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Optimization of chimeric antigen receptor (CAR) design strategy for a specific anti-CD123 targeted therapy in pediatric Acute Myeloid Leukemia (AML)

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Ai miei genitori, alle mie nonne ed a Dario,

che, insieme, mi hanno sostenuta durante tutto il dottorato e mi hanno permesso di arrivare serenamente fino alla fine.

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Chapter 1

General Introduction

1. Hematopoiesis and Acute Myeloid Leukemia (AML)

The process by which new blood cells are formed is referred to as hematopoiesis. Even if during embryonic development there is a change in the site of hematopoiesis occurrence, the location in which it principally takes place is represented by the Bone Marrow (BM).

In particular, mature blood cells own a finite life span, needing thus to be continuously replaced throughout life and the cell source that is principally responsible for their production over time is represented by Hematopoietic Stem Cells (HSCs). HSCs, which cover approximately 0.01-0.2% of total BM mononuclear cells in human (3), are self-renewing and multipotent stem cells located at the top of the hematopoietic hierarchy.

HSC cell divisions are infrequent and often asymmetric, giving rise to both identical stem cells and more specialized cells: Multipotent Progenitors (MPP) and Lineage Restricted progenitors, the latter characterized by an extensive proliferation and differentiation toward specific mature blood cells (3).

When the processes of self-renewal and differentiation, typical of HSCs, become deregulated or uncoupled, Acute Myeloid Leukemia (AML) can occur. Indeed, the accumulation of abnormal immature blasts, not more able to correctly differentiate into functional cells, results in AML development. Despite the clonal origin of AML blast cells, which makes them sharing several features, it has been shown

that the organization of the AML bulk population is hierarchical, being analogous to the normal hematopoietic system (4).

Indeed, according to the "Cancer Stem Cell hypothesis" AML can be described as an aberrant hematopoietic process (5), in which rare Leukemic Stem Cells (LSCs), that share many characteristics with normal HSCs, including quiescence, multipotency and self-renewal, give rise to the pathology. However, LSCs have been shown to have a phenotypical heterogeneity (6), given their derivation not only from mutations at the HSC level, but even from mutations occurring in progenitor cells, at different stages of the hematopoietic hierarchy. As a consequence, the ectopic reacquisition of the unique self-renewal properties typical of stem cells can occur, interfering also with the subsequent ability of progenitor cells to correctly differentiate (7) (Figure 1). In addition, both the apoptosis and senescence mechanisms, usually adopted by cells to prevent an uncontrolled proliferation, are efficiently evaded by LSCs. Moreover, LSC resistance against most of current cancer treatments, such as irradiation and chemotherapy, is higher compared to the total population of malignant blast cells, contributing thus to treatment failure. In this way, the quiescence of LSCs could be one of the reasons for drug resistance, being chemotherapy and irradiation active only on cycling cells. Furthermore, the expression of efflux pumps on the surface of LSCs, represents another mechanism involved in conferring multidrug resistance (8). Also the contribution of BM microenvironment is important in determining drug resistance, as in

the case of the so called environmental-mediated drug resistance (EMDR) (9).

The survival in a quiescent state, together with the acquisition of more complex drug resistance phenotypes (9), which contribute to the LSC protection from therapy, are promoted by signaling events mediated by environmental soluble factors and adhesion molecules.

Minimal residual disease (MRD) status, which refers to post-treatment persistence of leukemic cells, residual leukemic blasts and/or therapy resistant LSCs (3), is thought to be cause of relapse (Figure 1). Therefore, in order to achieve a remission and potentially cure the leukemic disease, it will be critical to design therapeutic strategies able to specifically target and eradicate both the leukemic bulk and LSCs.

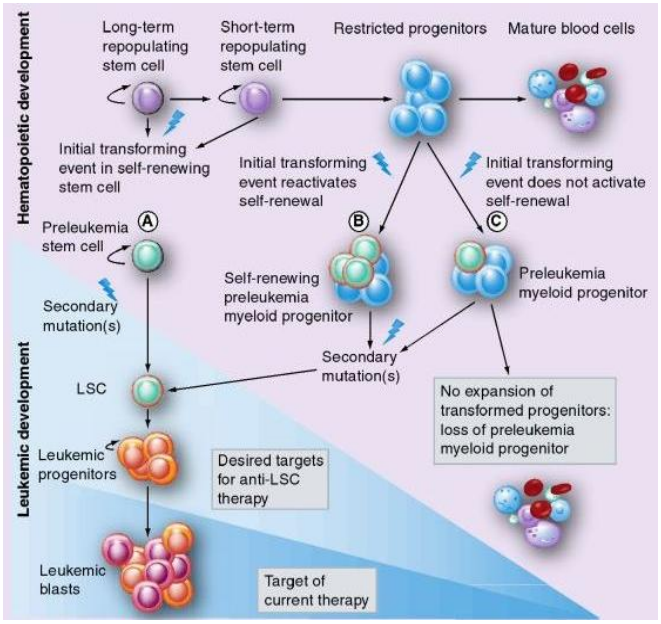

Figure 1. Model of leukemogenesis. Adapted from Gail J. Roboz 2009, Expert Rev Hematol. (10).

2. Childhood AML

AML is a heterogeneous clonal hematopoietic disorder, with maturation arrest of myelopoiesis, leading to an accumulation of myeloblasts in BM and/or Peripheral Blood (PB) (11), but also other tissues.

2.1 Epidemiology and Incidence

Although leukemia is the most common childhood cancer, accounting for about 30% of diagnoses (12), AML is less common, accounting for approximately 18% of childhood leukemias, with an incidence of 7 cases per million per year in children younger than 15 years (13) (14) (15).

2.2 Pathogenesis and risk factors

AML, like most cancers, can be defined as a multifactorial disease, since its origins depend not only on genetic and infectious events, but also on the combination of environmental and socioeconomic factors (16) (17) (18) (19). In particular, leukemia is the result of chromosomal rearrangements and multiple gene mutations that cooperate in permitting expansion of highly dysregulated cells.

A model by which AML development requires at least two types of genetic events is supported by several evidences. According to this pathological scheme, class I mutations involve genes in the kinase signaling pathways, promoting cell survival and proliferation or inhibiting apoptosis (*RAS, FLT3, JAK2* activating mutations, *P53*

inactivating mutations) and class II mutations, involving transcription factors or cofactors, lead thus to impaired hematopoietic differentiation (t(15;17)/*PML-RARα*, inv(16)/*CBFβ-MYH11* and t(8;21)/*RUNX1-RUNX1T1* fusions, *MLL*/*PTD*, *CEBPα* and *AML1*/*RUNX1* mutations) (20). As a result, it has been hypothesized that class I mutations would confer a proliferative advantage to hematopoietic cells, which are already blocked in differentiation by class II mutations, leading to leukemia development.

In fact, combination of class I and class II mutations has been shown to induce AML in a variety of mouse models (20). Several novel mutations, involving genes related to epigenetic modifications, have been also recently detected in AML (21). Examples of epigenetic regulatory genes carrying mutations in AML are: isocitrate dehydrogenase 1 (*IDH1*), *IDH2*, ten-eleven translocation 2 (*TET2*), DNA methyltransferase 3A (*DNMT3A*), additional sex comb-like 1 (*ASXL1*) and histone methyltransferase enhancer of zeste homologue 2 (*EZH2*). These mutations in epigenetic modifiers seem to be enriched in adult AML compared to pediatric AML (22) (23).

Epidemiological studies have identified that the development of childhood AML is associated with several risk factors (14), such as environmental factors or genetic disorders, which interact with genetic events. Some of these risk factors 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 use		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 (14).

2.3 Signs and symptoms

Diverse and not specific are the clinical signs and symptoms of AML, but they are usually directly attributable to the leukemic infiltration of the BM, with resultant cytopenia. Typically, patients present with signs and symptoms of fatigue, hemorrhage, or infections and fever due to decreases in red cells, platelets, or white cells, respectively. Moreover, leukemic infiltration of various tissues, including the liver (hepatomegaly), spleen (splenomegaly), skin (leukemia cutis), lymph nodes (lymphadenopathy), bone (bone pain), gingiva, and central nervous system, can produce a variety of other symptoms (24).

2.4 Diagnosis

Clinical and laboratory evaluation at diagnosis of AML in children is similar to the regular adult workup with some specifically pediatric considerations. Morphology, cytochemistry, immunophenotyping, molecular and cytogenetics of the BM aspirates are performed. In addition, and unlike adults, investigation of Central Nervous System (CNS) involvement is necessary in children. Indeed, CNS implication at diagnosis in pediatric AML is fairly common.

Morphologic analysis is used to detect the percentages of undifferentiated, granulated or atypical blasts, intracellular structures, such as Auer rods, and presence of myelodysplasia. Cytochemistry allows to distinguish between myeloid (myeloperoxidase (MPO) positive) and monoblastic differentiation (MPO negative and nonspecific esterase positive) and confirms lineage affiliation.

In children, a low percentage of blasts renders difficult the distinction between AML and advanced myelodysplastic syndrome (MDS). Indeed, while in adults it is sufficient to consider a threshold of 20% of BM blasts to differentiate between these diseases, blast percentages between 20 and 30 may be observed in children with MDS. However, some features allow to support AML diagnosis rather than MDS: AML specific genetics, hyperleucocytosis, extramedullary disease and progression within a short time interval (2-4 weeks). Children with Down syndrome (DS) are diagnosed with AML even if the blast threshold of 20% is not reached. The same exception is valid for AML with recurrent genetic abnormalities t(15;17), t(8;21), inv(16), or

t(16;16) and low blast counts (25). Regarding immunophenotyping, to date there is no standardization of antibody panels used among the large trial groups. However, it is suggested to use multicolor monoclonal antibody combinations that include CD45 for recognizing the AML blast population within the context of normal residual hematopoiesis (26). Indeed, leukemic blasts show a low expression of CD45, compared to normal hematopoietic cells, which are strongly positive for this marker.

Examples of antigens for immunophenotypic analysis during diagnosis of childhood AML are (13):

- Myelomonocytic markers: CD11b, CD11c, CD13, CD14, CD15, CD33, CD64, CD65, intracellular MPO (iMPO), i-lysozyme;
- Megakaryocytic markers: CD41, CD61;
- Erythroid marker: CD235a;
- Mixed phenotype acute leukemia (MPAL): B-lineage (CD19, iCD79a, iCD22, CD10), T-lineage (iCD3 or surface CD3), myeloid lineage (iMPO, CD11c, CD14, CD64).

Numerical and structural chromosomal abnormalities (e.g., translocations, inversions or deletions), which have been reported in 70-80% of children with AML (13), can be detected by conventional cytogenetic analysis and FISH, being useful for diagnosis as well as for AML classification and prognosis. Fusion genes and mutations can also be detected by reverse transcriptase-polymerase chain reaction (RT-PCR).

2.5 Classification

AML diagnosis is followed by its classification that has evolved from the primarily morphologic and cytochemical system of the early French-American-British Cooperative Group (FAB) (27) to the clinical, morphologic, immunophenotypic, genetic and clinical (28) system of the 4th edition of the *WHO Classification of Tumors of the Hematopoietic and Lymphoid Tissues*. According to a multidisciplinary approach, this classification recognizes different disease entities having an impact on prognosis and on selection of a specific treatment (table 2: FAB classification and table 3: WHO classification, 2016 revision).

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., 1985 (27).

-AML with recurrent genetic abnormalities

- AML with t(8;21)(q22;q22.1);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
- AML 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

-*Provisional entity: AML with BCR-ABL1*

- AML with mutated NPM1
- AML with biallelic mutations of CEBPA

-*Provisional entity: AML with mutated RUNX1*

-AML with myelodysplasia-related changes

- Following MDS or MDS/MPD
- Without antecedent MDS or MDS/MPD, but with dysplasia in at least 50% of cells in two or more myeloid lineages

-Therapy-related myeloid neoplasms

- Alkylating agent/radiation–related type
- Topoisomerase II inhibitor–related type
- Others

-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
    - Erythroleukemia, erythroid/myeloid
- Acute megakaryoblastic leukemia
- Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis

Table 3: WHO classification. Adapted from Vardiman et al. 2008 (28) and Arber et al., 2016 (29).

The last category of the WHO classification, AML NOS, is subclassified by criteria similar to FAB classification. There are also two other categories recently considered: acute undifferentiated leukemia (AUL) and mixed phenotype acute leukemia (MPAL).

2.6 Prognostic factors

Prognostic factors include host factors, disease characteristics and response to therapy. These factors are important determinants of clinical outcome and are used to stratify patients into risk categories influencing the choice of therapy.

-Host factors:

Several host factors, including age, gender, race, body mass index (BMI) and host polymorphisms, have been implicated in disease outcome (30) (31) (30-32). Several studies (33) (34) have demonstrated that racial differences are associated with disease outcome, as African American patients had a significantly worse outcome than their white counterparts, primarily for higher rates of relapse, probably due to host polymorphisms. Indeed, there are evidences that polymorphisms in genes involved in drug metabolism, DNA repair or regulation of hematopoietic development may contribute to variable response to therapy (35).

Variance from an ideal BMI in AML patients at the time of diagnosis has recently been shown to impact clinical outcome. Lange et al. (36) found that both extremely overweight and underweight children had survival inferior to that of children with BMI in the range of the 11^{th} to 94^{th} percentiles.

Age at diagnosis is also associated with patient survival: young patients with AML have better survival rates than older patients, the latter being more prone to treatment related mortality and resistance to therapy (37).

-Disease characteristics:

Disease characteristics which are relevant to the prognosis are represented by leukocyte count, cytogenetics and molecular aberrancies.

A leukocyte count <20,000 cells/ml^3 has been associated with a better prognosis while a count >100,000 cells/ml^3 has been linked to an unfavorable outcome (38).

The cytogenetic feature in AML is widely recognized as one of the most significant prognostic factors in all age groups and it has been the initial feature considered in the reclassification of AML by the WHO (30) (31) (39). As evidenced by several trials, the use of cytogenetics alone can divide AML into three broad and major risk groups impacting relapse risk (RR), disease-free survival (DFS), and overall survival (OS): favourable, intermediate and unfavourable (40). Favourable AML cytogenetics include the core binding factor (CBF) leukemias t(8;21) (*RUNX1-ETO*), inv(16) (*MYH11-CBFB*), t(15;17) (*PML-RARA*). Unfavourable cytogenetics comprise complex cytogenetics (three or more distinct cytogenetic abnormalities in a leukemic clone): monosomy 7, monosomy 5, del(5q), abnormal chromosome 3. The intermediate-risk cytogenetics enclose all the remaining chromosomal abnormalities commonly seen in AML, such

as +8, +21, 11q23 (*MLL*)-associated abnormalities, as well as patients with a normal karyotype (30) (41) (42). For AML patients with intermediate-risk cytogenetics, including those with normal karyotype, three gene mutations permit further risk stratification: *FLT3*, that indicates poor prognosis (43), *NPM1* and *CEBPA*, that suggest good prognosis (44) (45) (46). Overexpression and mutations of *WT1* gene are also typically considered as two different categories of molecular alterations in AML but the prognostic significance in pediatric AML is unclear. Indeed, recent studies, in contrast to reports mostly on adult AML, have demonstrated that *WT1* mutations and *WT1* overexpression at diagnosis have no independent prognostic significance in predicting outcome in pediatric AML (47) (48). However, this does not preclude an important role for *WT1* in AML biology, indeed *WT1* expression after treatment may be used as a marker for MRD.

LSC frequency is also an important prognostic factor. Recently, Witte et al. showed that high frequency at diagnosis of $CD34^{+}/CD38^{-}/CD45^{-/low}$ fraction, LSC enriched, correlates with poor clinical outcome in childhood AML (49). Future studies will have to confirm the correlation between high numbers $CD34^{+}/CD38^{-}/CD45^{-/low}$ cells, as surrogate for the LSC population at diagnosis, and poor clinical outcome in higher numbers of pediatric AML of different subtypes. Moreover, because LSCs are hypothesized to be chemotherapy resistant and, consequently, to grow out after

treatment and cause relapse, it is of great importance to study the frequency of these LSCs during follow-up.

-Response to therapy:

Response to therapy is an independent strong prognostic factor in AML to assess the quality of response after the induction therapy and to outline personalized treatment, based on the individual risk of relapse, avoiding the situation of under or over drug exposure. Historically, response to therapy has been measured by the morphologic presence of disease at defined periods after the start of induction therapy. However, the limit of detection of the morphologic analysis is not sufficient for identifying minimal residual leukemia, which may be more prognostic. MRD is defined as any measurable disease or leukemia detectable above a certain threshold, depending on the methodology applied. Detection of MRD in patients in morphologic remission may contribute to the improvement of standard risk-classification, identifying patients at high risk of relapse, thus resulting to be an additional independent prognostic factor (50). PCR and multiparametric flow cytometry (MPFC) are the most popular methods to investigate MRD, being adequately sensitive and specific. MRD monitoring by PCR relies on the detection of leukemia specific targets, such as: fusion genes (e.g., *RUNX1-RUNX1T1*, *CBFB-MYH11*, *PML-RARA*); mutations, (for example in *NPM1*) and over expression of genes such as *WT1*. In contrast, MRD monitoring by MPFC relies 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. Data published recently by many international groups indicate that in AML, MRD detection by PCR or MPFC is feasible, rapid and able to help the prediction of relapse, even though it is not yet widely used in clinical decision making. Among the several reasons to be considered there is the lack of large prospective studies, the absence of standardization among different laboratories concerning immunophenotypic stability, but also identifications of thresholds and time-points during follow-up (51).

2.7 Treatment of AML

Nowadays, complete remission (CR) rates have improved to as high as 93%, with overall survival (OS) rates now reaching 65% (13) (52) (53) (54) (55) (56). The reason has to be found not only in the use of more intensive chemotherapy regimens, but also by improvements in supportive care and better risk-group stratification. However half of patients continue to relapse.

Conventional treatment of AML is generally divided into two phases: induction of remission, with concurrent CNS prophylaxis for children with CNS involvement at diagnosis, and post-remission therapy for consolidation.

-Induction therapy:

The aim of induction therapy is the achievement of CR. The most common induction regimen adopted both in children and adults AML

patients is the so called ‘7+3’ or ‘10+3’ regimen, consisting in the continuous infusion for 7-10 days of the antimetabolite cytarabine and the concomitant use of an anthracycline drug for 3 days. Remission rates >85% for children and adolescents are generally reached during this phase, usually consisting of one or two cycles (13).

Despite the known efficacy of anthracyclines, cardiac toxicity represents an important side effect. A recent report showed that children with AML had the highest mortality rate related to heart disease, compared to the other pediatric cancers (57) (58) (59). For this reason, the promotion of strategies that give more attention to cardioprotection has been prompted, as in the case of the recent development of liposomal anthracyclines with the potential to reduce cardiotoxicity by modifying drug distribution, resistance mechanisms and bioavailability (60) (61) (62) (63).

-Post-remission therapy:

One of the major challenges in the treatment of children with AML is to prolong the duration of the initial remission with additional chemotherapy or Haematopoietic Stem Cell Transplantation (HSCT). Post remission therapy consists thus in consolidation chemotherapy, usually composed by high-dose cytarabine (HiDAC). The optimal number of post-remission courses of therapy remains unclear, but appears to require at least two courses (32).

Regarding HSCT, both autologous and allogeneic approaches have been studied. In an autologous transplant, the not leukemic cells of the patient, collected after CR, are employed, but several trials have found

no benefits for autologous HSCT over intensive chemotherapy (64) (65) (66). On the other hand, allogeneic transplantation consists in the infusion of stem cells derived from an HLA-matched healthy donor, ideally a sibling related donor (matched related donor, MRD). However, there is only a 25% chance for a sibling to be HLA matched, therefore there are only about 30% of patients who have a sibling matched donor. This number is expected to continue to drop as the average family size keeps going down. An alternative donor source is an unrelated HLA matched donor (MUD) and, in the absence of HLA matched donors, another possibility is the use of mismatched/haploidentical donors, that have one haplotype in common with the recipient, so they match in at least five out of ten HLA loci. These are most commonly relatives, such as parents, children or siblings. Prospective trials of transplantation in children with AML suggest that overall 60% to 70% of children undergoing allogeneic HSCT from an HLA-matched healthy donor experience long-term remission (65) (67). This is likely due to the myeloablative preparatory regimen that is used, as well as the immunotherapeutic anti-leukemic activity exerted by the donor cells, also known as the Graft Versus Leukemia (GVL) effect. However, the principal drawback of such an approach is represented by the potential development of Graft Versus Host Disease (GVHD) or other HSCT-related complications, which can lead to Transplant-Related-Mortality (TRM).

For this reason, when the preferable option of MRD is not available, T-cell depletion (TCD) of the donor graft offers the potential for GVHD prevention, without the morbidity associated with immunosuppressive drugs. However, the reduction in GVHD did not translate into improved overall survival because of unexpected high rates of graft failure, opportunistic infections due to a delayed immune reconstitution and disease recurrence after TCD BM transplantation, being T cells the major mediators of GVL effect after HSCT (68).

Some strategies have been developed in order to accelerate immune reconstitution and to prevent relapse. Among them it is possible to number donor lymphocyte infusion (DLI), also associated with GVHD, and depletion of donor alloreactive T cells from haploidentical grafts. The latter strategy would theoretically result in a significant reduction of GVHD rates without affecting both GVL and immune reconstitution (69) (70) (71).

Nowadays, there is consensus that HSCT should be offered to all children with relapsed AML in second CR. Moreover, many groups offer HSCT to children with high-risk or refractory disease (72), while HSCT is avoided in low-risk patients (73).

-Supportive care:

It has become the standard of care to include stringent supportive cares during the treatment of patients. These include mandatory hospitalization following each chemotherapy, until the absolute phagocyte count is rising for two successive days and the patient is

afebrile and clinically stable. Moreover, antifungal and antibacterial prophylaxes are also recommended.

-Special subgroups:

- Children under 2 years of age: these patients generally present features of high-risk AML (25), but the susceptibility to toxicities in infants younger than 1 year is increased due to immaturity of organs and differences in pharmacokinetic and pharmacodynamic profiles of certain drugs. For these reasons, special attention in drug dosages and high treatment expertise are greatly demanded.
- Children with Down syndrome: since these patients have a favorable prognosis, they can be treated with intensity-reduced chemotherapy and without HSCT, resulting in event-free survival and survival rates >85% (74) (75, 76).
- Childhood acute promyelocytic leukemia (APL): the treatment of this class of patients is similar to that used in adults and is based on all-trans retinoic acid (ATRA), that induces differentiation of APL cells by targeting the most common fusion product PML-RARα, in combination to chemotherapy.

An important problem in the treatment of pediatric AML remains the high frequency of treatment related deaths as well as the long term side-effects (52) (77) (78). For this reason, thanks to the continuous progresses in the knowledge of childhood AML biology, many efforts are focusing on the development of even more targeted therapeutic approaches.

Even though a large number of new therapeutic agents are currently under development, mainly in the adult clinical setting, in order to investigate preliminary toxicity and efficacy profiles before being adopted in children, in recent years novel classes of therapeutics have also been investigated in childhood AML, including purine nucleoside analogs, small molecule kinase inhibitors and immunomodulatory therapies (79).

-LSCs specific targeting:

AML-LSCs represent a rare population of AML cells residing within the osteoblastic niche of the bone marrow, which is responsible of perpetuating leukemia (80) (81). Being AML LSCs resistant to conventional chemotherapies, due to both chemotherapy resistance and enhanced cellular ability of detoxification (82, 83), LSC-directed therapies are emerging, in order to completely eradicate AML.

Indeed, several studies have shown that AML patients with high prevalence of LSCs at diagnosis have worse outcomes compared to those whit lower LSC component (84) (85).

Since their identification, several efforts have been made in order to better characterize the LSC population and it has been found that these cells share several phenotypic and molecular features with HSCs.

Initial *in vitro* and *in vivo* studies suggested that both normal and malignant stem cells were negative for the expression of lineage markers (Lin^-), and were $CD34^+$, $CD38^-$ (86) (87). However, with the development of more immunodeficient xenograft models, which have allowed the detection of smaller populations of LSCs, it has been

shown that LSCs are more heterogeneous (6) and present in several different phenotypic cell compartments. Indeed, it is currently known that LSCs can be positive or not for CD34 and/or CD38 expression (6).

Regarding molecular features, LSCs, like HSCs, are quiescent, multipotent and capable of self-renewal. However, differently from HSCs, the pathways governing these characteristics are often mutated or aberrantly activated. Thus, considering the heterogeneity of the LSC compartment and the potential toxicity against HSCs, developing a LSC targeted therapy is challenging. To overcome these obstacles current strategies consist in the targeting of conserved markers of the LSC population, which are also differentially expressed on LSCs compared to normal HSCs (i.e., deregulated molecular pathways and overexpressed surface antigens). However, the selectivity is not absolute and the risk of toxicity cannot be excluded. In this context, some LSC targets are represented by Hedgehog signaling pathway, NFκB signaling, BCL-2 and SRC Family Kinases (87) (88) (89), which could be targeted with small molecule inhibitors, but also many overexpressed surface antigens such as CD33, CD123, CLL-1, CD44, CD47, CD96, TIM3 (4) (90) (91) (92) (93) (94), which could be targeted with immunotherapeutic strategies. The table below shows the potential interventions and the current active and recruiting clinical trials targeting LSCs.

Putative LSC target	Potential interventions	Active and recruiting trials
Hedgehog signaling pathway	Small molecule inhibitors of pathway regulators (e.g. Smoothened)	NCT01841333, NCT01546038, NCT01842646
NFκB Signaling/induction of oxidative stress	Partenolide, Bortezomib	NCT01174888, NCT01861314, NCT01127009, NCT01534260, NCT01371981, NCT01736943, NCT01075425, NCT00410423
c-KIT; SRC Family Kinases	Dasatinib, RK-20449 (HCK inhibitor)	NCT00892190, NCT01876953
BCL-2	ABT-199, oblimersen sodium (inhibitors of BCL-2)	NCT01994837
CLL-1	Monoclonal antibody	
CD44	Monoclonal antibody	
CD47	Monoclonal antibody	
CD33	Antibody-based (Gentuzumab ozogamicin, SGNCD33A, Actinium-225 labeled HuM195), Chimeric Antigen Receptor (CART33), DARTs, BITEs, Triplebodies	NCT01902329, NCT01864902, NCT00672165, NCT01869803, NCT01864902
CD96	Monoclonal antibody	
IL3 Receptor-α (CD123)	Diphteria toxin-IL-3 fusion protein, CSL362 monoclonal antibody, CART123, DARTs, BITEs, Triplebodies	NCT00397579, NCT01632852

Table 4. Strategies targeting LSC and related Clinical Trials. Adapted from Polleya et al. and Larkin et al. *(95) (96)*.

2.8 Immunotherapy and AML

One emerging and promising novel therapeutic approach for both relapsed and refractory AML is represented by immunotherapy, which can improve the immune response against leukemic cells.

Theoretically, a competent immune system (IS) is capable of recognizing and eliminating transformed malignant cells through the exertion of the immune-surveillance mechanisms, but tumor cells are able to uncontrollably grow, employing a variety of strategies to elude the IS, by means of immune-escape induction. AML cells are indeed able to evade the immune control in several ways. Firstly, they induce a poor activation of the effector T cells, by aberrantly processing antigens, down-regulating MHC molecules and weakly expressing co-stimulatory signals. In addition, they influence the tumor microenvironment inducing immunosuppression, through the secretion of inhibitory cytokines, such as IL-10 and TGF-β, but also through the up-regulation of the costimulatory molecule CD200 or the constitutively expression of the enzyme indoleamine 2,3-dioxygenase (IDO) which, in turn, favors the regulatory T cells (Treg) differentiation (97). Moreover, AML cells overexpress molecules that inhibit T and NK cell activation and functions, such as CTLA-4, (Cytotoxic T-Lymphocyte Antigen 4) and PD1-L (Programmed cell death-1 ligand) (98) (99). Besides, inhibitory stimuli to the effector cells are provided not only by AML cells, but also by the microenvironment of the BM niche, which protects leukemic cells from effector T-cell infiltration. Indeed, it has been shown that stromal

cells of the niche produce soluble factors, such as PGE (Prostaglandin E), IL-10, TGF-β and HGF (Hepatocyte Growth Factor), that inhibit responding T cells or block dendritic cell (DC) maturation from BM progenitors into a tolerogenic phenotype. Moreover, stromal cells induce Treg that, in turn, can suppress effectors or block Antigen Presenting Cells (APCs) (100). Considering all these possible situations in the pathological context, with the aim of reinforcing the reaction of the immune system to AML, different immunotherapy approaches can be considered. Immunotherapy can be indeed subdivided into "active" immunotherapy (not-specific and specific) and "passive" immunotherapy (cellular and humoral).

2.8.1 Active immunotherapy

Active immunotherapy aims at spontaneously augmenting the host immune response against the tumor. Not specific strategies consist in enhancing the immune functions through the administration of adjuvants, e.g., cytokines (IL-2, IFN) and bacterial adjuvants. Specific strategies are represented by cancer vaccines, based on isolated tumor associated antigens (TAAs), inactivated tumor cells (unmodified or *in vitro* modified to express factors that increase their T-cell stimulatory capacity), nucleic acids (DNA or RNA) encoding for tumor antigens, but also DCs loaded with tumor antigens (101).

Active immunotherapy presupposes the existence of a competent immune system, however immune responsiveness is often impaired in AML patients due to chemotherapy regimens and immunosuppressive factors released by leukemic cells. Moreover, most TAAs or tumor

specific antigens (TSAs) are self-proteins to which T cells develop tolerance by clonal deletion or anergy. Therefore, while T cells against such antigens can be detected for at least some tumor types, they are represented by low avidity T cells, giving weak responses which are inadequate in decisively fighting tumor cells (102).

2.8.2 Passive immunotherapy

Passive immunotherapy consists in the direct administration of immune effector molecules, such as monoclonal antibodies (passive humoral immunotherapy) or tumor specific effector cells (passive cellular immunotherapy).

-Monoclonal antibodies (mAbs):

The therapeutic efficacy and specificity of mAbs against human cancers have been largely established so far and several mAbs have been approved for the treatment of solid and hematological tumors (103). The mechanisms of action of mAbs against cancer include antibody-dependent cellular cytotoxicity (ADCC), stimulation of complement-dependent cytotoxicity (CDC), cell mediated cytokine production or direct induction of apoptosis.

In order to improve their potency, mAbs manipulated in the Fc portion, bi-specific antibodies or mAbs conjugated to toxic agents (chemotherapeutic agents, bacterial and plant toxins, radioisotopes) have been generated. To date, several TAAs have been studied for the selective AML targeting with mAbs, such as CD33, CD123, CD44, CLL-1, CD96, CD47, CD32, and CD25 (104). In order to potentiate the anti-tumor responses, an emerging field of application of mAbs

consists in targeting co-stimulatory and co-inhibitory receptors expressed by immune cells (as in the case of mAbs against CTLA-4 or PD-1).

-Adoptive cell therapy (ACT):

Adoptive T-cell transfer refers to as the infusion of *ex-vivo* expanded autologous or allogeneic T cells into tumor-bearing patients (105). The first evidences of the potential therapeutic benefit of ACT have been represented by the pioneering discovery of Tumor Infiltrating Lymphocytes (TIL) by Rosenberg and colleagues (106) in patients with malignant melanoma (MM). TIL cells can be directly isolated from patient biopsies, expanded *ex-vivo* using high doses of IL-2 (6000 U/ml) and transferred back to the patients. Since then, TIL therapy has been practiced in the treatment of several malignancies such as MM, colorectal and ovarian carcinoma.

As already mentioned, another demonstration of the potential of the immune system in fighting tumor cells has been the observation of the GVL effect after HSCT and/or DLI.

In addition, adoptive transfer of immune cells, specific for antigens primarily expressed by tumor cells have been developed. T cells specific for TAAs can be isolated out of cancer patients (105), such as the above-mentioned TIL, or can be generated *in vitro* using TAA-loaded APCs, such as DCs or modified tumor cells (107) (108).

However, the isolation and expansion of antigen specific T cells is time and labor intensive, moreover the lack of T cells with high affinity for TAAs, due to T-cell tolerance, and tumor immune-escape

mechanisms hamper the activity and persistence of these patient derived T cells.

Thus, various efforts have been made overtime to overcome these limitations, improving the efficacy of 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 (109) (110) (111) (112), with the aim to identify an effector population easily expandable *in vitro* and effective once *in vivo*. Moreover, thanks to the progresses in the fields of synthetic biology and immunology, genetically modified cells expressing artificial T-cell receptors (TCRs) (113) and **Chimeric Antigen Receptors** (**CARs**) (114) have been generated.

3. Cytokine Induced Killer (CIK) Cells

Among the different effector populations widely studied for ACT, particular interest has been raised by the $CD8^+$ NKT cells, featuring a mixed T-NK cell phenotype, with a polyclonal TCR repertoire and a not-MHC-restricted cytolytic activity. Due to the paucity of $CD8^+$ NKT circulating cells in the human peripheral blood (accounting for 1 to 5% of peripheral mononuclear cells), in the '90s Lu and Negrin, together with the group of Schmidt-Wolf, developed a protocol for the *ex-vivo* $CD8^+$ NKT cell generation. Such cells were named "cytokine-induced killers", being generated by the addition of cytokines and being able of mediating a potent MHC-unrestricted cytotoxicity against a variety of malignant human cell lines. Moreover, they do not

require neither a previous target contact nor the exogenous administration of IL-2, which turned to be toxic at high dosages, to become fully activated once *in vivo* (115) (116) (117). CIK cells can be efficiently *in vitro* generated starting from peripheral blood mononuclear cells from healthy donors, after the stimulation with IFN-γ (Day 0), OKT-3 (an anti-CD3 mAb) and IL-2 24 hours later, reaching a ~ 1000 fold expansion in 14-21 days of culture, after the repeated administration of IL-2 every three days (118). The feasibility of the expansion protocol allows to obtain adequate number of CIK cells for clinical use. At the end of the differentiation process the population is extremely heterogeneous, being 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, that display the major cytotoxic properties. The addition of IL-2 and anti-CD3 after the prior administration of IFN-γ, in a decisive time-controlled manner, seems to be crucial for the achievement of an elevated cytotoxic potential, operating the signals at different levels (116). Indeed, the IFN-γ priming leads to the monocytes activation, which results in the production of important stimuli involved in the acquisition of a T_H1 phenotype and expansion of $CD56^+$ T cells (119), while mitogenic stimulants are subsequently produced through the successive administration of both IL-2 and anti-CD3 (120).

Within the fully differentiated CIK cell population, it is possible to find a $CD3^+/CD56^+$ counterpart (accounting for 40 to 80%), but also a $CD3^+/CD56^-$ subset (in the range of 20 to 60%) and finally a $CD3^-$

/$CD56^+$ small fraction (less than 10%). While the anti-tumour activity of CIK cells is principally exerted by the first $CD3^+/CD56^+$ subpopulation, the ability of proliferating and persisting for longer periods, conferring the whole population the capacity of producing a continuous source of cells, is rather dependent on the $CD3^+/CD56^-$ compartment (121) (122). Indeed, $CD3^+CD56^+$ CIK cells are more terminally differentiated with a limited proliferative potential, in contrast $CD3^+CD56^-$ CIK cells are early effector T cells with a greater proliferative aptitude (121). CIK cell phenotype also includes the HLA-DR, CD57, CD11b and CD5 molecules, while excluding CD16 (Fcγ receptor), the typical NK cell marker.

Once expanded, CIK cells produce high amounts of pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-2 and IL-6, typical of the T_H1 context, displaying very powerful proliferative and cytolytic activities against several hematological and solid tumor targets, both *in vitro* and *in vivo* (115) (123). MHC-unrestricted tumor antigen recognition operates principally through NKG2D (Natural Killer Group 2 member D) receptor (124). NKG2D, in fact, is responsible for the recognition of MHC-unrestricted ligands on tumor cells, such as MHC-class I-like molecules, MICA, MICB and members of the ULBP family, ULPB1-4, and has been demonstrated that expanded CIK cells up-regulate NKG2D, being the cytolitic activity of these cells mostly dependent on the NKG2D interaction rather than the TCR engagement (125). Since NKG2D ligands are often overexpressed by both solid and hematological tumor cells, CIK cells own a broad cytotoxic activity

against several tumors (122) (126) (127) (128). The killing mechanism is perforin-dependent, since CIK cells derived from perforin-deficient mice had no cytotoxic activity both *in vitro* and *in vivo* (129).

With the aim of generating higher numbers of $CD3^{+}CD56^{+}$ CIK cells some groups reported that IL-7, IL-12 or IL-15 can be used instead of IL-2 (130) (131) (132). Moreover it has been shown that CIK cells can also be expanded starting from granulocyte colony-stimulating factor (G-CSF)-mobilized bone marrow and from cord blood cells (118) (133).

Another important reasons for considering CIK cells for clinical settings is the possibility of obtaining these cells not only from healthy donors but also from a variety of clinical conditions. Indeed, CIK cells can be easily expanded starting from engrafted patients after allogeneic Hematopoietic Stem Cell Transplantation (HSCT), but also during immune-suppression treatments and finally from patients with newly diagnosed leukemia, with the ability of generating free-leukemic contamination cell cultures (118) (122) (133). The effectiveness of this strategy comes from experimental evidences obtained by different research groups, with CIK cells obtained from AML patients, but also from Chronic Myeloid Leukemia (CML) and Chronic Lymphocytic Leukemia (CLL) patients. In particular, with regard to their efficacy, CIK cells proved to have a strong and specific functionality against autologous and allogeneic AML targets, while showing a reduced activity against Acute Lymphocytic Leukemia (ALL) (134) (135) (136) (137) (138). In fact, a work published by

Linn and collaborators, aimed at defining the molecular mechanisms involved in the CIK cell anti-tumor functions against AML and ALL targets, clearly showed that in the context of ALL cytotoxicity there is a strong TGFβ1 upregulation by CIK cells, a cytokine with immune-inhibitory functions. This could be the explanation for ALL resistance to the CIK cell killing activity, since currently there is no evidence for the efficacy of these cells in the treatment of ALL (139) (140). Another important characteristic of CIK cells, mostly if considering these cells for an ACT anti-tumor approach, is their ability of reaching tumor infiltrating sites, where they demonstrated to exert a very effective GVL effect, while causing minimal GVHD (141). This feature is very important, underlining the possibility of employing CIK cells also in an allogeneic clinical setting. In fact, since no GVHD occurrence has been observed in the context of $CD3^+/CD56^-$ cell deletion, it has been hypothesized that $CD3^+/CD56^+$ CIK cells have the potential to separate GVT effects from GVHD (136). The ability of dissociating the anti-tumor response from the host-response is thought to be dependent on CIK cell capacity to produce high amounts of IFN-γ, that has been demonstrated to have a protective effect towards the GVHD. Even though the mechanisms involved in the GVHD occurrence are still not fully understood and the role played by IFN-γ in the pathogenesis of GVHD is quite complex and controversial, the protective effect due to IFN-γ production at early time points after bone marrow transplantation (BMT), compared to the exacerbating GVHD effect when administered late, has been

elucidated (142) (143). According to this model, GVHD onset could be prevented because of the CIK cell production of IFN-γ at very early time points after BMT. 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 (123). 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.

Furthermore, another important explanation for the reduced ability of causing GVHD by allogeneic CIK cells is to be found in the elimination of the dendritic cells (DCs) in 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 (144). 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 allow T cells to enter GVHD target organs (145).

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 bone marrow transplant

model, further accounting for the reduced capacity of allogeneic CIK cells of causing GVHD (145). Finally, preclinical studies in which emerged the strong anti-tumor activity and low toxicity of CIK cells, alongside the large-scale expansion of this effector population, paved the way for the employment of CIK cells in clinical trials for the treatment of both solid and hematological malignancies (116).

Among the different research groups who employed CIK cells for the treatment of relapsed hematological malignancies after HSCT, in 2007 our group participated in the first phase I study to date, 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 four patients developed GVHD, but at most with a second level severity (146). A phase IIA study has been recently concluded, by our group (NCT), with similar encouraging results regarding CIK cell safety profile and initial evidence of moderate efficacy (NCT01186809) (147). In order to report autologous or allogeneic CIK cell-based clinical trials against several types of tumors and to collect and interpret the results obtained worldwide, an *International Registry on CIK Cells* has been created (148). Until now, the current database adds up to 45 studies (24 phase

I and 21 phase II), investigating 22 different tumor types and disease stages, including a total number of ~ 2729 patients. The main findings number among a 39% response rate, but also an improvement in the quality of life and a significant increase in the OS (149). Overall, CIK cells emerged as a promising immunotherapeutic approach to be adopted in oncology, both as immunotherapy adjuvant in order to prevent recurrence and as a potential effector population to be genetically manipulated for the development of advanced targeted therapies.

4. Chimeric Antigen Receptors (CARs)

The birth of CAR technology occurred 28 years ago when it has been shown that antibody-derived variable regions of the desired specificity could be substituted for the corresponding regions within a TCR (114). Since T-cell activation follows an antibody recognition, Eshaar and collaborators coined the term ‘T-body’ to describe this technology (150). Initially, the approach was cumbersome since two chimeric genes, encoding for the variable light (V_L) and heavy (V_H) chain, needed to be delivered.

This drawback has been overcome by connecting the V_L and V_H chain through a peptide linker, thereby creating a single chain fragment variable (scFv) (150). Therefore, CARs combine the antigen-binding properties typical of antibodies with T-cell effector functions, such as cytotoxicity, migration capability and infiltration within the tumor sites, but also cytokine release with the subsequent recruitment of

other immune components and an overall sustained anti-tumor immune response (151). Indeed, CARs do not require antigen processing and presentation by HLA and are able to recognize any cell surface antigen (proteins, carbohydrates, glycolipids). The not HLA restricted recognition allows the use of CARs in a broad range of patients and enables the antigen recognition even in case of down regulation of HLA molecules on the cell surface, which is a mechanism of tumor immune-escape.

These recombinant receptors are generally comprised of an antigen binding domain, an extracellular spacer/hinge region, a transmembrane domain and an intracellular signaling domain resulting in T-cell activation following antigen binding (Figure 2).

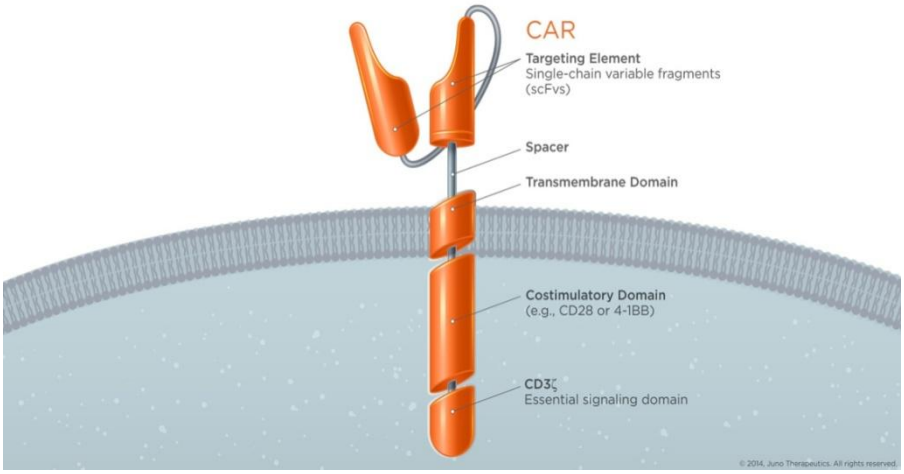

Figure 2. Schematic representation of a second generation CAR. Adapted from *Juno Therapeutics* 2014 (https://junotherapeutics.com/our-science/car-technology/).

-Antigen binding domain:

The antigen binding domain usually consists of scFv derived from mAbs. Alternatively, it can also be constituted by a ligand for a receptor that is expressed on tumor cells (152).

-Spacer/hinge domain:

The spacer/hinge domain, connecting the antigen binding domain and the transmembrane region, governs CAR flexibility, with a length that appears to be important for the CAR-mediated functions (153) (154).

-Transmembrane domain:

The transmembrane domain, which lies between the hinge and the signaling domain, consists of hydrophobic alpha helices, usually derived from CD3-ζ, CD4, CD8 or CD28 molecules. Like the hinge, this region has always been considered as a non functional structural element, but conversely, several studies have shown that the transmembrane domain has a significant influence on CAR surface expression and function (155) (156).

-Endodomain:

The endodomain is responsible for CAR signal delivering and it has undergone various manipulations in order to optimize engineered cell functionality. Indeed the ‘generations’ of CARs refer to as the number of intracellular signaling domains inside the CAR molecule. Based on this characteristic, current CARs are grouped into three generations. First generation CARs contain a single signaling element that is most commonly constituted by the CD3-ζ chain of the TCR complex. The CD3-ζ signal provides the required ‘signal 1’ for T-cell activation,

however the anti-tumor effect of first generation CARs is limited overtime because the ‘signal 1’ induces only transient cell divisions and suboptimal cytokine production, failing in promoting T-cell persistence *in vivo* and thus a sustained anti-tumor effect (157). For this reason, to overcome the limitations 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, since tumor cells and tumor microenvironment do not express co-stimulatory ligands, such as CD80 and CD86, that are ligands for the CD28 receptor, expressed by activated T cells. In the absence of costimulation, T cells undergo anergy and failure of *in vivo* expansion. The pioneering work performed by Finney et al. by adding the CD28 molecule in the CAR endodomain, showed that, while not influencing the cytotoxic activity, the main advantage were correlated to the induction of IL-2 secretion and T-cell proliferation (158). More recently, other co-stimulatory molecules have been described, including ICOS (inducible costimulatory), OX40 (CD134), 4-1BB (CD137), CD27, DAP10 or 2B4 (CD244) (159). Therefore, when compared to first generation CARs, the subsequent generations show increased proliferation, cytokine secretion, *in vivo* expansion and persistence, being able to eradicate systemic malignancies in mice models of disease (160) (161) (162).

4.1 Safety concerns

Before considering an ACT approach, several aspects need to be carefully examined, since there are fundamental differences between traditional drugs and T-cell therapies.

Among them are, for example, the intrinsical variability from patient to patient in the production of the effector T cells, the massive proliferation and the long persistence of T cells after the infusion, with a different and long-lasting exposure to therapy, virtually limitless in the case of T cells, compared to standard therapies. As an immediate result, these considerations further complicate the comprehension of the maximum tolerated dose to be applied in a more general cohort of patients. Differently from synthetic drugs, another complication of ACT concerns the termination of exposure to treatment, above all in the context of an amplification of the T-cell response, due to the recognition of healthy tissues expressing the target antigen, leading to a massive cross-specific cytotoxicity. In fact, while it is possible to clearly identify a therapeutic window in the case of standard therapies, in which the sensitivity of tumors to drugs is considerably higher than that of healthy tissues, both the presence of immune inhibitory factors in the tumor microenvironment and defects in antigen processing and presentation may render tumors less susceptible to T-cell mediated killing, compared to healthy tissues, and a therapeutic window is far from being recognized. Indeed, if such a therapeutic window exists the potential effectiveness of T-cell mediated therapy will be covered by the need of dosing T cells within a limited range, thus precluding the

implementation of discoveries coming from the necessity of increasing the potency of the adoptively transferred T cells (Figure 3).
In addition, the patients receiving these cells are frequently previously treated with lymphoconditionig chemotherapy, owing to absent or low circulating leukocytes, but also an extremely reduced number of regulatory T cells and an higher amount of cytokines able to promote T-cell survival than normal conditions, in a general non-physiological scenario (163) (164) (165).
In this way, more often than what expected, adverse events have not been offset by impressive anti-tumor activities, due to the recognition of normal cells expressing the target antigen by the genetically modified T cells (166) (167) (168) (169) (170) (171) (Figure 4).
Indeed, although the targeting of tumor-specific antigens would be more promising in avoiding auto-immune toxicities and in reducing the GVHD development, creating at the same time a broader therapeutic range, some safety concerns are associated with the use of CARs. Among them are the 'on-target-off-tumor' toxicity, the cytokine release syndrome (CRS) or 'cytokine storm' and the tumor lysis syndrome (TLS), which proved to be fatal in certain occasions.
'On-target-off-tumor' responses are due to the fact that most antigens targeted by CAR^+ cells are TAAs and not TSAs, therefore they are also expressed by normal tissues and, consequently, susceptible of recognition by CAR expressing cells. A serious adverse event caused by 'on-target- off-tumor' toxicity has been described by Morgan and colleagues, in which a patient with colorectal cancer, treated with T

cells expressing an anti-HER2 CAR, developed pulmonary toxicity within 15 minutes from the T-cell infusion in association with very high cytokine levels, followed by cardiac arrest, and died 4 days later (168). It has been postulated that the toxicity may have been due to the targeting of pulmonary vasculature expressing low levels of HER2. This epilogue was not foreseeable as it has not been observed in HER2 vaccine trials for most of breast cancer patients treated with trastuzumab, the HER2 monoclonal antibody.

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 (172).

However, sometimes, engineered T cells may kill healthy tissues or cells, without causing major morbidity.

An example is represented by the depletion of healthy B cells following adoptive transfer of CD19 CAR T cells in patients with B cell malignancies, resulting in B cell cytopenia and hypogammaglobulinaemia, which are well-tolerated, although immunoglobulin replacement is required (173).

The second major concern is represented by the ‘cytokine storm’ which manifests in high fevers, hypotension and hypoxia. It is related to the production of several proinflammatory cytokines including IL-6, TNF-α and IFN-γ, secondary to T-cell activation and, in general, it occurs in patients with large tumor burdens rather than in those with MRD (174) (175). A fatal event related to this type of toxicity has

been described by Brentjens and colleagues. Indeed, a patient with bulky chronic lymphocytic leukemia, who received autologous T cells transduced with a CD19 CAR, after cyclophosphamide administration for lymphodepletion, developed fever, hypotension and dyspnea 20 hours after infusion which rapidly progressed (173). However, this trial has been reopened because it has been postulated that the inflammatory cytokine cascade was primarily caused by cyclophosphamide and then worsened by T-cell infusion.

Therefore, in order to prevent life-threatening toxicities, while maximizing the potential antitumor effects, the group of Crystal Mackall adapted the CRS grading system designed by the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE v4.0), regardless of the inciting agent (1). Figure 5 shows the treatment algorithm for CRS managing, based on the revised CRS grading system.

TLS is a group of metabolic abnormalities that results from the rapid release of intracellular metabolites from lysed malignant cells and has been reported in patients treated with CD19 CAR T cells (176), however it has been managed successfully with standard supportive therapy (177).

Taken together, the serious adverse events related to CAR-based ACT require efforts in order to improve the safety of this approach. In addition to the judicious selection of the antigen, that is essential for controlling on-target toxicity, and to the careful dose escalation of CAR T cells, especially for controlling CRS, there are several

strategies that may be helpful for improving the safety of CAR technology, such as: achieving the optimal distance between target antigen and CAR (154); incorporating a suicide gene within the engineered T cells (178); developing immune inhibitory receptors (iCARs) to preemptively constrain T-cell responses (179); separating the T-cell signaling domains on two different CARs with different specificities (180); using engineered T cells modified with CAR encoding messenger RNA in order to obtain a transient expression of the receptors (181). Otherwise, another method to better control T-cell potency is the employment of CARs with reduced affinity for TAA, in order to prevent and diminish toxicities against healthy tissues, but owning at the same time the risk of eliminating or lessening the anti-tumor effects of the treatment (2) (182).

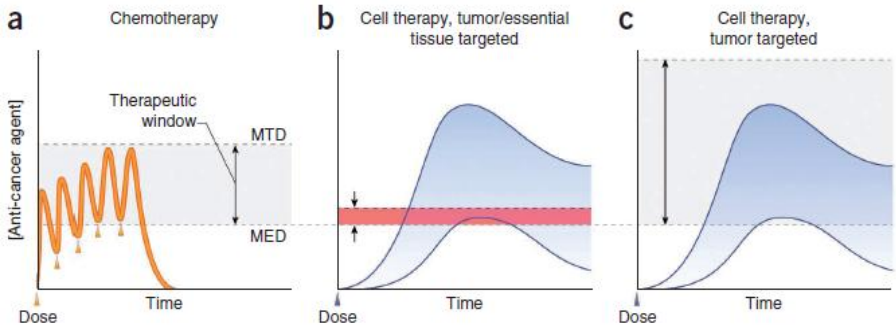

Figure 3. The determination of therapeutic range is importantly impacted by differences in the pharmacokinetics and mechanisms of action between cytotoxic chemotherapy and adoptive T-cell therapies. **a)** The orange line, representing patient-to-patient variation in drug levels, is small for chemotherapeutics. In this way, the area under the curve, representing the drug exposure, can be easily predictable and terminated by stopping drug dosing when toxicity events would occur. The therapeutic window (gray shaded area), representing difference in sensitivity between healthy tissues and tumors, is thus defined by the maximum tolerated doses (MTD), in which intolerable toxicities verify, and minimum effective dose (MED), in which tumor eradication takes place. **(b-c)** The blue shaded area represents ACT pharmacokinetics variability. Given these fluctuations, a therapeutic window (pink shaded area) has thus not far been identified for any cellular therapy directed against antigens expressed by tumors and essential healthy tissues. The therapeutic window can be opened by targeting an antigen that is not expressed by healthy tissues (gray shaded area), by increasing the MTD for ACT, because direct cytotoxicity to normal tissues does not occur, even at high doses of cells (**c**). Adapted from Hinrichs C.S. *et al.* 2013 (2).

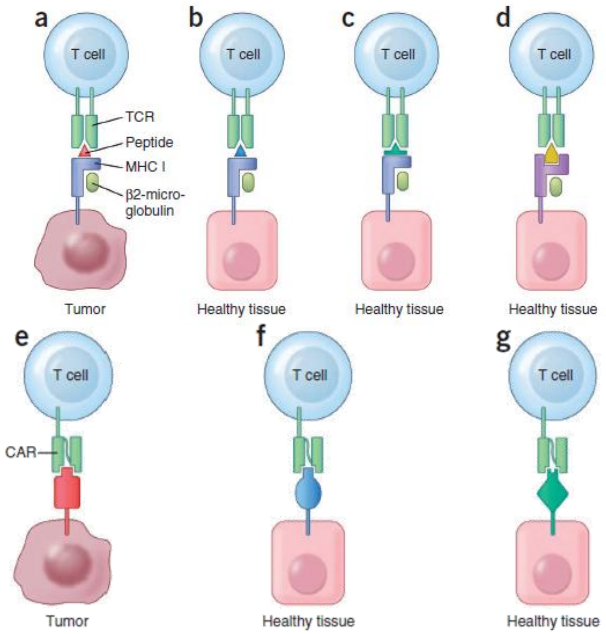

Figure 4. Untargeted antigens can be recognized by TCRs and CARs in a mechanism of cross-reaction. **(a-d)** A peptide-MHC complex that is expressed exclusively by tumor cells and is not expressed by healthy tissues represents the ideal T-cell target for ACT **(a)**. Healthy tissue may express antigens that contain peptide epitopes identical to the one recognized by the engineered T cells **(b)**, peptide epitopes that are different in sequence from that originally targeted by genetically modified T cells **(c)**, complexes of different MHC molecules with peptides from non-target antigens that are cross-recognized by the engineered TCR **(d)**. **(e–g)** Target antigens are directly engaged by CARs. An ideal CAR for ACT specifically recognizes a single epitope that is unique to a tumor-restricted antigen **(e)**, but identical epitopes owned by non-target antigens expressed on healthy cells can be recognized by CAR^+ T cells **(f)** as well as structurally similar epitopes (but different in sequence) comprised in non-antigens, because of an imperfect antibody specificity **(g)**. Adapted from Hinrichs CS *et al.* 2013(2).

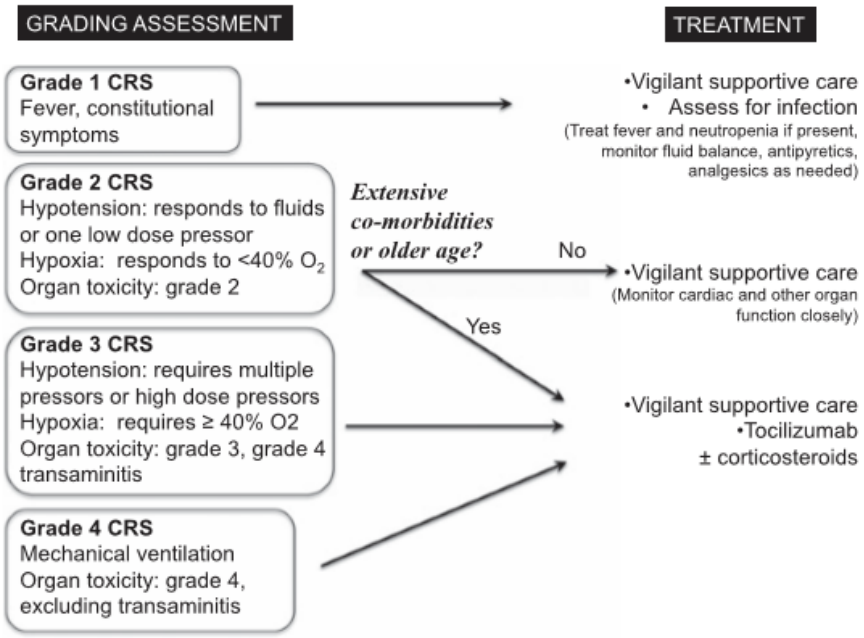

Figure 5. Revised CRS grading system and treatment algorithm related to CRS menagement. Vigilant supportive care, including empiric treatment of concurrent bacterial infections, together with maintenance of adequate hydration and blood pressure for every grade is recommended. In all patients with grade 3 or 4 CRS, immunosuppression should be employed, with the indication of being instituted earlier in patients with extensive comorbidities and or older age. CTCAE v4.0 dictates grades 2-4 organ toxicities. Adapted from Lee D.W. et al, Blood, 2014 (1).

5. CAR design implementation: the affinity issue

5.1 Canonical TCRs and affinity-related T-cell responses

Among the variables able to significantly affect the effector functions of the CAR-redirected T-cell populations, the affinity of the CAR binding domain for its target antigen is of particular interest, being an aspect still not fully characterized and completely understood.

The importance of studying affinity comes from evidences based on how high and low affinity endogenous TCRs play different roles in the immune response (183). Indeed, it has been observed that the efficiency of the triggered T-cell response is critically dependent on the efficacy and quality of the binding between the TCR and the pMHC (peptide-MHC) complex, with stronger interactions thought to be more powerful in causing a robust T-cell activation compared to weaker interactions (184, 185).

A way to describe the binding occurring between a TCR and its pMHC complex, is a one-to-one interaction, involving a bimolecular binding reaction, commonly used to describe the interaction between two free molecules in solution, with 3D mobility (1):

$$TCR + pepMHC \underset{Koff}{\overset{Kon}{\rightleftarrows}} TCR{:}\,pepMHC \quad (1)$$

In this equation, K_{on} refers to the association rate of the interaction, while K_{off} describes the dissociation rate of the interaction.

From this equation it is possible to derive the equilibrium binding constant or dissociation constant K_D ($K_D = 1/K_a = [TCR][pepMHC]/[TCR:pepMHC] = K_{off}/K_{on}$), that is a physical parameter generally used to describe the strength with which a TCR binds to a given pMHC complex, in other words a measure of the affinity between the TCR and the pMHC interaction. In this way, lower K_D values are indicative of higher affinity constructs compared to higher K_D values, because of the inverse correlation between K_{off} and K_{on} in the equation (183, 184). Even if it is wide accepted that T cells with high affinity TCRs dominate immune responses to pathogens, with effector functions thought to be superior than T cells with low affinity TCRs (186, 187), it is established as well that also lower affinity T-cell clones actively participate to an immune response. Indeed, in the context of anti-tumor responses, lower affinity T cells play the most important role, since most high affinity self-tumor-antigen specific T cells are usually prone to elimination by central and peripheral tolerance mechanisms.

A recent study demonstrated how the major difference between high and low affinity TCR clones, during an immune response, principally relies on the proliferation maintenance, a characteristic typically owned by high affinity T cells, because of the recruitment of specific transcription factors involved in T-cell clonal expansion and function, such as IRF-4, only after high affinity T-cell contacts (188).

Therefore, since the type of the response and thus the functional properties of the T cells are strongly affected by the affinity of the

binding between a TCR and its pMHC at the beginning of the interaction, several efforts have been made in order to fully unravel the relation between the strength of the binding and T-cell activation and later functions (189, 190). According to these studies, the minimal affinity required to obtain a full CD8 T-cell activation is in the range of 300 μM ($3x10^{-4}$ M) and an affinity threshold has been identified around the value of 10 μM (10^{-5} M), above which there is no further *in vitro* and *in vivo* improvement of the T-cell effector functions (182, 191-193). The biological meaning of a low affinity threshold relies on the need of avoiding self-damage, because of the tight correlation between antitumor activity and autoimmunity (182). In this way, strategies for increasing the affinity of TCRs have been undertaken in order to enhance the efficacy of passive immunotherapy with T-cell bearing tumor specific artificial T-cell receptors (194-196). However, this correlation is still controversial and it remains unclear if increasing the TCR affinity could or not render ACT more effective, leading to the need of performing a more precise quantitative analysis of TCR affinity and its relation to the *in vivo* antitumor activity.

In this context, the strength of the TCR-pMHC complex interaction is dependent not only on the TCR affinity, but also on other factors, such as TCR clustering and coreceptors. The combined effect arisen by the stabilization of the TCR-pMHC contacts governed by coreceptors, such as CD8, leads to what is commonly referred to as avidity (184), or rather additional cellular responses to the TCR-pMHC binding. Several models have been proposed in order to describe the

relationship between affinity and avidity. According to the “affinity-based model” (184, 191, 193, 197-200) the strength of T-cell signaling is determined by the number of TCR/pMHC complexes formed at the synapse. Thus, stronger responses in terms of distal events, such as cytokine production, can be achieved by high-avidity TCRs, through the engagement of a major number of pMHC ligands and their longer preservation (201). In contrast, only three pMHC complexes are needed in order to trigger T-cell killing of target cells (202), and the antigen density is not fundamental in determining the rapid delivery of lytic granules (203). Similar considerations can be applied to describe the contribution of the affinity to the avidity, with a threshold set up to 10 μM, showing that increasing the affinity over this value does not further improve the avidity and does not lead to a more potent antitumor activity (182). An interesting explanation of this phenomenon has been proposed by Zhong S. and colleagues, according to which at the level of the threshold all clustered TCRs may be occupied and thus only monovalent TCR-pMHC interactions are still possible when increasing the affinity above the threshold value (182). Moreover, the “kinetic proofreading” model proposes that upon pMHC dissociation, a series of accumulated biochemical modifications, generated after the TCR-pMHC binding, is lost, leading thus to impaired T-cell activation. In this way, there would exist a sufficiently long time for the TCR-pMHC binding, in order to trigger a critical modification of the TCR, able to produce a productive signal (204-206).

Conversely, the “serial triggering model” indicates that a single pMHC can bind to and trigger up to 200 individual TCRs. According to this model, both the interactions and the dissociation rates exhibit two requirements, respectively. In order to complete proximal signaling, the former need to be sufficiently long and the latter need to be sufficiently short, to allow multiple TCRs the opportunity to bind to the same pMHC complex. The immediate consequence of this theory is that longer interaction times would occur between high affinity TCRs and pMHC, resulting in a reduced number of TCRs available for the binding with the limiting number of pMHC complexes on the target cells (207, 208). In this way, the immediate result of this reduced interactions would lead to decreased cell functions, such as cytotoxicity and cytokine production.

To further complicate the scenario of the features determining T-cell activation, Schodin and coauthors proposed a model by which there would be a measurable relationship between the total number of surface TCR molecules required for T-cell activity and the TCR binding affinity. In a suboptimal activation context, that is lowest antigen densities, nearly all of the available ~ 100000 TCRs/cell are required, independently on their binding affinities. In contrast, when the amount of antigen is very high, the number of TCRs required to allow a complete T-cell activation inversely correlates with their binding affinities. In other words, high affinity TCRs need ~ 1000 receptors for T-cell activity, while low TCRs require more than 50000 TCRs for T-cell activation (209).

Whether the first models or the others could explain the exact relationship between T-cell activation and all the characteristics involved in the binding is not possible to be known at the moment, since experimental evidences obtained until now seem to contradict both the theories.

However, recently, Aleksic and coworkers proposed the "confinement time" model, which could account for the discrepancies within the experimental contexts hitherto found, offering at the same time a possible solution. Indeed, the confinement time can be described as the binding rate and consequent probability of re-association for the generation of a new binding between the interacting cells, within a precise radius of contact. According to empirical mathematical predictions, when the K_{on} is small, leading thus to little rebinding, the "intrinsic localized on rate" (K^*_{off}) can be approached to the K_{off}, whereas when the K_{on} is large, allowing for a frequent rebinding, K^*_{off} can be considered as the K_D. Therefore, a specific length of interaction time, until complete dissociation occurs, would govern the outcome of TCR engagement. In this way, repeated rebinding after TCR-pMHC chemical dissociation could be explained based on its dependence on the K_{on} (210).

Altogether, these aspects are important in defining the requirements that a TCR should have in order to be effective and safe for T-cell immunotherapy, taking into account in particular the subtle border existing between efficient tumor regression and minimal autoimmune response.

Indeed, even if increasing the affinity of the naïve TCR brings the big advantage of augmenting the functional and protective capacity of tumor-specific CD8 T cells (166, 192, 194, 211, 212), it could also result in the potential side effect represented by the possibility of harming the normal tissues. Indeed, it has been shown in several studies that increasing the TCR affinity up to very high values, such as K_D < 1 nM (10^{-9} M) results in loss of antigen-specificity, but also cross-reactions or allo-reactions (194, 200, 213). For instance, engineered Melan-A and MAGE-A3-specific T lymphocytes have been employed for the treatment of both metastatic melanoma and synovial cell sarcoma, finally leading to a powerfully harmful *in vivo* immune response against normal tissues, as in the case of melanocytes in the skin, eye and ear for Melan-A-specific T cells and neurons for MAGE-A3-specific T lymphocytes (166, 169). For this reason, several authors proposed not to optimize the affinity beyond the natural affinity range when designing improved self-tumor specific T cells for immunotherapy, in order to avoid the risk of cross-reactivity (183, 193, 214).

All of these observations are crucial in the context of the present investigation, since the CAR construct is different by definition from the TCR and confers dramatically increased effector properties to the CAR engineered T cells.

5.2 Affinity of CAR-redirected T-cell: an open question

As previously mentioned, CARs are molecules composed by an antigen binding domain, derived from a mAb specific for a precise tumor antigen, in the form of V_H and V_L linked together as a single chain fragment variable (scFv) and by an intracellular signaling moiety, usually the CD3-ζ complex, together with one or more co-stimulatory signaling modules (215, 216). The binding interaction occurring between a CAR^+ T-cell and its specific antigen can be seen as described in the equation (1), through the substitution of the terms referring to the TCR-pMHC complexes with those generated by the Ag-binding domain and the target antigen (as shown in (2)). This is due to the fact that the binding occurring via the CAR is direct and does not require the presence of a pMHC on the surface of the target cell.

$$CAR + Target\ Antigen \underset{Koff}{\overset{Kon}{\rightleftarrows}} CAR{:}Target\ Antigen \quad (2)$$

As it was for the equation (1), according to (2) the value reported by the K_D describes the affinity, referring to as the strength in the binding between the CAR^+ T-cell and the target antigen. The major difference compared to the TCR is given by the K_D value that is sensitively lower, because the Ag-binding domain is derived from a mAb, thus conferring a significant higher affinity, in the range of 10^{-7} - 10^{-11} M. Because of the much higher binding affinity of the CAR, compared to

the TCR, the potential for sustained signaling after the CAR engagement is potentiated, leading to still not well characterized consequences on the effector functions, cell survival and therapeutic efficacy (217, 218). This important difference is due to the fact that a CAR can engage a significantly greater number of antigens on the surface of the target cells, compared to the number of pMHC complexes available (218). As in the case of CD19 and ROR1 targeting, as examples of B-cell tumor antigens, recent estimations suggest a number of $>10^4$ molecules for CD19 and 10^3-10^4 for ROR1, that considerably exceed the capacity of presenting a single peptide by the reduced number of MHC molecules present at the same time (207, 219-221). Therefore, the probabilities that a serial triggering of the CAR could occur in the same fashion of the TCR are extremely low, suggesting that the CAR acts with completely different kinetics (217, 218, 222, 223). Recently, Watanabe and colleagues identified a "lytic threshold" of ~ 200 molecules, given by the number of antigens on the surface of the target cells required to produce a full cytotoxic activity in an anti-CD20 CAR model, and an "activation threshold" with an antigen density ~ 10-fold higher (a few thousand of molecules), necessary to induce the successive activation and expansion of the CAR^+ T cells (224). However, the authors state that a number of other factors, such as affinity (225), structure (226), epitope localization in the context of individual CAR-Ag pairs (227, 228), but also the expression of coreceptors on target cells, may influence the thresholds. In this way, it becomes evident that these "lytic" and

"activation" thresholds could change among different CARs and target antigens, needing for this reason a further investigation, in order to be converted into variables that can be fully characterized and thus considered when a CAR has to be designed. Moreover, since the minimum threshold number of surface target molecules that can be recognized and lysed by CAR^+ T cells is markedly low, compared to the mAb from which the same CAR has been derived, the Ag safety in the context of mAb therapy could not necessarily be translated in the same Ag safety, when employing the dramatically more sensitive CAR T-cell therapy (224, 229). All these considerations demand a careful evaluation of potential 'on-target-off-tumor' toxicities related to the recognition of target cells expressing low levels of the target antigens, indicating that off-tumor expression of the target molecules should be negative or at least at very low levels, being below the "lytic" threshold (224). Indeed, serious side effects have been reported in literature (168, 172), of which the worst cases led to death of the treated patients, suggesting the need to further implement the CAR design in a more context-dependent manner. One representative example of this phenomenon has been reported by Morgan and colleagues, of a patient died after the infusion of anti-ErbB2 CAR^+T cells, in which lung epithelial cells expressing low levels of ErbB2 have been rapidly recognized by CAR^+T cells (168). In this way, the CAR binding affinity to the target antigen represents an aspect worth to be investigated, since finding an affinity variant of a CAR able to specifically recognize and kill the tumor cells while sparing at the

same time the healthy cells, low Ag positive, could have an important impact on adoptive cell therapy strategies. However, the aspect of the affinity regarding CAR-based approaches is extremely controversial.

The affinity problem has been firstly raised by Eshhar and collaborators in 1997, postulating that, based on the natural affinity range of the TCRs, maybe a low to median affinity scFv is preferable to an high affinity scFv, since it allows better triggering and recycling of T cells, thereby increasing the efficiency of target cell lysis. Moreover, it has been proposed low affinity scFvs as preferable, being less vulnerable to low concentrations of circulating soluble antigens, thus resulting less inhibited compared to high affinity scFvs (230).

The first attempt to modify the affinity has been investigated in the context of TCR-like Abs, which differ from CARs in that they are antibodies specifically redirected to MHC-peptide complexes. Two variants of the anti-HLA-A1/melanoma-associated Ag (MAGE)-A1 TCR-like Ab were produced with the aim to verify if increasing the affinity of the receptor could enhance T-cell specific immune functions. In particular, the Fab (Fragment antigen binding)-G8 (2.5 x 10^{-7} M) and Fab-Hyb3 receptors have been generated, the latter with an affinity of 18-fold improvement, clearly influencing the effector properties in terms of higher cytotoxicity, but also sensitivity towards the melanoma associated Ag-A1 peptide-loaded cells and TNF-α production (231). According to these first findings, even if they are not proper CARs, increasing the affinity of the TCR-like receptors seems to result in enhanced T-cell effector functions.

Subsequently, Chmielewski and collaborators created different anti-ErbB2 CARs, with affinity ranging from 3.2 x 10^{-7} M to 1.5 x 10^{-11} M, showing that only high amounts of ErbB2 were able to activate T cells with low affinity receptors ($K_D > 10^{-8}$ M). This work unequivocally showed how an increase in recombinant-receptor affinity does not necessarily lead to a more potent T-cell activation, but rather to a reduction in T-cell discrimination between low and high Ag expressing cells. In fact, they found that target cell lysis efficiency was principally dependent on the amounts of Ag expressed on the surface of the target cells and it was only partially due to the affinity. Indeed, there was no augment in the cytotoxic activity by increasing the affinity ($K_D < 10^{-8}$ M) in the presence of medium and high ErbB2 expression target cells. On the other hand, to obtain the same cytolytic efficiency, T cells with lower affinity required higher E:T ratios, showing a reduced activity compared to the high affinity receptors. Analogous results have also been obtained concerning IFN-γ production. The major difference between the constructs seemed rather to rely on their kinetics of action, since medium and high affinity receptors lysed target cells faster as compared to low affinity receptors, with the latter demonstrating the ability of eradicating only tumor cells with the highest Ag expression levels. The conclusion of this work is that raising the affinity of the scFv domain could be ambiguous on adoptive immunotherapy for several reasons. Firstly, cellular activation and target cell lysis are more efficient with high affinity immune-receptors ($K_D < 10^{-8}$ M), but at the same time there is

no additional improvement of receptor-mediated cellular activation by increasing the affinity beyond 10^{-8} M ("affinity ceiling"). Moreover, there is less discrimination between high and low Ag expressing target cells by high affinity immune-receptors, compared to low affinity ones (232, 233).

Few years later, Turatti and colleagues tested other two affinity variants of the anti-ErbB2 CAR, previously studied by Chmielewski et al.. In their model, the high affinity variant (10^{-9} M) was already used by Chmielewski and collaborators, while the low affinity variant (1.6 x 10^{-6} M) had a binding affinity 10-fold lower as compared to their lowest affinity receptor. From this study it has been observed that the high affinity CAR had stronger capacity to bind and retain Ag than the low affinity CAR. However, in conditions of suboptimal activation, such as low levels of expression of both CAR and Ag, direct lysis of tumour cells was achieved with less efficacy by the high affinity CAR, in comparison to low affinity CAR.

Moreover, CAR and Ag expression levels seemed to be important for the control of tumor growth over time, as in the case of a maintained effector-target contact, losing the difference between high and low affinity constructs (225). Therefore, the most likely situation is the "rolling" of the low affinity receptors across the surface of target cells, because of their faster off-rates, compared to the slower off-rates of the high affinity immune-receptors.

Since both the affinity and the CAR expression profile, but also the Ag amounts on the target cells, have potent implications in the

effector activities of redirected T cells, the authors suggested to carefully modulate these features, in order to discriminate between two different biological contexts, represented by normal tissues, expressing low levels of Ag, and tumor variants able to down-modulate the target Ag.

Several years later, the group of Crystal Mackall published a paper discussing the strategies to optimize an anti-CD22 CAR, through the choice of target epitope binding site (i.e. distal or proximal with respect to the plasma membrane), the affinity maturation and the employment of a second, compared to a third, generation CAR. They demonstrated that anti-CD22 CAR activities were not enhanced by increasing the distance between the T-cell surface and the CD22 epitope targeted (by means of increasing the CH_2CH_3 spacer length). Moreover, they did not found differences in the two constructs created, bearing only a negligible difference in their affinity, the former with a K_D of 5.8×10^{-9} M and the latter with an affinity of 2.3×10^{-9} M respectively, hypothesizing the possibility of having reached the 10^{-8} M "affinity ceiling" mentioned by Chmielewski and colleagues. However, since all the cell lines tested had similar expression levels of the CD22 antigen, none of which below 2000 molecules per tumour cell, they could not assess if the effector functions might be affected by the affinity in the context of a suboptimal antigen distribution, being their model not suitable to see any divergence in this scenario (227). Furthermore, they unexpectedly saw a difference in the cytolytic activity when comparing signalling

domains in their CAR constructs, suggesting that the second generation CAR would be better in their therapeutic setting.

Recently, Hudecek and colleagues further described the relationship existing between the length of the CAR spacer and the affinity of its antigen binding domain. They started from the need of augmenting the potency of their anti-ROR1 CAR, initially targeting the ROR1 antigen in a distal position, with respect to the plasma membrane of the target cells. They decided to create different anti-ROR1 CARs, three of which with different lengths of the CH_2CH_3 spacer and two short variants with an affinity divergence of 50 fold (the 2A2 original CAR with a K_D of 1.2×10^{-8} M). In their context, by shortening the CAR construct, it was possible to increase the cytotoxic activity, but also the later effector functions of their original CAR. Since this construct proved to significantly enhance the anti-tumor effects desired, they decided to consider the affinity variation in the scenario of their more potent and short CAR. In this way, they showed how an affinity maturation was not important in determining the cytotoxic activities of both the CARs, but was rather determinant in causing a more sustained proliferation and cytokine production. This work demonstrated for the first time how high affinity receptors do not necessarily act by an increase in their lytic activities, but rather through an amplification of their cytokine production and proliferation rates (226).

With the aim of potentiating the discrimination between high Ag expressing tumour cells and low Ag positive healthy tissues, two

groups recently published on the effects of reducing the CAR construct affinity. The first attempt is represented by the work of Caruso and collaborators, who employed a 10-fold affinity reduced anti-EGFR CAR, to test the hypothesis of being more discriminative between $EGFR^+$ tumor and healthy tissues, both *in vitro* and *in vivo* (234). However, several drawbacks have emerged concerning their approach. Firstly, their lower affinity CAR derived from a different mAb clone, binding to an overlapping epitope region that required at least a bivalent biding event in order to be fully activated, compared to the high affinity CAR. Moreover, they encountered a marked reduction in the activation profile and also in the effectiveness of their lower affinity CAR, as compared to the high affinity receptor against high Ag positive cells. Finally, in their *in vivo* model, they made use of a tumor cell line, low Ag positive, in order to mimic the healthy tissue, in terms of Ag expression profile, being far away from a realistic comparison between the two tissues. Besides, the conclusion of their work is that a lower affinity CAR receptor should be greatly preferable compared to an high affinity one, in that the former demonstrated to be able to spare the tumor cell line, chosen as representative of the healthy tissue, without taking into consideration the possibility of tumor escape mechanisms due to the Ag down-modulation following therapy.

On the other hand, the work of Liu and co-workers addressed the same problem, with both anti-ErbB2 and anti-EGFR CARs, by performing an affinity reduction of ~3 orders of magnitude, ranging

from ~10^{-9} to ~10^{-6} M. Compared to the previous publications of both Chmielewski and Caruso et al., the authors found that a lower affinity CAR in both models was able to be as effective as higher affinity CAR constructs against high Ag positive tumor cells, showing at the same time a better sparing capability when challenged against low Ag positive healthy tissues (235). However, in their model it became evident the lack of sensitivity by the high affinity receptors to an Ag escalation, in terms of related functional responses, encountering as well a reduced effectiveness of low affinity CARs when challenged against low Ag positive tumor cell lines. However, even in this case, in the *in vivo* model proposed, the authors made use of a low Ag positive tumor cell line to represent the healthy tissue. This aspect limits their conclusion that by lowering CAR affinity there could be a benefit in terms of ameliorating the CAR therapeutic index. In this way, the inability of controlling the low Ag positive tumor growth, showed by lower affinity CARs, should be carefully evaluated, before proposing this strategy as therapeutically beneficial.

Ultimately, the last published work on CAR-affinity tuning concerns the anti-FRβ targeting, with the aim of comparing a high ($2.48x10^{-9}$ M) and a low ($5,43x10^{-8}$ M) affinity CAR, displaying a 10-fold affinity divergence. Given the need of increasing the potency of the original anti-FRβ CAR, the authors stated that a high affinity receptor could be preferable compared to the low affinity CAR, due to the greatly enhanced *in vitro* and *in vivo* antitumor activity against $FR\beta^{+}$ AML and reduced toxicity against low Ag positive HSCs. However,

the authors suggest the employment of a mRNA-based CAR strategy, in order to *in vivo* deliver such a high affinity potent CAR, with the extent of limiting the long term myeloid toxicity against healthy cells, low FRβ positive, such as monocytes (236).

In conclusion, after the examination of the affinity-based CAR approaches investigated until now, it appears evident the lack of universal accepted concepts, concerning the type of affinity (i.e. high or low) that is preferable to employ in a more generalized approach. What is unequivocally clear is that the affinity variable seems to affect redirected T-cell activities in a context-dependent manner, leading thus to the need of elucidating this important feature with regard to the specific Ag-CAR pairs studied and to the precise intent with which modifying the CAR antigen binding domain.

6. CAR design implementation: choice of the target antigen for AML immunotherapy

The clinical success of an immunotherapy approach strongly depends on the choice of the target antigen. Anguille and colleagues have adapted the criteria recently drawn up by the Translational Research Working Group of the National Cancer Institute to AML, so that according to these criteria a target antigen for immunotherapy should ideally be (237):

1. specific for leukemic cells with minimal to no expression in normal tissues;

2. overexpressed in the most cases of AML and on the majority of leukemic cells, including LSCs;
3. equipped with a role in the malignant phenotype;
4. immunogenic;
5. of therapeutic utility.

At this regard, lots of efforts have been made in order to identify surface expression markers fulfilling the majority of the above mentioned criteria (89, 104).

6.1 CD123: a good antigen for AML targeting

CD123 has emerged as a potential immunotherapeutic target, both in adult and childhood AML.

It belongs to the class I cytokine receptor superfamily and is a type I transmembrane glycoprotein with an extracellular domain at the N-terminus, a transmembrane domain and a cytoplasmic domain, without any intrinsic enzymatic activity. The receptors in this family share fibronectin type III domains, a common motif of four conserved cysteine residues in the amino-terminal portion of the ligand-binding domain, as well as a conserved stretch of amino acids (WSXWS = Trp-Ser-X-Trp-Ser; X representing a not-conserved residue) proximal to the membrane-spanning region.

In particular, CD123 is the α subunit of the human interleukin-3 receptor (IL-3R), binding IL-3 with low affinity, while together with the β subunit (βc or CD131, shared with IL-5 and GM-CSF) forms the functional heterodimeric high affinity IL-3R (238). 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 (239).

IL-3 is a cytokine involved not only in the proliferation and differentiation, but also in the viability maintenance (through the apoptosis suppression) of HSCs, neutrophils, basophils and monocytes. It also plays a role in mediating the maturation of both erythroid and megacariocytic precursors, being further implicated in the functional activation of neutrophils and monocytes (240).

Several studies have shown that CD123 is frequently overexpressed on AML blasts, $CD34^+$ progenitors and most importantly on LSCs, in comparison with normal HSCs (4, 241, 242). Moreover, recently, Ehninger and colleagues have evaluated the CD123 expression on AML blasts in a cohort of 298 patients, founding that this TAA is expressed at high level on different AML FAB and particularly overexpressed in patients with unfavorable cytogenetics and molecular aberrations (243). Testa and coworkers have investigated the effects of CD123 overexpression on leukemic cells and have shown that leukemic blasts overexpressing CD123 exhibit constitutive activation of STAT5, conferring to these cells a proliferative advantage and resistance to apoptosis (242). Moreover, a significant correlation between the CD123 levels and the number of leukemic blasts at the diagnosis was observed and this was associated with a negative prognosis (242).

In line with these findings reported for adult AML patients, Chávez-González and colleagues have observed more abundant CD123 expression in $CD34^+$ cells of BM from pediatric AML patients as compared to normal bone marrow samples (244).

All these evidences, consistent with the criteria mentioned above, make CD123 a promising AML-specific TAA. Indeed, several immunotherapeutic approaches targeting the CD123 have been developed. For example, in 1996, Sun and collaborators isolated the 7G3 mAb, 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 (245). Subsequently, Jin and coworkers confirmed these results both on AML blasts and LSCs (246). The same group, using a NOD/SCID xenograft mouse model, demonstrated that 7G3 mAb impaired also AML-LSCs *in vivo*, inhibiting their homing in the BM, reducing the AML engraftment capability in secondary-transplantation experiments and activating the innate immunity (mainly by NK cell-mediated ADCC). Moreover, 7G3 administration in NOD/SCID mice transplanted with normal human BM or cord blood did not inhibit normal human hematopoiesis. The same 7G3 clone has been recently linked to a nuclear translocation sequence (NLS) and (111)In, [(111)In-NLS-7G3], being therefore able to bind to AML cells and to be internalized. Moreover, Micro-SPECT/CT (micro-single photon emission computed tomography) revealed the ability of the mAb to reach the leukemia infiltrated tissues, i.e., BM, spleen and lymphnodes (247).

Considering the preclinical successes obtained with this clone, a recombinant chimeric IgG1 mAb derived from 7G3, CSL360, has been developed, which showed the same biological activity against the receptor (248). Preclinical toxicology studies have been carried out in cynomolgus monkeys and have shown no significant CSL360-related effects in clinical signs, hematology, chemistry, urinalysis, gross pathology or histopatology (249). In a phase I clinical trial, CSL360 was overall safe and tolerable, however, despite the blockade of CD123 and the subsequent IL-3 signaling inhibition, CSL360 was not able to induce anti-leukemic activity in the majority of the treated patients. Therefore, it has been deduced that the functional inhibition of the receptor is not sufficient to determine a clearance of CD123 expressing leukemic cells (248). For this reason, a second generation of CSL362 has been generated. It was humanized, affinity-matured and engineered in the Fc domain in order to enhance ADCC activity. A Phase 1 study of this CSL362 has been completed and a phase II/III is recruiting patients with CD123 positive AML (250) (NCT02472145).

Another immunotherapeutic approach targeting CD123 is represented by the use of the IL-3 cytokine conjugated to the diphtheria toxin (DT_{388}IL-3), which was found to be functional in preclinical studies. Toxicology studies have been conducted in cynomolgus monkeys and a phase I/II clinical trial has examined the use of DT_{388}IL-3 as a single agent in patients with relapsed/refractory AML or with de novo AML unfit for chemotherapy, in order to determine the maximum tolerated

dose and assess the antitumor activity. However, among 70 patients, only 2 durable complete responses and 5 partial responses were reported (251) (NCT00397579). More encouraging results were instead obtained in the treatment of the myeloid neoplasm blastic plasmacytoid dendritic cell neoplasm (BPCDN), indeed, among 9 patients, 5 complete responses and 2 partial responses were reported (251). Another phase I/II clinical trial is currently recruiting partecipants for testing the DT_{388}IL-3 as consolidation therapy in patients with adverse risk AML in first CR (NCT02270463).

Bi- and tri- functional antibodies have also been generated (CD123xCD3 bispecific scFv (252), CD3xCD123 DART (253) CD123xCD16 bispecific scFv (254) and CD123xCD33xCD16 trispecific scFv (255)) in order to induce a potent leukemic cell lysis.

Finally, the groups of Tettamanti, Mardiros and Gill (256-258) have applied the CAR technology for the targeting of this antigen, demonstrating killing of CD123-positive AML cell lines *in vitro* and anti-leukemic activity *in vivo* in immunodeficient mouse models. Given these promising results, the group of Lihua Budde (City of Hope) is recruiting patients for a phase I clinical trial with anti-CD123 autologous CAR T cells, *ex vivo* expanded and genetically modified using a self-inactivating lentiviral vector following lymphodepletion for patients with relapsed or refractory AML (NCT02159495). In this trial, anti-CD123 CAR T cells will be used as a salvage therapy, as a "bridge to transplant" approach. Moreover, in order to test the feasibility, safety and efficacy of electroporated mRNA anti-CD123

CAR autologous T cells, with 4-1BB costimulatory domain, also the group of Saar Gill (Abramson Cancer Center) opened a pilot open-label study, with AML patients under recruitment (NCT02623582).

Recently, Munoz and coworkers have showed that CD123 is also expressed in B cell lymphoproliferative disorders of mature B lymphocytes, opening the possibility to extend CD123 targeting also to other hematological malignancies (241).

However, it is known that the IL-3R is constitutively expressed on committed hematopoietic stem/progenitor cells (HSPCs); indeed IL-3 cytokine is primarily a hematopoietic growth factor. Different CD123 expression levels have been reported on $CD34^+$ normal cells at different stages of differentiation: the more primitive compartment shows negative/low expression of CD123, while the more mature progenitors (myeloid and B-lymphoid) show a higher expression of CD123 (238). CD123 is also expressed by monocytes, eosinophils, basophils (238) and endothelial cells (259), even if at substantial lower levels compared to leukemic cells. However, although normal cells express low levels of CD123, both in terms of percentage of positive cells and MFI (4, 260) (Figure 6), this expression represents a safety concern not negligible for a powerful immunotherapeutic approach like that of CARs. In this way, the above mentioned clinical trials will help in shedding new light on the potential side effects possibly emerging by CAR-based approaches targeting CD123 in humans.

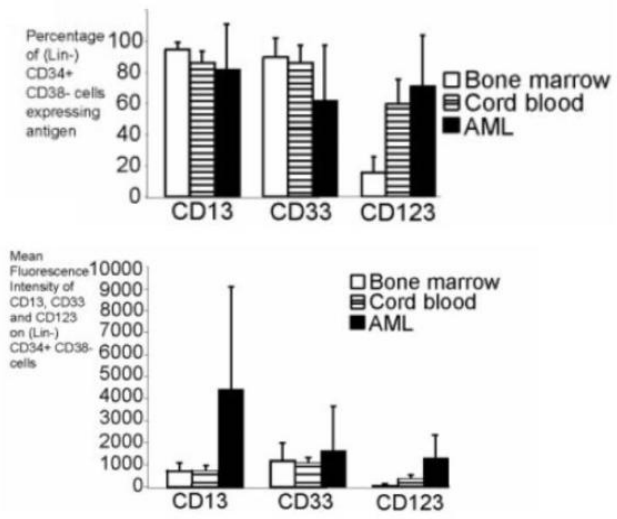

Figure 6. Percentage and MFI of (Lineage$^-$) CD34$^+$CD38$^-$ cells expressing CD13, CD33, and CD123 in bone marrow, cord blood, and AML bone marrow. Adapted from Taussig *et al.* 2005 (260).

7. *Sleeping Beauty*: a new approach of gene therapy for CIK cell engineering with CARs.

Among the different strategies for the delivery of CAR transgenes to T cells, an innovative non-viral gene transfer system, developed in the last years, exploits the use of transposons, which are mobile DNA sequences naturally occurring in the genome, that are able to 'jump' from a donor site to an acceptor site. Since, to date, no active vertebrate transposons are available, most of the transposon systems used for gene delivery have been reconstructed from fixed transposon fossils. In particular, the Sleeping Beauty (SB) platform, that belongs to the *Tc1/mariner* class of transposons, has been derived from salmonid fishes (261) and has been adapted for human gene therapy (262). It consists of two components: the transposon, containing the transgene, flanked by inverted repeat containing direct repeated sequences (IR/DR), and the transposase, a synthetic enzyme optimized for mammalian use. SB transposase recognizes the IR/DR sequences and, by a "cut and paste" mechanism, transposes 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. The original SB transposase has been mutagenized during time in order to increase the enzymatic activity, with SB10X, SB11X, SB100X mutants today available, which are 10, 11, 100 fold more potent than the original enzyme. The SB platform offers a series of advantages compared to viral vectors. Firstly, transposon based plasmids are less expensive and not time consuming

to produce, secondly they are less immunogenic and less prone to give oncogenic mutations (263), thanks to a close to random pattern of integration. Moreover transposons, per se, are not constrained by the size of the cargo load, although the efficiency of transposition and entry of the plasmids through cellular membranes appears to decrease with size (264). A disadvantage of this non viral technology is represented by the inability of the system to independently transduce the cells, posing thus the need of resorting to transfer techniques, such as nucleofection.

Thanks to promising preclinical data, the SB gene therapy approach is now also applicable in the clinical practice. Indeed, the Food and Drug Administration (FDA) has approved the first clinical trial using SB to generate T cells expressing CD19 CARs for B cell malignancies therapy (265) and the first preliminary results have been reported (266).

Scope of the thesis

The scope of the present PhD project was to characterize, in the context of AML (Acute Myeloid Leukemia), the impact of different variables involved in the anti-CD123 CAR design, such as CAR binding affinity and CAR expression, in the recognition of leukemic and normal tissues, through *in vitro* evaluation of CAR T-cell early and later effector functions.

The first chapter provides a general introduction on AML pathological features, as well as on current and promising therapeutic strategies, focusing in particular on recent advances in the field of CAR T-cell therapy and CAR design, supplying basic literature elements to better understand the developed project.

The second chapter presents our recent submitted study, focused on the *in vitro* characterization of CIK cells redirected with anti-CD123 CAR affinity mutants in terms of efficacy against tumor targets and safety towards healthy cells. Computational structural biology tools were exploited to design point mutations in the wild type anti-CD123 CAR antigen binding domain, in order to provide a CAR design system without interference coming from other elements, such as CAR spacer length and presence of costimulatory molecules. Introduction of single amino acid substitutions in the antigen binding loops allows evaluating the real effect of CAR affinity by tuning the

same scFv and not by comparing antibodies targeting different binding epitopes.

Finally, the third chapter reports the general conclusion of this study, summarizing the main findings derived and focusing on the future perspectives offered when dealing with CAR-design strategies.

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Chapter 2

Anti-CD123 CAR binding affinity and density balance in AML

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Abstract

Chimeric Antigen Receptors (CARs)-redirected T lymphocytes are a promising novel immunotherapeutic approach, nowadays object of accurate preclinical evaluation also for the treatment of Acute Myeloid Leukemia (AML). In this context, we recently developed a CAR against CD123, over-expressed on AML blasts and leukemic stem cells. However, the potential recognition of low CD123-positive healthy tissues, through the "on-target-off-organ" effect, limits the safe clinical employment of CAR-redirected T cells. Therefore, in search for a CAR design optimization, we here evaluated the effect of variables capable to modulate CAR T-cell functional profiles in a context-dependent manner, such as CAR binding affinity for the target antigen, CAR expression and target antigen density.

To study these variables in the absence of other interfering elements we exploited computational structural biology tools to design 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 were able to define both “lytic” and “activation” antigen thresholds, showing that whereas the early T-cell cytotoxic activity is not affected either by CAR expression or CAR affinity tuning, later effector functions are impaired by low CAR expression. Moreover, a promising balance in the efficacy and safety profiles of CAR T cells was observed in the lowest affinity mutant in response to targets with different antigen densities. Overall, the full dissection of all these variables offers additional knowledge for the

proper design of a suitable anti-CD123 CAR for the treatment of AML.

Introduction

In the last years, adoptive cellular immunotherapy employing T lymphocytes genetically modified with Chimeric Antigen Receptors (CARs) has demonstrated impressive anti-tumor effects in the context of high risk relapsed/refractory patients affected by B-cell origin neoplasms (1-3, 4) .These significant clinical results have paved the way towards the possibility to translate this approach to other aggressive hematological malignancies, such as Acute Myeloid Leukemia (AML), nowadays still associated with high rates of relapse when treated with conventional therapeutic regimens (5).

CARs are artificial molecules comprised of an extracellular antigen binding domain, usually derived from a monoclonal antibody (mAb) in the form of a single chain fragment variable (scFv), and by an intracellular signaling moiety, such as the CD3-ζ complex, together with one or more co-stimulatory signaling modules (6-8).

The choice of the target antigen for CAR-based therapies should be carefully evaluated, since drawbacks have emerged in the clinical translation of CARs. Indeed, serious side effects have been reported, leading in the worst cases to the death of the treated patients, due to the CAR mediated recognition of Tumor Associated Antigens (TAA) expressed at low levels also on normal tissues, a problem known as "on target off-tumor" effect (9, 10).

Indeed, the CAR T-cell technology is an extremely sensitive approach, since the minimum threshold number of surface target molecules that can be recognized is markedly low as 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. Thus, strategies aimed at ameliorating the CAR-based approach should focus on an off-tumor target antigen expression at very low levels, or 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 the similarities between Hematopoietic Stem Cells (HSCs) and Leukemia Stem Cells (LSCs), a rare population of cells which is responsible for AML emergence and maintenance, make the identification of a good target antigen a challenging aspect. In this scenario, our attention has been posed on the Interleukin-3 Receptor Alpha (IL3RA; CD123), which is a poor prognosis over-expressed marker on AML blasts and LSCs (11).

An anti-CD123 CAR previously generated by our group was able to eradicate AML blasts *in vitro* and *in vivo*, showing a safer profile towards normal Hematopoietic Stem/Progenitor Cells (HSPCs) when compared to the formerly studied anti-CD33 CAR (12, 13). However, a moderate *in vitro* toxicity was observed towards CD123-low-expressing endothelium and monocytes, suggesting to pose a higher level of caution for a future clinical translation of this approach to avoid a potential 'on-target off-tumor' effect.

Although the exact structural and signaling features for the design of an ideal CAR are still undefined, it has been demonstrated in several tumor models that it is possible to affect the functional activity of the

genetically modified T cells by tuning the binding properties between the CAR and its TAA (14-17). In particular, further investigation of the role played by the binding affinity in a more context-dependent manner is warranted. Indeed, each pathological situation is different in terms of percentage and density of antigen positive cells, but also antigen localization, accessibility of the targeted epitope and CAR design. As a matter of fact, in a model of anti-CD20 CAR, both a "lytic" and an "activation threshold" have been defined as the number of antigens on the surface of the target cells required to produce a full cytotoxic activity and activation/expansion of CAR^+ T cells, respectively (18). For this reason, we aimed to unravel how the interplay between CAR affinity, antigen density and CAR expression could impact on anti-CD123 CAR-redirected effector cell efficacy against AML cells and safety towards healthy cells.

We therefore generated and tested anti-CD123 lower affinity CAR mutants by single residues substitution on the wild type (wt) CAR scFv. In this way, the binding properties (affinity) should be the only variable in the system, while maintaining the same epitope binding site and the overall features within the CAR structure. Mutation of these residues resulted in lower affinity antibodies with K_{off} (dissociation constant) identical to the original molecule but slower K_{on} (association constant). This is expected to decrease the sensitivity towards low antigen concentrations, as it is the case in off-tumor organs with low target antigen expression levels, thus favoring the recognition of over-expressing tumor cells.

The CAR-redirected effector T-cell population has been represented by Cytokine Induced Killer (CIK) cells, an *ex-vivo* easily expandable population greatly heterogeneous, displaying a T-$CD3^+$/$CD56^+$ cell enrichment with natural killer (NK)-like properties. This population has shown very powerful cytolytic activity against both solid and hematological tumors (particularly for AML) and no graft versus host disease (GVHD) in allogeneic setting, equally *in vitro* and *in vivo* (19) (20) (21).

We show that the CAR expression profile is able to strongly influence the CAR-CIK cell effector functions, in particular their later properties, such as proliferation and cytokine production. Additionally, our set of anti-CD123 CAR affinity mutants allowed us to *in vitro* define antigen specific "lytic" and "activation" functional thresholds, influenced by the CAR binding kinetics.

Results

Structure based design of CAR mutants with lower affinity for the CD123 antigen

In order to better explore the role of the sole CAR binding affinity, we aimed to generate and compare a set of anti-CD123 CARs, derived from antibody mutants that would i) equally recognize the same epitope within the CD123 antigen; ii) have reduced binding affinity due to slower association rates.

We used a combination of computational tools and structural analysis to rationally design mutants of the anti-CD123 antibody clone 7G3 that, once conceived as a CAR, has been previously shown to be effective in redirecting CIK cells against AML targets, but marred by possible toxicity against non tumor targets (12).

We first obtained an atomic model of the antibody and of its complex with the CD123 target antigen by computational simulations, validated by available experimental information regarding the antibody binding site (22) (23). Computational and visual analysis allowed us to identify single antibody residues in the antigen binding loops that, once mutated, could decrease but not completely abrogate the antibody binding. Indeed, the point mutations should not alter the binding site or overall antibody properties. We then produced the mutated antibodies as single chain Fragment variable (scFv) versions (the same used in CAR-CIK cells) and measured their binding properties for the CD123 antigen with Surface Plasmon Resonance.

In addition to the wild type (wt) antibody, four mutants named CAMs (CAR Affinity Mutants), were selected for analysis 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 (KD $3x10^{-9}$ M for wt, $3x10^{-7}$ M for CAM-L (Low affinity), $3x10^{-8}$ M for CAM-M (Medium affinity)). Two further mutants with binding properties similar to the wt molecule were generated as controls (CAM-H1 (High affinity)=$1x10^{-9}$ M and CAM-H2=$2x10^{-9}$ M). All mutants had dissociation rate similar to the wt molecule but diverse association rates (k_{on} $1x10^{5}$ 1/Ms for wt, $8x10^{2}$ 1/Ms for CAM-L and $4x10^{4}$ 1/Ms for CAM-M; k_{off} $2x10^{-4}$ 1\s for wt, $2x10^{-4}$ 1\s for CAM-L and $9x10^{-4}$ 1\s for CAM-M) (Figure1).

A reduced CAR expression impairs anti-CD123 CAR-CIK cell later efficacy profile

In order to verify the impact of both the mutations and the genetic manipulation on CIK cell phenotype and functional efficacy, we genetically modified CIK cells with the above described CAM-H1 and CAM-H2 mutants and wt CAR. The typical CIK cell phenotype was minimally affected by the CAR engineering for all the tested conditions (wt CAR and CAMs), being comparable to the un-manipulated NO DNA control (Supplementary Figure 1A, 1B and 1C).

The percentage of CAR expression at the end of culture (day 21) 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 MFI in comparison to the other constructs (Figure 2,B).

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-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).

Evaluation of the lytic activity of all CARs tested revealed a basal killing capacity against the MHH-CALL-4 negative control, comparable to that of NO DNA, clearly indicating that the mutations introduced did not render them unspecifically active against a CD123 negative target. Furthermore, the CAR-engineered CIK cells showed a similar killing ability against the THP-1 positive target, compared to the NO DNA control, despite the markedly reduced expression of CAM-H2 (Figure 2C).

These results point out that at comparable binding affinity (K_D values), these CAMs induced a similar cytotoxic effect against the high $CD123^+$ THP-1 cell line, even if CAM-H2 has a much lower CAR expression profile.

By contrast, upon stimulation with THP-1 target cells the CAM-H2 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. Lack of proliferation activity was observed when CAR-CIK cells were exposed to the CD123 negative MHH-CALL-4 cells, confirming their specificity against the CD123 target antigen (Figure 2D).

All the CAR-redirected CIK cells were able to produce cytokines, such as IL-2 and IFN-γ, in response to THP-1 cell line and not to MHH-CALL-4. Similarly to the proliferation results, CAM-H2-CIK cells stimulated with the THP-1 cell line produced statistically significant lower levels of IFN-γ and IL-2 in comparison to wt- and CAM-H1-redirected CIK cells (Figure 2E and 2F).

Since wt CAR, CAM-H1 and CAM-H2 have comparable binding affinity but different expression profile on CIK cells, these results suggest that 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 impacts anti-CD123 CAR-CIK cell later efficacy compared to CAR affinity

In order to better characterize the biological effects of the affinity tuning on CAR-CIK cell functional properties, we focused our attention on the CAM-L and CAM-M mutants with binding affinity approximately 100 and 10 fold weaker than the wt (KD $3x10^{-9}$ M for

wt, $3x10^{-8}$ for CAM-M and $3x10^{-7}$ for CAM-L). As previuosly verified, both the genetic manipulation and the affinity tuning modification minimally affected the phenotype of engineered CIK cells, being comparable to the NO DNA control (Supplementary Figure 2A, 2B and 2C).

Wild type 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, Figure 3A and 3B).

The "double target challenge" cytotoxic assay showed that CIK cells engineered with the less affine CAM-M and CAM-L receptors maintained significant killing activity for highly CD123 positive cells, in comparison to the NO DNA control. All tested CAM-CIK cells showed comparable killing capacity for primary AML cells and no activity for the co-cultured healthy bone marrow cells (Figure 3C). Similarly, the highly positive CD123 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 the 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. It is worth reminding that, in comparison to wt, CAM-L has 100 fold weaker affinity for CD123 and CAM-M is 10 fold weaker, whereas CAR expression levels

appear lower for CAM-M. This strengthens the findings that CAR expression profile is more relevant than affinity in determining later effector functions (Figure 3D).

Indeed, 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, just as CAM-H2, produced significantly less IFN-γ and IL-2 than the other constructs; IL-2 levels, in particular, were not different from the NO DNA control for both primary AML and THP-1 cells (Figure 3E and 3F). Cytokine production was more abundant in the presence of CAM-L, further highlighting 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 further characterized the efficacy profile by measuring the killing of CD123 positive cells over time in 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). 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 the NO DNA control.

Taken together, the above 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 the binding affinity offers a potential balance between CAR efficacy and safety profiles

Anti-leukemia targeted therapies must recognize and suppress cancer cells while avoiding healthy cells expressing the target antigen at lower levels. 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 different CD123 expression and density (Figure 4A and 4B). We chose THP-1 and KG-1 AML cell lines as high $CD123^+$ target cells. The former had 99% of CD123 positive cells with 7435±1986 CD123 molecules per cell; the latter had 81% $CD123^+$ cells with 4550±559 CD123 molecules per cell. The AML U937 cell line and healthy endothelial 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=1603±215; TIME=1688±328).

As before, we confirmed both the efficacy and specificity of CAR-CIK cells when co-cultured with the $CD123^+$ THP-1 and the $CD123^-$ MHH-CALL-4 (Figure 5A).

Co-culture of the highly positive THP-1 cell line with CD123 low positive conditions showed preferential killing of THP-1 cells (Figure

5B and 5C), highlighting the fact that the presence of a second target, low $CD123^+$, does not inhibit the killing of a first intended highly $CD123^+$ target. CAM-L-CIK cells were as effective as wt- and CAM-H1-CIK cells in killing highly positive CD123 cells despite having a 100 fold lower binding affinity. The same was not true for low $CD123^+$ cells where CAM-L-CIK cells showed a trend of lower killing activity in comparison to the other constructs (Figure 5D).

In order to characterize the safety profile of CAM-L-CIK cells against the TIME healthy tissue we also 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 3B). 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, with improved or at least equal safety profile with regard to the healthy tissue.

Furthermore, since the killing activity exerted by CAR-CIK cells against the low $CD123^+$ cells was higher than the negative control, but significantly lower if compared to the high $CD123^+$ leukemic target, it appeared that the presence of approximately 1600 CD123 molecules per 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 (18).

By contrast, no proliferation activity was detected against the low $CD123^+$ cells in comparison to the spontaneous proliferative activity exerted by CAR-CIK cells alone (Figure 6A), while there was a strong and equal proliferative response obtained against the high $CD123^+$ targets for all the CARs studied. According to these findings, a number of ~1600 CD123 molecules is sufficient to trigger the lytic activity, but not to induce the proliferation of CAR-redirected CIK cells.

When considering the cytokine production in response to $CD123^+$ target cells, it emerged how the CAR-redirected CIK cell sensitivity to the target was clearly influenced by their CD123 positivity. A hierarchical cytokine production, from the THP-1 cells with the highest CD123 expression, 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. A significant amount of TNF-α and IL-6 was only produced in the presence of THP-1 cells, instead (Figure 6).

Notably, no activation was induced against the CD123 negative control and the healthy tissue by all the CAR-CIK cells tested and for all the cytokines analyzed.

Later effector functions and not early cytotoxic activity are proportional to CAR downmodulation

After antigen engagement by T cells with canonical 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).

For this reason, in order 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, CAM-M and CAM-H2 had progressively lower amount of downmodulation while having higher affinity than CAM-L (Figure 7A). Since we previously observed the impact of the CAR expression levels on CAR-CIK cell later effector functions, in particular the cytokine production, we sought to determine the impact of CAR downmodulation in this behavior. The mutants with lower downmodulation, CAM-M and CAM-H2, were also found to determine a lower IFN-γ production by CIK cells (Figure7B).

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).

We also analyzed the amount of CAR downmodulation as a result of stimulation with different CD123 antigen densities on the target cells (Figure 7D). We noted that the CAR down-modulation is related to the antigen density on the tested cell lines (THP-1, KG-1, U937, MHH-CALL4), accounting for a different strength of CAR^+ T-cell activation and thus functional related responses (Figure 6, Figure 7D). Increasing numbers of CD123 molecules per cell lead to a larger amount of CAR downmodulation, resulting in an escalating CAR^+ T-cell activation. The results are comparable between wt CAR, CAM-H1 and CAM-L, thus independent of binding affinity, and reflect the hierarchical trend previously observed with cytokine production.

Taken together the above results suggest that target antigen density and CAR expression profile are the primary determinants for CAR downmodulation. Lower downmodulation results in reduced cytokine production, whereas it does not affect early lytic activity.

Discussion

Among the innovative immunotherapeutic approaches for the treatment of AML, the use of anti-CD123 CAR-redirected T cells has shown to be a promising tool to selectively target AML cells and Leukemic Stem Cells (LSCs) (12, 13, 30, 31). However, targeting CD123 has potential safety concerns due to its low expression on healthy cells, especially monocytes and endothelial cells (12) (30).

Despite recent advances regarding CAR design strategies and improved knowledge about CAR T-cell related properties, the exact determinants leading to a balance between ideal anti-tumor responses and safety profiles remain partially unclear and disease-dependent. However, even starting from a specific pathological context, it would be helpful to define common trends to guide the general design of a CAR construct.

Therefore, in search for an optimization of the strategy leading to efficient CAR design, we investigated the effect of three of the main variables affecting CAR T-cell activity: (i) the CAR binding affinity for the target antigen, reported to modulate the effector functions of redirected T cells (14-17); (ii) the expression levels of CAR molecules on the redirected T cells and (iii) the target antigen density.

Amino acid single point anti-CD123 CAR affinity mutants (CAMs) were rationally designed on the basis of computational and structural investigation of the interaction between the CD123 antigen and the anti-CD123 antibody. The principle at the basis of this approach was to generate antibody mutants with slower association rate in

comparison to the wild type. Experimental SPR analysis confirmed that two anti-CD123 mutants had the desired characteristics, with 100 (CAM-L) and 10 (CAM-M) fold lower affinity in comparison to the wild type. Introduction of single amino acid substitutions in the antigen binding loops allows evaluating the real effect of CAR affinity by tuning the same scFv and not by comparing antibodies targeting different binding epitopes.

Altogether, the computational simulations allowed rapid and inexpensive rational design of a limited set of mutants, bypassing the need to generate and screen a large number of mutations in randomized sequence searches. The major advantage of this integrated approach lies in the fact that it 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 the CAMs tested were specific for $CD123^{+}$AML cells including AML primary samples. Differently from other published contexts (32) (15) (14) (17), even the weaker binders CAM-L and CAM-M showed enhanced redirected-CIK cell effector properties. However, two mutants had a lower CAR expression and MFI (CAM-H2 and CAM-M), which strongly affected the later functional properties of the genetically modified CIK cells, independently of CAR binding affinity. Indeed, 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 CAR expression profile was not a limiting factor in determining a

powerful cytotoxic activity against a highly CD123 positive leukemic target, since both CAM-M- and CAM-H2-CIK cells were equally lytic as the 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 the target cell lysis and the production of pro-inflammatory cytokines against a target cell with a high TAA expression (33).

In our model, a "CAR expression ceiling" of ~40% CAR^+ cells was observed. Indeed, 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 effector functions.

It is worth noting that although in laboratory settings the problems linked to low CAR expression could be avoided by sorting out the CAR^+ cells, this is not easily achievable in a Good Manufacturing Practices (GMP) grade clinical application. Furthermore, the low MFI values could not be restored by CAR^+ cell selection.

We then investigated the effect of CAR binding affinity and target antigen density on the efficacy and safety profiles of CAR redirected CIK cells. We considered THP-1 and KG-1 AML cell lines as highly CD123 positive models, having also a high number of CD123 molecules/cell. U937 (AML cell line) and TIME (healthy endothelial cell line) targets were representative of low CD123 expressing cells. No reduction of cytotoxic activity of redirected CIK cells against a

first intended target, highly $CD123^+$, in the presence of a second unintended target, low $CD123^+$, was detected. This is in contrast to what observed in an anti-CD20 CAR model described by James et al. (29).

We found that a value of approximately 1600 CD123 molecules per cell (in U937 and TIME) is sufficient to produce a detectable lytic activity, albeit significantly lower than the response to highly $CD123^+$ THP-1 cells. Watanabe and collaborators recently described this feature as the CAR "lytic threshold" (18). Notably, the "double target challenge" cytotoxic assay, which is at 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 equally 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} molar is sufficient to achieve a satisfactory CAR T-cell lytic activation. In contrast, differences were detected in the activity against the low CD123 positive U937 and TIME cells, where CAM-L-CIK cells showed a trend of inferior killing levels in comparison to CAM-H1- and wt CAR-redirected cells. Since a reduced killing activity was detected against low $CD123^+$ targets, our results point out that 10^{-7} M binding affinity could offer a better safety profile.

In a model of anti-ErbB2 targeting, Chmielewski and co-workers identified an affinity ceiling of 10^{-8} M, below which there is no additional improvement of receptor-mediated cellular activation. They

also noted less discrimination between high and low Ag expressing target cells by high affinity immune-receptors (15). However, in their model 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 that later effector functions are affected by target antigen density. A hierarchical trend is evident, particularly for IFN-γ and IL-2 production, from higher cytokine release against THP-1, to lower detection against KG-1 and even lower for U937 cells. A similar trend was not present in the proliferation activity. Indeed, CAR-CIK cell proliferative capability was not sensitive anymore to antigen escalation above ~5000 antigen molecules per cell (KG-1 values).

For the first time to our knowledge, we could observe that the triggering of cytokine production and T-cell proliferation, depends on different antigen thresholds. The definition of these thresholds is of fundamental importance to propose proper target antigens for a CAR-based adoptive cell immunotherapy approach.

Caruso and collaborators have recently proposed, in a model of anti-EGFR CARs, that it might be possible to limit the recognition of healthy tissues, while maintaining a potent anti-tumor activity, by lowering the CAR binding affinity for the target antigen. However, similar later effector functions were detected against either the low

$EGFR^+$ healthy tissue (~15000 molecules/cell) or the U87 tumor cell line (engineered to express ~30000 antigens/cell) by low affinity CARs. Thus, the lytic and activation thresholds might have been exceeded in the EGFR system (32), limiting the proper interpretation of a potential on-target off tumor effect on the healthy tissues.

It was shown that CAR antigen engagement can trigger CAR internalization at a specific rate, leading to reduced CAR availability on the surface of genetically modified T cells (29, 32). This observation is in line with the classical TCRs internalization following antigen binding, resulting in TCRs downmodulation proportional to the strength of the binding and thus T-cell activation (24) (27) (25, 26, 28). We noted that CAR binding affinity has no impact on CAR downmodulation when CAR-CIK cells are challenged with highly antigen positive THP-1 cells. The lytic activity does not appear related to CAR downmodulation, since equal killing activity (~70%) was detected for different mutants with downmodulation levels between ~35 and ~75%. The mutants with higher downmodulation, instead, appeared to have increased IFN-γ production. This is in line with the idea that downmodulation is mainly responsible for later effector functions rather than early lytic activity.

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 CAR molecules above such threshold would represent accessory signaling events that can be required to sustain different and later effector functions. 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 (more antigens per cell lead to more interactions and more CAR molecules internalized).

In conclusion, low levels of CAR expression resulted in impaired activity, especially with regards to later CIK cell effector functions, such as proliferation and cytokine production. This seems to arise from decreased levels of CAR internalization/downmodulation 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 towards the screening of optimal CAR expression profiles and not exclusively in the hunt for a high affinity antibody-derived CAR. In fact, CIK cells redirected with the lowest affinity mutant tested showed a trend of decreased killing of low positive target antigen cells, being equally active as high affinity CAR-CIK cells against highly antigen positive targets. This suggests that lower affinity mutants might actually lead to improved safety profiles. 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 anti-CD123 CAR redirected CIK cells towards AML cells and healthy tissues, expressing different levels of target antigen.

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 cell line (ATCC® CRL-4025™, dermal micro-vascular endothelial cell line), have been obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA).

The THP-1, U937 and KG-1 cell lines were maintained in culture with Advanced RPMI medium (Invitrogen, San Giuliano Milanese, Italy) 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, Bergamo, Italy). 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, rh_EGF, rh_FGF basic and rh IGF-1) and combined with 10 mM L-glutamine, heparin sulfate 0.75 Units/mL, Hydrocortisone hemisuccinate 1µg/mL, FBS 5%, ascorbic acid 50µg/mL (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 in protein data bank server (PDB code 4JZJ), while anti-CD123 antibody was obtained using RosettaAntibody software. PDB codes of the templates are as follows (sequence identity is indicated in parenthesis): 1MVU for the light-chain framework (96.77%) and 1MJ8 for the heavy-chain framework (91%); 1MVU for L1 (100%) and for L3 (88.89%), 1Q9R for L2 (100%); 1EGJ for H1 (100%) and for H2 (82.35%), 12E8 for H3 (same length, no identity). Best 10 antibody models out of 2100 were selected thanks to 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 Ab CDR loops facing the N-Terminal part of CD123 protein, since it is known that domain 1 of CD123 is fundamental for anti-CD123 binding (34), and then separated by 25Å. Docking runs of 10 different antibody models with its antigen were conducted as previously described (35). Best models of each simulation, selected thanks to their energetic score and by visual analysis, after discarding complexes with interface far away from 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 variants protein production

Human CD123 domain 1+2 nucleotidic sequence was cloned in frame into pET21a plasmid and expressed using *E.coli* system (Rosetta DE2 cells). Single clone cells were grown in LB AMP+CHA+ media until 0.6 O.D., then induced with 1mM IPTG and harvested after 3 hours. CD123 1+2 was found in the unsoluble fraction, so protein pellet was washed and solubilized using mild-denaturing buffer (100mM TRIS pH 12.5 2M UREA 5mM 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, 1% PEG3350. CD123 domain 1+2 elute at 68 mL according to its monomeric molecular weight.

Anti-CD123 single chain antibody nucleotidic sequence was cloned in frame into pET21a plasmid and expressed using *E.coli* system (Rosetta DE2 cells). Single clone cells were grown in LB AMP+CHA+ media until 0.6 O.D., then induced with 1mM IPTG and harvested after 3 hours. Single chain antibody was found in the unsoluble fraction, so protein pellet was washed and solubilized using denaturing buffer (50mM MES pH 6.5 1M NaCl 6M Guanidinium-HCl). Protein was loaded into an hitrap column (GE Healthcare) pre-equilibrated with solubilization buffer and eluted with same buffer plus 500mM Imidazole. Fractions containing antibody were refolded

using direct dilution into 20mM NaP pH10 150mM NaCl 200mM Arginine 1mM Glut Red 0.1 Glut Ox. Protein was concentrated by centrifugation (2000 rpm 4°C) with 10kDa Vivaspin (Sartorius), and loaded on size exclusion column (HiLoad 16/60 superdex 75, GE Healthcare) pre-equilibrated with 50mM TRIS pH9 50mM NaCl. Anti CD123 elute at 64mL according to its monomeric molecular weight.

Mutagenesis of anti-CD123 single chain Ab

Mutagenesis was performed using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Specific mutated primers were synthesized by Microsynth AG 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 minutes to remove the parental (i.e., the nonmutated) supercoiled ds DNA.

The DpnI treated DNAs (mutated plasmids) were used to transform XL10-Gold Ultracompetent Cells; the colonies obtained from the transformations were used to DNA amplification and extraction with Qiagen Maxi or Mini Prep Kit.

The sequence of each mutated plasmid was verified by DNA sequencing (Microsynth AG); the verified plasmids were used to 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

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

Surface Plasmon Resonance (SPR)

SPR experiments were performed in order 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 pH7.4, 150 mM NaCl, 3 mM EDTA, 0.005% TWEEN20). The injection of the antigen spanned a concentration range of 200-12.5 nM at flow rate of 70 µL/min. Data were fit using Langmuir equation.

Transposons plasmids

The wild type anti-CD123/pTMNDU3 SB transposon expresses the human 3rd generation anti-CD123CD28OX40 CAR under pTMNDU3 promoter. The construct has been derived as a SB expression plasmid, replacing the eGFP sequence from the pT-MNDU3-eGFP with the scFv CD123 (7G3 clone) previously cloned in frame with CH2CH3-CD28-OX40-ζ from SFG.aGD2 (kindly provided by Dr. Martin Pule, University College of London, London, UK). 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 University of Minnesota).

Generation of CIK cells genetically modified for the expression of the anti-CD123 CARs

CIK cells were generated starting from PBMCs from healthy subjects, obtained after centrifugation of fresh blood on a density gradient using Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden). Once collected, PBMCs were resuspended in Amaxa Nucleofector solution, provided with the P3 Primary Cell 4D-Nuceofector X kit (Lonza, Bergamo, Italy), together with SB11X transposase and DNA plasmid encoding for one of the anti-CD123 CAR mutants and transfected using the 4D-Amaxa Nucleofector^{TM} device (Lonza). Our controls included unmodified cells, being nucleofected with no DNA, and eGFP episomal transfected cells (with 2 μg 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 1000 U/ml of IFN-γ (Dompè Biotec S.p.A, Milano, Italy) were added to each well (Magnani C. F., et al, manuscript under revision). Twenty-four hours later, IL-2 (Chiron B.V, Emeryville, USA) and OKT-3 (Janssen-Cilag S.p.A., Cologno Monzese, Italy) 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 $0.75x10^6$ cells/ml. Cells were then cultured for 21 days.

Flow cytometry

Immunostaining and flow cytometric analysis were performed with the following antibodies: Allophycocianin (APC)-anti-CD123 (Becton Dickinson, BD, San Jose, USA), Phycoerythrin (PE)-anti-CD123 (BD), v-500 anti-CD45 (BD), Phycoerythrin-Cyanin 7 (Pe-Cy7)-anti-CD34 (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-IgG (H+L) (anti-Fc), PE-anti-IL-2, FITC-anti-IFN-γ, Pe-Cy7-anti-TNF-α, Horizon BV421-anti-IL-6, Horizon BV421-anti-Ki-67, FITC-anti-CD19 (BD), PE-anti-CD144 (BD). Cell death and apoptosis were detected using the GFP-Certified™ Apoptosis/Necrosis detection kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA), according to the manufacturer's instructions. Cell membrane labeling was also performed using two lipophilic fluorescent dyes: FITC- and PE- Cell Tracker (Life Technologies, Invitrogen division, Monza, Italy).

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 (36).

Flow cytometry was performed on FACSCanto 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 assessed by means of the "double target challenge", CIK cells were co-cultured for 4 hours with the CD123 positive targets (THP-1, primary AML cells, U937 and TIME cell line), together with the CD123 negative control (MHH-CALL-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 the apoptosis detection by flow cytometry, after Annexin V and 7-AAD staining, gating in the Cell Tracker PE^+ and $FITC^+$ cells. The percentage of killed cells was calculated according to the following formula:

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

Two long-term cytotoxicity assays were conducted at an Effector:Target (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 by co-culturing CIK cells with THP-1, MHH-CALL-4 and TIME cell lines for 72 hours. Target cell survival was then evaluated after a surface staining with PerCP-anti-CD3 (BD) antibody, in order to detect CIK cells, and by comprising the following antibodies: APC-anti-CD123 (BD), FITC-anti-CD19 (BD),

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:

$$\left(\frac{Number\ of\ targe\ cells\ after\ the\ co-culture\ with\ effector\ T\ cells}{Total\ number\ of\ target\ cell\ alone}\right) \text{x } 100$$

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 hour co-culture, the cells were collected, immunostained for intracellular Ki-67 and then analyzed by flow cytometry, by performing the detection of Cell Tracker negative-Ki-67^{+} cells.

Intracellular cytokine staining (ICS)

CAR-CIK cell ability of producing cytokines was evaluated following a stimulation with the various target cell conditions, at an E:T *ratio* of 1:3. After a 2 hours and 30 minute co-culture, BD GolgiStop™ 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 the anti-CD3 and anti-Fc surface molecules detection. Finally, an intracellular cytokine staining for IL-2, IFN-γ, IL-6 and TNF-α was performed using the BD Cytofix/Cytoperm kit, according to manufacturer's protocol. Specimens were then analyzed by flow cytometry.

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 downmmodulation percentage was calculated according to the following formula, as described in the work of James S. C. et al., (29):

$$\%\ of\ CAR\ downmodulation = 100\ x\ (\frac{1 - stimulated\ MFI}{Unstimulated\ MFI})$$

Statistical analysis

Data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc).

One Way ANOVA and Two Way ANOVA statistical tests were performed, for column statistics and grouped statistics respectively, making use of no matching and repeated measures criteria, according to the type of data set analyzed and presence or not 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.

Supplementary materials

Supplementary Figures 1 and 2. CAR-redirected CIK cell and NO DNA control phenotype characterization at day 21.

Supplementary Figure 3. Characterization of the efficacy and safety profiles of CAM-L-CIK cells over time, by means of long term cytotoxic assays.

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Author contributions: S.A., M.B., M.C.R., 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. **Competing interests:** The authors have no potential conflicts of interest.

Figures

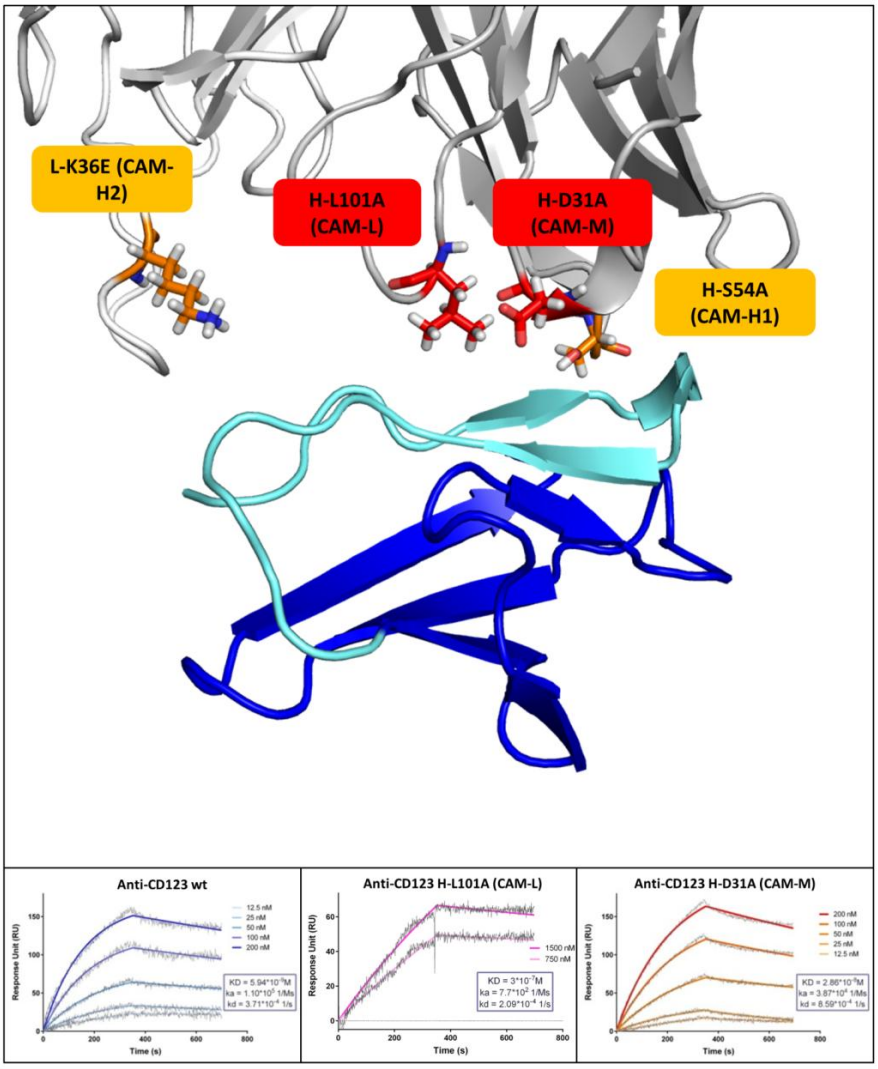

Fig. 1 Three-dimensional model of the anti-CD123/CD123 complex and SPR binding analysis. (**TOP**) Cartoon visualization of CD123 domain I (blue with the region important for anti-CD123 binding in

cyan) and anti-CD123 antibody (heavy and light chain in dark and light grey, 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 is 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.

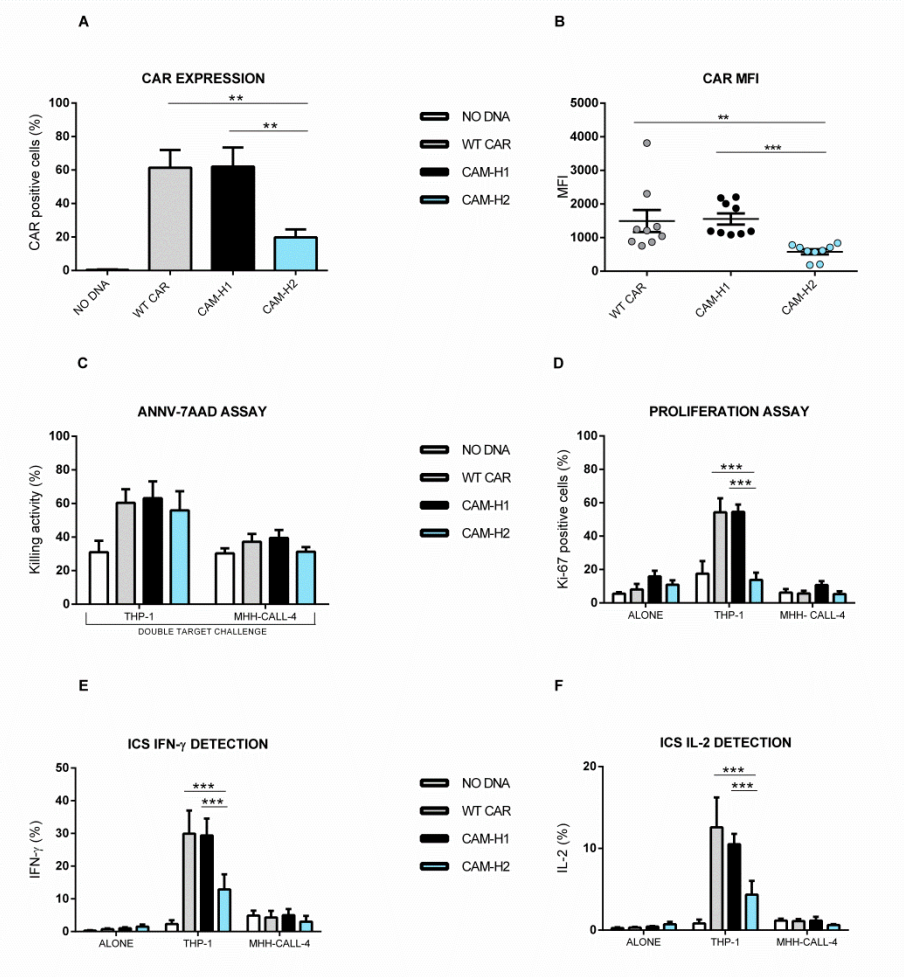

Fig. 2. Later effector functions and not early cytotoxic profile are impaired in CIK cells redirected with CAR mutants displaying identical binding affinity but reduced expression. CAM-H1 (black) and CAM-H2 (cyan), have the same binding affinity of wt anti-CD123 CAR (grey). (**A**) CAR expression and (**B**) MFI values at day 21 of anti-CD123 CAR affinity mutant redirected CIK cells and NO DNA

control. Data represented are the result of mean ± SEM, (A) n=6 (**p< 0.01; one way analysis of variance (ANOVA), Bonferroni test); (B) n=9 (**p<0.01; ***p<0.001; one way analysis of variance (ANOVA), Dunn's multiple comparison test). (**C**) 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-hour co-culture with both THP-1 and MHH-CALL-4 cell lines. No difference is detected among CIK-CAR mutants despite the reduced expression of CAM-H2-CIK cells. (**D**) Long term proliferation assay, performed through the detection of intra-cytoplasmic Ki-67^{+}/CIK-CAR^{+} cells after the co-culture of the effector cells with THP-1 and MHH-CALL-4 cell lines for 72 hours. (**E** and **F**) Intracellular cytokine staining of IFN-γ and IL-2. Effector cells and both THP-1 and MHH-CALL-4 target cells were co-cultured for 5 hours. (C, D, E and F) Data represented are the result of mean ± SEM, n=4 (***p< 0.001; two way analysis of variance (ANOVA), Bonferroni test).

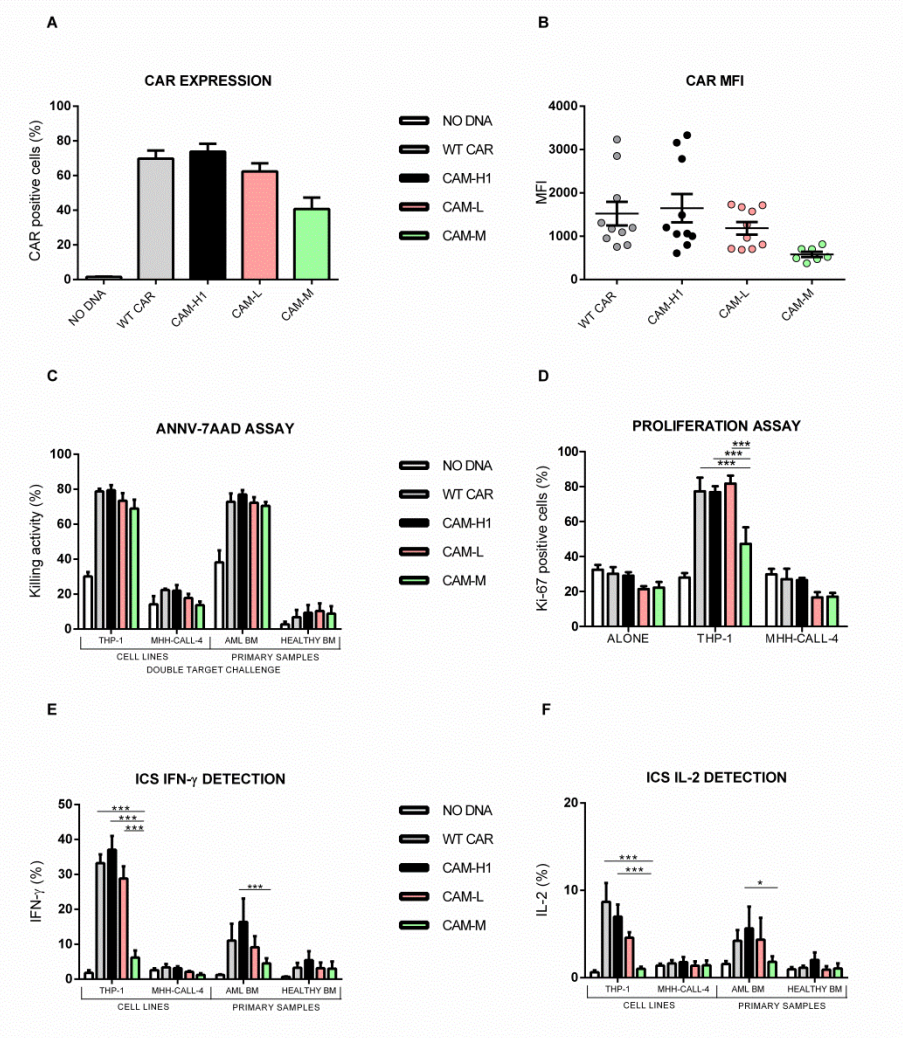

Fig. 3. Reduced CAR binding affinity does not affect CIK cell cytotoxic activity, proliferation and cytokine production in response to highly CD123 positive target cells. CAM-L (pink, K_D $3x10^{-7}$ M) and CAM-M (green, K_D $3x10^{-8}$ M) receptors have 100 and 10 fold reduced affinity in comparison to anti-CD123 wt CAR (K_D $6x10^{-9}$ M). (**A**)

CAR expression and (**B**) MFI values at day 21 of anti-CD123 CAR affinity mutant-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 analysis of variance (ANOVA), Bonferroni test). (**C**) AnnV-7AAD assay through "double target challenge", after co-culture with both THP-1 and MHH-CALL-4 cell lines and primary AML and healthy Bone Marrow samples, E:T ratio 5:1, n=4. No difference is detected among CIK-CAR mutants despite the reduced CAR affinity. (**D**) Long term proliferation assay, performed through intracytoplasmic Ki-67^{+}/CIK-CAR^{+} cell detection after the co-culture of the effector cells with THP-1 and MHH-CALL-4 cell lines for 72 hours. (**E** and **F**) Intracellular cytokine staining of IFN-γ and IL-2 upon challenge with THP-1, MHH-CALL-4 and primary AML and healthy BM samples. CAM-M, carrying a lower expression, shows the lowest proliferation and cytokine production. No difference in proliferation or cytokine production is detected for CAM-L despite its 100 fold reduced affinity. (C, D, E and F) 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 analysis of variance (ANOVA), Bonferroni test).

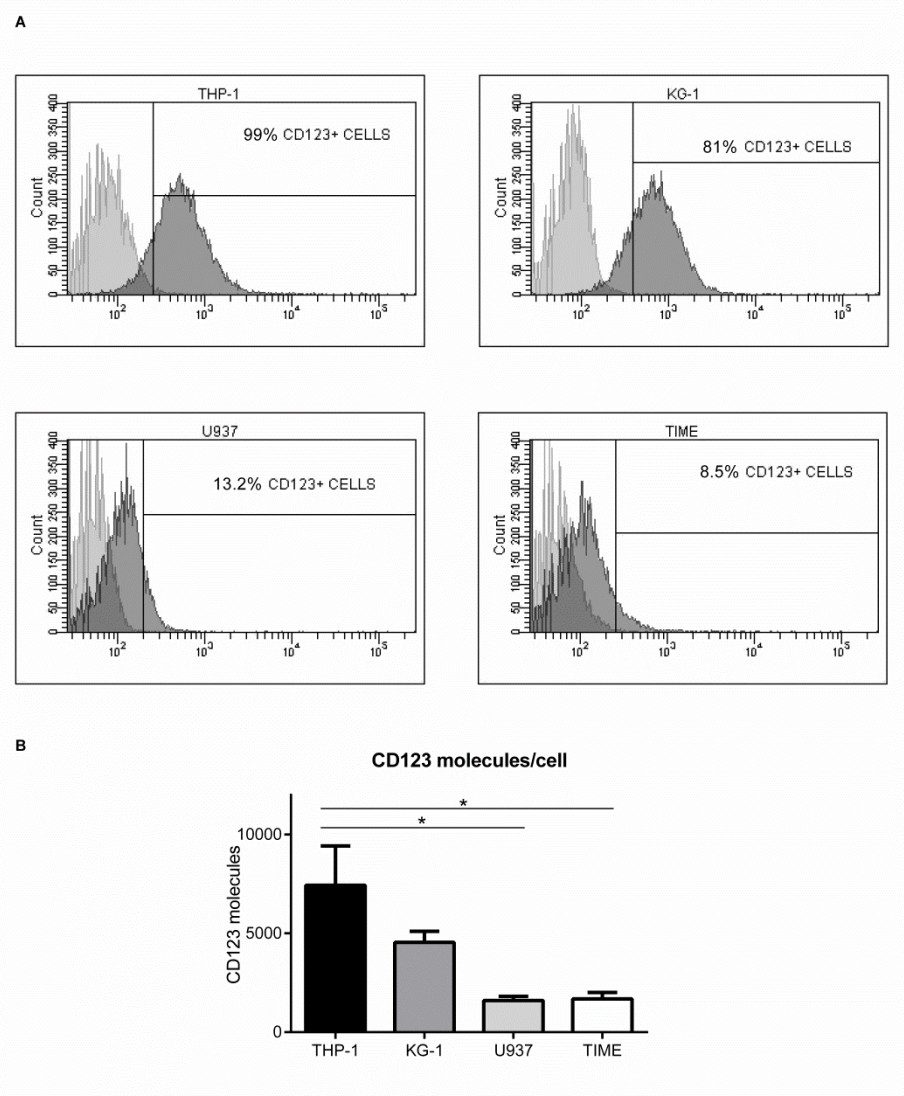

Fig. 4. CD123 expression in different target cell lines**.** (A) CD123 expression and (B) QuantiBrite quantification of CD123 molecules per cell in THP-1, KG-1, U937 and TIME cells. Data represented are the result of mean ± SEM, THP-1: n=6, KG-1: n=3, U937 and TIME

cell line: n=5 (*$p<0.05$; one way analysis of variance (ANOVA), Bonferroni test).

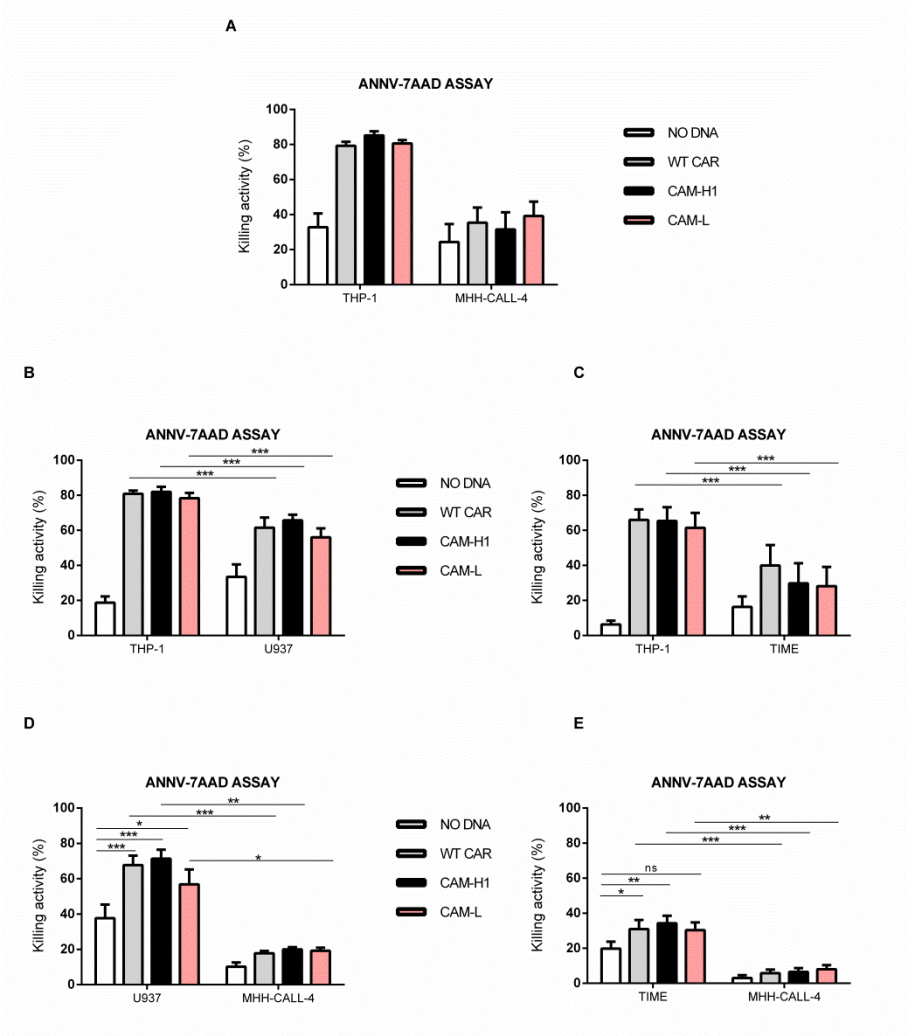

Fig. 5. Short term cytotoxic "Double target challenge". Cytotoxic "double target challenge" AnnV-7AAD assay, E:T ratio 5:1. (**A**) Highly CD123 positive THP-1 cells are killed by CAR-CIK cells when co-cultured with CD123 negative MHH-CALL-4 cells. No killing of MHH-CALL-4 is detected. (**B** and **C**) A reduced killing

activity for the low CD123 positive cells, U937 and TIME, is detected when co-cultured with THP-1 cells. (**D** and **E**) The lowest affinity mutant (CAM-L, pink) induces a killing activity comparable to wt-CAR by redirected CIK cells in the presence of highly positive THP-1 cells, but shows a trend of CAR-CIK cell reduced activity against low $CD123^{+}$ U937 cells and the TIME endothelial target. (A) THP-1/MHH-CALL-4 co-culture, n=5, CAM-H1: n=4; (B) THP-1/U937 co-culture, n=5, CAM-H1: n=4; (C) THP-1/TIME co-culture, n=6; (D) U937/MHH-CALL-4 co-culture, n=5, CAM-H1: n=4; (E) TIME/MHH-CALL-4 co-culture, n=6. Data represented are the result of mean ± SEM (*$p<0.05$; **$p<0.01$; ***$p<0.001$; two way analysis of variance (ANOVA), Bonferroni test).

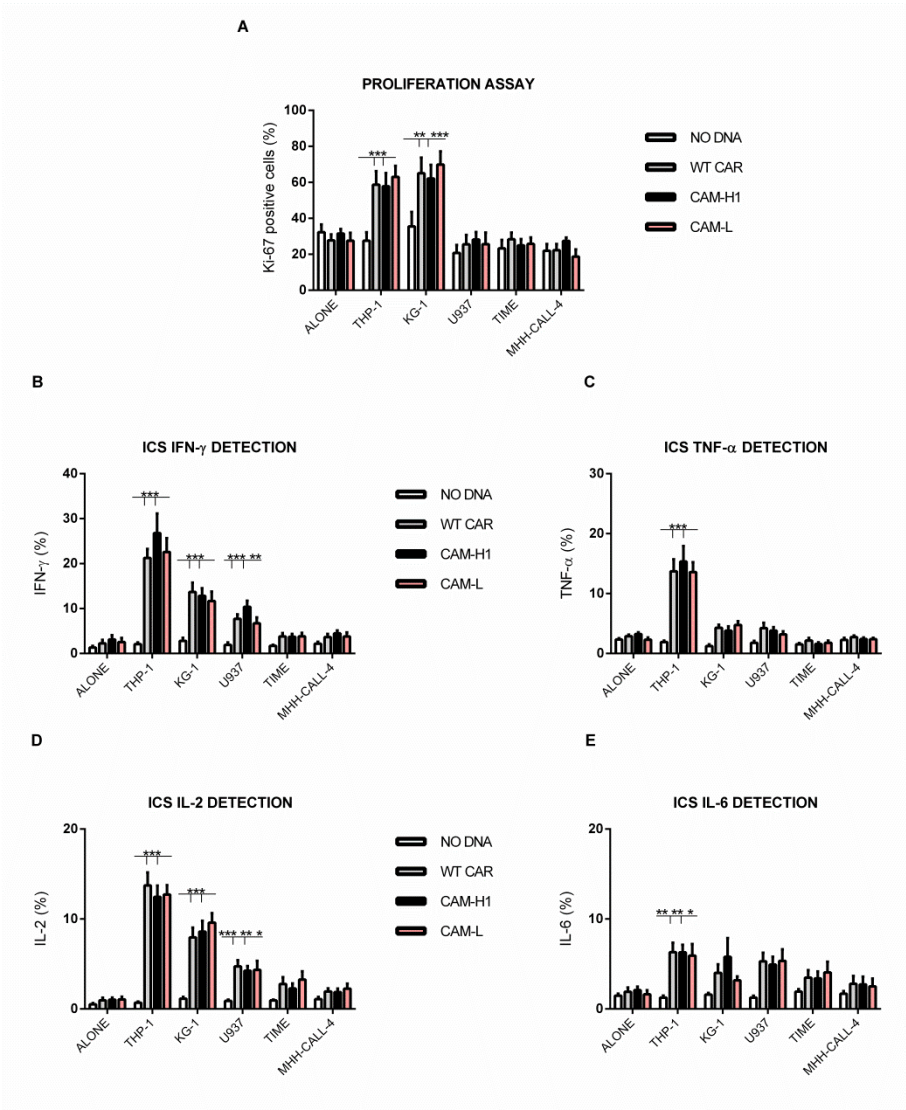

Fig. 6. Target antigen density, and not CAR binding affinity, affects later effector functions. (**A**) Long term proliferation assay, performed through the detection of intracytoplasmic Ki-67^{+}/CIK-CAR^{+} cells after the co-culture of the effector cells with the indicated cell lines for

72 hours. Low or no CD123 expressing cells (U937, TIME and MHH-CALL-4) do not stimulate redirected CIK cell proliferation. (**B**, **C**, **D** and **E**) Intracellular cytokine staining of IFN-γ, TNF-α, IL-2 and IL-6. Considering the lowest affinity condition, no difference is detected for CAM-L-CIK cells (pink). Similarly, high and low affinity CIK-CAR mutants produce comparable levels of cytokines. By contrast, cytokine production decreases at decreasing target antigen density. Effector cells were co-cultured for 5 hours with THP-1, KG-1, U937, TIME and MHH-CALL-4 cell lines. 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 the CARs tested against the KG-1 cell line (*$p<0.05$; **$p<0.01$; ***$p<0.001$; two way analysis of variance (ANOVA), Bonferroni test).

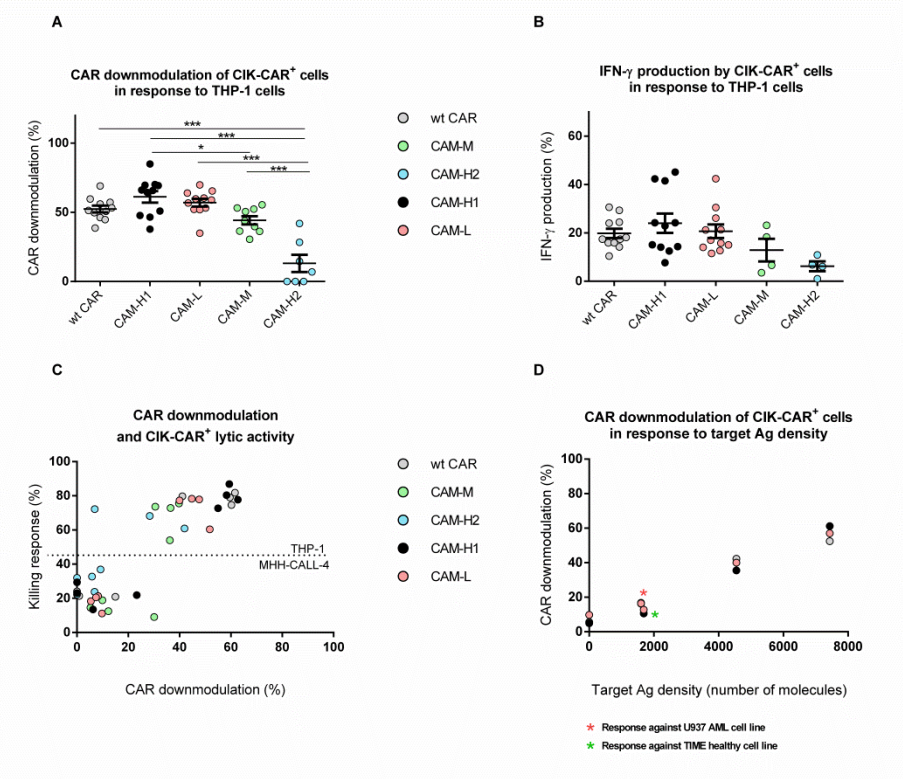

Fig 7. CAR downmodulation in redirected CIK cells. (**A**) Percentage of redirected CIK cell CAR downmodulation in response to THP-1 AML cells and (**B**) percentage of IFN-γ production when CAR-CIK cells are challenged against THP-1 cells. CAR redirected CIK cell downmodulation is not affected by CAR binding affinity since CAM-L (pink) shows CAR dowbmodulation levels comparable to wt CAR, despite a 100 fold reduced binding affinity. Lower CAR expression, instead, results in reduced CAR downmdulation levels, as seen in the lower CAR expressing CAM-M (green) and CAM-H2 (cyan; see figures 2 and 3 for CAR expression). (**C**) There is no apparent

correlation between early killing activity and CIK cell CAR downmodulation in response to THP-1 cells. A killing response of approximately 60-80% is detected at various CAR downmodulation levels. (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 analysis of variance (ANOVA), Bonferroni test). (B) wt CAR, CAM-H1, CAM-L: n=11, CAM-H2 and CAM-M n=4. (**D**) CAR-CIK cell CAR downmodulation as a function of target antigen density. Higher CD123 density on the target cells corresponds to higher levels of CAR downmodulation. Data represented are the result of mean, 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.

Supplementary Materials

Supplementary 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.

Supplementary 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 1A). 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 1, B panel).

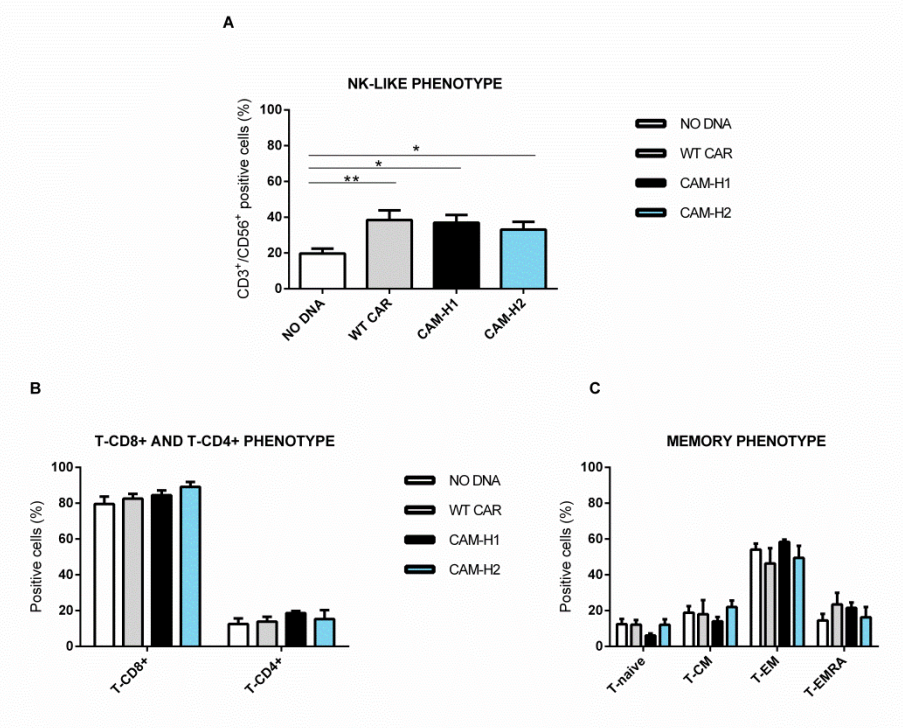

Fig. S1. CAR-redirected CIK cell and NO DNA control phenotype at the end of the differentiation period (Day21). *(***A**) NK-like, (**B**) T-CD8$^+$ and T-CD4$^+$ and (**C**) memory phenotype at day 21 of NO DNA, wt CAR-, CAM-H1- and CAM-H2-CIK cells. Data represented are the result of mean ± SEM, (A) 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).

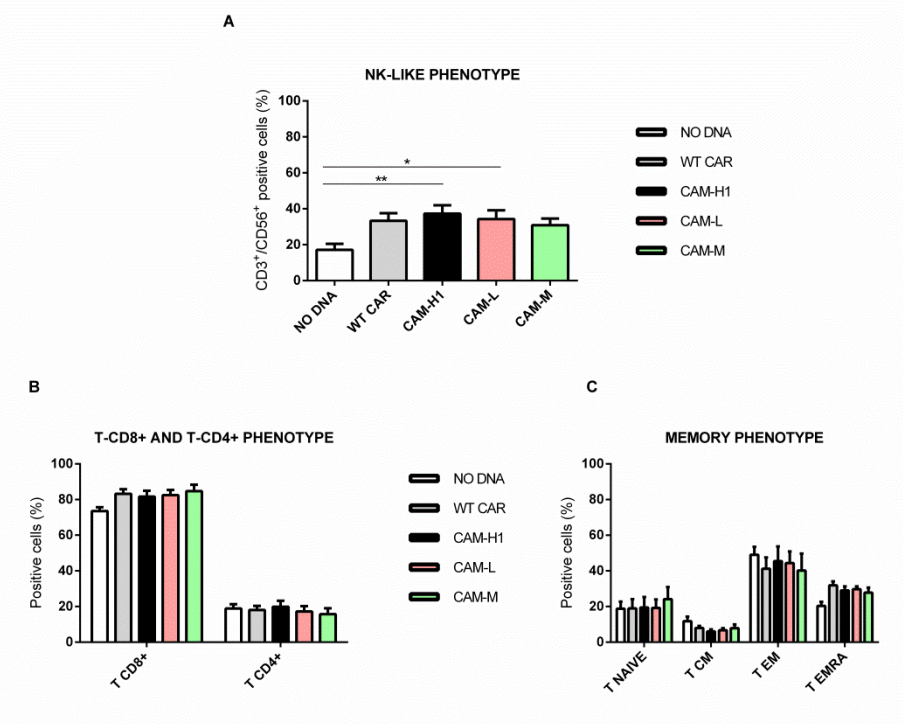

Fig S2. CAR-redirected CIK cell and NO DNA control phenotype at the end of the differentiation period (Day21). *(***A**) NK-like, (**B**) T-CD8$^+$ and T-CD4$^+$ and (**C**) memory phenotype at day 21 of NO DNA, wt CAR-, CAM-H1-, CAM-L- and CAM-M-CIK cells. Data represented are the result of mean ± SEM, (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).

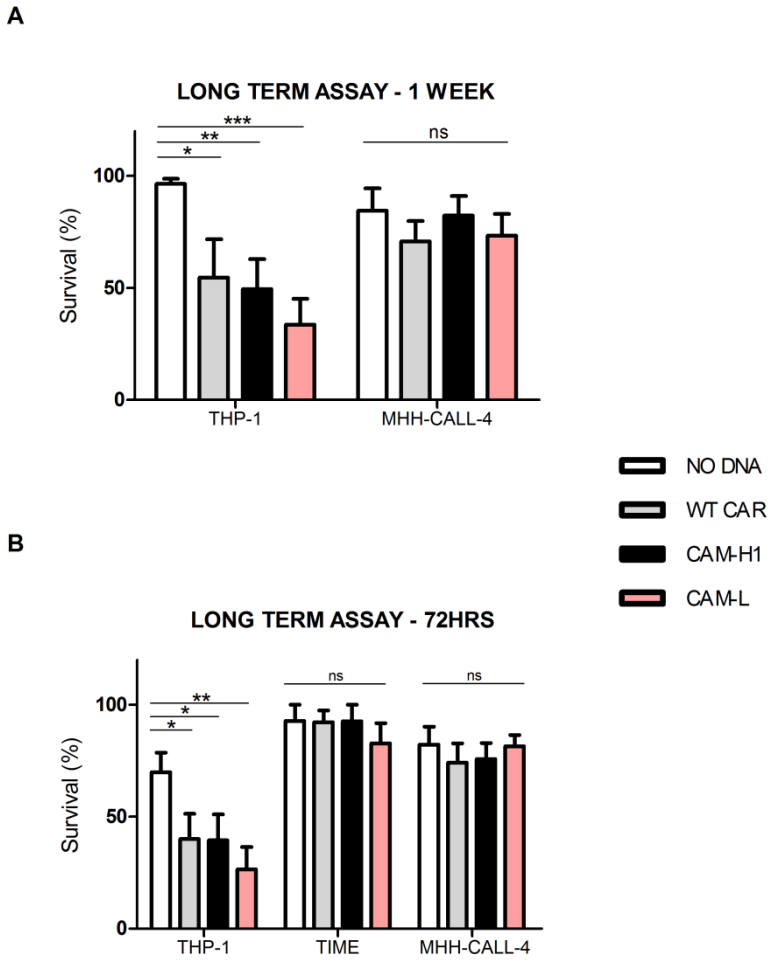

Fig. S3. CIK cells redirected with the low affinity CAM-L in vitro control the leukemic growth over time, while preserving its safety profile towards the healthy tissue. (**A** and **B**) Long-term cytotoxic assays performed with, wt CAR, CAM-H1 and CAM-L redirected CIK cells and NO DNA control for 1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

Chapter 3

Summary, Conclusions and Future perspectives

Summary, Conclusions and Future perspectives

Nowadays, the use of more intensive chemotherapy regimens, together with the indication for an allogeneic hematopoietic stem cell transplantation (HSCT) for high-risk acute myeloid leukemia (AML) patients, ensure high complete remission (CR) rates, but many patients eventually relapse or become refractory to the therapies (1), leading thus to the need of finding more effective therapeutic approaches.

In this context, adoptive immunotherapy with T cells engineered to express a Chimeric Antigen Receptor (CAR) targeting a specific tumour associated antigen (TAA) could represent a valid therapeutic alternative to be considered to increase the patient immune response against AML. CARs are synthetic receptors able to target sufarce antigens in their native conformation (2) and combine the antigen binding properties typical of antibodies with the T-cell effector functions. Therefore, both immune tolerance of the T-cell repertoire and MHC restriction are circumvented by T cells redirected with CARs, allowing the use of CARs in a broad range of patients.

The feasibility and the clinical impact of the CAR approach has been widely demonstrated in the preclinical setting, and more recently also in the clinical context, particularly in high risk relapsed and refractory patients with B cell malignancies (4) (5). Of note, after almost 30 years of preclinical careful decoding of the basic biology of the CAR approach, on July 2014, the United States Food and Drug Administration granted “breakthrough therapy” designation to anti-

CD19 CAR T-cell therapy, opening a new route of treating high-risk leukemia by redirecting the immune system against cancer (6) (6, 7). However, different drawbacks have emerged in the clinical translation of the CAR approach, such as unpleasant circumstances in which the occurrence of adverse events has not been offset by impressive anti-tumor activities. Serious side effects have been reported in literature, of which the worst cases led to death of the treated patients (10) (11), suggesting the need to further implement the CAR design in a more context-dependent manner. Indeed, being the adoptive cell therapy (ACT) with CAR engineered T cells an extremely powerful technology, a careful evaluation of potential 'on-target off-tumour' toxicities related to the expression of the targeted antigen also by healthy tissues is necessary. Hence, the target antigen choice is of primary importance. In particular, in the AML context, our group recently demonstrated the effectiveness and feasibility of a safer approach when employing T cells redirected with an anti-CD123 CAR, compared to an anti-CD33 CAR (12). CD123 is more differentially expressed between AML cells and Hematopoietic Stem/Progenitor cells (HSPCs) in comparison to CD33, allowing thus for a better discrimination between the two compartments. However, although CD123 represents a good target antigen because of its over-expression on LSCs, it is not immune from CAR T-cell recognition outside the leukemic context. Indeed, even if at low levels, CD123 is also expressed on other normal cell types, such as monocytes and endothelial cells. Although our preclinical results demonstrated a

moderated *in vitro* toxicity against endothelium and monocytes, envisaging a future clinical application of this targeting, a higher level of caution needs to be ultimately posed to avoid any potential 'on-target off-organ effect' *in vivo*.

This requirement is of particular importance, above all if considering that CAR T cells require a lower number of surface target molecules compared to the monoclonal antibodies (mAb) for their activation (13) (14).

Therefore, the preservation of the CAR anti-leukemic activity without leading to exacerbated toxic events would not be possible if not through a fine regulation of both its potency and specificity in the context of CAR-design.

Among the variables able to significantly affect the effector functions of CAR redirected T cells, the affinity of the CAR binding domain for its target antigen represents an aspect decisively worth to be investigated, being a feature not fully characterized and understood, enhancing the need to be further evaluated in a more context-dependent manner. For this reason, the aim of the present study was to *in vitro* characterize a panel of anti-CD123 CAR affinity mutants to further elucidate the mechanisms at the basis of CAR binding properties and CAR activation that are still poorly understood. Another key question was focused on unraveling how the CAR binding affinity tuning could influence the efficacy and the safety profiles of CAR redirected T cells, in terms of obtaining a good balance in specifically recognizing and killing the tumour cells, while

sparing the low Ag positive healthy cells. Our effector T-cell population has been represented by CIK cells, a really suitable cell source, particularly because of their intrinsic anti-tumor cytotoxicity, together with the advantages of being easily expandable *in vitro* and of showing a limited capacity of causing GVHD in an allogeneic setting (15).

The affinity mutants characterized in this study were identified through a computational docking analysis, which predicted single amino-acidic substitutions conceived in order to alter the binding strength and kinetics, without aletring the overall CAR design. The principle advantage of this approach relied in the choice of mutating the orginal scFv and not in considering another antibody clone, recognizing a different epitope binsing site, differentiating this study from the actual available literature. In this way, we could really investigate the real contribution of CAR binding affinity tuning in CAR T-cell functional properties.

In particular, two mutations led to a strong affinity reduction in comparison to anti-CD123 wt CAR. The reason at the basis of lowering CAR binding affinity came from evidences deriving from both TCR- and CAR-based approaches. Indeed, in order to accomplish a full T-cell activation, a larger dose of antigen is required for T cells expressing low affinity TCRs, compared to T cells with high affinity TCRs (16) (17). Moreover, unlike TCRs, in which T-cell activation and specificity is dictated by a narrow range of affinity, CAR T cells display a much greater affinity span and it has been

showed that antigen aspecificity can be encountered when increasing CAR binding affinity to extremely high levels (~10^{-12} M), even if CAR T-cell effector functions resulted not to be potentiated over ~10^{-8} M (18).

We found that killing of highly antigen positive target cells, reflecting the tumor conditions, is achieved even by lowering the CAR binding affinity by 100 folds, as in the case of our lowest affinity mutant (CAM-L). Even if it is important to bear in mind the specificity of the tumor context here evaluated, we showed that ~10^{-7} M corresponds to a CAR binding affinity sufficient to produce a complete CAR T-cell response, lowering the upper affinity threshold of ~10^{-8} M described by Chmielewski et al. as foundamental for CAR T-cell functions to be fully accomplished (18).

At the same time, the lowest affinity mutant showed decreased killing of low antigen positive cells, suggesting that its use might lead to a better safety profile.

Moreover, early cellular functions and activation of the lytic cell machinery were achieved even at very low antigen density on the target cells, leading to the identification of a “lytic threshold” around ~ 1600 CD123 moleucles/cell.

By contrast, CAR expression profile and target antigen density had a profound impact on later cellular functions, such as cytokine production and proliferation, which also turned out to be dependent on different target antigen densities for being respectively triggered. This seemed to arise from decreased levels of CAR internalization and

down-modulation when dealing with both low CAR and/or low target antigen densities, leading to a weak intracellular signaling intensity and related lower CAR T-cell potency.

Taken together, these results indicated that when designing CAR-redirected T cells within the specific tumor context, efforts should be better directed in the screening of optimal CAR expression profiles and not exclusively in the hunt for high affinity antibody-derived CARs. Efficient CAR expression, as well as sufficient antigen density, are also important requirements to achieve optimal later cellular responses. This feature has been also suggested by clinical evidences, in which both CAR expression at the cell surface and *in vivo* persistence of CAR T cells in the patients proved to be foundamental requirements to obtain durable anti-tumor responses (19).

Ongoing and future perspectives

When developing a CAR against a TAA, analysis of target antigen tumor expression, together with healthy tissue susceptibility of being killed by genetically modified T cells, could not be complete if not accompanied by an accurate *in vivo* characterization. Therefore, given the results obtained with CAM-L receptor, it will be interesting to test the hypothesis of CAM-L as responsible of a better balance between CAR T-cell efficacy towards tumor cells and safety against healthy tissues, through an *in vivo* model of NSG mice. However, the lack of NSG mice models to test the safety profile with respect to the endothelium, given the differences in the CD123 murine epitope

structure, compared to the human CD123 (20), limits the safety evaluation only towards HSPCs, which will be evaluated using $CD34^+$ CB (Cord blood) or bone marrow-derived cells.

Therefore, efforts are ongoing in order to reproduce a model of matrigel-embedded human endothelial cultured cells, subcutaneously implanted in NSG mice, for *in vivo* evaluation of endothelium-related toxicity by both wt CAR and CAM-L redirected CIK cells.

Moreover, in order to study hematopietic toxicities following CAR T-cell infusion, a more suitable model could be represented by NSG-S mice, bearing the advantage of developing human mature myeloid cells, such as monocytes and dendritic cells, also expressing CD123 (21).

Being another key aspect of CAR T-cell infusion in case of severe adverse events, in order to prevent long term persistence of redirected T cells, we will also evaluate the possibility of employing "suicide genes" to switch-off CAR^+ T cells for a personalized control of the CAR activity *in vivo*. In this sense, a suitable suicide gene is the iCasp9, shown to have a rapid mechanism of action upon administration of the AP1903 compound (22). To achieve in all the CAR^+ cells coexpression of both the CAR and the suicide gene, iCasp9 gene will be cloned in frame to anti-CD23 CAR, through the 2A-peptide (23), which ensures a stoichiometric production of the two proteins.

Another possibility will be also represented by the evaluation of an mRNA CAR-based delivery system, in order to time control CAR

expression on the surface of redirected CIK cells (24). These backup control systems, together with the lower CAR affinity variant selected, represent an additional safety level in case of potential occurrence of adverse events after infusion of CAR^+ CIK cells.

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PUBLICATIONS

Combining CD23 chimeric antigen receptor immunotherapy and lenalidomide as a novel therapeutic strategy for CLL

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Anti-CD123 CAR binding affinity and density balance in AML

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AML targeting by CARs: balancing efficacy and safety by novel strategies of gene engineering and CAR design

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