World Journal of Stem Cells

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DOI: 10.4252/wjsc.v7.i7.1022

World J Stem Cells 2015 August 26; 7(7): 1022-1038
ISSN 1948-0210 (online)

REVIEW

Adoptive immunotherapy for acute leukemia: New insights in chimeric antigen receptors

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Author contributions: Heiblig M designed and wrote this review; Elhamri M, Mauricette M and Thomas X provided a critical revision of the manuscript.

Conflict-of-interest statement: Authors declare that they have no competing interests.

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Received: September 5, 2014
Peer-review started: September 6, 2014
First decision: December 17, 2014
Revised: May 3, 2015
Accepted: June 18, 2015
Article in press: June 19, 2015
Published online: August 26, 2015

Abstract

Relapses remain a major concern in acute leukemia. It is well known that leukemia stem cells (LSCs) hide in hematopoietic niches and escape to the immune system surveillance through the outgrowth of poorly immunogenic tumor-cell variants and the suppression of the active immune response. Despite the introduction of new reagents and new therapeutic approaches, no treatment strategies have been able to definitively eradicate LSCs. However, recent adoptive immunotherapy in cancer is expected to revolutionize our way to fight against this disease, by redirecting the immune system in order to eliminate relapse issues. Initially described at the onset of the 90's, chimeric antigen receptors (CARs) are recombinant receptors transferred in various T cell subsets, providing specific antigens binding in a non-major histocompatibility complex restricted manner, and effective on a large variety of human leukocyte antigen-divers cell populations. Once transferred, engineered T cells act like an expanding "living drug" specifically targeting the tumor-associated antigen, and ensure long-term anti-tumor memory. Over the last decades, substantial improvements have been made in CARs design. CAR T cells have finally reached the clinical practice and first clinical trials have shown promising results. In acute lymphoblastic leukemia, high rate of complete and prolonged clinical responses have been observed after anti-CD19 CAR T cell therapy, with specific but manageable adverse events. In this review, our goal was to describe CAR structures and functions, and to summarize recent data regarding pre-clinical studies and clinical trials in acute leukemia.

Key words: Chimeric antigen receptors; Adoptive immunotherapy; Acute leukemia; T cells; Immune surveillance

Core tip: Leukemia cells ultimately escape to the immune system, due to various mechanisms such as limited availability of tumor specific T cells or down-regulation in major histocompatibility complex expression. Chimeric antigen receptor (CAR) T cell technology redirects immune reactivity towards a broad variety of chosen antigens in a human leukocyte antigen-independent manner. Recent introduction of co-stimulatory domains

in the CAR construct enhances significantly *in vitro* and *in vivo* expansion and persistence of these genetically modified T cells. First clinical trials, especially with anti-CD19 CAR T cells, report promising results in acute lymphoblastic leukemia.

Heiblig M, Elhamri M, Mauricette M, Thomas X. Adoptive immunotherapy for acute leukemia: New insights in chimeric antigen receptors. *World J Stem Cells* 2015; 7(7): 1022-1038 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i7/1022.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i7.1022

INTRODUCTION

Despite recent advances in therapeutics over the last decades, relapses remain a major concern in acute leukemia (AL). Despite complete remission (CR) achievement, leukemia stem cells (LSCs) resist to therapeutic strategies, hiding into bone marrow hematopoietic niches or other unknown sanctuaries[1]. More than evading apoptosis and self-sufficiency of growth signals, these leukemia cells are also characterized by their ability to evade the immune system. Malignant cells escape such immune surveillance through the outgrowth of poorly immunogenic tumor-cell variants, known as immune selection, and/or through disruption of the immune system[2,3]. A robust innate immune system is mandatory to avoid relapses by targeting chemoresistant malignant cells, underlining that bone marrow should be preserved as many as possible from aggressive chemotherapy agents. Allogeneic stem cell transplantation (ASCT) is a potential way to restore the tumor cell immunogenicity by transferring a brand new immune system. However, ASCT is largely unspecific and the benefit of graft versus leukemia is offset by the potential complications related to graft versus host disease (GVHD)[4].

In order to achieve long-term survival and good quality of life, other types of immunotherapy have been developed, such as treatments using tumor-associated antigen (TAA)-monoclonal antibodies (mAbs) and more recently adoptive cellular therapies. Adoptive transferred tumor reactive T cells compared favorably with mAbs. They display direct tumor lysis, enhanced bio-distribution and synergism with the immune system through release of cytokines, and long-term memory properties. Cytokine induced killer (CIK) cells are *in vitro* manufactured T lymphocytes with natural killer (NK) cell properties. They can be extracted from human peripheral blood, bone marrow or cord blood mononuclear cells[5]. They showed a non-major histocompatibility complex (MHC)-restricted lysis function on a broad spectrum of tumor targets *in vitro*, which was confirmed *in vivo*[6,7]. However, first clinical results were not convincing, probably due to a lack of specificity, with a limited basal anti-leukemia activity and a rapid exhaustion of these cells[8,9]. Adoptive transfer of autologous or allogeneic manipulated T cells has proven to be safe and extendable in clinical practice. In patients presenting prolonged lymphopenia, adoptive multi-virus specific T cell transfers have showed promising data in reconstituting anti-viral immunity after SCT or in patients infected by human immudeficiency virus[10,11]. Another approach is to genetically engineer lymphocyte subsets to redirect their natural immune response, correct impaired immunity, and improve T cell anti-tumor effector response.

First described in 1989, the concept of the genetic redirection of T cells is based on chimeric antigen receptors (CARs), which are recombinant receptor molecules genetically transferred, redirecting T cells against a specific TAA[12]. CARs are composed of 3 distinct domains, each displaying their own functions. The extra-cellular domain is generally constituted by a single chain variable fragment (scFv). Its targeting moiety derived from the fused variable heavy and light chains of an antibody. The trans-membrane domain is connected to the scFv through a "spacer" to provide flexibility and stable expression of the extracellular moiety. The intra-cellular signaling domain, usually derived from the CD3ζ-chain of the T cell receptor (TCR)-CD3 complex, mediates activation of CAR T cells. CAR T cells overcome some primordial limitations of TCR by targeting antigens in a non-MHC manner, and can recognize tumor independently of human leukocyte antigen (HLA) molecules[13]. After years of investigations to implement gene transfer tools and codify good manufacturing practices, CARs have been considered for human application. The first clinical trials have shown promising results in hematological diseases[14-17].

In this review, we focused on the CAR backbone technology and its application in the setting of human AL therapy.

CHIMERIC ANTIGEN RECEPTOR T CELLS: STRUCTURE AND FUNCTIONS

CAR is an artificial T cell surface receptor that simulates the physiological response of a T cell receptor and targets native cell surface antigens. However, CARs have the ability to target molecules that can be recognized without requiring peptide processing or HLA presentation. Unlike regular T cells, CAR T cells can restore immunogenicity of tumor cells. They recognize antigens in any HLA background, independently of the patient haplotype, and without any cross-reactive action toward endogenous antigens[18,19]. With a capacity of binding to any cell surface antigens, including proteins, carbohydrates and glycolipids, CARs can respond to a broader range of targets than native TCR. However, antibody-mediated recognition by CARs is not restricted to peptide antigens and does not exclude targeting MHC presented peptides. Engineered NK cells harboring a scFv specific for HLA*A2 (MHC class I) carying a peptide derived from the Epstein-Barr virus latent

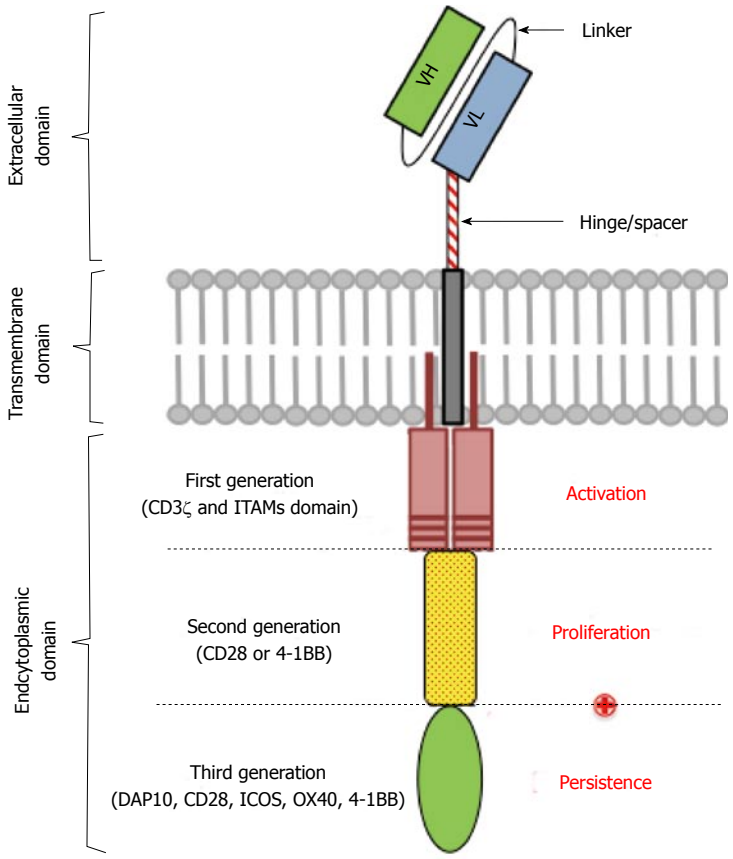

Figure 1 Schema of the general structure of a chimeric antigen receptor. The antigen recognition moiety is composed of the variable domain of heavy (VH) and light (VL) chains of the antibody, specific for a native tumor-associated antigen expressed on the surface of malignant cells. This structure is connected to the cytoplasm by a spacer, generally derived from CD8 or CD28 subunits, and a transmembrane domain. First generation chimeric antigen receptor T cells were solely composed of a single intracellular domain generally derived from CD3ζ subunit and its immunoreceptor tyrosine-based activation motifs (ITAMs), essential for Lck recruitment and full downstream T cell receptor-like signaling transduction. In order to improve engineered T cells proliferation and persistence, investigators introduced successively one (second generation) or ⩾ 2 additional intracellular signaling domains (third generation). ICOS: Inducible costimulator.

protein EBNA3C have been recently developed[20]. These CAR NK cells showed substantial cytolytic activity against peptide-pulsed HLA-A2$^+$ antigen-presenting lymphoblastic B-cell line in a peptide-specific, HLA-restricted manner without cross reactivity with native HLA*A2, paving the way toward highly sensitive and specific target cell killing[21].

CARs engage the target *via* their extra-cellular recognition subunits, usually a scFv, but other strategies are actually explored, such as antigen-binding domains derived from natural ligand receptors (*i.e.*, NKG2D)[22,23]. These TAA extra-cellular moieties are either derived from murine or humanized Fab's (variable fragment of an antibody) or synthesized *via* phage display libraries (Figure 1). Because of their accessibility, murine scFvs are the most frequently used, but they are considered more immunogenic than those derived from human libraries. The major risk is to induce humoral and/or cell mediated immune responses as previously reported[24]. There is currently broad evidence that distinct epitopes of a same antigen, as well as their distance to the cell membrane, have not the same potential upon T cell activation. Based on the kinetic segregation model (KSM) relating TCR activation and ligands size-sensitivity, several reports support that this also occurs in CAR T cells[25]. The KSM implied that TCR engagement through distal epitopes binding creates larger TCR-MHC-peptide complexes and that close-contact zone displays the synapse to phosphatase action such as CD45 or CD148 repressing TCR signaling. Conversely, targeting more proximal epitopes favored more potent TCR-MHC interaction and more efficient downstream signaling[26]. In a study assessing the anti-leukemia effect of anti-CD22 CAR T cells, it was showed that proximal targets have superior anti-leukemia effects[27]. This was confirmed by further published data[28]. An increased affinity for the target was not necessarily associated with an increased cytotoxicity. CARs with high affinity for an antigen had a higher proliferation rate than CARs with lower affinity, although this increased affinity was not correlated with higher T cell effector functions[29,30]. Similarly, the effects of antigen density are not well understood but it seems that CARs exert various cytolytic activities according to antigen expression. In a CD123 CAR T cells model, it was demonstrated that engineered T cells eliminated CD123^{high} cells, while CD123^{low} targets survived in co-culture[31]. A high affinity and density regarding a specific antigen is not an absolute condition for an optimal anti-tumor activity, but this should be considered as an advantage in order to reduce the severity of the "on target/off tumor" effect. Despite technical considerations, identifying the perfect target remains a matter of debate, specifically in AL. The ideal antigen target should be homogenously expressed on malignant cells without ubiquitous expression on healthy tissues, and should be critical for tumor cell survival. CD19 is currently the best candidate in B cell precursor acute lymphoblastic leukemia (ALL). The situation is more complex in acute myeloid leukemia (AML), for which the best target still remains to be defined.

A short spacer region, which is the least aspect

of CAR design under discuss, generally follows the scFv's moieties. With the trans-membrane domain, these structures contribute to flexibility, accessibility and synapse formation of the extra-cellular complex. They also seem to influence CARs specificity[32,33]. The simplest form of spacer region is that derived from human immunoglobulin (Ig) Fc (constant fragment) (*i.e.*, IgG4) potentially linked with CH_2CH_3 molecule(s). However, constant regions from human CD8α and CD28 have been until now the most frequently used in pre-clinical assays (Figure 1). Following scFv binding moiety interaction with its target, CAR T cells need an active signaling transfer through their intra-cellular domain for proliferation, cytokine production, and acquisition of effector functions. In order to enhance T cell persistence and anti-tumor functions *in vivo*, research in CAR T cells has been focused on co-stimulation over the last decade.

CO-STIMULATORY DOMAINS IN CAR T CELLS: FIRST TO THIRD GENERATION

First generation CARs were composed only of one signaling domain such as the CD3ζ-chain of the TCR-CD3 complex or the γ-chain of the high affinity IgE Fc receptor (FcεRI) initially associated with antigen-specific scFv derived from a murine antibody (Figure 1). They were associated with the activation of the phosphatidyl inositol and tyrosine kinase pathways. However, they failed to mediate persistent and robust anti-tumor activity[15,34,35]. Clinical trials with first generation CAR T cells have been conducted in different settings. Some of them are still ongoing, but first results were rather disappointing (Table 1). In a study exploring CD20 CAR T cells in 7 patients with relapsed or refractory indolent lymphoma, only a clinical partial response was obtained in one patient. Moreover, after three infusions of autologous CD20 specific T cells, T cell persistence was less than 10 wk (15-65 d), even after interleukin (IL)-2 administrations[36]. In a cohort of 4 patients with non-Hodgkin lymphoma (NHL) treated by CD19-CD20 CAR T cells, no objective response and a very limited persistence of T cells was observed. In other patients, cellular anti-transgene immune rejection responses (specific immune response against transfected T cells) have been observed, showing that the immunogenicity of the transgene used is of major importance[37]. In ALL, there is no clinical data available with first generation CAR T cells. However, two clinical trials based on CD19 scFv-CD3ζ (19z1) CARs with or without IL-2 supplementation are currently recruiting (Table 1). Nevertheless, CD3ζ alone is able to induce a TCR-like signal through immunoreceptor tyrosine-kinase based activation motif (ITAM) phosphorylation and lymphocyte-specific protein tyrosine kinase (Lck) recruitment, which are indistinguishable from those generated by an intact TCR currently used to provide signal one.

It is well known that TCR engagement (signal one) without co-stimulatory signal (signal 2) lead to T cell anergy and rapid activated induced cell death (AICD). Signal 2 is provided by engagement of co-stimulatory domains, mediated essentially by the CD28 superfamily, involving receptors for the agonistic CD80 (B7.1) and CD86 (B7.2) ligands. However, these ligands are generally missing in most cancer cells. This plays a major role in the immune surveillance escape. In this setting, second generation CAR T cells have been designed by adding an additional intra-cellular co-stimulatory domain such as CD28 (forming at the end one polypeptide single chain) to the CAR backbone (Figure 1)[38,39]. CD28, a disulfide-linked homodimer of the immunoglobulin superfamily is activated by binding either B7.1 or B7.2, expressed on the surface of antigen presenting cells. This represents one of the most potent co-stimulatory pathways. It facilitates Lck recruitment to ITAMs and the formation of the linker activation (LAT) complex. This structure is necessary for signal transduction through phospholipase C gamma (PLCγ) leading to IL-2 promoter activation and full T cell activation. Other co-stimulatory domains have been tested, such as members of the tumor necrosis factor (TNF)-receptor family [4-1BB (CD137) or OX40 (CD134)], ICOS or DAP10[28,40-42]. Second generation CAR T cells generally show sustained polyclonal proliferation without B7-CD28 engagement, enhanced IL-2, interferon-gamma (IFNγ), TNF-alpha (α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) cytokine production and enabled resting primary T cells to survive[43]. In two recent clinical studies, it was reported that CAR (scFv-4-1BB-CD3ζ) T cells undergo 1000-fold amplifications *in vivo* compared to only 3.75-fold reported in first generation CAR clinical trials[44,45]. This may be related directly on the co-stimulatory signal that seems to counteract the inhibition effect of tumor growth factor β1 (TGF-β1) secreted by regulator T cells (T_{reg}) on proliferating CAR T cells[46]. Generally, TGF-β1 represses IL-2-dependent T cell amplification, CD122 up-regulation (IL-2R-α), and IFNγ production. Dual signaling provided by second-generation CAR T cells enhanced engineered T cell persistence comparatively to their first generation counterparts. This was demonstrated in patients treated with either CD3ζ or CD3ζ-CD28 CARs[47]. Besides overcoming PD1-mediated inhibition (an activation-induced inhibitory receptor expressed on T cells), co-stimulation through CD28 induces intrinsic survival signals independently of the exogenous survival signals mediated by IL-2. *In vitro*, CD28-B7 interaction leads to an up-regulation of Bcl_{XL}, an anti-apoptotic protein of the Bcl2 family, and to resistance to apoptosis by Fas (CD95, APO-1) cross-linking[48]. This interaction enable protection from intrinsic and extrinsic cell death signals and promotes survival of the expanding lymphocyte population[49]. Surprisingly, it seems that the adjunction

Table 1 Ongoing clinical trials recruiting patients with acute leukemia

Clinical trial.gov ID	Center	Disease	Antigen (scFv)	CAR signaling domain	Vector	Transplantation history	Origin	Lymphocyte depletion	Patients age
Acute lymphoid malignancies									
NCT01044069	MSKCC	B-ALL	CD19	CD3ζ-CD28	γ-retrovirus	Autologous	Autologous	Chemo	⩾ 18 yr
NCT01860937	MSKCC	B-ALL	CD19	CD3ζ-CD28	γ-retrovirus	Autologous	Autologous	Cy	up to 26 yr
NCT01430390	MSKCC	B-ALL	CD19 VST (EBV)	CD3ζ	Lentivirus	Relapse post-ASCT	Allogenic	Chemo	up to 19 yr
NCT01626495	CHOP	B-ALL	CD19	CD3ζ-4-1BB *vs* CD3ζ	Lentivirus	Relapse after ASCT	Autologous	Chemo	1-24 yr
NCT02030847	University of Pennsylvania	B-ALL	CD19	CD3ζ-4-1BB	Lentivirus	Relapse ± after ASCT	Autologous	NS	⩾ 18 yr
NCT00840853	BCM	B-ALL/NHL/CLL	CD19 VST (EBV)	CD3ζ	γ-retrovirus	Post-ASCT	Allogenic	No	No limit
NCT00586391	BCM	B-ALL/NHL/CLL	CD19	CD3ζ-CD28	γ-retrovirus	No ASCT	Autologous	NS	⩾ 18 yr
NCT02132624 (not open)	Uppsala University	B-ALL/NHL	CD19	CD3ζ-4-1BB-CD28	γ-retrovirus	Relapse ± after ASCT	Autologous	NS	⩾ 18 yr
NCT01593696	NCI	B-ALL/NHL/CLL	CD19	CD3ζ	γ-retrovirus	Relapse ± after ASCT	Autologous	Flu/Cy	1-30 yr
NCT01087294	NCI	B-ALL/NHL/CLL	CD19 VST (EBV)	CD3ζ	γ-retrovirus	Relapse post-ASCT	Allogenic	Cy	18-75 yr
NCT01683279	Seattle Children Hospital	B-ALL	CD19 EGFRt$^+$	CD3ζ-CD28	Lentivirus	No ASCT	Autologous	Cy	1-26 yr
NCT02028455	Seattle Children Hospital	B-ALL	CD19 EGFRt$^+$	CD3ζ-4-1BB	Lentivirus	Relapse after ASCT *vs* no ASCT	Autologous	Cy/Flu/TBI	1-26 yr
NCT01865617	FHCRC	B-ALL/NHL/CLL	CD19	CD3ζ	Lentivirus	> 1st relapse	Autologous	NS	⩾ 18 yr
NCT01475058	FHCRC	B-ALL/NHL/CLL	CD19 VST (CMV, EBV)	CD3ζ	Lentivirus	Post-ASCT	Allogenic	No	18-75 yr
NCT02051257	City of Hope Medical Center	B-ALL/NHL/CLL	CD19 EGFRt$^+$	CD3ζ-CD28	Lentivirus	Post-ASCT	Autologous	Chemo	⩾ 18 yr
NCT02146924 (not open)	City of Hope Medical Center	B-ALL	CD19 EGFRt$^+$	CD3ζ-CD28	Lentivirus	Relapse/ progression ± after ASCT	Autologous	Cy	⩾ 18 yr
NCT01195480	London University College	B-ALL	CD19 (EBV-CTL)	CD3ζ	γ-retrovirus	Relapse after 1st ASCT	Allogenic	NS	Up to 18 yr
NCT01864889	Chinese PLA General Hospital	B-ALL/NHL/CLL	CD19	CD3ζ-4-1BB *vs* CD3ζ	γ-retrovirus	Relapse ± after ASCT	Autologous	NS	5-90 yr
NCT01735604	Chinese PLA General Hospital	B-ALL/NHL/CLL	CD20	CD3ζ-4-1BB *vs* CD3ζ	γ-retrovirus	Relapse ± after ASCT	Autologous	NS	18-90 yr
NCT01362452	MDACC	B-ALL/NHL/CLL	CD19 (cord blood derived)	CD3ζ	Transposon	Relapse post-ASCT	Allogenic	No	1-75 yr
NCT01497184	MDACC	B-ALL/NHL/CLL	CD19	CD3ζ	Transposon	Post-ASCT	Allogenic	No	1-65 yr
Acute myeloid malignancies									
NCT01864902	Chinese PLA General Hospital	AML	CD33	CD3ζ-4-1BB *vs* CD3ζ	γ-retrovirus	Relapse ± after ASCT	Autologous/ Allogenic	NS	5-90 yr
NCT02159495	City of Hope Medical Center	AML	CD123 EGFRt$^+$	CD3ζ-CD28	γ-retrovirus	Relapse/ refractory AML	Autologous	Cy	⩾ 18 yr

CAR: Chimeric antigen receptor; AML: Acute myeloid leukemia; ASCT: Allogeneic stem cell transplantation; B-ALL: B-lineage acute lymphoblastic leukemia; BCM: Baylor College of Medecine; CHOP: Children Hospital of Philadelphia; Chemo: Chemotherapy prior CAR T cells infusion; CLL: Chronic lymphoid leukemia; Cy: Cyclophosphamide; FHCRC: Fred Hutchinson Cancer Research Center; Flu: Fludarabine; MDACC: MD Anderson Cancer Center; MSKCC: Memorial Sloan-Kettering Clinical Center; NCI: National Center Institute; NS: Not specified; NHL: Non Hodgkin lymphoma; VST: Virus specific T lymphocytes; EBV: Epstein-barr virus; CTL: Cytotoxic T-cell lymphoma.

of a co-stimulatory domain (*i.e.*, CD28) do not modify the threshold of antigen-dependent MHC-independent T cell activation[50]. It seems therefore that incorporation of at least one co-stimulatory domain in a CAR cons-

truct is mandatory for complete T cell activation. However, a major remaining issue concerns the potential superiority of one endodomain comparatively to another. Because of their heterogeneity, some CD28 or 4-1BB-based second generation CARs have been compared, essentially in mice models, and showed contradictory results. Regarding cell proliferation and cytokine production, second generation CD28 transfected CAR T cells were superior to CD19z1 (first generation CAR T cells), but also to constructs with 4-1BB, OX40 or DAP10 endodomains[51]. Conversely, it was showed that $CD28^-$ and 4-1BB-based CAR T cells, directed against the same epitope (SS1 scFv-based chimeric receptor), have the same anti-tumor activity[52]. Other pre-clinical studies, containing 4-1BB signal transduction endodomain, exhibited a greatest anti-leukemia activity and prolonged *in vivo* survival[53]. In AL, most of the current clinical trials use indistinctly CD28 or 4-1BB endodomains. Comparisons between intra-cellular signaling domains should take into account all representatives of distinct antigen epitopes, their location, their density, and their affinity for the target in order to clarify their exact contribution in the anti-tumor activity.

More recently, third generation CAR T cells have been developed, including more complex structures with three or more signaling domains, enabling wider T cell effector function in a specific fashion (Figure 1). In mice models, the inclusion of multiple endodomains showed enhanced functionality and greater potency of tumor targeted T cells *in vivo* and *in vitro.* However, this was not confirmed in other models[54-56]. In a study comparing the consequences of CD28, OX40 and 4-1BB co-stimulation, it was showed that third generation CD3ζ-CD28-OX40 CARs prevent AICD in memory T cells, and substantially improve effector functions in both naive and memory CD4 and CD8 T cells. They also increase cytokine production comparatively to second generation CAR T cells combination[57]. Considering these results and the substantial improvement in terms of efficacy, the addition of a third endomain should be associated with increased long-term persistence and should delay anergy. However, a pilot study, using CD28-4-1BB-based CARs in 4 NHL patients, reported one partial response, one disease progression, and 2 cases free of disease at 12 and 24 mo, respectively. In this study, engineered T cells were detectable up to 12 mo after infusions by quantitative polymerase chain reaction (PCR) at low levels (< 1%)[58]. Because of discrepancies among pre-clinical and clinical results, further investigations are warranted to optimize our understanding regarding CAR T cell signaling. Because massive activation and uncontrolled proliferation of genetically modified T cells has been associated with serious adverse events, investigators should take into account these features and find the right balance between efficiency of these engineered T cells and an acceptable tolerance.

CARS MANUFACTURING: A PERSONALIZED ASSEMBLY-LINE WORK

Investigators developed distinct approaches to introduce efficiently CAR constructs into T cells. Actually, most of them utilized viral transduction systems (mostly γ-retroviral) leading to permanent sequence integration into T-cell DNA. However, this kind of vehicle presents disadvantages: (1) high costs related to the production process; (2) risks of CAR expression silencing due to terminal repeat alterations; (3) risks of insertional mutagenesis; and (4) production of replication competent virus. Alternatively, lentiviral vectors, which are theoretically less genotoxic, can permanently transduce T cells, but display inferior yields of transgene integration[59,60]. However, no genotoxic-related events related to viral vectors have been reported until now in human clinical trials using manipulated T cells[61]. Moreover, residual vector sequences present in the genome lead to immunogenic epitope expression and can increase anti-CAR mediated response. First clinical trials in B-cell lineage ALL using viral vectors reported very limited gene transfection efficiency (5%-15%), without impairing anti-leukemia effect[62,63].

Non-viral based DNA transfection represents an interesting alternative because of its low cost and its theoretically limited insertional mutagenesis [insertion at thymidine-adenosine (TA) dinucleotide base pairs and non-preference of integration into transcriptional units]. DNA plasmids were the first non-viral vehicles that have been tested. They seemed less immunogenic and independent of the sequence size comparatively to viral vectors. However, because of low chromosomal intake and sustained transgene expression, long-term cultures are required to obtain a sufficient number of CAR-modified T cells, despite a potential negative effect on T cell activity and *in vivo* expansion. One major issue with plasmid vectors is represented by the genomic integration of multiple copies and transfected DNA hypermethylation, leading to transgene silencing[64]. Originally described in 1996, "Sleeping Beauty (SB)" transposase/transposon, based on the concept of "jumping genes" discovered by McClintock, enabled to overcome these issues and to restart non-viral DNA vectors[65]. Generally, one plasmid is loaded with a transgene, named transposon, surrounded by inverted repeats that contain short direct repeats (Figure 2). These sequences are recognized by an enzyme (transposase) transported in a second plasmid, which cut the transposon out of the plasmid. The genetic cargo is then delivered into the targeted cell cytoplasm by any of the established non-viral delivery techniques, and inserted randomly into TA dinucleotide base pairs of the recipient (Figure 2). Costly but safer, the SB platform displays attractive features for human gene therapy, to such an extent that many protocols for manufacturing clinical grade T cells recently came to light[66]. At the last European Bone Marrow Transplantation (EBMT)

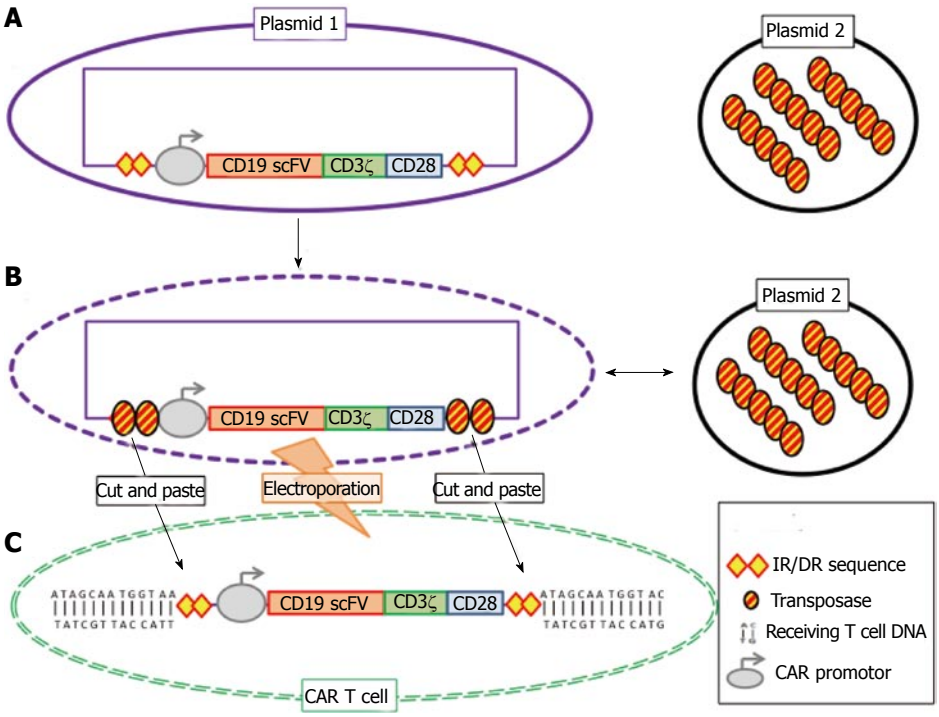

Figure 2 Schema of the transposon/transposase "sleeping beauty" system. A: The genetic cargo of a first plasmid (plasmid 1) is the chimeric antigen receptor (CAR) (anti-CD19 CD3ζ-CD28) flanked by inverted/short direct repeats (IR/DR). The whole set composed the transposon. The enzyme transposase is loaded in a second plasmid, which is specific of the IR/DR sequence; B: Materials of the two plasmids are fused together by electroporation, an electric current opening pores or channels at the cell surface. Transposase binds to the IR/DR sequences; C: The enzyme cut out of the plasmid the flanked sequences (CAR transgene with its promotor) and transfect the genetic cargo into a random DNA sequence of the recipient (T cell).

meeting, promising results were reported about the production of third generation CD19/CD123 CIK CAR cells. With about 50% CAR transfection yields, investigators are now able to greatly expand CAR T cells and produce clinical grade within 3 wk (*vs* 90 d with retroviral vectors). To our knowledge, two human clinical trials using the SB system are currently ongoing at the MD Anderson Cancer Center (Table 1)[67].

Clinical trials have reported serious adverse events during CAR T cells therapy in early clinical phase trials, which were related to uncontrolled T cells proliferation, cytokine storm, or "off tumor" effect[68,69]. In this context, strategies to incorporate abortive mechanisms inside the genomic load have emerged. Based on *ex vivo* genetic modification of donor T lymphocytes, suicide gene therapy with the Herpes simplex thymidine kinase (Hsv-tk)/ganciclovir (GCV) suicide system in the context of ASCT has demonstrated its safety and efficacy[70]. In order to facilitate its clinical development, this technology has been applied to CAR T cell development. Despite successes in the treatment of severe GVHD, Hsv-tk/GCV system showed some limitations: (1) the HSV-TK protein can be immunogenic in an immunocompetent situation; (2) the system can lead to unexpected elimination of modified T-cells in case of treatment with GCV; and (3) the system can show efficacy only on dividing cells[71]. Other non-immunogenic suicide systems have been developed, especially through a modified caspase 9 (iCasp9) member of the intrinsic apoptosis pathway[72]. A modified FK506-binding protein (humanized FKBP12), belonging to a family of protein which display a propyl isomerase activity, has been combined with caspase 9. Infusion of a synthetic drug, AP1903, allows the dimerization of iCasp9 and activates the apoptosis cascade. Beside optimal bio-distribution of the dimerization inducer, iCasp9-based cell safety switch offers superior pharmacodynamical properties than the HSC-tk system, and leaves GCV available for antiviral therapy[73]. The major issue of this suicide gene strategy is that anti-tumor T cells could be definitely eliminated, impairing the efficacy of cellular therapy. However, to date, no suicide gene therapy has been already used in human clinical trials with engineered CAR T cells. Although not as effective as CAR using integrating vectors technology, transient CAR expression is an interesting alternative approach currently under investigation. According to a recent study published by the University of Pennsylvania, CAR mRNA can be efficiently transduced into T cells by *in vitro* repeated electroporation procedure and administrated repeatedly safely, avoiding the risk of CAR T cells accumulation. This approach precludes transgene persistence for one week or two, and a rapid decrease of toxicity in case of adverse events after T cell discontinuation[73]. The current stocks and production rates of adequate serum for good manufacturing procedure (GMP) are insufficient regarding the outgrowing demand, suggesting a further development of serum-free conditions for cell therapy cultures[74].

CAR'S CULTURE: EXPANSION, PERSISTENCE AND TRAFFICKING

The technique and the duration of CAR T cell cultures prior CAR transfection and infusion of the product may be also critical in the setting of clinical activity. Autologous/allogeneic non modified-T cells are harvested from peripheral blood by leukapheresis, with heterogeneous threshold according to the underlying pathology. Then, naive T cells are expanded *ex vivo* generally with an artificial-antigen presenting cell system or anti-CD3/CD28 coated beads in combination with various cytokines (IL-2, IL-15, IL-17, IFNγ). As T cells are grown under GMP, expansion protocols and manufacturing input may vary among centers, depending on transduction efficiency yields, CD4/CD8 ratio, and final T cell phenotype. Most of culture protocols support the acquisition of a central memory phenotype ($CD45RA^+/CD122^{low}/CD62L^{low}/CCR7^+$), which is associated with self-renewal, high proliferation potential, and increased longevity. However, it was highlighted that proliferation, cytolytic activity, and persistence are markedly hampered by long-term *ex vivo* cultures, likely due to T cell exhaustion and telomere length shortening[36,58]. In addition, the T cell subtype transduced with CARs seems to play a key role in the clinical efficacy of adoptive immunotherapy. Due to their well-known cytolytic activity through granzyme/perforine and Fas/FasL complexes, αβ $CD8^+$ CAR T cells have been considered as the effective component of CAR T cell-based therapy and have been the major T cell subset used in pre-clinical trials. However, recent data revealed that $CD4^+$ T cell subset transfected with CARs showed cytolytic activity similar to that of $CD8^+$ T cells toward their targeted antigen. They are also useful on CAR mediated activation[75,76]. All T cell subsets play a key role in the anti-tumor immune response. The most powerful subset depends likely on tumor phenotype, its accessibility, and the tumor-cytokine microenvironment. In this setting, γδ, Th17, central memory, stem cell like memory, virus specific T cells and hematopoietic stem cells are also a part of CAR-based therapy[77-80].

For a long-term disease control by adoptive immune surveillance, engineered T cell persistence is highly warranted. Preclinical studies indicate that tumor burden and the degree of lymphocyte depletion prior T cell infusion are likely to be critical requirements for proper T cell expansion and persistence. First clinical trials indicate that persistence and *in vivo* expansion of adoptively transferred T cells is strongly correlated with treatment outcome, as previously documented with tumor-infiltrating lymphocytes (TILs) [45,69]. Conditioning chemotherapy is a way to enhance persistence and expansion of transferred CAR T cells. However, the optimal regimen is still a matter of debate. Although underlying mechanisms are not fully understood, this model probably involves homeostatic proliferation. Actually, T cell populations are tightly regulated by homeostatic mechanisms that maintain the T cell pool at a near-constant level. These mechanisms mimic chronic T cell activation, depend upon TCR/MHC self-peptide interaction, and involve cytokines, such as IL-7 and IL-15. During the first clinical trials in chronic lymphoid leukemia (CLL) patients, anti-CD19 CAR T cell infusions without conditioning chemotherapy showed limited T cells persistence and disappointing results[45]. Conversely, in a cohort of 5 relapsed ALL patients treated with 19-28z CAR T cells after lymphocyte depletion by cyclophosphamide, it was reported long-term CAR modified T cells persistence for 3 to 8 wk. Four of the five patients underwent ASCT and remained in minimal residual disease (MRD)-negative status. This study was the first to suggest an inverse relationship between CAR T cells clinical efficiency and initial tumor burden at the time of infusion. It was also shown that lymphocyte depletion prior CAR T cells infusion enhanced CAR T cells persistence[62]. Lymphocyte depletion following chemotherapy (cyclophosphamide/fludarabine) or total body irradiation (TBI) suppresses repressive cell populations, such as T_{reg}, and cells competing for stimulatory cytokines (IL-2, IL-7, IL-15, IL-21), preventing rapid anergy of adoptively transferred T cells and transient "cytokine sink". "Cytokine sink" corresponds to the competition between transferred and host T cell subsets regarding homeostatic cytokines, and can decrease *in vivo* T cell expansion[81]. IL-2, which has been widely used for adoptive T cell expansion *in vivo*, is essential for the maintenance of peripheral self-tolerance and is able to promote T cell effector functions. However, the administration of exogenous cytokines remains highly controversial. It has been shown that homeostasis and efficient suppressor functions of T_{reg} were essentially mediated by IL-2 receptor signaling. The infusion of anti-IL-2 plus IL-15 in tumor bearing mice increases effector functions of adoptively transferred T cells[82]. Although IL-2 represents a key factor in the induction of terminal differentiation of effector T cells, its use for *in vivo* expansion may impair anti-tumor immunity. More recently, cyclophosphamide lymphocyte depletion has been shown to significantly reduce FoxP3 T_{reg} and to induce IL-12 and IFNγ secretion[83]. IL-12 is a heterodimeric cytokine secreted by APCs, known to enhance T cell clonal expansion and T cell effector function in concert with TCR complex signaling (signal 1 and 2), serving as signal 3. This cytokine favors NK recruitment, but also avoid T cell anergy and T_{reg} action on effector T cells. This was illustrated by CAR targeted T cells (first generation 19z1) modified to produce autocrine IL-12 and to manage B lymphocyte depletion associated with tumor eradication[84]. The exact schedule for infusing CAR T cells after lymphocyte depletion is still a matter of debate. Nevertheless, recent evidences support the superiority of a rapid transfer in term of T cell engraftment and immune reconstitution[85,86].

AL is defined by an erratic clonal proliferation of immature hematopoietic stem cells, essentially localized in the bone marrow. Despite 60%-80% of CR

achievement after a first induction course of chemotherapy, most of AL patients will ultimately relapse, due to LSCs or persistence of resistant clones in unreachable reservoirs to standard chemotherapy. CAR T cells have to traffic in the entire body, after *ex-vivo/in vivo* activation, to clear central (bone marrow) and peripheral reservoirs. In solid tumors and lymphomas, preclinical studies and clinical trials have established that CAR T cells, specifically when using second generation CARs, can accumulate and persist over time at the site of disease[47,62]. In a recent phase I study using anti-LeY CAR T cell therapy in AML, it was demonstrated by detection of a transgene derived PCR signal and by radiolabeled cells that CAR T cells have a systemic distribution and migrate to the bone marrow[87]. Genetically engineered T cells seem to naturally traffic to the bone marrow, but also to the central nervous system (CNS), with however 5 to 10-folds less than what is observed in blood[17,44]. The reasons why CAR T cells naturally cross the meningeal barrier remain intriguing. However, this is a major concern regarding B cell lineage-ALL, for which CNS involvement could have dramatic repercussions on treatment outcome. Lymphocyte depletion by cyclophosphamide or TBI stimulate the production of chemo-attractants by the microenvironment, and thus favor CAR T cells migration and engraftment in the bone marrow[88]. Because chronic activation is associated with down-regulation of homing receptors such as CD62L, investigators are currently exploring genetic expression of chemokine/cytokine receptors (*i.e.*, CXCR2) and *ex vivo* cell surface glycan engineering (*i.e.*, modifications of fucosyltransferase, an enzyme involved in hematopoietic stem cell homing) in order to enhance bone marrow trafficking, which is critical for successful leukemia cell eradication[89,90].

CARS IN ACUTE LEUKEMIA: A MATTER OF TARGET

Acute lymphoblastic leukemia

Historically, the first hematological malignancies treated with CAR T cells were CLL and lymphomas from the B cell lineage. As mentioned above, several issues have to be considered to select a target for CAR-mediated tumor cell recognition and unfortunately most of the TAA are self-antigens expressed on healthy tissues. In this setting, CD19 arise as a perfect target antigen, since it is expressed in almost all B cell malignancies (except 5% of undifferentiated immature B cell lineage-ALL) and long-term B cell depletion is generally well tolerated (*i.e.*, chimeric monoclonal antibody rituximab). CD19 is expressed on normal B lineage cells from pro-B-cells to mature B cells and plasma cells, but hematopoietic stem cells and other tissues lack this antigen expression. CD19 is thought to play a role in the balance between self-tolerance and antigen activation of the B cell receptor (BCR) complex in a specific and sensitive manner. Another hypothetical advantage of CD19 is that CD19-positive cells are constantly produced in the bone marrow, thus providing an inexhaustible source of antigen stimulation. It was shown that CD19 was involved in Myc driven B cell oncogenesis in a BCR-independent manner, through paired box protein 5[91]. Although they are sharing the same target, B cell lineage malignancies may respond differentially to adoptively transferred T cells. Based on promising preclinical data with first and second-generation CARs, first trials with anti-CD19 CARs were designed in patients with recurrent indolent NHL or CLL. They showed promising results with prolonged CR[37,47,69]. However, the initial enthusiasm was hampered by further trials showing no CR achievement and very limited CAR T cells persistence[47,62]. Discrepancies among studies could be attributed to the suppressor role of the tumor microenvironment, differences in treatment history, pretreatment tumor burden, potential tumor resistance to the lymphocyte depleting agent, and/or impaired T cell immunological function in lymphomas[92,93]. CD-19-CAR T cells appeared as the best choice for the treatment of B-cell lineage-ALL with an overall CR rate of 80% (Table 2). The first case of treatment by CD19-CAR T cells in B cell lineage-ALL has been published by the Memorial Sloan Kettering Cancer Center. The patient was in early relapse and has been treated with second generation 1928z CAR T cells (second generation CAR T cells anti-CD19 scFv CD28-CD3ζ). After CR achievement, the patient received cyclophosphamide (3.0 g/m^2) followed after 2 d by a split dose of autologous 1928z T cells (1.8 × 10^8 cells/kg). He underwent ASCT 8 wk after T cell infusion. Prolonged B lymphocyte depletion was observed and related to 1928z CAR T cells persistence confirmed by immunohistochemistry on bone marrow aspirates through 6 to 8 wk post-infusion[62]. This trial has been recently updated reporting about 13 patients (including the first one) with relapsed/refractory B cell lineage-ALL (of whom 3 patients with Philadelphia-positive B cell lineage-ALL). CR rate was 85% (10/13 patients) with complete molecular responses obtained 7 to 14 d after T cell infusion. Cytokine release syndrome (CRS) was observed in 6/13 patients and was controlled by corticosteroids or anti-IL-6R antibody therapy. Nine of the 13 patients underwent ASCT[94]. Another phase 1 trial was reported in 5 adults with relapsed/refractory B cell lineage-ALL. They received one single dose of autologous 1928z-CAR T cells (1.5 to 3 × 10^6 cells/kg) after administration of high-dose cyclophosphamide. All patients became MRD-negative after CAR T cell infusion. Four of them underwent ASCT and were still MRD-negative at the time of the last report. The last patient, ineligible for ASCT and further CAR T cell infusion, relapsed 13 wk after T cell administration. Relapse was due to abrogation of CAR T cells persistence[63]. Another phase 1 study, using virus-specific 1928z-CAR T cells (CD19 CAR-VSTs, cytotoxic T cells with a native receptor specificity directed to persistent human viruses) in patients relapsing after ASCT or with high-risk B cell malignancies, included 3 patients with B cell lineage-

Table 2 Completed chimeric antigen receptor T cells trials including acute leukemia patients

Indications	CAR construct	Vector	Cell dose	Pre-treatment	Patients	Responses	Relapses	CAR persistence (days median)	Toxicities	Ref.
Acute lymphoid malignancies										
Relapsed B-ALL ± post-ASCT	Anti-CD19 scFv 4-1BB-CD3ζ	Lentiviral	10^6-10^7 cells/kg	Cy	30	90% CR (27/30)	6 relapses (one $CD19^-$)	145	CRS for all responding patients (fever, ARDS. MODS)	[17,97,98]
Relapsed B-ALL post-ASCT	Anti-CD19 scFv CD28-CD3ζ	γ-retroviral	10^6 cells/kg	Cy + flu	2	100% CR (5/5)	1 transient CR	ND	Mild CRS, no GVHD	[117]
B-ALL relapsed	Anti-CD19 scFv CD28-CD3ζ	γ-retroviral	3×10^6 cells/kg	Chemo	13 (3 Ph^+ B-ALL)	85% CR (10/13)	1 NR, 1 relapse	ND	6/13 CRS	[62,94]
B-ALL relapsed without prior ASCT	Anti-CD19 scFv CD28-CD3ζ	γ-retroviral	$1.5\text{-}3 \times 10^6$ cells/kg	Cy	5	100% CR (5/5)	1 relapse (no ASCT)	ND	3/5 mild CRS (MRD^+ or bulk)	[63]
Relapsed B-ALL/ CLL post-ASCT	Anti-CD19 scFv CD28-CD3ζ VST (CMV, EBV, ADV)	γ-retroviral	1.5×10^7-1.2×10^8 cells/m^2	No	4 (4/8)	75% CR (3/4), 25% PD (1/4)	1 relapse (no ASCT)	80	No CRS, No GVHD	[95]
Acute myeloid malignancies										
Relapsed AML	Anti-LeY scFv CD28-CD3ζ	γ-retroviral	1.3×10^9 cells	Chemo	4	25% CR, 50% SD	4 relapses	14-120	Neutropenia, skin "flare" reaction, fever, rigors	[87]

CAR: Chimeric antigen receptor; ADV: Adenovirus; AML: Acute myeloid leukemia; ARDS: Acute respiratory distress syndrom; ASCT: Allogeneic stem cell transplantation; B-ALL: B-lineage acute lymphoblastic leukemia; Chemo: Chemotherapy prior CAR T cells infusion; CLL: Chronic lymphoid leukemia; CMV: Cytomegalovirus; CR: Complete remission; CRS: Cytokine release syndrome; Cy: Cyclophosphamide; EBV: Epstein Barr virus; GVHD: Graft versus host disease; MODS: Multi-organ dysfunction syndrome; ND: Not determined; NR: No response; PD: Progressive disease; SD: Stable disease; VST: Virus specific T lymphocytes.

ALL. No pre-conditioning regimen was administered. CD19 CAR-VSTs required several rounds of expansion (5-6 wk of cell culture) before infusion in order to reach clinical CAR T cell threshold. No GVHD was observed and all B ALL patients achieved CR, but relapsed between 2 to 8 mo after T cell infusion (Table 2)[95]. The largest cohort was initially reported by the Children Hospital of Philadelphia in 2013 and updated at the 2014 EBMT meeting. The study included 30 patients (25 children and 5 adults) with chemo-refractory B cell lineage-ALL or ALL relapsing after ASCT. Patients received CTL019 CARs (second generation anti-CD19-scFv CAR T cells) coupled with 4-1BB endodomain and lentivirus transfected. The median dose of CTL019 was 3.7×10^6 cells/kg administrated in 3 doses with 5×10^9 total cells as a target dose. Lymphocyte depletion regimens varied among patients, but were mostly based on cyclophosphamide administered during the week prior T cell infusion. Patients were at least in second relapse or were refractory to several lines of treatment. Overall, 90% of them achieved CR, and half of them underwent subsequently ASCT. With a median follow-up of 3.4 mo (range: 2-18 mo), only 6 patients relapsed. In one case, relapse occurred with emergence of CD19 negative blast cells. As previously reported with blinatumomab (a bi-specific T-cell engager antibody designed to redirect $CD3^+$ cytotoxic T cells to $CD19^+$ malignant B cells), this relapse from CD19 negative leukemia cells illustrates the impact of such a targeted therapy on leukemia sub-clones[96]. No such cases have been reported in CLL[93]. CRs were observed independently of the level of tumor burden before T cell infusion. Among the 16 patients treated after ASCT, T cells were efficiently collected from recipient. No GVHD recurrence was observed after CAR T cell infusions. CTL019 cells expanded to levels that were more than 1000 to 10000 times as high as the initial levels. CAR T cell persistence was observed until 6 to 18 mo. This was concomitant of B lymphocyte depletion in responding patients, as previously reported in CLL patients treated with CTL019. Long-term persistence ($\geqslant$ 145 d) was significantly associated with CR achievement. All responding patients developed some degree of delayed CRS, which was concomitant to T cell expansion and increased levels of IL-6. This seemed in relationship with the tumor burden prior CAR T cell infusion (Table 2)[17,97,98]. Overall, these first results suggest that CD19 gene-modified T cell therapy is likely not enough efficient by itself, but it should be considered as a potentially life-saving bridge to ASCT. Recently, a multi-center clinical consortium was proposed in order

to harmonize practices and further export this new technology to academic institutions.

Alternative targets are likely to be developed. CD22, a type Ⅰ trans-membrane sialo-glycoprotein expressed specifically on B lineage cells, is closely related to the BCR pathway. Moxetumomab pasudotox, an anti-CD22 covalently fused to a pseudomonas exotoxin, has shown promising results[99], and could be considered as a suitable possible choice. On the other hand, in order to prevent antigen loss through selective clonal pressure and to improve tumor specificity, investigators are developing combinatorial antigen recognition strategy, and oncogene targeted CARs[100,101].

Acute myeloid leukemia

Treatment of AML remains a great challenge. Whereas combined efforts in the field of intensified chemotherapy, SCT, and supportive care have yielded to improve survival, more than 50% of patients will relapse. Because 70% of AML patients are over 60 years, many of them are not eligible for ASCT[102]. Recent preclinical reports demonstrated that CAR T cells have the potential to effectively and durably eradicate primitive myeloid blast cells[103,104]. Although two trials are currently recruiting in China and in Australia, only one phase 1 trial, targeting Lewis Y (LeY) antigen coupled with cytoplasmic CD28 and CD3ζ chain, has been reported so far in the literature regarding AML (Table 1). LeY antigen is a difucosylated carbohydrate antigen, part of the human blood system. It is widely expressed on AML cells, but has a limited expression in healthy tissues. However, its exact role and its functional significance for survival of the leukemia cells have to be elucidated. Four patients received 1.5 to 4.7 $\times$ 10^6 cells/kg CAR T cells 6 wk after fludarabine-based chemotherapy regimen. A modest T cell expansion and persistence (1 to 10 mo) was observed and resulted in limited advantages. Only 2 out of four treated patients showed a reduction of leukemia cells. Beside these disappointing results, this study conveys major endpoints. Infusions were well tolerated with no CRS even in cases presenting with a high tumor burden. Second, it was demonstrated by SPECT imagery that anti-LeY CAR T cells can target bone marrow leukemia cells, but also peripheral lesions (leukemia cutis). The absence of down-regulation of LeY antigen after CAR T cell infusions suggests that this target is suitable for long-term immune control of the disease[87]. However, the weak anti-leukemia activity of this antigen-based therapy should be balanced with combinatorial recognition strategy or loaded in more potent effectors such as CIK cells. Other antigens are also on the bench, such as CD33, CD123 or CD44v6, but only pre-clinical data are currently available, demonstrating a potential efficacy against AML blasts. CD33 is a member of the sialic acid-binding receptor family and is highly expressed on myeloid progenitor cells, and on the surface of 90% of AML blasts. Gemtuzumab ozogamicin (Mylotarg), an anti-CD33 humanized mAb conjugated to calicheamicin, was the first agent of its class and one of the first targeted therapies in AML. However, initial enthusiasm was tempered by inadequate efficacy, severe hepatotoxicity (*i.e.*, veino-occlusive disease) related to accumulation of the drug acting like an intercalating agent, and prolonged neutropenia[105]. Moreover, drug resistance rapidly may occur by active efflux from leukemia cells through the P-glycoprotein pump. Because of the very strong non HLA-restricted NK-like cytotoxicity and the lack of allogeneic activity in the ASCT setting, CIK have been largely used in pre-clinical studies of CAR redirected T cells in AML. Anti-CD33 CAR-T cells might have several potential advantages over gemtuzumab ozogamicin. First pre-clinical data using CIK and EBV-specific T cells endowed promising features[104,106]. Beside the anti-leukemia activity and its expected myelotoxicity, *in vitro* colony forming unit (CFU) assays showed remnant clonogenic activity of hematopoietic progenitors suggesting that toxicity is reversible. Furthermore, it was shown that anti-CD33 CAR T cells with CD28-OX40 endodomains exert cytolytic activity on KG-1 cell line, known to be resistant to gemtuzumab ozogamicin[106]. In order to avoid off-target toxic effect on hematopoietic stem cells (HSC), CD123, also known as IL-3 receptor α-subunit, appears as an attractive target. Its functional role in AML is still unknown. CD123 is widely over-expressed among AML blasts and LSCs, while its expression is lower on HSCs, monocytes, and endothelial cells. The expression of CD123 at the time of diagnosis has been associated with a poor prognosis and resistance to apoptosis. Preliminary *in vivo* data showed that one single administration of anti-CD123 CAR T cells led to an immunologic memory associated with a specific anti-tumor response, yielding to long-term survival in mice engrafted with AML cell lines or fresh AML cells[107]. Results of CFUs, regarding distinct anti-CD123 CAR constructs, were heterogeneous. However, one study revealed a limited activity against normal $CD123^{low}$ expression on endothelial cells and monocytes[31,103,104]. It is therefore hypothesized that suboptimal affinity of the scFv for CD123 allows recognition of targeted cells by CAR T cells, according to their antigen density and their expression of co-stimulatory molecules[108]. The hyaluronase receptor CD44, a ligand for E-selectin, is broadly expressed on malignant cells in hematological malignancies. It plays a crucial role in the bone marrow homing of initiating leukemia cells and in interactions with the microenvironment. It was recently showed that CD44 inhibition drives leukemia cells into differentiation and apoptosis by dislodging them from the osteogenic niche[109]. The isoform variant 6 (CD44v6) recently emerged as one of the most promising TAA for AML. It is absent on normal HSCs, but is over-expressed in 80% of AML cells[110,111]. It has been demonstrated that anti-CD44v6 redirect T cells were able to efficiently kill leukemia cells, while sparing HSCs and keratinocytes that expressed low levels of CD44v6[72]. These findings suggest that "off tumor" target expression levels do

not accurately predict susceptibility to CAR T cells[72]. All together, these results suggest that anti-CD123 and anti-CD44v6 CARs are safer than anti-CD33, but *in vitro* data have to be confirmed in clinical practice (Table 1).

TOXICITY: NEW INSIGHTS IN CYTOKINE RELEASE SYNDROME MANAGEMENT

With the development of new therapeutic reagents, physicians have to face previously unknown toxicities. Immunotherapy adverse events are mainly related to autoimmune direct toxicity, known as "on tumor/off target effect", and to an indirect cytokine-associated toxicity, called CRS.

Indirect toxicity: Cytokine release syndrome

CRS has been initially described following mAb infusions (*i.e.*, anti-CD52 or anti-CD20) and more recently with bi-specific antibodies and CARs[112,113]. This syndrome is characterized by a massive non-antigen specific inflammatory response. CRS shares features with macrophage activation syndrome, such as cytopenia, fever, hyperferritinemia and hypofibrinogenemia. Regardless the TAA target, it seems that T cell expansion activates other hematological effectors (B cells, neutrophils, macrophages), and favors release of inflammatory cytokines, such as TNF-α, IL-2, IL-6, IL-13 and IFNγ[44,98]. Symptom onset has been reported with a widely variable timing among clinical trials, ranging from 1 d to 3 wk for anti-CD19 CARs. All CRS reported with anti-CD19 in ALL trials were related to the tumor burden prior the first infusion and corresponded with maximal *in vivo* T cell expansion. Symptoms of CRS were drastically mild or even absent with anti-LeY in AML and with anti-CD19 CARs in CLL[45,87]. The magnitude of immune activation after engineered T cell infusion is therefore dependent upon the underlying condition, the remaining lymphocyte pool, and CAR T cell features. Symptoms are not specific and confusion can be made with those of infections. Fever appears as the hallmark of CRS, and arises generally concomitantly to rigors, myalgia, and gut disorders. However, other life threatening complications can occur. Recently, IL-6, a pleiotropic cytokine with ambivalent functions, emerged as the gatekeeper in the pathophysiology of CRS. In this setting, tocilizumab, an anti-IL-6R mAb initially developed for rheumatologic autoimmune diseases, has proven its efficacy in severe CRS. This agent showed a safe profile with few side effects at the recommended dose (one injection at 8 mg/kg for children and 4 mg/kg for adults). It did not seem to alter anti-leukemia functions of the transferred T cells. Alternative therapy in non-responders could involve anti-TNF-α mAb (infliximab), soluble TNF-α receptor (etanercept), or corticosteroids especially in patients presenting neurological symptoms[114]. Cardiac complications (similar to stress cardiomyopathies) are non frequent and generally reversible events, although potentially fatal. Neurological symptoms include mainly headache, dysphasia, confusion, seizure. Magnetic resonance imaging has shown abnormalities consistent with a mild encephalopathy with reversible splenial lesion syndrome, as observed in severe viral infections. The exact pathophysiology of neurological features during CRS is not fully understood, but seems to be related to a direct neurotoxicity of IL-6[115]. IL-6 levels in cerebrospinal fluid have been monitored during CRS and have shown high levels. It can be hypothesized that systemic inflammatory response after CAR T cells infusion could lead to permeable blood-brain barrier, yielding trafficking of IL-6 and activated immune cells to CNS. At least for CTL019, CAR T cells revealed to cross the blood-brain barrier and to be evolve in the neurological symptoms, which can be overcome by tocilizumab therapy. However, the occurrence of neurological symptoms can be enhanced by the IL-6R inhibitor. Tocilizumab may inhibit the IL-6 receptor mediated clearance and may allow transient increase of IL-6 levels. In patients with severe neurological symptoms, but without other life-threatening organ failure, it has been recommended to treat with corticosteroids (especially dexamethasone)[116]. Acute respiratory distress syndrome, hepatic/renal failure, disseminated coagulopathy have also been reported.

Direct toxicity: "On tumor/off target effect"

Prolonged B lymphocyte depletion illustrates perfectly the antigen driven direct toxicity, provided by anti-CD19 CAR T cells and hence a theoretical immunodeficiency. Although B lymphocyte depletion has been profound in reported clinical studies, its durability appears as a sign of prolonged anti-tumor response. This condition is very close to that of patients with X-linked agammaglobulinemia[97]. While B lymphocyte depletion increases the risk of opportunistic infections, this may be improved by intravenous immunoglobulin therapy. Although patient follow-up is relatively short, patients treated with anti-CD19 CAR T cells did not show until now any increase of bacterial infections. However, the development of this new technique will certainly be accompanied by the description of further new adverse events.

CONCLUSION

Immunotherapy represents certainly a great step forward in the treatment of AL. Because of the complexity of T cell repertory and its interaction with the immune system, there is, however, a long way to go before achievement of a complete and optimal understanding of this technology, as illustrated by the "on tumor/off target" paradigm. Furthermore, production of CAR T cells is time consuming, and still requires tremendous financial resources. The next challenges will be to improve cell isolation and culture of CAR-modified T cell production, to reduce the cost of the technique,

and therefore facilitate CAR T cells production and delivery in most institutions, beyond the academic research environment.

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P- Reviewer: Shimoni A **S- Editor**: Gong XM **L- Editor**: A
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