Allogeneic chimeric antigen receptor T cells for children with relapsed/refractory B-cell precursor acute lymphoblastic leukemia

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Abstract

Chimeric antigen receptor (CAR) T-cell therapy has emerged as a breakthrough cancer therapy over the past decade. Remarkable outcomes in B-cell lymphoproliferative disorders and multiple myeloma have been reported in both pivotal trials and real-word studies. Traditionally, the use of a patient's own (autologous) T cells to manufacture CAR products has been the standard practice. Nevertheless, this approach has some drawbacks, including manufacturing delays, dependence on the functional fitness of the patient's T cells, which can be compromised by both the disease and prior therapies, and contamination of the product with blasts. A promising alternative is offered by the development of allogeneic CAR-cell products. This approach has the potential to yield more efficient drug products and enables the use of effector cells with negligible alloreactive potential and a significant CAR-independent antitumor activity through their innate receptors (i.e., natural killer cells, γδ T cells and cytokine induced killer cells). In addition, recent advances in genome editing tools offer the potential to overcome the primary challenges associated with allogeneic CAR T-cell products, namely graft-*versus*-host disease and host allo-rejection, generating universal, off-the-shelf products. In this review, we summarize the current pre-clinical and clinical approaches based on allogeneic CAR T cells, as well as on alternative effector cells, which represent exciting opportunities for multivalent approaches and optimized antitumor activity.

Introduction

Autologous, patient-derived T cells engineered to express a chimeric antigen receptor (CAR) represent a breakthrough treatment that has revolutionized the management of patients with B-cell malignancies.[1-5] In 2018, the results of the ELIANA study, a global trial testing the use of tisagenlecleucel in pediatric patients with relapsed/refractory B-cell acute lymphoblastic leukemia (B-ALL), showed that a substantial proportion of these patients in whom many lines of previous therapies had failed, including allogeneic hematopoietic stem cell transplantation (HSCT), can be rescued with the approach, leading to the first ever approval of a CAR T-cell product by the American and European Regulatory Agencies, the Food and Drug Administration and European Medicines Agency, respectively.[1] Recently, the 3-year update of the ELIANA study was published and confirmed an overall remission rate of 82%, with a 3-year event-free survival of 44%.[2] After the approval of tisagenlecleucel, several real-word studies confirmed the extraordinary ability of these autologous, CD19-directed, second-generation CAR T cells to induce high rates of minimal residual disease (MRD)-negative complete remission (CR) in children with relapsed/refractory B-ALL and provided further evidence on their therapeutic potential. In particular, several studies documented that tisagenlecleucel was able to induce CR in 85-87% of the patients, with the remission being MRD-negative in most of the cases, and 1-year and 2-year event-free survival rates of 50-52.4% and 45.3%, respectively.[3-5] A high response rate can also be achieved in patients relapsing after receiving allogeneic HSCT, with the time to relapse after the transplant being a strong predictor of outcome.[4] Importantly, CAR T cells are able to induce similar results across cytogenetic cat-

egories and are associated with promising outcomes also in infants, with an unclear impact on the risk of myeloid switch.[6-8] In addition, some preliminary evidence of efficacy of CD19-CAR T cells in patients with central nervous system involvement has been reported, especially if disease control is achieved before infusion of the cell product.[9-12] Despite the success of CD19-specific CAR T-cell therapy, several limitations of the approach have emerged and will be discussed in this review, together with the possible solutions offered by the use of allogeneic CAR T cells.

Limitations of autologous chimeric antigen receptor T cells

The manufacture of autologous CAR T cells involves a complex productive procedure that starts with the collection of the patient's peripheral blood mononuclear cells to obtain viable T cells. The quality of the leukapheresis product is key in ensuring the success of the entire manufacturing process (Figure 1). Thus, collecting a sufficient number of T lymphocytes with preserved cytotoxic functions is a crucial prerequisite in autologous CAR T-cell therapy. The timing of this procedure may present a considerable challenge when dealing with heavily pre-treated patients, who are experiencing rapid disease progression and have been exposed to agents impairing T-cell function. As previously reported, the collection of peripheral blood mononuclear cells in pediatric patients poses some peculiar technical challenges, especially in, but not limited to, infants, including: the difficulty of obtaining an adequate venous access; a relatively large extracorporeal volume of the cell separator device, occasionally requiring priming with packed red blood cells; and metabolic complications due

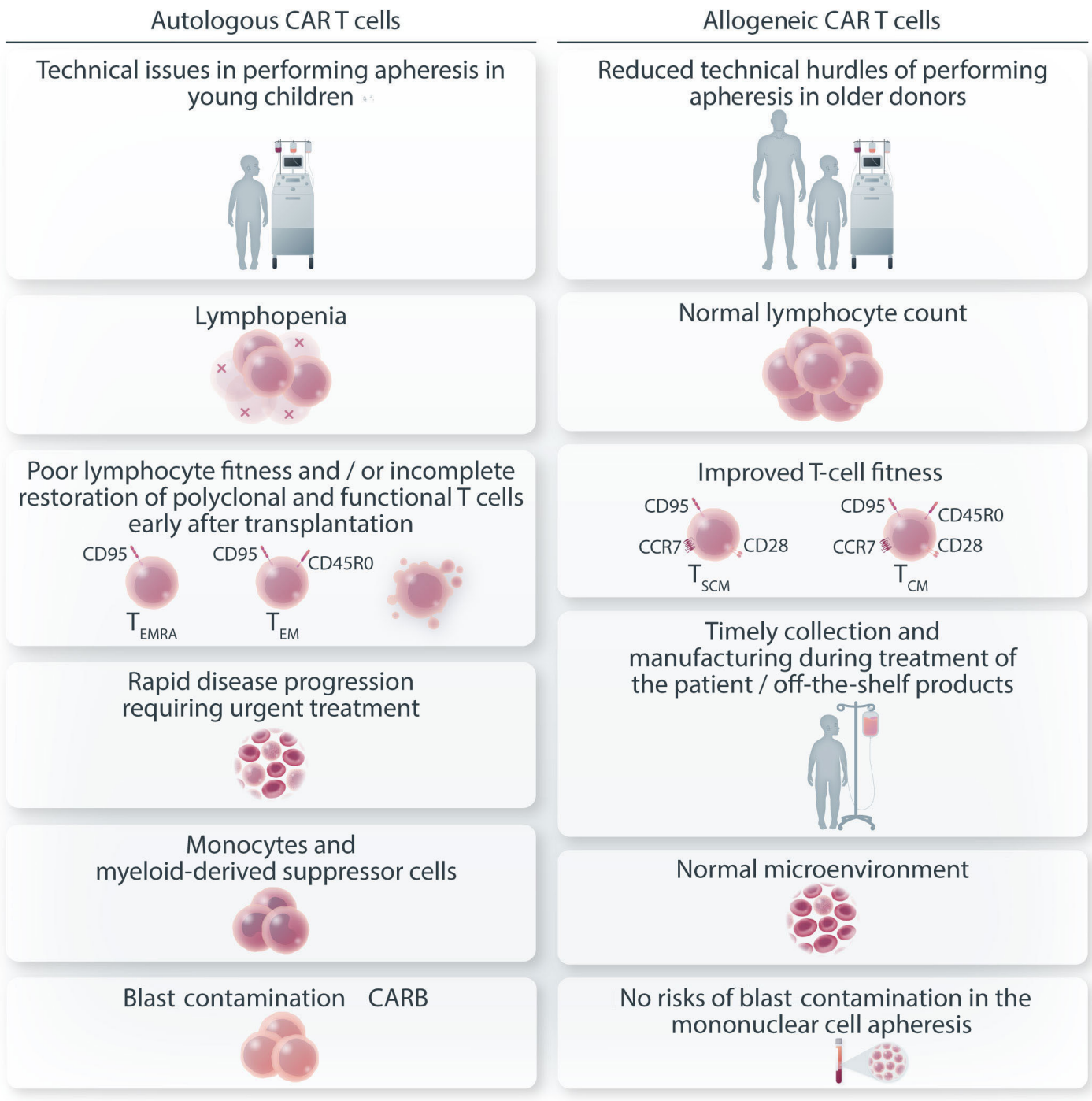

Figure 1. Limitations of autologous chimeric antigen receptor (CAR) T cells and possible solutions offered by allogeneic CAR T cells.

to citrate toxicity.[13] Although reports on the use of CAR T-cell therapies in the real-world setting have shown that most centers have gained extensive experience in the performance of leukapheresis, demonstrating the ability to obtain peripheral blood mononuclear cells from both very young/infant patients[7,8,14] and heavily pre-treated adults (all patients receiving CAR T cells as third or further lines of treatment), some patients are unfortunately not eligible for this step, due to marked lymphopenia. Data on the actual percentage of those patients for whom a sufficient number of T cells cannot be harvested are limited and these patients are often excluded from studies on CAR T cells.[15] The use of autologous lymphocytes also has another relevant clinical limitation: patients with rapidly progressing disease, a scenario frequently associated with relapsed/refractory B-ALL, require urgent treatment and may not be able to undergo the collection and manufacturing; alternatively, these procedures can ultimately be performed, but not in a timely manner before patients experience massive progression or disease-related complications, preventing the opportunity to receive CAR T cells. These important considerations are reflected in the difference in the overall CR rates in the intention-to-treat analysis of the ELIANA study, which dropped from 81% to 66%, with one patient not enrolled for apheresis-related issues and seven additional patients who died after enrollment, during the manufacturing period.[1]

Beyond lymphocyte count, other cellular components in the apheresis product can affect CAR T-cell production, including both circulating blasts and non-malignant cells, such as myeloid cells (Figure 1). Cells of myeloid origin with immunosuppressive properties have been found in patients with cancer and have been associated with manufacturing failures related to their inhibitory effects on T-cell proliferation.[16,17] Contamination of the apheresis product with blasts represents another relevant limitation to the use of autologous products, since blasts can be inadvertently transduced and aberrantly express the CD19-CAR on their surface (generating the so-called CARB). Although rare, such an event has been described and is associated with binding *in cis* of the CD19 epitope leading to its masking from recognition by CD19-CAR T cells, thereby conferring an intrinsic mechanism of resistance to the therapy.[18]

The quality of the lymphocytes used as starting material for CAR T-cell manufacturing is extremely important for the quality of the final product and, therefore, for its subsequent clinical activity, especially in the long-term. In patients with cancer, both transcriptional and functional heterogeneities have been identified in T cells, leading to the classification of these cells into distinct categories, including pre-dysfunctional, early dysfunctional, and late dysfunctional T cells.[19] These T-cell categories are characterized by elevated expression of inhibitory receptors on the cell surface, including programmed cell death-1 (PD1), lymphocyte activation gene 3 protein (LAG3), T-cell immunoglobulin mucin receptor 3 (TIM3; encoded by *HAVCR2*), 2B4, CD200, and cytotoxic T-lymphocyte antigen 4 (CTLA4). Additionally, these cells exhibit a reduced capacity to perform classical $CD8^+$ T-cell effector functions, such as the ability to produce cytokines including tumor necrosis factor, interleukin (IL)-2, and interferon-γ. This reduction in functionality is observed directly *ex vivo* or after stimulation with cognate antigens or low doses of anti-CD3 antibodies. It is intuitive that the clinical efficacy of CAR T cells generated by cancer patients may be limited by the high frequencies of these dysfunctional T cells present in both the starting material and the final CAR T-cell product. The overall fitness of patient-derived T cells is influenced by several impairing factors, including age, disease burden, and prior cancer treatments and is reasonably a constraining factor for CAR T-cell therapy. By contrast, it has been consistently demonstrated that individuals whose leukapheresis products contain a higher proportion of proliferative, less differentiated T-cell subsets tend to achieve more favorable outcomes. Indeed, the use of T cells with central memory or stem cell-like memory phenotypes for manufacturing CAR T cells has been associated with greater expansion in culture and final products characterized by superior antitumor activity and persistence than products obtained from bulk T cells.[20-22] Considering that approximately 50% of patients treated with CAR T cells experience a relapse of disease, in several cases characterized by loss of persistence of the engineered T cells and $CD19^+$ blasts, identifying potential approaches able to improve the persistence of the engineered cells remains crucial.

In addition to the abovementioned limitations, a recent German real-world experience highlighted another important obstacle to the efficacy of autologous CAR T cells. Interestingly, the German study showed that in patients relapsing after allogeneic HSCT, the use of tisagenlecleucel within 6 months after transplantation was associated with significantly worse long-term clinical outcomes and shorter duration of B-cell aplasia than those of patients relapsing >6 months after the transplant.[4] These findings suggest a lower persistence of CAR T cells generated early after allogeneic HSCT and the authors speculated that this may be associated with an incomplete restoration of polyclonal and functional T cells early after transplantation.[4]

The use of mononuclear cells obtained from healthy donors, i.e. allogeneic CAR T cells, could be a way of tackling all these issues (Figure 1). Allogeneic CAR T cells have many potential advantages, including: greater fitness, since the T cells have not been exposed to either chemotherapy, or to the immune-suppressive effects of myeloid cells and blasts; they can be obtained from all donors, overcoming the potential issue of lymphopenia, in a timely manner without the need of a wash-out windows from the therapies that would require an halt in the treatment of a patient; and they do not run the risk of being contaminated by blasts. The creation of universal, off-the-shelf products

also has the advantage of being able to build a bank of drug products that can be available anytime upon clinical need, also reducing manufacturing costs by scaling up a single process.
Several allogeneic approaches, tested in the preclinical and clinical settings, are discussed in this review.

Allogeneic chimeric antigen receptor T cells: clinical experience with donor-derived and gene-edited approaches

The clinical use of allogeneic CAR T cells has been limited by the potential occurrence of graft-*versus*-host disease (GvHD) leading, on the one hand, to the underdevelopment of this strategy and prompting, on the other hand, the identification of strategies to develop universal CAR T cells, engineered to abrogate the alloreactivity. The results of the clinical studies testing allogeneic, donor-derived CAR T cells are reported in Table 1.
The first experience on the use of allogeneic, donor-derived CAR T cells was reported in 2013 by Cruz *et al.*[23] who gave donor-derived, virus-specific cytotoxic T cells genetically modified to express a second-generation (incorporating CD28 as a costimulatory domain) CD19-specific CAR (CD19.CAR-VST) to patients with B-cell malignancies who either had disease relapse or were at high risk of disease relapse after allogeneic HSCT. Six adults and two children (9 and 12 years of age) with either chronic lymphoblastic leukemia or B-ALL were treated with CD19.CAR-VST, generated from either an unrelated donor or an HLA-matched sibling donor. An objective response was observed in two of six patients, lasting for 3 months and 8 weeks. Importantly, none of the patients developed GvHD.[23] In the same year, Kochenderfer *et al.*[24] published their preliminary experience on ten adults affected by chronic lymphoblastic leukemia, diffuse large B-cell lymphoma or mantle cell lymphoma persisting after allogeneic HSCT and at least one donor lymphocyte infusion. CAR T cells were generated from either an unrelated donor, 10/10 HLA-matched, or matched sibling donor, using a second-generation (CD28), CD19-directed construct, and infused at doses of 0.4-7.8x10^6 CAR^+ T cells/kg recipient body weight, without prior lymphodepletion. Three patients had regression of their tumors and, importantly, none of the ten patients developed GvHD.[24] In 2016, the same group reported updated results on a cohort of 20 adult patients with B-cell malignancies, including five patients with B-ALL.[25] Interestingly, but not surprisingly, patients with B-ALL had the best response to treatment, with four out of the five (80%) obtaining an MRD-negative CR after infusion; overall, eight of the 20 patients obtained either CR or partial remission. Also in the updated cohort, no new onset GvHD was observed in any of the patients.
In 2015, Dai *et al.*[26] reported on an adolescent and a young adult (15 and 23 years of age) treated with allogeneic, donor-derived, second-generation (4.1bb) CD19-CAR T cells at the doses of 4.5 and 4.2x10^6 CAR^+ T cells/kg of recipient body weight, respectively, for B-ALL. No details on preparation with lymphodepletion or on the characteristics of the donors were reported. Neither patient had a sustained response and both developed grade 2-3 acute GvHD, requiring short-term use of corticosteroids and/or cyclosporin A. Chen *et al.*[27] treated five adults and one child who had relapsed after allogeneic HSCT with allogeneic, third-generation (CD28-CD27) CAR T cells derived from haploidentical donors and infused after administration of lymphodepleting chemotherapy, mostly fludarabine and cyclophosphamide-based. The characteristics of the CAR construct employed in the study were not reported. MRD-negative CR was obtained by 83% of the patients and 50% of the patients developed grade 2-3 GvHD after infusion, which is similar to the rate previously reported by the same group with the use of donor lymphocyte infusion from haploidentical donors.[28] All patients had a short duration of remission with relapses occurring 2-7 months after response.[27]
Using a different transduction system, namely the Sleeping Beauty transposon/transposase system, Kebriaei *et al.*[29] treated 19 adults with either ALL (n=17) or diffuse large B-cell lymphoma (n=2) with second-generation (CD28) CAR T cells generated by the lymphocytes of their matched sibling donors (n=11) or haploidentical donors (n=8). CAR T cells were infused at doses ranging from $10^6/m^2$ to $10^8/m^2$, without prior lymphodepletion, at a median of 64 days after allogeneic HSCT. The 1-year progression-free and overall survival rates were 53% and 63%, respectively. Three patients developed GvHD, so the rate of this complication was comparable to that expected after allogeneic HSCT and lower than that observed after donor lymphocyte infusion.[29]
Very limited experience has been reported to date in the pediatric population. In 2021, Zhang *et al.*[30] described their experience using second-generation (either CD28 or 4.1bb) CD19-CAR T cells in 43 subjects, aged from 4 up to 60 years old, with B-ALL relapsing after allogeneic HSCT: 79% had a morphological CR and there were only two cases of acute GvHD, both grade ≤2. Unfortunately, no specific details on the outcome of the children were provided. Recently, we reported our experience on the use of donor-derived, second-generation (4.1bb) CD19-CAR T cells in 13 children and young adults with B-ALL that had relapsed after allogeneic HSCT or was extremely refractory.[31] Doses ranged between 1×10^6 and 3×10^6 CAR^+ T cells/kg and all patients underwent fludarabine/cyclophosphamide-based lymphodepletion before infusion. Only one patient developed acute GvHD, namely a young adult who was treated early after allogeneic HSCT and who had previously developed grade 2 acute GvHD.[31] Together with a mild toxicity profile, we obtained 100% MRD-negative CR in the bone marrow

Table 1. Studies describing clinical experience with the use of allogeneic, donor-derived, CD19-chimeric antigen receptor T cells.

Population	Disease	N of pts	CAR	Gene delivery	Prior LD	Cell dose	Donor	Prior DLI	GvHD	Clinical responses	Ref
Adults and children	CLL B-ALL	8 (6 adults and 2 children)	2nd gen, CD28	Retrovirus	No	$5.8x10^7$ - $1.13x10^8$ (5 pts multiple infusions)	URD, MSD	Yes (2 pts only)	No	1 CR; 2 pts treated in CR and maintaining it 1 PR 1 SD 3 PD	23
Adults	CLL B-NHL	10	2nd gen, CD28	Retrovirus	No	0.4-7.8x10^6/kg	URD, MSD	Yes	No	1 CR 1 PR 6 SD 2 PD	24
Adults	B-NHL B-ALL	2	2nd gen, 4.1bb	Lentivirus	Not reported	4.5x10^6/kg 4.2x10^6/kg	Not reported	Yes	G2-3 GvHD in both	1 CR in BM and PR in EM 1 PR (2)	26
Adults	B-ALL B-NHL CLL	20	2nd gen, CD28	Retrovirus	No	0.4-7.8x10^6/kg	URD 10/10 or 9/10; MSD	Yes	G1-2 GvHD in 2 pts (pre-existing)	6 CR (4/6 MRD-neg) 2 PR 8 SD 4 PD	25
Adults	B-ALL (>5% blasts)	6	3rd gen (CD28/ CD27)	Lentivirus	Flu-Cy	0.38-4.1x10^8/ kg (4 pts: 2 infusions)	Haplo	Yes	50% GvHD (G2-3)	4 CR (all relapsed subsequently) 1 not evaluable 1 NR	27
Adults	B-NHL B-ALL	19	2nd gen, CD28	Sleeping Beauty	No	$10^6/m^2$-$10^8/m^2$	MSD, haplo	No	G1-4 GvHD in 3 pts	9 CCR 1 CR 2 AWD 2 DIR 5 DOD	29
Adults and children	B-ALL	43	2nd gen, CD28 or 4.1bb	Lentivirus	Mainly Flu-CY	0.4-12×10^6/kg	MSD, haplo	Yes	G1-2 GvHD in 2 pts	2 died of toxicity 34 morph. CR	30
Children and young adults	B-ALL	13	2nd gen, 4.1bb	Retrovirus or lentivirus	Flu-Cy	1-3x10^6/kg	MSD, URD, haplo	Yes	G3 GvHD in 1 pt	12 CR in BM and EM 1 CR in the BM and PR in EM	31

Pts: patients; CAR: chimeric antigen receptor; LD: lymphodepletion; DLI: donor lymphocyte infusion; GvHD: graft-*versus*-host disease; Ref: references; CLL: chronic lymphocytic leukemia; B-ALL: B-cell acute lymphoblastic leukemia; B-NHL: B-cell non-Hodgkin lymphoma; gen: generation; URD: unrelated donor; MSD: matched sibling donor; CR: complete remission; PR: partial response; SD: stable disease; PD: progressive disease; BM: bone marrow; EM: extramedullary; G: grade; MRD: measurable residual disease; Flu: fludarabine; Cy: cyclophosphamide; NR: non-response; haplo: haploidentical donor; CCR: continuous complete remission; AWD: alive with disease; DIR: dead in remission; DOD: dead of disease; morph.: morphological.

and CR in five of the six cases of extramedullary diseases which, with a median follow-up of 12 months, was maintained in 62% of the patients.[31]

Overall, all these studies, although extremely heterogeneous in terms of patient population, diseases, CAR constructs and lymphodepletion, highlight the feasibility of the use of allogeneic, donor-derived CAR T cells, with a low incidence of GvHD. Together with the encouraging data on the antileukemic activity of allogeneic products, these reports suggest that the approach deserves further investigation. Interestingly, none of the group experienced rejection of the allogeneic, donor-derived CAR T cells. It has been extensively shown, in the context of organ and umbilical cord blood transplantation, that matching of HLA-A, HLA-B and HLA-DR alleles is sufficient to significantly reduce the incidence of allograft rejection.[32,33] The use of donor-derived T cells for the generation of allogeneic CAR T cells overcomes the issue of rejection thanks to the HLA-compatibility between donor and recipient of the cells, in the case of unrelated and matched sibling donors, and to the immune-reconstitution of the patient with the immune system of the donor before generation and infusion of allogeneic CAR T cells. This aspect also contributes to the long-term persistence of these cells. The role of the possible occurrence of donor-specific antibodies will have to be further investigated in this setting, since it may impair the persistence of allogeneic, donor-derived CAR T cells.

In order to develop a universal, off-the-shelf CAR T-cell product for the timely treatment of patients, readily available when clinically needed, some groups have focused

on different gene-editing approaches aimed at disrupting the endogenous T-cell receptor (TCR) locus, to abrogate the potential alloreactivity of allogeneic T cells. Moreover, to overcome the second obstacle associated with the potential rejection of the product by the patient, these approaches have simultaneously disrupted the CD52 locus in the donor cells in order to induce resistance to the CD52-directed monoclonal antibody alemtuzumab, which can then be introduced in the lymphodepleting regimen of the patients to ablate the endogenous T cells before infusing allogeneic CAR T cells.[34] The clinical studies testing these approaches are summarized in Table 2. The clinical proof of concept of the potential of such an approach (named UCART19), using transcription activator-like effector nuclease (TALEN) technology for gene editing of the TCR α chain (to abrogate alloreactivity) and CD52 (to induce resistance to alemtuzumab, used in the lymphodepleting chemotherapy) gene loci, was provided by two infants.[35] Both patients achieved MRD-negative CR within 28 days after infusion, enabling consolidation with allogeneic HSCT. Subsequently, two phase I studies evaluated the feasibility, safety and antileukemia activity of UCART19 in adults (CALM trial, n=14) and children (PALL trial, n=7).[36] All patients received lymphodepletion with fludarabine and cyclophosphamide with (n=17) or without (n=4) alemtuzumab; then, children received 1.1-2.3×10^6 UCART19 cells/kg and adults received doses of 6×10^6, 6-8×10^7 or 1.8-2.4×10^8 UCART19 cells in a dose-escalation study. Overall, 67% of patients achieved CR after infusion and alemtuzumab proved essential for allowing UCART19 expansion and anti-leukemia activity. Subsequently, 71% of the patients proceeded to allogeneic HSCT. At data cut-off, only two children and three adults were alive and in CR.[36] Among the seven children treated, six (86%) obtained CR and five subsequently underwent allogeneic HSCT. UCART19 expanded with kinetics comparable to that of autologous CAR T-cell products, but had low persistence, generally up to 28 days. With regard to toxicity, one child and one adult developed grade 1, skin acute GvHD and the toxicity profile was comparable to that reported with autologous CAR T cells.[36] More recently, Ottaviano *et al.*[37] described the results obtained with the use of TT52CAR19 T cells, universal, CD19 CAR T cells generated by disrupting the TCR α chain (TRAC) and CD52 loci with next-generation CRISPR-Cas9 editing, for the treatment of six children with relapsed/refractory B-ALL in a phase I clinical trial. Patients underwent lymphodepletion with fludarabine, cyclophosphamide and alemtuzumab, followed by a single infusion of 0.8-2.0x10^6 TT52CAR19 T cells/kg. TT52CAR19 T cells expanded after 7-14 days in four of the six children and induced clearance of the leukemia, enabling consolidation with allogeneic HSCT; in two children, no CAR T-cell expansion was observed and both patients experienced progression of their disease. One patient developed skin GvHD.[37] In 2021, Hu *et al.*[38] reported on the first six patients treated with CTA101, a universal, second-generation (4.1bb) CD19/CD22 tanCAR, obtained by disrupting TCR α chain (TRAC) and CD52 loci with CRISPR-Cas9 editing. Patients underwent fludarabine-cyclophosphamide-alemtuzumab lymphodepletion followed by the infusion of either 1x10^6 or 3x10^6 CTA101 cells/kg. No case of GvHD was reported and five of the six (83%) patients obtained MRD-negative CR, which was sustained in three of the five patients with a median follow-up of 4.8 months (range, 2-8). The median duration of CTA101 persistence, measured by quantitative polymerase chain reaction, was 42 days (range, 21-114).[38]

Table 2. Studies describing the clinical experience with the use of universal, gene-edited CD19-chimeric antigen receptor T cells.

Population	Disease	N of pts	CAR	Gene-editing strategy	Prior LD	Cell dose	GvHD	Clinical responses	Ref
Children (infants)	B-ALL	2	UCART19	TALEN	Flu-Cy-alemtuzumab	4.6 and 4.0 × 10^6/kg	G2 GvHD in 1 pt	2 MRD-negative CR	35
Adults and children	B-ALL	14 and 7	UCART19	TALEN	Flu-Cy (N=4) Flu-Cy-alemtuzumab (N=17)	Ped: 1.1-2.3 × 10^6 cells/kg Adults: 6×10^6, 6-8×10^7 or 1.8-2.4×10^8	G1 GvHD (1 child and 1 adult)	14 CR (71% MRD-negative)	36
Children	B-ALL	6	TT52CAR19T	CRISPR-Cas9	Flu-Cy-alemtuzumab	0.8-2.0x10^6 cells/kg	G1 GvHD	4 CR (3 MRD-negative) 2 NR	37
Adults	B-ALL	6	CTA101 (CD19/CD22 dual targeting)	CRISPR-Cas9	Flu-Cy-alemtuzumab	1x10^6 or 3x10^6 cells/kg	No	5 MRD-negative CR 1 NR	38

Pts: patients; CAR: chimeric antigen receptor; LD: lymphodepletion; GvHD: graft-*versus*-host disease; Ref: references; B-ALL: B-cell acute lymphoblastic leukemia; TALEN: transcription activator-like effector nucleases; Flu: fludarabine; Cy: cyclophosphamide; G: grade; MRD: measurable residual disease; CR: complete remission; CRISPR: clustered regularly interspaced short palindromic repeats; Ped: pediatrics; NR: non-response.

Interestingly, viral and/or fungal infections were reported in all the studies using universal CAR T cells, suggesting that the prolonged lymphodepletion induced by alemtuzumab may expose patients to a higher risk of infections. However, as mentioned, the use of alemtuzumab was shown to be crucial for allowing the expansion of universal CAR T cells.[36] Moreover, the curtailed persistence of these cells, linked to both rejection by the host and the complex and prolonged T-cell manipulation during manufacturing that impairs the function of T cells, remains a limitation of the approach.

Alternative cell sources for allogeneic chimeric antigen receptor cells: induced pluripotent stem cell approaches

In addition to conventional effector cells, allogeneic CAR cells can be produced using alternative cell sources, which encompass stem cell and progenitor cell populations, such as induced pluripotent stem cells (iPSC) and precursor T cells. iPSC, with their nearly limitless proliferative potential, can be directed to differentiate into various cell types, including T and NK cells. This unique feature makes iPSC an excellent renewable source for potentially standardized cell-based immunotherapies. Moreover, iPSC can be genetically modified to potentiate the characteristics of the resulting immune cells. The possibility of generating CAR T cells from iPSC was confirmed by the demonstration of the successful transduction of iPSC derived from peripheral blood lymphocytes with a lentiviral vector encoding for a second-generation CD19-directed CAR.[39] In detail, after hematopoietic differentiation, the authors employed the T-lymphoid commitment co-culture protocol to generate anti-CD19-CAR-T-iPSC-T cells. These iPSC-derived CAR T cells were directly compared with TCR-$\alpha\beta$ and TCR-$\gamma\delta$ peripheral blood lymphocytes from the same donor, transduced with the same CAR. The study demonstrated that iPSC-derived CAR T cells exhibited anti-cancer activity comparable to that of CAR TCR-$\gamma\delta$ cells in an immunodeficient mouse xenograft tumor model.[39] T-cell-derived iPSC hold promise as a source for producing allogeneic 'off-the-shelf' CAR T cells, but their *in vitro* differentiation often leads to effector cells with less-than-ideal characteristics. Several approaches have been developed recently to overcome this limitation. It has been demonstrated that inducing premature expression of the TCR or a continuously active CAR in T-cell-derived iPSC promotes the development of an innate-like phenotype. This issue can be circumvented by deactivating the TCR and relying on the CAR to drive differentiation instead. By delaying CAR expression and fine-tuning its signaling strength in T-cell-derived iPSC, human TCR$^-$ CD8$\alpha\beta^+$ CAR T cells with a similar activity compared to standard CD8$\alpha\beta^+$ CAR T cells derived from peripheral blood can be successfully generated. Indeed, these iPSC-derived CAR T cells demonstrated the ability to effectively control tumors when administered systemically in a mouse model of leukemia, without triggering GvHD.[40] Moreover, an approach to optimize the proliferation and persistency of allogeneic CAR T cells derived from iPSC has been recently published and is based on the genetic knockout of diacylglycerol kinase, which inhibits antigen-receptor signaling, and the transduction of cells with genes encoding for membrane-bound IL-15 and its receptor subunit IL-15Rα.[41] Another great innovation in the field of iPSC-derived CAR T cells is represented by artificial thymic organoids, used to facilitate the specific differentiation of CAR-transduced human iPSC into CAR T cells.[42] This innovative approach has enabled the production of human CD19-CAR T cells closely resembling conventional CAR T cells. Remarkably, these engineered CAR-T cells have proven to be highly effective in controlling human CD19$^+$ leukemia in animal models.[42] Both approaches, leveraging CAR-driven maturation in T-derived iPSC, could pave the way for the large-scale production of potent allogeneic $\alpha\beta^+$ CAR T cells.

Alternative cell sources for allogeneic chimeric antigen receptor cells: non-T-cell-based platforms

Another strategy for developing allogeneic CAR-cell products involves bypassing the use of $\alpha\beta$ T cells and genetically modifying different cell types to express a CAR. The ideal cell suitable for CAR adoptive therapy should be: endowed with inherent cytotoxic abilities that can be redirected towards a cancer cell through the expression of a cell surface receptor; amenable to collection from accessible sources such as peripheral blood or the umbilical cord; and relatively straightforward to transduce and expand.
NK cells, originally recognized for their tumor-killing capabilities, play a central role in natural tumor immunosurveillance mechanisms and meet all the above-mentioned criteria. Several different sources of NK cells have been considered for clinical development of allogeneic CAR NK cells, including peripheral blood, umbilical cord blood, iPSC or immortalized cell lines. Umbilical cord blood-derived NK cells genetically modified using an inducible caspase 9/CAR.19/IL-15 vector have demonstrated promising efficacy in treating relapsed or refractory CD19$^+$ cancers (non-Hodgkin lymphoma or chronic lymphoblastic leukemia).[43] CAR-NK cells were administered in a single infusion at one of three doses (1×10^5, 1×10^6, or 1×10^7 CAR-NK cells/kg) after fludarabine/cyclophosphamide-based lymphodepleting chemotherapy and obtained a 73% response rate, with seven of eight responders showing CR.[43] Although this study paved

the way for the clinical use of allogeneic CAR-NK cells, it also showed the difficulties in generating an off-the-shelf product, as well as underscoring a limited persistence of the engineered NK cells. With regard to the first issue, several pre-clinical models are under development to overcome this limitation, including the large expansion of allogeneic CAR.CD19 NK cells starting from either peripheral blood mononuclear cells or leukapheresis, from which a larger number of NK cells can be derived, or from iPSC.[44] The allogeneic iPSC approach was exploited to differentiate NK cells, subsequently engineered with CAR characterized by standard constructs or by the NK co-stimulatory machinery represented by the transmembrane domain of NKG2D and the co-stimulatory domain of 2B4.[45-47] These preclinical models have shown that iPSC-derived CAR-NK cells hold the potential to serve as an effective off-the-shelf product. In the search of a strategy to improve the long-term persistence of allogeneic CAR NK cells, memory-like NK cells (MLNK) have been explored. MLNK cells, induced by the presence of IL-12, IL-15, and IL-18, display enhanced and prolonged responses upon tumor re-stimulation, making them an appealing choice for adoptive cellular immunotherapy. In a recent study, researchers demonstrated efficient and stable gene delivery of a CD19-CAR construct to MLNK cells using retroviral vectors, achieving a transduction efficiency comparable to that seen in conventional NK cells.[48] When compared to conventional CAR NK cells, CAR-MLNK cells exhibited significantly increased production of interferon-γ and degranulation in response to $CD19^+$ target cells, resulting in enhanced cytotoxic activity against $CD19^+$ leukemia and lymphoma cells. Moreover, the memory-like properties induced by IL-12, IL-15, and IL-18 led to prolonged persistence of effector cells in the $CD19^+$ tumor-bearing mice.[48]

NK T cells (NKT cells) represent another source of allogeneic effector cells that could be used in alternative to conventional T cells. They are a subset of T lymphocytes that exhibit NK-cell surface markers. Within NKT cells, 'invariant NKT cells' (iNKT cells) are characterized by a TCR that recognizes particular lipid antigens presented by CD1d, a non-polymorphic HLA class I-like molecule found on various cells, including B cells, antigen-presenting cells, and select epithelial tissues. In preclinical models, iNKT cells engineered with a CD19-targeted CAR displayed potent anti-lymphoma properties by simultaneously targeting CD19 and CD1d molecules expressed on lymphoma cells.[49] Moreover, it has been shown that allogeneic CAR iNKT cells, in addition to their established direct cytotoxic activity, trigger host CD8 T-cell antitumor responses. This activation has a robust and enduring antitumor effect, lasting even beyond the persistence of the allogeneic cells.[50]

Cytokine-induced killer (CIK) cells constitute a heterogeneous cell population that includes NK, NKT, and T cells. They are generated through the culture of peripheral blood mononuclear cells in the presence of interferon-γ, anti-CD3 antibody, and IL-2 for 2-3 weeks. CIK cells possess a unique combination of phenotypic and functional characteristics combining the advantages of both T and NK cells. This makes them capable of recognizing and eliminating tumor targets through mechanisms that involve both MHC-dependent and MHC-independent pathways. Previous clinical data have confirmed the safety of donor-derived CIK-cell therapy, without occurrence of GvHD and with demonstration of CR in eight of 13 patients 28 days after infusion.[51]

The concept of enhancing the efficacy and reducing the GvHD potential of allogeneic CAR T-cell therapy has also been explored by the use of γδ T cells. The first description of CAR-modified γδ T cells was by Rischer *et al.*[52] who demonstrated specific *in vitro* tumor cell lysis using zoledronate-expanded Vγ9Vδ2 T cells equipped with CD19- or GD2-directed CAR. Subsequent studies confirmed these findings, showing the potential of γδ T cells bearing CAR against a variety of targets.[53,54] Notably, CAR-modified Vγ9Vδ2 T cells retained their ability to cross-present tumor antigens to αβ T cells *in vitro*, suggesting the possibility of prolonging anti-tumor efficacy. Furthermore, γδ T cells with a CD19-CAR, unlike conventional CD19-αβ CAR T cells, exhibited reactivity against both $CD19^+$ and $CD19^-$ cells *in vitro* and *in vivo*. This effect was augmented by zoledronate, indicating that CD19-directed γδ CAR T cells might be effective against leukemic cells even after antigen loss, maintaining their specificity against phospho-antigens through their TCR.[55,56] More recently, it has been demonstrated that γδ CAR T cells can also be derived from iPSC. Wallet *et al.* introduced iPSC-derived γδ CAR T cells (γδ CAR-iT)[57] and demonstrated that, in the presence of IL-15, γδ CAR-iT cells exhibited sustained *in vitro* tumor cell killing, while producing significantly less interferon-γ and other inflammatory cytokines compared to conventional αβ CAR-T cells. This suggests a potentially lower risk of cytokine release syndrome. Moreover, a single dose of γδ CAR T cells resulted in potent inhibition of tumor growth in a xenograft mouse model.[55]

Conclusions and future directions

Allogeneic CAR platforms are gaining increasing attention thanks to their capability to overcome most of the main limitations of autologous CAR T cells. Three major strategies have made their way to the clinics showing promising results: the use of donor-derived allogeneic T cells, the use of off-the-shelf, gene-edited universal T cells and the use of CAR-NK/CIK cells. Donor-derived CAR T cells are associated with no rejection by the patient, promising long-term persistence and encouraging antitumor efficacy, but are limited by the availability of a cell donor. The experiences on the use of T cells derived from haploidentical donors suggest comparable results, in terms of risks of either GvHD or rejection, to those from unrelated donors and matched sibling donors, significantly enlarging the

range of patients who could potentially benefit from such an approach. Moreover, our experience showed that in the case of a matched sibling donor, donor-derived CAR T cells can potentially be used also in patients with highly refractory diseases who did not receive a prior allogeneic HSCT.[31] Universal, off-the-shelf CAR T cells represent a fascinating approach, potentially readily available for every patient upon clinical need, and have shown an acceptable toxicity profile and promising efficacy. Nevertheless, their limited long-term persistence indicates that this approach needs further optimization. Preclinical translational data and shortened, optimized manufacturing strategies will be needed to determine the most efficient approach to avoid rejection of allogeneic CAR T cells and prolong their persistence. Moreover, the requirement for lymphodepletion that ensures prolonged ablation of the patient's T cells increases the risk of infections, limiting the approach to well-selected patients. CAR-NK/CIK cells have shown antitumor activity with negligible toxicity in the first clinical trials, paving the way for their development also for other clinical indications. Multiple infusions will probably be required to ensure long-term control of the disease, considering the limited persistence of these cells. Preclinical studies have shown promising results also with alternative cell platforms and the clinical testing of these strategies will provide crucial information on their potential. Important restrictions to the use of these approaches are limited frequency and yield and optimal strategies for expansion or differentiation systems are under investigation.

Disclosures

No conflicts of interest to disclose.

Contributions

FdB, CQ and FL contributed equally to this manuscript, reviewing the available literature, supervising the preparation of the paper and writing the manuscript. The final version was approved by all the authors.

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