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Review Article

CAR-T Cell Therapy in B-Cell Acute Lymphoblastic Leukemia

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Abstract. Treatment of refractory and relapsed (R/R) B acute lymphoblastic leukemia (B-ALL) is an unmet medical need in both children and adults. Studies carried out in the last two decades have shown that autologous T cells engineered to express a chimeric antigen receptor (CAR-T) represent an effective technique for treating these patients. Antigens expressed on B-cells, such as CD19, CD20, and CD22, represent targets suitable for treating patients with R/R B-ALL. CD19 CAR-T cells induce a high rate (80-90%) of complete remissions in both pediatric and adult R/R B-ALL patients. However, despite this impressive rate of responses, about half of responding patients relapse within 1-2 years after CAR-T cell therapy. Allo-HSCT after CAR-T cell therapy might consolidate the therapeutic efficacy of CAR-T and increase long-term outcomes; however, not all the studies that have adopted allo-HSCT as a consolidative treatment strategy have shown a benefit deriving from transplantation.
For B-ALL patients who relapse early after allo-HSCT or those with insufficient T-cell numbers for an autologous approach, using T cells from the original stem cell donor offers the opportunity for the successful generation of CAR-T cells and for an effective therapeutic approach.
Finally, recent studies have introduced allogeneic CAR-T cells generated from healthy donors or unmatched, which are opportunely manipulated with gene editing to reduce the risk of immunological incompatibility, with promising therapeutic effects.

Keywords: CAR T; Acute lymphoid leukemia; Allogeneic CAR-T; Autologous CAR-T.

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Introduction. Chimeric antigen receptor (CAR) T cells are engineered fusion proteins targeting T lymphocytes to a specific membrane antigen expressed on the surface of cancer cells, thus generating a specific antitumor immune response.[1]

CAR-T cells targeting membrane antigens expressed on B-lymphoid cells, such as CD19, CD20, or CD22, were shown to have significant therapeutic activity in the treatment of patients with refractory and/or relapsed B-cell malignancies, including B-acute lymphoblastic leukemia (B-ALL) and B-cell lymphomas, such as mantle cell lymphoma, diffuse large B-cell lymphoma and indolent B-cell lymphomas.[2]

Patients with refractory/relapsed (R/R) B-ALL have a dismal prognosis (overall survival of only 6 months), with a remission rate ranging from 20 to 40%. The

introduction of new treatment strategies based on either inotuzumab ozogamicin (a humanized anti-CD22 antibody conjugated with calicheamicin) or blinatumomab (bispecific T cell antibody binding to both CD3 on T lymphocytes and CD19 on B cells) have improved the progression-free survival and overall survival of R/R B-ALL; however durable remissions usually require allo-HSCT consolidation.[3-4]

CD19 CAR-T Cells

Two CD19-targeted CAR-T cell products are currently approved by the FDA for the treatment of R/R B-ALL patients: Tisagenlecleucel (Tisa-cel) and Brexacabtagene autoileucel (Brexa-cel). These two products are second-generation constructs, including an antigen-binding domain (anti-CD19), hinge and transmembrane domains (derived from CD8a in Tisa-cel and CD28 in Brexa-cel), co-stimulatory domain (derived from 4-1BB in Tisa-cel and from CD28 in Brexa-cel) and a T cell activation domain (derived from CD3ζ). Another notable difference between these two CAR-T cell products is that Brexa-cel is delivered using a gammaretrovirus, while Tisa-cel is delivered using a lentivirus.

Tisagenlecleucel. Tisa-cel (CTL019) was evaluated in B-ALL patients. An initial study using Tisa-Cel evaluated 35 adult B-ALL patients with R/R disease, aged 20-70 years, treated with Tisa-Cel at three different doses: the complete remission (CR) rate was 69% (90% in the patients treated at the highest dose of Tisa-cel); in the whole-treated population the EFS was 5.6 months; in the cohort of 20 patients treated with the highest dose the 2-year OS was 73% and EFS 49.5%; 38% of patients achieving CR were treated with allo-HSCT.[5]

Other studies have evaluated the safety and efficacy of Tisa-Cel in young pediatric B-ALL patients. The primary analysis of the phase II ELIANA trial, involving 75 pediatric (children, adolescent) and young R/R B-ALL patients, showed an ORR of 81% and an EFS of 76%, with 69% of responder patients remaining relapse-free at 12 months.[6] This study led to FDA approval of Tisa-Cel for pediatric/young R/R B-ALL patients. A more recent study evaluated the safety profile and efficacy in 79 pediatric and young adult patients with R/R B-ALL with a median follow-up of 38.8 months.[7] The overall remission rate was 82%, with an EFS of 24 months and the median overall survival not reached.[7] At a 3-year follow-up, the EFS was 44%, OS 63%, and RFS 52%.[7] Grade 3-4 adverse events were observed in 29% of patients.[7] 17% of patients in CR received consolidative allo-HSCT.[7] In 46 responding patients, a consistent improvement in quality of life was observed.[7]

Few studies have evaluated the safety and efficacy of Tisa-Cel in children and infant B-ALL patients. Ghorasian et al. reported the results of a retrospective study based on the treatment of 35 children and infants younger than 3 years with R/R B-ALL: 76% of these patients have *KMT2A*-rearranged B-ALL and 66% relapsed after previous allo-HSCT.[8] The patients received a single infusion of Tisa-cel; 18% of patients previously received inotuzumb and 37% blinatumomab.[8] After a median follow-up of 14 months, the OS at 12 months was 84%, EFS 69%.[8] Adverse events grade 3 or more consisting of CRS were observed in 14% of cases; no severe neurotoxicity was observed.[8]

Makop and coworkers retrospectively analyzed 14 infant R/R B-ALL patients who received Tisa-Cel: 64% of these patients achieved MRD-negative remission after CAR-T therapy, and 50% remained in remission at the last follow-up (median duration of follow-up 231 days).[9] 86% of these patients had *KMT2A* rearrangements and 29% had prior HSCT.[9] The treatment was well tolerated, and 3/4 of patients displayed grade 3 cytokine release syndrome.[9] The estimated post-CAR-T 6-month EFS and OS were 48% and 71%, respectively.

Studies performed in a real-world setting confirmed the efficacy of Tisa-Cel in the treatment of R/R B-ALL patients. Thus, the Center for International Blood and Marrow Transplant Research (CIBMTR) performed a retrospective analysis of 255 pediatric patients with R/R B-ALL treated with Tisa-Cel; after a median follow-up of 13.4 months, an initial CR rate of 85.5% was observed, with a 12-month duration of response, EFS and OS rates of 60.9%, 52.4%, and 77.2%, respectively.[10] An updated analysis of the CIBMTR registry of real-world data, presented at the 2021 ASH (American Society of Hematology) meeting, displayed the outcomes of 451 R/R B-ALL children /young patients (≤25 years-old) treated with Tisa-Cel.[11] With a median follow-up of 21 months, the ORR was 86.8%, the mDOR (median Duration of Remission) was 23.9 months, mEFS was 14 months and mRFS was 23.9 months; 12-month EFS and RFS were 54.3% and 62.3%, respectively; mOS was not reached.[11] Grade 3 CRS and neurotoxicity events were observed in 17.8% and 10% of patients, respectively.[11]

Fabrizio et al. have retrospectively evaluated 184 R/R B-ALL patients treated in the context of the Pediatric Real World CAR Consortium (PRWCC) with the specific aim of assessing the efficacy of this therapy in patients with extramedullary disease, subdivided into those with central nervous system (CNS) and non-CNS involvement.[12] In patients with CNS disease, 88% achieved a CR, compared to 66% in those with non-CNS disease.[12] The 24-months OS and 11-month RFS were similar in patients with CNS and non-CNS disease and in those with bone marrow-only disease.[12]

Other studies have explored some biomarkers or clinical parameters associated with the clinical response to Tisa-Cel in R/R B-ALL patients. A retrospective analysis carried out in a total of 200 R/R B-ALL patients treated with Tisa-Cel, involving 15 USA institutions,

Table 1. CAR-T cell therapy of B-ALL involving Tisagelecleucel. The most relevant clinical studies are reported.

Clinical study	Patients	Median follow-up (mo)	ORR (%)	CR (%)	mEFS (mo)	mOS (mo)	Patients in CR receiving allo-HSCT (%)
Frey et al. 2020 NCT 01029366	25 adult R/R B-ALL (20-70 years)	13 months	75	69	5.6 2-yr at highest dose: 49.5%	2-yr at highest dose: 73%	38
Schultz et al. 2021 Retrospective	200 children and young R/R B-ALL (0-26 years)	335 days	88	85	HDB: 31% LDB: 70% UD: 72%	1-yr: 72% HDB: 58% LDB: 85% UD: 95%	28
John et al. 2021 Retrospective	451 children and young ≤25 years	21 months	86.8	87	14 mRFS: 23.9	Not reached	NR
Ghorasian et al. 2022	35 infants and children <3 years (0-3 years)	14 months	90	86	1-yr: 69%	1-yr: 84%	17
Laetsch et al. 2023 ELIANA, phase-II, single-arm	79 children and young adult R/R B-ALL (3-24 years)	39 months	90	82	24 3-yr EFS: 44% 3-yr RFS: 52%	At 2-yr, not reached 3-yr OS: 63%	17

Abbreviations: HDB: high disease burden; LDB: low disease burden; UD: undetectable disease; NR: not reported.

reported a CR of 85%, with 12-month OS of 72%.[13] Univariate and multivariate analyses showed an association between high disease burden (defined by ≥5% bone marrow leukemic blasts and presence of extramedullary disease) with inferior outcomes with a 12-month OS of 58% and EFS of 31%, compared with patients with low-disease tumor burden, exhibiting OS of 95% and EFS of 72%.[13] Furthermore, the high-burden tumor was also associated with increased toxicity (grade 3 cytokine release syndrome and neurotoxicity).[13]

Pulsipher and coworkers have evaluated the predictive role of the persistence of B-cell aplasia and detection of minimal residual disease (MRD) by NGS in B-ALL patients undergoing treatment with Tisa-Cel in the context of the ELIANA and ENSIGN trials.[14] In these studies, the large majority of relapses occurred within the first year after CAR-T cell infusion.[14] B-cell aplasia was used as a pharmacodynamic marker of the persistence of CAR-T functional activity. An association was observed between B-cell recovery and shorter EFS; however, the measurement of B-cell aplasia after CAR-T cell treatment by itself is not sufficient to predict relapse since CD19-negative relapse can occur early and at higher frequency in patients with persistence of B-cell aplasia.[14] The study of MRD by NGS was the most sensitive technique for defining the risk of relapse after CAR-T cell therapy. The evaluation of MRD at day 28 post-infusion showed that a small percentage of patients who have NGS MRD positivity display long-term responses, thus suggesting that in these patients at day 28, the response is not complete and is continuing.[14] Therefore, repeated NGS-MRD evaluations are required for an adequate prediction of the relapse risk in these patients. Importantly, clonality analyses comparing MRD clones with the corresponding baseline clones showed no differences in IgH rearrangements, thus suggesting that the relapsing clones evolved from the original clones.[14]

CAR-T cell expansion and/or persistence are major determinants of response to CAR-T cell treatment. An appropriate lymphodepletion prior to CAR-T cell infusion is required for optimal CAR-T cell expansion and survival. Particularly, the addition of fludarabine to cyclophosphamide as a lymphodepletion regimen significantly improved CAR-T cell expansion and persistence in children and young adult B-ALL patients receiving Tisa-Cel.[15] A retrospective analysis carried out on 28 B-ALL patients treated with Tisa-Cel showed a significantly improved probability of leukemia-free survival, a lower incidence of CD19-positive relapsed, and a delayed B-cell recovery in patients receiving high fludarabine regimens compared to those receiving low-fludarabine regimens.[15]

Dourthe and coworkers have explored the factors associated with outcome in 51 relapsed-refractory B-ALL patients undergoing treatment with Tisa-Cel: 49/51 patients achieved CR/Cri with an 18-month overall survival of 74%; 22/49 patients relapsed with a median time of 3.7 months: 12 had CD19-positive relapse and 8 CD19-negative relapse.[16] Factors associated with a high tumor burden, such as cytokine release syndrome and prior blinotumumab therapy, were associated with an increased risk of relapse and a reduced EFS and OS.[16] Pre-lymphodepletion high disease burden and detectable MRD at day 28 correlated with an increased risk of CD19-negative relapse, while low disease burden and loss of B-cell aplasia predicted an increased risk of CD19-positive relapses.[16] These observations supported

an important role of prior therapy on patient outcomes.

Other CAR-T Cell Products with 4-1BB Co-Stimulatory Domain. Hay et al. have explored the safety and the efficacy of CAR-T cells manufactured using a CAR composed of a single-chain variable fragment (scFv) derived from an anti-CD19 monoclonal antibody fused to an IgG4 hinge region, CD28 transmembrane domain, 4-1BB co-stimulatory domain, and CD3ζ signaling sequence.[17] CAR-T cells were infused in a defined $CD4^+$:$CD8^+$ ratio to improve uniformity and maximize the potency of the infused product, according to preclinical studies.[17] Using these CAR-T cells, a phase I/II study was carried out, involving the enrollment of 53 R/R adult B-ALL patients; at a follow-up of 31 months, 89% of patients achieved a CR/CRi response, with a mEFS of 6 months among patients achieving MRD-negative status.[17] 40% of patients who achieved a CR with MRD-negativity were processed for allo-HSCT.[17] With a median follow-up of 28.4 months after allo-HSCT, the EFS and OS rates were 61% and 72%, respectively; allo-HSCT after CAR-T cell therapy was associated with longer EFS compared with no allogeneic HSCT.[17] mEFS and mOS were significantly better among patients achieving CR with MRD-negativity compared to those with MRD-positivity.[17] In patients achieving CR with MRD-negative condition, lower LDH levels, higher platelet counts, incorporation of fludarabine in the lymphodepletion regimen, and allo-HSCT after CAR-T cell therapy were associated with improved EFS.[17]

An et al. reported the results of a multicentre phase II study involving the treatment of 47 R/R children and adult B-ALL patients (aged 3-72 years) using Sino CD19 CAR-T cells obtained using a CAR composed of scFv, IgG4 hinge, CD28 transmembrane domain and 4-1BB co-stimulatory domains.[18] 81% of patients achieved a CR, and after a follow-up of 12 months, the RFS was 10.5 months, and 26% of patients in CR received consolidative allo-HSCT.[18] 19/28 of the patients achieving allo-HSCT relapsed after CAR-T cell therapy; patients who underwent allo-HSCT after CAR-T cell therapy had a lower risk of relapse and death, but not statistically significant when compared with those who did not.[18] Factors associated with poor outcome were the presence of high-risk cytogenetic factors, the presence of extramedullary disease, and higher levels of circulating T-reg cells.[18]

CAR-T Cells with CD28 Co-Stimulatory Domains. Several studies involved CAR-T cell products based on hinge, transmembrane and co-stimulatory domains derived from CD28; these CAR-T cell products include the commercialized product Brexu-Cel. Pivotal studies carried out by Hollyman and coworkers have described the development of CAR-T cells obtained by the genetic manipulation of T cells with a replicon-defective gamma retroviral vector derived from Maloney murine leukemia virus encoding a CAR targeted to CD19 (19-28z), containing mouse scFv, CD28 H/T and CD28 co-stimulatory domains.[19] Using this CAR-T cell product, Park et al. have evaluated 53 adult R/R B-ALL patients (23-74 years) heavily pretreated and reported their evaluation at a median follow-up of 29 months: the median EFS was 6.1 months; the mOS was 12.9 months (**Table 2**). Patients with a low disease burden prior to CAR-T cell therapy (<5% BM blasts) displayed enhanced remission duration and survival, with a mEFS of 10.6 months and a mOS of 20.1 months compared to 4 and 12 months, respectively, in those with high-disease burden (>5% BM blasts or extramedullary disease).[20] Furthermore, patients with high disease burden showed a significantly increased incidence of CRS and neurotoxic events.[20] A parallel study evaluated the safety and efficacy of 19-28z CAR-T cells in 25 pediatric/young adult R/R B-ALL patients.[21] The study of these patients supported the conclusion that the dose intensity of conditioning chemotherapy and low pretreatment disease burden displayed a positive impact on response without a negative impact on treatment-associated toxicity.[21] Finally, a retrospective study evaluated 38 adult B-ALL patients who progressed after 19-28z CAR-T cell therapy; the median time to progression after CAR-T cell therapy in these patients was 5.5 months, with a mOS of 7.5 months.[22] A high pretreatment disease burden was associated with the risk of disease progression after CAR-T cell therapy.[22]

Several studies have explored autologous CAR-T cells manufactured using the product KTE-X19 (commercialized as Brexu-Cel). In a first phase I study (ZUMA-3 study), 45 adult R/R B-ALL patients with age comprised between 18 and 77 years were treated with Brexu-Cel at a dosage of $2x10^6$ cells per Kg (6 patients) or $1x10^6$ (23 patients) or $0.5x10^6$ (16 patients).[23] Grade ≥3 CRS was observed in 31% of patients and grade ≥3 neurologic events in 38% of cases.23 At a median follow-up of 22.1 months, the ORR was 69%, with 53% of patients achieving CR and 16% achieving Cri; the median duration of response for the patients achieving a CR/Cri was 14.5 months (18 months for patients treated at $1x10^6$ dose).[22] MRD was undetectable in all responding patients; 13% of patients received allo-HSCT.[23]

The ZUMA-3, phase II study involved the treatment of 55 adult (28-52 years old) B-ALL patients with R/R B-ALL patients treated with Brexu-Cel; at a median follow-up of 16.4 months, 71% of treated patients displayed a CR, with a median duration remission of 12.8 months; mRFS was 11.6 months and mOS was 18.2 months.[24] Among responders, mOS was not reached. 18% of patients received allo-HSCT treatment after CAR-T cell therapy infusion.[24] The most common

Table 2. CAR-T cell therapy of B-ALL involving Brexucabtagene. The most relevant clinical studies are reported.

Clinical study	Patients	Median follow-up (mo)	ORR (%)	CR (%)	mEFS (mo)	mOS (mo)	Patients in CR receiving allo-HSCT (%)
Park et al. 2018 Phase I 19-28z CAR-T	53 adult R/R B-ALL (23-74 years)	29 months	88	83	6.1 LDB: 10.6	12.9 LDB: 20.1	39
Shah et al. 2021 Phase I (ZUMA-3 trial) KTE-X19	45 adult R/R B-ALL (18-72 years)	22 months	80	69	7	12.1	13
Shah et al. 2021 Phase II (ZUMA-3 trial) KTE-X19	55 adult R/R B-ALL (28-52 years)	16 months	81	71	11.6 mDOR: 12.8 months	18.2 Not reached among responders	13
Shah et al. 2021 Phase I CD10.28ξ CART	50 children R/R B-ALL (4-30 years)	56 months		62	3.1	10.5	75
Wayne et al. 2022 Phase I/II (ZUMA-4 trial) KTE-X19	24 children and young adult R/R B-ALL (3-20 years)	36 months	92	6.7 75% MRD-negative	mRFS: 5.2 months		**88**
Jacoby et al. 2022 Phase II CAR-T19	37 children R/R B-ALL (1-36 years)	36 months	90 All MRD-positive patients at day 28 relapsed	86	17 41%	Not reached 56%	83
Ceolin et al. 2023 Retrospective studies	39 pediatric B-ALL (1.4-23 years) After inotuzumab ozogamicin	18 months	90	87	12-months: 53%	12-months: 78%	NR

Abbreviations: DOR: duration of remission; MRD: minimal residual disease; NR: not reported; mRFS: median relapse free survival.

adverse events were grade 3 or higher anemia (49%), pyrexia (36%), and infections (25%); CRS or neurological events of grade 3 or higher occurred in 24% and 25% of cases, respectively.[24]

On the basis of the results of the ZUMA-3 trial, Bruxa-Cel received approval from the FDA for the treatment of adult patients with R/R B-ALL.[25]

A longer follow-up confirmed the consistent therapeutic efficacy of Brexu-Cel in R/R B-ALL patients, with a CR rate of 75%, median duration of remission, and OS of 14.6 and 25.4 months, respectively.[26] Furthermore, in the SCHOLAR-3 study, 49 patients treated with Brexu-Cel in the context of the ZUMA-3 trial were matched with 40 treated patients from historical clinical trials, with a comparative mOS of 25.4 and 5.5 months, respectively.[26] A detailed comparative analysis of the results obtained in the SCHOLAR-3 study showed that outcomes of patients treated in historical standard-of-care trials were poor, irrespectively of the prior treatment (blinatumomab/inotuzumab-treated or naïve) with a mOS of <60 months, compared with a mOS of >25 months in matched patients enrolled in the ZUMA-3 trial.[27]

The phase I ZUMA-4 trial explored the safety and the efficacy of Brexu-Cel in 24 pediatric/adolescent R/R B-ALL patients; the overall CR rate was 67%, and MRD-negativity was 100% among responders.[28] 85% of responders underwent subsequent allo-HSCT; the median duration of remission at HSCT time and median OS were not reached.[28] Grade 3 CRS was 33%, and grade ≥3 neurologic events were observed in 11-27% of patients following the dose of CAR-T cells.[28]

Other studies have explored the efficacy of CAR-T cells manufactured using vectors similar to those used in Brexu-Cel in infants/children patients with R/R B-ALL. Thus, Jacoby et al. reported the long-term response of 37 infants/children and young adult patients (1-36 years) with R/R B-ALL treated with CD19 CAR-T cells in the context of a phase II study; the CR rate was 86%, with 71% of the responding patients achieving an MRD-negative status; 83% of the patients in CR proceeded to allo-HSCT; all MRD-positive patients at day 28 post-CAR-T cell infusion relapsed.[29] mEFS was 17 months, and mOS was not reached, with 56% of patients surviving at 3 years.[29] A prior HSCT did not affect the response to CAR-T cell therapy, but a consolidation with allo-HSCT after CAR-T cell therapy improved long-term survival.[29]

Shah et al. reported the long-term results of a single-center phase I study involving the treatment of 50 children and young adult (4-30 years) patients with R/R B-ALL treated with CD19-28z CAR-T cells; at a follow-up of 4.8 years, 62% of patients displayed a CR, with a mEFS of 3.1 months and a mOS of 10.1 months.[30] 56% of patients displayed a CR with an MRD-negative status, and 75% of these patients were processed for consolidative allo-HSCT.[30]

Ceolin et al. have retrospectively analyzed the response to CD19 CAR-T cell therapy in 39 pediatric R/R B-ALL patients (1.4-23 years old) previously treated with inotuzumab ozogamicin; at an 18-month follow-up, an ORR of 53% and a mOS of 78% were observed.[31] These results were comparable to those previously reported for patients without prior inotuzumab ozogamicin exposure.[31]

CD19-Targeted CAR-T Cells Engineered with Lower Affinity Anti-CD19. Ghorashian et al. have explored whether a lower CAR binding affinity could improve CAR-T activity and reduce the toxic effects induced by CAR-T cell therapy.[32] To this end, these authors have generated a novel CD19 CAR (CAT) with a lower affinity than FMC63, the high-affinity anti-CD19 scFv used in most CD19 CAR constructs.[32] CAT CAR-T cells exhibited increased proliferation and cytotoxicity *in vitro* and enhanced proliferative and *in vivo* antitumor activity compared with FMC63 CAR-T cells.[32] The safety and the efficacy of CAT-CAR-T cells were explored in 17 pediatric patients with high-risk R/R $CD19^+$ B-ALL; 14 patients received an infusion of CAR-CAR-T cells, and 12 had a molecular complete response after 3 months; the expansion of CAT-CAR-T cells observed in these patients was about threefold higher than that reported for Tisa-Cel at 28 days post-infusion.[32] At 1 year, the OS was 63%, and the event-free survival was 46%.[32] No patients required tocilizumab or intensive care support for grade ≥3 CRS.[32]

A subsequent study evaluated the safety and efficacy of CAT19-CAR-T cells (AUTO1) in 20 patients with R/R adult B-ALLs; 65% of these patients received prior allo-HSCT.[33] No patient experienced grade ≥3 CRS, and 3 of 20 patients had grade neurotoxicity that resolved within three days of treatment with steroids.[33] 85% of patients achieved a complete MRD-negative response at 1 and 3 months post-infusion; 17 patients underwent allo-HSCT while in remission.[33] The event-free survival was 68% and 48%, respectively. A high level of CAR-T cell expansion was observed in 15 of 20 patients.[33] It is interesting to note that risk-adaptive and split-dosing were incorporated into the design of this study.

Based on these results, the pivotal FELIX study was proposed; this is a phase Ib/II study enrolling R/R B-ALL patients with >5% of bone marrow blasts (cohort A), MRD-positivity (cohort B), or extramedullary disease (cohort C): The patients received a target dose of $4x10^6$ CAR-T cells as a split dose on day 1 and day 10; the dosing schedule is based on the percentage of bone marrow blasts.[34] A first interim analysis involved 50 patients enrolled in cohort A of the study, with a median number of prior lines of treatment, including 42% of patients prior to transplant; at screening, patients had 55% of BM blasts.[34] 70% of patients achieved a CR; 3% of treated patients had a CRS of grade ≥3.[34] In this study, AUTO1 CAR cell treatment received the commercial designation of abecabtagene autoleucel.[34]

Interestingly, a recent study reported the single-cell transcriptomic analysis of CD19 CAR-T cells of 10 children enrolled in the initial CARPALL study, studied at the moment of infusion and 1-3 months, 4-6 months, and after 7 months.[35] 87% of patients achieved complete remission; 46% of responding patients subsequently relapsed, while the remaining 54% of patients achieved long-lived remissions maintained by detectable CAR-T cells and concomitant B-cell aplasia.[35] All patients with long-lived CAR-T cells developed a CD4/CD8 double negative phenotype with an exhausted-like memory state and a distinct transcriptomic signature.[35] Interestingly, this "persistence" signature was also observed in two adult patients with chronic lymphocytic leukemia with decade-long remission following CD19 CAR-T cell therapy.[35]

CD22-Targeted CAR-T Cell Therapy of B-ALL. CD22 is expressed in the large majority of pediatric and adult B-ALL and, therefore, represents a suitable target for CAR-T cell therapy of these leukemias.

Initial studies were based on the use of CD22-targeted/4-1BB cells. The initial phase I study reported the results on 21 children and adult B-ALL patients treated with CD22-CAR-T cells. The response to this treatment was dose-dependent, with 1/6 patients and 10/11 patients responding with $3x10^5$ and $1x10^6$ CD22-CAR-T cells.[36] An updated report of this study showed the results obtained in 55 B-ALL patients, with a CR rate of 70%, a median duration of remission of 6 months, and a mOS of 13.4 months.[37] 24% of patients in CR received allo-HSCT.[37] 63% of patients with CR achieved an MRD-negative status. The rate of responses and duration were lower in patients with prior CD22-targeted therapy (inotuzumab).[37] A third of treated patients developed hemophagocytic lymphohistiocytosis about 2 weeks after CAR-T cell infusion.[37]

Pan et al. evaluated the safety and efficacy of CD22-41BB CAR-T cells in 34 pediatric/adult R/R B-ALL patients who, in large majority (91% of cases, have received prior CD19 CAR-T cell therapy).[34] 70.5% of the enrolled patients achieved a CR, and 11 of these patients were bridged to allo-HSCT.[38] Leukemia-free survival at 12 months for patients who achieved CR was 58%, and for those receiving allo-HSCT was 71.6%.[38] CD22

antigen loss or mutation was not associated with disease relapse.[38]

Tan et al. reported the development of a new CD22-CAR construct with low immunogenicity and potent activity for treating B-ALL patients who have failed previous CD19- or CD22-targeted CAR therapies. This construct was based on the fusion of a full-human anti-CD22 scFv to the intracellular 4-1BB co-stimulatory and CD3ζ signaling domains to generate CD22-CAR^{FH80} T cells.[39] These CAR-T cells were evaluated in 8 patients who were refractory or relapsed after previous CD19- and CD22-CAR-T cell therapies: 7/8 patients achieved a response to treatment, and 4 responding patients were bridged to allow HSCT.[39] The follow-up of these patients was limited to 6 months.[39]

Multitargeting of CD19 and CD22. Targeting both CD19 and CD22 can be accomplished by four different approaches, based on: (i) the generation of two cell populations expressing different CARs and infusion of these cells together (simultaneous coadministration) or sequentially (sequential coadministration); (ii) the simultaneous engineering of T cells with two different CAR constructs (co-transduction), thus generating three different CAR-T cells, represented by single-expressing and dual-expressing CAR-T cells; (iii) the development of bicistronic vectors that encode two different CARs on the same cells; (iv) the encoding in the same chimeric protein of two different CARs, using a unique expression vector (bispecific or tandem).[40]

Several studies have explored the safety profile and efficacy of the coadministration of CD19 and CD22 CAR-T cells to R/R B-ALL patients. Wang et al. have performed a phase I study involving the sequential infusion of a cocktail of anti-CD19 and anti-CD22, 2 single-specific, third-generation CAR (CAR 19/22) T cells in 51 patients R/R with R/R B-ALL; a CR/CRi was observed in 96% of patients with a PFS of 13.6 months and an OS of 31 months. High-grade CR and neurotoxic events were observed in 22 and 1 of the cases, respectively.[41] Liu et al. have investigated in phase I clinical study the therapeutic efficacy of the combination of CD19 and CD22 CAR-T cell therapy in 27 B-ALL patients who relapsed post-transplant; 27 patients received the first CD19 CAR-T cells, and 85% achieved a CR; 21 of these patients received the second CD22 CAR-T cells and were followed by a median of 19.7 months: 14 patients remained in CR, and 7 patients relapsed.[38] EFS and OS at either 12 or 18 months were 67.5% and 88.5%, respectively.[42]

A single-arm, multicenter phase II study involved 195 childhood patients (≤20 years) with R/R B-ALL treated with a protocol involving coadministration of CD19 and CD22 CAR-T cell therapy; 99% of these patients achieved a CR, with MRD negativity.[42] 12-month EFS was 73.5%; relapse occurred in 43 patients (mostly with $CD19^+/CD22^+$ or $CD19^-/CD22^+$ relapse).[38] Seventy-eight patients received allo-HSCT and displayed a 12-month EFS of 85%, while 116 patients were non-transplanted and showed a 12-month EFS of 69.2%.[42] Favorable outcomes were seen for patients with consistent B-cell aplasia at 6 months.[43] The 12-month EFS was 95% for patients with isolated testicular relapse and 68.6% for patients with CNS relapse.[43]

Several studies reported the development and the clinical use of tandem CD19/CD22 bispecific CAR-T cells. Preclinical studies have supported the efficacy of tandem CD22/CD19 CAR-T cells in mediating the killing of leukemia cells with low CD19 and CD22 antigen density.[44] Dai et al. reported the development of bispecific CD19/CD22 CAR-T cells generated using a tandem CD19/CD22 vector.[41] In the clinical study of CAR-T cells generated using this vector, all 6/6 R/R B-ALL patients attained CR, with the achievement of MRD negativity.[45] Three of these patients relapsed within the first year after CAR-T cell therapy, and one of them with CD19-negative leukemia cells.[45] Cui et al. reported the results of an open-label, single-center clinical trial involving the investigation of the safety and efficacy of tandem CD19/CD22 dual targets CAR-T cells in 47 R/R B-ALL patients (44% with primary refractory B-ALL and 57% with high disease burden).[46] 100% of patients responded to treatment, and 85% had MRD-negative status; grade ≥2 CRS occurred in 17% of patients and neurotoxicity events only in 1 patient; leukopenia was the most severe common hematological abnormality.[46] At a follow-up of 21.8 months, the mOS and mLFS were not reached; at 2 years, the OS was 74.5%, and at 1 year, the leukemia-free survival (LFS) was 68%; 72% of the patients proceeded to bridge allo-HSCT, with 1-year OS of 80.4%; at 1 year, the cumulative incidence of relapse was 23%.[46]

Liu et al. have performed a comparative analysis of the efficacy of single-target (CD19) or dual-target (tandem or sequential CD19/CD22) CAR-T cell therapy for R/R B-ALL patients.[47] In this retrospective analysis, a total of 219 patients, subdivided into single CD19 CAR-T (147 patients), tandem CD19/CD22 CAR-T (51 patients), and sequential CD19/CD22 CAR-T (21 patients), all tested at the same institution, were included.[43] The CR rates in the single-CD19, tandem CD19/CD22 and sequential CD19/CD22 were 83%, 98% and 95%, respectively.[47] A higher proportion of patients treated with tandem CD19/CD22 CAR-T (70.5%) was bridged to allo-HSCT compared to those treated with single CD19 (39%) or sequential CD19/CD22 (28.5%) CAR-T cell therapy.[47]

EP300-ZNF384 is originated by a cryptic t(12;22)(p13;q13) chromosome translocation and is associated at phenotypic level with high CD19 and CD22 expression. This translocation is observed in 4-6% of B-ALL patients; Zhang et al. reported the successful

treatment of two R/R AP300-ZNF384 B-ALL patients with tandem CD19/CD22 CAR-T cell therapy, with bridging to allo-HSCT.[48]

Spiegel and coworkers have generated a CD19-22.BB,z-CAR comprising a single cistron encoding the anti-CD19 murine FMC63 scFv and fully human anti-CD22 m971 scFv, followed by human CD8 hinge and transmembrane domains, 4-1BB co-stimulation and CD3ζ activation domains.[49] The study enrolled 17 R/R B-ALL patients, all responding to the treatment (88% CR and 12% PR); all patients with CR achieved an MRD-negative status.[49] After a median follow-up of 9.3 months, mOS was 11.8 months, and PFS was 5.8 months.[44] Ten patients displayed disease progression after CAR-T cell therapy, and 5 of these patients had low-negative CD19 expression and maintained CD22 expression on relapsing leukemic cells.[49]

Cordoba et al. developed AUTO3, a CAR-T cell-based treatment with dual specificity (CD19 and CD22) generated through transduction of autologous T cells with a bicistronic vector encoding humanized anti-CD19 and anti-CD22 CARs, both incorporating tumor necrosis factor receptor co-stimulatory domains.[50] The AMELIA phase I clinical trial was performed using AUTO-3: at 1 month after AUTO-3 infusion, 86% of patients achieved a CR, with 80% of patients showing MRD negativity; 9 of the responding patients relapsed, and many of these patients had low CAR-T cell numbers, thus suggesting that a low persistence *in vivo* of these cells could be the predominant cause of treatment failure.[50]

Annesley and coworkers have explored the safety and the efficacy of CAR-T cells targeting both CD19 and CD22, generated through a co-transduction approach: autologous T cells were double transduced with lentiviral vectors encoding for either a CD19-specific or a CD22-specific CAR, both with 4-1BB co-stimulation.[51] Two types of CAR-T cell products were explored: SCRI-CAR19x22v1 (leading to prevalent engraftment of CD19 CAR population, with unsuccessful eradication of $CD19^-/CD22^+$ leukemic cells) and SCRI-CAR19x22v2 /leading to prevalent engraftment of CD22 CAR-expressing cells).[47] With SCRI-CAR19x22v2, a 91% CR rate was observed, with 100% of MRD negativity in 12 R/R B-ALL patients.[46] It is of interest to note that the SCRI-CAR19x22v2 product is predominantly composed of $CD22^+$ and $CD19^+/CD22^+$ CAR-T cells, with few $CD19^+$ cells, while *in vivo* engraftment is predominated by single CD22 CAR-expressing T cells.[51]

Lucchini et al. developed AUTO1/22, an autologous CAR-T cell product co-transduced with two different lentiviral vectors encoding a previously described fast-off rate CD19 CAr and a novel CD22 CAR designed to recognize targets with low antigen density.[52] Safety and efficacy of AUTO1/22 in a phase I study in 12 children young adults with R/R B-ALL; the enrolled patients had a median of 3 prior lines of therapy; six of these patients had relapsed post-allo-HSCT; six patients had an extramedullary relapse, and 3 had detectable CD19-negative disease.[52] 10 of the 12 evaluable patients (83%) achieved MRD-negative complete remission at 1 month post-infusion; with a follow-up of 8.7 months, 50% of patients remained alive in MRD-negative CR; the median duration of response in responding patients was 9.9 months; the OS rate at 6 and 12 months was 75%; Event-free survival was 75% and 60% at 6 and 12 months, respectively. No patient experienced grade 3 CRS.[52]

Relapse after CAR-T Cell Therapy for B-ALL. The relapse after single-targeted CD19 CAR-T cell therapy can be subdivided into three subgroups, differentiated according to CD19 expression on relapsing leukemic cells and differentiation status: $CD19^+$ relapse and $CD19^{-/low}$ relapse; a third type of relapse is related to lineage switch (LS) from a lymphoid to a myeloid phenotype. During the treatment of R/R B-ALL patients with CD19-directed CAR-T cells, most of the relapses occurring soon after CAR-T cell therapy were composed of $CD19^+$ leukemic cells.[53]

The $CD19^+$ relapse usually results from the low potency or short persistence of CD19 CAR-T cells.[6] In $CD19^-$ relapses, which account for about 40-50% of total relapses, B leukemic cells lose CD19 membrane expression and escape CAR-T cell-mediated recognition and killing. Molecular studies have shown that the occurrence of *de novo* frameshift/missense CD19 mutations, alternative splicing of CD19 mRNA, and hemizygous deletions spanning the CD19 locus cause impaired CD19 mRNA expression.[54-56]

CAR-T cell therapy is not responsible for a dysregulated CD19 transcription but favors the emergence of minor $CD19^-$ clones preexisting in the patients and escaping CD19-targeted therapy.[57] This mechanism was directly supported by single-cell profiling of leukemic cells of patients with $CD19^-$ relapse after CAR-T cell therapy.[57]

Pan et al. have analyzed the outcome of 68 R/R B-ALL children treated with CD19 CAR-T cells and with a consolidation therapy based either on allo-HSCT (34 patients) or CD22 CAR-T cells (30 patients): the DFS at 1 year was 79.6%, with 12 relapsing patients with a median time of 6.3 months.[58] 8 of the 12 relapsing patients were characterized by the presence of *TP53* mutations in their pre-therapy leukemic cells; 7/8 of these patients displayed CD19 negativity on their relapsed leukemic cells.[58]

As discussed above, for Tisa-Cel-treated patients, high-disease burden and prior failure in response to blinatumomab therapy are associated with a reduced response to CAR-T cell therapy.[16] These observations were confirmed and extended in a wide analysis involving 420 children/young adult B-ALL patients

undergoing treatment with Tisa-Cel and other CD19 CAR-T treatments; 18% of these patients received prior treatment with blinatumomab.[59] Blinatumomab nonresponders had worse EFS and RFS compared to responders or blinatumomab-naïve patients.[54] A high disease burden was associated with inferior EFS.[59]

A second study based on the analysis of these 420 patients further extended the analysis of clinical, genetic, and biochemical factors associated with the relapse of these patients following their treatment with CAR-T cells. Clinical characteristics associated with worse EFS included high tumor burden, circulating peripheral blasts, CD19/28ζ CAR construct type, and poor response to blinatumomab.[60] Of 420 R/R children/young adult B-ALL patients treated with CD19 CAR-T cells, 39.5% relapsed; the relapsing patients were 50% $CD19^+$, 41% $CD19^-$ and 7.2% LS relapses.[60] A greater number of prior complete remissions was associated with $CD19^+$ relapses; high preinfusion disease burden, prior blinatumomab nonresponse, older age, and 4-1BB construct were all associated with $CD19^-$ relapses; the presence of *KMT2A* rearrangements was the only preinfusion risk factor associated with LS relapses.[60] The median overall survival following a post-CAR-T relapse was 18.9 months for $CD19^+$ relapses, 9.7 months for $CD19^-$ relapses, and 3.7 months for LS relapses.[60]

Primary Resistance to CAR-T Cell Therapy. In addition to acquired resistance observed in patients initially responding to CAR-T cell therapy, primary resistance (PR) is observed in about 10-20% of patients undergoing CD19 CAR-T cell therapy. PR therapy is characterized by CD19-positive progressive disease. It is associated with the increased expression of exhaustion markers (LAG3, TIM3, and PD-1) in the apheresis product used for CAR-T cell manufacturing or a decreased rate of CAR-T cell expansion *in vivo*.[61] Using a genome-wide loss-of-function screening provided evidence that impaired death receptor signaling in B-ALL leads to rapidly progressive disease in CD19 CAR-T-treated patients: reduced expression of death receptor genes was associated with worse overall survival and reduced T-cell fitness.[62]

A recent study identified a gene expression profile that correlates with primary resistance to CD19 CAR-T cell therapy in B-ALL samples, related to the expression of genes typically expressed in hematopoietic stem/progenitor cells while maintaining a pre-B cell phenotype.[63] This finding is important because it identified a mechanism of resistance intrinsic to leukemic cells, preexisting to CAR-T cell therapy, and may provide a tool to define the eligibility of B-ALL patients to CAR-T cell therapy.

Expansion and Persistence of CAR-T Cells after in Vivo Infusion. Optimal *in vivo* expansion and persistence are two additional important determinants of the therapeutic efficacy of CAR-T cells in B-ALL patients.

The kinetics of CAR-T cells after their *in vivo* infusion shows an initial expansion after cell infusion, followed by a peak level and then a decline with persistence at variable levels for years after treatment. Higher peak CAR-T cell levels and CAR-T cell area under the curve within the first month of treatment have been associated with response in most of the studies carried out in B-ALL patients.[64]

In addition, the studies carried out using Tisa-Cel in pediatric and young adult patients have clearly supported the role of long-term CAR-T cell persistence for a durable response.[6-7] In the ELIANA study, the time to B-cell recovery among responders was 35.3 months, and the probability of persistent B-cell aplasia (evidencing persistent functional CAR-T cells) at 12 and 24 months after the infusion, was 71% and 59%, respectively.[6-7] Furthermore, the duration of response for allo-HSCY patients with onset of B-cell recovery at <6 months was clearly shorter compared to those with onset of B-cell recovery at >6 months.[7]

The Role of Allo-HSCT as a Consolidation Therapy after CAR-T Cell Therapy in B-ALL Patients. Allo-HSCT represents an important option to consolidate the therapeutic results obtained using CAR-T cells in B-ALL patients. This consolidation strategy was adopted in several clinical trials involving CD19 or CD22 CAR-cell therapy in B-ALL patients. The results observed in these studies on the capacity of allo-HSCT to improve the results of CAR-T cell therapy, in terms of EFS and OS, are variable. It is important to note that, to date, no clinical trial has been specifically designed to evaluate the role of allo-HSCT after CAR-T cell therapy.

The results obtained in the various clinical trials showed variable results related to the efficacy of allo-HSCT to consolidate the results achieved by CAR-T cell therapy in terms of EFS and OS. Thus, in the ELIANA trial carried out in children and young adults treated with Tisa-Cel, only 15% of the patients who achieved a response underwent subsequent allo-HSCT with apparent no benefit in EFS related to transplant.[6-7] In contrast to these findings, Shah et al., in the National Pediatric trial carried out using the CD19.28ζ CAR-T, reported consolidative allo-HSCT in 75% of patients achieving a CR with MRD-negative status, with a mOS of 70.2 months, a 5-yr EFS rate of 61.9% and a cumulative incidence of relapse of 9.5% at 24 months; 7 patients with MRD-negative CR not undergoing allo-HSCT relapsed with a median time of relapse of 152 days.[30] Thus, this study concluded that consolidative allo-HSCT is required following CAR-T cell therapy with CD19.28ζ construct.[30]

Zheng et al. reported a retrospective analysis on 52

R/R B-ALL patients undergoing remission following treatment with CD19 or CD22-targeted CAR-T cell therapy and processed for allo-HSCT after myeloablative reduced intensity conditioning; after a median follow-up of 334 days, 1-year OS and EFS were 87.7% and 73%, respectively, with 1-year relapse rate and transplantation-related mortality of 24.7% and 2.2%, respectively.[65]

The ZUMA-3 trial with Axu-Cel[24,26-27] and the study of Park et al. with CD19.28ζ CAR-T of Park et al.[20] carried out in adult R/R B-ALL patients failed to show any significant effect of allo-HSCT in improving EFS and overall survival in patients who achieved a CR, with a MRD-negative status. However, Hay and coworkers, in their study with CD19 CAR-T cells with 4.1BB co-stimulatory domain, carried out on 53 adult R/R B-ALL patients, showed that patients achieving a CR with MRD negativity and undergoing allo-HSCT displayed a longer EFS compared to patients with CR and MRD negativity not undergoing allo-HSCT.[17]

Cao et al. have retrospectively analyzed long-term follow-up data concerning 97 R/R B-ALL patients who relapsed after a first HSCT and who have received either CD19- or CD22-targeted CAR-T cell therapy followed by consolidation with a second allo-HSCT.[66-67] The second transplant was performed using donors different from the first transplant. These patients' 4-year OS and OS were 52.6% and 49.8%, respectively.[66-67] These observations support the view that CAR-T cell therapy followed by consolidation with a second HSCT for B-ALL patients who have relapsed after first transplantation may improve long-term survival.

ALLOGENEIC CAR-T CELL THERAPY

Since 2017 (FDA) and 2018 (EMEA), autologous CAR-T cells have been approved for commercialization to treat many lymphoid hematological malignancies, showing impressive clinical efficacy in patients with relapsed or refractory advanced-stage tumors. However, using the patient's T cells as starting material (i.e., autologous use) has important limitations since patients' T cells may be dysfunctional and exhausted, influencing CAR-T cell products' potency and variability. This can be caused by patient age, the number of previous lines of treatment, the disease itself, and, in solid tumors, local immune suppression and the effects of prolonged T-cell stimulation. In addition, autologous CAR-T cell therapies are individualized products, thus entailing theoretically higher costs and manufacturing time, usually around 2–3 weeks. Moreover, in patients with refractory leukemias, there are often large numbers of circulating leukemic cells that can be extracted along with healthy lymphocytes and thus contaminate the product. It has been suggested that CAR-transduced cancer cells present on therapy may be associated with down-regulation of the target antigen, leading to patient relapse by this newly generated population.

Other causes of strains could be manufacturing delays, high production costs, and difficulties in standardizing the preparation process. Furthermore, the number of T-cells is too low to be harvested in some circumstances.[68]

To take the next step and reach much larger numbers of patients, you should adopt treatments "off-the-shelf" offering a standardized, consistent, and cost-effective product to patients.

The use of allogeneic CAR T cells from donors has many potential advantages over autologous approaches, such as the immediate availability of cryopreserved batches for patient treatment, possible standardization of the CAR-T cell product, time for multiple cell modifications, redosing or combination of CAR T cells directed against different targets, and decreased cost using an industrialized process. Furthermore, allogeneic CAR-T cells are the only ones that can be utilized when the subject is lymphopenic. All autologous and allogeneic CAR-T cells are genetically modified T cells to express the specific CAR molecule. Moreover, one of the main strategies to enable the allogeneic use of CAR-T cells manufactured from healthy donor T cells involves the addition of extra genetic modifications in the manufacturing process. The host immune system may rapidly eliminate the allogeneic T-CAR cells; therefore, the infusion of allogeneic T-CAR cells must be preceded by a lymphodepletion regimen comprising fludarabine (F, 90mg/m2) and cyclophosphamide (C,1,500mg/m2) with or without alemtuzumab (A, 1 mg/kg, or 40mg, or 60mg flat dose), to improve CAR-T cell engraftment and expansion. However, UCART19 expansion rates in the clinical trials confirm the need for alemtuzumab to observe UCART19 expansion (along with fludarabine cyclophosphamide).[69]

Furthermore, the allogeneic CAR T cells may cause life-threatening graft-versus-host disease. Developing next-generation allogeneic CAR T cells to address these issues is an active area of research.

In recent years, a wide range of different approaches have been studied to achieve the production of allogeneic CAR-T cell therapies, which could be classified into two main categories: those involving extra genetic modifications in addition to CAR transgene introduction and those relying on the selection of alternative cell sources/subpopulations as starting material.

Different sources of T cells for optimal allogeneic CAR-T cell therapy and different technological approaches, mainly based on gene editing, have been settled to produce allogeneic CAR-T cells with limited potential for graft-versus-host disease.[70]

Novel strategies, many of which have been reported in the last 5 years, include the use as cell sources of γδ T cells, Induced pluripotent stem cells (iPSCs), Umbilical cord blood T (UCB T) cells, memory T cell

Autologous CAR-T	Allogeneic Matched Donor CAR-T	Allogeneic Unmatched CAR-T
PRO	**PRO**	**PRO**
Established Clinical Efficacy	Good persistence	Available right away
Greater Persistence	Modest GVHD	No leukapheresis needed
No GVHD	Feasible with low Lymphocytes	Product uniformity
CON	**CON**	Healthy, no Dysfunction T-cells
Time from Leukaferesis to Infusion	Donor availability	Likely decreased cost
Need for bringing chemotherapy	Product variability	**CON**
Not feasible with low lymphocytes	Expensive	Limited clinical data
Product variability		Limited persistence
Expensive		Risk of GVHD
		Lymphodepletion with Alemtuzumab (Infections?)

subpopulations, Virus-Specific T (VST) cells, and Cytokine-induced killer cells (CIK) cells. Although genetic modification of T cells is the most widely used approach, new strategies combining both methods have emerged. However, further preclinical and clinical research is needed to establish the most appropriate strategy for producing allogeneic CAR-T cells, which should minimize the major risks of this therapy: GvHD and immune rejection. Commercializing this promising antitumor therapy could extend the availability of CAR-T cells to a larger number of patients.[70-72]

As previously mentioned, the two main potential problems of the allogeneic use of T cells are GvHD and immune rejection. The former can be avoided by eliminating the TCR, usually through the knockout of the constant domain of one of its chains (α or β), or by replacing some TCR subunits that impede its antigen recognition function.

Regarding immune rejection, it is avoided by preventing the expression of HLA class I (HLA-I) molecules by knocking out their common subunit β2-microglobulin (encoded by the B2M gene), which prevents the recipient's T cells from recognizing the therapeutic cells as foreign through their TCR.

In the context of relapsed and refractory childhood pre-B cell acute lymphoblastic leukemia (R/R B-ALL), CD19-targeting chimeric antigen receptor (CAR)-T cells often induce durable remissions, which requires the persistence of CAR-T cells.[68]

The approach is simpler by utilizing T cells obtained from a compatible donor as starting material for allogeneic CAR-T cell manufacturing. However, it depends on donor availability and maintains the main disadvantages of autologous therapies, such as high cost, lack of standardization, and manufacturing time. It is not a truly "off-the-shelf" therapy but has been used experimentally in some patients.[73-79] Additionally, several studies investigate using specific cell sources or subpopulations to produce "off-the-shelf" CAR-T cell therapies. In the following, we describe these different strategies that, in most cases, are based on selecting a specific subpopulation that would allow allogeneic use without causing GvHD.

So far, most clinical experience is concerned with using T cells of compatible donors.[76-80]

T-Cells from Compatible Donors. CAR-T cells from compatible donors demonstrated their efficacy in relapsed patients after allogeneic stem cell transplantation, increasing disease-free survival.

The first demonstration that donor allogeneic anti-CD 19 CAR-T cells were able to induce regression of B-cell Lymphoid malignancy in relapse after allogeneic transplantation was reported by Kochenderfer et al. (2013), who conducted a clinical trial of allogeneic T cells genetically modified to express a chimeric antigen receptor (CAR) targeting the B-cell antigen CD19. T cells for genetic modification were obtained from each patient's allo-HSCT donor. All patients had a B cell malignancy that persisted after allo-HSCT and standard donor lymphocyte infusions (DLIs). Patients did not receive chemotherapy prior to the CAR T-cell infusions and were not lymphocyte-depleted at the time of the infusions. The 10 treated patients received a single infusion of allogeneic anti-CD19-CAR T cells. Three patients had regressions of their malignancies. One patient with chronic lymphocytic leukemia (CLL) obtained an ongoing complete remission after treatment with allogeneic anti-CD19-CAR T cells, another CLL patient had tumor lysis syndrome as his leukemia dramatically regressed, and a patient with mantle cell lymphoma obtained an ongoing partial remission—none of the 10 patients developed graft-versus-host disease (GVHD). Toxicities included transient hypotension and fever. Cells containing the anti-CD19-CAR gene were detected in the blood of 8 of 10 patients. These results showed for the first time that donor-derived allogeneic anti-CD19-CAR T cells can cause regression of B-cell malignancies resistant to standard DLIs without causing GVHD.[73] A similar experience was reported by Cruz et al. (2013) with an infusion of donor-derived CD19-redirected virus-specimen in patients who relapsed after

allogeneic BMT B-cell malignancies.[74] Eight patients were treated with allogeneic (donor-derived) CD19. CAR-VSTs 3 months to 13 years after HSCT. There were no infusion-related toxicities. VSTs persisted for a median of 8 weeks in blood and up to 9 weeks at disease sites. Objective antitumor activity was evident in 2 of 6 patients with relapsed disease during CD19.CAR-VST persistence, whereas 2 patients who received cells while in remission remained disease-free.[74]

Good results were also obtained by Brudno et Al. (2016) with the T cells obtained from each recipient's allo-HSCT donors. Eight of 20 treated patients obtained remission, which included six complete remissions (CRs) and two partial remissions. The response rate was highest for acute lymphoblastic leukemia, with four of five patients obtaining minimal residual disease-negative CR. None of the patients developed new-onset acute graft-versus-host disease after CAR T-cell infusion. Toxicities included fever, tachycardia, and hypotension.[75]

Good results have also been obtained by the sequential infusion of allogeneic donors followed by autologous and CAR-T cells in a child suffering from relapsed and refractory B-ALL, severely lymphoid-depleted.[76]

Donor-derived CAR-T infusions after allogeneic transplantation compared with donor lymphocyte infusions. Three Chinese papers[77-79] demonstrated the superiority of donor-derived anti-CD19 CAR T cells vs Donor lymphocytes (DLI) for the management of relapsed B-cell acute lymphoblastic leukemia (B-ALL) after allo-hematopoietic stem cell transplantation (HSCT)

Hua et Al. (2021) compared B-ALL patients who relapsed after allo-HSCT; 13 were treated with donor-derived anti-CD19 CAR T-cell (study group), and 15 were treated with DLI (DLI group). The rates of MRD-negative complete remission (61.5%) in the study group were significantly higher than those in the DLI group (13.3%) ($p = 0.02$). The complete remission duration in the study group and DLI group were median of 8.0 months (range, 3–25 months) and 4.4 months (range, 1–25 months; $p = 0.026$), respectively. The overall survival of patients in the study group was superior to that of the DLI group: 9.5 months (range,3–25 months) versus 5.5 months (range, 1–25 months; $p = 0.030$). The study group identified one patient with grade 1 acute graft-versus-host disease (aGVHD). At the same time, five (33.3%) patients in the DLI group developed grades III–IV aGVHD. Three patients (23.07%) developed grade 3 or 4 cytokine release syndrome in the study group. This study suggested that donor-derived anti-CD19 CAR T-cell therapy is a promising, safe, and potentially effective treatment for relapsed B-ALL after allo-HSCT and may be superior to DLI.[77] The efficacy of anti-CD19-CAR T-cell therapy can be improved by the donor hemopoietic stem cell infusion (DSI) more than donor lymphocyte infusion (DLI) therapy, as reported by Li et al. (2023). In total, 22 B-ALL patients who relapsed after allo-HSCT received anti-CD19-CAR T-cell therapy. Patients who responded to CAR T-cell therapy received DSI or DLI as maintenance therapy. The two groups' clinical responses, acute graft versus host disease (aGVHD), expansion of CAR-T-cells, and adverse events were compared. In our study, 19 patients received DSI/DLI as maintenance therapy. After DSI/DLI therapy, progression-free survival and overall survival were higher in the DSI group than in the DLI group at 365 days. The aGVHD, grades I and II, was observed in four patients (36.4%) in the DSI group. Only one patient developed grade II aGVHD in the DLI group. IL-6 and TNF-α levels increased again in nine of 11 patients after DSI but not in the DLI group. These findings indicate that for B-ALL patients who relapse after allo-HSCT, DSI is a feasible maintenance therapy if CR is obtained with CAR-T-cell therapy.[78]

A single-center retrospective study was conducted comparing 12 patients treated with DLI (control group) and 12 patients treated with donor-derived CD19 CAR-T cells. The median age of patients was 31. The event-free survival (EFS) of patients in the experimental group was longer than that of the control group: 516 days versus 98 days ($p = 0.0415$). No significant difference in the incidence of infection was identified between these two groups. Three patients developed GVHD after CAR-T therapy, including 2 cases of aGVHD (grade 2 and grade 3) and one severe cGVHD, and were effectively controlled by combinational therapy with steroids. Among patients of the DLI group, 2 (16.7%) and 7 (58.8%) developed grades I–II and III–IV aGVHD, respectively. Most patients in the experimental group had only mild cytokine release syndrome, and post-transplantation relapse was associated with better EFS. There was no significant difference in EFS between patients treated with dual-target CAR-T and those with single CD19 CAR-T. In this study, data supported that donor-derived CAR-T therapy is a safe and potentially effective treatment for relapsed B-ALL after HSCT and may be superior to DLI.[79]

Another experience with donor-derived allogeneic CD-19-directed CAR-T cells in relapsed/refractory B-cell precursor acute lymphoblastic leukemia (BCP-ALL) has been reported by Del Bufalo et al. from the Bambino Gesù Hospital in Rome. Donor-derived T cells were transduced with a second-generation(4.1BB) CD19-directed CAR manufactured in the site place of care. Thirteen children/young adults (median age 15 years) received ALLO–CAR-T cells between March 2021 and October 2022. Doses ranged between 1.0×10^6 and 3.0×10^6 CAR-T cells per kg. The toxicity profile was comparable with that of autologous CAR-T cells,

characterized mainly by cytopenia, cytokine release syndrome (maximum grade 1), and grade 2 immune-effector cell–associated neurotoxicity syndrome. One case of acute graft-versus-host disease (GVHD) occurred and was rapidly controlled with steroids and ruxolitinib. None of the other patients, including 3 treated with ALLO–CAR-T cells from an HLA-haploidentical donor, experienced GVHD. Two patients received ALLO–CAR-T cells before HSCT and showed a significant expansion of CAR-T cells without any sign of GVHD. All patients obtained complete remission (CR) without minimal residual disease in the bone marrow. With a median follow-up of 12 months (range, 5-21), 8 of 13 patients maintain CR.[80]

T-Cells from Unselected Healthy Donors.

UCART19 engineered. Allogeneic CAR-T cells, not HLA compatible with the recipient, can be utilized only by performing a Genome editing of allogeneic T cells by the disruption of T cell receptor α chain (TRAC) to prevent graft-versus-host disease (GVHD) and removal of CD52 (cluster of differentiation 52) by using transcription activator–like effector nuclease (TALEN) for obtaining a survival advantage in the presence of alemtuzumab.[81] The mRNA encoding TALENs is used to knock out the genes encoding the TCR α constant chain and CD52 to minimize the risk of GVHD by reducing the number of TCRαβ-positive T cells and to confer resistance to the anti-CD52 monoclonal antibody alemtuzumab.

This method was utilized by the UCART19 Group to treat CD 19 positive ALL. Qasim et Al. (2017) first treated two infants with relapsed refractory CD19+ B cell acute lymphoblastic leukemia receiving lymphodepletion chemotherapy and anti-CD52 serotherapy, followed by a single-dose infusion of UCART19 cells. Molecular remissions were achieved within 28 days in both infants, and UCART19 cells persisted until conditioning ahead of successful allogeneic stem cell transplantation. This bridge-to-transplantation strategy demonstrates the therapeutic potential of gene-editing technology.[78]

Benjamin et Al. (2020) reported phase 1 trials in pediatric and adult patients with late-stage relapsed or refractory B-cell acute lymphoblastic leukemia treated with UCART19.

Pediatric or adult patients were enrolled in two ongoing, multicenter, phase 1 clinical trials to evaluate the safety and antileukemic activity of UCART19. All patients underwent lymphodepletion with fludarabine and cyclophosphamide with or without alemtuzumab, then children received UCART19 at $1{\cdot}1–2{\cdot}3 \times 10^6$ cells per kg, and adults received UCART19 doses of 6×10^6 cells, $6–8 \times 10^7$ cells, or $1{\cdot}8–2{\cdot}4 \times 10^8$ cells in a dose-escalation study. Patients not receiving alemtuzumab (n=4) showed no UCART19 expansion or antileukemic activity. The median duration of response was 4·1 months, with ten (71%) of 14 responders proceeding to a subsequent allogeneic stem-cell transplant. Progression-free survival at 6 months was 27%, and overall survival was 55%.

The primary outcome measure was adverse events in the period between the first infusion and data cutoff.

Cytokine release syndrome was the most common adverse event and was observed in 19 patients (91%); three (14%) had grade 3–4 cytokine release syndrome. Other adverse events were grade 1 or 2 neurotoxicity in eight patients (38%), grade 1 acute skin graft-versus-host disease in two patients (10%), and grade 4 prolonged cytopenia in six patients (32%). Two treatment-related deaths occurred: one caused by neutropenic sepsis in a patient with concurrent cytokine release syndrome and one from pulmonary hemorrhage in a patient with persistent cytopenia. 14 (67%) of 21 patients had a complete response or complete response with incomplete hematological recovery 28 days after infusion

Of seven children in the PALL study, 1 was lost to follow-up, 2 are alive and in remission, 4 died, 3 from progressive disease, and 1 from infection after stem-cell transplant.

Of 14 adults in the AALL study, 3 are alive and in remission, 1 relapsed, 11 died, 7 from progressive disease, 2 from non-treatment-related infection, 1 from treatment-related infection plus cytopenia, 1 from treatment-related infection plus cytokine release syndrome.

These two studies showed, for the first time, the feasibility of using allogeneic, genome-edited CAR T cells to treat patients with aggressive leukemia. UCART19 exhibited in-vivo expansion and antileukemic activity with a manageable safety profile in heavily pretreated pediatric and adult patients with relapsed or refractory B-cell acute lymphoblastic leukemia. The results of this study were considered an encouraging step forward for the field of allogeneic CAR T cells.[83]

This unicentric experience was repeated in a polycentric study., with the same modality.[84] A phase 1 open-label study was conducted at eight centers across France, the UK, the USA, and Japan. Adult patients aged 16–70 years with CD19-positive relapsed or refractory B-cell acute lymphoblastic leukemia who had a morphological relapse or a minimal residual disease level of at least 1×10^{-3} and had exhausted standard treatment options were enrolled in the study, which comprised a dose-escalation phase of up to three UCART19 doses followed by a safety expansion phase. Patients underwent lymphodepletion, and then $1{\cdot}8–2{\cdot}4 \times 10^8$ total CAR T cells were infused intravenously, followed by safety evaluation and disease response assessments. The primary endpoint was the incidence and severity of adverse events. Secondary endpoints

were the overall response rate, duration of response, relapse-free survival, progression-free survival, and overall survival. Between Aug 1, 2016, and June 30, 2020, 25 patients were enrolled in the study and treated with UCART19. The median duration of follow-up was 12·8 months (IQR 2·8–24·8). The median age was 37 years (IQR 28–45). 14 (56%) patients were male and 11 (44%) females. Seventeen (68%) patients were White, two (8%) were Black, two (8%) were Asian, and four (16%) were from other racial or ethnic groups. Three patients developed dose-limiting toxicities (one at each dose level); one had grade 4 cytokine release syndrome, and two had grade 4 prolonged cytopenia. Grade 3 or higher cytokine release syndrome was reported in six (24%) patients, and grade 3 or higher neurological toxicity in one (4%) patient. Grade 3 or higher infections occurred in seven (28%) patients, and grade 4 prolonged cytopenia in four (16%) patients. Two (8%) patients developed grade 1 acute cutaneous graft-versus-host disease. 14 patients died, nine from progressive disease and five from infections or other complications, of which four were considered to be related to UCART19, lymphodepletion, or both. After a median of follow-up of 12·8 months (IQR 2·8–24·8), overall response rate was 48% (95% CI 28–69; 12 of 25 patients), duration of response and median relapse-free survival were 7·4 months (95% CI 1·8 to not calculable), progression-free survival was 2·1 months (95% CI 1·2–2·8), and overall survival was 13·4 months (95% CI 4·8–23·0).

UCART19 had a manageable safety profile and showed evidence of antileukemic activity in heavily pretreated adult patients with relapsed or refractory B-cell acute lymphoblastic leukemia. This study shows that allogeneic off-the-shelf CAR T cells can be used safely to treat patients with relapsed B-cell acute lymphoblastic leukemia.[84]

Dupouys et Al. (2022) reported twenty-five adult patients with CD19-positive R/R B-ALL, enrolled from August 2016 to July 2020 in an open-label nonrandomized phase I/II study conducted in eight clinical centers across Europe, USA, and Japan. This trial, CALM study, comprised two phases: a dose escalation investigating three dose levels of UCART19 (6×10^6,6–8×10^7, or 1.8–2.4 $\times 10^8$ total CAR+ cells) followed by a dose expansion at the recommended dose (6–8 $\times 10^7$ total CAR+ cells). All patients received a 6-day lymphodepletion regimen prior to UCART19 infusion (day 0) consisting of fludarabine (F) 30 mg/m2/day i.v. for 3 days (day-7 to day-5) and cyclophosphamide (C) 500 mg/m2/day i.v. for 3 days (day-4 to day-2), with or without alemtuzumab (A) 1 mg/kg, 40 or 60 mg flat doses (day-7 to day-3). The dose of alemtuzumab was modified during the trial to balance the infectious complications related to alemtuzumab use and UCART19 efficacy. An allogeneic stem cell transplantation (allo-SCT) could be performed at any time following disease evaluation on day 28 after the UCART19 infusion. In this trial, UCART19 was administered to 25 adult patients with relapsed or refractory (R/R) B-cell acute lymphoblastic leukemia (B-ALL). All patients underwent lymphodepletion with fludarabine and cyclophosphamide ± alemtuzumab and received one of three ascending doses of UCART19. Given the allogeneic nature of UCART19, we analyzed the impact of lymphodepletion, HLA disparities, and host immune system reconstitution on its kinetics, along with other factors that affect autologous CAR-T cell clinical pharmacology. Responder patients (12/25) had higher UCART19 expansion (Cmax) and exposure (AUCT last) than no responders (13/25), as measured by transgene levels in peripheral blood. The persistence of CAR+ T cells did not exceed 28 days in 10/25 patients and lasted beyond 42 days in 4/25.

No significant correlation was found between UCART19 kinetics and administered cell dose, patient and product characteristics, or HLA disparities. However, the number of prior lines of therapy and the absence of alemtuzumab negatively impacted UCART19 expansion and persistence. Alemtuzumab exposure positively affected IL7 and UCART19 kinetics while negatively correlating with host T lymphocyte AUC0-28.

UCART19 expansion is a response driver in adult patients with R/R B-ALL. These results shed light on the factors associated with UCART19 kinetics, which remain highly affected by the impact of alemtuzumab on IL7 and host-versus-graft rejection. This study represents the first description of the clinical pharmacology of a genome-edited allogeneic anti-CD19 CAR-T cell product, showing the crucial role of an alemtuzumab-based regimen in sustaining UCART19 expansion and persistence through increased IL7 availability and decreased host T lymphocyte population.[85]

To better understand and quantify the impact of the preconditioning regimen on the engraftment and proliferation of CAR-T cells, Derippe et Al. built a population-based mechanistic pharmacokinetic-pharmacodynamic model describing the complex interplay between lymphodepletion, host immune system, homeostatic cytokines, and pharmacokinetics of UCART19, an allogeneic product developed against $CD19^+$ B cells. Data were collected from a phase I clinical trial in adult relapsed/refractory B-cell acute lymphoblastic leukemia. They revealed three different UCART19 temporal patterns: (i) expansion and persistence, (ii) transient expansion with subsequent rapid decline, and (iii) absence of observed expansion. On the basis of translational assumptions, the final model was able to capture this variability through the incorporation of IL-7 kinetics, which is thought to be increased owing to lymphodepletion, and through an elimination of UCART19 by host T cells, which is specific to the allogeneic context. Simulations from the

final model recapitulated UCART19 expansion rates in the clinical trial, confirmed the need for alemtuzumab to observe UCART19 expansion (along with fludarabine cyclophosphamide), quantified the importance of allogeneic elimination, and suggested a high impact of multipotent memory T-cell subpopulations on UCART19 expansion and persistence. In addition to supporting the role of host cytokines and lymphocytes in CAR-T cell therapy, such a model could help optimize the preconditioning regimens in future clinical trials.[86]

In conclusion, UCART19 was shown to proliferate and induce responses in adult patients with B-ALL following a lymphodepletion regimen, including fludarabine, cyclophosphamide, and alemtuzumab. Several factors potentially influencing UCART19 cellular kinetics were identified, highlighting areas for improvement. Further efforts are needed to optimize the therapeutic window, allowing appropriate expansion and persistence of allogeneic CAR-T cells, among which optimization of the chosen lymphodepletion regimen and strategy of redosing are key to making allogeneic CAR-T cell therapy a success.

CRISPR/Cas9 Technology. The utility of CRISPR-Cas9 technology has led to a surge in applying genome editing approaches to combat various genetic disorders and cancers. Inherited genetic diseases with known gene mutations can be corrected in these cases. CRISPR/Cas9 technology applications are being explored in T-cell-based immunotherapies to improve T-cell effector function and persistence, reduce treatment toxicity, and increase patient product availability.[87-90]

Compared with TALEN gene editing technology, CRISPR/Cas9 technology has a simpler design, higher editing efficiency, and wider versatility. Clinical data from trials of the product generated using CRISPR/Cas9 gene editing technology have demonstrated its safety and feasibility, including through single-antigen targeting (CD7) in R/R T cell ALL.

Hu et al. developed (2021) CRISPR-edited universal off-the-shelf CD19/CD22 dual-targeted CAR-T cells as a novel therapy for r/r ALL. In their open-label dose-escalation phase 1 study, universal CD19/CD22-targeting CAR-T cells (CTA101) with a CRISPR/Cas9-disrupted *TRAC* region and *CD52* gene to avoid host immune-mediated rejection were infused in patients with r/r ALL. Safety, efficacy, and CTA101 cellular kinetics were evaluated.

Six patients received CTA101 infusions at doses of 1 (3 patients) and 3 (3 patients) × 10^6 CAR^+ T cells/kg body weight. Cytokine release syndrome occurred in all patients. No dose-limiting toxicity, GvHD, neurotoxicity, or genome editing-associated adverse events have occurred. The complete remission (CR) rate was 83.3% on day 28 after the CTA101 infusion. With a median follow-up of 4.3 months, 3 of the 5 patients who achieved CR or CR with incomplete hematologic recovery (CR/CRi) remained minimal residual disease (MRD) negative. The authors concluded that CRISPR/Cas9-engineered universal CD19/CD22 CAR-T cells exhibited a manageable safety profile and prominent antileukemia activity. Universal dual-targeted CAR-T cell therapy may offer an alternative therapy for patients with r/r ALL.[91]

Ottaviano et Al. deployed next-generation CRISPR-Cas9 editing and linked CAR expression to multiplexed DNA editing of TRAC and CD52 through the incorporation of self-duplicating CRISPR guide RNA expression cassettes within the 3' long terminal repeat of a CAR19 lentiviral vector. Three cell banks of TT52CAR19 T cells were generated and cryopreserved. A phase 1, open-label, nonrandomized clinical trial was conducted and treated six children with relapsed/refractory CD19-positive B cell acute lymphoblastic leukemia (B-ALL) (NCT04557436). Lymphodepletion included fludarabine, cyclophosphamide, and alemtuzumab and was followed by a single infusion of 0.8 × 10^6 to 2.0 × 10^6 CAR19 T cells per kilogram with no immediate toxicities. Four of six patients infused with TT52CAR19 T cells exhibited cell expansion, achieved flow cytometric remission, and then proceeded to receive allogeneic stem cell transplantation. Two patients required biological intervention for grade II cytokine release syndrome, one patient developed transient grade IV neurotoxicity, and one patient developed skin GVHD, which resolved after transplant conditioning. Other complications were within expectations, and primary safety objectives were met.[92]

This study provides a demonstration of the feasibility, safety, and therapeutic potential of CRISPR-engineered immunotherapy, and none developed neurotoxicity.

Conclusions. CAR-T cell therapy has made an important contribution to the therapy of B-ALL patients, particularly those with R/R disease. Thus, in 2017, Tisa-Cel was approved for treating pediatric and young adult patients; in 2021, Brexu-Cel received approval for adult patients with R/R B-ALL.

Real-world studies have confirmed the high rates of CR observed using these agents in patients with R/R B-ALL.[93] Particularly, a real-world report of CAR-T cell treatment in B-ALL patients from Germany showed that patients with refractory or relapsed disease also relapsed after allo-HSCT could be rescued with an infusion of CAR-T cells (Tisa-Cel): patients who relapsed ≥6 months after HSCT have a good chance to survive their disease (45% of patients).[94]

However, adverse events such as cytokine release syndrome and immune effector cell-associated neurotoxicity represent significant challenges to CAR-T cell therapy. The severity of these adverse events

correlates with the pretreatment tumor burden; this observation supports CAR-T cell therapy early at low tumor burden and debulking therapies prior to CAR-T cell infusion to reduce the severity of these adverse events.

Furthermore, a significant proportion of patients rapidly relapse after an initial response due to early CAR-T cell loss and antigen downregulation and to enhance CAR-T cell persistence *in vivo*.

The role of allo-HSCT after CAR-T cell therapy remains an undefined issue; specific randomized trials are required for the different CAR-T constructs and for pediatric/young adult and adult B-ALL patients to assess the role of allo-HSCT in consolidating the therapeutic effects achieved through CAR-T cell therapy. However, the donor-matched T-CAR lymphocytes have had a major utilization and should be proposed whenever the number of patient's lymphocytes is too low to allow sufficient harvesting. Their superior efficacy in comparison to simple lymphocytes has been demonstrated.

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