells were detectable in all the districts analyzed and maintained the CAR expression (Figure 4E-F).

When exploiting the xenograft chemotherapy model we confirmed that the “5+3” chemotherapy induction protocol was able to induce a remission-like status in the BM (Figure 5) and produced mild and temporary side effects only, thus allowing novel agents to be combined for investigation. Further studies are ongoing to test the efficacy of CD33.CAR-CIK cells in this setting.

DISCUSSION

CAR-T cell therapy is now a real therapeutic option for certain types of B-ALL and large-B-cell lymphoma and holds great potential for treating other types of cancer (Wohlfarth et al., 2018). Nevertheless, several limitations need to be overcome before its widespread clinical use, including the development of simplified and cost-effective production protocols, the generation of "off-the-shelf" CAR-T cells, and the identification of suitable target antigens to grant a proper balance between efficacy and safety profiles, in order to move beyond B cell malignancies.

In the attempt to address these issues, we preclinically assessed the feasibility of engineering CIK cells with a CD33.CAR through the non viral SB transposon system for the treatment of AML.

Currently, CAR-T cells are typically generated by employing viral gene transfer systems that are however associated to high manufacturing costs (Milone & O'Doherty, 2018). Alternatively, CAR engineering can be performed by using non viral SB vectors (P. B. Hackett, Largaespada, & Cooper, 2010) which, being inexpensive, could bring down costs and democratize this kind of immunotherapy. In addition to the advantage of being cheaper, integration data clearly indicate the superior safety profile of SB vectors with respect to retroviral and lentiviral ones (Field et al., 2013) (Vargas et al., 2016). The major impediments to a broad diffusion of the SB transposon system have been represented by low gene transfer efficiency and significant T cell toxicity associated with

both electroporation and high plasmid doses, demanding repeated stimulation cycles with feeder cells presenting the CAR target antigen to achieve sufficient number of CAR^+ T cells (Huang et al., 2006). Consequently, T cell terminal differentiation represents a possible drawback of such protocols and may limit the anti-tumor activity of CAR-T cell therapy once *in vivo*.

Our group has recently optimized the SB platform, in the version of SB11-pT, in order to achieve stable and efficient expression of different CARs on the surface of CIK cells by using γ-irradiated autologous PBMCs as a single stimulation step (Magnani et al., 2016), thus simplifying the culture process usually exploited with the non viral system and potentially allowing shortening the culture period to preserve minimal differentiated T cells.

In this study, we demonstrated that CIK cell CAR expression could be further increased by using the latest optimized version of the Sleeping Beauty transposon system SB100X-pT4 (Mátés et al., 2009; Y. Wang et al., 2017) that, additionally, offers the advantage of reducing the amount of transposase DNA required for the transposition. This should prevent the possibility of transposase integration or transgene remobilization, ensuring genomic stability. Thus, the SB100X-pT4 molecular evolution provides a more effective non-viral SB-transposition that could represent a valid alternative to viral vectors due to its superior safety profile, easy large-scale production, and low manufacturing costs.

Commercially available CAR-T cell therapies rely on engineering patients' own T cells, representing bespoke products that cannot be mass-produced to guarantee a ready accessibility.

As can be expected, expansion and manufacturing of autologous CAR-T cells is sometimes unsuccessful due to low lymphocyte counts and poor quality of patient cells (U.S. Food & Drug Administration: KYMRIAH (tisagenlecleucel); See August 30, 2017 Summary Basis for Regulatory Action- KYMRIAH, available from https://www.fda.gov/biologicsbloodvaccines/cellulargenetherapyproducts/approvedproducts/ucm573706.htm). Additionally, autologous products are intrinsically heterogeneous in terms of CD4:CD8:naïve:central memory cell ratios. Autologous products are also associated to the risk of residual contaminating tumor cells during CAR-T cell manufacturing which can acquire resistance to CAR therapy inducing relapse (Ruella et al., 2018). Therefore, there is active investigation in moving from autologous to allogeneic CAR-T cells. The use of donor derived cells for CAR immunotherapy offers the advantage of overcoming the immune defects associated with autologous cells following cancer treatment and could provide “off-the-shelf” products which would be reproducible, immediate available and less expensive. However, this strategy has to face the risk of graft-versus-host disease (GVHD). To circumvent this issue, genome editing techniques such as zinc finger nucleases or CRISPR/Cas9 have been exploited to delete the endogenous TCR (Eyquem J, Mansilla-Soto J,

Giavridis T, van der Stegen SJ, Hamieh M, Cunanan KM, Odak A, Gönen M, 2017; Torikai et al., 2012). In a pilot trial, Qasim et al. generated functional allogeneic CD19.CAR-T cells (UCART19) that have been TALEN (transcription activator– like effector nuclease) gene edited to disrupt both TCRαβ expression, for abolishing the risk of GVHD, and CD52 expression, to make them resistant to the anti-CD52 lymphodepleting conditioning. These "off-the-shelf" CAR-T cells have been used to treat two infants with relapsed/refractory B-ALL ahead of allogeneic HSCT. This approach has been proposed as a bridge to transplant strategy due to concerns related to the risk of TALEN induced translocations or off target effects and of GVHD occurrence given by residual αβ-TCR cells.

Another approach consists in investigating other types of effector cells for CAR engineering including ϒδ-T cells (Deniger et al., 2013) or NK cells (Mehta & Rezvani, 2018) which do not cause GVHD, however, if such populations will be effective as conventional T cells still needs to be proven.

Along this line, our group focused the attention on an alternative T cell population represented by the so called Cytokine-Induced Killer (CIK) cells, heterogeneous polyclonal T-effector cells with both T and NK cell phenotypic and functional properties (Schmidt-Wolf, Negrin, Kiem, Blume, & Weissman, 1991). CIK cells are therefore able to mediate a HLA-independent NK-like cytotoxicity against different types of tumors with a minimal capacity of causing GVHD in allogeneic settings

(Gao et al., 2017), thus widening the number of patients that could benefit of CIK cell-based therapy as well as overcoming the tumor immune escape mechanism of HLA downregulation. Furthermore, CIK cells expansion protocol is easy, it makes use of GMP compliant reagents and it is inexpensive. A growing number of clinical trials have been performed by using CIK cells, in both solid and hematological tumors, alone or in combination with conventional therapy or, more recently, with immune checkpoints inhibitors (Poh & Linn, 2016), demonstrating CIK cell potential in improving patient clinical outcomes in absence of severe adverse events (Schmeel, Schmeel, Coch, & Schmidt-Wolf, 2015).

Additionally, we and others have shown that CIK cell intrinsic antitumor activity can be redirected and further improved by CAR engineering (Marin et al., 2006) (Zuo S, Wen Y, Panha H, Dai G, Wang L, Ren X, 2017). More recently, we described the large-scale production and preclinical evaluation, in terms of both efficacy and toxicity, of donor-derived SB engineered CD19.CAR-CIK cells for a pilot Phase I/II trial to treat relapsed B-ALL (Magnani et al., 2018) (NCT03389035).

With the aim of translating this approach also to AML, here we provide the preclinical evaluation of CD33.CAR-CIK cell anti-tumor activity.

The employment of CAR-T cells for the treatment of AML is still in its infancy and the first pioneering clinical trials have not produced the same impressive results experienced when dealing with B-cell

malignancies. However, the myeloid setting is more challenging than the lymphoid one and thus more difficult to recapitulate in preclinical contexts, hampering a proper and more accurate prediction of CAR-T cell efficacy.

In this scenario, our attention has been posed on targeting the CD33 molecule, one of the main validated target antigen in AML so far, broadly expressed on AML blasts in almost 90% of the patients, with a differential expression between leukemic cells and normal Hematopoietic Stem/Progenitor Cells (HSPCs) (Ehninger et al., 2014) .

We found that CD33.CAR expressing CIK cells exhibited a potent anti-leukemic activity *in vitro* being able to specifically kill, proliferate and produce inflammatory cytokines in a target specific fashion. Moreover, CD33.CAR-CIK cells were able to respond to repetitive antigen exposure maintaining a significant cytotoxic activity when re-challenged with the CD33 target following a previous stimulation.

CD33.CAR CIK cells were also able to mediate a potent anti-leukemic activity *in vivo*, being effective against Leukemia Initiating Cells (LICs) when administered as an “early treatment” approach in a secondary transplanted PDX, likely selected for dominant AML subclones originated from the residual LIC. CD33.CAR-CIK cells were also able of significantly delaying the progression of established disease in mice. Importantly, CD33.CAR-CIK cell treatment has been well tolerated and no emergence of $CD33^-$ AML cells have been detected following up to three CD33.CAR-CIK cells infusions. Furthermore, to implement the

preclinical *in vivo* efficacy evaluation, CD33.CAR-CIK cells will be tested also in combination with standard treatment by exploiting the already established xenograft chemotherapy model which mimics human induction therapy in AML xenograft mouse models. Being the CAR-T cell therapy nowadays still considered as a second-line treatment, this model coupled to PDX provides a much more relevant platform to study the real potential benefit of combining standard-of-care treatment with most innovative targeted therapies, allowing to evaluate the CD33.CAR-CIK cell contribute in the elimination of the chemotherapy resistant/residual AML cells.

Concerning the safety aspect, the CD33 expression on normal HSPCs raises concerns for potential myelotoxicity. Whereas B-cell aplasia following CD19.CAR-T cell treatment can be managed with intravenous immunoglobulin administration, a similar approach is not conceivable to cope with sustained myelosuppression. Nevertheless, the potential CD33.CAR-CIK cell long-term persistence still needs to be properly assessed due to both CIK cell intrinsic features and immunosuppressive behavior of AML (Isidori et al., 2014). In this sense, envisaging the use of CD33.CAR-CIK cells in a "bridge to transplant" setting could offer the therapeutic safe window in which evaluating the myelosuppression potential.

To date, the few published results of early phase clinical trials exploiting CAR-T cell immunotherapy for the treatment of relapsed/refractory AML did not report long-term disease control and

thus T cell persistence (Ritchie et al., 2013; Q. S. Wang et al., 2015). However, to control sustained myelosuppression, safeguards strategies need to be envisaged for the clinical translation, such as HSCT, incorporation of a suicide gene, drug-mediated T cell depletion, or transient CAR expression using mRNA for engineering T cells.

In conclusion, the described protocol of SB- modified CD33.CAR-CIK cells accomplishes and synergizes some features still in implementation in the CAR-T cell field, such as the simplification of culture processes, by means of reduced costs and manipulation steps, improvements of CAR gene transfer, and use of a suitable and well tolerated T cell source alternative to the autologous setting. Moreover, to bridge the gap towards the clinic, the use of an *in vivo* model recapitulating the AML disease resistance to chemotherapeutic drugs will offer a more accurate preclinical evaluation of anti-AML CAR-T cell therapy.

MATERIALS AND METHODS

Plasmids

The high-affinity, humanized rat anti-human CD33, 113, in single chain fragment variable -Fv-(L-(gly4ser)4-H generated using UCB's Selected Lymphocyte Antibody Method (kindly provided by Dr. Helene Finney, UCB Celltech, Slough, UK), was codon optimized and cloned in frame with CH2CH3-CD28transmembrane-CD28-OX40-ζ (Marin et al., 2010) in the SB transposon plasmids. The SB pT donor vector and SB11 transposase were kindly provided by Laurence JN Cooper. The SB pT4 donor vector and SB100X transposase were kindly provided by Zsuzsanna Izsvak.

Generation of CD33.CAR genetically modified CIK cells

CD33.CAR CIK cells were generated as previously described (Magnani et al., 2016). In brief, on day 0, 10^7 peripheral blood mononuclear cells (PBMCs) from healthy subjects were nucleofected with SB transposon and transposase DNA plasmids according to the manufacturer's instructions using 4D-NucleofectorTM device (Lonza). After nucleofection, cells were transferred into a 6-well plate containing 4 mL of pre-warmed medium (advanced RPMI supplemented with 20% of heat-inactivated FBS and 1% of L-glutamine) and autologous γ-rad PBMCs ($1,5*10^6$) and IFN-γ (1000 U/ml Dompè Biotec) were added to each well. Twenty-four hours later, IL-2 (Chiron) and OKT-3 (Takara) were added at 300 U/mL and at 50 ng/mL, respectively. Cells were

then cultured for 21 days, fresh medium and IL-2 were added twice a week and cell concentration was maintained around 0.75 10^6 cells/mL.

Cell lines and AML primary cells

THP-1 and MHH-CALL4 cell lines have been obtained from the American Type Culture Collection (ATCC). THP-1 cell line was maintained in culture with Advanced RPMI medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 25 IU/mL of penicillin and 25 mg/mL of streptomycin (Lonza). MHH-CALL-4 cell line was maintained in culture with 20% FBS Advanced RPMI complete medium. MA9-NRas AML cells were kindly provided by Professor James C. Malloy (University of Cincinnati College of Medicine) and were maintained in culture with 20% IMDM (Invitrogen) complete medium. Primary AML cells were obtained from bone marrow and PBMCs collected from children or adults with AML. The Institutional Review Board of the Ethical Committee of San Gerardo Hospital approved this study and informed consent was obtained from patients or their guardians.

Flow Cytometry

CIK cells were tested for the expression of CD3 (clone SK7), CD8 (clone RPA-T8), CD4 (clone SK3), CD56 (clone B159), CD62L (clone DREG-56) and CD45R0 (clone UCHL1) using specific antibodies (BD Bioscience). CAR expression was detected using an anti-Human IgG (H+L) specific antibody (Jackson ImmunoResearch). Target cells were assessed for

CD45 (clone 2D1), CD33 (clone HIM3-4) CD123 (clone 7G3) and CD19 (clone HIB19) expression. Anti-human IFN-γ (clone B27), IL-2 (clone MQ1-17H129) and Ki67 (clone B56) mAbs (BD) have been used to assess CIK cell cytokine production and proliferation. Cell death and apoptosis were detected using the GFP-Certified™ Apoptosis/Necrosis detection kit (Enzo Life Sciences), according to the manufacturer's instructions. CIK cell proliferation was also assessed by measuring Carboxyfluorescein succinimidyl ester (CFSE) dilution (Invitrogen). Target cells were also labeled with FITC- and PE- Cell Tracker (Invitrogen). Human grafts in mice were assessed using anti-human CD45, CD33 and CD123 and anti-mouse CD45 (clone 30-F11) (eBioscience) mAbs. Samples were acquired using the FACS Canto II flow cytometer (BD) and data were analyzed using BD FACS DIVA software version 6.1.3.

Short- and Long-Term Cytotoxicity Assays

To evaluate the killing ability of both unmodified and CAR-redirected CIK cells, short- and long-term cytotoxicity assays were performed. In the short-term cytotoxic assay CIK cells were co-cultured for 4 hours with the target cells (previously labeled with PE-Cell Tracker) at an effector-target (E:T) ratio of 5:1. At the end of the incubation, target cell killing was measured through apoptosis detection by flow cytometry, after Annexin V and Necrosis Detection Reagent (NDR) staining. The percentage of killed cells was determined adding the percentage of PE^+/Annexin V^+/NDR^- cells to that of PE^+/Annexin

V^+/NDR^+ cells in co-culture with the effectors compared to target cells alone. In the rechallenging assays, after 4 hours co-culture of CIK cells with PE labeled target cells, fresh FITC labeled target cells were added to the culture that was maintained for additional 4 hours. The percentage of killed cells was determined for both PE^+ and $FITC^+$ cells. In the long-term cytotoxic assay CIK cells were co-cultured with THP-1 cells for 1 week at E:T ratio of 1:10 without exogenous IL2, on a human bone marrow-derived stromal mesenchymal cell layer, as previously described (Marin et al., 2007). After 1 week, cells were harvested, passed through a 19-gauge needle to disrupt residual mesenchymal-cell aggregates, stained with anti-CD33 mAb and flow cytometry-based quantitative analysis was employed to determine the percentage of viable target cells recovered from the culture.

Proliferation assay

The proliferation ability of CD33.CAR redirected CIK cells was evaluated after co-culture with the various target cells irradiated or not (E:T ratio of 1:1). After 72 hours co-culture, the cells were collected, surface stained for both CD3 and CAR. Then, intracellular staining for Ki67 was performed using the BD Cytofix/Cytoperm kit. Specimens were then analyzed by flow cytometry. Alternatively, CIK cell proliferation was determined by staining with 1 μM CFSE. After 72 hours, the cells were collected, surface stained for both CD3 and CAR and analyzed by flow cytometry.

Intracellular cytokine staining

CAR redirected CIK cell ability of producing cytokines was evaluated following a stimulation with the various targets, E:T ratio of 1:3. After 2 hours and 30 minutes co-culture, BD GolgiStop^{TM} was added. The co-culture was then maintained for an additional period of 2 hours and 30 minutes after which the cells were collected and stained for CD3 and CAR surface molecules. Then, intracellular cytokine staining for IL-2 and IFN-γ was performed using the BD Cytofix/Cytoperm kit. Specimens were then analyzed by flow cytometry.

Mouse models

Human AML PDX models were generated by transplanting NOD-SCID IL2Rγ null (NSG) mice (The Jackson laboratory) with splenocytes and/or bone marrow cells from primary recipients. Then, mice received once weekly for 3 weeks an intravenous infusion of 10^7 CD33.CAR-CIK cells. CIK cell administration started on day 5 after AML cell transplantation, in the “early treatment approach”, or after clear leukemia engraftment in the bone marrow for the “AML treatment model”. The xenograft chemotherapy model was established as previously described (Wunderlich et al., 2013). After leukemia engraftment (≈20% in the bone marrow) mice were treated for 5 consecutive days with 50 mg/kg cytarabine along with 1.5 mg/kg doxorubicin for 3 days (day 1 to 3). The study was approved by the Italian Ministry of Health (approval no.102/2013-B, issued on the April 23, 2013). Procedures involving animal handling and care were

conformed to protocols approved by the Milano-Bicocca University in compliance with national and international law and policies.

Statistical analysis

Data were analyzed using GraphPad Prism 6 software (GraphPad). One-way ANOVA analysis with Turkey's multiple comparisons test, Two-way ANOVA analysis with Bonferroni's multiple comparison test and unpaired t-test were performed to determine statistical significance of the *in vitro* data; the *in vivo* experiments were analyzed by the Mann–Whitney U-test or unpaired t-test. Reported values of the statistical analyses are the result of the evaluation of mean ± SEM.

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FIGURES

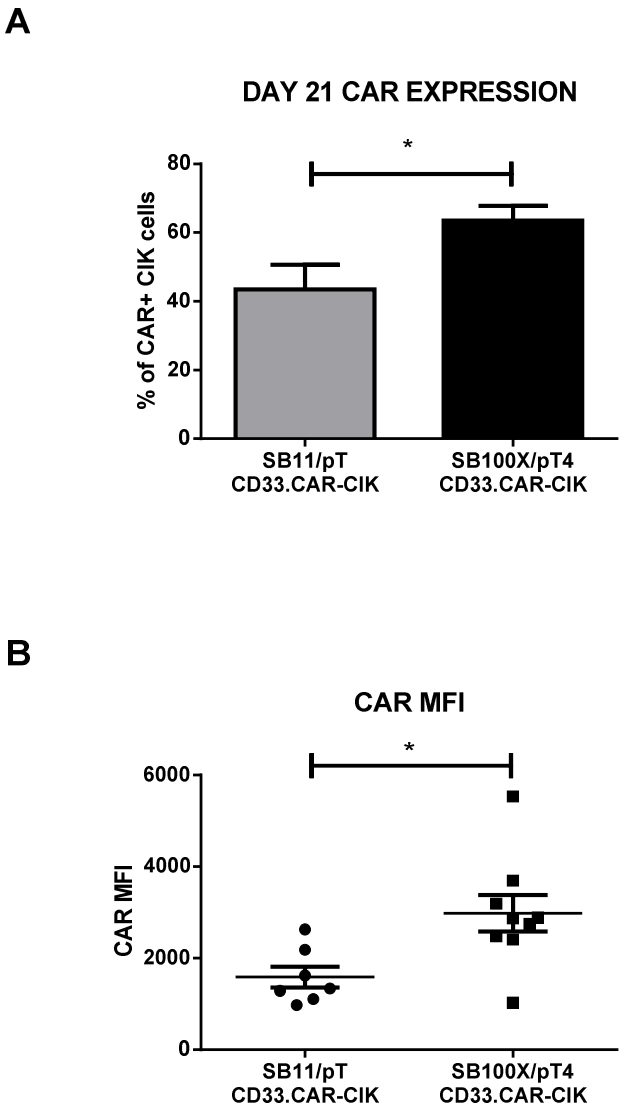

Figure 1. CAR expression in SB-modified CD33.CAR-CIK cells at day 21.
A) Percentage of CAR expression; B) MFI values. Data represented are the result of Mean ± SEM (n=7 for SB11/pT; n=9 for SB100X/pT4; *P<0.05 Unpaired t-test).

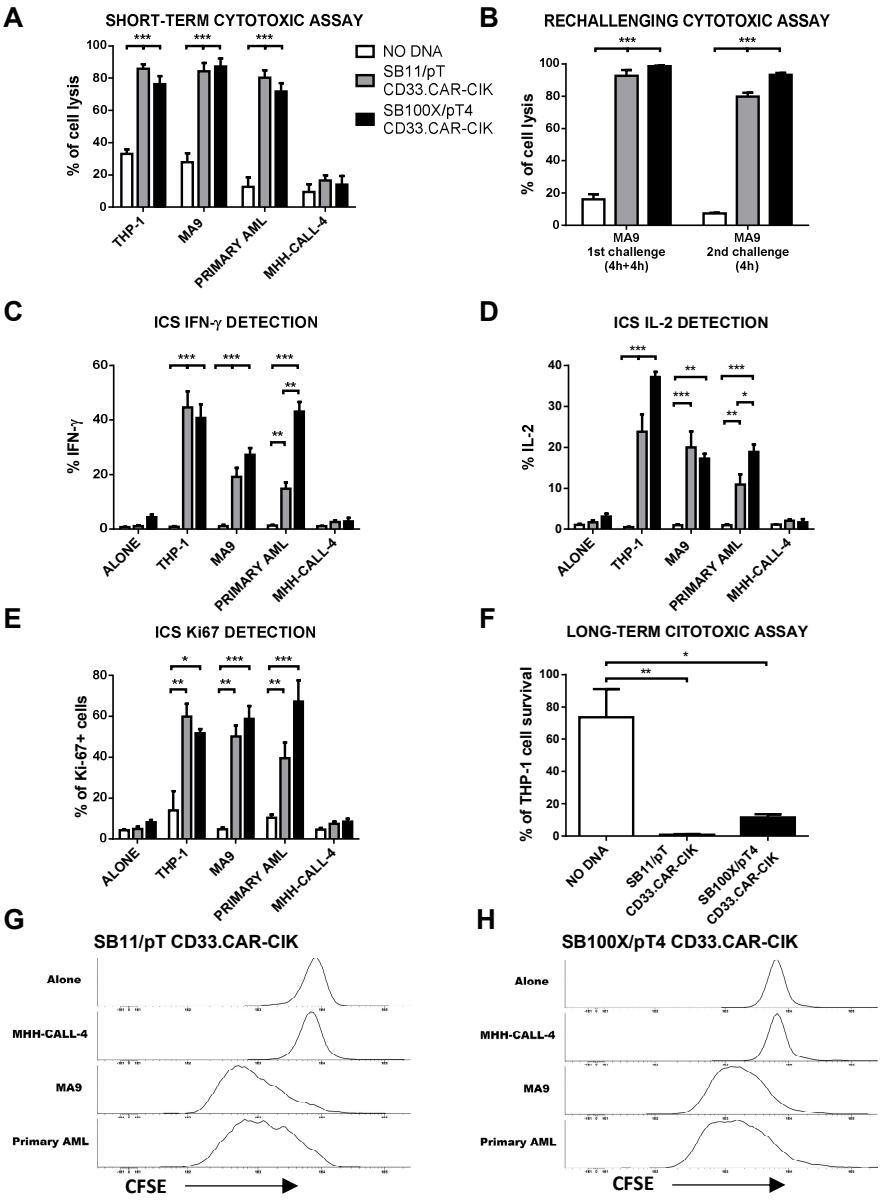
A
SHORT-TERM CYTOTOXIC ASSAY
% of cell lysis
NO DNA
SB11/pT CD33.CAR-CIK
SB100X/pT4 CD33.CAR-CIK
THP-1
MA9
PRIMARY AML
MHH-CALL-4
B
RECHALLENGING CYTOTOXIC ASSAY
% of cell lysis
MA9 1st challenge (4h+4h)
MA9 2nd challenge (4h)
C
ICS IFN-γ DETECTION
% IFN-γ
ALONE
THP-1
MA9
PRIMARY AML
MHH-CALL-4
D
ICS IL-2 DETECTION
% IL-2
E
ICS Ki67 DETECTION
% of Ki-67+ cells
F
LONG-TERM CITOTOXIC ASSAY
% of THP-1 cell survival
G
SB11/pT CD33.CAR-CIK
Alone
MHH-CALL-4
MA9
Primary AML
CFSE
H
SB100X/pT4 CD33.CAR-CIK

Figure 2. *In vitro* characterization of SB-modified CD33.CAR-CIK cells.
A) Short-term cytotoxic assay E:T 5:1 (THP-1: n=7 for NO DNA and SB11/pT, n=3 for SB100X/pT4; MA9: n=7 for NO DNA and SB11/pT, n=6 for SB100X/pT4; PRIMARY AML: n=6; MHH-CALL-4: n=7 for NO DNA and SB11/pT, n=4 for SB100X/pT4). B) Rechallenging assay: after 4h co-culture with MA9, CIK cells were re-challenged with fresh MA9 for additional 4h (n=2). C) Intracellular staining for IFN-γ and IL-2 (D) after 5h co-culture of CIK cells with the various targets E:T 1:3 (THP-1: n=7 for NO DNA and SB11/pT, n=3 for SB100X/pT4; MA9: n=7 for NO DNA and SB11/pT, n=4 for SB100X/pT4; PRIMARY AML: n=7; MHH-CALL-4: n=7 for NO DNA and SB11/pT, n=3 for SB100X/pT4). E) Intracellular staining for Ki67 after 72h co-culture of CIK cells with the various targets E:T 1:1 (THP-1: n=7 for NO DNA and SB11/pT, n=3 for SB100X/pT4; MA9: n=3 for NO DNA and SB11/pT, n=2 for SB100X/pT4; PRIMARY AML: n=7 for NO DNA and SB11/pT, n=2 for SB100X/pT4; MHH-CALL-4: n=7 for NO DNA and SB11/pT, n=3 for SB100X/pT4). F) One week cytotoxic assay towards THP-1 cell line E:T 1:10 (n=3). G-H) CFSE Proliferation assay after 72h co-culture of CIK cells with the various targets E:T 1:1. The result of a representative experiment out of 3 is shown.
Data represented are the result of Mean ± SEM (*P<0.05, **P<0.01, ***P<0.001, One-way ANOVA analysis with Turkey's multiple comparisons test).

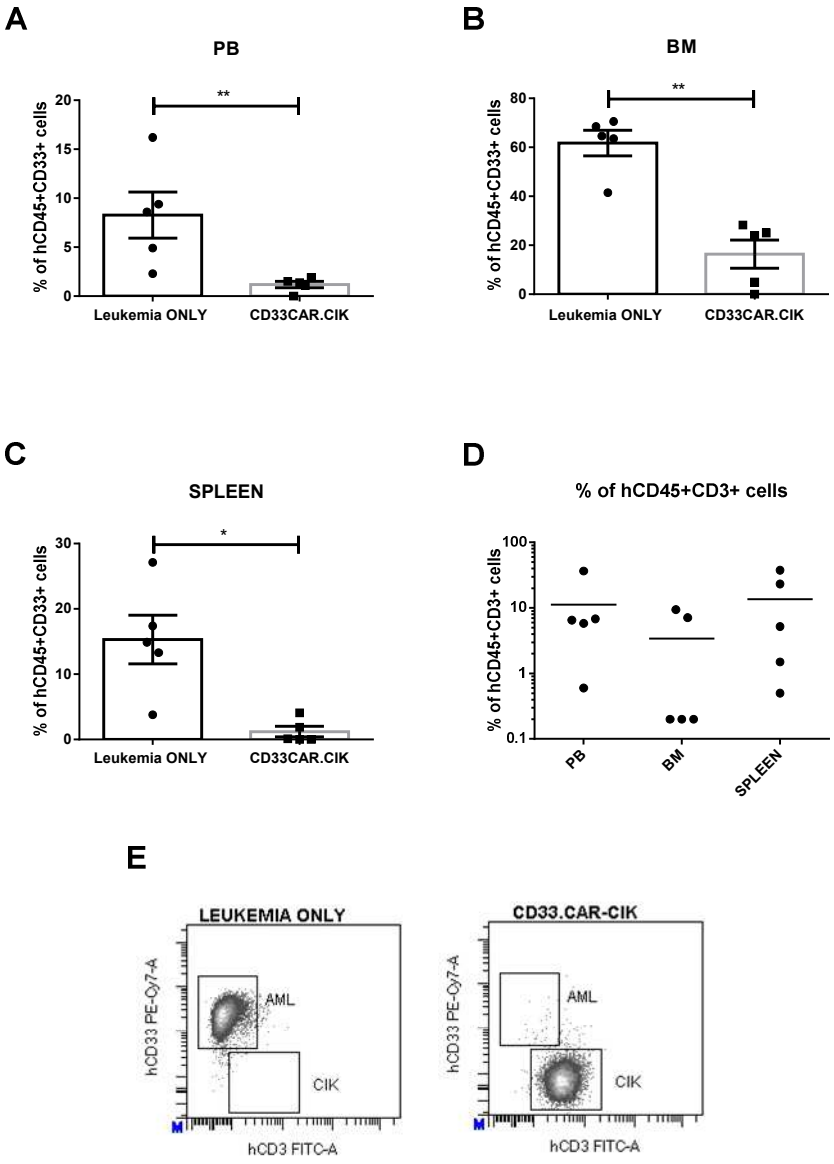

Figure 3. *In vivo* antitumor efficacy of SB100X/pT4 CD33.CAR-CIK cells in an "early treatment" model. NSG mice were inoculated with 10^6 cells of a secondary PDX. Starting from day 5, mice received once-weekly, for 3 weeks, 10^7 CD33.CAR-CIK cells. Mice were sacrificed 7 days after the last injection.

A) Percentage of leukemia engraftment in the peripheral blood, in the bone marrow (B) and spleen (C). D) Percentage of $CD3^+$ cells in the different districts detected in each of the treated mouse at the end of the experiment. E) A representative plot from each group is shown.
Each dot represents a mouse. Data represented are the result of Mean ± SEM (n=5 NSG mice per group, *P<0.05, **P<0.01, Mann-Whitney test).

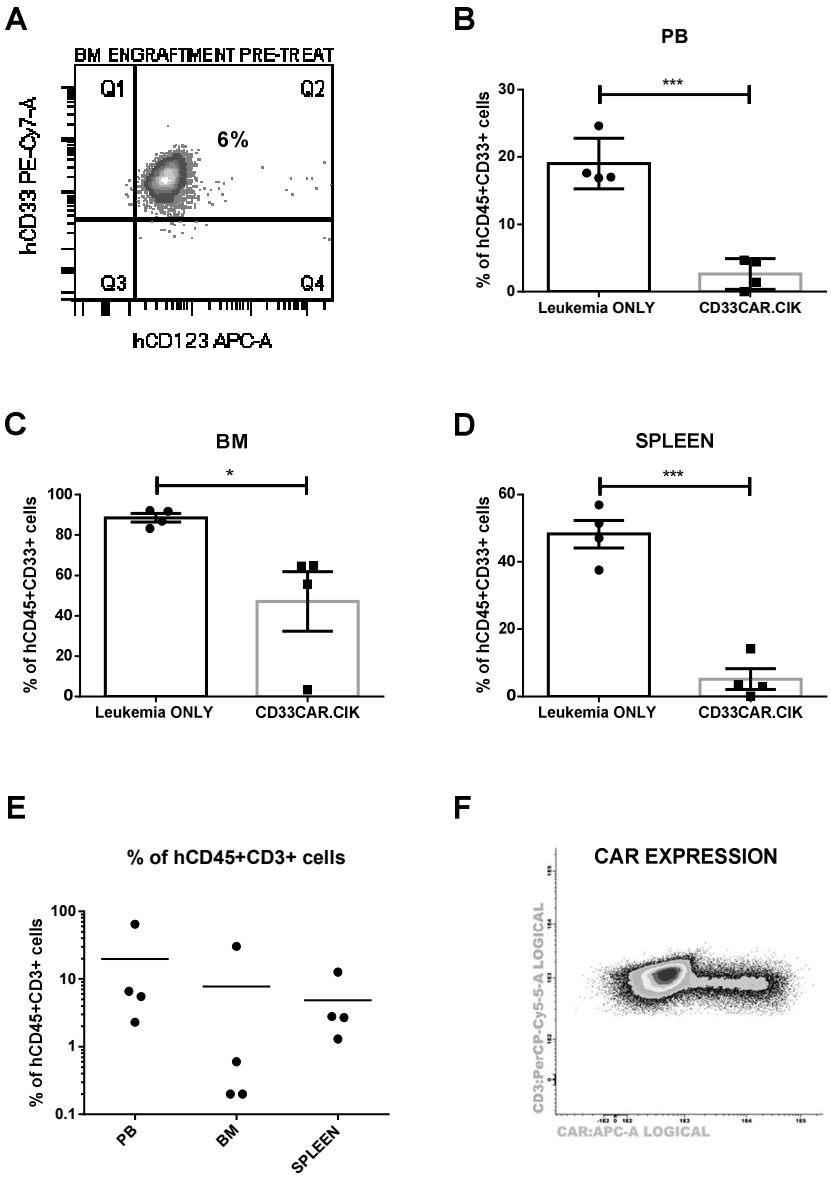
A
BM ENGRAFTMENT PRE-TREAT
Q1
Q2
Q3
Q4
6%
hCD33 PE-Cy7-A
hCD123 APC-A
B
PB
***
% of hCD45+CD33+ cells
30
20
10
0
Leukemia ONLY
CD33CAR.CIK
C
BM
*
% of hCD45+CD33+ cells
100
80
60
40
20
0
Leukemia ONLY
CD33CAR.CIK
D
SPLEEN
***
% of hCD45+CD33+ cells
60
40
20
0
Leukemia ONLY
CD33CAR.CIK
E
% of hCD45+CD3+ cells
% of hCD45+CD3+ cells
100
10
1
0.1
PB
BM
SPLEEN
F
CAR EXPRESSION
CD3:PerCP-Cy5-5-A LOGICAL
CAR:APC-A LOGICAL

Figure 4. *In vivo* antitumor efficacy of SB100X/pT4 CD33.CAR-CIK cells in an "AML treatment" model. NSG mice were inoculated with 10^6 cells of a secondary PDX. When a substantial tumor burden was evident in the bone marrow, mice received once-weekly, for 3 weeks, 10^7 CD33.CAR-CIK cells. Mice were sacrificed 7 days after the last injection

A) Representative dot plot of bone marrow engraftment before treatment. B) Percentage of leukemia engraftment in the peripheral blood, in the bone marrow (C) and spleen (D). E) Percentage of CD3+ cells in the different districts detected in each of the treated mouse at the end of the experiment. F) A representative dot plot of CD3 and CAR expression from peripheral blood of a treated mouse is shown.

Data represented are the result of Mean ± SEM (n=4 NSG mice per group, *P<0.05, **P<0.01, ***P<0.01 Unpaired t-test).

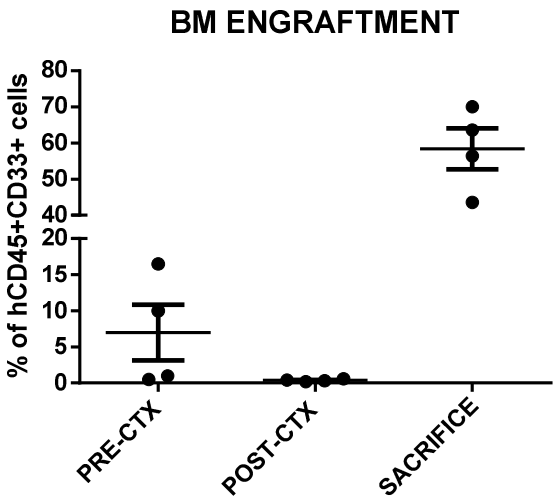

Figure 5. Leukemia engraftment in a "xenograft chemotherapy" model. NSG mice were inoculated with 10^6 cells of a secondary PDX. When a substantial tumor burden was evident in the bone marrow, mice received the "5+3" chemotherapy induction protocol resulting in a remission-like status with disease recurrence wihin short time.

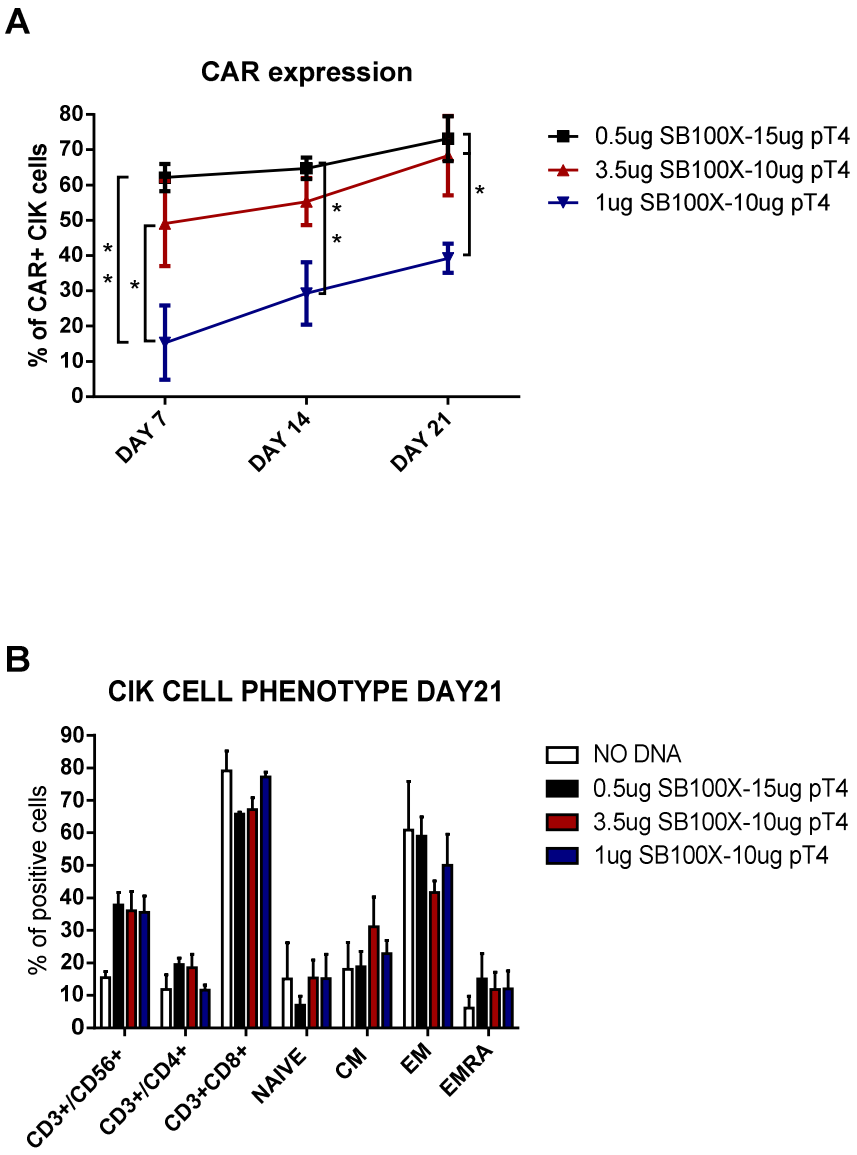

Figure S1. Comparison of transposition rate and stability after transfection with different ratios of SB100X transposase and pT4 transposon encoding for the CD33.CAR. A) Percentage of CAR expression during CIK cell culture;

B) CIK cell phenotype at the end of the differentiation (day 21). CM = central memory, EM = effector memory, EMRA = Terminally differentiated effector memory cells.
Data represented are the result of Mean ± SEM (n=3, *P<0.05, **P<0.01, Two-way ANOVA with Bonferroni's multiple comparison test).

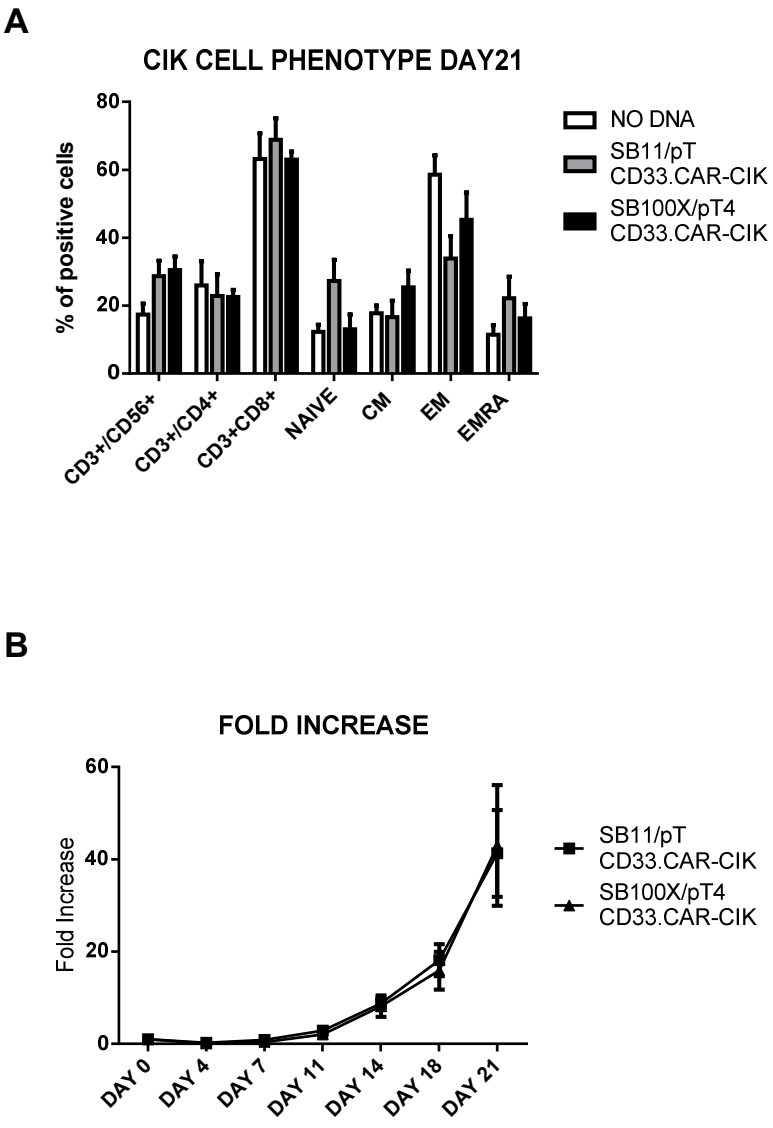

Figure S2. CIK cell phenotype and fold increase at the end of the differentiation (day21). A) $CD3^+/CD56^+$, CD3+/CD4+, CD3+/CD8+ and memory phenotype, n=6; B) Fold increase of SB-modified CIK cells during the differentiation culture, n=4. Data represented are the result of Mean ± SEM.

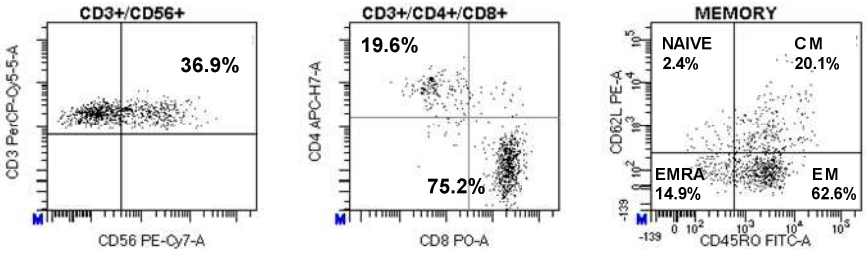

Figure S3. ***Ex vivo*** **CIK cell phenotype.** Representative dot plots of $CD3^+/CD56^+$, CD3+/CD4+, CD3+/CD8+ and memory phenotype from spleen of a treated mouse are shown.

Chapter 4

Summary, Conclusions and Future perspectives

Acute myeloid leukemia (AML) still represents one of the major unmet clinical need in the field of oncohematology. For almost 40 years AML treatment relied on a “one size fits all” approach conceived to be directly effective against actively proliferating AML blasts. However, only 35-40% of patients younger than 60 and less than 20% of elderly patients benefit from conventional treatments, including intensive chemotherapy and hematopoietic stem cell transplantation (HSCT) (Döhner, Weisdorf, & Bloomfield, 2015). In recent years, the expanding knowledge of AML genetic alterations better uncovered the disease heterogeneity providing the basis for a more differentiated risk stratification as well as for the development of targeted therapies focused towards tailored treatment approaches (Coombs, Tallman, & Levine, 2015).

The evidence of the graft versus leukemia (GVL) effect in patients receiving allo-HSCT advances the concept that leukemia immunogenicity could be exploited to develop novel therapeutic strategies aimed at enhancing the anti-leukemia immune response. In this context, immunotherapy adopting T cells engineered to express tumor-directed chimeric antigen receptors (CARs) offers the potential to more specifically target the leukemic cells, including the leukemic stem cells (LSCs).

CARs are synthetic receptors owning the advantage of combining the broader antigen specificity of antibodies with T cell effector functions such as cytotoxicity, biodistribution and immunological memory

(Gross, Waks, & Eshhar, 1989). In the last decade, CAR-T cell therapy has shown striking results in clinic particularly in the context of B-cell malignancies (Lichtman & Dotti, 2017), sparking a keen interest in extending this approach also to other hematological malignancies, such as AML. However, the first pioneering clinical trials on myeloid malignancies have not produced the same impressive results experienced when dealing with lymphoid malignancies (Ritchie et al., 2013; Wang et al., 2015). Of note, the myeloid setting is more challenging than the lymphoid one due to intrinsic characteristics of the disease, including heterogeneity, lack of tumor specific antigens that could lead to off-target toxicity and tolerance and/or exhaustion induced by immunosuppressive factors within the AML microenvironment. Thus, the employment of more predictive preclinical models, better recapitulating the AML disease complexity, would contribute to the ongoing efforts of designing an optimized anti-AML CAR immunotherapy approach appropriately matching efficacy with safety.

In this scenario, our CAR based strategies consist in the recognition of the CD123 and CD33 molecules, two of the main validated target antigens in AML so far, broadly expressed on AML blasts in almost 90% of the patients, with a differential expression between leukemic cells and normal hematopoietic stem/progenitor cells (HSPCs) (Ehninger et al., 2014).

Together with the proper target antigen choice, the effector T cell population, the cell-engineering technique and the CAR design structure are other important aspects that need to be fully characterized and optimized in the development of CAR-based approaches. Our T cell population is represented by cytokine-induced killer (CIK) cells, heterogeneous polyclonal T effector cells with both T and NK cell phenotypic and functional properties. CIK cells are therefore able to mediate a HLA-independent NK-like cytotoxicity against different types of tumors with a minimal capacity of causing graft versus host disease (GVHD) in allogeneic settings (Gao et al., 2017), as demonstrated also by our group (Introna et al., 2007). Furthermore, CIK cell expansion protocol is easy, Good Manufacturing Practices (GMP) compliant and inexpensive. All these features make CIK cells a really suitable cell source to be employed for CAR engineering. Importantly, CAR redirection of donor-derived CIK cells allows to overcome costs, manufacture and safety issues associated with autologous products.

For CAR gene transfer, our group has recently optimized a non-viral *Sleeping Beauty* (SB) transposon platform to redirect CIK cells with different CARs achieving, with a single stimulation step, a stable and efficient CAR expression while preserving phenotype, viability, and expansion of transduced CIK cells (Magnani et al., 2018, 2016). The SB system is composed by two elements: the transposon plasmid, which contains the transgene of interest and the transposase plasmid that

through a “cut and paste” mechanism, mediates the excision and stable genomic integration of the transgene. This strategy allows to overcome the main limitations associated with viral vectors, such as potential genotoxicity, immunogenicity, high manufacturing costs, regulatory hurdles and scale-up complexities. Furthermore, we here demonstrated that CIK cell CAR expression could be further increased by using the latest optimized SB version of transposase SB100X-transposon pT4. Indeed, the novel SB100X-pT4 combination has allowed a better CD33.CAR transfer in CIK cells compared to the previously optimized system. Integration site analysis are currently ongoing to compare SB genomic distribution between the two SB combinations employed.

Concerning the CAR architecture, in the context of CD123 targeting, we focused our attention on dissecting the effect of several variables involved in the CAR design, known to modulate CAR T-cell functional profiles, such as CAR binding affinity and expression in relation to the target antigen density. Indeed, while the “on target-off tumor” effect associated to the CD33 targeting is mostly limited to the hematological compartment, the CD123 targeting demands a higher level of caution, due to the potential recognition of low CD123-positive endothelial tissue. Thus, computational structural biology tools were exploited to design point mutations in the wt CD123.CAR antigen binding domain. In this way, we were able to maintain the same epitope binding site and the overall features within the CAR structure. We generated CAR

mutants (CAM) with a binding affinity in the range of the wt CAR (CAM-H1 and CAM-H2) and with a lower affinity (CAM-M and CAM-L). Some point mutations also resulted in CAR mutants with an expression profile inferior than the wt (CAM-H2 and CAM-M).

By challenging CAM redirected CIK cells with target cell lines bearing a diverse CD123 surface density, we were able to define in our affinity range (10^{-9}-10^{-7}M) both "lytic" and "activation" antigen thresholds defined as the number of cell surface antigens required to produce full cytotoxic activity and activation or expansion of CAR-T cells, respectively (K. Watanabe et al., 2015). These context dependent thresholds are worth to be investigated when assessing the suitability of the selected target antigen in order to find a therapeutic margin which allows CAR-T cells to be more discriminative between tumor cells with high target antigen density and normal cells expressing low levels of target antigen. Moreover, a rational tuning of both CAR affinity and density should allow the modulation of these thresholds in order to improve CAR-T cell therapeutic index.

Our *in vitro* characterization of wt and mutant CD123.CARs confirmed CD123 as a safe target antigen for CAR-CIK cell approach, being the CD123 antigen density on the endothelial cell line below the "activation" threshold. Furthermore, we found that i) early cytotoxic activity is not affected by either CAR affinity or expression (within the tested range); ii) later effector functions are not affected by CAR affinity but rather by CAR density; iii) the lower affinity mutant (10^{-7}

M) is just as effective as the high affinity ones (10^{-9}M) while remaining safer or, at worst, equally safe. Overall, a combination of computational structural biology and cellular assays allowed us to characterize the role and interplay of CAR binding affinity and CAR expression in the efficacy and safety profiles of CD123.CAR redirected CIK cells towards AML cells and healthy tissues, expressing different levels of target antigen. These results supply relevant notions for the proper design of a suitable CD123.CAR for the treatment of AML, which can grant a proper balance between efficacy and safety profiles. The target antigen safety should be ultimately tested *in vivo*. However, in our context, the lack of homology between the murine and human CD123 antigen (Hara T, 1992) has limited a proper safety evaluation with respect to the endothelium. Future studies could be implemented by exploiting xenoreceptors humanized mouse models, carrying the CD123 human gene within the equivalent murine locus, although considering the possibility of differences in CD123 expression between the mouse endothelium and the human one.

In parallel, a proper preclinical assessment of novel therapies also demands for accurate efficacy evaluation, particularly considering the AML disease complexity, such as the heterogeneity and the immunosuppressive myeloid microenvironment.

Thus, since CAR-T cells are currently exploited as a second line treatment, we will exploit an already established AML xenograft chemotherapy model (Wunderlich et al., 2013) mimicking the

standard induction therapy in order to better resemble the human clinical setting. This model coupled to patient-derived xenografts (PDX) will provide a much more relevant platform to study the real potential benefit of combining standard-of-care treatment with most innovative targeted therapies, allowing to evaluate the anti-AML CAR-CIK cell contribute in the elimination of the chemotherapy resistant/residual AML cells.

Envisaging a future clinical translation of anti-AML CAR CIK cells at our institution, we will focus our efforts in applying the above described model in the investigation of the CD33 target, being nowadays the most known and characterized AML antigen exploited in clinic also with other kind of immunotherapeutic approaches.

We found that CD33.CAR expressing CIK cells exhibited a potent anti-leukemic activity *in vitro* being able to specifically kill, proliferate and produce inflammatory cytokines in a target specific fashion. Moreover, CD33.CAR-CIK cells were able to respond to repetitive antigen exposure maintaining a significant cytotoxic activity when re-challenged with the CD33 target following a previous stimulation.

CD33.CAR CIK cells were also able to mediate a potent anti-leukemic activity *in vivo*, being effective against Leukemia Initiating Cells (LICs) when administered as an early treatment approach in a secondary transplanted PDX, likely selected for dominant AML subclones originated from the residual LIC. CD33.CAR-CIK cells were also able of significantly delaying the progression of established disease in mice.

Importantly, CD33.CAR-CIK cell treatment has been well tolerated and no emergence of $CD33^-$ AML cells have been detected following up to three CD33.CAR-CIK infusions. Ongoing optmization of our approach include the incorporation of alternative costimulatory signals within our CD33.CAR construct, currently relying on CD28.OX40 combination, and the investigation of spacer regions other than the IgG1-derived one. Indeed, based on lessons learned thus far from CD19.CAR clinical experiences, the success of CAR-T cell therapy is associated with expansion and long-term persistence of the infused product. Loss of CAR-T cell persistence can be due to the development of immune responses towards the CAR transgene or to CAR-T cell activation-induced cell death (AICD) or senescence (Hay & Turtle, 2017). In this sense, improvements in CAR design may enhance CAR-T cell persistence and hence clinical activity. Indeed, it has been shown how providing CARs with different costimulations profoundly affects tumor rejection kinetics and persistence of CAR T cells. Of note, depending on the T cell population engineered, certain costimuli may work better than others (Hombach, Rappl, & Abken, 2013; Long et al., 2015; Zhao et al., 2015). Additionally, inclusion of Fc domains as spacer domains can support binding of CARs with IgG Fc gamma receptors (FcγRs) on myeloid cells, thereby leading to an unintended “off-target” activation of both innate cells and T cells together with the initiation of activation induced cell death (AICD) through overstimulation of the CAR-T cell (A Hombach, 2010; Hudecek et al., 2015).

In light of these evidences, we aim to further define which are the proper modular components that in our context ensure the maximum performance and fitness of SB modified CD33.CAR-CIK cells. Furthermore, with the aim of improving the AML selectivity whilst reducing the potential off-target effects, a dual CAR targeting strategy is currently object of evaluation. Indeed, taking advantage of our long lasting experience on both CD33 and CD123 targeting, we plan to exploit the transsignaling strategy, by which CIK cells are modified to express both a CD123.CAR providing the activation signal 1 (CD3ζ) and a CD33 chimeric costimulatory receptor (CCR) to give the costimulatory signal 2. To ensure successful CIK cell activation only after simultaneous engagement of both receptors, a suboptimal primary signaling CD3ζ has to be pursued (Kloss, Condomines, Cartellieri, Bachmann, & Sadelain, 2013). Thus, current efforts are aimed at identifying a CD123.CAR which is responsible for limited CIK cell activation. In particular, we are verifying if the already characterized CAM-L, which was almost as effective as wt CAR when considered as 3rd generation CAR, can satisfy this requirement when conceived as 1st generation CAR.

In conclusion, the deconvolution of all the variables implicated in the preclinical characterization of efficacy and safety profiles of anti-AML CAR-CIK cells performed in this study offers the proper background to build up and optimize a CAR-T cell therapy approach that could be rationally translated to the clinic.

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