PhD PROGRAM in TRANSLATIONAL and MOLECULAR MEDICINE

DIMET

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Chimeric antigen receptors: a cell therapy based approach for the treatment of acute myeloid leukemia

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"It is the time you have wasted for your rose
that makes your rose so important"

(Antoine De Saint-Exupéry)

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Chapter 1

Introduction

HEMATOPOIETIC STEM CELLS

Most mature cells in normal blood are relatively short lived and need to be constantly replaced. The generation of a sufficient number of red and white cells is maintained via the homeostatic process of *hematopoiesis*, which yields approximately 10^{10} cells per day with the capability to dramatically increase this production in response to physiological stress. At the basis of this remarkable turnover is a small population of hematopoietic stem cells (HSCs) that have the ability to self-renew and differentiate into highly proliferative and specialized cells, making them able to sustain the hematopoiesis throughout the lifetime of an individual.

In the early 1960's, Till and McCulloch gave the first evidence and definition of a HSC, observing that bone marrow (BM) cells injected into irradiated mice gave rise to colonies of proliferating cells, termed spleen colony forming units (CFU-S), in the spleen of recipient animals. They demonstrated that each colony was derived from a single cell and these clonally derived colonies contained all the blood cell types at various stages of maturation [1, 2]. Furthermore, they demonstrated

that CFU-S had the capacity to self-renew by injecting dissected primary colonies in to secondary recipients [3].

While the work by Till and McCulloch laid the foundation of HSC research, the establishment of mouse xenotransplantation models and the development of FACS technology revolutionized the field of stem cell research, allowing the identification and purification of HSCs. Today we know that no single marker specifically identifies HSCs, but a high degree of enrichment can be achieved by combining several antigens: HSCs can be defined by the absence of lineage markers and CD38, and the expression of CD34, CD90 and CD49f (Lin^- $CD34^+CD38^-CD90^+CD49f^+$) [4-8].

HSCs can be divided into functionally different sub-fractions based on the presence of specific antigens expressed on their cell surface. Long-term HSCs (LT-HSCs) are able to self-renew and maintain repopulation potential through serial transplantations, while short-term HSCs (ST-HSCs) have a limited self-renewal ability that is lost over time.

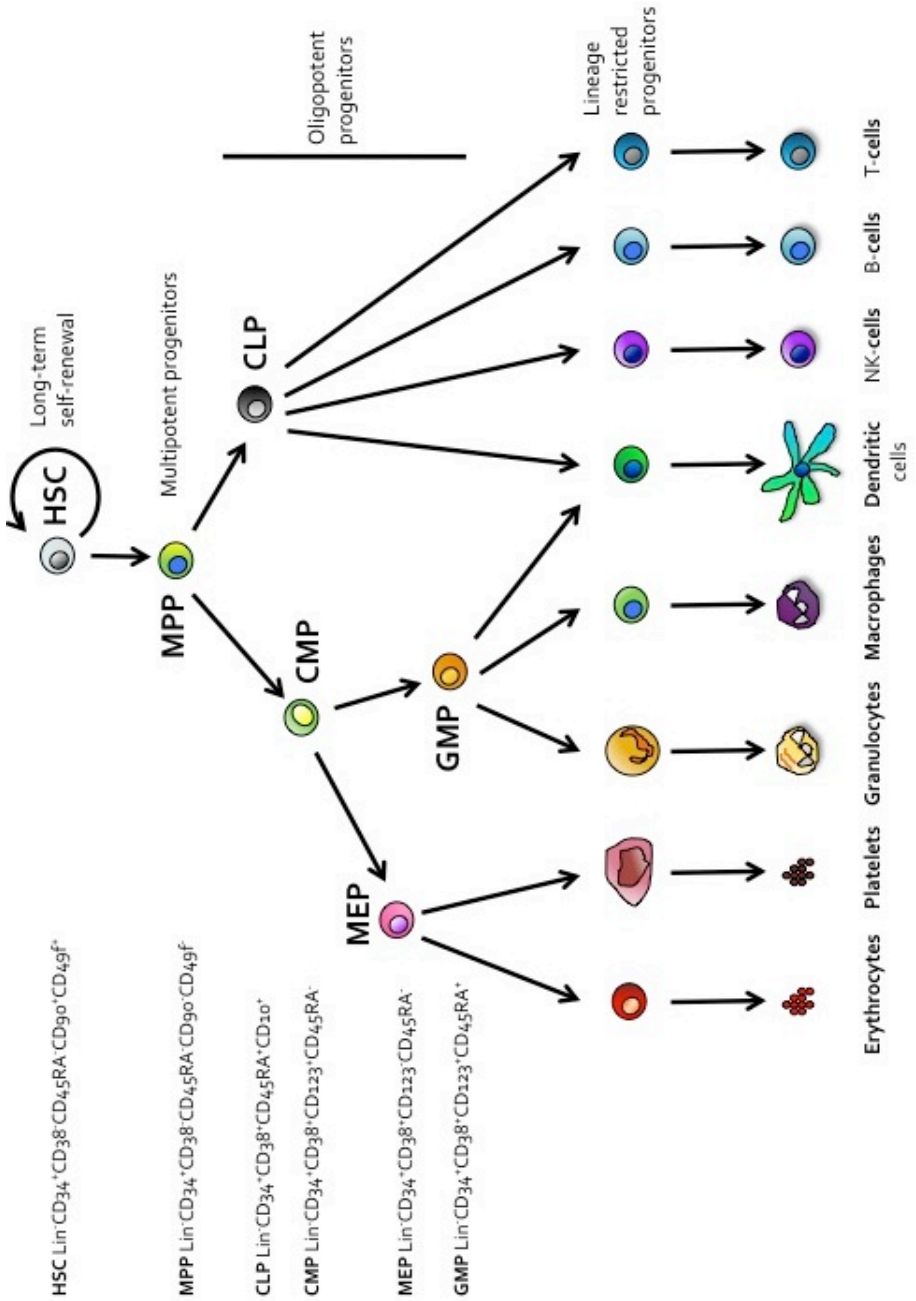

FIGURE 1: The hematopoietic system.

According to the classic accepted model, hematopoietic differentiation into the different lineages proceeds in a hierarchical manner (FIGURE 1). At the apex is the HSC compartment, which gives rise to multipotent progenitors (MMPs) with the potential to differentiate into oligopotent progenitors with a more restricted differentiation ability. At this level the first lineage branching point occurs between myelopoiesis and the lymphopoiesis, as supported by the identification of common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). The CLPs give rise to the effector cells of the system (dendritic, B-, T-, NK-cells), while the CMPs differentiate either into the granulocyte-monocyte progenitors (GMPs), which subsequently produce mature granulocytes (basophils, eosinophils and neutrophils) and monocyte/macrophages, or into the megakaryocyte-erythrocyte progenitors (MEPs), giving rise to platelets and red cells [9-11]. However, numerous recent studies have proven the existence of a developmental relationship between different lineages and a remarkable degree of plasticity in the early stages of maturation, suggesting hematopoietic differentiation is much more complex that initially proposed.

ACUTE MYELOID LEUKEMIA

Acute Myeloid Leukemia (AML) is a clonal disorder characterized by the aberrant accumulation of immature myeloid cells that are defective in their maturation and function in the bone marrow, subsequently interfering with the normal hematopoietic process.

The invasion of the bone marrow by leukemic cells can produce anemia resulting in pallor and fatigue, and thrombocytopenia, which can cause bleeding diathesis of the skin and/or mucosae. Moreover, patients can be highly susceptible to infections. The leukemic blasts can also invade the periosteal, often leading to sub-periosteal haemorrhages resulting in muscle and bone pain. The impairment of the central nervous system, due to the presence of blasts in the cerebrospinal fluid and/or leukemic infiltration in nervous tissue, may be silent, cause headaches, loss of balance and impaired vision.

AML accounts for 70% of all acute leukemia diagnoses, with an incidence of 3.5 new cases per 100,000 inhabitants per year in Europe and the United States. This disease is not very common in children where it accounts for 10-20% of pediatric leukemias.

Associations with environmental, socioeconomic, infectious and genetic events have been extensively studied, but the aetiology of AML is still unknown; it is likely that both genetic

and environmental factors play important and different roles in the development of the disease. However, patients with genetic syndromes such as Down syndrome, Fanconi anemia, Shwachman-Diamond and Blackfan-Diamond syndrome show a 10-20 fold increase in the risk of developing AML than the general population.

Similar to normal hematopoiesis, AML originates from a primitive cell residing at the top of a hierarchy. These primitive leukemic cells, defined as leukemic stem cells (LSC), are responsible for maintaining the disease. For the first time in 1997, using the NOD/SCID mouse model, Bonnet and Dick [12] identified human AML LSCs. They demonstrated that only the cells contained in the $CD34^{+}CD38^{-}$ subpopulation were able to initiate leukemia in recipient mice, using a small number of cells. In contrast, $CD34^{+}CD38^{+}$ or $CD34^{-}$ cells from primary human AML samples failed to engraft despite the transplanting more than 10^{6} cells. Furthermore, the $CD34^{+}CD38^{-}$ subpopulation was able to recapitulate the entire population seen in the original sample, while at the same time maintaining itself.

However, more recently the concept that all LSCs are contained within the $CD34^{+}CD38^{-}$ population has been challenged. It has now been shown that some clones of

antibodies against CD38 inhibit the engraftment of $CD34^+CD38^+$ leukemic cells in an Fc-dependent clearance by residual innate immunity in NOD/SCID mice [13]. By abrogating the effect of innate immunity, AML repopulating activity was detected in the $CD34^+CD38^+$ as well as the $CD34^+CD38^-$ population. Subsequent studies have specifically investigated AML with nucleophosmin mutations (NPM), and have determined that LSCs exist in both the $CD34^+$ and $CD34^-$ fractions [14].

Today we know that AML is a very heterogeneous disease and the identification of LSCs may vary between AML samples where the stem cell activity may reside in more than one population [15-17]. An additional consideration is that the LSCs phenotype may be not be stable over the course of the disease. Moreover, LSCs share several properties with normal HSCs, such as surface antigen phenotype, self-renewal capacity and quiescence, suggesting that they might arise from normal stem cells through the accumulation of oncogenic insults [18, 19]. In fact, clonal chromosomal abnormalities have been observed in the stem cell compartment of the large majority of AML cases [19-21]. More recently, a different model has been proposed suggesting that mature leukemia cells, that have undergone molecular transforming events, can de-differentiate and reacquire LSCs properties [22, 23]. Regardless of this continued

debate, it is clear that a rare population of leukemic cells can be isolated and is uniquely responsible for initiating and sustaining the disease in human-mouse xenografts. In addition, LSCs are quiescent cells characterized by an aberrant apoptotic response that might confer them resistance to standard chemotherapy [24, 25]. Hence, to improve cure rates of AML, it appears critical to design novel therapeutic strategies aimed at eliminating LSCs.

DIAGNOSTIC PROCEDURES and CLASSIFICATION

The diagnosis of AML is defined by the presence ≥ 20% of cells with the morphological characteristics of myeloid blasts in the bone marrow.

The study of membrane antigens and cytoplasmic markers expressed by leukemic cells constitutes an essential element in the confirmation of a morphological and cytochemical diagnosis. Of particular interest are a number of antigens, including CD34, CD38, CD13 and HLA-DR that are expressed on precursor cells; CD11b, CD14 and CD15 that are known monocytic markers; CD41 and CD61 that are typically expressed by megakaryoblasts; and glycophorin-A expressed on erythroblasts. Traditionally, AMLs were classified on the basis of the morphological and immunohistochemical features

of blast cells found in the bone marrow and in the peripheral blood of the patient. In 1976, the Cooperative Group Franco-American-British (FAB) developed the most comprehensive classification system of AML. According to the FAB scheme, AML can be divided into eight subtypes (M0-M7) based on the type of cells from which AML develops and the corresponding block in differentiation (TABLE 1).

TABLE 1

FAB Subtype	FREQUENCY	DESCRIPTION
M0	2-3%	Undifferentiated
M1	20%	Myeloblastic without maturation
M2	30-40%	Myeloblastic with maturation
M3	5-10%	Promyelocytic (APL)
M4	15%	Myelomonocytic
M5	10%	Monocytic
M6	5%	Erythroleukemia
M7	1%	Megakaryoblastic

Chromosomal abnormalities are present in approximately 55% of adult and in 70-80% of childhood AML cases making conventional cytogenetic analysis (structural and numerical) and molecular genetics mandatory components in the diagnosis of AML. Fusion proteins, proteins from cryptic translocations, and the loss of chromosomes can be detected using FISH. Additionally, several gene mutations and the aberrant expression of genes have been identified in AML, including those patients with a normal karyotype, allowing for further classification and prognostic stratification. The leukemic blasts affected by genetic abnormalities are categorized where class I mutations promote cell proliferation and survival (such as abnormities in tyrosine kinase FLT3) and class II mutations induce differentiation arrest (AML1, CEBP-alpha and WT1) and alter the mechanisms of apoptosis (TP53 and NPM1) [26] .

TABLE 2

PROGNOSIS	GENETICS
Favorable	- t(8;21)(q22;q22)/ RUNX1-RUNX1T1 - inv(16)(p13.1q22) - t(16;16)(p13.1;q22)/CBFB-MYH11 - t(15;17)(q22;q21)/PML-RARA - Molecular (in CN-AML) - NPM1-mutated AML - CEBPA double mutation -t(1;11)(q21;q23)/MLL-MLLT11 (AF1Q) - GATA1s
Intermediate	Cytogenetic abnormalities not classified as favourable or adverse
Adverse	- -7, -5 or del(5q) - inv(3)(q21;q26.2) or t(3;3)(q21;q26.2) - t(6;9)(p23;q34)/DEK-NUP214 - t(7;12)(q36;p13)ETV6-HLXB9 - t(4;11)(q21;q23)/MLL-MLL T2 (AF_4) - t(6;11)(q27;q23)/MLL-MLL T_4 (AF6) - t(10;11)(p12;q23)/MLL-MLL T10 (AF10) - t(9;22)(q34;q11.2) - complex karyotype - WT1 mut/FLT_3-ITD

PROGNOSTIC FACTORs

In AML, there are only a few clinical and biological parameters that are used as prognostic factors. These parameters are used in clinical practice to assign patients into prognostic groups: favourable, intermediate or adverse. The prognostic assignment subsequently determines the different treatment options that are likely to be pursued. This has the aim of maximizing the effectiveness of the treatment while reducing any side effects through varying the intensity of the treatment based of the probability of disease relapse [27].

Prognostic factors can be divided in different categories:

1. CYTOGENETICS and MOLECULAR GENETICS: The cytogenetics and molecular genetics of leukemic cells are the strongest prognostic factors for the patient's response to induction therapy. They are summarized in TABLE 2.

2. PATIENT RELATED FACTORS:

- *FAB classification*: M0, M5, M6, M7 are associated with a poor prognosis;
- *Age*: older patients have a worse prognosis;
- *Response to therapy*: response to the first and second course of treatment (for example, on day 15 and on day 28), and the achievement of a minimal residual disease (MRD) state are strongly predictive of a positive outcome;

- *Blast count at diagnosis*: hyperleukocytosis (WBC>100x10^9 cells/L) is associated with poor response to induction therapy.

TREATMENT of AML

Treatment of AML typically consists of two phases: induction and post-remission.

INDUCTION: Induction treatment aims to achieve complete remission (CR). CR for AML is defined as blasts count lower that 5% of the total bone marrow population, with recovery of normal hematopoiesis (TABLE 3).

Standard induction therapy comprises three days of anthracycline treatment and seven to ten days of cytarabine treatment. A third drug, such as 6-thioguanine or epipodophyllotoxins (VP-16), is commonly administered in combination. Today, as a result of the optimization of different induction protocols, CR is achieved in 80-90% of patients.

TABLE 3

CATEGORY	DEFINITION
Complete Remission (CR)	- Bone marrow blasts <5%; - Absence of blast with Auer rods; - Absence of extramedullary disease; - Absolute neutrophil count > $1x10^9$/L; - Platelet count > $80x10^9$/L; - Independence of red cell transfusions
CR with incomplete recovery	All CR criteria except for residual neutropenia or thrombocytopenia
Relapse	- Bone marrow blasts >5%; or reappearance of blast in the blood; or development of extramedullary disease;

POST-REMISSION STRATEGIES: The primary objective of post-remission therapies is to consolidate and maintain the CR. It can be realized by additional chemotherapy cycles (conventionally five to six) or repeated high-dose chemotherapy followed by allogeneic or autologous hematopoietic stem cell transplantation (HSCT).

In order to avoid pharmaco-resistance mechanisms, chemotherapy post-remission is based on a non-cross-resistant drugs combination consisting of high-dose cytarabine combined with anthracycline. Consolidation chemotherapy is given for approximately five to six months. The age of the patient and cytogenetic risk classification are important clinical parameters used to determine the intensity of this consolidation phase.

A therapeutic approach radically different from chemotherapy is represented by HSCT. HSCT, and in particular allogeneic HSCT, is considered a good alternative therapy in the consolidation phase once remission through induction treatment has been attained. The benefits of HSCT are attributable to both high dose chemotherapy (in the last years myeloablative chemotherapy is preferred to total body irradiation, still associated with more late side effects and an increased risk of secondary malignancies) and a potent graft versus leukemia (GvL) effect [28].

This approach is still controversial for patients with a good or intermediate risk prognosis who achieve CR after induction treatment. In this situation, the risk of AML relapse has to be balanced with the mortality rate associated with transplantation. However, allogeneic HSCT is a viable option in the treatment of high risk patients in first remission or patients who are not able to achieved CR after two cycles of induction treatment [29].

Allogenic transplants can be obtained from an HLA identical sibling donor or a matched unrelated donor. Unfortunately, due to the HLA allelic variation, such donors are not available for 40% of patients and alternative donors need to be considered. In recent years, Velardi and collaborators have shown that in the absence of a suitable donor, the infusion of high doses of T-cell depleted hematopoietic stem cells obtained from a haploidentical family donor (HLA compatible to 50%), would still constitute a valid therapeutic strategy for patients with a poor prognosis AML [30].

Due to improved treatment strategies, the prognosis of AML patients has greatly improved over the last 30 years; the probability of long-term survival has increased from 10% in the early 1970s to 60-70% using current therapeutic protocols. However, despite these advances, 5-10% of patients are

resistant to treatment, where they do not achieve CR after two cycles of induction therapy. Moreover, in ~30% of patients that do achieve an initial morphologic and cytogenetic CR following induction and consolidation therapies, the leukemia will usually recur within one year. In relapsed patients the prognosis is very poor, with a probability of survival between 15-30% and the treatment options remain unsatisfactory. The inability to induce CR of AML in a large percentage of patients together with the side effects and toxicity of the adopted regimes has created a strong interest in alternative therapeutic approaches that could be more effective and less toxic. Among the novel therapeutic approaches, immunotherapy has the potential to maintain AML remission once the bulk of the disease has been reduced with conventional chemotherapy.

IMMUNOTHERAPY with MONOCLONAL ANTIBODY

With respect to immunotherapy as an alternative or additional therapy in treating AML, particular attention has been paid to monoclonal antibodies directed against antigens expressed primarily or exclusively by leukemic cells. Approximately 90% of myeloblasts in patients with AML express the CD33 molecule [31, 32], an antigen that could serve as a potential target for the development of a selective therapy. CD33 is a 67 kDa transmembrane glycoprotein belonging to the sialic acid-binding immunoglobulin (Ig)-like lectins (Siglec; Siglec-3) superfamily involved in cell-cell interactions. It is abundantly expressed on hematopoietic cells, recognizing differentially linked terminal sialic acids on glycoproteins and glycolipids. CD33 is highly expressed on myeloid-committed cells in the bone marrow and on circulating monocytes, is progressively down-regulated on peripheral granulocytes and resident macrophages, and constitutively expressed on dendritic cells (DC). As previously mentioned, CD33 is widely expressed both by AML blasts and leukemic stem cells [31], wherein the level of CD33 expression of does not appear to be associated with a particular karyotype.

Gemtuzumab ozogamicin (GO) is a humanized IgG4 anti-CD33 antibody chemically linked to calicheamicin (FIGURE 2). Upon binding to CD33, the linked drug is rapidly internalized and, at the lysosomal level, calicheamicin is released from the antibody in a pH-dependent hydrolysis leading to the formation of the reactive drug. Calicheamicin binds at specific sequences in the minor groove of DNA where it induces the breakage of the double helix through an aromatization reaction [33]. This leads to the production of radicals responsible for the cleavage of the phosphodiesteric link, leading to cell death by apoptosis.

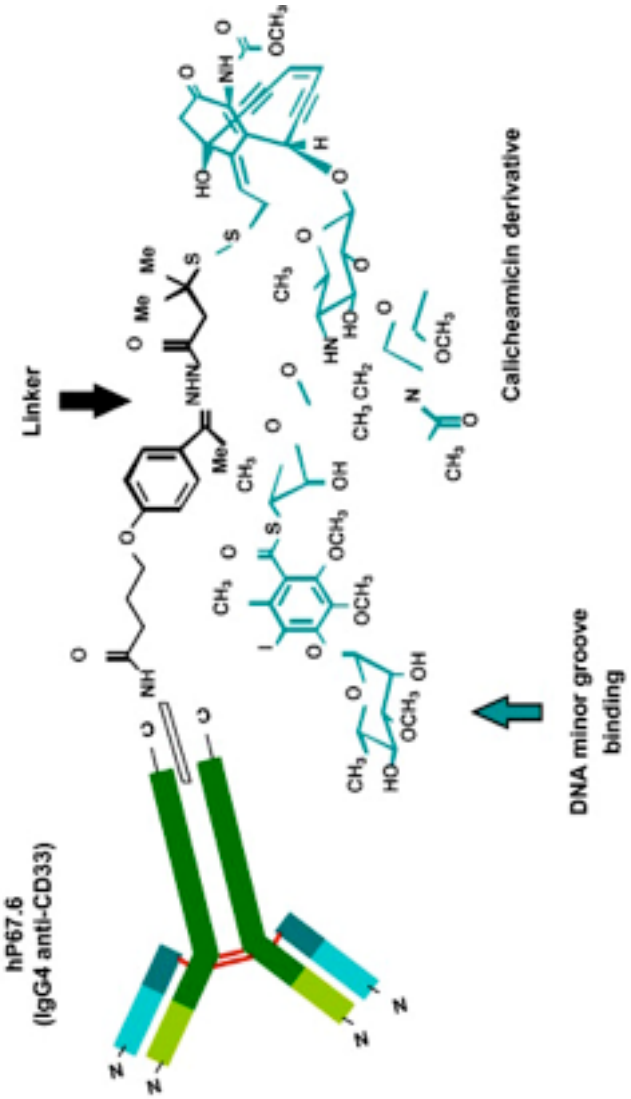

FIGURE 2: Schematic structure of *Gemtuzumab Ozogamicin* [33].

Based on results obtained from several non-randomized clinical studies conducted in the late 1990s, in May 2000 the Food and Drug Administration (FDA) approved the administration of GO as a single agent for the treatment of CD33-positive AML in relapsed patients over the age of 60 for whom intensive cytotoxic chemotherapy treatment was not suitable [34]. However, clinical trials since approval have not demonstrated any benefit from the addition of GO to induction or maintenance therapies; there has been no improvement in survival outcomes and an increased toxicity to the patients has been observed.

The most significant side effect induced by GO is myelosuppression due to the expression of CD33 by HSCP cells. Neutropenia, thrombocytopenia and anemia were observed in a very high percentage of treated subjects, with a recovery of platelet and neutrophil counts ~20 days after ceasing treatment. Outside of the hematopoietic system, a common adverse effect associated with GO is hepatotoxicity. This results in an initial transient and reversible increase in transaminases and bilirubin, which in some patients can degenerate into veno-occlusive disease (VOD) syndrome. The high frequency of hepatotoxicity may be due to the expression of CD33 on Kupffer and sinusoidal cells, the infiltration of leukemic blasts into the liver and the presence of not

conjugated calichemicin at the sinusoidal level. Moreover, it has been demonstrated that GO can be taken up and metabolized by human hepatocyte cells, despite being CD33 negative [35].

As a result of the clinical outcomes where no improvement in survival outcomes was seen and a high level of toxicity resulted in increased side effects, in June 2010, GO was voluntarily removed from the market and it is now only available as an investigational agent in clinical trials. Following this, in 2011, a phase III clinical study conducted in adults by the Medical Research Council, AML15, demonstrated no overall difference in the frequency of achieving CR between regimes with or without GO. However, this study did show a significant benefit in terms of disease-free survival in patients that were classified as intermediate risk.

All of the reports show that GO has limited activity as a single agent in front line therapies, with the exception of Minimal Residual Disease (MRD) eradication in acute promyelocytic leukemia (APL) as demonstrated by Breccia and colleagues [32]. In that study, all treated patients responded with durable molecular remission. However, other clinical trials are currently ongoing with the aim of identifying possible beneficial effects of GO in the different contexts of induction, consolidation or *in vivo* purging before HSCT. Now, the focus of immunotherapy

has shifted towards the optimization of new therapeutic approaches, such as cellular immunotherapy, that are able to combine target-specific recognition with a more physiological anti-tumor response which could potentially be more effective and selective with a better safety profile.

CELLULAR IMMUNOTHERAPY

Cellular immunotherapy is a therapeutic strategy that works by exploiting the functional properties of effector immune cells. It has been proposed that such a strategy can boost a specific and potent anti-tumor response capable of overcoming the mechanisms generally used by tumor cells to escape immune surveillance.

AML cells are known to avoid immune control by various means, particularly by inducing a poor and inefficient long-lasting activation of effector populations. Additionally, AML cells have a defective mechanism for processing and presenting antigens, resulting in the down-regulation or absence of expression of MHC molecules. Often, AML blasts will weakly express co-stimulatory molecules (CD80 and CD86) that can favour their escape from T-cell mediated killing. These molecules are also important in supporting the complete

activation of T lymphocytes, thereby preventing their anergy or clonal deletion. Moreover, an increased number of regulatory T-cells has been found in AML patients together with the constitutive expression of the tryptophan-depleting enzyme, indoleamine 2,3-dioxygenase (IDO). Leukemic cells can also inhibit the immune response by secreting immunosuppressive factors such as IL-10 and TGF-β, or overexpressing molecules that negatively regulate T- or NK-cell activation such as CTLA-4, PD1-L, FasL and KIR 2DL2. Finally, the microenvironment can protect leukemic cells. Stromal cells can provide an immunosuppressive milieu that can prevent effector cell infiltration (FIGURE 3).

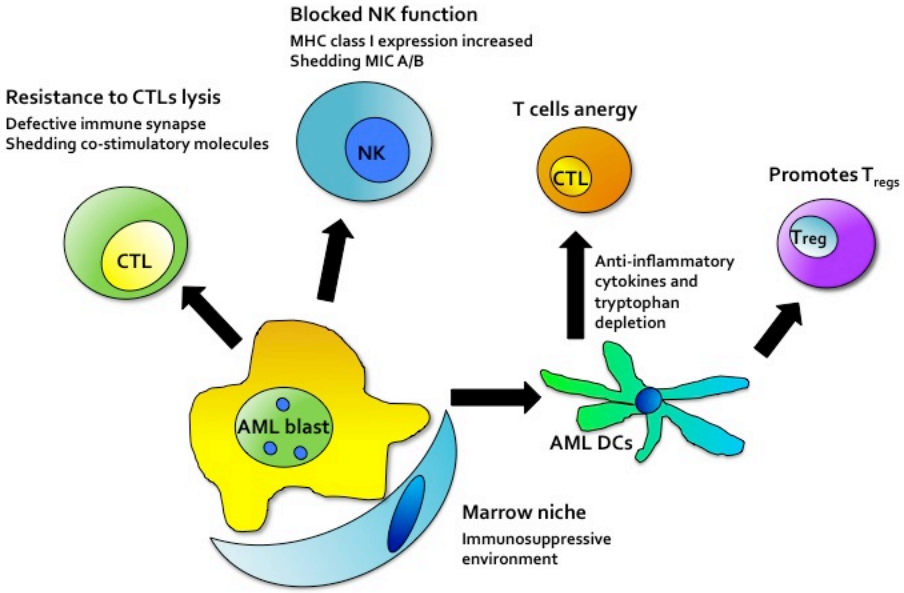

FIGURE 3: Tumor escaping mechanisms.

Understanding the mechanisms of tumor evasion together with recent advances in the techniques of manipulation of the immune system have led to the development of different approaches to cellular immunotherapy, including:

- *Active immunization* intended to restore the responsiveness of the immune system against tumor cells by "anti-cancer vaccines";
- *Passive immunization* through the infusion of ex-vivo expanded tumor specific effector T-cells (adoptive immunotherapy).

ACTIVE IMMUNIZATION: TUMOR VACCINEs

The aim of active immunization is the induction of an anti-tumor immune response after exposure to tumor-specific immunogenic antigens. Vaccination should be able to generate a long-lasting humoral and cell-mediated response, capable of controlling and eliminating any relapse of the disease. The production of an ideal anti-cancer vaccine consists of the generation of strongly immunogenic cellular products. One of the best-documented approaches to creating such vaccines involves the use of DCs.

DCs are the most effective antigen-presenting cells of the immune system. They circulate in the peripheral blood in

immature forms where they take up antigens by phagocytosis. They efficiently process the antigens, mature and subsequently migrate to secondary lymph nodes.

In the lymph nodes, they present the tumor antigen fragments via MHC molecules, priming naïve T-cells and inducing the activation and proliferation of antigen-specific Cytotoxic T-Lymphocytes (CTLs).

DCs represent a rare population in PB, accounting for approximately 0.1% of leucocytes. With such a low number of cells, different protocols have been developed for the *in vitro* generation of functional DCs. DCs can be easily generated *in vitro* starting from $CD14^+$ monocytes or $CD34^+$ progenitor cells derived from the patient's bone marrow or peripheral blood, and maturation can be obtained *ex vivo* with a cocktail of cytokines. Mature DCs can then be loaded with Tumor Associated Antigens (TAAs) expressed as a single peptide or as a fully processed protein. These are presented on the surface of DCs in the context of MHC class I and II molecules. DCs can then be genetically modified to express tumor specific antigens through the use of plasmid vectors, bacteria, or electroporation with mRNA.

Recently, electroporation has been used to efficiently pulse DCs with mRNA encoding WT-1, a TAA aberrantly overexpressed in the majority cases of AML. In a phase I/II

vaccination trial with AML patients that had achieved complete or partial remission after chemotherapy but remained at high risk for relapse, DCs have proven effective in stimulating a tumor-specific response [36]. DC vaccination was associated with the achievement of molecular remission in five out of 10 patients. Moreover, in two of these patients partial remission with morphological evidence of disease was converted into complete remission, demonstrating the clinical efficacy of this strategy.

When the identification of a specific TAA is not possible, DCs can be pulsed with apoptotic tumor cells or tumor lysates that allow the presentation of a broad spectrum of antigens, which are useful for inducing polyclonal lymphocyte activation.

A further mechanism developed to induce the presentation of tumor antigens by DCs consists in the generation of hybridomas derived from the fusion of dendritic and tumor cells. The product of this fusion combines the functional properties of DCs and the antigen expression of the phenotypic characteristics of tumor cells [37].

Finally, it has recently been discovered that AML blasts, when cultured in the presence of cytokines, can develop into immature DCs. These cells can then undergo maturation with one or two days in culture using different stimuli. A number of pilot clinical trials demonstrated that these AML-derived DCs

(AML-DC) express a wide range of patient-specific TAAs and possess the capacity to prime naïve $CD8^+$ cells into antigen specific CTLs.

Although such approaches may be able to generate an immunological response, it does not necessarily confer any significant advantage from a clinical point of view.

Due to the limitations observed with active immunotherapy approaches that are mostly attributable to serious deficits in the immune system that occur post HSCT, several researchers have now focused on passive immunization strategies.

ADOPTIVE IMMUNOTHERAPY

Adoptive immunotherapy involves the transfer of *ex vivo* expanded effector cells to generate effective anti-tumor responses.

DONOR LYMPHOCYTE INFUSION (DLI)

The demonstration that infusion of donor lymphocytes could eradicate the recurrence of leukemia after HSCT provides the first evidence of graft-versus-leukemia (GvL) effect [38].

Effector cells of the donor are in fact able, not only to reconstitute immunity protective, but can also recognize and eliminate neoplastic cells survived to the myeloablative regimen, bypassing cellular mechanisms of drug resistance.

One approach to enhance GvL response has been the donor lymphocyte infusion (DLI).

In 1990, Kolb demonstrated that in patients with chronic myeloid leukemia (CML), following a bone marrow transplant marrow, DLI could be used to generate an immune response against the tumor, resulting in a complete remission of the disease: almost 80% of patients with relapsed stable-phase CML after HSCT, get a complete hematological and cytogenetic response to unmanipulated DLI [16, 39].

Patients with other hematopoietic malignancies respond less to DLI. Response rate of 25-50% have been reported for relatively indolent diseases, such as multiple myeloma (MM), chronic lymphocyte leukemia (CLL) and myelodysplasia (MDS), while in acute lymphoblastic and myeloid leukemia (ALL and AML) clinical remission have been even less frequently using DLI as a single agent. In particular for AML a large retrospective study published in 2007 [17] estimated that survival at 2 years was superior in patients receiving DLI compared to patients who did not received DLI (21% vs 9%). Moreover subgroup analysis revealed that, if DLI was performed in patients with favorable cytogenetic and low bulk disease at the relapse, the overall survival after 2 years was 56%.

However, the results obtained in terms of clinical response are not yet fully satisfactory.

It is possible that the lower response rates reflect the rapid kinetics of leukemia cells growth and the relative slow response time to DLI, so patients die before the GvL reaction may have a clinical effect [16].

Resistance to therapeutic effect of DLI may occur due to the failure of complete activation of donor cells. Different studies have reported that stimulation of lymphocytes with IL-2 can

improve clinical responses. However non-specific stimulation may be not sufficient to initiate and sustain anti-tumor activity.

A possible approach to improving DLI responses has been to prime cells before infusion. Porter and colleague reported that infusion of *ex-vivo* activated DLI using anti-CD_3 anti-CD_{28} coated beads led to responses in patients with different haematological disorders where conventional DLI had been inadequate [40].

However, a serious limitation is the cross reactivity of donor lymphocytes with alloantigens, which may be associate to the development of GvHD, potentially responsible of the mortality associated with this procedure. GvHD still represents a major complication of this therapeutic strategy (GvHD develops in about 50-60 % of the patients receiving DLI [18]) and often limits its use, particularly when high cell doses are needed

These limitations have led to the development of new therapeutic strategies aimed to increase the efficacy and safety of the approaches of adoptive therapy, through the selection and manipulation *ex vivo* tumor-specific lymphocyte populations, equipped with anti-leukemic properties and reduced or absent reactivity towards normal tissues.

ANTI-LEUKEMIA CTLs

In recent years, several researchers have investigated the possibility of generating T-cells specifically directed against leukemic cells. This field of research has been supported by the identification of TAAs that can be used for pulsing DCs *in vitro*, priming tumor-specific cytotoxic T lymphocytes from PBMCs.

Montagna and colleagues recently demonstrated that in the absence of tumor-associated antigens, irradiated leukemic blasts could be used as a tumor antigen source [41]. Using this approach, the generated CTLs have a potent lytic activity against the leukemic blasts of the patient, with a reduced or absent reactivity towards non-leukemic cells. With this methodology it was possible to generate and expand CTLs *in vitro* that were specific for different hematological malignancies.

Despite encouraging results, many difficulties still hinder the possibility of using leukemia-specific CTLs in clinical practice. TAAs are frequently expressed in normal tissue and can be recognized as self-antigens, thereby limiting the response of CTLs because of mechanisms of tolerance, clonal deletion or anergy. In this case, high-avidity CTLs may be deleted during the tolerance process and the remaining isolated anti-leukemic CTLs may possess only low affinity TCRs and be less effective at tumor killing.

Alternately, if the TAA is not expressed on normal tissue, CTLs specific for the TAA are immunologically naïve and very rare. These factors limit the number of specific CTLs that can be isolated and the generation of an adequate number of cells for clinical practice then requires a long and complicated process of selection, characterization and expansion *in vitro*. This extensive manipulation results in a loss of functionality and reduced survival of T-cells *in vivo*. Another consideration is that such a long generation time is incompatible with the rapid progression of the disease, especially in cases of relapse post-transplant. Moreover, the inability to fully characterize the selected CTLs impedes further rational improvements of this methodology.

The current limitations of this strategy could be successfully overcome by the production of genetically modified T-cells with the aim of improving the functionalities *in vivo* and increasing the desired specificity against cancer cells.

GENETICALLY MODIFIED T-CELLs

A promising strategy to overcome the problem of generating a sufficient number of T-cells with specific anti-tumor-activity is to produce genetically modified T-cells.

The advantages of genetic modification are not limited to conferring new antigen reactivity to recipient T-cells, but can also be used to improve the efficacy and the persistence of transduced T-cells.

It is possible to insert genes encoding molecules involved in: co-stimulation; the prevention of apoptosis and senescence (Bcl-2, $BCl\text{-}_{XL}$, siRNA against Fas); autocrine growth signalling that enhances homeostatic proliferation (IL-7, IL-15 or IL-21); the stimulation of the immune system through cytokines (IL-2 or IL-12); or the promotion of T- cell homing towards specific tumor infiltrating sites (CXCR2, CCL5) through chemokine receptors [42].

The two the most common approaches focus on redirecting T-cell specificity using conventional T-cell receptors (TCRs) and chimeric antigen receptors (CARs).

T-cell receptors , TCRs, are composed of a heterodimer made up of an alpha and beta chain that recognize TAAs present on a cell surface MHC molecules and cloned into recipient T-cells. Redirected T-cells acquire the specificity of the original T-cell clone, can easily be expanded and are able to induce tumor lysis both *in vitro* and *in vivo*. This approach has already been widely applied in cases of melanoma [43] sarcoma [44] and leukemia [45, 46].

Morgan and colleagues demonstrated the first successful application of this strategy in 2006 [47]. Patients with advanced metastatic melanoma received T-cells modified with genes encoding the alpha and beta chains of the anti–MART-1 TCRs. Two out of 15 patients showed full, sustained (> 6 years) clinical regression of the disease after the transfer of genetically engineered T-cells. Gene modified lymphocytes demonstrated prolonged persistence, where one year after treatment both responding patients still had high detectable levels of engineered cells.

Despite this promising data, two main problems limit the use of TCR gene transfer in the clinic. The first concern is the possibility that the transferred chains can mispair with the endogenous TCR, a problem that, unless the two native TCR chains are selectively ablated, can occur when native and transgenic TCR chains are expressed in the same cell. Pairing of

a native chain with a transgenic chain can lead to decreased antigen recognition by the transgenic receptor and to off-target effects resulting in the recognition of unintended molecules or unexpected autoimmune reactions. Moreover, the effectiveness of this approach is limited by the fact that the cloned TCR recognizes only a single HLA, precluding its use for the treatment of patients with different MHCs and introducing the risk of tumor escape due to the well-known mechanism of MHC molecules being down-regulation on the surface of tumor cells.

To overcome these difficulties, researchers have been investigating alternative approaches to modify the specificity of T-cells using CARs.

CHIMERIC ANTIGEN RECEPTORS

(As general references for this paragraph see [48, 49]).

Chimeric Antigen Receptors (CARs) have recently emerged as a powerful and attractive tool to redirect the activity of immune cells towards specific molecular targets expressed on the cell surface of various tumors, rendering CAR-manipulated T-cells potent players in cancer adoptive immunotherapy.

CARs are artificial T-cell receptors generated by joining an extracellular antigen-binding domain to an intracellular-signalling region that is immediately triggered in response to recognition of its target.

CARs exploit the non Major Histocompatibility Complex (MHC)-restricted binding property of monoclonal antibodies with T-cell properties. This results in strong cytotoxicity, homing to and penetration of tumors, the release of cytokines that recruit additional components of the immune system, as well as an amplified and sustained anti-tumor response [50].

In a schematic way, the main structure of a CAR can be divided into three domains: the ectodomain, the transmembrane domain and the endodomain (FIGURE 4).

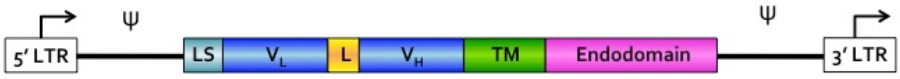

FIGURE 4: Schematic rapresentation of CAR molecules.

1) ECTODOMAIN: the ectodomain represents the extracellular portion of the CAR. It is generally composed of the following:
   - **Signal peptide**: directs the nascent protein into the endoplasmic reticulum. This is essential if the receptor needs to be glycosylated and anchored in the cell membrane leading to the expression of the CAR.
   - **Antigen recognition domain**: most commonly, it is derived from the antigen binding fragment (Fab) of a monoclonal antibody linking the variable regions of the heavy (V_H) and light (L_H) chains expressed as a single chain fragment variable (scFv) and joined together with a short linker. The linker is usually a serine-glycine rich motif, which allows the V_H to fold over the L_H into a native configuration with the flexibility that allows the molecule a high level of mobility. When the use of the scFv is not available or suitable, there are alternatives. The antigen recognition domains of CARs can be

generated from hormones or cytokine molecules to redirect specificity against their receptor, such as the IL-13Rα2-specific "zetakine"[51].

- **Spacer region (Hinge)**: joins together the antigen binding portion and the transmembrane domain. It should be flexible enough to facilitate the orientation of the CAR in different directions to allow optimal T-cell activation after antigen recognition [52]. The most common form is usually the CH_2-CH_3 region of immunoglobulins [53].

2) TRANSMEMBRANE DOMAIN: the transmembrane domain is a hydrophobic alpha helix that spans the membrane. Different transmembrane domains result in different receptor stability. For example, the CD28 transmembrane domain results in a more stable and highly expressed receptor [53].

3) ENDODOMAIN: the endodomain is the effector arm of the receptor and it is responsible for the transduction of the signal after antigen recognition. The most commonly used intracellular domain component is the zeta chain of the TCR/CD_3 complex, which by the phosphorylation of the

ITAM and recruitment of Src and Syc kinases, transmits an activation signal to the T-cell after antigen is bound. However, several studies demonstrated that these "first generation" CARs, containing solely the CD3-zeta chain were associated with limited clinical benefits where T-cells undergo rapid death after an initial proliferation (2-3 cells divisions). Tumors often down-regulate ligands for co-stimulatory molecules and the engagement of first-generation CARs leads to anergy with a very short persistence *in vivo* due to an ineffective or incomplete activation of the cells. The introduction of the intracytoplasmic portion of co-stimulatory molecules such as CD28, CD134, CD137 or ICOS into the endodomain prevents this potential anergy and apoptosis resulting from a single signal. These "second generation" CAR transduced T-cells are able to successfully reactivate and proliferate after a second exposure to the antigen. More recently, "third generation" CARs linking multiple signalling domains *in cis* have been shown to further improve T-cell effector functions, and due to the to higher levels of anti-apoptotic signalling elements (bcl-xL, pAKT), have an extended persistence *in vivo*.

Recently additional genetic approaches have developed to enhance the in vivo antitumor efficacy of CAR-modified T

cells. Specifically, the additional genetic modification of CAR-modified T cells to express proproliferative T cell–costimulatory ligands (4-1BBL) [38] or proinflammatory cytokines (IL-12) [40], resulting in "armored" fourth-generation CAR-modified T cells. These cells have shown enhanced *in vivo* antitumor efficacy in preclinical tumor models compared with T cells modified to express the tumor-targeted CAR alone [39].

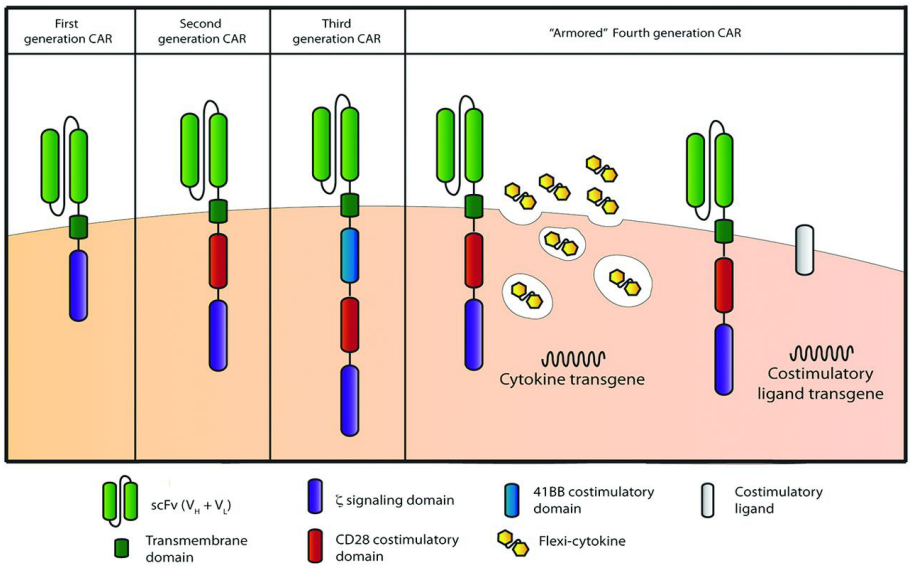

FIGURE 5: CARs evolution [39].

One of the main advantages of the CAR strategy is that it overcomes the major limitations associated with the use of "classical" TCR transgenic molecules. Target recognition by the CAR is non-MHC restricted and independent of antigen processing and presentation, therefore allowing for its use in patients with different haplotypes. This widens eligibility to a larger group of patients and bypasses tumor escape due to MHC-molecule down-regulation.

This important feature allows CAR-expressing cells to attack tumors that are poorly immunogenic.

CARs can also be targeted toward protein-derived peptides or non-protein molecules, such as carbohydrates and glycolipids. Additionally, there is no risk of unpredictable and potentially harmful aberrant recognition, as can happen with transduced TCRs when they form hybrids with the endogenous TCR. As CARs do not interact with either of the native TCR chains they are not subject to mispairing with complementary chains. Gene transfer introduces a counter measure to tumor immune evasion while also improving the survival of the engineered T-cells in a hostile tumor milieu by the addition of endogenous growth factors, co-stimulation signals or blocking T-cell inhibitory or pro-apoptotic pathways [50, 54, 55]

GENE TRANSFER METHODs

Today, the gold standard strategy in genetic modification of T-cells involves the use of gamma-retroviruses or lentiviruses that are engineered to encode the CAR molecule. Upon infection of the cell, the provirus is randomly integrated into the host, becoming part of the genome. Lentiviruses are capable of infecting quiescent cells, while retroviruses require the cells to be proliferating for successful integration. The viruses used are replication-defective due to the lack of key genes necessary to complete their life cycle through proliferation and the infection of other cells [56]. The main advantage of this viral approach is the very high efficiency of transduction that shortens the time of cell culture required to produce clinically significant number of cell. However, viral production under good manufacturing practise (GMP) is a very expensive method and requires highly specialized facilities. It also carries the risk of insertional mutagenesis and the possible occurrence of malignant transformation of the transduced cells, as well as the risk that replication competent viruses might be generated where viral genes could be immunogenic and thereby reduce cell survival.

Recent papers describe the use of transposon, such as *Sleeping Beauty* (SB) [57] or *PiggyBac* (PB) [58], as a way in which to stably introduce CARs into T-cells. Transposon-based gene

vectors consist of two components: the transposon, consisting of the transgene of interest flanked by inverted repeats containing short direct repeats (IR/DRs); and the transposase that recognizes and binds the IR/DR and catalyses transposition. During transposition, the transposase excises the target sequence from a plasmid and stably inserts it at a genomic target site, usually into TA dinucleotide sites.

On a genomic scale, transposase insertion profiles show no pronounced preference for insertion into transcriptional units or transcriptional regulatory regions of genes [59]. Even though chromosomal integration of SB transposons is precise, like other random integrating methods, it carries the potential risk of insertional mutagenesis occurring.

In order to avoid or limit potentially hazardous insertions and improve the safety profile of SB, the concomitant use of transposons with zinc-finger nuclease (ZFN) technology could guide SB transposon insertions to "safe harbors" in the human genome, as recently demonstrated by Voigt and colleagues [60]. Transposons provide a good level of transfection and reduced costs in comparison to viral approaches. Moreover, after electroporation the number of T-cells can be rapidly and specifically increased in a CAR-dependent manner by culture PBMC on irradiated artificial APC [61], and thereby avoiding

long periods of culture during which T-cells may became terminally differentiated and senescent.

Finally, as demonstrated in a recent report, T-cells can be genetically modified by mRNA electroporation [62] where CAR expression was detected up to a week post electroporation. However, as this method provides only a temporary expression of the transgenes, multiple injections are necessary to obtain anti-tumor effect *in vivo*. This is the first report regarding the feasibility of this approach, showing that optimized RNA electroporation could be a novel and cost-efficient method for adoptive cell transfer precluding long term toxicities and being able to increase the therapeutic index of modified T-cells for the treatment of cancer without the associated safety concerns of integrating gene vectors.

SAFETY MECHANISM

An important consideration for the clinical applicability of the CAR strategy is represented by the safety issues concerning the use of integrating vectors for gene transfer and their potential risk of uncontrolled proliferation of the infused T-cells, as well as the potential reactivity of CAR-transduced cells against normal host cells expressing the target antigen ("on-target but

off-organ" effect). These limitations can potentially be overcome by introducing a suicide gene that would serve as a back-up control, allowing the elimination of the genetically modified T-cells in cases of unexpected reactivity [63].

Several inducible suicide gene strategies, which can be encoded *in cis* with the CAR sequence, have been described so far. The most described approach has been based on the thymidine kinase gene of the Herpes simplex virus (HSV-TK). Expression of the HSV-TK gene by transduced T-cells renders them susceptible to gancyclovir (GCV) [64]. However, HSV-TK is highly immunogenic in humans. Other potentially less immunogenic strategies have been recently described, including the inducible Casp9 suicide system that is currently beingused in clinical trials. This system is based on a fusion protein constituted of human caspase-9 and a modified human FK506 binding protein (FKBP) that allows conditional dimerization triggered by a chemical inducer of dimerization (CID) [65], human CD20 that is targeted by the monoclonal antibody Rituximab [66], and a mutant human thymidylate kinase (mTMPK) that renders transduced cells susceptible to zidovudine (AZT) [67].

Our group recently demonstrated [68] that HSV-TK, iCasp9 and CD20 have equal levels of activity, but iCasp9 may be more suitable because it is highly effective in producing rapid

apoptosis (50% of the cells undergo to apoptosis in 30 minutes) even in non-dividing cells. This suggests a role for it in increasing the safety of CAR-modified T-cells.

CYTOKINE INDUCED KILLER CELLS

A fundamental step towards the success of CAR T-cell based immunotherapy largely depends on the cell population that is chosen as the anti-tumoral effector. The optimal effector T-cell population should be easy to expand and to transduce *in vitro*, should be able to migrate towards sites of tumor infiltration, and persist in a functional state *in vivo* thus providing specific and potent anti-tumor activity and negligible toxicity against other tissues. Different effector T-cell populations have been described in the literature as potential candidates for a CAR-mediated immunotherapy strategy; one such population is the Cytokine Induced Killer (CIK) cells.

CIK cells are heterogeneous immune-effector T-cells with an NK-T cell phenotype and function, enriched in the $CD3^+CD56^+$ subpopulation [69]. They represent a minor subset of total circulating lymphocytes (from 1% to 5% of peripheral blood mononuclear cells) that can be easily and rapidly expanded up to 1000 fold *in vitro* in 21 days of culture after activation with

IFN-g and OKT3, followed by repeated addition of high doses of IL-2 [70].

At the end of their expansion, CIK cells are a heterogeneous $CD3^+$population with two main subsets: the $CD3^+CD56^+$ and the $CD3^+CD56^-$ populations (FIGURE 6).

The $CD3^+CD56^+$ population is responsible for cytotoxic activity and the less potent $CD3^+CD56^-$ subset has the ability to proliferate and persist for longer periods, providing a continuous source of cells [71].

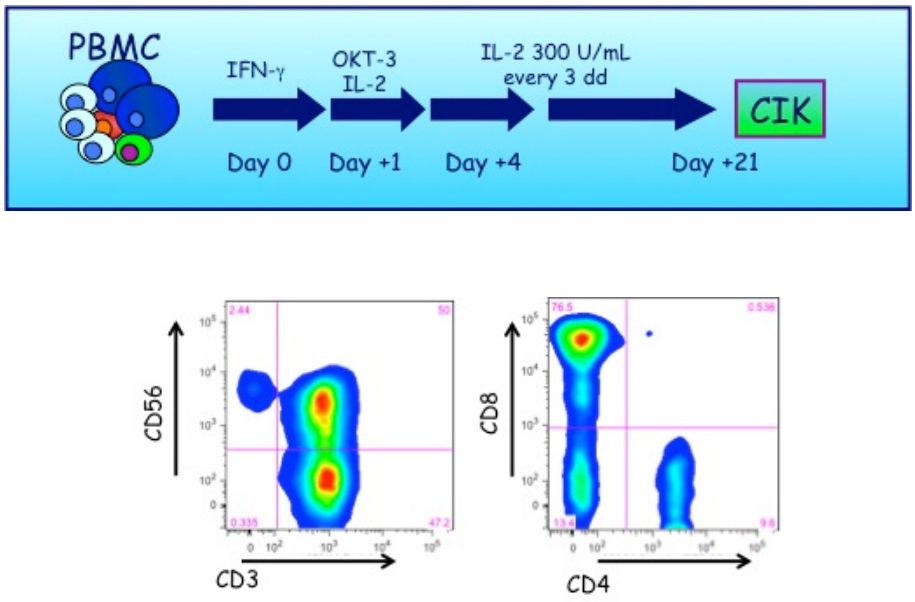

FIGURE 6: CIK cells generation and typically phenotype.

It has been reported that CIK cells are terminally-activated cytotoxic T-EMRA lymphocytes [72]. FACS analysis has demonstrated that CIK cells are a mixture of cells containing naïve ($CD62L^+CD45RA^+$), central memory ($CD62L^+CD45RA^-$), effector memory ($CD62L^-CD45RA^-$) and effector memory RA^+ (T-EMRA) ($CD62L^-CD45RA^+$) phenotypes.

CIK cells have been shown to have cytotoxic activity against several tumor cell targets, including acute and chronic myeloid leukemia, B- and T-cell lymphoma and renal cell carcinoma, metastatic renal cell carcinoma, both *in vitro* and *in vivo* although in a variable manner [71, 73-75]. The killing mechanism has been demonstrated to be MHC-independent and similar to natural killer (NK) cells, where immunogenic priming is not required for the observed cytotoxicity. On the contrary, CIK cells do not express CD16, so their effector functions do not rely on antibody-dependent cell cytotoxicity (ADCC).

The exact mechanisms involved in tumor recognition and killing by CIK cells are still not fully understood, but a large role seems to be played by the NKG2D receptor [76]. Its artificial inhibition with blocking antibodies or siRNA leads to a significant reduction of the anti-tumor cytotoxic activity of CIK cells and further experiments have confirmed that CIK-induced

cytolysis is exerted through NKG2D rather than TCR engagement [75, 77].

NKG2D activity is associated with up-regulation of the adaptor molecule DAP-10, which is induced by high doses of IL-2 present in the culture [76]. The main ligands recognized by NKG2D are the MHC class I-related molecules A and B (MIC A/B), members of ULBP (ULBPs 1,2 and 3) and stress-induced proteins expressed by a wide range of malignant tumor cells, regardless the original histotype [78, 79]. The final tumor lysis 39induced by CIK cells is mediated by a perforin/granzyme-dependent mechanism [76, 80].

As CIK cells express receptors for adhesion molecules and chemokine receptors, such as CD49d, CD11a, CXCR4 and CCR6 [81], they are potentially able to migrate towards the bone marrow, lymph nodes, spleen and other organs that represent the main sites of leukemic infiltration and proliferation.

In addition, CIK cells secrete different inflammatory cytokines, such as IFN-γ, IL-2 and TNF-α, [82] and once activated by Fas-ligand they are able to secrete different chemokines (CCL3, CCL4, and CCL5) that can amplify the anti-tumor response of Fas-Ligand positive tumor cells by recruiting into the tumor-rejection site various immune-effector cells implicated in the innate response, including granulocytes, monocytes and NK cells [83].

Different phase I studies have been performed with CIK cells where no toxicity was observed and a partial response was detected [70, 84, 85]. Moreover, compared to DLI or allogeneic splenocytes, CIK cells show a low propensity to induce both acute and chronic Graft versus Host Disease (GvHD) [86, 87], probably due their capability to secrete IFN-γ.

Moreover, compared to the classical lymphokine-activated killer (LAK) cells, once infused CIK cells do not require the exogenous administration of IL-2.

From the above observations, cultured CIK cell may represent an ideal population for adoptive immunotherapy.

Aim of the study

Despite the progress in the treatment of acute myeloid leukemia (AML) achieved in the last decades, a significant number of patients are still refractory to or relapse after standard cures. Hence, to improve cure rates of AML, it is crucial to develop novel therapeutic strategies.

Immunotherapy with T cells genetically modified to express chimeric antigen receptors (CARs) represent a valid option in this sense. CARs are artificial molecules constituted by an extracellular-antigen-binding domain derived from a monoclonal antibody and an intracellular-signalling region that is immediately triggered after antigen recognition.

Different CARs have been generated so far, against a wide range of surface molecules expressed by many tumors and, currently, several clinical trials are undergoing and the results obtained so far are very encouraging.

The CARs approach can be employed to selectively target AML cells due to the overexpression of myeloid antigens, like CD33 and CD123.

We recently demonstrated that expression of CD33-specific CARs in a population of ex-vivo activated T cells, called

"cytokine induced killer" (CIK) cells, confers them potent *in-vitro* anti-leukemic functions.

However, since CD33 antigen is also expressed on normal haematopoietic stem/progenitors cells (HSPCs) resulting in a potential severe impairment of normal myelopoiesis, CD123 has recently been proposed as a new potential attractive molecule based on its differential expression pattern, being widely overexpressed by AML population and at the same time less expressed on HSPCs.

In order to improve the safety profile against these cells we developed and tested a novel CAR specific for the CD123 antigen.

Here we describe the efficacy and safety of this approach based on CIK cells genetically modified to express CAR molecules specific for the CD33 or CD123 antigen.

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Chapter 2

Cytokine Induced Killer (CIK) cells for cell therapy of acute myeloid leukemia (AML): improvement of their immune activity by expression of CD33-specific chimeric receptors (CARs)

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ABSTRACT

CIK cells are *ex-vivo* expanded cells with potent antitumoral activity. CIK cells infusion in acute myeloid leukaemia patients relapsing after allogeneic hematopoietic stem cell transplant is well tolerated, but limited clinical responses were observed. To improve their effector functions against acute myeloid leukemia, we genetically modified CIK cells with CARs specific for the CD33 myeloid antigen.

SFG-retroviral vectors coding for anti-CD33-ζ and anti-CD33-CD28-OX40-ζ CARs were used to transduce CIK cells. Transduced cells were *in vitro* characterized for their ability to lyse leukemic targets (4- hours-^{51}Chromium-release and 6-days co-cultures assays on stromal mesenchymal cells), to proliferate (^{3}H-thymidine-incorporation assay) and secrete cytokines (Flow cytomix assay) after contact with acute myeloid leukemia cells. Their activity against normal $CD34^{+}$ hematopoietic progenitors was evaluated by analysing the colony-forming unit capacity after co-incubation.

CIK cells were efficiently transduced with the anti-CD33.CARs, maintaining their native phenotype and functions and acquiring potent cytotoxicity (up to 80% lysis after 4-hours) against different acute myeloid leukaemia targets, as also confirmed in long-term killing experiments. Moreover,

introduction of the anti-CD33.CARs was accompanied by a prominent CD33-specific proliferative activity, with a release of high levels of immunostimulatory cytokines. The presence of CD28-OX40 in CAR endodomain was associated with a significant amelioration of CIK cells anti-leukemic activity. Importantly, anti-CD33.CARs-transduced CIK cells showed a transient toxicity against normal hematopoietic $CD34^+$ progenitors.

Our results indicate that anti-CD33.CARs strongly enhance anti-leukemic CIK functions, suggesting that CIK cells transduced with these molecules might represent a promising optimized tool for acute myeloid leukemia immunotherapy.

INTRODUCTION

Acute myeloid leukemia (AML) is the most common form of leukemia in adults and accounts for 30% of leukaemia-related deaths in children [1]. Current chemotherapy regimens ensures long-term remission in only 30 to 50% of patients and prognosis of relapsed patients is very poor, with a low survival probability [2, 3]. Therefore it is envisaged to develop novel alternative approaches for refractory patients. Immunotherapy with T cells using unmanipulated donor lymphocyte infusion (DLI) for the treatment of leukaemia recurrence in hematopoietic stem cell transplant (HSCT) recipients, has had some success in AML, but the use of DLI carries a significant risk of inducing graft versus host disease (GvHD) [4]. Cytokine induced killer (CIK) cells are T cells enriched in $CD3^+CD56^+$ cells [5, 6], that can be easily and rapidly expanded *in vitro* from either human peripheral blood, bone marrow or cord blood mononuclear cells [7, 8] with the sequential addition of IFN-γ, OKT-3 and high-doses of IL-2 [9, 10]. It has been demonstrated that CIK cells can lyse a broad array of tumor targets in a non-MHC-restricted manner [5, 9, 11], have the capacity to migrate toward tumor sites [12, 13]and display anti-tumoral activity *in vivo* [9, 12]. On the contrary, they show negligible alloreactivity and minimal tendency to induce GvHD compared to allogeneic

splenocytes [14, 15]. The clinical applicability of CIK cells has been proved by various phase I studies performed so far [16-19], including our published experience, where we investigated the safety and toxicity profile of donor-derived CIK cells in patients relapsing after allogeneic HSCT. Our study clearly indicated that good manufacturing practice (GMP)-grade allogeneic CIK cell generation and subsequent infusion is feasible and well tolerated. However, we registered only limited clinical responses [19]. Several reasons could be taken into account, but one of the most relevant might be related to the limited basal anti-leukemic activity of CIK cells, that showed, in *in vitro* testing, only a mean lytic activity of 40% against patients leukemic cells with a wide donor-dependent variability [19].[19] Moreover, being CIK cells terminally differentiated T-EMRA lymphocytes [20], they might have a restricted survival *in vivo*.

Therefore, novel strategies need to be conceived to increase CIK cell efficacy and persistence after injection. Chimeric receptors (CARs) represent an innovative technology to redirect T-cell activity against tumors. We previously demonstrated the potency of the CAR approach in redirecting CIK cells against B-lineage acute lymphoblastic leukaemia (ALL) and we highlighted, as demonstrated by others [21, 22], the crucial role exerted by costimulatory molecules in the CAR

signalling domain, which significantly improves the anti-tumoral effector functions of CAR-expressing CIK cells. Furthermore, a recent study indicated that inclusion of a tripartite CD28-OX40-ζ cytoplasmic domain into a CAR lead to a considerable higher proliferation and cytokine release of *in vitro* activated T cells [23], than what observed with the CAR containing only one costimulatory domain. The so-called "third-generation" CARs might conceivably represent optimal constructs, as also recently shown in in vivo tumor models [24]. With this study we aimed at improving CIK cells activity against AML through their genetic modification with two different CARs specific for the CD33 myeloid antigen, containing respectively the ζ or the CD28-OX40-ζ signalling domain. Here we show that CIK cells expressing anti-CD33.CARs acquire more potent and consistent anti-leukemic effector functions against AML cells, when compared to unmanipulated cells, with a substantial improvement when the CD28-OX40 domain is present. Moreover, when we analyzed their safety profile against normal hematopoetic $CD34^{+}CD33^{+}$ progenitors, we observed that, although anti-CD33-CARs-transduced CIK cells exerted a significant cytotoxic activity, a reduced, but consistent number of clonogenic progenitors could be recovered in *in vitro* colony forming-assays, thus indicating a reversible toxic effect.

DESIGN AND METHODS

Cells

Bone marrow and peripheral blood cells were collected from children with AML at diagnosis. Flow cytometry analysis showed that, between 80% and 98% of the blasts expressed the CD_{33} antigen. All leukemia samples were cryopreserved and subsequently thawed for each experiment. The Institutional Review Board approved this study and informed consent was obtained from patients or their guardians. The human B-lineage ALL cell line (SUP-B15) was kindly provided by Dr. Claudia Rossig (University Children's Hospital, Muenster, Germany), the human acute myeloid cell lines HL-60 and KG-1 and the human chronic myelogenous leukemia cell line K562 were purchased from American Type Culture Collection (ATCC) and were maintained in RPMI-1640 supplemented with 10% FCS, L-glutamine and antibiotics (complete RPMI medium) (Lonza, Bergamo, Italy). The hTERT+ bone marrow derived mesenchymal cell line was kindly provided by Prof. Dario Campana (St. Jude Children Research Hospital, Memphis, USA) and was maintained in complete RPMI medium with the addition of 10-6 M hydrocortisone (Sigma Aldrich, Milano, Italy). The human renal epithelial cell line 293T was kindly provided by Dr. Martin Pule (University College of London,

London, UK) and it was maintained in DMEM highglucose (Lonza), supplemented with 10% FCS, L-glutamine and antibiotics.

Generation of CIK cells

CIK cells were prepared as previously described [13]. Briefly, peripheral blood mononuclear cells (PBMCs) of healthy subjects were obtained after centrifugation of fresh blood on a density gradient using Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden). Cells were then resuspended in complete advanced RPMI medium (Invitrogen, San Giuliano Milanese, Italy). At the beginning of the culture, IFN-γ (Dompè Biotec S.p.A, Milano, Italy) was added at 1000 U/ml. The next day, 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, and cells were kept at the initial concentration of 3x106 cells/ml. Cells were then cultured for 21 days. Fresh medium and IL-2 were added weekly during culture and cell concentration was maintained around 0.5x106 cells/ml.

FACS analysis

Aliquots of cells were analyzed for the expression of various surface markers using Fluorescein isothiocyanate (FITC)-anti-

CD8 (Exalpha Biologicals, USA), phycoerythrin (PE)-anti-CD4, (Exalpha Biologicals), Peridinin-chlorophyll-protein Complex (PerCP)-anti-CD3 (Becton Dickinson, BD, San Jose, CA), PE-anti-CD56 (IQ product, The Nederlands), FITC-anti-CD45RA (BD), PE-streptavidin—anti-mouse biotin-anti-CCR7 (BD) and anti-CXCR4 (BD), PE-anti-CD33 (Becton Dickinson), allophycocianin-anti-CD34 (Becton Dickinson) with isotype-matched antibodies (Becton Dickinson), as controls. CAR expression has been detected with a cyanine 5 (Cy5) anti-Fc-specific antibody (Jackson ImmunoResearch,West Grove, PA), as previously described [25]. A FACScan (Becton Dickinson) flow cytometer device was used to analyze the samples.

Chemotaxis assays and trans-Matrigel migration

Chemotactic migration assays was performed as previously described with 96-well Transwell insert (5-µm pore size; Corning Costar, Corning, Amsterdam, The Netherlands), adding 300 ng/ml of the chemokine CXCL12 (PeproTech, Rocky Hill, USA).

Migrated cells in bottom wells were enumerated after 1 hour by quantitative flow-cytometry analysis.

Results are expressed as the migration index of CIK cells in response to the chemokine versus the basal condition. Trans-Matrigel migration assay were performed as previously

described [26]. Migrated cells in bottom wells were enumerated after 3 hours by quantitative flow-cytometric analysis.

Plasmids, retrovirus production and retroviral transduction of CIK cells

The high-affinity, humanized rat anti-human CD33, 113, in single chain Fv (L-(gly4ser)4-H generated using UCB's Selected Lymphocyte Antibody Method (kindly provided by Dr. Helene Finney, UCB Celltech, Slough, UK), was cloned in frame with CH_2CH_3-CD28tm-z or CH_2CH_3-CD28tm-CD28-OX40-z in the SFG-retroviral construct (kindly provided by Dr. Martin Pule). The retroviral supernatant was produced by FuGENE 6 (Roche Diagnostic S.p.A., Italy)-mediated cotransfection of 293-T cells with the MoMLV *gag-pol* expression plasmid pEQ-PAM3(-E) (kindly provided by Dr. Martin Pule), the RD114 *env* expression plasmid pRDF (kindly provided by Dr. Yasu Takeuchi, Cancer Research Technology, London, UK) and the SFG-anti-CD33.CAR vectors. Supernatants containing retrovirus were harvested 48 hours and 72 hours after transfection, immediately frozen in dry ice, and stored at –80 °C until further use. 293T cells were used to titrate virus concentration. For transduction, $0.5x10^6$ CIK cells at day 5 of culture, were resuspended in 2.5 ml of thawed viral supernatant and seeded

on RetroNectin (TaKaRa BioEurope, Gennevilliers, France) coated 24-well non-tissue culture plates (Becton Dickinson). CIK cells were then spin infected in the presence of IL-2 (600 U/ml) at 1600rpm for 40 minutes and incubated for 72 hours in a humidified incubator at 37 °C, 5% CO_2.

Short-term cytotoxicity assay

The cytotoxicity of unmanipulated and anti-CD33.CARs-modified CIK cells against leukemic cells was evaluated as previously described [13] with a standard 4-hours ^{51}Cr-release cytotoxicity assay. Radioactivity was detected by a β-scintillation counter (PerkinElmer Life Science, Boston, MA), as counts per minutes (CPM) and the percentage of specific lysis was calculated as previously described [27]. Experiments were performed in triplicates.

Long-term cytotoxicity assay

The killing activity of unmanipulated and anti-CD33.CARs-modified CIK cells toward leukemic cells was also determined after 6 day co-cultures in duplicates at 1:100 and at 1:200 effector:target (E:T) ratios, without exogenous IL-2, on a human bone-marrow derived stromal mesenchymal cell layer, as previously described [21]. After 6 days, cells were harvested, passed through a 19-gauge needle to disrupt residual

mesenchymal-cell aggregates, stained with PE-anti-CD33 antibody and assayed by flow cytometry with a method specifically designed to enumerate leukemic cells recovered from culture, as previously described [21].

Cell proliferation assay

CD33-specific proliferation was evaluated by ^{3}H-thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) incorporation. At 15 days of culture, 1x10^{5} unmanipulated and anti-CD33.CARs-modified CIK cells were co-cultured for 72 hours with irradiated HL-60 or primary AML cells, in triplicates at 1:1 ratio, without the addition of IL-2. Counts per minute were measured in a β-scintillation counter (Beckman Coulter, LS6500 multipurpose scintillation counter, USA). Data are expressed as proliferation index (PI), calculated as the ratio of cpm stimulated/cpm unstimulated conditions. As a control, the same assay was performed using the CD33-negative SUP-B15 cell line.

Cytokine production detection

For cytokine production, 2x105 unmanipulated and anti-CD33.CARs-expressing CIK cells were stimulated with γ-irradiated HL-60 cells and primary AML cells at 1:5 E:T ratio for 24 hours. Levels of INF-γ, IL-2, TNF-α, TNF-β cytokines in

culture supernatants were determined with a Flow Cytomix assay (Multiplex Bender MedSystem, Wien, Austria).

Colony-Forming-Unit (CFU)-assay

CD34+ cells were purified from cord blood by immunomagnetic selection with anti-CD34 beads (Miltenyi Biotech, Bergisch Gladbach, Germany), incubated for 4-16-24-48 hours with unmanipulated or anti-CD33.CARs-transduced CIK cells at an E:T ratio of 10:1. Residual CD34+CD33+ progenitor cells were enumerated by flow cytometry. CD34+ cells were then plated at 5x102 cells/well in a methylcellulose-based medium (MethoCult H4434; StemCell Technologies Inc., Vancouver, Canada). After 14-21 days, colonies were counted as previously described [28].

Statistical analysis

The results were compared by using the paired Student *t* test. A p value ≤ 0.05 was considered to be significant.

RESULTS

Generation and characterization of anti-CD33.CAR-transduced CIK cells

Healthy donor (HD)-derived CIK cells were efficiently generated and transduced with the SFG-anti-CD33–ζ and SFG-anti-CD33-CD28-OX40-ζ retroviral vectors (Figure 1A), with a mean CAR expression of 64% (range, 47%-95%; n=20) and 65% (range, 41%-89%; n=20), respectively (Figure 1A). The transduction process did not alter CIK cells native phenotype and functions. Transduced CIK cells in fact, displayed a comparable phenotype to unmanipulated cells (Figure 1B), with a typical enrichment in the $CD3^+CD56^+$ population (mean % $CD3^+CD56^+$ cells at day 21 of culture, 42%, range, 21%-79%; n=20 for anti-CD33-ζ and 45%, range, 23%-78%; n=20 for anti-CD33-CD28-OX40-ζ expressing CIK cells vs 40%, range, 22%-73%; n=22 of untransduced CIK cells). Similar expansion rate was registered in anti-CD33.CARs-transduced and unmanipulated CIK cells, with a mean fold increase of 31 (range, 6-69; n=23), 25 (range, 6-52; n=22) and 24 (range, 6-89; n=26) respectively for anti-CD33-ζ, anti-CD33-CD28-OX40-ζ and untransduced cells (Figure 1C). Moreover, anti-CD33.CARs-transduced CIK cells showed similar or higher killing of the K562 cell line compared to non-transduced cells (Figure 2A)

with a mean lysis at effector:target -E:T- ratio of 5:1of 53% (range, 26%-74%; n=5) for anti-CD33-ζ and 58% (range, 48%-73%; n=5) for anti-CD33-CD28-OX40-ζ expressing CIK cells, respectively, compared to a mean lysis of 31% (range, 22%-54%; n=5; p≤0.05) of untransduced cells, which is likely related to the expression of the CD33 antigen on these cells. [29] Finally, analogous chemotactic activity and trans-matrigel migration in response to the CXCL12 chemokine, was observed, with a mean migration index (ratio of migrated cells in presence of CXCL12/migrated cells in presence of medium alone) of 4.3 (range, 2.1-8.1; n=5), 4.4 (range, 2.1-7.1; n=5) and 4.4 (range, 1.9-8.9; n=5) respectively for anti-CD33-ζ, anti-CD33-CD28-OX40-ζ and untransduced CIK cells (Figure 2B, upper panel), and a mean migration index trough Matrigel of 3.9 (range, 1.7-8.9; n=4), 3.8 (range, 1.8-8.3; n=4) and 3.7 (range, 1.7-8.1; n=4) respectively for anti-CD33-ζ, anti-CD33-CD28-OX40-ζ and untransduced CIK cells (Figure 2B, lower panel).

Figure 1. Transduction with the anti-CD33.CARs does not alter CIK cells native phenotype and in vitro expansion capability. (**A**) The expression of the anti-CD33.CARs on the surface of CIK cells was evaluated by flow cytometry with a Cy5-conjugated-anti human-Fc antibody after 21 days of culture. A representative experiment is shown. (**B**) The expression of anti-CD33.CARs (Fc+) and of CD_3 along with CD56, CD8, CD_4 and $CXCR_4$ on the surface of CIK cells and their memory phenotype (evaluated with CCR_7/$CD_{45}RA$ staining) was evaluated after 21 days of culture by flow cytometry. Data shown are mean ± SD of 20 separate experiments. (**C**) Expansion of CIK cells was calculated and expressed as the fold increase in cell number at 14 days after transduction versus the day of transduction. Data shown are mean ± SD of 20 separate experiments.

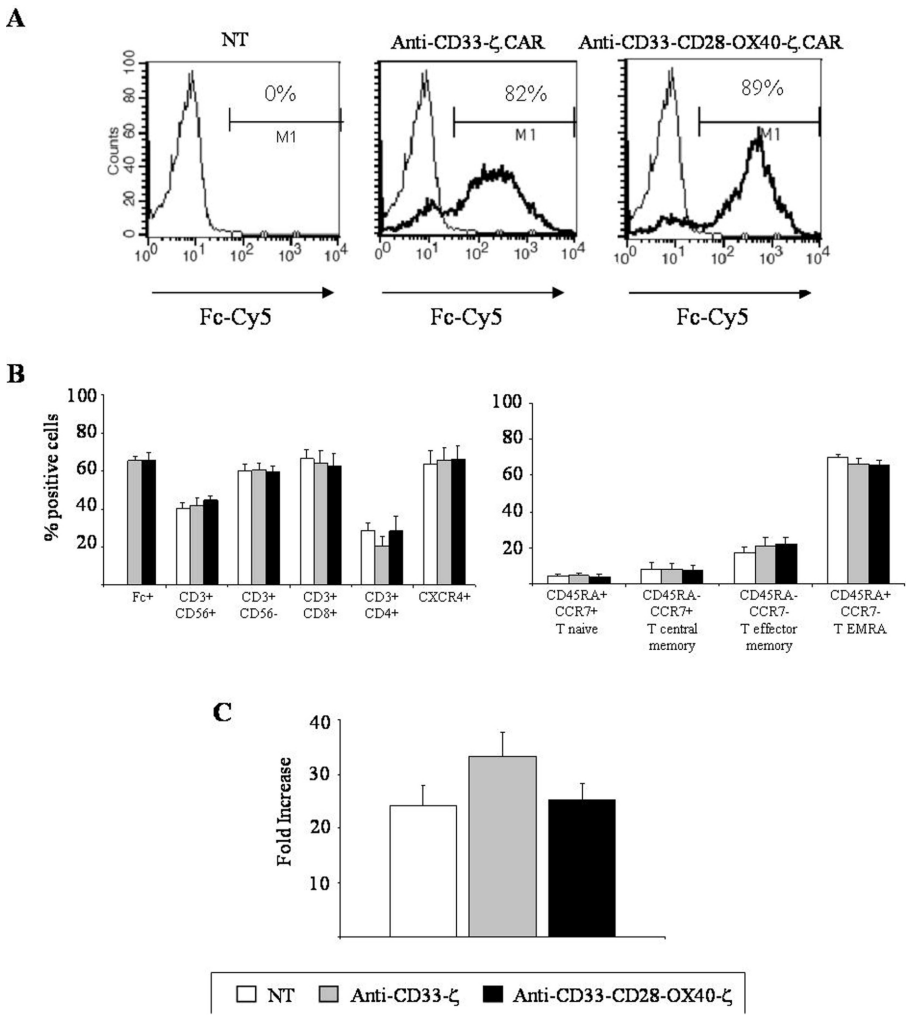
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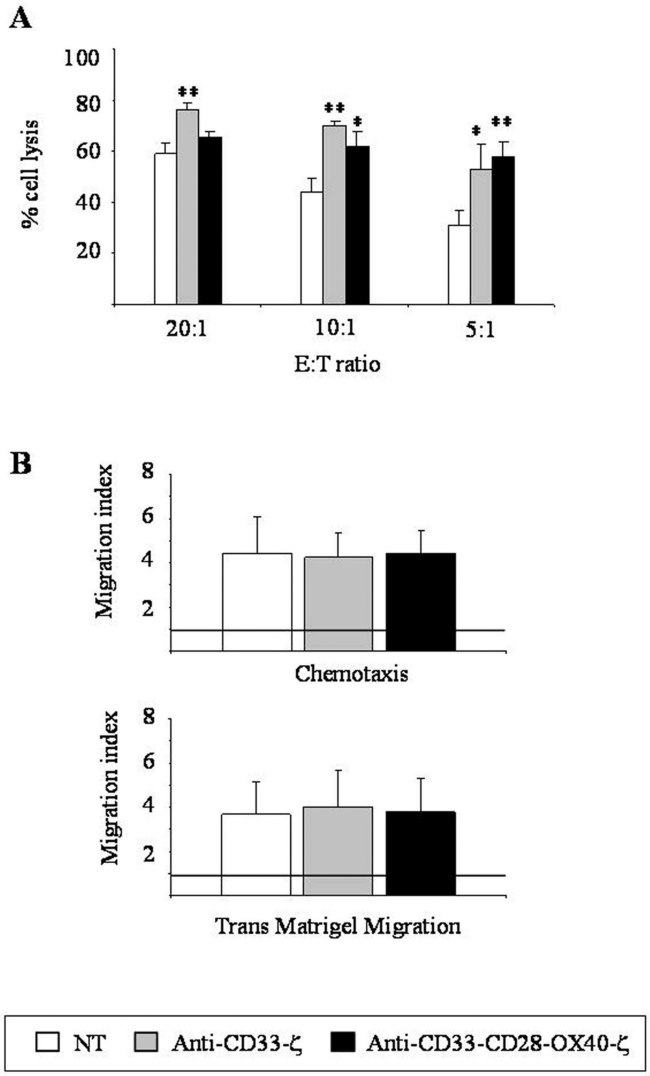
Figure 2. Transduction with the anti-CD33.CARs does not alter native CIK cells functions. (A) Reactivity of CIK cells against the control K562 cell line cells was determined after 21 days of culture with a standard 4 hours ^{51}Cr-release assay. Data shown are mean ± SD of 5 separate experiments. Anti-CD33.CARs-transduced CIK cells displayed similar or higher killing of the K562 cell line compared to unmanipulated cells, consistently with the reported expression of CD33 on this cell line (**p≤0.005 and *p≤0.005 *versus* unmanipulated CIK cells). **(B)** The migratory activity of CIK cells in response to the CXCL12 chemokine was determined by an *in vitro* chemotactic assay either in the absence (*upper panel*) and in the presence of reconstituted basement membrane (Matrigel) (*lower panel*) (4 donors were evaluated). The horizontal line at the migration index 1.0 indicates lack of chemotaxis.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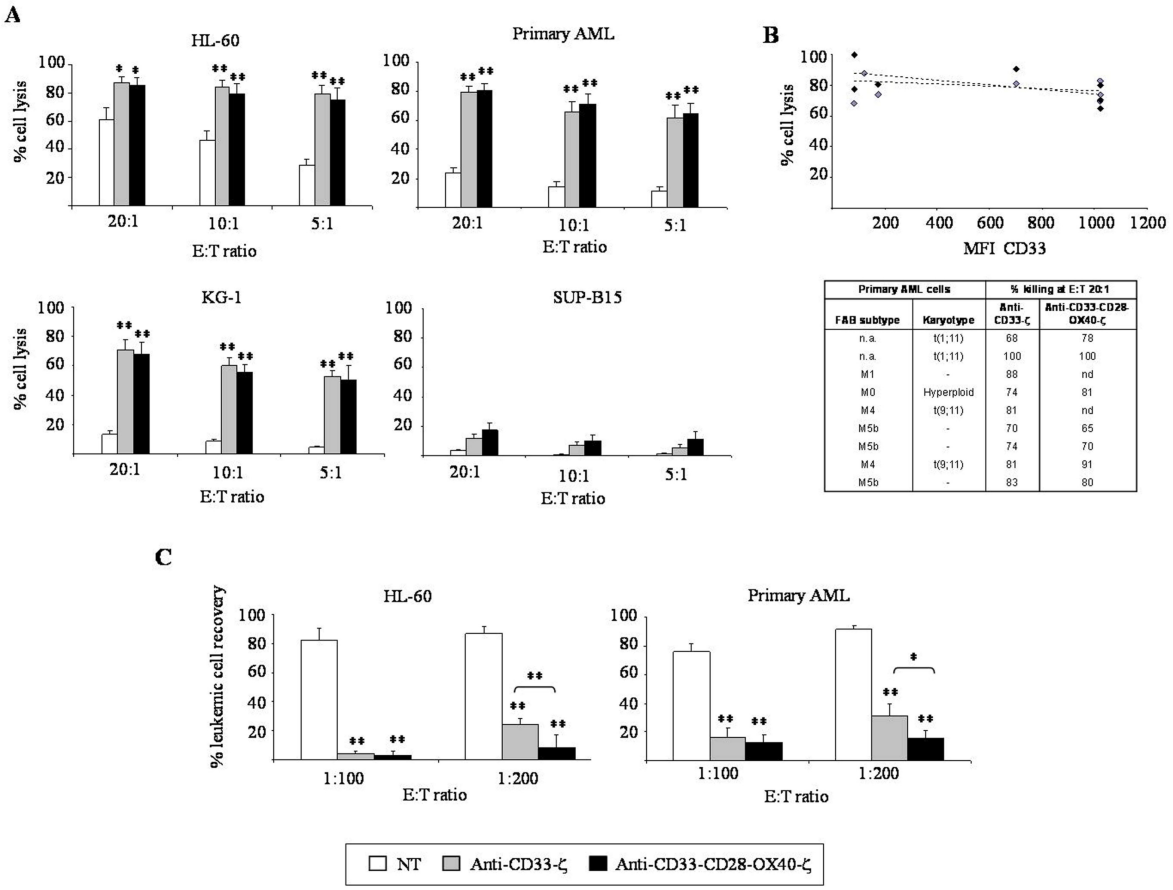
Anti-CD33.CAR-redirected CIK cells acquire potent lytic activity against different AML targets.

Anti-CD33.CARs redirected CIK cells showed a strong cytotoxic activity in classical 4-hours ^{51}Chromium-release assays (short-term cytotoxicity) against different $CD33^{+}$ targets, including the HL-60 cell line (mean lysis, 79%, range, 58%-100%; n=7, for anti-CD33-ζ and mean lysis, 75%, range, 50%-100%; n=7, at E:T ratio of 5:1 for anti-CD33-CD28-OX40-ζ CIK cells), the KG-1 cell line (mean lysis, 53%, range, 43%-63%; n=4 for anti-CD33-ζ and mean lysis, 50%; range, 40%-70%; n=4, at E:T ratio of 5:1 for anti-CD33-CD28-OX40-ζ CIK cells) and primary AML samples (mean lysis, 61%, range, 17%-100%; n=8 and mean lysis, 65%, range, 37%-100%; n=8, at E:T ratio of 5:1 for anti-CD33-CD28-OX40-ζ CIK cells) (Figure 3A). Cytotoxicity of anti-CD33.CARs-transduced CIK cells against AML cells was not dependent on CD33 expression levels and on the sybtype of AML, with up to 100% of lysis even in case of CD33-low expressing AML cells (Fig.3B). On the contrary, untransduced cells were minimally cytotoxic against the same targets (mean lysis, respectively, of HL-60, 28%, range, 17%-43%; n=7; $p \leq 0.005$; mean lysis of KG-1 cell line, 5%, range, 3%-8%; n=4; $p \leq 0.005$ and mean lysis of primary AML, 11%, range, 2%-23%; n=8 at E:T ratio of 5:1; $p \leq 0.005$) (Figure 3A). The killing activity of transduced cells was specific for CD33-positive targets, as

demonstrated by the negligible levels of cytotoxicity of anti-CD33.CARs-transduced CIK cells against the $CD19^{+}/CD33^{-}$ cell line SUP-B15 (Figure 3A).

The capacity of anti-CD33.CARs-transduced CIK cells to kill leukemic cells over time was then evaluated, in co-culture experiments on mesenchymal stromal cells, at low E:T ratios (1:200), in the absence of exogenous IL-2. The presence of the CD28-OX40 costimulatory moiety in the anti-CD33.CAR was able to potently boost the cytotoxicity of CIK cells, to levels significantly higher than what observed with anti-CD33-ζ CIK cells, that anyway displayed, in these assays, a strong lytic activity (Figure 3C). In fact, almost all leukemic blasts were eliminated from anti-CD33-CD28-OX40-ζ-transduced CIK cells, with a mean leukemic cell recovery of 4% (range, 0.1%-8%; n=3) in the case of co-culture with HL-60 cells and of 16% (range, 1%-36%; n=7) in the case of co-culture with primary AML blasts, toward respectively, a mean lysis of 87% (range, 82%-92%; n=3; $p \leq 0.005$) and 91% (range, 83%-100%; n=7; $p \leq 0.005$) for unmanipulated CIK cells and of 25% (range, 19%-32%; n=3; $p \leq 0.005$) and 31% (range, 1%-60%; n=7; $p \leq 0.05$) for anti-CD33-ζ-transduced CIK cells.

Figure 3. CIK cells expressing the anti-CD33.CARs acquire potent cytotoxic activity against AML. (A) Short-term cytotoxicity of CIK cells was evaluated by a standard 4 hours ^{51}Cr release assay after 21 days of culture at effector:target ratio of 20:1, 10:1 and 5:1. Anti-CD33.CARs-transduced CIK cells showed a consistent cytolytic activity against the $CD33^+$ targets HL-60, KG-1, and primary AML compared to unmanipulated cells (*p≤0.05;**p≤0.005), whether negligible killing, similar to those exerted by untransduced cells, was registered against the $CD33^-$ cell line SUP-B15. Data shown are mean ± SD of 7, 4, 8 and 4 separate experiments respectively. **(B)** Correlation of CD33 expression levels on primary AML cells (evaluated as mean fluorescent intensity -MFI-) and cytotoxicity of anti-CD33.CARs-transduced CIK cells at effector:target ratio of 20:1 (*upper panel*) and lytic efficiency (expressed as % cell lysis at effector:target ratio of 20:1) of anti-CD33.CARs-redirected CIK cells against different AML subtypes (*lower panel*) **(C)** CIK cells were co-cultured with HL-60 or primary AML cells at effector:target ratio of 1:100 and 1:200 for 6 days on human stromal cell layer in the absence of IL-2. Leukemic cell recovery was evaluated by flow cytometry (*p≤0.05;**p≤0.005). Data shown are mean ± SD of 3 and 7 independent experiments, respectively.



Primary AML cells		% killing at E:T 20:1	
FAB subtype	Karyotype	Anti-CD33-ζ	Anti-CD33-CD28-OX40-ζ
n.a.	t(1;11)	68	78
n.a.	t(1;11)	100	100
M1	-	88	nd
M0	Hyperploid	74	81
M4	t(9;11)	81	nd
M5b	-	70	65
M5b	-	74	70
M4	t(9;11)	81	91
M5b	-	83	80

Anti-CD33.CAR-redirected CIK cells proliferate and release immunostimulatory cytokines after specific CD33-stimulation, with a more pronounced effect in the presence of CD28-OX40 domain

The ability of anti-CD33.CARs-transduced CIK cells to expand *in vitro* after CD33 engagement was measured after 4 days co-culture with either irradiated HL-60 and primary leukemic cells, in the absence of exogenous IL-2, by a ^{3}H-thymidine incorporation-assay. As outlined in figure 4, the anti-CD33.CAR containing just the ζ-chain confers a significant CD33-specific proliferative activity to transduced CIK cells, with a mean proliferation index -calculated as the ratio of stimulated/unstimulated cell proliferation- of 2.2 (range, 1.4-4.1; n=13) after HL-60-mediated stimulation and 2.4 (range, 0.9-7.2; n=11) after primary AML cells-mediated stimulation, compared to 0.9 (range, 0.1-1.7; n=13; p≤0.005;) and 1.4 (range, 0.6-2.7; n=11; p≤0.05), respectively, for untransduced CIK cells. The introduction of the CD28-OX40 domain in the CAR intracellular signalling region resulted in an even higher expansion rate, with a mean proliferation index of 4.4 (range, 2.5-7.1; n=13; p≤0.005) after HL-60-mediated stimulation and 3.7 (range, 1.3-8.9; n=11; p≤0.005) after primary AML cells-mediated stimulation. No proliferation was registered when

cells were co-cultured with the $CD19^{+}$-$CD33^{-}$ SUP-B15 cell line (data not shown).

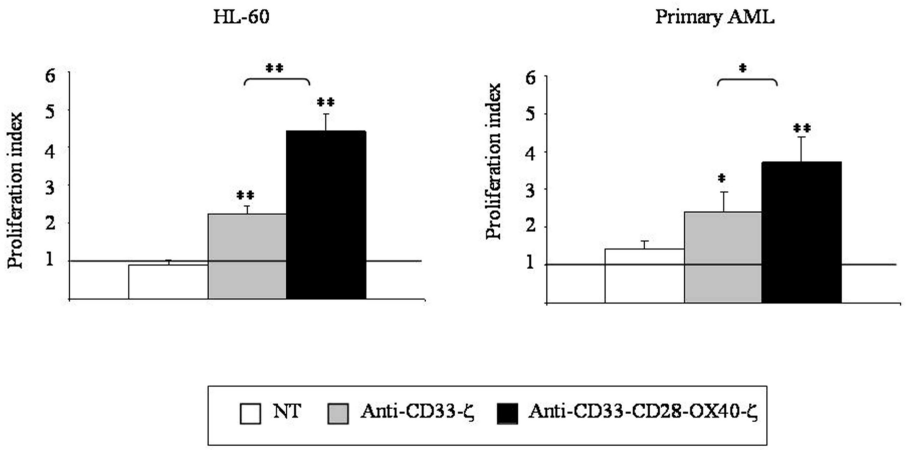


Figure 4. Anti-CD33.CARs expression on CIK cells confers them a CD33-specific proliferation activity. CIK cells were stimulated with irradiated HL-60 or primary AML cells at effector:target ratio of 1:1 for 72 hours and proliferation was estimated by ^{3}H-thymidine incorporation. Data shown are mean ± SD of 13 and 11 independent experiments, respectively (*p≤0.05; **p≤0.005).

Expression of the anti-CD33.CARs on CIK cells resulted in a consistent improvement in the release of a panel of immunostimulatory cytokines, including IFN-γ, TNF-α, TNF-β and IL-2 after 24-h stimulation with irradiated HL-60 and primary AML at 1:5 E:T ratio. HL-60 and primary AML cells alone did not produced TNF-β, and released only low levels of IFN-γ, TNF-α, and IL-2 (data not shown), which were subtracted from those measured in CIK-stimulated cultures. After HL-60-mediated stimulation, anti-CD33-ζ and anti-CD33-CD28-OX40-ζ CAR-transduced CIK cells secreted 11-fold and 10-fold more amount of IFN-γ (mean release, 8236pg/ml, range, 6367pg/ml-11165pg/ml; n=6; p≤0.005 for anti-CD33-ζ and mean release, 7040pg/ml, range, 5523pg/ml-8921 pg/ml; n=6; p≤0.005 for anti-CD33-CD28-OX40-ζ respectively), 120-fold and 180-fold more TNF-α (mean release, 11126pg/ml, range, 4079pg/ml-15283pg/ml; n=6; p≤0.005 for anti-CD33-ζ and mean release, 16622pg/ml, range, 12470pg/ml-24000pg/ml; n=6; p≤0.005 for anti-CD33-CD28-OX40-ζ respectively), 250-fold and 600-fold more TNF-β (mean release, 243pg/ml, range, 25pg/ml-498pg/ml; n=6; p≤0.005 for anti-CD33-ζ and mean release, 624pg/ml, range, 500pg/ml-778pg/ml; n=6;p≤0.005 for anti-CD33-CD28-OX40-ζ respectively), 1400-fold and 3800-fold more IL-2 (mean

release, 1481pg/ml, range, 390pg/ml -3923pg/ml; n=6; p≤0.05 for anti-CD33-ζ and mean release, 3855pg/ml, range, 273pg/ml-10323pg/ml; n=6; p≤0.05 for anti-CD33-CD28-OX40-ζ respectively), compared to unmanipulated cells (Figure 5A). When primary AML cells were used as stimulators, except for INF-γ, which was released at high levels also by untransduced CIK cells (Figure 5B), as previously described, [30] a significant improvement in the production of TNF-α (mean release, 8773pg/ml, range, 597pg/ml-21013 pg/ml; n=6; p≤0.05 for anti-CD33-ζ and mean release, 10371pg/ml, range, 183pg/ml - 27793pg/ml; n=6; p≤0.05 for anti-CD33-CD28-OX40-ζ respectively compared to 143pg/ml, range, opg/ml-921pg/ml; n=6 for unmanipulated CIK cells), TNF-β (mean release, 289pg/ml, range, 26pg/ml-630pg/ml; n=6; p≤0.05 for CD33-ζ and mean release, 340pg/ml, range, 11pg/ml-727 pg/ml; n=6; p≤0.05 for anti-CD33-CD28-OX40-ζ respectively compared to 3pg/ml, range, opg/ml-15pg/ml; n=6 for unmanipulated CIK cells), and IL-2 (mean release, 2220pg/ml, range, opg/ml-6686pg/ml; n=4; p≤0.05 for anti-CD33-ζ and mean release, 3882pg/ml, range, 64pg/ml-13000 pg/ml; n=4;p≤0.05 for anti-CD33-CD28-OX40-ζ respectively compared to 0 pg/ml; n=6 for unmanipulated CIK cells) was detected in anti-CD33.CARs-

expressing CIK cells, without major differences between the two receptors (Figure 5B).

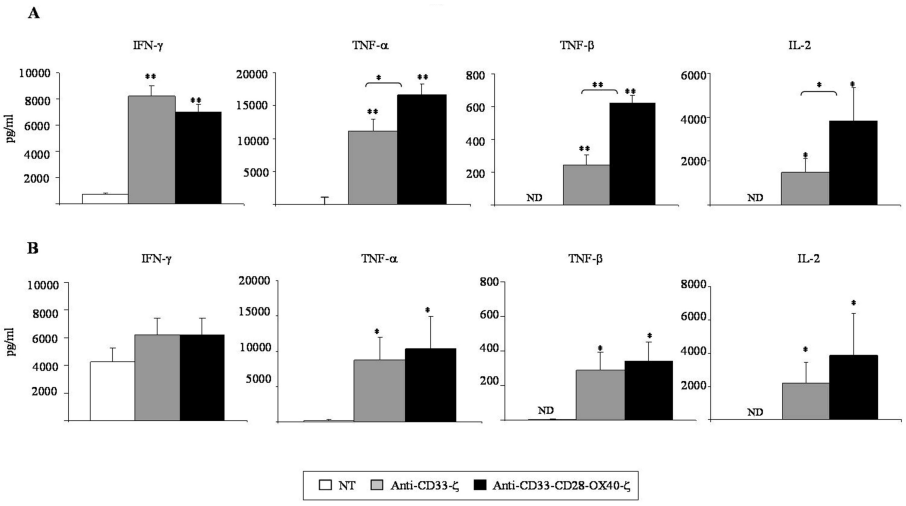


Figure 5. Anti-CD33.CARs expression on CIK cells results in a consistent release of inflammatory cytokines after specific CD33-stimulation. CIK cells were stimulated with irradiated HL-60 (A) and primary AML cells (B) at effector:target ratio of 1:5 for 24h. Release of IFN-γ, TNF-α, TNF-β and IL-2 was detected in the culture supernatants by flow cytometry. Data shown are mean ± SD of 6 independent experiments (*p≤0.05; **p≤0.005).

In vitro colony-forming capacity of normal hematopoietic progenitors is preserved after co-culture with anti-CD33.CAR-transduced CIK cells

To assess the toxicity of anti-CD33.CAR-redirected CIK cells against normal $CD34^+CD33^+$ progenitors, transduced CIK cells were incubated with cord-blood derived-$CD34^+$-immunopurified precursors at a 10:1 effector:target ratio and at different time-points (4h-16h-24-48h) residual $CD34^+CD33^+$ were enumerated and subsequently seeded for colony-forming assays. Anti-CD33.CARs-expressing CIK cells showed a significant cytotoxic activity against normal $CD34^+CD33^+$ cells, with a mean survival at 48h of co-culture of 17% (range, 7%-27%; n=3; $p \leq 0.05$) for anti-CD33-ζ and 14% (range, 6%-22%; n=3; $p \leq 0.005$) for anti-CD33-CD28-OX40-ζ, compared to 67% (range, 66%-69%; n=3) for unmanipulated cells (Figure 6A). In three different experiments, numbers of colony forming unit-granulocyte, macrophage -CFU-GM-, CFU-granulocyte, erythrocyte, macrophage, megakaryocyte -CFU-GEMM-, colony forming unit erythroid -CFU-E- and burst-forming-unit erythroid -BFU-E- colonies derived from $CD34^+$ cord blood cells (previously incubated for 48h with anti-CD33-ζ and anti-CD33-CD28-OX40-ζ CAR-expressing CIK cells) were reduced (mean number of CFU-GM+CFU-GEMM, 4, range, 1-7; n=3; $p \leq 0.05$ for anti-CD33-ζ and 11, range, 2-18; n=3; $p \leq 0.05$ for anti-CD33-

CD28-OX40-ζ and mean number of CFU-E+BFU-E, 10, range 4-20; n=3; p≤0.005 for anti-CD33-ζ and 16, range, 4-29; n=3; p≤0.05 for anti-CD33-CD28-OX40-ζ), compared to co-cultures with untransduced CIK cells (mean number of CFU-GM+CFU-GEMM, 21, range, 16-30; n=3 and mean number of CFU-E+BFU-E, 52, range 45-59; n=3) (Figure 6B). Total numbers of colonies were, however, in the range of normality according to the assay utilized [31, 32].

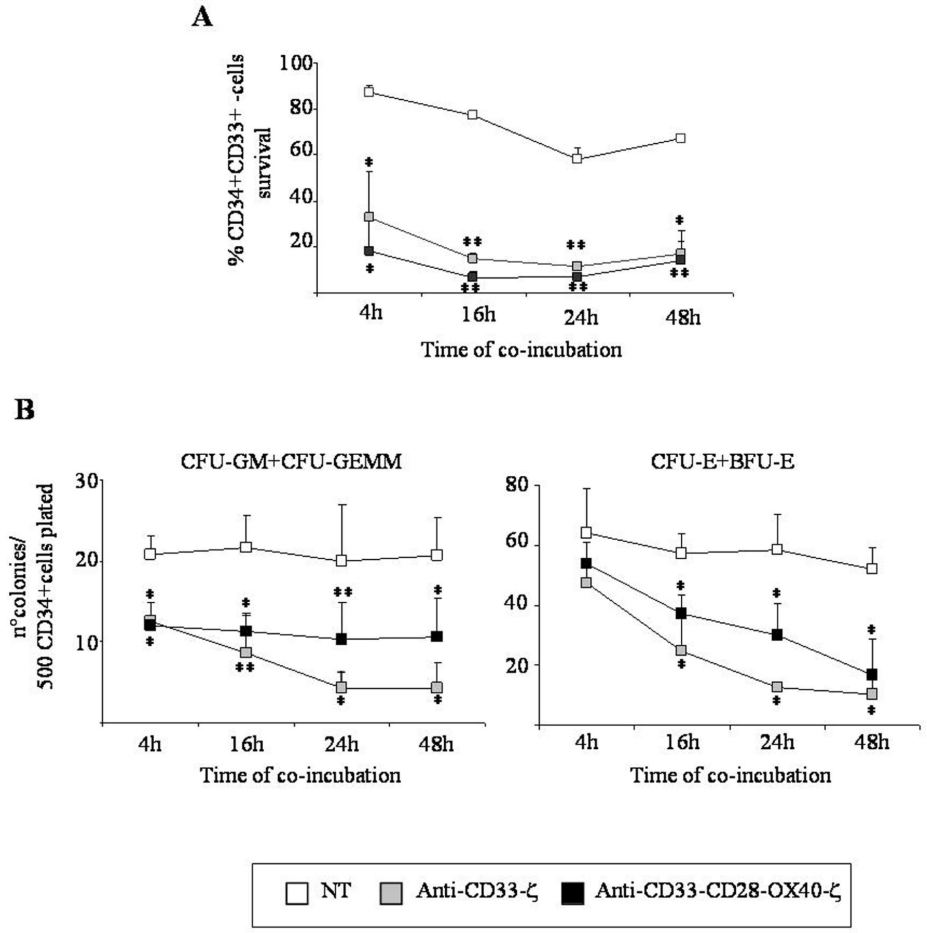


Figure 6. Cytototoxicity of anti-CD33.CARs-transduced CIK cells against normal myeloid progenitors is temporary. (A) CIK cells were incubated with cord-blood purified $CD34^+$ progenitors at effector:target ratio of 10:1 and at different time-points residual $CD34^+CD33^+$ cells were enumerated by flow cytometry. **(B)** After co-incubation with CIK cells, $CD34^+$ progenitors were seeded in methylcellulose-based medium and after 14-21 days colony were counted. Data shown are mean ± SD of 3 independent experiments (*p≤0.05; **p≤0.005).

DISCUSSION

In this study we demonstrated that the introduction of anti-CD33-specific CARs into CIK cells is able to significantly improve their effector functions against AML cells in vitro, without affecting their native phenotype and characteristics. In particular, CIK cells acquired a considerably higher lytic activity, compared to unmanipulated cells, toward different AML cell lines and primary leukemic samples, including those with a more aggressive subtype. It is noteworthy that the killing efficiency was not dependent on CD33 expression level on target cells, as expected with the high-affinity 113-anti-CD33 scFv [33], thus suggesting that this approach might be very effective also in cases of low antigenic expression. We observed, as well, that anti-CD33.CARs-transduced CIK cells were able to maintain the anti-leukemic cytotoxicity over time in the absence of exogenous cytokines, as demonstrated by the 6-day co-culture assays at low E:T ratios and this phenomenon might be explained by their acquired CD33-specific proliferative potential. Moreover, the expression of the anti-CD33.CARs on CIK cells resulted, after stimulation with CD33-positive targets, in an important release of IFN-γ, TNF-α, TNF-β and IL-2, which have been extensively demonstrated to have a crucial role in tumor eradication [34].

It is conceivable that the observed potency of the anti-CD33.CAR containing the ζ-chain only, compared to what reported in other studies [21, 23, 35], might be related to various combined factors, including the high-affinity of the scFv, the choice of the spacer region -human Fc, reported to improve CAR's activity by limiting the formation of heterodimeric complexes with endogenous T cell molecules compared to CD8α spacer-, and the activation state induced in CIK cells by the high-doses of IL-2.

No direct comparison was performed in our experimental setting between the ζ-chain alone and "second generation" CARs containing the CD28-ζ and OX40-ζ domains separately, since many data have been already produced in the literature with these molecules [22]. Instead, we focused on a CAR containing a tripartite endodomain, based on the consideration that inclusion of a primary domain (ζ), an early signalling Ig superfamily member (CD28), with a later signalling TNF superfamily (OX40) member, could be considered an optimal construct to fully activate T cells [36]. In line with these considerations, it is interesting to note that the addition of the CD28-OX40 costimulatory endodomain to the anti-CD33-ζ.CAR significantly improved CIK cells effector functions that might be relevant to sustain the activity of anti-CD33.CAR-transduced CIK cells in vivo upon adoptive transfer,

including CD33-specific proliferation and cytokine secretion. Besides, the benefit deriving from CD28-OX40 endodomain was particularly evident in the long-term cytotoxicity assay, which most likely reflects the potential clinical situation, where leukemic cells should be in numeric advantage over effector cells and no exogenous IL-2 is administered to the patient. The in vivo efficacy of a CAR-based approach could be knocked down by its immunogenicity: CARs are artificial proteins that might be abnormally processed and presented, thus generating new epitopes to which the immune system is not tolerant. However, the major cause of immunogenicity is likely to be represented by murine sequences present in the scFv domain of the CAR molecules: anti-CAR antibody responses have been seen with some CARs, but this could be encompassed by using humanized scFv or scFv derived from human monoclonal antibodies [22, 24, 36] as we did in our study.

The important functional ameliorations in CIK cells activity against AML, obtained through anti-CD33.CARs expression, are not trivial. In the phase I trial previously published by our group [19], where patients with different hematological disorders received CIK cells infusions after allogeneic HSCT, no evidence of anti-tumoral activity of CIK cells in vivo could be found, neither in those subjects receiving the higher cell doses.

The sole patients showing a clinical response where those with indolent disease (and not acute leukemia), who had also been previously treated with DLI, thus rendering impossible to dissect the real contribution of the two cell populations infused. Despite these limited results in terms of clinical efficacy, CIK cells still remain an interesting tool to be used for leukemia cell therapy, for the possibility to be simply generated and expanded to high numbers under GMP conditions, and for their low propensity to cause GvHD. Therefore, our proposed strategy was specifically designed to implement their capability to kill leukemia, but more importantly to persist through leukemia-specific proliferation and secretion of IL-2, and to further amplify the anti-leukemia response through IFN-γ, TNF-α and TNF-β release. Our results are very promising in this sense and might, at least partially, address the issue related to the limited efficacy of unmanipulated CIK cells observed in vivo in our previously published phase I study [19].

The idea to target the CD33 molecule was first exploited by gemtuzumab-ozogamicin (GO), an anti-CD33 monoclonal antibody (mAb) conjugated with calicheamycin, which has shown in its clinical use a certain degree of activity, but also a suboptimal safety profile. Even though GO is highly effective as single treatment for patients with molecular relapse of

acute promyelocytic leukemia [37], the complete response (CR) rate in AML, registered in different studies, is around 30% [38]. In addition, it has been reported that treatment with GO is associated with myelosuppression, neutropenia and thrombocytopenia, in almost all treated patients, and severe hepatotoxicity, with elevations of liver transaminases, hyperbilirubinemia and hepatic veno-occlusive disease, in approximately 20% of patients [38]. CAR-mediated cell therapy might have several potential advantages over GO. Anti-CD33.CAR-expressing CIK cells should be able, once infused, to better infiltrate the tumor and exert a more physiological anti-leukemic response compared to GO, activating and intensifying a tumor-specific immune response. Moreover, CIK cells genetically modified with a CD33-specific CAR should avoid the drug-resistance mechanisms typical of GO, like dependence on CD33 expression levels [39], and capacity of the cells to internalize the drug or to mediate the efflux from the cells through P-glycoprotein [40, 41]. In this study, in fact, we demonstrated that anti-CD33.CARs-redirected CIK cells are able to kill at high levels the KG-1 cell line, known to be resistant to GO activity. In addition, we showed that cytotoxicity against all the $CD33^+$ leukemic cells, differently from what described for GO, is not influenced by

the expression intensity of CD33 on the targets, with strong lysis even in samples with low antigenic expression.

The use of CD33 as target antigen is not devoid of problems. This antigen, in fact, is expressed on the surface of normal hematopoietic precursors (myeloid, erythroid and megakaryocytic), and on Kuppfer cells. This panel of expression might partially explain the toxicity profile of GO. The CAR-CIK-based immunization strategy, instead, might have interesting advantages also on the safety profile. As we demonstrated in our study, in fact, even if anti-CD33.CARs-transduced CIK cells showed a significant killing against normal human hematopoietic progenitor cells, a reduced, but consistent, number of clonogenic progenitors were recovered in in vitro colony forming unit assays. This phenomenon could be explained by the fact that not all normal CD34+ CD38- human hematopoietic progenitors express CD33 at their surface, as reported by Taussig et al. [42]. It is likely that these residual CD34+ CD33- stem cells, which will not be targeted by our treatment, will be enough to reconstitute the bone marrow compartment overtime. Anyway, we are well aware that only in vivo data could finally demonstrate the safety profile on normal hematopoietic progenitors. It is also to be noted that CIK cells could be further manipulated to introduce safety systems that might prevent undesired toxic effects or that

might render cells rapidly eliminable from the organisms, in case of unwanted events occurrence. CIK cells, in fact, might be manipulated to prevent expression of adhesion molecule or chemokine receptors mediating migration/extravasation to the liver [43] or trough the introduction of a suicide gene. Many suicide gene systems are available [44], and we are currently comparing their efficacy (manuscript under preparation). Our preliminary data clearly show a potent and rapid clearance capacity of the inducible Caspase-9 system, which is at least equally effective as the Herpes Simplex Virus-Thymidine Kinase (HSV-TK) approach, it is clinically applicable [45], and it presents the important advantage of being not immunogenic. Generally speaking, the addition of a suicide gene could be considered an ideal strategy to optimize the safety of our proposed approach. This system, in fact, can be exploited to prevent also the risks associated with the use of retroviral integrating vectors, that always raises concerns related to the described episodes of leukemia occurrence [46].

However it is to be noticed that with terminally differentiated cells, as already widely discussed in other contexts, the likeliness of tumorigenic events is virtually absent [46]. Moreover, other kinds of transduction approaches are under evaluation, such as selfinactivating (SIN) vectors and non-viral methods (like transposones). Major technical developments

are under constant investigation for a safer clinical translation [47].

Finally, with this approach it is completely abrogated the CD33-independent toxicity caused by free calicheamicin, which is the main toxic mechanism due to GO. It has been described, in fact, that this toxin is accumulated in the hepatocytes inducing apoptosis and therefore significantly contributing to the hepatic damage [48], which is among the limiting side-effects observed in patients treated with GO, particularly those receiving a HSCT. This aspect is of great relevance, taking into account that anti-CD33.CAR-expressing cells might likely be used in a post-HSCT setting in presence of molecular relapse of AML.

Other than GO, anti-CD33 unconjugated-antibody-based approaches are actually under development, like the Micromet's anti-CD33-T cell receptor engager BiTE and the Seattle Genetics' Lintuzumab. Whether no preclinical and /or clinical data concerning the first strategy are yet available, the latter has been already tested in clinical studies -where it showed a moderate success [49, 50] - and its mechanisms of action are currently under analysis [51]. We believe however that a T cell-based-CAR-mediated targeting of AML might be a potentially better approach. T cells, in fact, might have superior homing capabilities, can amplify the anti-tumoral

immune responses trough target-specific cytokine release, and, last but not the least, can proliferate after contact with tumor cells, thus ensuring a boosted and persistent anti-tumoral activity and hopefully the complete eradication of the disease. Obviously, this must be counterbalanced with possibly greater toxic effects towards the normal hematopoietic compartment, and only *in vivo* data will finally solve this issue. Noteworthy, in case of unwanted or limiting side effects, they can be potentially controlled by the mechanisms of safety-manipulation mentioned above.

Even though we are aware that we need to have final proof of the efficacy and safety of our anti-CD33.CARs-transduced CIK cells in a murine xenogenic model, that we are currently setting up, our results indicate that CD33-redirection of CIK cells might not only improve their effector functions against AML, but also represent, when compared to the unmanipulated counterpart, a step forward in the immunotherapy of resistant and relapsed AML.

Authorship and Disclosures

VM, EB and AB designed the research, critically analyzed the data and wrote the paper. VM, IP and VA performed the experiments. HF and AL contributed in the analysis of the data and in the writing of the paper. MP performed the cloning of the constructs. RR contributed in critically revising the paper. The authors reported no potential conflict of interest.

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Chapter 3

Targeting of acute myeloid leukaemia by cytokine-induced killer cells redirected with a novel CD123-specific chimeric antigen receptor

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ABSTRACT

Current therapeutic regimens for acute myeloid leukaemia (AML) are still associated with high rates of relapse. Immunotherapy with T-cells genetically modified to express chimeric antigen receptors (CARs) represents an innovative approach. Here we investigated the targeting of the IL-3Rα (CD123) molecule, which is overexpressed on AML bulk population, $CD34^+$ leukaemia progenitors, and leukaemia stem cells (LSC) compared to normal haematopoietic stem/progenitor cells (HSPCs), and whose overexpression is associated with poor prognosis. Cytokine-induced killer (CIK) cells were transduced with SFG-retroviral-vector encoding an anti-CD123 CAR. Transduced cells were able to strongly kill $CD123^+$ cell lines, as well as primary AML blasts. Interestingly, secondary colonies experiments demonstrated that anti-CD123.CAR preserved *in vitro* HSPCs in contrast to a previously generated anti-CD33.CAR, while keeping identical cytotoxicity profile towards AML. Furthermore, a limited killing of normal monocytes and CD123-low-expressing endothelial cells was measured, thus indicating a low toxicity profile of the anti-CD123.CAR. Taken together, our results indicate that CD123-specific CARs strongly enhance anti-AML CIK functions, while sparing HSPCs and normal low-expressing antigen cells, paving

the way to develop novel immunotherapy approaches for AML treatment.

INTRODUCTION

Acute myeloid leukaemia (AML) is a heterogeneous clonal disorder characterized by the accumulation of abnormal immature blasts, still associated with high rate of recurrence when treated with conventional regimens (Estey and Dohner 2006). In the last years, great interest has been focussed on the identification of surface molecules that are preferentially expressed by AML cells and leukaemia stem cells (LSCs), in order to selectively target the tumour population, whilst sparing the normal counterpart of haematopoietic stem/progenitor cells (HSPCs) (Majeti 2011), and possibly impeding disease recurrence.

Cytokine-induced killer (CIK) cells expressing chimeric antigen receptors (CARs) could be a promising tool to target AML in humans. CIK cells are an *ex-vivo* expanded population with natural killer (NK)-like cytotoxic activity, enriched in $CD3^+/CD56^+$ cells, having an intrinsic property to kill AML blasts, although in a variable manner (Introna *et al,* 2007). CIK cells are used for a variety of tumours but, as recently reported by the Negrin group in Stanford (Laport *et al,* 2011), they likely

find their best application in the context of myeloid malignancies after allogeneic bone marrow transplantation, without causing any significant toxicity towards normal myelopoiesis or Graft versus Host Disease (GvHD) occurrence.

CARs are molecules constituted by an extracellular-antigen-binding domain (consisting of the variable chains of a monoclonal antibody linked together as a single chain Fragment variable-scFv-) and an intracellular-signalling region (usually the ζ chain of the TCR/CD3 complex) that is triggered after antigen recognition, leading to T-cell activation, with consequent killing of target cells and cytokine release (Biagi *et al,* 2011; Biagi *et al,* 2007; Gross *et al,* 1989; Kohn *et al,* 2011).

We have previously demonstrated that targeting the CD33 antigen by anti-CD33.CAR-expressing CIK cells leads to a robust killing of primary AML samples, but at the same time, at least in *in vitro* assays, the cell fraction containing HSPCs is significantly impaired (Marin *et al,* 2010), due to high expression of CD33 on HSPCs surface. In order to avoid off-target toxic effects on HSPCs, a proper antigen to be further investigated is represented by the CD123 molecule, the α subunit of the IL-3 receptor (IL-3R) that, together with CD131 (β), forms the functional heterodimeric high-affinity IL-3R (Hara and Miyajima 1996). The binding of the IL-3 cytokine to the IL-3R mediates the differentiation of multipotent HSPCs

into myeloid progenitor cells, as well as signals of proliferation and survival of myeloid cells (Blalock *et al,* 1999). CD123 is normally expressed at low levels by HSPCs, monocytes, a subset of dendritic cells (DC), named plasmacytoid DC – pDC (Li *et al,* 2011) and endothelial cells (Korpelainen *et al,* 1996; Taussig *et al,* 2005). In contrast to the low expression on HSPCs, AML cells widely overexpress CD123, rendering this receptor an attractive target for novel treatments of AML (Munoz *et al,* 2001; Testa *et al,* 2002). Moreover, CD123 overexpression on AML cells is associated with resistance to apoptosis, higher proliferating potential and poor prognosis (Graf *et al,* 2004; Testa *et al,* 2002; Vergez *et al,* 2011). Treatment with anti-CD123 mAb 7G3 has been shown to be effective in reducing AML burden in the bone marrow and periphery of NOD-SCID mice with pre-established disease and in impairing secondary transplantation upon treatment (Jin *et al,* 2009). Notably, CD123 is selectively over-expressed not only by AML blasts, but also by a rare quiescent population of AML leukaemia stem cells (LSCs), which has been recently shown to be one of the major players in chemotherapy drug resistance (Bonnet and Dick 1997; Guan and Hogge 2000; Guzman *et al,* 2001; Hope *et al,* 2004; Jordan *et al,* 2000; Lapidot *et al,* 1994; Wang and Dick 2005). Here we describe the efficacy and the safety profile of a new immunotherapeutic approach based on

CIK cells genetically modified to express CAR molecules specific for the CD123 antigen. We found that anti-CD123.CAR rendered CIK cells able to robustly kill $CD123^+$ leukaemia cells *in vitro*, while keeping a safer profile, preserving a normal HSPCs reconstitution in colony assays. Moreover, anti-CD123.CAR^+CIK cells showed *in vitro* a limited toxicity against normal CD123 expressing cells, such as endothelial cells and monocytes. At our knowledge, no previous report has ever described such an approach based on CAR-transduced effector cells targeting the CD123 molecule in AML, thus deeply supporting its further investigation *in vivo* as a novel immunotherapy approach to selectively target AML.

MATERIALS and METHODS

Cell lines, HSPCs and AML primary cells

The THP-1 and CEM cell lines were obtained from the American Type Culture Collection (ATCC). In order to create the CEM-123 cell line, the CD123 cDNA was synthesized from the total RNA of THP-1 cell line and cloned within an SFG-retroviral vector, subsequently used to stably transduce the CEM cell line. All cell lines were maintained in culture with Advanced RPMI medium (Invitrogen, San Giuliano Milanese, Italy) supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), 2mM L-

glutamine, 25 IU/ml of penicillin and 25 mg/ml of streptomycin (Lonza, Bergamo, Italy). The hTERT^{+} bone marrow derived mesenchymal cell line was kindly provided by Prof. Dario Campana (St. Jude Children Research Hospital, Memphis, USA) and was maintained in RPMI-1640 supplemented with 10% FCS, L-glutamine and antibiotics (complete RPMI medium, Lonza) with the addition of 10^{-6} M hydrocortisone (Sigma Aldrich, Milano, Italy). The human renal epithelial cell line 293T was kindly provided by Dr. Martin Pule (University College of London, London, UK) and maintained in DMEM high glucose (Lonza), supplemented with 10% FCS, L-glutamine and antibiotics. Primary AML cells were obtained from bone marrow and peripheral blood mononuclear cells (PBMCs) collected from children or adults with AML at diagnosis. A detailed description of primary AML samples characteristics is provided in Table 1. The Institutional Review Board approved this study and informed consent was obtained from patients or their guardians. HSPCs (Lin^{-}), were isolated from healthy donor cord blood cells performing a negative selection with the Human Hematopoietic progenitor enrichment cocktail, StemSep (STEMCELL technologies, Vancouver, Canada), that allowed the removal of lineage-committed cord blood cells with Tetrameric Antibody Complexes recognizing CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, glycophorin A and

dextran-coated magnetic particles (Liu *et al,* 2010). The isolated cells were also stained with anti-Human CD41 TAC (STEMCELL technologies) in order to remove residual platelets and megakaryocytes. Human Umbilical Vein Endothelial Cells (HUVEC), Human Dermal MicroVascular Endothelial cells (HMVEC-D) and Human Lung MicroVascular Endothelial cells (HMVEC-L) were obtained from Lonza and maintained in formulated EGM-2 medium (Lonza). Healthy donor-derived monocytes were isolated from frozen PBMCs using the anti-Human CD14 MicroBeads (Miltenyi Biotec S.r.l., Bologna, Italy).

Generation of CIK cells

CIK cells were prepared as previously described (Marin *et al,* 2006). Briefly, PBMCs of healthy subjects were obtained after centrifugation of fresh blood on a density gradient using Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden). Cells were then resuspended in complete Advanced RPMI medium (Invitrogen). At the beginning of the culture, IFN-γ (Dompè Biotec S.p.A, Milano, Italy) was added at 1000 U/ml. The next day, 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, and cells were kept at the initial concentration of $3x10^6$ cells/ml. Cells were then cultured for 21

days. Fresh medium and IL-2 were added weekly during culture and cell concentration was maintained around $0.5x10^6$ cells/ml.

Flow cytometric analysis

Immunophenotypic analysis was performed on aliquots of cells for the detection of various surface markers using Fluorescein isothiocyanate (FITC)-anti-CD8 (Exalpha Biologicals, Shirley, USA), Phycoerythrin (PE)-anti-CD4 (Exalpha Biologicals), Peridinin-chlorophyll-protein complex (PerCP)-anti-CD3 (Becton Dickinson, BD, San Jose, USA), PE-anti-CD56 (IQ product, Groningen, The Netherlands), PE-anti-CD62L (BD), FITC-anti-CD45RO (BD), PE-anti-CD33 (BD), PE-anti-CD123 (BD), APC-anti-CD123 (BD), Allophycocianin-anti-CD34 (BD), PE-anti-CD38 (BD), PE-anti-CD144 (BD), FITC-anti-Lineage cocktail 1 (BD), with Isotype-matched antibodies (BD) as controls. CAR expression has been detected with a cyanine 5 (Cy5) anti-Fc specific antibody (Jackson ImmunoResearch, Suffolk, UK), as previously described (Vera *et al*, 2006). A FACScan (BD) flow cytometer device was used to acquire the samples that have been subsequently analyzed with FlowJo 7.5.5. flow cytometry analysis software (TreeStar, Inc.).

Cloning of the anti-CD123.CAR, retroviral supernatant production and CIK cells transduction

The scFv for CD123 was generated starting from the DNA amplification of the variable regions of the light chain (VL) and heavy chain (VH) of the mAb 7G3 (Sun *et al,* 1996) (kindly provided on behalf of CSL Research by Gino Vairo, CSL Limited Australia), separated by a DNA sequence encoding for a linker region $(Ser\text{-}Gly_4)_3$ to allow proper orientation of the two variable units. The scFv-CD123 was cloned in frame with CH_2CH_3-CD28transmembrane-ζ in the SFG-retroviral construct (kindly provided by Dr. Martin Pule, University College of London, London, UK) (Fig 1A). The retroviral supernatant was produced by TransIT-2020 (Mirus BIO, Madison, WI, USA) mediated co-transfection of 293T cells with the MoMLV *gag-pol* expression plasmid pEQ-PAM3(-E) (kindly provided by Dr. Martin Pule), the RD114 *env* expression plasmid pRDF (kindly provided by Dr. Yasu Takeuchi, Cancer Research Technology, London, UK) and the SFG-anti-CD123.CAR vector. Supernatants containing retroviral particles were harvested 48 and 72 hours after transfection, immediately frozen in dry ice, and stored at -80°C until further use. 293T cells were used to titrate virus concentration. For transduction $0.5x10^6$ CIK cells at day 5 of culture were resuspended in 2.5 ml of thawed viral supernatant supplemented with IL-2 (600 U/ml), seeded on

RetroNectin (TaKaRa BioEurope, Gennevilliers, France)-coated 14ml tubes and incubated for 24 hours in a humidified incubator at 37°C, 5% CO_2. The day after, the viral supernatant was removed and CIK cells were then resuspended in 2.5 ml of newly thawed viral supernatant supplemented with IL2 (600 U/ml), and incubated for 72 hours in a humidified incubator at 37 °C, 5% CO_2.

Short-term co-culture experiments

- *^{51}Chromium-release cytotoxicity assay*

Cytotoxic activity of non-transduced (NT) CIK cells and CAR^+CIK cells was measured using a standard ^{51}Chromium-release assay after 4-hour incubation with target cells as previously described (Marin *et al,* 2006). The targets tested included THP-1, CEM, CEM-123 cell lines and adherent HUVEC, HMVEC-D and HMVEC-L cells. Radioactivity was detected by a β-scintillation counter (PerkinElmer Life Science, Boston, USA), as counts per minute (CPM) and the percentage of specific lysis was calculated as previously described (Montagna *et al,* 2001). Experiments were performed in triplicates.

- *Flow cytometry-based cytotoxic assay*

The cytotoxic activity of NT and CAR^+CIK cells towards primary AML cells was evaluated in a 4-hour co-culture assay. NT and

CAR^{+}CIK cells were co-cultured with target cells at an Effector:Target (E:T) *ratio* of 3:1. Cells were collected, stained with PE-anti-CD33 to detect primary AML cells, analyzed by flow cytometry, with a method specifically designed to enumerate the percentage of viable AML cells recovered from culture, as previously described (Marin *et al,* 2007). In brief, the final percentage of killing activity is calculated as:

$$\left(1 - \frac{\text{Total numer of cells after the co-culture(effect+ target cells)}}{\text{Number of target cells}}\right) \times 100$$

We performed the same assay using as targets HSPCs (E:T *ratio* of 3:1) and monocytes (E:T *ratios* of 5:1 and 2.5:1), staining the former with FITC-anti-Lineage cocktail 1 (BD) and the latter with Carboxyfluorescein Succinimidyl ester (CFSE, eBioscience, San Diego CA).

Long-term co-culture experiments

The killing activity of NT and CARs-modified CIK cells toward leukaemia cells was also determined after a 6-day co-culture in duplicates at 1:10 and at 1:100 E:T *ratios*, without exogenous IL-2, on a human bone-marrow derived stromal mesenchymal cell layer, as previously described (Marin *et al,* 2007). After 6 days, cells were harvested, passed through a 19-gauge needle to

disrupt residual mesenchymal-cell aggregates, stained with PE-anti-CD33 antibody and measured as mentioned in the paragraph above.

Colony-forming cell (CFC) assays

To determine the reconstitution potential of HSPCs after short-term co-culture with effector CIK cells, the residual Lin- were sorted (FACSAria III, BD) and used for the CFC assay. Briefly, cells were resuspended in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) and mixed with methylcellulose-based semisolid medium (MethoCult H4434; StemCell Technologies, Vancouver, Canada). 500 Lin^- cells per ml of media mixture were plated on 35-mm petri dish. Cultures were plated in duplicate. Plates were incubated at 37°C, 5% CO_2 for 14 days and colonies were counted and scored according to standard morphologic criteria (BFU-E: burst-forming unit-erythroid, CFU-G: colony-forming unit-granulocyte, CFU-M: colony-forming unit-macrophage, CFU-GM: colony-forming unit-granulocyte/macrophage, CFU-GEMM: colony forming units Granulocyte, Erythrocyte, Macrophage, Megakaryocyte). For secondary colony assay, dishes were washed with PBS, duplicated cultures were pooled and $0.1x10^6$ cells were reseeded in MethoCult. The number of colonies was counted 14 days after replating.

Cytokine release assay

For cytokine production, $1x10^5$ NT and CAR^+CIK cells were stimulated with γ-irradiated CEM cells, THP-1 cells, primary AML cells, HUVEC cells and monocytes at 1:5 E:T ratio for 24 hours. Levels of INF-γ, TNF-α, TNF-β, IL-10 cytokines in culture supernatants were determined with a Flow Cytomix assay (Multiplex Bender MedSystem, Wien, Austria).

Statistical analysis

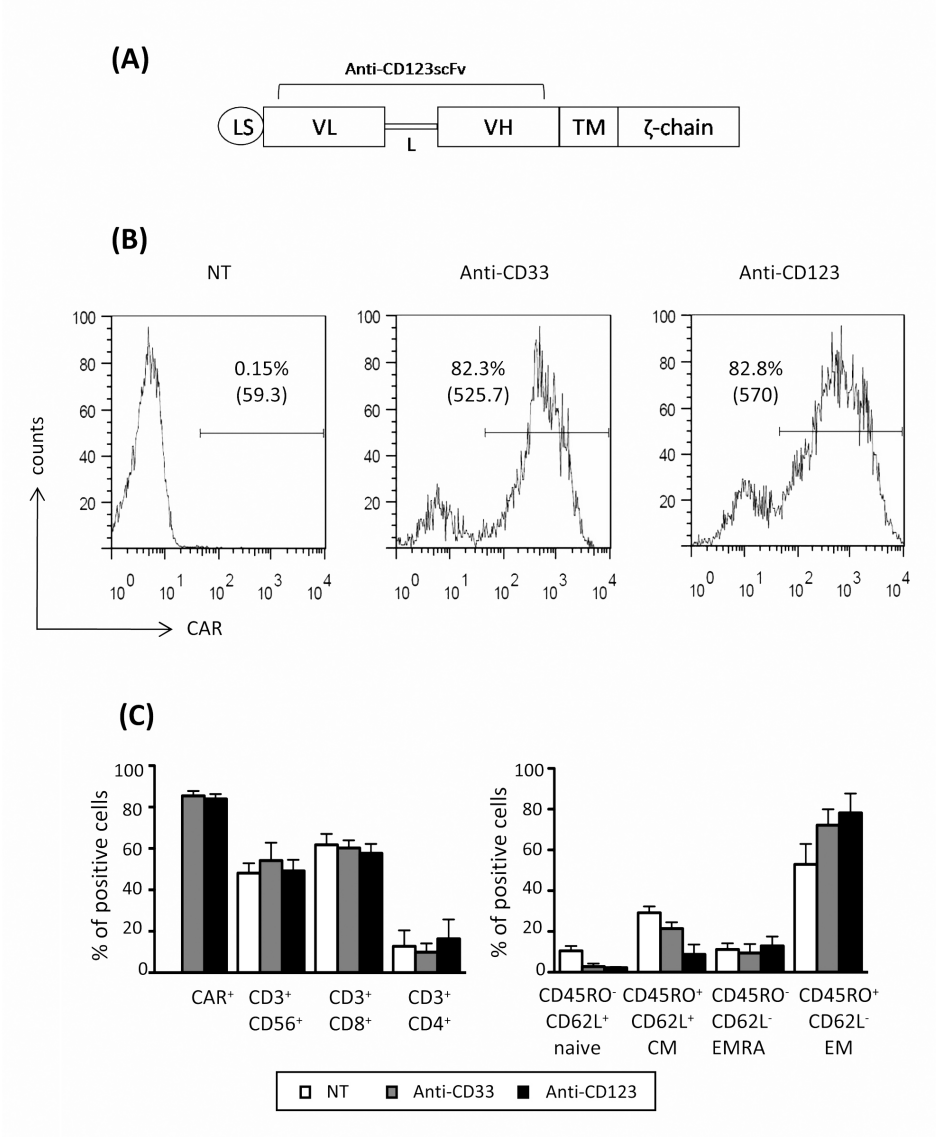
Mean values were reported as Mean±Standard Error (SE). Mann Whitney test was used to determine the statistical significance of the data. Two-tailed analysis was performed, unless not specified in the text. Significance was defined as *$p \leq 0.05$; **$p \leq 0.005$; and ***$p \leq 0.0005$. Statistic calculations were performed with the Prism program 5.0 (GraphPad Software, Inc.).

RESULTS

Generation and characterization of CIK cells transduced with anti-CD123.CAR

We first evaluated whether anti-CD123.CAR could be stably expressed on the surface of CIK cells, without altering the typical CIK cell phenotype. Anti-CD33.CAR$^+$CIK cells, previously characterized in our laboratory (Marin *et al,* 2010), were used as positive control. Healthy donor-derived CIK cells were generated and at day 6 and 7 were transduced either with an anti-CD33.CAR or an anti-CD123.CAR. After 21 days of culture, as shown in Fig 1B, CAR molecules were stably expressed on CIK cells surface (average 85.5±2.3%, n=18, and 84±2.4%, n=7, for anti-CD33.CAR and anti-CD123.CAR, respectively). Phenotypic analysis performed at the same time-point showed that transduced CIK cells were comparable to NT CIK cells in terms of enrichment in $CD3^+/CD56^+$ cells, percentage of CD3 along with $CD4^+$ and $CD8^+$, and memory phenotype (Fig 1C).

Figure 1: CIK cells efficiently express both anti-CD123.CAR and anti-CD33.CAR, keeping their typical phenotype. (**A**) Structure of anti-CD123.CAR. LS, secretion leader sequence from the human IgH; VL and VH, variable region light and heavy chains of mAb 7G3; L, (Ser-Gly4)3 linker; TM, CD28 transmembrane domain; ζ chain of the TCR/CD3 complex. (**B**) The expression of anti-CD33.CAR and of anti-CD123.CAR on the surface of CIK cells was evaluated by flow-cytometry with a Cy5-conjugated-anti-human-Fc antibody (FC) after 21 days of culture (numbers represent percentages of positive cells and median fluorescence intensity -MFI- in bracket). The results of a representative experiment are shown (NT= non-transduced). (**C**) The expression of anti-CD33.CAR and anti-CD123.CAR (CAR+), CD3 along with CD56, CD8, CD4 on CIK cell surface and their memory phenotype after 21 days of culture are shown. Data represented are mean±Standard Error (SE) of 7 independent experiments. CM= Central Memory; EMRA= Effector Memory RA; EM= Effector Memory.


(A)
Anti-CD123scFv
LS
VL
L
VH
TM
ζ-chain
(B)
NT
Anti-CD33
Anti-CD123
0.15%
(59.3)
82.3%
(525.7)
82.8%
(570)
counts
CAR
(C)
% of positive cells
CAR+
CD3+ CD56+
CD3+ CD8+
CD3+ CD4+
CD45RO- CD62L+ naive
CD45RO+ CD62L+ CM
CD45RO- CD62L- EMRA
CD45RO+ CD62L- EM
NT
Anti-CD33
Anti-CD123

Anti-CD123.CAR-redirected CIK cells are highly lytic against the CD123$^+$ THP-1 leukaemia cell line and primary AML blasts

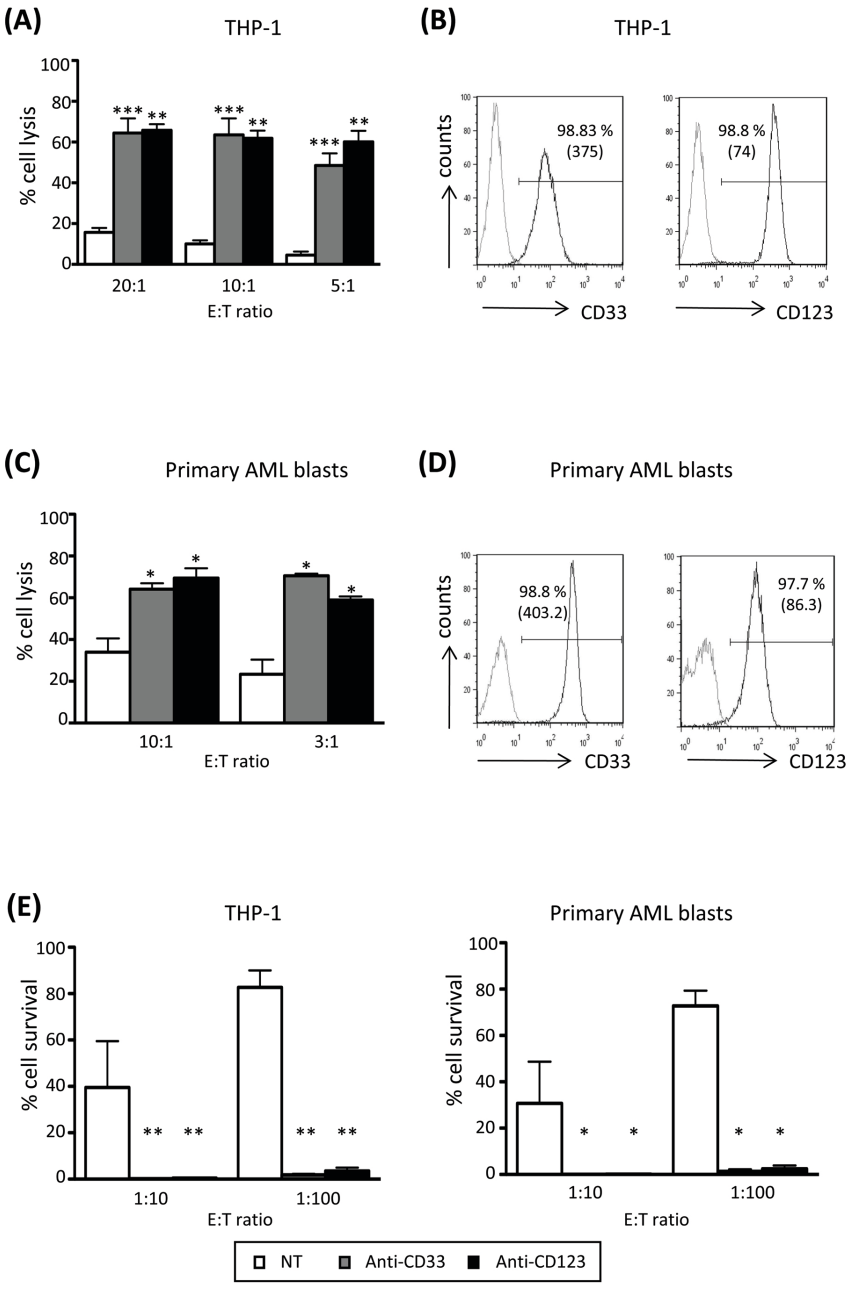
Anti-CD123.CAR-expressing CIK cells were able to robustly kill the CD123$^+$ THP-1 leukaemia cell line in classical 4-hour ^{51}Chromium-release assay (short-term cytotoxicity, mean lysis of 60.1%±5.4%, n=3 for CIK cells expressing anti-CD123.CAR, at an E:T *ratio* of 5:1, compared to an average killing of NT CIK cells of 4.5%±1.8%, n=10, p≤0.005). The killing efficiency of CIK cells transduced with anti-CD123.CAR was comparable to anti-CD33.CAR$^+$CIK cells (with no statistically significant difference, Fig 2A-B).

Short-term flow cytometry-based assay for the quantitative analysis of primary AML blasts elimination confirmed the efficacy of anti-CD123.CAR$^+$CIK cells. Analysis of the cytotoxicity at an E:T *ratio* of 10:1 showed an average killing of 69.4%±4.7%, n=3 for CIK cells expressing anti-CD123.CAR, compared to an average killing of NT CIK cells of 33.9%±6.6%, n=3, p≤0.05. The strength of this CAR molecule against primary AML blasts could be equally appreciated also using a less favourable E:T *ratio* of 3:1 (Fig 2C). The same results were observed with anti-CD33.CAR$^+$CIK cells, as expected according to the overexpression of both target antigens (Table 1, Fig 2C-D). With the aim to better characterize the ability of anti-CD123.CAR$^+$CIK cells to kill leukaemia cells over time we

performed long-term cytotoxicity assay at low E:T *ratios* (1:10 and 1:100), in the absence of exogenous IL-2. We used as targets the THP-1 cell line and primary AML blasts, observing that anti-CD123.CAR-transduced CIK cells, equally to CD33.CAR$^+$CIK cells, were lytic against the target populations. Indeed, we observed at an E:T *ratio* of 1:100 an average THP-1 survival of 3.5%±1.5% (n=5, p≤0.005 one-tailed test) and a primary AML blasts survival of 2.4%±1.4% (n=3, p≤0.05 one-tailed test), for co-cultures with CIK cells expressing anti-CD123.CAR, compared to an average survival of 82.7%± 7.4% (THP-1) and of 72.8±6.6 (AML blasts) when co-cultured with NT CIK cells (Fig 2E).

To assess the specificity of the anti-CD123.CAR molecule, we artificially introduced the CD123 antigen in the CD123 negative CEM cell line (Fig 3A). In contrast to NT CIK cells, anti-CD123.CAR$^+$CIK cells were specifically active against CEM-CD123$^+$ cells with an average killing of 59.4%±10.1%, n=3, at an E:T *ratio* of 2.5:1, compared to an average killing against CEM cells of 18.8%±5.8%, n=3, p≤0.05 one-tailed test. Moreover, no statistically significant difference was observed between NT and anti-CD123.CAR$^+$CIK cells when killing was measured versus wild-type un-manipulated CEM cells (Fig 3B).

Figure 2: Anti-CD123.CAR+CIK cells are potently cytotoxic against AML targets, like anti-CD33.CAR+CIK cells. (**A**) Short-term cytotoxicity of CIK cells was evaluated by a standard 4-hour ^{51}Chromium-release assay after 21 days of culture at E:T ratios of 20:1, 10:1 and 5:1. Data represent the mean±SE of 10 (NT), 7 (anti-CD33.CAR) and 3 (anti-CD123.CAR) independent experiments against THP-1 (**p≤0.005; **p≤0.0005). (**B**) Flow cytometric analysis of CD33 and CD123 surface expression on THP-1 cells (numbers represent percentages of positive cells and MFI in bracket). The results of a representative sample are shown. (**C**) CIK cells from 4 donors (NT, anti-CD33.CAR and anti-CD123.CAR) were co-cultured with primary AML cells from 4 patients at E:T ratios of 10:1 and 3:1. AML cell lysis was evaluated by flow cytometry. Data shown are mean±SE of 4 independent experiments (*p≤0.05, one-tailed test). (**D**) Flow cytometric analysis of CD33 and CD123 surface expression on primary AML cells (numbers represent percentages of positive cells and MFI in bracket). The results of a representative sample out of 7 AML patient cells are shown. (**E**) CIK cells were co-cultured with THP-1 and primary AML blasts at E:T ratios of 1:10 and 1:100 for 6 days on a human stromal mesenchymal cell layer in the absence of IL-2. Leukaemia cell recovery was evaluated by flow cytometry (*p≤0.05, **p≤0.005). Data shown are mean±SE of 5 and 3 independent experiments, for THP-1 and primary AML respectively.


(A)
THP-1
% cell lysis
E:T ratio
20:1
10:1
5:1
(B)
THP-1
counts
98.83 %
(375)
98.8 %
(74)
CD33
CD123
(C)
Primary AML blasts
% cell lysis
E:T ratio
10:1
3:1
(D)
Primary AML blasts
counts
98.8 %
(403.2)
97.7 %
(86.3)
CD33
CD123
(E)
THP-1
Primary AML blasts
% cell survival
E:T ratio
1:10
1:100
NT
Anti-CD33
Anti-CD123

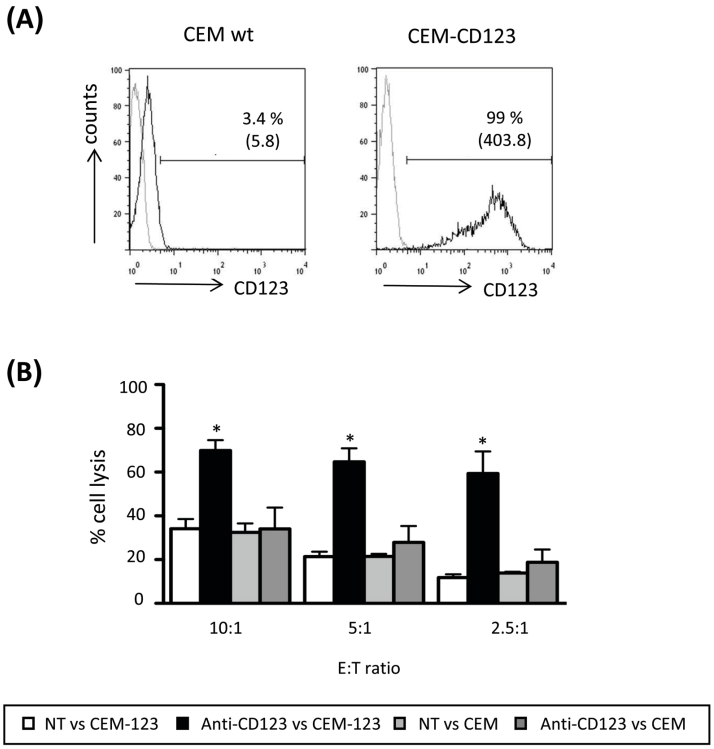


Figure 3: Anti-CD123.CAR+CIK cells are specifically active against artificially-expressing CD123+ CEM cell line. (**A**) Flow cytometric analysis of CD123 surface expression on wild type CEM and CEM-CD123+ cell lines (numbers represent percentages of positive cells and MFI in bracket). The results of a representative sample are shown. (**B**) Short-term cytotoxicity of CIK cells was evaluated by a standard 4-hour ^{51}Chromium-release assay after 21 days of culture at E:T ratios of 10:1, 5:1, 2.5:1. Data represent the mean±SE of 6 (NT) and 3 (anti-CD123.CAR) independent experiments against the artificially-expressing CD123+ CEM cell line (*p≤0.05, one-tailed test). The CEM CD123-negative wild type cell line was introduced as control for measuring basal activity of both NT and transduced CIK cells.

Safety profile of anti-CD123.CAR: anti-CD123.CAR^{+}CIK cells have a better profile in preserving in vitro colony-forming capacity (CFC) of normal haematopoietic progenitors in comparison to anti-CD33.CAR^{+}CIK cells

In order to test the toxicity of anti-CD123.CAR^{+}CIK cells against normal HSPCs, two-stage CFC assay was performed, in which primary single cell-derived colonies were detached and reseeded again at clonal density in a semisolid medium. In our context, we started the CFC assay after 4 hours of co-culture with NT CIK cells and CIK cells transduced with both anti-CD33.CAR and anti-CD123.CAR together with Lin^{-} targets at an E:T *ratio* of 3:1 (mean lysis of 36.9%±6.2%, 33.3%±3.7%, respectively for CIK cells expressing anti-CD33.CAR and anti-CD123.CAR, compared to an average killing of NT CIK cells of 24.2%±1.2%, n=4, see Fig S1). 500 Lin^{-} sorted cells were plated to form primary colonies. After two weeks, we enumerated the numbers of BFU-E, CFU-G, CFU-M, CFU-GM and CFU-GEMM, observing a general reduction in the number of colonies for NT CIK cells and for both anti-CD33.CAR^{+}CIK cells and anti-CD123.CAR^{+}CIK cells in respect to Lin^{-} cells seeded alone, as shown in Fig 4A. Interestingly, the number of colonies preserved by CIK cells transduced with anti-CD123.CAR and anti-CD33.CAR became significantly different in the secondary colony setting, revealing a higher HSPCs recovery potential

when using anti-CD123.CAR^{+}CIK cells. Indeed, the mean total number of secondary colonies was 191.3±8.4 for Lin^{-} cells seeded alone, compared to 146.8±6.6, 66.4±5.1, 117.6±4.6, for Lin^{-} cells co-cultured with NT CIK cells, anti-CD33.CAR^{+}CIK cells, anti-CD123.CAR^{+}CIK cells respectively (Fig 4B, $p \leq 0.005$ when comparing NT CIK cells to Lin^{-} cells, $p \leq 0.0005$ when comparing NT CIK cells to anti-CD33.CAR^{+}CIK cells, $p \leq 0.005$ when comparing NT CIK cells to anti-CD123.CAR^{+}CIK cells, and $p \leq 0.0005$ when comparing anti-CD123.CAR^{+}CIK cells to anti-CD33.CAR^{+}CIK cells). Even though the statistical analysis demonstrated, as expected, a basal level of toxicity of anti-CD123.CAR compared to NT CIK cells, the recovery of the secondary colonies was higher when compared to anti-CD33.CARs.

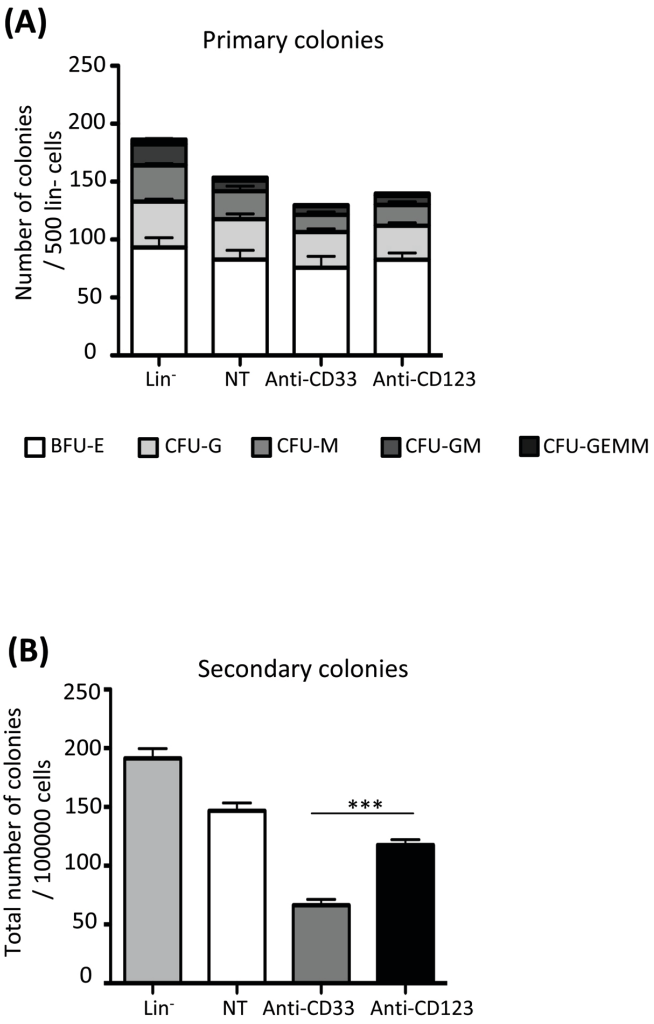


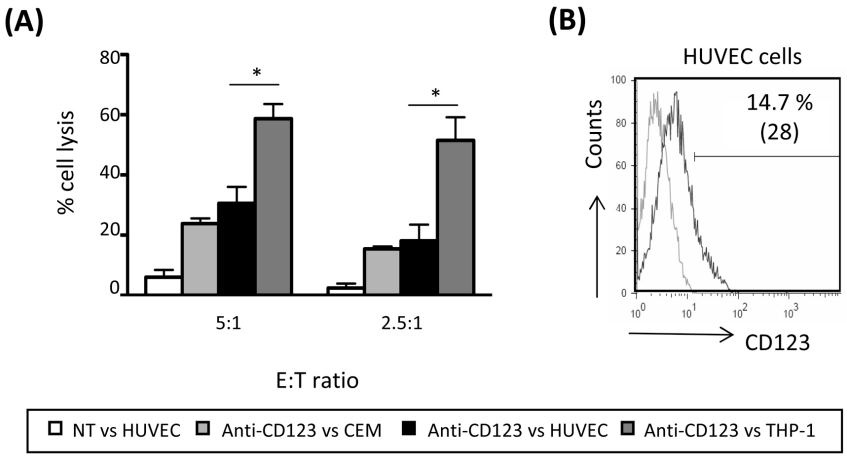
Figure 4: Anti-CD123.CAR+CIK cells display limited toxicity against normal HSPCs. (**A**) 500 Lin- progenitors, sorted after a 4-hour co-incubation with NT and transduced CIK cells at an E:T ratio of 3:1, were seeded in methylcellulose-based medium and after 14 days CFU-GM-, CFU-GEMM-, CFU-E- and BFU-E- were counted. Data shown are mean±SE of 4 independent experiments. (**B**) 0.1x106 were reseeded in methylcellulose based medium and after 14 days secondary colonies were counted (*** p≤0.0005).

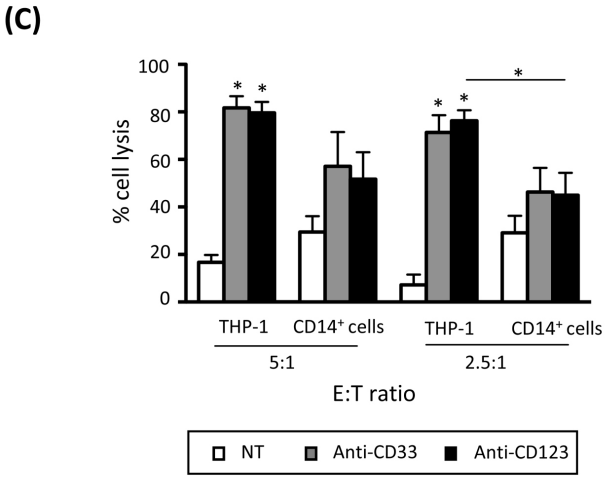
Safety profile of anti-CD123.CAR: anti-CD123.CAR$^+$CIK cells show limited activity against normal CD123 low-expressing endothelial cells and monocytes

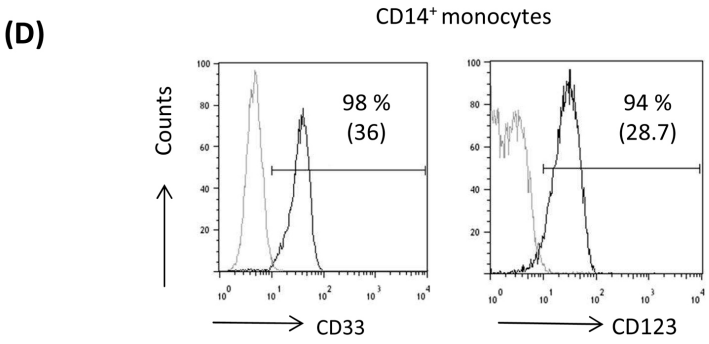
Since it has been reported that recognition by CAR molecules of normal tissues expressing low levels of the target antigen might cause severe toxicity (Morgan *et al*, 2010), we investigated the potential on-target but off-organ effect that CIK cells expressing anti-CD123.CAR might further display *in vivo.* We performed cytotoxicity assays against CD123 low expressing endothelial cells and CD123$^+$ monocytes. Thus, we checked by short-term ^{51}Chromium-release assay if anti-CD123.CAR-transduced CIK cells could kill HUVEC primary endothelial cells. We observed that anti-CD123.CAR$^+$ CIK cells, while being still lytic against the leukaemic target control THP-1 cell line at low E:T *ratio* of 2.5:1, exerted a limited activity against HUVEC cells, showing levels of cytotoxicity similar to those detected against the CD123-negative CEM cell line (mean lysis of 18.5%±5.2%, n=7, 15.5%±0.8%, n=3, and 51.5%±7.7%, n=4, for anti-CD123.CAR$^+$CIK cells against HUVEC, CEM and THP-1, respectively; $p \leq 0.05$ when comparing anti-CD123.CAR$^+$CIK cells killing of HUVEC cells in respect to the killing of THP-1, Fig 5A). The low toxicity profile against primary endothelial cells has been strengthened by additional experiments performed on other human primary endothelial

cells (see Fig S2). Quantitative cytotoxicity assay was performed to measure the potential killing activity of anti-CD123.CAR^{+}CIK cells against monocytes, which express CD123 at higher levels compared to endothelial cells (Fig 5B-D). THP-1 cell line was included as a positive control. While we confirmed the potent lytic activity of anti-CD123.CAR^{+}CIK cells against THP-1 in respect to NT CIK cells (mean lysis at E:T *ratio* of 2.5:1 of 76.3%±4.4% for CIK cells expressing anti-CD123.CAR and mean lysis of 7.2%±4.2% for NT CIK cells, n=3, p≤0.05 one-tailed test), we did not observe any statistically significant difference concerning the killing against monocytes (mean lysis of 45%±9.4%, for CIK cells expressing anti-CD123.CAR and mean lysis of 29.2%±7% for NT CIK cells, n=3, p≥0.05 one-tailed test). Moreover, anti-CD123.CAR^{+}CIK cells showed limited activity against monocytes in comparison to a high killing activity towards THP-1 (E:T *ratio* of 2.5:1, p≤0.05 one-tailed test)(Fig 5C).

Figure 5: Anti-CD123.CAR+CIK cells exert minimal lytic activity against CD123 low-expressing HUVEC and monocytes. (**A**) Short-term cytotoxicity of CIK cells (NT and anti-CD123.CAR) was evaluated by a standard 4-hour ^{51}Chromium-release assay after 21 days of culture at E:T ratios of 5:1 and 2.5:1. Data represent the mean ± SE of independent experiments against HUVEC (n=7), THP-1 (n=4) and CEM (n=3), this latter used as control for basal aspecific cell lysis measurement of transduced CIK cells (*p≤0.05). (**B**) Flow cytometric analysis of CD123 surface expression on HUVEC cells (numbers represent percentages of positive cells and MFI in bracket). The results of a representative sample out of 7 are shown. (**C**) CIK cells from 3 donors (NT, anti-CD33.CAR and anti-CD123.CAR) were co-cultured with healthy donor-derived CFSE+ CD14+ monocytes (n=3) at E:T ratios of 5:1 and 2.5:1. CD14+ cell lysis was evaluated by flow cytometry. Data shown are mean±SE of 3 independent experiments (*p≤0.05, one-tailed test). CFSE+ THP-1 cells were used as positive control. (**D**) Flow cytometric analysis of CD33 and of CD123 surface expression on monocytes (numbers represent percentages of positive cells and MFI in bracket). The results of a representative sample out of 3 are shown. CD33 and CD123 surface expression on THP-1 are shown in Fig 2B.


(A)
(B)
HUVEC cells
14.7 %
(28)
Counts
CD123
% cell lysis
5:1
2.5:1
E:T ratio
NT vs HUVEC
Anti-CD123 vs CEM
Anti-CD123 vs HUVEC
Anti-CD123 vs THP-1


(C)
% cell lysis
THP-1
CD14+ cells
THP-1
CD14+ cells
5:1
2.5:1
E:T ratio
NT
Anti-CD33
Anti-CD123


(D)
CD14+ monocytes
Counts
98 %
(36)
94 %
(28.7)
CD33
CD123

Anti-CD123.CAR^{+}CIK cells release significant levels of immuno-stimulatory cytokines after specific CD123-stimulation

The cytokine-release of anti-CD123.CAR^{+}CIK cells upon CD123 antigen recognition was investigated after 24-hour stimulation with γ-irradiated CD123-expressing cells, at 1:5 E:T *ratio*, such as THP-1 and primary AML cells. We included CD123-negative CEM cells as control. In addition, the cytokine release upon stimulation with HUVEC and monocytes at 1:5 E:T *ratio* was measured. All the stimulator cells alone did not produce TNF-β and IL-10; CEM and HUVEC cells released only low levels of IFN-γ and TNF-α, while primary AML cells secreted only low levels of TNF-α (data not shown), which were subtracted from those measured in CIK-stimulated cultures. After THP-1-mediated stimulation, anti-CD123.CAR^{+}CIK cells secreted 7-fold more amount of IFN-γ ($n=3$, $p\leq0.005$), 19-fold more TNF-α ($n=3$, $p\leq0.005$) and 175-fold more TNF-β ($n=3$, $p\leq0.005$), compared to NT CIK cells. After primary AML-mediated stimulation, anti-CD123.CAR^{+}CIK cells secreted 5-fold more amount of IFN-γ ($n=3$, $p\leq0.05$), 3-fold more TNF-α ($n=3$, $p\leq0.05$) and 59-fold more TNF-β ($n=3$, $p\leq0.05$), compared to NT CIK cells. We did not observe any statistically significant difference in the release of immuno-stimulatory cytokines when both NT and anti-CD123.CAR^{+}CIK cells were stimulated with CEM cells. Although

we could observe a statistically significant difference in the release of immuno-stimulatory cytokines when comparing NT and anti-CD123.CAR^{+}CIK cells stimulated with HUVEC and monocytes, the absolute levels of secreted cytokines from anti-CD123.CAR^{+}CIK cells were lower in respect to those released after stimulation with CD123-overexpressing cells, THP-1 and primary AML cells. Specifically, the production of TNF-α from anti-CD123.CAR^{+}CIK cells stimulated with THP-1 or primary AML cells was significantly higher compared to their stimulation with monocytes or HUVEC (n=3, p≤0.05 one-tailed test), while the amounts of IFN-γ and TNF-β were significantly higher comparing the stimulation of THP-1 or primary AML cells with that of monocytes (n=3, p≤0.05 one-tailed test). At the same time, we did not observe any significant increase in the release of IL-10 from anti-CD123.CAR^{+}CIK cells compared to NT CIK cells, when stimulating them with all the targets considered in our analysis (Fig 6).

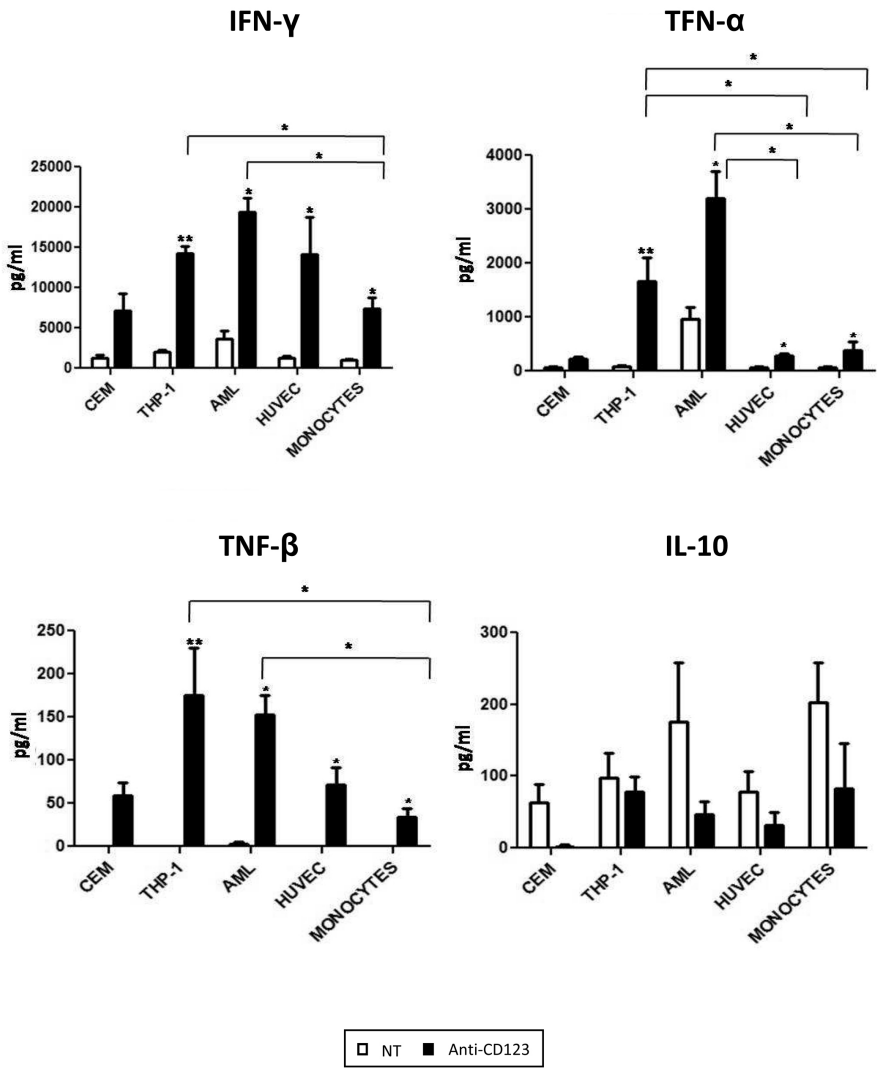


Figure 6: Cytokines release by anti-CD123.CAR+CIK cells after stimulation with leukaemic targets, HUVEC and monocytes. CIK cells were stimulated with γ-irradiated CEM cells, THP-1 cells, primary AML cells, HUVEC cells and monocytes at 1:5 E:T ratio for 24 hours. Release of INF-γ, TNF-α, TNF-β and IL-10 was detected in the culture supernatants by flow cytometry. Data shown are mean±SE of 3 independent experiments (*P≤0.05; **P≤0.005).

Table I. AML patients' characteristics

	SUBTYPE	CD33%	CD123%	KARYOTYPE and GENE MUTATIONS	PROGNOSIS
UPN1	M4	89	84	46,XY,t(9;11)(p22;q23); normal FLT3-ITD*,NPM1a	HR
UPN2	M4	85	81.7	46, XX, inv(16)(p13q22)[16]/46,XX[4]; normal FLT3-ITD, NPM1a	SR
UPN3	M1	84	98.8	46,XY; FLT3-ITD positive, NPM1a positive	HR
UPN4	M5a	98.8	97.7	47-48,XX,del(2)(p12),del(5)(p12),?t(6;7)(q21;q32),t(9;?)(q34;?),-11,del(12)(p11),+19,+4markers[cp9]/46,XX[3]; normal FLT3-ITD, NPM1a	HR
UPN5	M4	97	98	46,XX,inv(16)(p13q22)[16]/46,XX[4]; normal FLT3-ITD, NPM1a	HR
UPN6	M0	98	73	46,XX,t(10;21)(q24;q22)[5]/46,XX[25]; FLT3-ITD positive, normal NPM1a	HR
UPN7	M2	99.4	99	46,XX,t(8;21)(q22;q22)[20]; normal FLT3-ITD	SR

*ITD= internal tandem duplication, HR= high risk, SR= standard risk

DISCUSSION

New treatments of AML are conceived to more selectively target the AML cells and to eliminate a resistant subpopulation of LSCs. In the current study we present a novel immunotherapeutic approach using CIK cells engineered with CAR molecules that are specific for the CD123 antigen, selectively overexpressed not only by the bulk AML population, but also by LSCs (Munoz *et al,* 2001; Testa *et al,* 2002).

CIK cells are an *ex-vivo* easy expandable population, that has been used in a number of clinical trials on AML and Non-Hodgkin Lymphoma (NHL) (Introna *et al,* 2006; Sangiolo 2011). In this context, at our Institution a phase I clinical trial has been concluded (Introna *et al,* 2007) and a phase II trial is currently

ongoing (Introna *et al,* 2011), administering un-manipulated CIK cells in patients with haematological malignancies after allogeneic Haematopoietic Stem Cell Transplantation (HSCT). Even though feasibility and safety have been largely proven, some clinical benefits have been observed only at higher cell dosage and after multiple CIK administrations (Introna *et al,* 2011; Introna *et al,* 2007), envisaging the need of new strategies to improve their killing capacity towards AML.

The strength of our strategy consists into taking advantage of effector CIK cells, which are already prone to kill malignant cells, and potentiate them with antigen-specific CAR molecules, as we previously demonstrated using the CD33 antigen as AML target (Marin *et al,* 2010). Since a robust level of toxicity against HPSCs was observed in that study, here we developed a novel CAR construct targeting the IL3-Rα (CD123) with the aim to be more selective in the elimination of AML cells, while sparing the normal counterpart of HSPCs. Indeed, the main characteristic that makes CD123 an interesting target molecule is the differential expression pattern, being overexpressed by the AML population (Biagi *et al,* 2007; Brentjens *et al,* 2010; Jin *et al,* 2009), and at the same time poorly expressed on normal HSPCs (Gal *et al,* 2006; Jin *et al,* 2009; van Rhenen *et al,* 2007). Our study shows that anti-CD123.CAR transduced CIK cells specifically kill $CD123^{+}$ cell

lines and primary AML cells. In fact, as anti-CD33.CAR molecule, anti-CD123.CAR provides CIK cells a highly effective activity against leukaemia targets, but at the same time results to be significantly less toxic than anti-CD33.CAR against normal HSPCs, as demonstrated by secondary CFC assays, showing a level of toxicity slightly higher than un-manipulated CIK cells, which have never been associated to HSPC impairment after *in vivo* infusion in humans (Laport *et al,* 2011). Great scientific and clinical interest has been focussed on CD123 antigen and many other research groups invested in the CD123 targeting, using different immunotherapeutic strategies (Du *et al,* 2007; Frankel *et al,* 2008; Jin *et al,* 2009; Leyton *et al,* ; Liu *et al,* 2004). In particular, the monoclonal Antibody (mAb) 7G3 which targets CD123 and blocks IL-3 binding showed in a phase I clinical trial an optimal profile of safety, with a limited therapeutic effect (http://clinicaltrials.gov/ct2/show/NCT00401739?term=CSL360&rank=1). It is likely that this anti-CD123 mAb triggered a low antibody-dependent cell-mediated cytotoxicity (ADCC) (Roberts *et al,* 2010; Sun *et al,* 1996). To overcome these obstacles new-generation anti-CD123 mAbs have been developed (Nievergall *et al,* 2011), in some cases producing bi- or tri-specific antibodies conjugating the anti-CD123 scFv with other scFv specific for other antigens (Kugler *et al,* 2010; Stein *et al,* 2010). Regarding this aspect, one of the main advantages

of using CAR-transduced T cells, compared to mAbs, is represented by their enhanced biodistribution and tumor sites infiltration, their synergism with the immune system through the release of cytokines, that recruit other immune cellular components, and the potential development of a long-lasting cell-mediated immune response (Cartellieri *et al,* 2010). In fact, in our experiments, as previously described for the anti-CD33.CAR (Marin *et al,* 2010), we could observe a pronounced release of IFN-γ, TNF-α and TNF-β against leukaemia targets by the CD123.CAR-expressing CIK cells, when compared to un-manipulated ones.

In the last few years, a number of clinical trials with CAR-redirected T cells have been carried out (Kershaw *et al,* 2006; Lamers *et al,* 2006; Park *et al,* 2007; Porter *et al,* 2011). Notably, even though the efficacy of CAR-redirected T cells has been largely demonstrated (Biagi *et al,* 2011; Biagi *et al,* 2007; Gross *et al,* 1989; Kohn *et al,* 2011), particularly targeting the CD19 antigen on B-lymphoid origin malignancies, this approach is not devoid of safety concerns, since serious adverse events (SAE) with fatal outcome occurred in two different patients (Brentjens *et al,* 2010; Morgan *et al,* 2010). In one case, the treatment with anti-HER2 CAR-redirected T cells in a patient affected by metastatic colon cancer caused an "on-target but off-organ" effect, presumably because of the CAR recognition

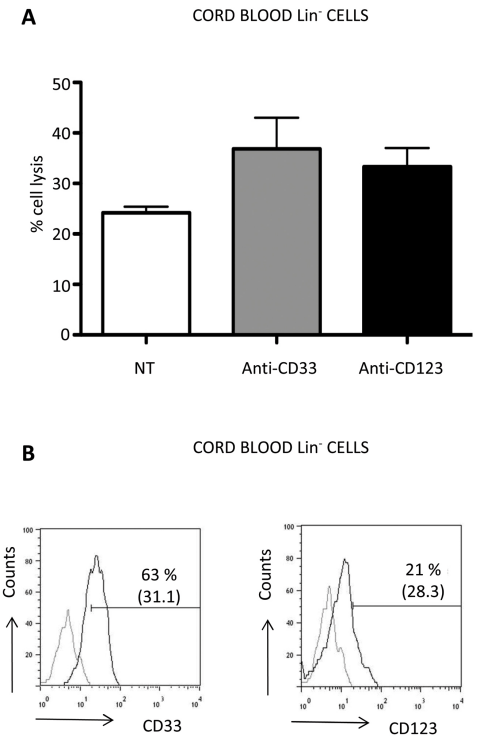
of low HER2-expressing lung endothelial cells, finally causing a lethal acute respiratory distress syndrome (Brentjens *et al,* 2010; Heslop 2010; Morgan *et al,* 2010). The potential risk of CAR-redirected T-cell adoptive transfer envisages the need to carefully evaluate the activity of newly generated CARs on normal tissues. Indeed, several factors may influence the potency of CAR-mediated response, such as the binding affinity of the scFv itself, the levels of CAR expression on effector cell surface, the presence of co-stimulatory molecules within the CAR construct (Casucci and Bondanza 2011; Heslop 2010), the density of the targeted antigen (Alvarez-Vallina and Russell 1999; Chmielewski *et al,* 2004; Hombach *et al,* 2007; Turatti *et al,* 2007), the absolute number of transduced infused cells and the pre-conditioning regimen, as widely discussed elsewhere (Heslop 2010). For this reason, in our study we considered that CD123 antigen is expressed not only by HSPCs, but also by endothelial cells and monocytes, and we performed cytotoxicity assays against HUVEC cells and healthy donor-derived $CD14^+$ monocytes, in order to better evaluate the safety profile of anti-CD123.CAR^+CIK cells. In our hand, anti-CD123.CAR^+CIK cells displayed a limited activity against normal CD123-expressing cells at least *in vitro*. It may be that CD123-specific CAR is constituted by a scFv with suboptimal affinity which could make it able to discriminate between cells with low

and high density surface antigen expression, these latter being represented by the AML tumour cells. In line with this hypothesis, we could observe a limited cytotoxic activity against CD123-low expressing endothelial cells and against monocytes, probably due to their lower CD123 antigen surface density compared to leukaemic cells, measured by the difference in median fluorescence intensity (MFI). Moreover, the release of potentially dangerous cytokines (such as TNF-α and TNF-β) by CD123.CAR-expressing CIK cells was extremely low against both HUVEC and monocytes. Even though encouraging, it will be necessary to confirm the *in vitro* data presented in an *in vivo* setting in humans, posing a high level of caution when transferring this strategy in the clinical setting. It has been described that a subset of DC producing large amounts of type I IFNs in response to viruses, named pDC (Colonna *et al,* 2004), highly expresses surface CD123 molecule (Li *et al,* 2011). It is therefore likely that our approach will target this population. Only *in vivo* human study will address the relevance of this safety issue in terms of increased susceptibility to viral infections. Moreover, it is to be noted that CIK cells could be further manipulated to introduce safety systems, such as suicide genes (Di Stasi *et al,* 2011), that might rapidly eliminate them from the organisms in case of unwanted events occurrence. Actually, many suicide gene systems are

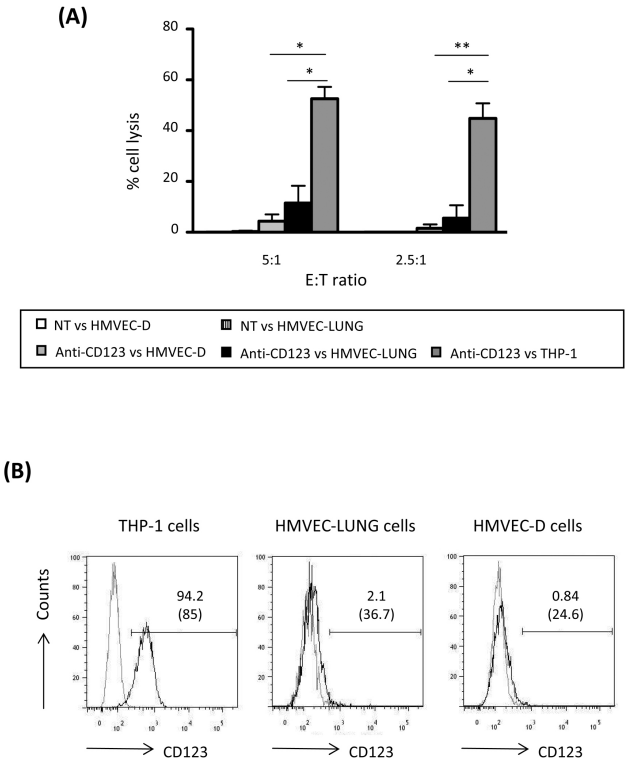
available (Bonini *et al,* 2007), and we have compared their efficacy in a recent study (Marin *et al,* 2012).

In conclusion, we demonstrated, for the first time at our knowledge, that anti-CD123.CAR-transduced CIK cells are effective *in vitro* against AML cells, whilst having an improved safety profile on normal HSPCs compared to anti-CD33.CAR and limited "on-target but off-organ" effects. Although further *in vivo* assays are needed to assess the validity of our *in vitro* findings and the safety of this approach, CD123.CAR-expressing T cells represent anyway an intriguing immunotherapy approach that could be tested in clinical trials for the treatment of resistant forms of AML, particularly for a precocious intervention in presence of minimal residual disease, in the context of early relapse after HSCT.

SUPPLEMENTAL FIGURES



Supplementary Figure 1: Cytotoxic activity of Anti-CD123.CAR+ CIK cells against normal HSPCs. (**A**) CIK cells were co-cultured with cord-blood purified Lin- progenitors at an E:T *ratio* of 3:1 for 4 hours. Lin^- cell recovery was evaluated by flow cytometry. Data shown are mean±SE of 4 separate experiments. (**B**) The panel represents flow cytometric analysis of CD33 and CD123 surface expression on Lin- progenitors (numbers represent percentages of positive cells and MFI in bracket). A representative sample out of 4 is shown.



Supplementary Figure 2: Anti-CD123.CAR+CIK cells exert minimal lytic activity against CD123 low ex pressing HMVEC-D and HMVEC-L. (**A**) Short-term cytotoxicity of CIK cells (NT and anti-CD123.CAR) was evaluated by a standard 4-hour 51Chromium-release assay after 21 days of culture at E:T *ratios* of 5:1 and 2.5:1. Data represent the mean ± SE of independent experiments against THP-1 (n=3), HMVEC-L (n=3) and HMVEC-D (n=3), (*p≤0.05; ** p≤0.005). (B) Flow cytometric analysis of CD123 surface expression on THP-1, HMVEC-L and HMVEC-D cells (numbers represent percentages of positive cells and MFI in bracket). The results of a representative sample out of 3 are shown.

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Chapter 4

In vitro comparison of three different chimeric receptor-modified effector T-cell populations for leukemia cell therapy

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ABSTRACT

The identification of the optimal T-cell effector subtype is a crucial issue for adoptive cell therapy with chimeric receptor-modified T-cells. The ideal T-cell population must be able to home toward tumor site, exert prolonged anti-tumoral activity and display minimal toxicity against normal tissues. Therefore we characterized the *in vitro* anti-tumoral properties of three effector T-cell populations: Epstein-Barr virus-specific cytotoxic T-lymphocytes (EBV-CTLs), cytokine induced killer (CIK) cells and $\gamma_9\delta_2$ T (GDT) cells, after transduction with a chimeric receptor specific for the CD33 antigen, broadly expressed on acute myeloid leukemia cells (AML).

EBV-CTLs, CIK and GDT cells were generated and transduced with high efficiency with a retroviral vector coding for an anti-CD33-z chimeric receptor without alterations of their native phenotype. Anti-CD33-ζ chimeric receptor-redirected T-cells displayed analogous *in vitro* chemotactic activity toward CXCL12. In addition, anti-CD33-ζ chimeric receptor-expressing EBV-CTLs, CIK and GDT cells showed potent and similar cytotoxicity against several $CD33^+$-leukemic targets both in short-term 4-hours-^{51}Chromium-release assays (mean killing vs primary leukemic cells at effector:target ratio of 5:1, 50%, 61% and 50%, respectively for EBV-CTLs, CIK and GDT cells

respectively) and in long-term assays, where they were co-cultured with leukemic cells for 6 days on stromal mesenchymal cells (mean survival of primary leukemic cells at effector:target ratio of 1:100, 18%, 16% and 29% respectively for EBV-CTLs, CIK and GDT cells). Moreover, all effector cells acquired consistent capability to proliferate *in vitro* after contact with $CD33^+$-cells and to release high and comparable levels of immunostimulatory cytokines, while secreting similar low amount of immunoregulatory cytokines as the unmanipulated counterpart.

Our results indicate that expression of an anti-CD33-ζ chimeric receptor potently and similarly increase the anti-leukemic functions of different effector T-cell subtypes, underlying the impossibility to identify a more potent T-cell population through *in vitro* analysis and, consistently with recent observations that have emerged from clinical trials with chimeric receptor-modified T-cells, suggesting the need to perform such type of studies in the human setting.

KEYWORDS: chimeric receptors, tumor immunotherapy, T-cells.

INTRODUCTION

Chimeric receptors (CAR) molecules have recently emerged as a powerful and attractive tool to redirect T-cell specificity and functional activity against tumors, rendering CAR-manipulated T cells potent players in cancer adoptive immunotherapy.

CAR are artificial molecules generated by joining the heavy and light chain variable regions of a monoclonal antibody, expressed as a single-chain Fv (scFv) molecule, to an intracellular signalling domain, usually the zeta chain of the TCR/CD_3 complex, that is immediately triggered after antigen recognition [1]. Therefore, CARs combine the antigen binding -non MHC-restricted- properties of monoclonal antibodies, to the typical T-cell mediated effector functions and capacity to efficiently home and infiltrate tumor sites. Different chimeric receptors have been generated so far [2], against a wide range of surface molecules expressed by many solid tumors and hematological malignancies. Recently, the efficacy of this approach has been reported in a phase I clinical study where autologous Epstein Barr Virus cytotoxic T-cells (EBV-CTLs) expressing a CAR specific for the tumor associated antigen diasialoganglioside GD2, were adoptively transferred in patients with advanced neuroblastoma. The authors demonstrated that the infusion of GD2.CAR-genetically

modified EBV-CTLs was associated with tumor regression or necrosis in half of the subjects tested [3]. Very hopeful are also the results obtained in a patient with follicular lymphoma after treatment with autologous T cells genetically-engineered with a CAR targeting the CD19 molecule [4].

A fundamental step to warrant the success of a CAR-T cells-based immunotherapy approach largely depends on the cell population that is chosen as anti-tumoral effector. The optimal effector T-cell population should be easy to expand and to transduce *in vitro*, should be able to migrate towards sites of tumor infiltration, and persist in a functional state *in vivo* thus providing specific and potent anti-tumor activity and negligible toxicity against other tissues. Different effector T-cell populations have been described in the literature so far as for being potential good candidates for a CAR-mediated immunotherapy strategy, including, other than EBV-CTLs, cytokine induced killer (CIK) cells [5] and $\gamma_9\delta_2$ T (GDT) cells [6].

EBV-CTLs, generated by repetitive stimulation of peripheral blood mononuclear cells with autologous EBV-immortalized B cells (lymphoblastoid cell lines, LCLs), are the most well characterized T-cell subtype for cell therapy approaches. In fact, they have been widely employed for the treatment of EBV-associated lymphoproliferative disorders in patients

receiving allogeneic hematopoietic stem cell transplant (HSCT) and they have been shown to be very successful and safe [7], with a low-risk of inducing graft-versus-host disease (GvHD). When considering their use for a CAR-based approach, EBV-CTLs are a very promising tool, since they have a dual-specificity: one that is conferred by the CAR and redirect their activity against the tumor and another that is conferred by the natural TCR, which is EBV-specific and hence could be easily triggered *in vivo* in EBV-seropositive individuals, thus sustaining the long-term functionality of the cells. As previously mentioned, the efficacy of CAR-expressing EBV-CTLs in human subjects has been proved by a recent phase I clinical trial [3].

CIK cells are immune-effector T-cells with NK-T cell phenotype and function, enriched in $CD3^+CD56^+CD8^+$ subpopulation. They represent a rare population in peripheral blood that can be easily expanded from human peripheral blood mononuclear cells or cord blood up to 1000 fold in 21 days of culture after activation with IFN-g and OKT_3 followed by culture with high-doses of IL-2 [8, 9]. It has been demonstrated *in vitro* that expanded CIK cells show potent non-MHC-restricted cytotoxicity against several tumor cell lines and primary tumor cells [8, 9] and also display potent anti-tumoral activity *in vivo* [8, 9], while having reduced propensity to induce GvHD [9] CIK cells are potentially able to migrate towards bone marrow,

lymph nodes and spleen because they express receptors for adhesion molecules and chemokines such as CD49d, CD11, CXCR4 and CCR6 [10]. Phase I studies have been performed with CIK cells, in various contexts, where no toxicity was observed and several partial responses were detected [8].

Similarly, peripheral blood GDT cells represent a potential alternative to EBV-CTLs. The vast majority of peripheral blood GDT cells in humans expresses TCR composed of a restricted set of variable regions called Vg_9 and Vd_2, that represent 0.5 to 5% of whole peripheral blood T cells and participate with dendritic cells, NK and NK-T cells in the innate immunity response to pathogens or cancer cells [11]. Beside their known anti-infectious activity, in particular following allogeneic HSCT [12], it has been shown that GDT cells are able to produce IFN-γ and to kill a wide variety of tumor cell lines [13]. GDT cells can be selectively expanded *in vivo* or *in vitro* in the presence of aminobisphosphonates without prior antigen priming [14].

In our study, we analyzed and compared *in vitro* the anti-tumoral properties of these three different effector T-cell populations, after transduction with a CAR specific for the CD33 antigen, which is widely expressed on acute myeloid leukemia (AML) cells, with the final aim of identifying the optimal T-cell subset for CAR-mediated tumor immunotherapy. We demonstrated that EBV-CTLs, CIK and GDT cells, genetically

modified with an anti-CD33-ζ.CAR, maintained their typical phenotype and killing capacity and have similar *in vitro* chemotactic activity toward CXCL12. In addition, the expression of the anti-CD33-ζ.CAR provides them with a strong and analogous cytotoxic activity against different leukemic targets, including primary leukemic samples, but also with the ability to similarly proliferate and secrete a panel of immunostimulatory cytokines after specific CD33-dependent stimulation, whereas the release of immunoregulatory cytokines was comparable to the unmanipulated counterpart.

These results suggest that CARs are extremely potent tools to improve the anti-tumor effector functions of T-cells, independently on their nature and derivation. Moreover, *in vitro* analysis is not appropriate to discriminate and identify a more efficient T-cells subset, but rather *in vivo* studies, in humans, might finally solve this issue.

METHODS

Cells

Bone marrow cells and peripheral blood cells were collected from children with AML at diagnosis. Flow cytometry analysis showed that between 80% and 98% of the blasts expressed the CD33 antigen. All leukemia samples were cryopreserved and subsequently thawed for each experiment. The Institutional Review Board approved this study and informed consent was obtained from patients or their guardians. The human B-lineage ALL cell line (SUP-B15) was kindly provided by Dr. Claudia Rossig (University Children's Hospital Muenster, Muenster, Germany), the human AML cell lines HL-60 and KG-1, the human chronic myelogenous leukemia cell line K562 and the Human Burkitt's lymphoma cell line DAUDI were purchased from American Type Culture Collection (ATCC) and were maintained in RPMI-1640 (Lonza, Bergamo, Italy) supplemented with 10% FCS, L-glutamine and antibiotics (complete RPMI). The $hTERT^{+}$ bone marrow-derived mesenchymal cell line was kindly provided by Prof. Dario Campana (St. Jude Children Research Hospital, Memphis, USA) and was maintained in complete RPMI medium with the addition of 10^{-6} M hydrocortisone (Sigma-Aldrich, Milano, Italy). The EBV-producing marmoset B-cell line (B95-8), used as

a source of EBV supernatant for transformation of human B-cells, was purchased from "Istituto Zooprofilattico di Brescia" and maintained in complete RPMI. The human renal epithelial cell line 293T was kindly provided by Dr. Martin Pule (UCL, London, UK) and was maintained in DMEM high-glucose (Lonza), supplemented with 10% FCS, L -glutamine and antibiotics.

Generation of effector T-cells

Peripheral blood mononuclear cells (PBMCs) of healthy donors were obtained after centrifugation of fresh blood on a density gradient using Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden). EBV-CTLs, CIK cells and GDT cells were generated as previously described [10, 15, 16]. For more details see supplementary **(Supplemental File 1)**.

Flow cytometric analysis

Aliquots of cells were analyzed for the expression of various surface markers using Fluorescein isothiocyanate (FITC)-anti-CD8 (Exalpha Biologicals, Shirley, USA), phycoerythrin (PE)-anti-CD4, (Exalpha Biologicals), PE-anti-CD33 (Becton Dickinson, BD, San Jose, USA), PE-anti-CD56 (BD), PE-anti-TCRgd (BD), Peridinin-chlorophyll-protein Complex (PerCP)-anti-CD3 (BD), PE-streptavidin-anti-mouse biotin anti-CXCR4

(BD), with isotype-matched antibodies (BD), as controls. CAR expression has been detected with a cyanine-5 (Cy-5) anti-Fc-specific antibody (Jackson ImmunoResearch, West Grove, USA). A FACScalibur (BD) flow cytometer device was used to analyze the samples.

Chemotaxis and trans-Matrigel migration assays

Chemotactic migration assays were performed as previously described [10] with 96-well Transwell insert (5-µm pore size; Corning Costar, Corning, USA), adding 300 ng/ml of CXCL12 (PeproTech). For more details see supplementary **(Supplemental File 1)**.

Plasmids, retrovirus production and retroviral transduction of cells

The high-affinity, humanized rat anti-human CD33, 113, in single chain Fv (L-(gly4ser)4-H generated using UCB's Selected Lymphocyte Antibody Method (kindly provided by Dr. Helene Finney, UCB Celltech, Slough, UK), was cloned in frame with CH_2CH_3-CD28tm-z in the SFG-retroviral construct (kindly provided by Dr. Martin Pule). The retroviral supernatant was produced by FuGENE 6 (Roche Diagnostic S.p.A., Monza, Italy)-mediated cotransfection of 293T cells with the MoMLV gag-pol expression plasmid pEQ-PAM3(-E) (kindly provided by Dr.

Martin Pule), the RD114 env expression plasmid pRDF (kindly provided by Dr. Yasu Takeuchi, Cancer Research Technology, London, UK) and the SFG-anti-CD33-z.CAR retroviral vector. Supernatants containing retrovirus were harvested 48 hours and 72 hours after transfection, immediately frozen in dry ice, and stored at -80 °C until further use. 293T cells were used to titrate virus concentration. The transduction has been synchronized at day 5 of culture for CIK and GDT cells, and at day +1 after the 3rd LCL-stimulation for EBV-CTLs. For the transduction 0.5x10^{6} effector T-cells, were resuspended in 2.5 ml of thawed viral supernatant and seeded on RetroNectin (TaKaRa BioEurope, Gennevilliers, France) coated 24-well non-tissue culture plates (BD). Cells were then spin infected in the presence of IL-2 and incubated for 72 hours in a humidified incubator at 37 °C, 5% CO_2.

Short-term and long term cytotoxicity assay

The cytotoxicity of unmanipulated and anti-CD33.CAR-z.CAR-modified T cells against leukemic cells was evaluated as previously described [10]. For more details see supplementary **(Supplemental File 1)**.

Cell Proliferation Assay

CD33-specific proliferation was evaluated by ^{3}[H]-thymidine (Amersham Pharmacia Biotech, Piscataway, USA) incorporation as described in the supplementary **(Supplemental File 1)**.

Cytokine Production Detection

For cytokine detection, $2x10^{5}$ EBV-CTLs, CIK and GDT cells expressing the anti-CD33-ζ.CAR were stimulated with γ-irradiated HL-60 cells at 1:5 E:T ratio for 24 hours. Levels of INF-γ, TNF-α, TNF-β, IL-2, IL-10 and TGF-β cytokines in culture supernatants were determined by a Flow Cytomix assay (Multiplex Bender MedSystem, Wien, Austria).

Statistical analysis

Mean values ± SD are given unless otherwise stated. The results were compared by using the paired Student *t* test. A p value ≤ 0.05 was considered to be significant.

RESULTS

Ex-vivo generation of EBV-CTLs, CIK and GDT cells

We set up the *ex-vivo* generation of EBV-CTLs, CIK and GDT cells from the same healthy donor-derived PBMC, according to standardized and published protocols.

EBV-CTLs after the 5^{th} stimulation were constituted mainly by $CD3^+CD8^+$ cells (mean $CD3^+CD8^+$, 71% ± 4%; n=15) (Fig. 1a) and they were not contaminated by residual LCLs, as indicated by the absence of $CD19^+$ cells (data not shown). EBV-CTLs showed a high cytotoxic activity against their autologous LCLs, with a mean lysis at E:T ratio of 5:1 of 59% ± 10% (n=8) and minimal alloreactivity against allogeneic LCLs (Fig. 1b).

After 21 days of culture CIK cells showed a typical selective enrichment in the $CD3^+CD56^+$ subpopulation, (mean $CD3^+CD56^+$, 40% ± 3%; n=22) and in $CD8^+$ cells (mean $CD3^+CD8^+$, 67% ± 5%; n=11) (Fig. 1c). In addition, they demonstrated a consistent and characteristic lytic activity against the K562 cell line (mean lysis, 31% ± 5%; n=5 at E:T ratio of 5:1, Fig. 1d).

As indicated by the immunophenotypic analysis, after 21 days of culture, most of the expanded GDT cells expressed the gdTCR (mean $gdTCR^+$, 70% ± 4%; n=14) (Fig. 1e). Moreover,

GDT cells were able to efficiently kill their native target, DAUDI cell line (mean lysis at E:T ratio of 5:1, 38% ± 6%; n=4; Fig. 1f).

EBV-CTLs, CIK and GDT cells could be efficiently transduced with the anti-CD33-ζ.CAR without alteration of their native phenotype and killing capacity

EBV-CTLs, CIK and GDT were transduced with the SFG-retroviral vector coding for the anti-CD33-ζ.CAR with high and similar efficiency, with a mean level of CAR expression of 71% ± 4% (n=15) respectively for EBV-CTLs, 65% ± 2% (n=25) for CIK cells and 67% ± 4% (n=14) for GDT cells (Fig. 2a). The transduction procedure didn't alter EBV-CTLs, CIK and GDT cells immunophenotype (Fig. 1a, 1c and 1e) and capacity to kill their natural targets (Fig. 1b, 1d and 1f), which were totally comparable to those of unmanipulated cells. When we analyzed the expansion rate of the three effectors cells in the 2-weeks culture period after the transduction process, we observed that CIK cells showed a significantly higher growth compared to EBV-CTLs and GDT cells, with a mean fold increase of 33 ± 4 (n=24) compared to 13 ± 2 (n=27; p≤0.005) for EBV-CTLs and 8 ± 1.2 (n=18; p≤0.005) for GDT cells, respectively (Fig. 2b).

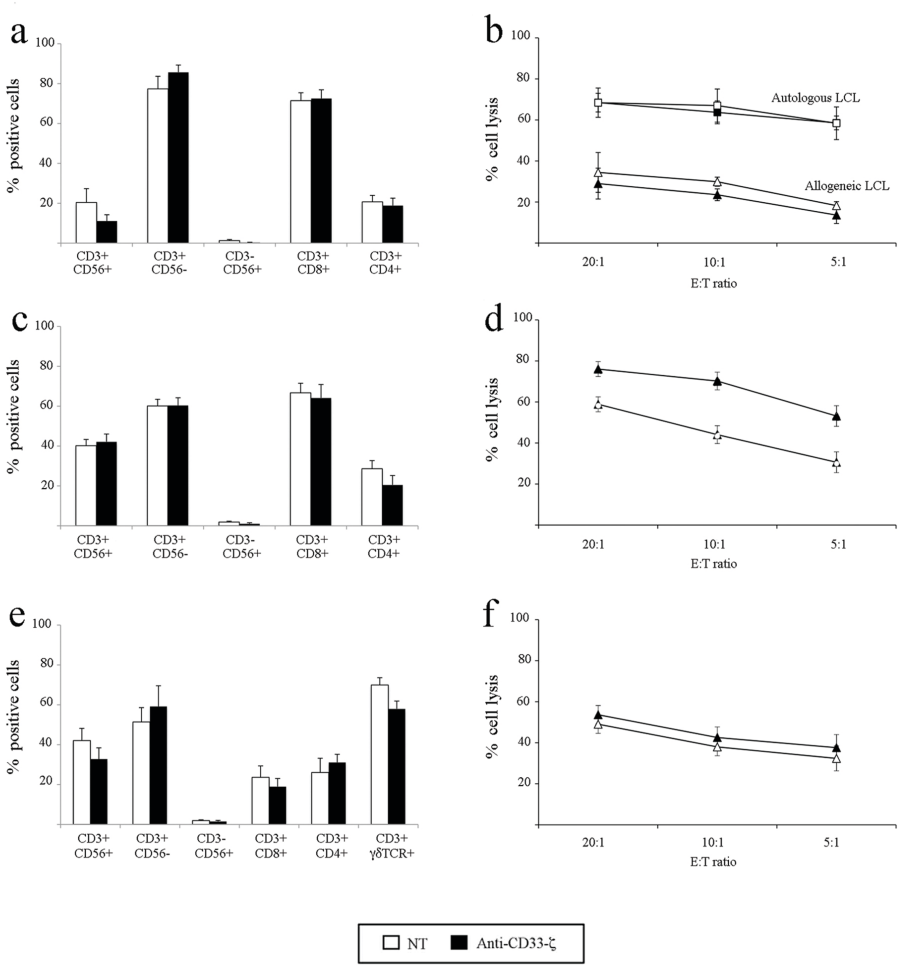


Figure 1. Transduction with the anti-CD33-ζ.CAR does not alter native effector cells phenotype and functions. Immunphenotypic analysis of EBV-CTLs (**a**), CIK cells (**c**), GDT cells (**e**). Reactivity of EBV-CTLs against autologous or allogeneic LCLs (**b**) of CIK cells against the K562 cell line (**d**) or of GDT cells against the DAUDI cell line (**f**) was evaluated 14 days after transduction by a classical 4-hours ^{51}Chromium release assay.

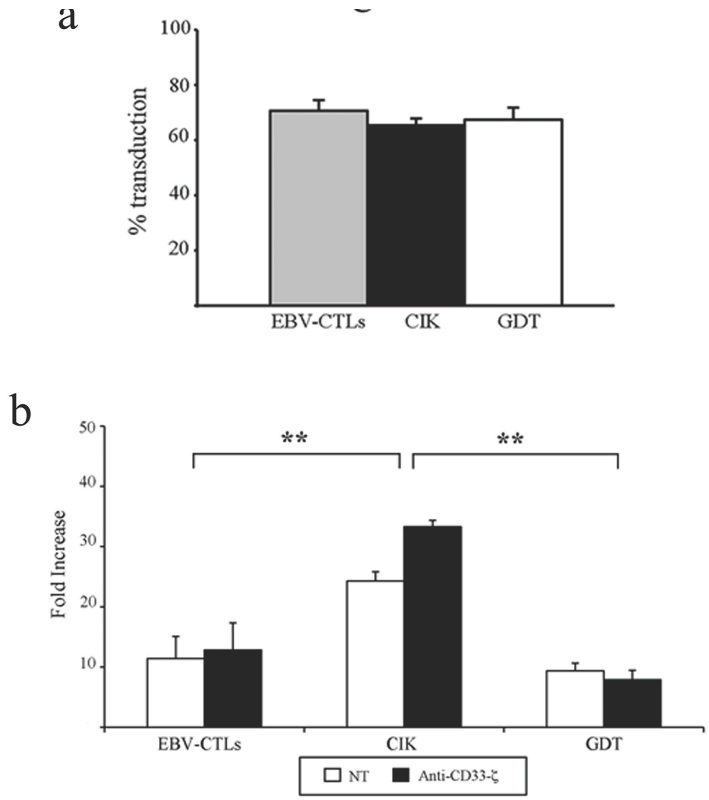


Figure 2. EBV-CTLs, CIK cells and GDT cells are efficiently and similarly transduced with the anti-CD33-ζ.CAR, without any impairment in their expansion capability *in vitro*. (**a**) Anti-CD33-ζ.CAR expression on the surface of effector T-cells was evaluated by flow cytometry with a Cy-5-conjugated anti-human Fc antibody 14 days after transduction. (**b**) Expansion of EBV-CTLs, CIK and GDT cells was calculated and expressed as the fold increase in cell number 14 day after transduction versus the day of transduction.

Chemotactic activity of EBV-CTLs, CIK and GDT cells

EBV-CTLs, CIK and GDT cells were analyzed and compared for their expression of the chemokine receptor CXCR4 and for their *in vitro* migration toward its corresponding ligand, CXCL12, which regulates trafficking to bone marrow, the principal site infiltrated by leukemia. CXCR4 could be detected at high levels on all the effector cells, with a mean % of $CXCR4^+$ cells of 76% ± 5% (n=10) for EBV-CTLs, 63% ± 8% (n=12) for CIK cells and 52% ± 7% (n=10) for GDT cells (Fig. 3a). When we analyzed the *in vitro* chemotaxis of these cells in response to 300 ng/ml CXCL12, in 4 different experiments, it was evident that EBV-CTLs, CIK and GDT display similar migratory activity, with analogous mean migration index -calculated as the ratio of migrated cells in the presence of the chemokine/migration in the presence of medium alone- of 5.6 ± 1.7, 4.4 ± 1.6 and 3.5 ± 0.7, respectively (Fig. 3b). Similarly, the capacity to transmigrate through a reconstituted basement membrane (Matrigel) was equivalent, with a mean migration index of 3.1 ± 1 for EBV-CTLs, 3.7 ± 1.5 for CIK cells and 2.1 ± 0.5 for GDT cells (Fig. 3c). Moreover, the expression of the anti-CD33-ζ.CAR did not alter the trafficking machinery of effector cells, which showed analogous CXCR4 expression and chemotactic activity as unmanipulated cells (Fig. 3a, 3b and 3c).

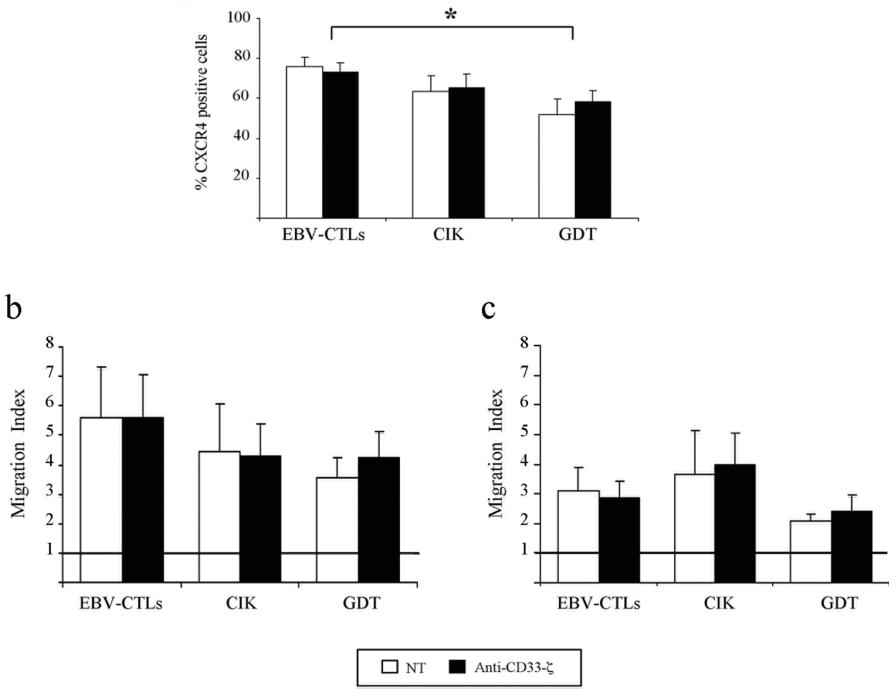


Figure 3. EBV-CTLs, CIK and GDT display a similar trafficking machinery. The expression of CXCR4 on the surface of EBV-CTLs, CIK and GDT cells was evaluated by immunostaining (**a**). The migratory activity of the three effector T-cells toward CXCL12 was determined both with transwell assays (**b**) and with trans-Matrigel experiments in the presence of reconstituted basement membrane (**c**). The expression of the anti-CD33-ζ.CAR didn't alter the trafficking capability of effector cells, which showed comparable CXCR4 expression and chemotactic activity as unmanipulated cells. The horizontal line at the migration index 1.0 indicates lack of chemotaxis.

Anti-CD33-ζ.CAR transduced EBV-CTLs, CIK and GDT cells acquire potent lytic activity against several CD33$^+$ targets

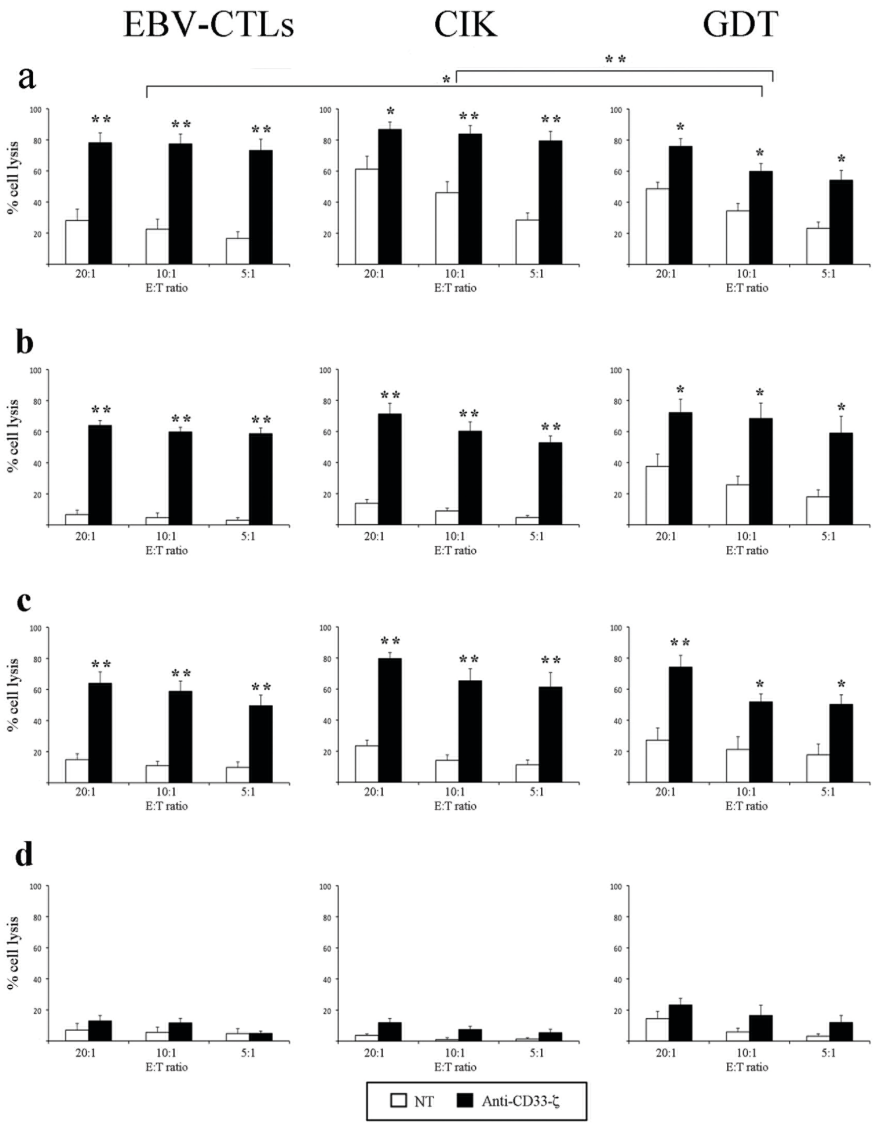
After transduction with the anti-CD33-ζ.CAR, EBV-CTLs, CIK and GDT cells showed potent and similar killing activity against different CD33$^+$ targets, including HL-60 and KG-1 AML cell lines and primary AML samples in classical 4-hours (short-term) cytotoxic ^{51}Chromium-release assays. In particular, the mean lysis of HL-60 at E:T ratio of 5:1 was 73% ± 7% (n=8) for anti-CD33-ζ.CAR-expressing EBV-CTLs, 79% ± 6% (n=7) for anti-CD33-ζ.CAR expressing-CIK cells and 54% ± 6% (n=7) or anti-CD33-ζ.CAR expressing-GDT cells compared to 16% ± 4% (n=8; p≤0.005) for unmanipulated EBV-CTLs, 28% ± 5% (n=7; p≤0.005) for unmanipulated CIK cells and 23% ± 4% (n=7; p≤0.05) for unmanipulated GDT cells, respectively (Fig. 4a). Similar level of lytic activity was registered against the KG-1 cell line, with a mean lysis at E:T ratio of 5:1 of 59% ± 4% (n=3) for anti-CD33-ζ. CAR expressing-EBV-CTLs, 53% ± 4% (n=4) for anti-CD33-ζ.CAR-expressing CIK cells and 59% ± 11% (n=5) for anti-CD33-ζ.CAR-expressing GDT cells compared to 3% ± 2% (n=3; p≤0.05) for unmanipulated EBV-CTLs, 4% ± 1% (n=4; p≤0.005) for unmanipulated CIK cells and 18% ± 4% (n=5; p≤0.05) for unmanipulated GDT cells, respectively (Fig. 4b). As outlined in Fig. 4c, transduced effector cells acquired strong

cytoxicity against blasts isolated from primary AML leukemias, with a mean lysis at E:T ratio of 5:1 of 50% ± 7% (n=9) for anti-CD33-ζ.CAR-expressing EBV-CTLs, 61% ± 9% (n=8) for anti-CD33-ζ.CAR-expressing CIK cells and 50% ± 6% (n=6) for anti-CD33-ζ.CAR-expressing GDT cells, compared to 9% ± 4% (n=8; p≤0.005) for unmanipulated EBV-CTLs, 11% ± 3% (n=8; p≤0.005) for unmanipulated CIK cells and 18% ± 7% (n=6; p≤0.05) for unmanipulated GDT cells, respectively. Importantly all the effectors showed limited killing (<15%) against the $CD33^-$ SUP.B15 cell line, to levels similar to unmanipulated cells (Fig. 4d).

To better characterize their anti-leukemic killing activity, we co-cultured for 6 days unmanipulated and anti-CD33-ζ.CAR-transduced effector cells with HL-60 and primary AML cells on human stromal cells at low E:T ratios and in the absence of exogenous IL-2. In these long-term assays, it was evident that redirection of effectors with the anti-CD33-ζ.CAR significantly improved the killing efficacy. Mean survival of HL-60 and primary AML after co-culture with transduced EBV-CTLs at E:T ratio of 1:100 was 39% ± 13% (n=3) and 18% ± 6% (n=4), compared to 84% ± 8% (n=3; p≤0.05) and 83% ± 7% (n=4; p≤0.05) of unmanipulated cells (Fig. 5a and 5b). Similarly, mean survival of HL-60 and primary AML after co-culture with anti-

CD33-ζ.CAR-transduced CIK cells at E:T ratio of 1:100 was 4% ± 2% (n=4) and 16% ± 7% (n=6), compared to 82% ± 9% (n=4; p≤0.005) and 76% ± 6% (n=6; p≤0.005) for unmanipulated cells (Fig. 5a and 5b). Analogous efficiency was registered with transduced GDT cells, with a mean survival of HL-60 and primary AML of 40% ± 16% (n=3) and 29% ± 4% (n=4) compared to 84% ± 7% (n=3; p≤0.05) and 72% ± 6% (n=4; p≤0.05) for unmanipulated cells (Fig. 5a and 5b). CIK cells displayed a more prominent effect compared to the other effector cells toward the HL-60 cell line.

Figure 4. EBV-CTLs, CIK and GDT cells expressing the anti-CD33-ζ.CAR acquire potent and analogous cytotoxic activity against AML in short-term killing experiments. Short-term cytotoxicity of EBV-CTLs, CIK and GDT cells was evaluated by a standard 4-hours ^{51}Chromium release assays 14 days after transduction at different effector:target (E:T) ratio (20:1, 10:1 and 5:1). Anti-CD33-ζ.CAR expression significantly increases the cytolitic activity of EBV-CTLs, CIK and GDT cells against the $CD33^+$ targets HL-60 cell line (**a**), KG-1 cell line (**b**), and primary AML (**c**) compared to unmanipulated cells. Negligible killing, similar to unmanipulated effector T-cell, was instead observed against the $CD33^-$ SUP.B15 cell line (**d**) (*p≤0.05; **p≤0.005).


EBV-CTLs
CIK
GDT
a
b
c
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% cell lysis
E:T ratio
20:1
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NT
Anti-CD33-ζ

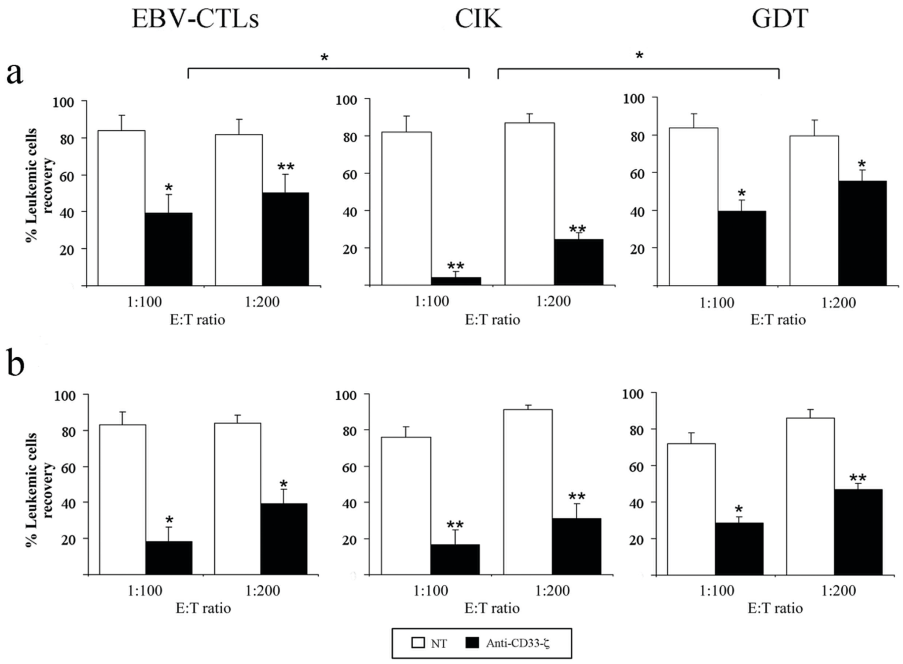

Figure 5. EBV-CTLs, CIK and GDT cells expressing the anti-CD33-ζ.CAR acquire strong and similar cytotoxic activity against AML in long-term killing experiments. EBV-CTLs, CIK and GDT cells were co-cultured for 6 days with HL-60 (**a**) or primary AML (**b)** cells at low E:T ratio (1:100 and 1:200) on human stromal cell layer in the absence of exogenous IL-2. Leukemic cell recovery was evaluated by flow cytometry (*p≤0.05; **p≤0.005).

Anti-CD33-ζ.CAR-transduced EBV-CTLs, CIK and GDT cells release substantial amount of immunostimulatory cytokines after CD33-specific stimulation but do not secrete significant amount of immunoregulatory cytokines

We have extended our functional *in vitro* characterization to the evaluation of immunostimulatory and immunoregulatory-cytokine release of anti-CD33-ζ.CAR-expressing effector cells after CD33 engagement. We observed that, after 24h stimulation with g-irradiated HL-60 at 1:5 E:T ratio, transduced cells release high levels of IFN-γ, with a mean release of 7108 pg/ml ± 1141 pg/ml (n=3) for EBV-CTLs, 8336 pg/ml ± 798 pg/ml (n=6) for CIK cells and 7452 pg/ml ± 371 pg/ml (n=3) for GDT cells, which are consistently higher than what observed in unmanipulated cells, where the mean release of IFN-γ was 0 pg/ml (n=3; p≤0.005) for EBV-CTL, 747 pg/ml ± 133 pg/ml (n=6; p≤0.005) for CIK cells and 2495 pg/ml ± 1887 pg/ml (n=3; p≤0.05) for GDT cells, respectively (Fig. 6a).

In addition, high secretion of TNF-α cytokine has been registered also after CD33-mediated stimulation with HL-60 at 1:5 E:T ratio from transduced effectors cells, with a mean release of 5232 pg/ml ± 1943 pg/ml (n=3) for EBV-CTLs, 11137 pg/ml ± 1764 pg/ml (n=3) for CIK cells and 6394 pg/ml ± 1200 pg/ml (n=3) for GDT cells compared to a mean release from unmanipulated cells of 40 pg/ml ± 21 pg/ml (n=3; p≤0.05) for

EBV-CTLs, 102 pg/ml ± 63 pg/ml (n=6; p≤0.005) and 399 pg/ml ± 155 pg/ml (n=3) for GDT cells (Fig. 6a). TNF-α levels after HL-60 stimulation were significantly higher in anti-CD33-ζ.CAR-transduced CIK cells than in the two other T cell populations (p≤0.05). Anti-CD33-ζ.CAR-transduced EBV-CTLs, CIK and GDT cells showed an important release of TNF-β, with 178 pg/ml ± 130 pg/ml (vs 0 pg/ml of unmanipulated cells; n=3) after HL-60 stimulation for EBV-CTLs; 243 pg/ml ± 62 pg/ml (vs 0.2 pg/ml ± 0.2 pg/ml of unmanipulated cells; n=3; p≤0.005) for CIK cells and 178 pg/ml ± 68 pg/ml (vs 0 pg/ml of unmanipulated cells; n=3; p≤0.05) for GDT cells (Fig. 6a).

Moreover, the specific CD33-stimulation resulted in a significant secretion of IL-2 from anti-CD33-ζ.CAR-transduced cells, with a mean release of 622 pg/ml ± 269 pg/ml (vs 11 pg/ml ± 7 pg/ml of unmanipulated cells; n=3; p≤0.05) from transduced EBV-CTLs, 1501 pg/ml ± 625 pg/ml (vs 0 pg/ml of unmanipulated cells; n=6; p≤0.05) from transduced CIK cells and 367 pg/ml ± 192 pg/ml (vs 12 pg/ml ± 3 pg/ml of unmanipulated cells; n=3; p≤0.05) from transduced GDT cells (Fig. 6a).

Ultimately, we did not observe any significant increase in the release of either TGF-β or IL-10 after the specific CD33-stimulation from anti-CD33-ζ.CAR-transduced cells. In

particular for TGF-β we observed a mean release of 25 pg/ml ± 16 pg/ml (vs 58 pg/ml ± 27 pg/ml for unmanipulated cells; n=6) from transduced EBV-CTLs, 240 pg/ml ± 89 pg/ml (vs 155.8 pg/ml ± 43.9 pg/ml for unmanipulated cells; n=6) from transduced CIK cells and 167 pg/ml ± 39 pg/ml (vs 85 pg/ml ± 40 pg/ml for unmanipulated cells; n=6) from transduced GDT cells. Similarly, transduced cells secreted IL-10 with a mean release of 418 pg/ml ± 212 pg/ml for EBV-CTLs, 65 pg/ml ± 35 pg/ml for CIK cells and 185 pg/ml ± 161 pg/ml for GDT cells, which is not statistically different to what observed in unmanipulated cells (3 pg/ml ± 3 pg/ml for EBV-CTL, 2 pg/ml ± 2 pg/ml for CIK cells and 0.6 pg/ml ± 0.6 pg/ml for GDT cells, respectively (n=6).

Anti-CD33-ζ.CAR- transduced EBV-CTLs, CIK and GDT cells acquire CD33-specific proliferative activity

To further characterize the functional improvements conferred by the anti-CD33-ζ.CAR to effector cells, we analyzed the proliferative activity induced by $CD33^+$-targets. After stimulation with irradiated HL-60 and primary AML at 1:1 E:T ratio, without addition of exogenous IL-2, in 8 different experiments, transduced EBV-CTLs displayed a mean proliferation index -calculated as the ratio of stimulated/unstimulated cell proliferation- of 1.5 ± 0.8 and 2.1 ± 1.7 compared to 0.7 ± 0.1 ($p \leq 0.05$) and 1.2 ± 0.5 ($p \leq 0.05$) of

unmanipulated cells (Fig. 6b). Similarly, after transduction with the anti-CD33-ζ.CAR, CIK cells showed a mean proliferation index of 2.2 ± 0.8 (n=13) and 2.4 ± 1.8 (n=11) compared to 0.9 ± 0.4 (n=13; p≤0.005) and 1.4 ± 0.7 (n=11; p≤0.05) of unmanipulated cells (Fig. 6b). Analogous effect induced by anti-CD33-ζ.CAR was registered on GDT cells, with a mean proliferation index of 2.1 ± 0.9 (n=13) and 3.1 ± 1.1 (n=13) compared to 0.9 ± 0.3 (n=13; p≤0.005) and 1.5 ± 0.3 (n=13; p≤0.005) of unmanipulated cells (Fig .6b). No proliferation was registered when cells were co-cultured with the $CD33^-$ SUP-B15 cell line (data not shown).

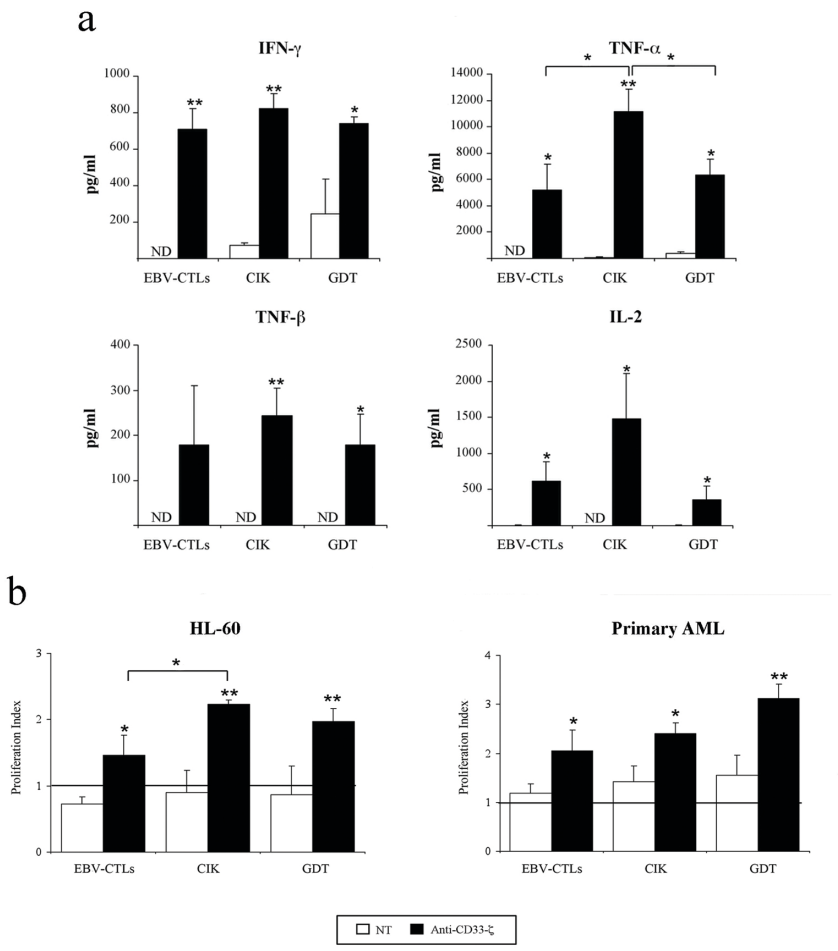

Figure 6. Anti-CD33-ζ.CAR expression results in a consistent and analogous release of inflammatory cytokines after specific CD33-stimulation and confers to EBV-CTLs, CIK and GDT cells a comparable CD33-specific proliferation activity *in vitro*. (a) EBV-CTLs, CIK and GDT cells were stimulated with γ-irradiated HL-60 at E:T ratio of 1:5 for 24 hours. Release of IFN-γ, TNF-α, TNF-β and IL-2 was detected in the culture supernatants by flow cytometry. **(b)** EBV-CTLs, CIK and GDT cells were stimulated with g-irradiated HL-60 or primary AML cells at E:T ratio of 1:1 for 72 hours and the proliferation was evaluated by ^{3}H-thymidine incorporation assays (*p≤0.05; **p≤0.005).

DISCUSSION

In this study we generated EBV-CTLs, CIK and GDT cells from the same healthy donor-derived PBMCs and efficiently transduced them with a retroviral vector coding for an anti-CD33-ζ.CAR. Anti-CD33-ζ.CAR expression was able to potently and similarly boost their anti-leukemic activity. In fact, as demonstrated by the classical short-term cytotoxic assays, several $CD33^+$-leukemic targets, including cell lines (HL-60 and KG-1) and primary AML samples, could be efficiently lysed by the anti-CD33-ζ.CAR-transduced effectors, with no major advantage of one population over the others. Analogous results were obtained in long-term killing experiments, which simulate a more physiological situation, with low number of effector cells interacting with a higher proportion of leukemic cells on a stromal layer. In these assays, again, it was evident that anti-CD33-ζ.CAR-expressing EBV-CTLs, CIK and GDT cells were equally effective, with an almost complete elimination of leukemic blasts. When HL-60 cell line was used as a target in these experiments, CIK cells showed a significant advantage over EBV-CTLs and GDT, however, this could be a cell-line restricted phenomenon. In fact, it has been reported that HL-60 cells are particularly susceptible to CIK cells activity [17, 18].

In addition, it is noteworthy that the same benefit was not observed with primary AML samples.

Other important parameters have to be considered besides the cytotoxic potential to clearly identify the optimal population for a CAR-based tumor immunotherapy approach, i.e. the capacity to reach the tumor site *in vivo*, the ability to sustain and amplify the anti-tumor immune response through the release of immunostimulatory cytokines and last, but not least, the capacity to survive and proliferate *in vivo* in order to exert a long-term tumor control. Therefore, we carried out our *in vitro* characterization of anti-CD33-ζ.CAR-transduced EBV-CTLs, CIK and GDT cells by analyzing and comparing the expression of the chemokine receptor CXCR4, as well as the chemotaxis and trans-migration toward a gradient of CXCL12, which represents the principal chemokine regulating the bone marrow trafficking. In addition, we studied the secretion of immunostimulatory and immunoregulatory cytokines (IFN-γ, TNF-α, TNF-β, IL-2, TGF-b and IL-10) and proliferation after contact with $CD33^{+}$-targets. In none of these experiments it was possible to discriminate a more efficient T-cell subset, since all the three subpopulations showed analogous responses, except for CIK-cell proliferation and TNF-α release after stimulation with HL-60, that could be a cell-line restricted phenomenon, as mentioned above.

Overall, our results clearly point out the limitation of the *in vitro* assays for this kind of analysis and demonstrate that it is not possible to identify a more potent effector T-cell population, since all of them, after transduction with a specific leukaemia-targeting CAR, appear completely well equipped to potentially reach the tumor site, to recognize and kill the target cells, to intensify the immune response trough cytokine secretion and to proliferate when encountering the tumor.

But then, how can an investigator decide which T-cell subtype is more advantageous to employ for clinical use? One possibility would be to consider this issue from a practical point of view, which would be to choose the T-cell subtype which would require the simplest and less costly production method, and which would expand to the required number of cells in the shortest timeframe. Therefore, if this criterion is used, it appears obvious that EBV-CTLs are automatically excluded, since it takes a long-time to produce them (up to two months) and they require special manipulation conditions, as live virus (EBV) is employed, whereas CIK cells might be the ideal candidate, given that they reach high numbers in only 21 days of culture with clinical-grade growth factors with easy manipulation methods. However, it is becoming clear that one property that would really help in discriminating a better T-cell type is the persistence of the T-cells post-infusion. The lack of

persistence could be related to the absence of co-stimulation delivered by tumor cells [19]. Therefore, several groups tried to modify chimeric receptor structure in order to enhance the signal delivered by their activation, by introducing costimulatory regions in the intracellular domain, such as CD28, 4-1BB or OX-40, generating "second-generation" CARs [20]. A plethora of data produced *in vitro* and *in vivo* in mouse models, supports the efficacy of this approach, and different clinical analysis are currently ongoing [4] (http://clinicaltrials.gov/ct2/show/NCT00586391, http://clinicaltrials.gov/ct2/show/NCT01044069 and others listed on the http://clinicaltrials.gov).

In our *in vitro* analysis we performed the same experiments described using the anti-CD33-ζ.CAR, with a so-called "third-generation" CAR, containing other than the CD3-ζ intracellular region, the CD28-OX-40 costimulatory domain. We decided not to show the results, since the resulting message is the same: the introduction of the costimulatory domain significantly and similarly boost all the effector functions of EBV-CTLs, CIK and GDT cells compared to anti-CD33-ζ.CAR-transduced and unmanipulated cells, but no difference emerged among the three populations, thus underling again the restriction of the *in vitro* model.

Recent studies indicate that the different nature of the T-cell subsets employed for a CAR-based approach might influence their persistence and hence their activity. Several reports have demonstrated for instance that the acquisition of a T-effector memory (T_{EM}) phenotype, through associated with strong effector functions *in vitro*, results in their persistence for short periods after adoptive transfer, while administration of central memory T-cells (T_{CM}) reflects in long term memory response with tumor eradication [21-23]. All the three T-cell subtypes analyzed in this study were characterized by a T_{EM}/ Teffector memory $CD45RA^+$ (T_{EMRA}) phenotype [3, 24, 25], therefore suggesting that they might have a limited anti-tumoral action *in vivo* due to their "exhausted" state. However, it is remarkable that in the neuroblastoma clinical trial published by Pule et al., infusion of EBV-CTLs expressing the GD2-specific CAR was very effective in terms of clinical responses [3], and that their efficacy correlated with their capacity to survive *in vivo*. The peculiarity of EBV-CTLs is that, even though they have a terminal differentiated phenotype, they could be restimulated *in vivo* trough their native TCR in a physiological manner, by EBV-infected cells in EBV-seropositive individuals, which besides constitute the majority of the population.

Other approaches might be as well employed to promote the persistence and anti-tumoral activity of CAR-transduced T_{EM}

cells. Lymphodepletion of the recipient might render the environment more prone to sustain the proliferation of infused T cells, since homeostatic cytokines are made available and T regulatory/suppressor cells are removed [26]. In addition, cells might be further manipulated to express either cytokines genes or their receptors [27, 28], or costimulatory molecules [29], or anti-apoptotic proteins [30], or patients receiving T-cell infusions might receive direct systemic administration of IL-2, IL-15 or IL-21 [31-33] . This could further ameliorate CIK cells profile for *in vivo* use, possibly augmenting their chances of survival in a human setting after intravenous administration. An other option, available for GDT cells alone, might be the i*n vivo* restimulation with zelodronic acid [34], already used for the treatment of osteoporosis and other disorders of bone resorption, which showed to sustain GDT persistence in a functional active state for long period after the infusion. It remains then to be elucidated which of these conditions might lead to the stronger effect.

It is hard to believe that preclinical *in vivo* animal studies might definitely unravel this issue. The readability of the results obtained in a xenogenic model in fact, is often controversial, in particular when mouse models are exploited. Persistence relies on different parameters, among which stromal support and cytokines are relevant factors. In addition, the murine

microenvironment might not be permissive for the long-term maintenance of T-cells, due to the xenogenic barriers [35, 36]. More physiologic studies might be performed in non-human primates [37], but this would be not be easily achievable by most of the academic investigators, may not anyway reveal a potential toxicity against human tissues [38] and may not be necessary, if phase I clinical trials are conceived in an intelligent manner so to limit possible side effects and, simultaneously, if reliable assays to detect and measure the *in vivo* activity of modified T-cells are available. The relevance of studies in the human setting aimed at assessing not only the efficacy, but also the safety of a CAR-based approach is supported by the recent description of serious adverse events occurred in subjects enrolled in two clinical trials with CAR-modified T cells [31, 39]. These reports taught us in fact, that second and third-generation CARs are extremely potent and dangerous tools if combined with high T-cell doses and lymphodepletion and clearly suggest the need to test more restricted dose-escalation/lymphodepletion schemes and conceivably to introduce a safety "switch" such as a suicide gene [40, 41].

We can conclude that the preliminary clinical testing of CAR-T-cells performed and documented so far has contributed in a significant manner in the disclosure of the possible toxicity of this approach -otherwise difficultly predicted through in *in vitro*

assays and *in vivo* preclinical models- as well its efficacy [3, 31, 39] and, as supported by the study of Pule et al. [3], might be definitely envisaged and sustained to identify which T-cell subset, and in which conditions, might be the best to obtain a clinical relevant and safe benefit.

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AUTHORSHIP AND DISCLOSURES

IP, VM, EB and AB designed the research, critically analyzed the data and wrote the paper. IP, VA, EC and VM, performed the experiments. HF and AL contributed in the analysis of the data and in the writing of the paper. MP performed the cloning of the construct. RR contributed in critically revising the paper. The authors reported no potential conflict of interest.

SUPPLEMENTAL FILE LEGENDS

Supplemental File 1. In the supplemental file a detailed description of the methods employed to generate effector T-cells and to analyze their functionality is provided.

METHODS - supplementary appendix

Generation of effector T-cells

Peripheral blood mononuclear cells (PBMCs) of healthy donors were obtained after centrifugation of fresh blood on a density gradient using Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden). EBV-CTLs, CIK cells and GDT cells were generated as previously described (10, 15-16).

Briefly, to generate EBV-transformed B cell lines (LCLs), PBMCs (5×106) were incubated with 50 µl of concentrated supernatant from the EBV producer cell line B95-8 for 60 min. The cells were then plated at 106 cells per well in a flat-bottomed 96-well plate in complete RPMI medium (Lonza), added with 1 µg/ml of cyclosporin A (Sandoz Pharmaceuticals, Washington, USA). Cells were fed weekly until LCLs were established. EBV-CTLs were expanded by co-culture of PBMC (2×106), resuspended in complete advanced RPMI medium (Invitrogen, San Giuliano Milanese, Italy) with 5×104 γ-irradiated (40 Gy) autologous LCLs. Starting on day 11, the responder cells were weekly re-stimulated with irradiated LCLs

at a responder:stimulator ratio of 4:1. Two weekly doses of IL-2 (40 IU/ml) were added from 3rd stimulation.

To generate CIK cells, PBMCs (3x106 cells/ml) were resuspended in complete advanced RPMI medium (Invitrogen). At the beginning of the culture, IFN-γ (Gammakine, Dompè Biotec S.p.A, Milano, Italy) was added at 1000 IU/ml. The next day, IL-2 (Proleukin, Chiron B.V, Emeryville, USA) and OKT-3 (Janssen-Cilag S.p.A., Cologno Monzese, Italy) were added at 300 IU/ml and at 50 ng/ml, respectively. Fresh medium and IL-2 (300 IU/ml) were added twice weekly during culture. Cells were cultured for 21 days.

To generate GDT cells, PBMCs (2x106 cells/ml) were resuspended in complete advanced RPMI medium with the addition of 100 IU/ml of IL-2 and 10 ng/ml of IL-15 (Peprotech, London, UK). The next day the cells were stimulated with 1 µg/ml of zelodronate (Zometa, Novartis Pharma S.p.A., Origgio, Italy). Two weekly doses of IL-2 (100 IU/ml) and IL-15 (10 ng/ml) were added. Cells were cultured for 21 days.

Chemotaxis and trans-Matrigel migration assays

Chemotactic migration assays were performed as previously described (10) with 96-well Transwell insert (5-µm pore size; Corning Costar, Corning, USA), adding 300 ng/ml of CXCL12 (PeproTech). After 1 hour migrated cells in bottom wells were

enumerated by quantitative flow-cytometry analysis. Results are expressed as the migration index of effector T-cells in response to the chemokine versus the basal condition. Migrated cells in bottom wells were enumerated after 3 hours by quantitative flow-cytometry analysis.

Short-term cytotoxicity assay

The cytotoxicity of unmanipulated and anti-CD33.CAR-ζ.CAR-modified T cells against leukemic cells was evaluated as previously described (10) with a standard 4-hours ^{51}Chromium-release cytotoxicity assay. Radioactivity was detected by a β-scintillation counter (PerkinElmer Life Science, Boston, USA), as counts per minutes (CPM) and the percentage of specific lysis was calculated as previously described (5). Experiments were performed in triplicates.

Long-term cytotoxicity assay

The killing activity of unmanipulated and anti-CD33-ζ.CAR-modified T cells toward leukemic cells was also determined after 6 days co-cultures at 1:100 and at 1:200 Effector:Target (E:T) ratios, without exogenous IL-2, on a bone marrow derived mesenchymal cell layer. After 6 days cells were harvested, passed through a 19-gauge needle to disrupt residual mesenchymal cell aggregates, stained with PE-anti-CD33

antibody and assayed by flow cytometry with a method specifically designed to enumerate leukemic cells recovered from culture.

Cell Proliferation Assay

CD33-specific proliferation was evaluated by 3[H]-thymidine (Amersham Pharmacia Biotech, Piscataway, USA) incorporation. At 10 days after transduction, 1x105 unmanipulated and anti-CD33-ζ.CAR-modified EBV-CTLs, CIK and GDT cells were co-cultured for 72 hours with γ-irradiated HL-60 or primary AML cells, at 1:1 E:T ratio, without the addition of IL-2. Counts per minute were measured in a β-scintillation counter (Beckman Coulter, LS6500 multipurpose scintillation counter, Brea, USA). Data are expressed as proliferation index (PI), calculated as the ratio of CPM stimulated/CPM unstimulated conditions. As a control, the same assay was performed using the CD33- SUP-B15 cell line.

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Chapter 5

Summary, Conclusion and future directions

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults and it accounts for 30% of leukemia-related deaths in children. Current chemotherapy regimens ensure long-term remission only in 50% of patients and prognosis of relapsed cases is very poor, with a low survival probability.

Novel therapeutic strategies are urgently needed to treat these patients. In these studies we propose to augment the immune response against leukemia using the novel approach of chimeric receptor transfer.

Chimeric antigen receptors (CARs) are a fascinating technology in the field of immunotherapy for orienting the activity of immune cells towards specific molecules expressed on the cell surface of tumors. CARs are artificial T cell receptors generated by joining the heavy and light chain variable regions of a monoclonal antibody, expressed as a single-chain Fv (scFv) molecule, to an intracellular signalling domain, usually the zeta-chain of the TCR/CD_3 complex. T lymphocytes genetically engineered to express CARs exhibit specific lysis and cytokine secretion upon exposure to tumor cells expressing the target

antigen. The CAR-mediated effector function produces sustained tumor cell lysis, more likely than humoral immune responses alone, based on the use of monoclonal antibodies. The perforin/granzyme killing mechanism is effective against cells that are relatively resistant to antibody and complement, while cytokine secretion recruits additional components of the immune system, amplifying and prolonging the anti-tumor immune response. Moreover, T cells display efficient tumor penetration and homing capabilities.

In our study we demonstrated that the introduction of anti-CD33 and anti CD123 specific CAR into CIK cells is able to improve their effector functions against AML, rendering CAR manipulated T cells potent players in cancer adoptive immunotherapy.

Since CD33 is also expressed on normal hematopoietic stem/progenitors cells (HSPCs) resulting in a potential severe impairment of normal myelopoiesis, CD123 has recently emerged as new potential attractive molecules based on its differential expression pattern. The increased expression of CD123 on AML cells compared with HSPCs presents an opportunity for selectively targeting AML cells, sparing the normal hematopoiesis.

A possible limitation of a CAR approach, as recently reported

from a serious adverse effect reported in a patient with metastatic colon cancer treated with ERBB2-specific CAR-transduced T cells [1], is that, due to the elevated affinity of interaction typical of the scFv, the CAR would not be able to discriminate among over-expressing tumor cells and normal tissue expressing basal levels of the antigen.

Similarly CD123 antigen is also express at low level by normal HSPCs, endothelial cells and monocytes.

In order to avoid possible side effects one possibility would be to modulate the affinity of the anti-CD123 CAR, by generating CARs mutants, and to identify the optimal affinity associated with a good lytic efficiency against tumor cells and no impact on normal cells.

Another option could be represented by the "tumor-sensing" strategy recently proposed by Sadelain to render genetically modified T cells specific for a tumor even in the absence of a specific tumor-restricted antigen [2].

We hope in this way to have the possibility to modulate the specificity of the CARs for its ligand in order for it to be able to discriminate among low expressing (usually healthy tissues) and high-expressing targets (tumor cells), and hence to render CARs safer instruments and helping to broaden their applicability and avoid some of the side effects of targeted T-cell therapies.

An important consideration for the clinical application of the CARs technology is represented by two major issues: safety and clinical applicability in terms of efficiency and costs.

Firstly, the safety issues concern the use of integrating vector for gene transfer, as well as the potential reactivity of CARs-transduced cells against host-normal cells expressing the target antigen. These limitations can be overcome by introducing suicide genes to be used in case of unexpected reactivity after administration of transduced cells.

Several suicide gene strategies have been described so far, but we recently demonstrated that inducible Casp9 could be the best option as the suicide gene to be used, given its rapid effectiveness and recent encouraging clinical data.

Finally, the transfer of CARs approach from the bench to the bedside might be delayed by the elevated costs associated with the production of the vector for clinical use. Transposones represent a good option in this context. They are natural DNA transfer vehicles that are capable of stable genomic insertion and are capable to give high efficient long term-transgene expression, comparable to that of viral strategies. An important advantage of this approach is represented by the reduced cost associated with manufacturing clinical-grade DNA plasmids, compared with manufacturing of recombinant viral vectors that renders this system more interesting for the clinical

applicability of any gene transfer methods.

In summary, we demonstrated that CARs is a promising tool to eradicate human AML. We strongly believe that the development and the optimization of the proposed strategy could be a good potential therapeutic tool in the context of minimal residual disease in high-risk transplanted AML patients. Moreover, CAR approach could be potentially used to treat patients resistant to conventional chemotherapeutic approaches or for whom high dose chemotherapy treatment could not be proposed.

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