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New Frontiers in gene-modified T cell technology

Edited by
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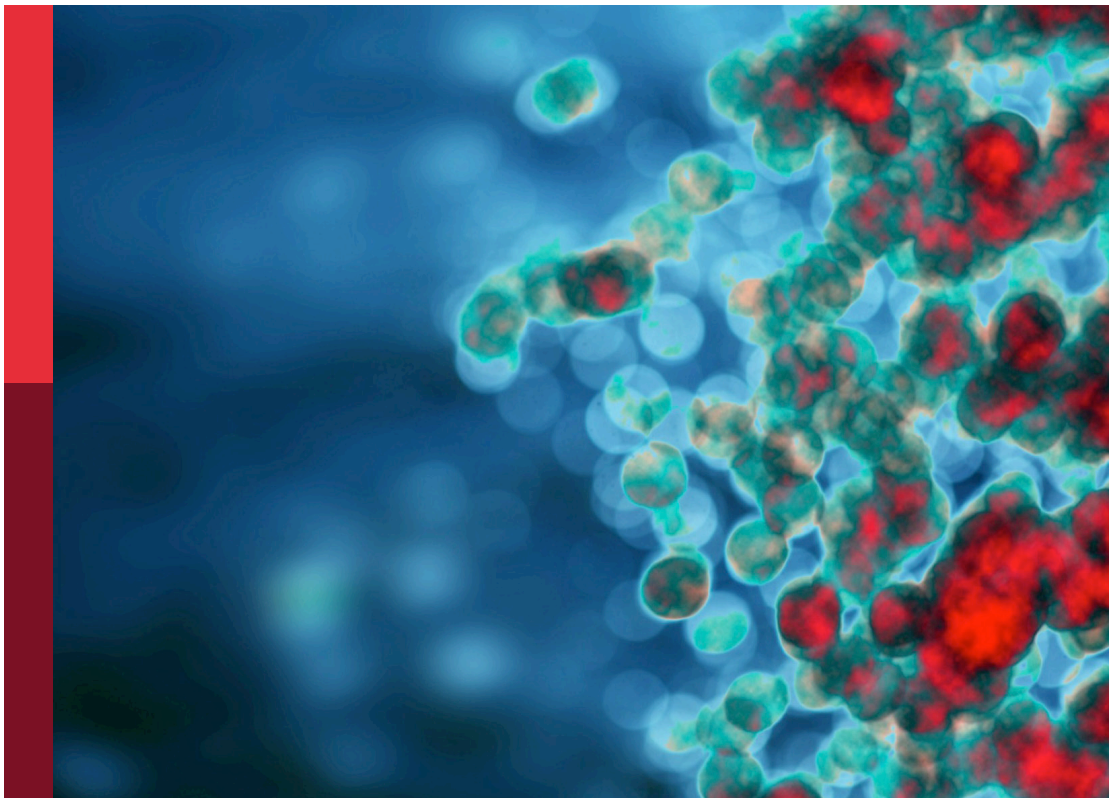

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New Frontiers in gene-modified T cell technology

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Humanized Anti-CD19 CAR-T Cell Therapy and Sequential Allogeneic Hematopoietic Stem Cell Transplantation Achieved Long-Term Survival in Refractory and Relapsed B Lymphocytic Leukemia: A Retrospective Study of CAR-T Cell Therapy

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Early response could be obtained in most patients with relapsed or refractory B cell lymphoblastic leukemia (R/R B-ALL) treated with chimeric antigen receptor T-cell (CAR-T) therapy, but relapse occurs in some patients. There is no consensus on treatment strategy post CAR-T cell therapy. In this retrospective study of humanized CD19-targeted CAR-T cell (hCART19s) therapy for R/R B-ALL, we analyzed the patients treated with allogeneic hematopoietic stem cell transplantation (allo-HSCT) or received a second hCART19s infusion, and summarized their efficacy and safety. We retrospectively studied 28 R/R B-ALL patients treated with hCART19s in the Affiliated Hospital of Xuzhou Medical University from 2016 to 2020. After the first hCART19s infusion, 10 patients received allo-HSCT (CART+HSCT group), 7 patients received a second hCART19s infusion (CART2 group), and 11 patients did not receive HSCT or a second hCART19s infusion (CART1 group). The safety, efficacy, and long-term survival were analyzed. Of the 28 patients who received hCART19s treatment, 1 patient could not be evaluated for efficacy, and 25 (92.6%) achieved complete remission (CR) with 20 (74.7%) achieving minimal residual disease (MRD) negativity. Seven (25%) patients experienced grade 3-4 CRS, and one died from grade 5 CRS. No patient experienced ≥3 grade ICANS. The incidence of second CR is higher in the CART+HSCT group compared to the

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CART2 group (100% *vs.* 42.9%, p=0.015). The median follow-up time was 1,240 days (range: 709–1,770). Significantly longer overall survival (OS) and leukemia-free survival (LFS) were achieved in the CART+HSCT group (median OS and LFS: not reached, p=0.006 and 0.001, respectively) compared to the CART2 group (median OS: 482; median LFS: 189) and the CART1 group (median OS: 236; median LFS: 35). In the CART+HSCT group, the incidence of acute graft-*versus*-host disease (aGVHD) was 30% (3/10), and transplantation-related mortality was 30% (3/10). No chronic GVHD occurred. Multivariate analysis results showed that blasts ≥ 20% in the bone marrow and MRD ≥ 65.6% are independent factors for inferior OS and LFS, respectively, while receiving allo-HSCT is an independent factor associated with both longer OS and LFS. In conclusion, early allo-HSCT after CAR-T therapy can achieve long-term efficacy, and the adverse events are controllable.

Keywords: chimeric antigen receptor T cell therapy (CAR-T), hematopoietic stem cell transplantation, relapsed/refractory B cell lymphoblastic leukemia, overall survival, leukemia free survival, minimal residual disease (MRD)

INTRODUCTION

Patients with relapsed or refractory (R/R) acute B-cell lymphoblastic leukemia (B-ALL) progress rapidly, and the overall 5-year survival rate is only 10–20% (1, 2). The response rate of salvage chemotherapy is low. At present, allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only cure for patients with R/R B-ALL. However, only a few patients have the opportunity to undergo allo-HSCT.

As a novel therapeutic strategy, rapid progress has been made in chimeric antigen receptor T-cell (CAR-T) therapy in recent years, especially in hematological malignancies, which showed a high remission rate (3, 4). We previously reported that the effective rate of CAR-T cell therapy targeting CD19 was 93% in R/R B-ALL patients (5, 6). These findings were consistent with those of Davila et al. (3), who reported that the complete remission (CR) rate of CD19 CAR-T on R/R B-ALL can reach 88%, and the adverse events can be tolerated. On the other hand, CAR-T cell therapy has shown significant therapeutic efficacy in turning MRD negativity. A few large-sale studies (7, 8) also indicated that CAR-T cell therapy promoted negative MRD (range: 67%–87%). Notably, pre-transplantation status, especially MRD status, is related to long-term survival after HSCT (9, 10).

Although a high MRD-negative CR rate was achieved after CAR-T therapy, the long-term efficacy was unsatisfactory due to loss of the CAR-T cells resulting from the limited long-term persistence, the immune-suppressive microenvironment, and exhaustion of CAR-T cells (11, 12). It is necessary to optimize the strategy of treatment to further improve long-term efficacy after CAR-T cell infusion. However, either bridging HSCT or second CART is controversial in improving long-term efficacy. Gauthier et al. (13) noted that only 21% of patients obtained CR after second infusion of anti-CD19 CAR-T cells, while the median duration of response was merely 4 months. On the other hand, Zhang et al. (14) demonstrated that 184 patients who underwent allo-HSCT had better 2-year OS and LFS than patients who did not (68% *vs.* 28.3, 60.4% *vs.* 27.8%, respectively, p<0.001), but lack longer follow-up. This is consistent with the findings of Hay et al., who found that allo-HSCT after anti-CD19 CAR-T cell therapy was associated with a better LFS (15). On the contrary, Park et al. (16) reported that relapse and transplant-related toxicities were the main causes of death for 17 patients who underwent allo-HSCT after CAR-T therapy, suggesting that the patients seemed not to benefit from allo-HSCT.

Therefore, we conducted long-term follow-up and retrospectively analyzed the efficacy and safety of patients who received HSCT or a second hCART19s infusion as a sequential treatment after the first hCART19s infusion.

PATIENTS AND METHODS

Patients

We retrospectively reviewed the data on CD19+ R/R B-ALL patients who received hCART19s therapy at the Affiliated Hospital of Xuzhou Medical University from May 2016 to May 2020. All the enrolled patients had relapsed or refractory disease. The eligibility criteria were age less than 70 years; good organ function and evaluation of a survival longer than 3 months; and Eastern Cooperative Oncology Group (ECOG) performance status <2. All patients provided signed informed consent before the hCART19s therapy and allo-HSCT. Patients received HSCT or a second infusion of hCART19s after hCART19s infusion depending on patients' choice, disease status, and their affordability to the treatment. Before HSCT, all patients were in MRD-negative CR after the first hCART19s infusion. In some cases of relapse after the first hCART19s infusion, the second hCART19s was infused as soon as the relapse was clinically confirmed (the detail is listed in **Supplementary Table 2**). Patients were divided into three groups: the first infusion of hCART19s without transplant or a second infusion of hCART19s (CART1 group), a second

infusion of hCART19s without transplant (CART2 group), and the first infusion of hCART19s followed by HSCT (CART+ HSCT group).

The study protocol was approved by the human studies review board at the Affiliated Hospital of Xuzhou Medical University (ClinicalTrials.gov # NCT02782351). The clinical investigation was conducted according to the principles of the Declaration of Helsinki.

CAR-T Cell Treatment Protocol

HCART19s constructed with 4-1BB costimulatory domain were generated *via* a lentiviral vector as previously reported (6). All the hCART19s required quality control before discharge. After a lymphocyte-depleting chemotherapy with a fludarabine and cyclophosphamide (FC) regimen (fludarabine at 30 mg/m^2 per day for 3 days and cyclophosphamide at 300 mg/m^2 per day for 3 days), all patients provided signed informed consent before the hCART19s therapy and allo-HSCT and received a single dose of autologous hCART19s infusion at 1×10^6 CAR-T cells/kg (**Supplementary Table 1**). No patient experienced bridged chemotherapy from preparation of CAR-T cells to infusion, and lymphodepletion was also conducted prior to the second CAR T infusion.

Transplant Protocol

All patients received marrow ablative regimens using BU/CY or modified BU/CY strategies for matched related transplantation, and anti-thymocyte globulin (ATG, rabbit) was administered for haploidentical or unrelated transplantation as a prophylactic against graft-*versus*-host disease (GVHD). Prophylactic regimens of GVHD were determined by the individual transplant physician based upon disease-related and transplant-related considerations composed of cyclosporine, methotrexate, and mycophenolate mofetil.

Assessment of Toxicity

The cytokine release syndrome was graded according to the cytokine release syndrome grading system. The cytokine release syndrome was considered to be severe if it was of grade 3 or higher. Neurotoxic effects were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.03. Severe neurotoxic effects were defined as a seizure of any grade or a toxic effect of grade 3 or higher.

Assessment of Response

Response to therapy was assessed using morphological analysis and multicolor flow cytometry. CR was defined as less than 5% bone marrow blasts, the absence of circulating blasts, and no extramedullary sites of disease (as assessed by means of computed tomography or positron-emission tomography), regardless of cell count recovery. MRD negativity was defined as less than 0.01% bone marrow blasts for all samples analyzed by multicolor flow cytometry. MRD detection was performed at 1, 2, 3, 6, 12, 18, 24, 36, and 48 months after hCART19s therapy or HSCT. Relapsed disease was defined as the reappearance of blasts in blood or bone marrow or in an extramedullary site after a CR. Overall survival (OS) was defined as the time from infusion to the date of death from any cause. Leukemia-free survival (LFS) was calculated from the date of CR to the date of relapse, death, or the last follow-up.

Statistical Analyses

All measurement data were described using median and range and compared using Mann–Whitney U tests. Enumeration data were presented as frequency (%) and compared using chi-square tests or Fisher's exact test. Follow-up time was estimated using the Kaplan–Meier method, whereas OS and LFS were estimated using the Kaplan–Meier method. A Cox regression model was used to obtain the hazard ratio (HR) estimates and corresponding 95% confidence intervals (CIs) for OS and LFS. The X-tile 3.6.1 software was used to determine the optimal cutoff values for MRD. All tests were two-sided, and $p< 0.05$ was considered statistically significant. Data were analyzed using SPSS version 26.0 and Graphpad Prism version 8.

RESULTS

Patient Characteristics

From May 2016 to May 2020, a total of 28 patients were enrolled in this study, including 12 males and 16 females, with a median age of 22 years. The characteristics of the patients are shown in **Table 1**, and detailed information is listed in **Supplementary Table 1**. There was no statistical difference among the baseline data of the three groups.

Efficacies of CAR-T Cell Therapy

Of the 28 patients receiving hCART19s infusion, 27 patients were evaluated for response at 14 or 28 days, and 1 patient died at day 14 before being evaluated. The complete remission rate was 92.6% (25/27), with 20 patients having MRD negativity. The rates of CR did not differ significantly among the aforementioned three groups after the first hCART19s infusion. Seven patients received a second hCART19s infusion, three of whom achieved second CR with MRD negativity (42.9%). Notably, as shown in **Figure 1**, compared to the first infusion, the proportion of patients with CR is lower after second infusion (92.6% *vs.* 42.9%, respectively, p=0.02).

CAR-T Cell Toxicities

CRS was the most common nonhematological adverse event after the infusion of hCART19s, which occurred in 27 of the 28 patients, including 8 patients experiencing severe CRS. The median time of CRS occurrence was 6 days (range: 1–20). Patients with CRS were treated with nonsteroidal anti-inflammatory drugs, glucocorticoids, and tocilizumab. One patient died from grade 5 CRS at day 14 after infusion of hCART19s, and one patient died from encephalorrhagia at 24 days after infusion of hCART19s. The peak serum levels of IL-6 in patients who developed grade 3–5 CRS were higher than those with grade 0–2 CRS (n=20). There was no statistical difference in serum ferritin levels between patients with grade 0–2 CRS and grade 3–5 CRS (**Figure 2**). Neurotoxicity occurred in one patient in the CART+HSCT group, with symptoms of convulsion. In

TABLE 1 | Patient characteristics.

Characteristics	CART+HSCT (n = 10)	CART2 (n = 7)	CART1 (n = 11)	*P* a	*P* b	*P* c
Age, years				0.291	0.807	0.687
Median (range)	19 (6-54)	8 (6-68)	33 (5-70)			
Sex: Male, n (%)	5 (50)	4 (57)	3 (27)	0.581	0.268	0.22
BCR-ABL1, n (%)	2 (20)	1 (14)	2 (18)	0.64	0.669	0.674
Prior intensive therapies				0.475	0.349	0.930
Median (range)	4 (2-11)	7 (2-17)	6 (2-16)			
Primary refractory to chemotherapy, n (%)	1 (10.0)	1 (14)	2 (18.2)	1	1	1
Number of relapses						
Median (range)	1 (1-2)	1 (1-2)	1 (1-3)	0.864	0.4	0.529
MRD at infusion				0.27	0.349	0.659
Median (range)%	15.7 (0.1-71.9)	36.7 (10-72.2)	29.7 (0.1-96.9)			
BM blasts before CAR-T				0.421	0.654	0.724
Median (range)%	14 (0-86)	42 (0-95)	18 (0-92)			
Volume of CAR-T cells				0.699	0.918	0.724
Median (range)	50 (50-100)	50 (20-100)	50 (50-100)			
Time from CAR-T to last chemotherapy				0.949	0.659	0.637
≤3months	7	5	6			
>3months	3	2	5			

CART, Chimeric Antigen Receptor T-Cell (CAR-T) therapy; CART+HSCT group, patients who received allogeneic hematopoietic stem cell transplantation after CAR-T; CART2 group, patients who received a second hCART19s infusion after CAR-T; CART1 group, patients who did not receive HSCT or a second hCART19s infusion; MRD, minimal residual disease; BM, bone marrow; a=CART+HSCT group vs. CART2 group; b= CART+HSCT group vs. CART1 group; c=CART2 group vs. CART1 group.

addition, all the patients developed B cell dysfunction, manifested as CD19+ B cell deletion and hypogammaglobulinemia. III–IV hematological toxicity and other adverse events are shown in **Table 2**.

The incidence of CRS after the second infusion was 7/7 (100%). No grade 3–5 CRS and neurotoxicity occurred in these patients.

Engraftment and GVHD

Ten patients received allo-HSCT after the first hCART19s infusion, with a median time of leukocyte engraftment of 15.5 days (range: 11–25) and platelet engraftment of 20 days (range: 12–36), respectively. All patients obtained a second CR. The incidence of second CR is higher in the CART+HSCT group compared to the CART2 group (100% *vs.* 42.9%, p=0.015) (**Figure 1**). One patient experienced grade 1–2 aGVHD, and two patients experienced grade 3–4 aGVHD, with a median time of 40 days (range: 26–45). No patient experienced cGVHD.

Survival Outcome

At the cutoff (January 1, 2021), the median follow-up time was 1,240 days (95% CI, 709 to 1,770). Among the 10 patients receiving allo-HSCT, the median time from the first hCART19s infusion to transplantation was 63 days. The 1-year OS rates were 70.0% (95%CI, 33.0 to 90.2), 57.1% (95% CI, 17 to 85.1), and 36.4% (95% CI, 11.2 to 62.4) in the CART+HSCT, CART2, and CART1 groups, respectively. Patients in the CART+HSCT group had a higher OS than those in the CART1 group (p=0.0063, **Supplementary Figure 1A**) but did not differ from that in the CART2 group (p=0.0893, **Supplementary Figure 1B**). The 1-year LFS rates were 80.0% (95%CI 40.9 to 94.6), 28.6% (95% CI, 4.1 to

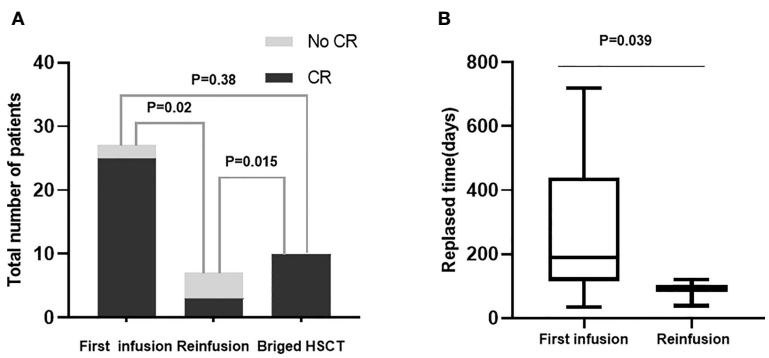

FIGURE 1 | **(A)** The influence of different times of infusion and receving allo-HSCT on CR rate. First infusion (n = 27); Reinfusion (n = 7); Bridge HSCT (n = 10). **(B)** The relapsed days according to times of infusion. First infusion (n = 14); Reinfusion (n = 3).

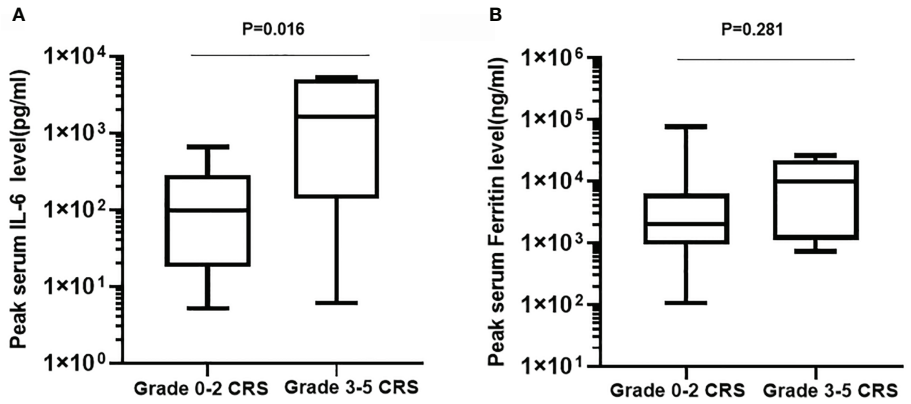

FIGURE 2 | Peak serum levels of IL-6 **(A)** and ferritin **(B)** in patients who developed grade 3-5 CRS (n=8) compared with those with grade 0-2 CRS (n=20).

61.1), and 33.3% (95% CI, 4.6 to 67.6) in the CART+HSCT, CART2, and CART1 groups, respectively. Patients in the CART +HSCT group had a higher LFS than those in the CART1 group (p=0.0018) and the CART2 group (p=0.0183) (**Supplementary Figures 1C, D**). OS and LFS in the CART1 and CART-2 groups did not differ significantly (p= 0.2327, p=0.1818, respectively, **Supplementary Figures 1E, F**). When it comes to long-term survival, higher rates of long OS and LFS were achieved in the CART+HSCT group at 3 years compared to the CART2 group (58.3% *vs.* 0, p<0.001; 66.7% *vs.* 0, p<0.001) (**Figure 3**). Similar results were obtained when the CART+HSCT group was compared to the CART1 group (58.3% *vs.* 0, p<0.001; 66.7% *vs.* 0, p<0.001) (**Figure 3**).

In addition, three patients relapsed at 30, 44, and 399 days after allo-HSCT. For the CART2 group, the median time to first relapse was 189 days (range: 92 to 719). On the other hand, three patients gained CR after a second hCART19s infusion, of whose median time to second relapse was 92 days (range: 38–120). For the CART1 group, the median time to relapse was 149 days (range: 30–449). Between the first hCART19s infusion and the second infusion, the median relapse time was much shorter after the second infusion (92 *vs.* 178, p=0.039) (**Figure 1**). Moreover, one patient relapsed with CD19 negative in the CART1 group and the CART2 group, respectively. All patients who relapsed were CD19 positive after CART+HSCT.

Considering all patients possessing positive MRD before hCART19s infusion, we used X-tile to determine the optimal cutoff value for MRD. The MRD cutoff value was with a maximum χ^2 log-rank value of 5.59 (p=0.017) (**Supplementary Figure 2**). Univariate analyses revealed that age, complex chromosome set, recurrence times, treatment times, volume, and the number of infused cells had no significant effects on OS and LFS. The disease burden significantly affected OS and LFS (**Figures 4A–D**). There was also a trend toward better OS (p=0.064) for patients with MRD-negative CR *versus* MRD-positive CR, even when no significant difference was obtained

TABLE 2 | Treatment-emergent adverse events.

Adverse events	All grades	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
CRS grade	27	14	5	3	4	1
CRS, specific symptoms						
Fever	27	14	5	3	4	1
Hypotension	11	0	4	2	4	1
Hypoxemia	3	0	1	1	1	0
Neurotoxicity	1	0	0	0	1	0
Muscle weakness	0	0	0	0	0	0
Nausea	3	1	0	0	2	0
Vomiting	2	0	0	0	2	0
Myalgias	1	1	0	0	0	0
Lung infection	4	1	3	0	0	0
Cerebral hemorrhage	1	0	0	0	1	0
Laboratory abnormalities						
ALT increased	15	6	5	1	2	1
Cr increased	1	0	1	0	0	0
APTT prolonged	11	3	4	1	2	1
Fib decreased	8	2	3	1	2	0

ALT, aminotransferase; Cr, creatinine; APTT, activated partial thromboplastin time; Fib, fibrinogen.

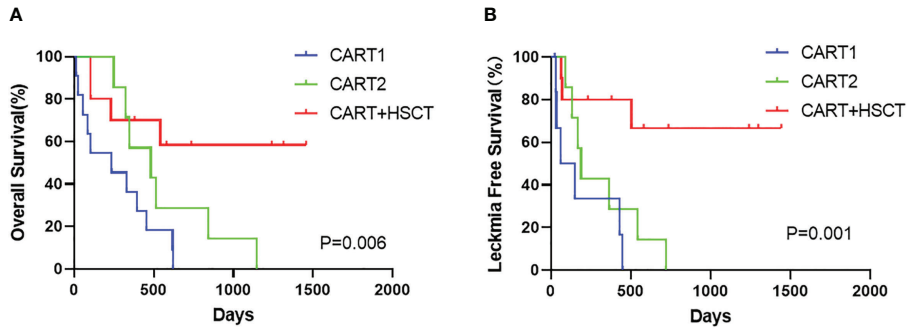

FIGURE 3 | Prognosis of patients after hCART19s therapy. **(A)** The overall survival (OS) of all patients after the infusion of hCART19s according to 3 groups. **(B)** The Leukemia-free survival (LFS) of complete remission (CR) patients according to 3 groups.

(**Figure 4E**). Patients with MRD-negative CR achieved a longer LFS than those with MRD-positive CR (p=0.032) (**Figure 4F**). Candidate variables with a p value <0.1 on univariate analysis were included in multivariate analysis. In multivariate analyses, the proportion of BM blasts (HR 6.055; 95% CI 1.624–18.933; p=0.004) and receiving allo-HSCT (HR 0.250; 95% CI 0.068–0.915; p=0.036) were independent predictors for OS. MRD<65.6% at infusion (HR 7.905; 95% CI 1.016-61.505-0.977; p=0.048) and receiving HSCT (HR 0.139; 95% CI 0.031–0.619; p=0.010) were independent risk factors for LFS. This result showed that receiving HSCT is predictive of both OS and LFS than a second infusion of hCART19s (**Table 3**).

DISCUSSION

Recently, results from clinical trials of CD19 CAR-T cell therapy have shown that patients with R/R B-ALL can achieve a high response rate; however, there is a high incidence of early relapse. We retrospectively analyzed the outcomes of patients treated with allo-HSCT or a second CAR-T cell infusion after CAR-T cell therapy, aiming at characterizing the safety and efficacy, and with a goal of identifying an optimal strategy after infusion of CAR-T cells.

The overall response rates of targeted CD19 CAR-T cells are 68%–93% in patients with R/R B-ALL (16, 17). In our study, the CR rate of R/R B-ALL after the first hCART19s infusion was 92.6% (25/27), which was comparable to the results in the previous study. CRS and CRES remain the major adverse events. Consist with early clinical trials, of whose incidence of severe CRS ranged from 26.67% (8/30) to 43.75% (7/16), the incidence of severe CRS after the first hCART19s infusion in our study were seven cases (25%). Only one (2.6%) developed CRES, and the clinical manifestation was epilepsy. The patient's symptoms were controlled after treatment with dexamethasone and antiepileptics. Some studies have shown that glucocorticoids may affect the efficacy of CAR-T cells and inhibit the proliferation of CAR-T cells. However, other studies reported the opposite results. Therefore, it is necessary to further explore whether glucocorticoids affect the efficacy of CAR-T cells.

Relapse remains a major obstacle after CAR-T cell therapy (18). Anagnostou et al. (19) and Lee et al. (17) reported recurrence rates of 43%–55% in patients achieving CR within 1 year after CAR-T cell treatment. The results of this study showed that the relapse rate in patients who did not receive allo-HSCT was 93.3% (14/15). Relapse can be divided into CD19 positive relapse and CD19 negative relapse, which may be attributed to CAR-T cell exhaust, loss, or mutation of target antigen. The possible mechanisms include growth of CD19-negative cells, lineage switching, cellular gnawing, increased expression of (progressed death) PD-1 in leukemia cells, etc. Seven patients who relapsed after the first hCART19s infusion received a second hCART19s infusion. We identify statistically significant differences in CR rates between the first infusion and the second infusion, suggesting that the second infusion with CAR-T cells had less efficacy. Moreover, the median relapse time was much shorter after the second hCART19s infusion (92 *vs.* 178, p=0.039). This result suggests that the second infusion had less efficacy to obtain remission again and failed to achieve durable CR.

It remains to be clarified whether patients may benefit from allo-HSCT after CAR-T cell therapy (8, 16, 20, 21). Consistent with the findings of Hay et al. (8) and Jiang et al. (21), our results supported the fact that a consolidative allo-HSCT after CD19 CAR-T-cell therapy may prolong OS and LFS in patients with R/R B-ALL. Specifically, 10 patients sequentially received allo-HSCT during the CR stage after the first hCART19s infusion, 6 of whom obtained long-term survival, with 1 patient's survival time reaching 4 years. It is well known that CAR-T cell therapy could achieve deep remission (MRD negative), potentially reverse chemotherapy resistance, and overcome adverse molecular genetic prognosis. However, there was still some recurrence. Moreover, HSCT has the effect of graft-*versus*-leukemia (GVL), which could act as a cure for B-ALL, especially for patients with MRD-negative status before HSCT. In our study, the 1-year OS rates were 70.0%, 57.1%, and 36.4% in the CART+HSCT, CART2, and CART1 groups, respectively. For long-term follow-up, in our CAR-T+HSCT group, the OS rate at 3 years was 58.3% for patients with R/R B-ALL. Since we did not conduct HSCT only when patients were R/R B-ALL, we compared the data from other centers. Duval et al. (22) reported that the OS rate at 3 years was 16% for patients with R/R ALL.

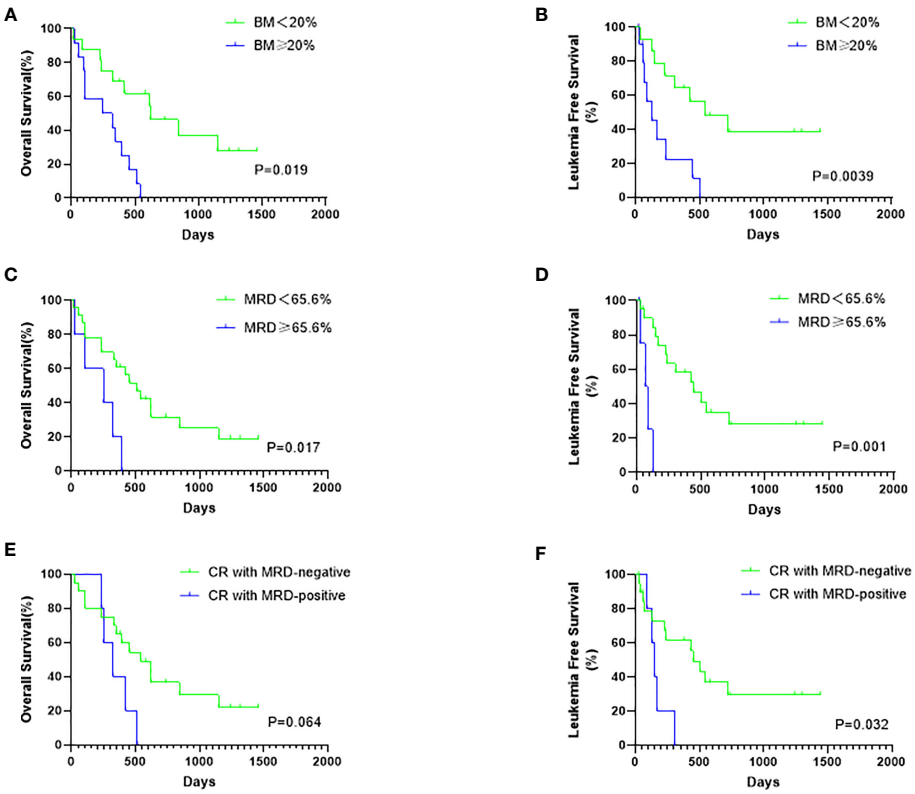

FIGURE 4 | The influence of disease burden at infusion and MRD status after infusion on survival. **(A)** The OS rates of all patients according to the bone marrow (BM) blasts status at infusion. **(B)** The LFS rates of CR patients according to the BM blasts at infusion. **(C)** The OS rates of all patients according to the mRD status at infusion. **(D)** The LFS rates of CR patients according to the MRD status at infusion. **(E)** The OS rates of all 28 patients according to the MRD status after infusion **(F)** The LFS rates of 25 CR patients according to the MRD status after infusion.

TABLE 3 | Multivariate Cox regression analysis for OS and LFS of CR patients (n = 25).

Subgroup	HR	95% CI	*P*
OS			
MRD[#] ≥65.6% *vs* < 65.6%	1.644	0.443-6.099	0.457
BM blasts[#] ≥20% *vs* <20%	6.055	1.624-18.933	**0.004**
MRD* positive *vs* negative	2.364	0.482-11.602	0.289
Group			0.109
CART1	reference		
CART2	0.556	0.193-1.600	0.276
CART+HSCT	0.250	0.068-0.915	**0.036**
LFS			
MRD[#] ≥65.6% *vs* < 65.6%	7.905	1.016-61.505	**0.048**
BM blasts[#] ≥20% *vs* <20%	1.950	0.554-6.867	0.310
MRD* positive *vs* negative	1.505	0.273-8.306	0.639
Group			0.031
CART1	reference		
CART2	0.290	0.068-1.235	0.904
CART+HSCT	0.139	0.031-0.619	**0.010**

*OS, overall survival; LFS, leukemia-free survival; CART, Chimeric Antigen Receptor T-Cell (CAR-T) therapy; [#]before CAR-T therapy; *after CAR-T therapy; MRD, minimal residual disease; BM, bone marrow; CART+HSCT group, patients who received allogeneic hematopoietic stem cell transplantation after CAR-T; CART2 group, patients who received a second hCART19s infusion after CAR-T; CART1 group, patients who did not receive HSCT or a second hCART19s infusion.*
Bold values, statistical significance.

Moreover, the mortality rate at 100 days after transplantation was 41% in ALL. Okamoto et al. (23) reported a 3-year OS of 22% in children and adolescents with nonremission ALL. We believe that combined CAR-T cell therapy and HSCT has a synergistic effect. Our data strongly suggest that allo-HSCT is needed to improve the durability of responses after CAR-T therapy. This was a preliminary retrospective clinical study, in which the treatment after the first CAR-T infusion was mainly determined by the disease state and willingness of patients, and thus there was a certain bias. Now, a prospective study of random control trial is warranted based on the clinical study.

In addition, the median time of neutrophil and platelet engraftment was 15.5 days (range: 11–25) and 20 days (range: 12–36), respectively; there was no significant prolongation compared with Luznik's study (24). At the same time, there was no increase in the incidence of aGVHD (30%) or extensive cGVHD (0%). These results suggest that bridging allo-HSCT after CAR-T cells infusion does not increase the risk of transplantation-related complications or the mortality related to transplantation.

There are also some shortcomings in this study. For example, the number of cases is small, and it is not a prospective study. The timing of transplantation can be further optimized. Further studies with more patients are needed to be conducted in the future. We recognized that it is possible that there is a survival advantage for HSCT because of a selection bias, which could skew the survival analysis in favor of the patients who got HSCT since the HSCT group would be selected for patients who had at least a frank relapse-free survival that exceeded the time to HSCT. Overall, the results of this study demonstrate that CAR-T therapy bridging to HSCT is a feasible, safe, and effective treatment for patients with R/R B-ALL.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study protocol was approved by the human studies review board at the Affiliated Hospital of Xuzhou Medical University (ClinicalTrials.gov # NCT02782351). The clinical investigation was conducted according to the principles of the Declaration of Helsinki. All patients provided signed informed consent before the hCART19s therapy and allo-HSCT.

AUTHOR CONTRIBUTIONS

WS, JZ and KX designed the research; WC, YM, ZS, HC, and RM collected the data and designed the figures. DY, MS, XW, XS, CS, JC, HC, FZ, HS, DL and ZL analyzed and interrupted the results; WC wrote the first draft of manuscript. WS, WC, YM and ZS wrote the revised manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.755549/full#supplementary-material

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PiggyBac Transposon-Mediated CD19 Chimeric Antigen Receptor-T Cells Derived From CD45RA-Positive Peripheral Blood Mononuclear Cells Possess Potent and Sustained Antileukemic Function

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The quality of chimeric antigen receptor (CAR)-T cell products, namely, memory and exhaustion markers, affects the long-term functionality of CAR-T cells. We previously reported that *piggyBac* (PB) transposon-mediated CD19 CAR-T cells exhibit a memory-rich phenotype that is characterized by the high proportion of $CD45RA^+$/C-C chemokine receptor type 7 $(CCR7)^+$ T-cell fraction. To further investigate the favorable phenotype of PB-CD19 CAR-T cells, we generated PB-CD19 CAR-T cells from $CD45RA^+$ and $CD45RA^-$ peripheral blood mononuclear cells (PBMCs) (RA^+ CAR and RA^- CAR, respectively), and compared their phenotypes and antitumor activity. RA^+ CAR-T cells showed better transient gene transfer efficiency 24 h after transduction and superior expansion capacity after 14 days of culture than those shown by RA^- CAR-T cells. RA^+ CAR-T cells exhibited dominant CD8 expression, decreased expression of the exhaustion marker programmed cell death protein-1 (PD-1) and T-cell senescence marker CD57, and enriched naïve/stem cell memory fraction, which are associated with the longevity of CAR-T cells. Transcriptome analysis showed that canonical exhaustion markers were downregulated in RA^+ CAR-T, even after antigen stimulation. Although antigen stimulation could increase CAR expression, leading to tonic CAR signaling and exhaustion, the expression of CAR molecules on cell surface after antigen stimulation in RA^+ CAR-T cells was controlled at a relatively lower level than that in RA^- CAR-T cells. In the *in vivo* stress test, RA^+ CAR-T cells achieved prolonged tumor control with expansion of CAR-T cells compared with RA^- CAR-T cells. CAR-T cells were not detected in the control or RA^- CAR-T cells but RA^+ CAR-T cells were expanded even after 50 days of treatment, as confirmed by sequential bone marrow aspiration. Our results suggest that PB-mediated RA^+ CAR-T cells exhibit a memory-rich phenotype and superior antitumor function, thus

CD45RA$^+$ PBMCs might be considered an efficient starting material for PB-CAR-T cell manufacturing. This novel approach will be beneficial for effective treatment of B cell malignancies.

Keywords: CAR-T cells, CD45RA, CAR-T cell therapy, *piggyBac* transposon, naïve/stem cell memory-like T cells

INTRODUCTION

Although chimeric antigen receptor (CAR)-T cell therapies targeting CD19 have achieved spectacular success for B-cell malignancies, long-term remission occurs only in half of the patients with B-cell malignancies (1–6). Therefore, enhancing the long-term functionality of CAR-T cells without affecting their anti-tumor potency is important. Although the antitumor efficacy of CAR-T cells depends on various host factors, namely, the disease status or actively hostile tumor microenvironment, the recent clinical studies on CAR-T cell therapies have reported that the quality of CAR-T cell products, namely, T-cell memory signatures and exhaustion-related markers, is critical for the function and antitumor efficacy of CAR-T cell therapies (7–9). The presence of naïve and stem cell memory-like CAR-T cells in the final product is correlated with the response of CAR-T cell therapies in B-cell malignancies (7). Therefore, the manufacturing process of CAR-T cells should be optimized to prevent early T-cell exhaustion and to maintain the memory phenotype during the expansion step. Pre-activation of T cells by anti-CD3 and CD28 antibodies—an indispensable step for retroviral or lentiviral gene transfer into T cells—strongly induces T-cell differentiation and exhaustion; therefore, various efforts to obtain memory-rich CAR-T cells have been attempted, namely, the use of interleukin (IL)-7 and IL-15 cocktails instead of IL-2 or transient stimulation with anti-CD3 and CD28 antibodies (7, 10).

Non-viral gene transfer using *piggyBac* (PB) transposon-based genetic modifications is a potentially effective strategy for CAR-T cell manufacturing (11–16). Our previous study and also other studies have reported that PB-CAR-T cells exhibit an enriched memory fraction and less exhaustion-related markers, regardless of the type of CAR constructs, electroporation conditions, or expansion protocols (17, 18). PB-CAR-T cells that redirected CD19, HER2, or ephrin type-B receptor 4 precursor (EPHB4) molecules were dominant in the naïve/stem cell memory-like T-cell fraction, which was characterized as CD45RA/C-C chemokine receptor type 7 (CCR7) double positive and is related to long-term functionality.

As the nature of T cells in the starting peripheral blood mononuclear cell (PBMC) materials affects the phenotype and function of CAR-T cells in the final product (19), the composition of the starting PBMC materials would contribute to the maintenance of the memory phenotype of PB-CAR-T cells in the final product. The phenotype of patient PBMCs might be greatly affected by other diseases or prior chemotherapeutic agents (19, 20), and is associated with the manufacturing success rate, phenotype, and functionality of autologous, patient-derived CAR-T cell products. Therefore, the optimization of the composition of starting PBMC materials would be important for the stable manufacturing of memory-rich PB-CAR-T cells. To improve the manufacturing success rate and the functions of CAR-T cells, previous studies have reported that the enrichment of whole T cells by elimination of monocytes and granulocytes from starting materials would improve T-cell activation and transduction efficiency during virally-engineered CAR-T cell processing (21–23); however, optimal PBMC subpopulations for PB-CAR-T cell processing are unknown. In this study, we aimed to generate PB-CD19 CAR-T cells using the subpopulations of PBMCs based on CD45RA expression and to investigate the usefulness of CD45RA positive PBMC subpopulation as the starting material for PB-CD19 CAR-T cell manufacturing.

MATERIALS AND METHODS

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine (Approval Numbers: ERB-C-669 and ERB-C-1406) and the recombinant DNA experiments were approved by the safety committee of the recombinant DNA experiment of Kyoto Prefectural University of Medicine (Approval Numbers 2019-111 and 2019-112). All experiments involving human participants were performed in accordance with the Declaration of Helsinki guidelines. All animal experiments and procedures were approved by the Kyoto Prefectural University of Medicine Institutional Review Board (Permit No.: M2020-13).

Blood Donors and Cell Lines

Blood samples from healthy donors were obtained with a written informed consent, and PBMCs were isolated from the whole blood samples by density gradient centrifugation using Lymphocyte Separation Medium 1077 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), followed by multiple washes with Dulbecco's phosphate-buffered saline (D-PBS; Nakarai Tesque, Kyoto, Japan). The number of live cells was determined by standard trypan blue staining and using an automated cell counter model R1 (Olympus, Tokyo, Japan). The human lymphoblastic leukemia cell line (REH) was purchased from the American Type Culture Collection (Manassas, VA). REH-expressing firefly luciferase (FFLuc) and green fluorescent protein (GFP) (REH-FFLuc-GFP) were obtained by introducing PB-based pIRII-FFLuc-puroR-GFP (18) in REH cells and subsequent fluorescent-activated cell sorting. REH and REH-FFLuc-GFP cells were cultured in

Roswell Park Memorial Institute-1640 medium (Nacalai Tesque) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc. Waltham, MA) and maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

Plasmid Construction

The PB transposase plasmid, pCMV-piggyBac (24), which contained ~2.4 kb of transposase elements with identical 13 base pair (bp) terminal inverted repeats and additional asymmetric 19 bp internal repeats (25, 26), was artificially synthesized (Mediridge Co., Ltd, Tokyo, Japan). CAR construct for CD19– CAR-T cells which encodes the CD19 specific scFv, followed by a short hinge, the transmembrane and signaling domain of the costimulatory molecule CD28, and the ζ signaling domain of the TCR complex, was kindly provided from Dr. Cliona M. Rooney (Baylor College of Medicine) and was subcloned into pIRII transposon vector backbone (11) (pIRII-CD19-28z) as described previously (27). For the generation of antigen-presenting feeder cells for the stimulation of CD19-CAR-transduced T cells, we used a plasmid containing sequences encoding the extracellular, transmembrane, and 20 amino-acid-long intracellular portion of CD19 protein (tCD19) driven by CMV promoter, followed by CD80 and 4-1BBL (CD137L) with TA2 and P2A self-cleaving sites that enabled independent gene expression. The tCD19-CD80-4-1BBL sequence was artificially synthesized (Fasmac Inc., Kanagawa, Japan) and cloned into a pIRII PB transposon vector (pIRII-tCD19-CD80-4-1BBL) (**Supplementary Figure S1**) (17, 18).

Manufacturing of PB-Mediated CAR-T Cells

$CD45RA^+$ and $CD45RA^-$ PBMCs were isolated by magnetic selection from the whole PBMCs using CD45RA MicroBeads, human (Miltenyi Biotec, Bergisch Gladbach, Germany) (**Supplementary Figure S2A**). The CD19-CAR transgene was then transduced into these cells using the PB transposon system, as described previously (17, 18). Briefly, pCMV-piggyBac (7.5 μg per 100 μl of electroporation buffer) and pIRII-CD19-28z (7.5 μg per 100 μl) (**Supplementary Figure S1**) were introduced into about 4×10^6 $CD45RA^+$ or $CD45RA^-$ PBMCs, respectively, using the P3 Primary Cell 4D-Nucleofector™ X kit (Lonza, Program; FI-115) or MaxCyte ATX® (MaxCyte Inc) with the optimized protocol for introduction of DNA plasmid into resting T cells (Protocol; RTC 14-3). Concurrently, an antigen-presenting feeder plasmid (pIRII-tCD19-CD80-41BBL; 15 μg per 100 μl) (**Supplementary Figure S1**) was introduced into approximately 1×10^6 whole PBMCs by electroporation. After electroporation, the CAR-T cells and feeder cells were cultured in complete culture medium consisting of ALyS™705 Medium (Cell Science & Technology Institute) supplemented with 5% artificial serum (Animal-free; Cell Science & Technology Institute), IL-7 (10 ng/ml; Miltenyi Biotec), and IL-15 (5 ng/ml; Miltenyi Biotec). The feeder cells were irradiated with ultraviolet light for inactivation 24 h after electroporation and co-cultured with CAR-T cells for 14 days, as described previously (17, 18). CAR-T cells redirected to the EPHB4 receptor were manufactured using the PB transposon system as described previously (17) for its use as the control CAR-T cells in the *in vivo* stress test (**Supplementary Figure S1**).

Flow Cytometry

Expression of CD19-CAR molecules on T-cell surface was measured by flow cytometry using the recombinant human CD19 Fc chimera protein (R&D Systems, Minneapolis, MN, USA) and goat anti-human immunoglobulin (Ig)-G Fc fragment specific antibody conjugated to fluorescein isothiocyanate (FITC) (Merck Millipore, Burlington, MA). Allophycocyanin (APC) or phycoerythrin (PE)-conjugated anti-CD3 antibody, FITC-conjugated anti-CD19 antibody, PE-conjugated anti-CD56 antibody, FITC-conjugated anti-CD15 antibody, APC-conjugated anti-CD14 antibody, APC-conjugated anti-CD8 antibody, PE-conjugated CD4 antibody, PE-conjugated anti-CD45RA antibody, and APC-conjugated anti-CCR7 antibody (all from BioLegend, San Diego, CA, USA) were used to characterize the phenotypes of CAR-T cells. APC-conjugated anti-programmed cell death protein-1 (PD-1) antibody, APC-conjugated anti-T cell immunoglobulin mucin-3 (TIM-3) antibody, Alexa Fluor 647-conjugated anti-CD223 (LAG-3) antibody, and Peridinin-Chlorophyll-Protein (PerCP)/Cyanine5.5-conjugated anti-CD57 antibody were used as the exhaustion and senescence markers of CAR-T cells (all from BioLegend). FITC-conjugated anti-CD19 antibody was also used to determine the phenotype of REH cells. All flow cytometry data were acquired using BD Accuri™ C6 Plus or BD FACSCalibur™ (BD Biosciences, Franklin Lakes, NJ) and analyzed using the FlowJo™ software (BD Biosciences).

Transgene Copy Number Analysis

After 14 days of culture, 1×10^5 CAR-positive T cells were isolated using a Cell Sorter SH800 (SONY, Tokyo, Japan), and total DNA was then extracted using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Quantitative PCR was carried out using the total DNA from 1×10^3 CAR-positive T cells (equivalent to 1 μl of DNA extract) and custom primer/Taqman probe set specific for the CD19-CAR transgene at the junction of CD28 cytoplasmic domain and CD3ζ by a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). To measure DNA copy number for absolute quantification, pIRII-CD19-CAR plasmid was used (**Supplementary Figure S3A**). The relevant primers and Taqman probe are shown (**Supplementary Table S1**).

Analysis of Exhaustion-Related Markers Expressed on T Cell Surface After Electroporation

The GFP plasmid was introduced into the whole PBMCs by electroporation using the same protocol as that used for CAR-T manufacturing. After electroporation, the GFP-introduced PBMCs were cultured in complete culture medium consisting of the same components used for CAR-T manufacturing. PBMCs without electroporation were cultured in the same medium and was used as the control. After 48 h of incubation,

the expression of PD-1, TIM-3, and LAG-3 on T cell surface was analyzed by flow cytometry by gating GFP-positive and CD3-positive T cells.

Sequential Killing Assay

We co-cultured 1×10^5 REH cells and 1×10^5 CD19 CAR-T cells derived from CD45RA$^+$ or CD45RA$^-$ PBMC (RA$^+$ CAR or RA$^-$ CAR, respectively) in 24-well cell culture plates. Three days later, the CD19 CAR-T cells were collected, counted, and treated and reconstituted with fresh REH cells at a ratio of 1:1. Cell counting and treatment with fresh REH cells were repeated every three days for a total of three iterations. The killing effect of these CD19 CAR-T cells was evaluated by counting the number of residual REH cells by flow cytometry. The mean fluorescence intensity (MFI), exhaustion-related markers, cytokine production, and proliferation of the CAR-T cells were analyzed by flow cytometry.

Cytokine Production Assay

The levels of interferon (IFN)-γ, tumor necrosis factor (TNF), and IL-6 were measured using a Cytometric Bead Array (CBA) Kit (BD Biosciences). Briefly, CAR-T cells were co-cultured with tumor cells at a ratio of 1:1. After 3 days of co-culture, the cell culture supernatant was collected and cytokine levels were determined and analyzed. Data were acquired with a BD Accuri C6 Plus (BD Biosciences) and analyzed with FCAP Array v.3.0 (BD Biosciences).

CytoTell™ Dilution Assay

We examined the proliferation of CAR-T-cells using CytoTell™ Red 650 (AAT Bioquest Inc) dye dilution after serial stimulation with tumor cells. Briefly, CAR-T cells were incubated with CytoTell™ Red 650 dye at 37°C for 30 min, and the dye working solution was removed. Then, the CAR-T cells were co-cultured with tumor cells at a ratio of 1:1 or without tumor cells (no stimulation). After 3 days of co-culture, the proliferation of CAR-T cells was analyzed by flow cytometry, and the experiments were repeated for serial three rounds of antigen stimulation.

IL-2-Dependent Proliferation Assay

We cultured 1×10^6 RA$^+$ CAR-T or RA$^-$ CAR-T cells in the presence or absence of IL-2 (final concentration; 100 IU/ml) in 24-well cell culture plates. IL-2 was supplemented weekly, and the numbers of live cells were determined every 7 days.

RNA-Sequencing and Bioinformatics Analysis

Total RNA was isolated from RA$^+$ CAR-T cells and RA$^-$ CAR-T cells after 14 days culture (these CAR-T cells were not sorted for CAR-positive population), with or without antigen stimulation, by co-culturing these cells with REH cells at an effector:target ratio of 1:1 for 3 days (RA$^+$/Stimulation$^+$, RA$^-$/Stimulation$^+$, RA$^+$/Stimulation$^-$, and RA$^-$/Stimulation$^-$, respectively), using RNeasy Mini Kit (Qiagen, Venlo, Netherlands). The concentration of total RNA was measured using NanoDrop 2000 (Thermo Fisher Scientific). Library preparation and high-throughput sequencing were performed using Eurofins Genomics (Ebersberg, Germany). In brief, mRNAs were enriched and their strand-specific library was prepared. Sequencing was performed using a NextSeq 500/550 system (Illumina, San Diego, CA, USA) and NextSeq 500/550 Mid Output Kit v2.5 150 cycles (Illumina). Adapter sequences and low-quality reads were removed using fastp version 0.21.0 (28). Filtered reads were aligned to the human reference genome (GRCh38.p13) using STAR version 2.7.6a (29). Counts per gene and transcripts per million were calculated using RSEM version 1.3.3 (30). Calculation of counts per million and differential expression analysis were performed using edgeR version 3.32.0 R package (31) and R v4.0.3 environment (https://www.R-project.org/). Pathway analysis was performed using R package for the Reactome Pathway Analysis (32). Differential gene expression profiles between RA$^+$ and RA$^-$ CAR were also analyzed and visualized using Morpheus (https://software.broadinstitute.org/morpheus) and specific gene signatures related to T cell activation, exhaustion, and differentiation (33).

In Vivo Stress Test Using a Murine Systemic Tumor Model

Female 8-week-old NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA), and housed at the Kyoto Prefectural University of Medicine for more than a week before starting the experiment. Food and water were provided *ad libitum*. REH-FFLuc-GFP cells suspended in D-PBS were infused into mice *via* the tail vein. Six days later, RA$^+$ CAR-T, RA$^-$ CAR-T, or irrelevant CAR-T cells, which redirected the EPHB4 receptor (17) as a control, were infused *via* the tail vein, and tumor burdens were monitored using the IVIS Lumina Series III system (PerkinElmer, Inc.). The regions of interest on the displayed images were quantified in photons per second (ph/s) using Living Image v2 (PerkinElmer, Inc.) as described previously (17). Bone marrow (BM) cells were obtained by sequential BM aspiration from tibias at several time points. The BM cells were stained with a PE-conjugated anti-human CD3 antibody and APC-conjugated anti-human PD-1 antibody (BioLegend), and the long-term persistence of human T cells was evaluated by flow cytometry. The mice were euthanized at predefined endpoints, under conditions that met the euthanasia criteria given by the Center for Comparative Medicine at the Kyoto Prefectural University of Medicine.

Statistics

Statistical comparisons between two groups were determined by two-tailed parametric or non-parametric (Mann–Whitney *U*-test) tests for unpaired data or by two-tailed paired Student's *t*-test for matched samples. All data are presented as mean ± standard deviation. The log-rank test was used to compare survival curves obtained using the Kaplan–Meier method. A P-value of <0.05 was considered statistically significant. All the statistical analyses were performed using the GraphPad Prism 9 software.

RESULTS

RA^+ CAR-T Cells Exhibited Superior Transduction Efficiency and Expansion Capacity, Dominant CD8 Expression, Enriched Stem Cell Memory Fraction, and Lower Expression of Exhaustion-Related Markers Than RA^- CAR-T Cells

First, we isolated $CD45RA^+$ or $CD45RA^-$ subpopulations from whole PBMCs using human CD45RA-targeted magnetic separation. We observed two peaks of CD45RA high and negative populations in the lymphocyte fraction (**Supplementary Figure S2A**). By magnetic bead sorting, $CD45RA^+$ and $CD45RA^-$ PBMCs were efficiently separated with >98% of CD45RA positive cells in the $CD45RA^+$ fraction and >93% of CD45RA negative cells in the $CD45RA^-$ fraction (**Supplementary Figure S2A**). Both RA^+ and RA^- PBMCs consisted of about 60% CD3-positive cells, and the rest of CD3-negative cells were positive for CD56 in RA^+ PBMCs, while CD56, CD14 and CD15 were negative in RA^- PBMCs (**Supplementary Figure S2B**). Moreover, electroporation did not greatly influence the CD3 positivity of both RA^+ and RA^- PBMCs (**Supplementary Figure S2C**). Indeed, RA^+ and RA^- PBMCs both contained approximately 40% CD3-negative cells, which included NK cells and other myeloid cells, but this percentage of these cells decreased at 24 h after electroporation and more decreased after 14 days. Based on this observation, CD3-negative T cells were likely destroyed during electroporation since the optimized protocol for introducing the DNA plasmid required a relatively high voltage. We then evaluated the transient gene transfer efficiency of CD19 CAR transgene into $CD45RA^+$ or $CD45RA^-$ PBMCs 24 h after electroporation. When the CD19 CAR transgene plasmid was introduced into unstimulated, magnetically sorted $CD45RA^+$ or $CD45RA^-$ PBMCs by electroporation, $CD45RA^+$ PBMC subpopulation exhibited higher transient gene transfer efficiency than $CD45RA^-$ PBMC subpopulation 24 h after electroporation (**Figure 1A**). Furthermore, CD19 CAR-T cells derived from $CD45RA^+$ PBMCs (RA^+ CAR-T) exhibited higher expansion capacity 14 days after culture compared with CD19 CAR-T cells derived from $CD45RA^-$ PBMCs (RA^- CAR-T) (**Figure 1B**). After 14 days of expansion, we determined the CAR positivity, phenotype, and exhaustion-related marker PD-1 expression on these CAR-T cells by flow cytometry. RA^+ CAR-T cells consisted of about 95% CD3-positive cells and a small number of CD3-negative/CD56-positive NK cells, while RA^- CAR-T cells consisted of about 85% CD3-positive cells and about 13% CD3-negative/CD56-positive cells, suggesting that residual NK cells could affect the immune function of these populations (**Supplementary Figure S2D**). Compared with RA^- CAR-T, RA^+ CAR-T cells exhibited higher CAR positivity, lower expression of exhaustion-related marker PD-1 and T-cell senescence marker CD57 (34, 35), and enriched naïve/stem cell memory fraction, which were associated with the longevity of CAR-T cells (**Figures 1C, D**). The copy number of the integrated CAR transgene was calculated by qPCR, and these CAR-T cells had ~20 copies of the CAR transgene (16.5 ± 1.1 in RA^+ CAR-T cells, 5.9 ± 0.3 in RA^- CAR-T cells, and 20.2 ± 1.3 in CD19 CAR-T cells derived from whole PBMCs (**Supplementary Figure S3B**)), which was consistent with the previous report (11). Furthermore, RA^+ CAR-T cells were markedly CD8-dominant, whereas the CD4/8 ratio of both RA^+ and RA^- PBMCs, the starting material, was CD4-dominant. These results suggest that CD8-positive stem cell-like T cells are primarily amplified in RA^+ CAR-T cells (**Supplementary Figure S1B**). By contrast, RA^- CAR-T cells are CD4-dominant in the final product, which may be detrimental to CAR-T cell function as RA^- CAR-T cells may harbor more regulatory CD4 CAR-T cells. Other activation/exhaustion-related markers such as TIM-3 and LAG-3 were highly expressed in both the types of CAR-T cells (**Figure 1D**), and this finding is consistent with that of previous studies on PB-CAR-T cells (14, 16–18, 36). As most PB-CAR-T cells were engineered by electroporation, we hypothesized that the high expression of TIM-3 and LAG-3 would be induced by the stimulation of electroporation. However, PBMCs 48 h after electroporation barely expressed PD-1, TIM-3, or LAG-3 on T cells (**Supplementary Figure S3**). Therefore, the expression of TIM-3 and LAG-3 but not PD-1 on CAR-T cells was induced during the incubation period and not by the stimulation of electroporation.

To further investigate the effect of RA^+ CAR-T or RA^- CAR-T cells at the molecular level and to identify the pathways involved in the favorable phenotype of RA^+ CAR-T cells, we performed genome-wide transcriptional profiling by focusing on immunogenic gene signatures. A total of 29 genes were identified with higher expression and 78 genes with lower expression in RA^+ CAR-T cells compared with $CD45RA^-$ CAR-T cells (**Figure 1E**). Reactome pathway analysis showed that the differential gene expression profiles observed in RA^+ CAR-T cells were related to the non-canonical NF-κB pathway and co-stimulation by the CD28 family pathway; PD-1 signaling pathway was significantly downregulated in RA^+ CAR-T cells (**Figure 1F**). Transcriptome profiling showed that RA^+ CAR-T cells exhibited activated but less exhausted profiles characterized by the upregulation of T-cell activation-like markers including transcription factor 7 (TCF7), lymphoid enhancer-binding factor 1 (LEF1), CCR7, and IL7R and the downregulation of canonical exhaustion-related markers including PD-1, T-cell immunoreceptor with Ig and ITIM domains (TIGIT), and Eomesodermin (EOMES) in RA^+ CAR-T cells (33) (**Figure 1G**, left), even after antigen stimulation (**Figure 1G**, right). LAG3 expression was also downregulated in RA^+ CAR-T cells, but flow cytometry results did not show statistical differences between RA^+ and RA^- cells (**Figure 1D**). These gene expression analysis data suggest that RA^+ CAR-T cells have an abundant naïve/memory phenotype even when stimulated by antigen-positive tumor cells, corroborating the phenotype of RA^+ CAR-T cells assessed by flow cytometry.

Analysis of the Function of CAR-T Cells Using the *In Vitro* Serial Tumor Challenge Assay

To evaluate the antileukemic activity of RA^+ CAR-T and RA^- CAR-T cells, we performed the tumor re-challenge assay in

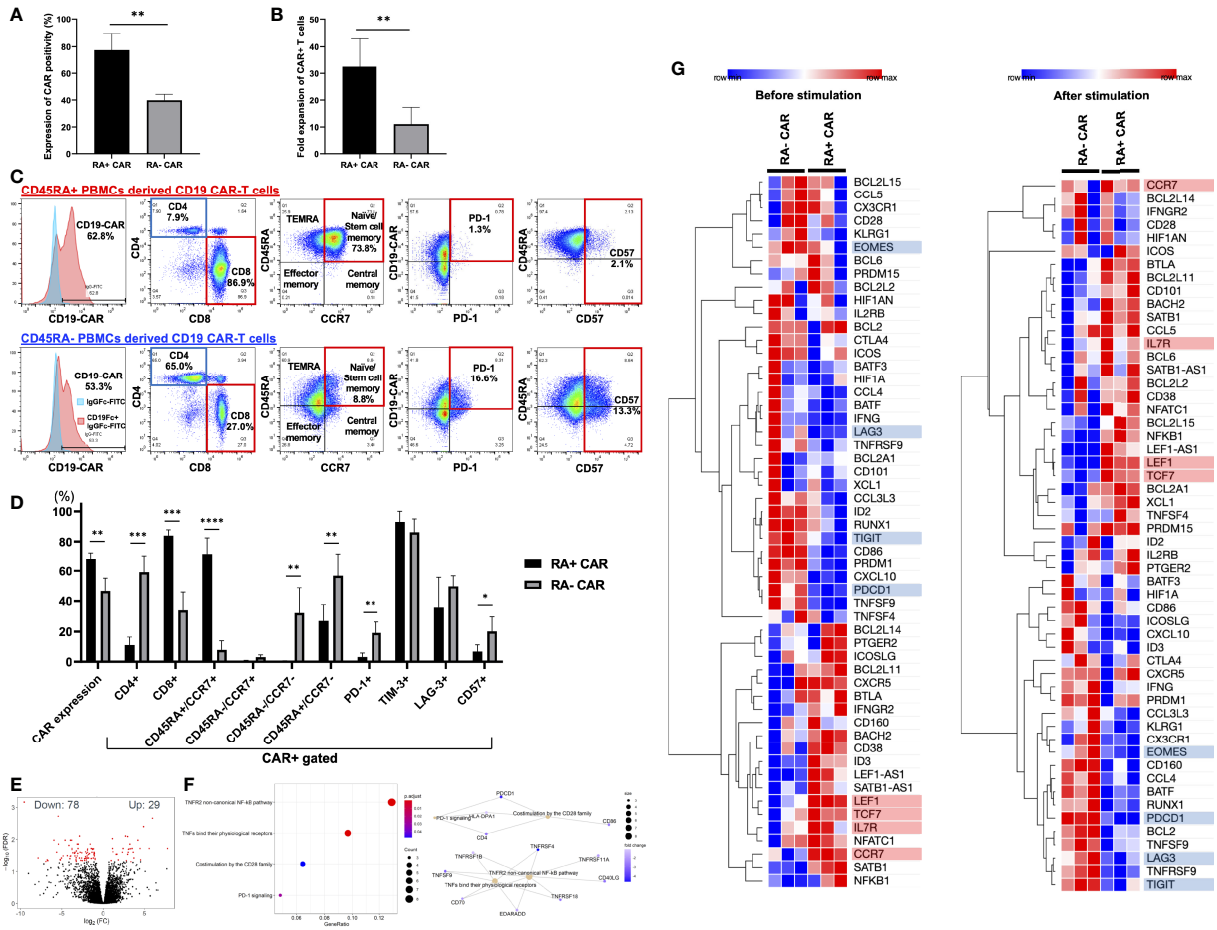

FIGURE 1 | CD45RA$^+$ chimeric antigen receptor (CAR)-T cells showed better transient gene transfer efficiency and expansion capacity, dominant CD8 expression, enriched stem cell memory fraction, and less expression of exhaustion-related markers than CD45RA$^-$ CAR-T cells. **(A)** The transient expression of CAR transgene 24 h after gene transfer (n = 3, 3 donors). **(B)** Number of CAR positive T cells at day 14 (n = 5, 3 donors). **(C)** Representative expression and phenotypes of CD19 CAR-T, and expression of exhaustion markers on CAR-T cells assessed by flow cytometry. The gating control (left, blue) for CAR expression showed CAR-T cells only, stained by anti-human IgG Fc fragment specific antibody conjugated to FITC, the gating of CAR expression (left, red) showed CAR-T cells combined with the recombinant human CD19 Fc chimera protein and secondary stained by anti-human IgG Fc fragment specific antibody conjugated to FITC. **(D)** The phenotype and exhaustion marker of CD19 CAR-T cells are represented (n = 3–6, 3 donors). **(E)** A volcano plot showing genes with adjusted FDR <0.05 that are differentially expressed in CD45RA$^+$ CAR-T compared with CD45RA$^-$ CAR-T. **(F)** Reactome pathway analysis showed that several gene pathways were significantly downregulated in CD45RA$^+$ CAR-T cells compared with CD45RA$^-$ CAR-T. Both red and blue circles showed downregulation of gene pathways in CD45RA$^+$ CAR-T, and their colors represented adjusted p-values. **(G)** Transcriptome profiling about expression of T-cell activation genes (highlighted by red underline) and exhaustion genes (highlighted by blue underline) in CD45RA$^+$ CAR-T and CD45RA$^-$ CAR-T, before antigen stimulation (left) and after antigen stimulation (right). Row min denotes lowest Z-score and row max denotes highest Z-score. All data are presented as means ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

which fresh REH cells were added to CAR-T cells every three days. Both the types of CAR-T cells achieved complete killing of REH cells even after multiple rounds of tumor re-challenge (**Figure 2A**). Interestingly, the expression of CAR molecules on the cell surface after antigen stimulation in RA$^+$ CAR-T cells was controlled at a relatively lower level than that in RA$^-$ CAR-T cells (**Figure 2B**), which suggested less tonic CAR signaling and exhaustion of RA$^+$ CAR-T cells compared with RA$^-$ CAR-T cells (37, 38). PD-1 expression in RA$^+$ CAR-T cells was lower than that in RA$^-$ CAR-T cells during multiple rounds of antigen stimulation (**Figure 2C**). In contrast, the expression of LAG-3 was similar in RA$^+$ CAR-T and RA$^-$ CAR-T cells, whereas TIM-3 expression in RA$^+$ CAR-T cells was higher than that in RA$^-$ CAR-T cells; these expressions gradually decreased during multiple rounds of antigen stimulation in RA$^+$ CAR-T and RA$^-$ CAR-T cells, and relatively high expression of TIM-3 and LAG-3 did not impair the killing efficacy of CAR-T cells (**Figure 2C**). We also evaluated the production of inflammatory cytokines in the cell culture supernatant in response to serial co-culture with tumor cells. The secretion of IFN-γ was higher in RA$^+$ CAR-T cells than in RA$^-$ CAR-T cells, although transcriptome profiling showed that IFNG expression has been already downregulated in RA$^+$ CAR-T cells on day 3 of co-culture with tumor cells (**Figure 1G**). Interestingly, the levels

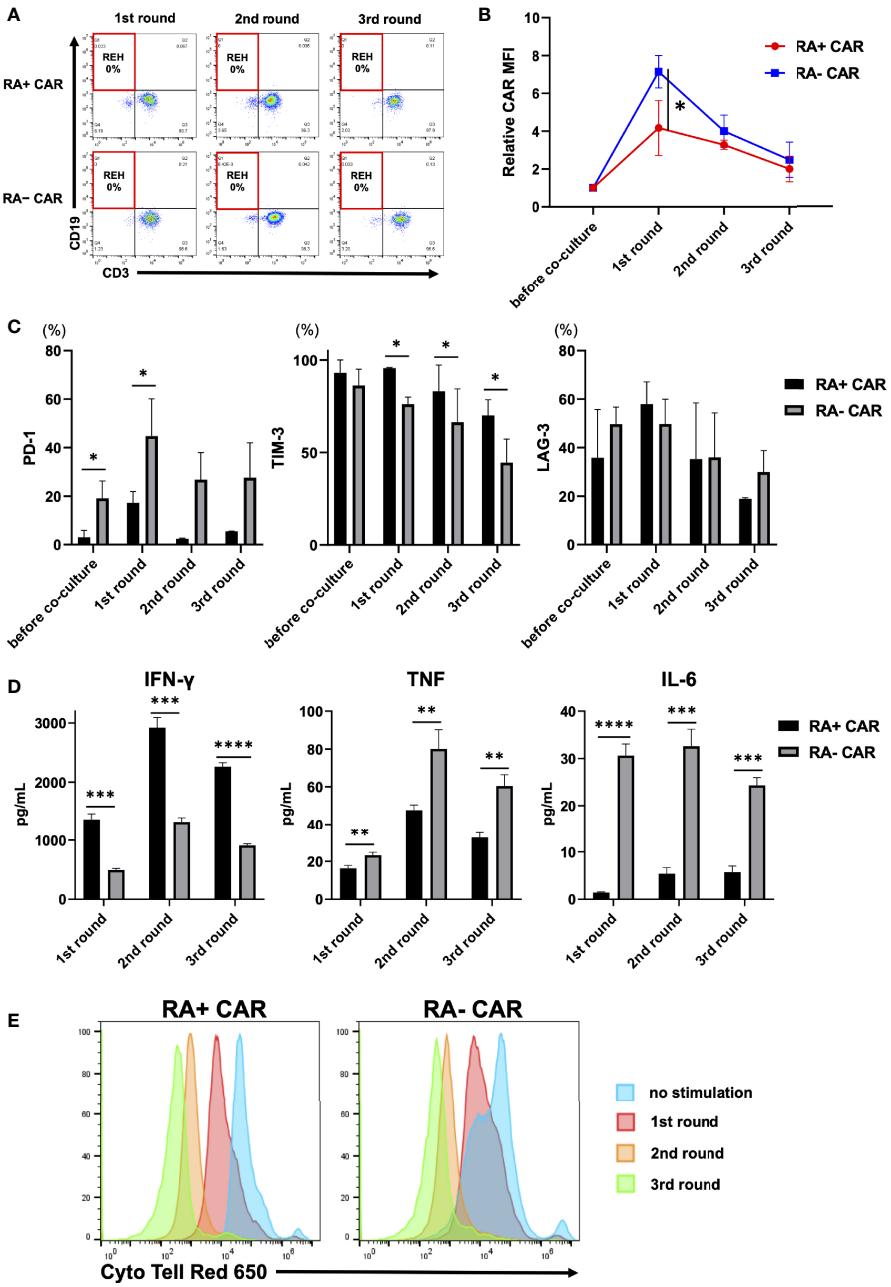

FIGURE 2 | Analysis of chimeric antigen receptor (CAR) function by *in-vitro* serial tumor challenge assay. **(A)** The expression of CAR-T and REH cells during sequential co-culture. Representative dot plots are shown. **(B)** Relative CAR mean fluorescence intensity (MFI) (MFI = 1, before co-culture) of CAR-T cells during sequential co-culture (n = 3). CAR MFI of CAR-T cells were calculated after gating on the CAR positive population. **(C)** The expression of PD-1, TIM-3, and LAG-3 on CAR-T cells during sequential co-culture (n = 3). **(D)** The level of cytokines in the co-culture supernatant containing CAR-T cells with REH cells after sequential co-culture (n = 3). **(E)** Cell division of CAR-T cells upon repeated REH cells stimulations or no stimulation. All data are presented as means ± standard deviation. *P < 0.05, ***P < 0.01, ***P < 0.001, ****P < 0.0001. PD-1, programmed cell death protein-1; TIM-3, T cell immunoglobulin mucin-3.

of TNF and IL-6 were significantly lower in RA^+ CAR-T cells than in RA^- CAR-T cells (**Figure 2D**). Furthermore, the proliferation of RA^+ CAR-T and RA^- CAR-T cells during serial stimulation was evaluated by CytoTell^{TM} dye dilution with tumor cells. Although RA^- CAR-T cells proliferated faster than RA^+ CAR-T cells without stimulation, there was no significant difference between the proliferation of RA^+ CAR-T cells and that of RA^- CAR-T cells after serial tumor stimulation (**Figure 2E**). Notably, the proliferation of both RA^+ and RA^- CAR-T cells did not occur spontaneously, but was dependent on

antigen stimulation or cytokine supplementation, as confirmed by an IL-2-dependent proliferation assay (**Supplementary Figure S4**), indicating no disordered proliferative potential in these cells.

RA^+ CAR-T Cells Showed Better Tumor Control With Long-Term Expansion of CAR-T Cells Than RA^- CAR-T Cells or Unsorted CAR-T Cells in *In Vivo* Stress Test

To evaluate the *in vivo* antitumor efficacy of RA^+ CAR-T and RA^- CAR-T cells, we performed the *in vivo* stress test in which CAR-T cell dosage was lowered to the functional limits, so that these CAR-T cells were maintained and expanded *in vivo* to achieve antitumor efficacy. Because CAR expression in RA^+ CAR-T and RA^- CAR-T cells was not exactly the same, we injected 1×10^5 CAR-positive T cells in each group. RA^+ CAR-T cells induced greater tumor reduction and prolonged median survival than those of RA^- CAR-T cells (**Figures 3A–C**). On day15, bone marrow in the RA^+ CAR group exhibited abundant human CD3 positive T cells with lower expression of PD-1 and a relatively smaller number of REH cells than that in the RA^- CAR group (**Figure 3D**). Furthermore, in two of the long-lived mice in the RA^+ CAR group, human $CD3^+$ T cells were expanded even after 50 days of treatment as confirmed by sequential bone marrow studies (**Figure 3E**), which indicated antigen-induced proliferation and long-term functionality of RA^+ CAR-T cells *in vivo*. The gating strategy of the bone marrow study was shown in **Supplementary Figure S5**.

To evaluate whether the selection of $CD45RA^+$ PBMCs as a starting material would facilitate highly effective PB-CAR-T cell manufacturing, we performed the *in vivo* stress test in RA^+ CAR-T and PB-CD19 CAR-T from unsorted (both $CD45RA^+$ and $CD45RA^-$) PBMCs (unsorted CAR-T). We infused 1×10^6 REH-FFLuc cells into NSG mice *via* the tail vein, and six days later, 1×10^5 CAR-positive T cells were infused into each group. RA^+ CAR-T cells achieved greater tumor reduction and prolonged median survival than unsorted CAR-T cells (**Figures 4A–C**). Sequential bone marrow studies in the RA^+ CAR group showed abundant human CD3 positive T cells with lower expression of PD-1 on day 15 (**Figure 4D**), lower expression of PD-1 in human CD3 positive T cells, and a relatively smaller number of REH cells than the unsorted CAR group (**Figure 4E**).

DISCUSSION

In this study, we generated PB-CD19 CAR-T cells using magnetically-isolated $CD45RA^+$ and $CD45RA^-$ PBMCs as the starting materials. We found that $CD45RA^+$ PBMCs were susceptible to the introduction of CAR transgene by electroporation, and RA^+ CAR-T cells exhibited superior expansion capacity than RA^- CAR-T Cells. RA^+ CAR-T cells were less vulnerable to T-cell exhaustion-related markers by multiple antigen stimulation, as evidenced by genome-wide transcriptome profiling, and RA^+ CAR-T cells demonstrated prolonged tumor control than RA^- CAR-T cells or even bulk CAR-T cells from unsorted PBMC in the *in-vivo* stress test. Therefore, CD45RA-positive PBMC selection from PBMCs starting from PB-CAR-T cell manufacturing would be important to improve the efficacy of the therapy.

Tonic-antigen stimulated or antigen-independent early T-cell exhaustion greatly impairs the function of CAR-T cells. Previous studies have shown that retrovirally-engineered CAR-T cells exhibit strong *in vitro* killing activity; however, the cells exhaust soon and fail to control tumor growth (38–40). In the clinical setting, differentiation and exhaustion profiles of T cells in the final products are associated with their clinical response and antitumor efficacy (8, 9). Various modifications reported in CAR-T engineering to prevent early T-cell exhaustion are as follows: use of endogenic promoters for stable expression of the CAR transgene (37), use of a combination of internal ribosome entry site constructs into the CAR transgene to reduce CAR-transgene expression (40), or modification of immunoreceptor tyrosine-based activation motifs in the CD3ζ chain (41). The PB-transposon system is an effective and adaptable tool for transgene delivery as an alternative for viral vectors, not only because of its cost-effectiveness and simple T-cell engineering process (42) but also the preferred T-cell phenotype and less exhausted profiles (16–18). Indeed, the present study and other studies have reported that PB-CAR-T cells exhibit a memory-rich CAR-T cell phenotype regardless of the target antigen or the manufacturing procedure; these CAR-T cells long-lived when infused in the tumor-bearing murine model and exhibited prolonged antitumor potency (17, 18). In the present study, PB-transgenes were preferentially introduced into $CD45RA^+$ PBMCs, and the memory-rich CD19 CAR-T cells were preferably enriched by PB-based manufacturing, which would be associated with the long-term functionality of PB-CAR-T cells. Indeed, IFN-γ in RA^+ CAR-T cells was greatly increased by antigen stimulation, and thus IFN-γ reached a high level 3 days after stimulation (**Figure 2D**). However, the activation of RA^+ CAR-T cells was transient and soon normalized, as evidenced by the downregulation of the IFN gene in RA^+ cells (**Figure 1G**). These results suggest that RA^+ CAR-T cells are not over-activated by antigen stimulation which would be related to the lower expression of immune exhaustion-related markers and long-term functionality.

PD-1 is an important exhaustion-related marker expressed on CAR-T cells that limits their function (43); CAR-T cell therapy combined with PD-1 blockade can be a potential strategy in cancer treatment (44–47). In this study, RA^+ CAR-T cells scarcely expressed PD-1, even after multiple rounds of antigen stimulation. In contrast, other canonical exhaustion-related markers such as TIM-3 and LAG-3 were highly expressed in both RA^+ and RA^- CAR-T cells, although their expression gradually decreased during antigen stimulation. Genome-wide transcriptome profiling showed that RA^+ CAR-T cells, despite of highly expressed TIM-3 and LAG-3, exhibited enriched expression of memory-T cell-like genes and less exhausted profiles. TIM-3 was initially identified as a molecule expressed by dysregulated, chronically-activated T cells and is generally

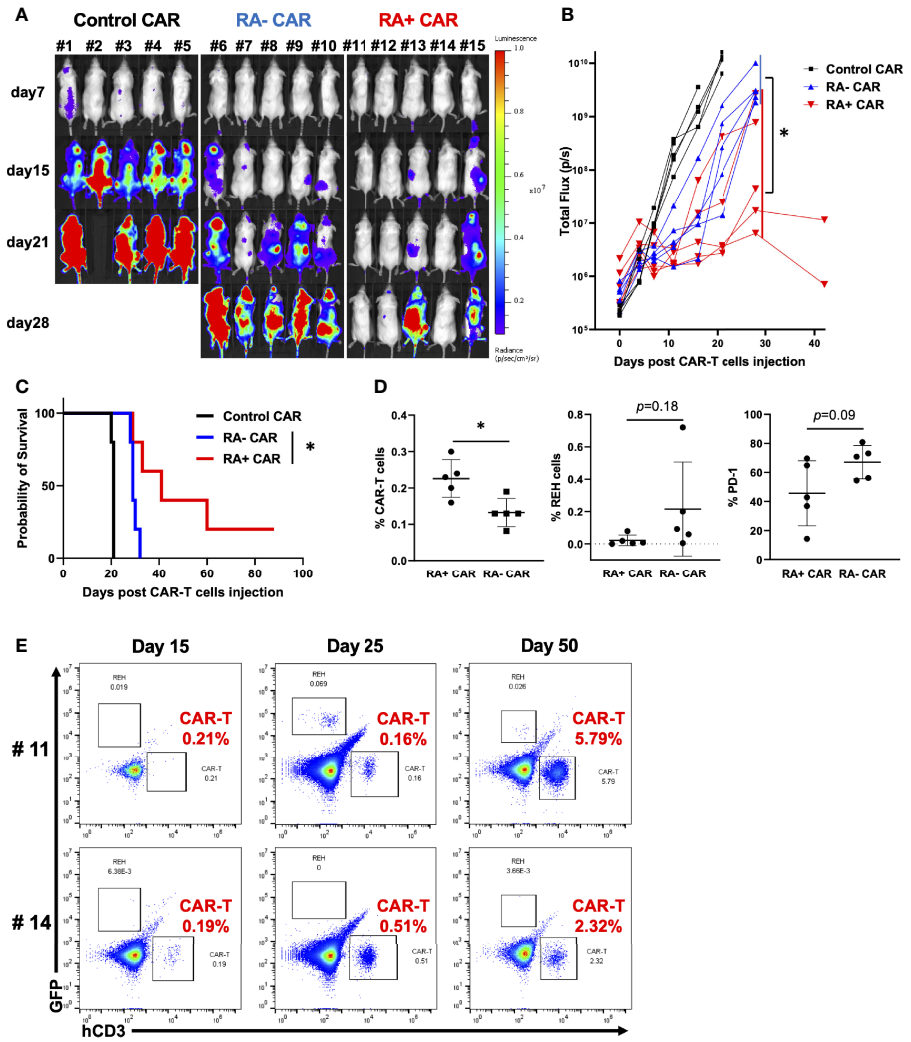

FIGURE 3 | CD45RA$^+$ chimeric antigen receptor (CAR)-T cells achieved prolonged tumor control with long-term expansion of CAR-T cells *in vivo*. We infused 5 × 10^5 REH-FFLuc cells into NSG mice *via* the tail vein. Six days later, 1 × 10^5 RA$^+$ CAR-T, RA$^-$ CAR-T, or control (EPHB4) CAR-T positive cells were infused into the tail vein of each mouse. **(A)** Bioluminescence images of groups of five NSG mice after intravenous CAR-T cell infusion. **(B)** The tumor volumes of each mouse measured as total flux (p/s) are shown. The CD45RA$^+$ CAR-T group showed a statistically significant tumor reduction, measured as the mean total flux at day 28, compared with the CD45RA$^-$ CAR-T group. **(C)** The Kaplan–Meier plot of overall survival (each group, n = 5). The CD45RA$^+$ CAR-T group achieved prolonged tumor control compared with the CD45RA$^-$ CAR-T group. Log-rank test: *P < 0.05. **(D)** On day 15 after CAR-T cell injection, bone marrow analysis showed CAR-T cells (left), REH cells (middle), and PD-1 expression on CAR-T cells (right) by flow cytometry. **(E)** In most long-lived mice infused with RA$^+$ CAR-T cells, CAR-T cells, and REH cells in the bone marrow of mice on days 15, 25, and 50. Representative dot plot data are shown. All data are presented as mean ± standard deviation. *P < 0.05.

considered to be a T-cell inhibitory protein (48, 49). However, the recent studies have indicated that TIM-3 exerts paradoxical costimulatory effect on T cells, including enhancement of the phosphorylation of ribosomal S6 protein, which is present downstream of T-cell receptor signaling (50, 51). We did not investigate the co-stimulatory function of TIM-3 on PB-CAR-T cells; however, based on the *in vitro* and *in vivo* potency of TIM-3 positive RA$^+$ CAR-T cells, the high expression of TIM-3 and LAG-3 on PB-CAR-T cell surface might not induce exhaustion which could impair the function of PB-CAR-T cells.

The expression profiles of several cell surface molecules are associated with the memory phenotype. We selected CD45RA as the marker for starting material selection, because clinical-grade CD45RA (or CD45RO) selection has been established and already used in the clinical setting (52). Therefore, the isolation of CD45RA$^+$ PBMC from the leukapheresis product could be easily translated into the clinical setting for PB-CAR-T cell manufacturing. Nevertheless, other memory-related T-cell surface molecules-positive PBMCs, such as CCR7 or CD62L-positive PBMCs, may also be potential starting materials for PB-CAR-T cell manufacturing. Moreover, previous studies have

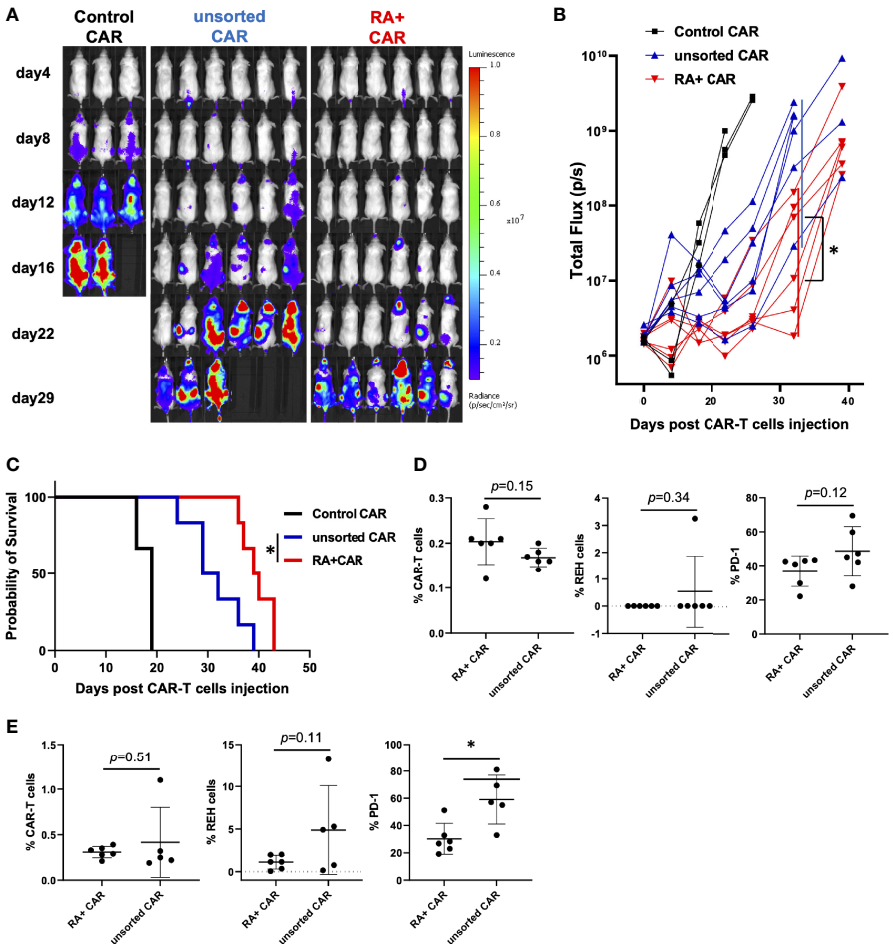

FIGURE 4 | CD45RA$^+$ chimeric antigen receptor (CAR)-T cells achieved prolonged tumor control than unsorted CAR-T cells *in vivo* stress test. We infused 1 × 10^6 REH-FFLuc cells into NSG mice *via* the tail vein. Six days later, 1 × 10^5 RA$^+$ CAR-T (n = 6), unsorted CAR-T (n = 6), or control (EPHB4) CAR-T (n = 3) positive cells were infused into the tail vein of each mouse. **(A)** Bioluminescence images of groups of NSG mice after intravenous CAR-T cell infusion. **(B)** The tumor volumes of each mouse measured as total flux (p/s) are shown. The CD45RA$^+$ CAR-T group showed a statistically significant tumor reduction, measured as the mean total flux at day 22, compared with the unsorted CAR-T group. **(C)** The Kaplan–Meier plot of overall survival. The CD45RA$^+$ CAR-T group achieved prolonged tumor control compared with the unsorted CAR-T group. Log-rank test: *P < 0.05. **(D)** On day 15 after CAR-T cell injection, bone marrow analysis showed CAR-T cells (left), REH cells (middle), and PD-1 expression on CAR-T cells (right) by flow cytometry. **(E)** On day 25 after CAR-T cell injection, bone marrow analysis showed CAR-T cells (left), REH cells (middle), and PD-1 expression on CAR-T cells (right) by flow cytometry. All data are presented as mean ± standard deviation. *P < 0.05.

reported that a specific formulation of post-manufactured CAR-T cell products would enhance antitumor efficacy (53); however, the cellular preparation of starting materials or post-manufacturing products has not been optimized yet. Nevertheless, CD45RA-positive selection of starting PBMCs would be beneficial in reducing the risk of manufacturing failure, which sometimes occurs in the clinical setting.

In a recent clinical trial conducted in Australia, two patients developed PB-mediated CAR-T cell-derived lymphoma (54). Although the researchers did not find transgene incorporation into known oncogenes and could not identify a unifying pathogenic mechanism, they determined a relatively high CAR transgene copy number in the malignancies and insertion of the CAR transgene into the BACH2 locus in both malignancies. The PB transposase they used was called "hyperactive PB", which was engineered to have a higher incorporation capacity and therefore would be related to the insertional mutagenesis. In the present study, we used an originally-developed PB transposase (24), and the copy number of the CAR transgene was relatively low compared to data from patients who developed lymphoma in a previous clinical trial (54). Nevertheless, there have been a number of clinical trials of CAR-T cells using hyperactive PB systems, but no malignant transformation has been reported except in a recent trial (54). It is also possible that the incorporation profile of CAR transgene may affect the malignant transformation. We did not evaluate the integration profile of CAR-T cells on a genome-wide basis, which is a limitation of our study. Similar to genetic manipulation by retroviruses, PB-mediated genetic modification is executed non-randomly in terms of integration sites, such as by favoring integration near the transcription start site (24). In fact, a

previous report demonstrated that the transgene profile of PB-mediated CAR-T cells generated by the exactly the same PB system that we used is similar to that of clinically accepted retroviral CAR-T cells (55). These suggested that not only the PB system but also the entire manufacturing processes, including plasmid and manufacturing reagents, or the patients background, might have contributed to malignant transformation in the previous clinical trial (54). Therefore, the safety of genetically modified T cells, including the long-term toxicity, should be thoroughly evaluated before clinical application.

In conclusion, PB-mediated RA^+ CAR-T cells exhibited a memory-rich phenotype and superior antitumor function *in vivo*, thereby indicated that the selection of $CD45RA^+$ PBMCs as a starting material would be useful for efficient PB-CAR-T cell manufacturing. The development of clinical grade and automatic cell isolation technologies may further facilitate genetically modified T-cell engineering with greater functionality and simplicity.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Kyoto Prefectural University of Medicine Institutional Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Kyoto Prefectural University of Medicine Institutional Review Board.

AUTHOR CONTRIBUTIONS

Conceptualization, SY. Methodology, MS, SY, NN, SK, YS, and MT. Investigation, MS, SY, NN, SK, and YS. Writing, Original Draft, MS and SY. Writing, Review & Editing, MS, SY, and TI. Funding Acquisition, SY. Resources, SY and TI. Supervision, SY, YN, and TI. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.770132/full#supplementary-material

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MINI REVIEW
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Adoptive Cell Therapy in Pediatric and Young Adult Solid Tumors: Current Status and Future Directions

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Advances from novel adoptive cellular therapies have yet to be fully realized for the treatment of children and young adults with solid tumors. This review discusses the strategies and preliminary results, including T-cell, NK-cell and myeloid cell-based therapies. While each of these approaches have shown some early promise, there remain challenges. These include poor trafficking to the tumor as well as a hostile tumor microenvironment with numerous immunosuppressive mechanisms which result in exhaustion of cellular therapies. We then turn our attention to new strategies proposed to address these challenges including novel clinical trials that are ongoing and in development.

Keywords: adoptive cell immunotherapy, solid tumor, tumor microenvironment, immune evasion, CAR (chimeric antigen receptor)

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INTRODUCTION

Immunologically "hot" solid tumors (e.g. melanoma) (1) with a tumor microenvironment (TME) marked by infiltrating CD8+ T-cells (2, 3), high programmed death ligand 1 (PD-L1) expression (4), or a high tumor mutational burden have shown remarkable responses to immunotherapy including immune checkpoint inhibitors (ICIs) (5). Unfortunately, these benefits have not extended to "cold" tumors (e.g. prostate or pancreatic cancer) (1) where T-cells are either entirely absent ("immune desert") or sequestered at the periphery ("immune-excluded") (3, 6). Many pediatric/adolescent and young adult solid tumors are cold tumors (7, 8) and have failed to respond to ICIs (9).

Several approaches have attempted to harness cellular therapy to cure these tumors. Autologous hematopoietic stem cell transplant (HSCT) has enabled maximal chemotherapy dosing in susceptible tumors with varying levels of effectiveness in neuroblastoma (10), Ewing sarcoma (11), breast cancer (12), retinoblastoma (13), hepatoblastoma (14), and other diseases. Recently some groups have piloted allogeneic HSCT to treat solid tumors. Though durable responses are rare, evidence for graft-vs-tumor effect has been observed (15). Finally, as adoptive cellular therapy (ACT) has proven transformative for leukemia and lymphoma, the development of novel ACT for

solid tumors has exploded (**Figure 1**). In this review, we discuss ACT in solid tumors in clinical development, consider challenges plaguing the field, and highlight proposed strategies which will be tested in future clinical trials.

T-CELL BASED THERAPIES

T-cells are critical in immune surveillance for cancer. The T-cell receptor (TCR) can recognize cancer-specific antigens processed by major histocompatibility complex (MHC) and presented on the cell surface. TCR engagement by MHC-presented non-self antigens leads to activation and T-cell mediated killing (16). T-cell cytotoxicity in solid tumors has been leveraged using both native T-cells and autologous T-cells genetically engineered to express a specific TCR. Future efforts in allogeneic "off-the-shelf" approaches are being actively studied.

Tumor Infiltrating Lymphocytes (TILs)

Early studies demonstrated that heterogeneous tumor infiltrating lymphocytes (TILs) collected from a freshly-resected tumor and expanded *in vitro* were able to specifically lyse autologous tumor (17). Subsequent clinical investigations showed transient responses in patients with metastatic melanoma after TIL infusion, typically under high IL-2 conditions (17). Early TIL trials reported responses in 49-72% of patients with melanoma (18, 19). Pretreatment with lymphodepleting chemotherapy led to improved TIL persistence (18) and recent advances include selection of TILs that recognize patient-specific tumor antigens using single cell sequencing (20). Selected autologous TILs have shown activity in several epithelial malignancies (21, 22).

While advances using TILs continue, the inability to isolate and effectively expand TILs from some solid tumors remains a challenge.

Engineered TCR-Based ACT

Initial attempts at engineering T-cells for ACT concentrated on genetic engineering of specific TCRs into autologous T-cells collected *via* peripheral blood apheresis with subsequent reinfusion.

Expression of cancer/testis antigens (CTAs) including melanoma antigen gene (MAGE) family proteins, synovial sarcoma X breakpoint (SSX) family proteins, and New York esophageal squamous cell carcinoma (NY-ESO-1) is normally restricted to the germline. However, solid tumors including melanoma, SS, myxoid/round cell liposarcoma (MRCL), and osteosarcoma express CTAs. Robbins and colleagues targeted NY-ESO-1 using a transduced TCR recognizing the peptide epitope SLLMWITQC in the context of HLA-A*02. Transgenic T-cells combined with IL-2 following lymphodepletion led to responses in 5/11 patients with SS and 2/11 patients with melanoma (23). A subsequent study showed responses in 6/12 patients with NY-ESO-1+ SS in an initial cohort (24), with one complete response (CR) and 14 partial responses (PR) in the first 42 patients (25). This response rate represents a potentially significant improvement over previous therapies for SS (26). These T-cells maintained clonal diversity over time and persisting cells were primarily of central memory

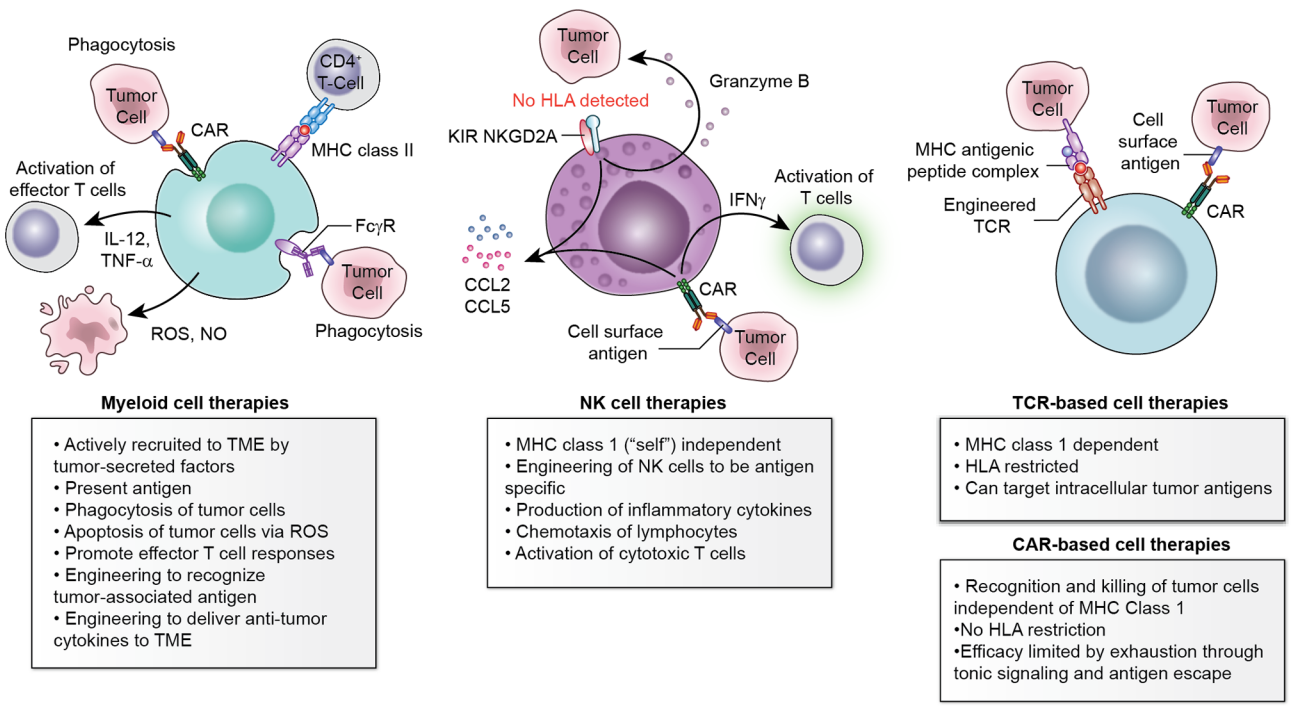

FIGURE 1 | Multiple cell types available to engineer for adoptive cellular therapy. Myeloid cells, NK cells, and T-cell-based therapies each have advantages and disadvantages which should be considered within the context of the histology to be targeted.

and stem cell memory populations (24). Ongoing trials are further investigating genetically engineered NY-ESO-1 targeting autologous T-cells in solid tumors including SS, MRCL and non-small cell lung cancer (NCT02992743, NCT03967223, NCT03709706).

Other successfully targeted CTAs include MAGE-A3 and MAGE-A4. Seventeen patients were treated in a dose escalation study of autologous T-cells genetically modified to express an MHC class II-restricted TCR recognizing MAGE-A3 combined with IL-2 (27). One patient with cervical cancer had a CR and several PRs were observed in patients with esophageal cancer, urothelial cancer, and osteosarcoma. Despite encouraging responses, the significant neurotoxicity observed in this and a subsequent trial targeting MAGE-A3 has hampered development of this strategy (28). In a phase I trial of a TCR developed in a transgenic murine model and recognizing residues 112-120 (KVAELVHFL) of MAGE-A3, 3/9 patients developed significant neurotoxicity (29). Preliminary data for the SURPASS trial utilizing autologous T-cells transduced with a MAGE-A4 TCR and CD8a co-receptor reported 2/5 patients with PRs (30). A MAGE-A4 targeting TCR is also being evaluated in a phase II study for patients with SS and MRCL (NCT04044768). Additional TCR-based strategies targeting CTAs are in development (31).

Viral antigens have also been successfully targeted for treating solid tumors using ACT. TILs targeting human papillomavirus (HPV) antigens E6 and E7 have shown efficacy in early phase clinical trials in HPV-associated carcinomas with responses in 5/18 patients with cervical cancer and 2/11 with head and neck cancer (32). Subsequent work identified TCRs recognizing epitopes of HPV16 E6 and E7 in the context of HLA-A*02:01 and T-cells genetically engineered to express these TCRs led to responses in two early phase studies (33, 34). Epstein-Barr virus (EBV) is associated with several solid tumors (e.g., nasopharyngeal carcinoma [NPC] and post-transplant lymphoproliferative disorder [PTLD]). EBV-specific cytotoxic T-lymphocytes (CTLs) were tested to treat PTLD following HSCT (35). EBV-specific CTLs resulted in PR for 2/10 patients with EBV-associated NPC (36). A phase 3 trial comparing chemotherapy with EBV-specific CTLs combined with chemotherapy for NPC is underway (NCT02578641). TCR-based therapy has also been explored for Merkel cell carcinoma, a skin cancer associated with Merkel cell polyomavirus (37). Autologous T-cells with TCRs recognizing an epitope of Merkel cell polyomavirus, large T antigen and small T antigen, led to durable regression of metastatic lesions in several patients (38).

CART-Based ACT

HLA-restriction (limiting patient access) and reliance on tumor MHC expression have limited TCR-based therapy utility. Chimeric antigen receptor T-cells (CART) are autologous T-cells engineered *ex vivo* to enable MHC-independent tumor cell killing without HLA restriction. First-generation CAR have 3 components: a specific antibody-derived single chain variable fragment (scFv), a hinge/transmembrane domain, and a T-cell signaling (CD3ζ) domain. Second-generation CAR incorporate one additional co-stimulatory domain, while third-generation CAR incorporate 2 additional co-stimulatory domains. Fourth-generation CAR, also known as TRUCKs (T cells redirected for antigen unrestricted cytokine-initiated killing) include a CAR-inducible transgene product, often pro-inflammatory cytokines which may enhance CART cytotoxicity and activate other immune cells in an immunosuppressive TME (39).

Human epidermal growth factor receptor 2 (HER2) is expressed on several solid tumor types and has attracted interest as a CART target. A clinical trial utilizing 10^{10} of a third-generation CART incorporating a scFV derived from the humanized monoclonal antibody trasztuzumab following lymphodepletion for HER2+ solid tumors. A patient with metastatic colorectal cancer developed fatal respiratory failure 15 minutes after CART infusion. This was thought to be due to massive cytokine release upon recognition of HER2 at low levels on lung epithelium and prompted concerns about the safety of HER2-CART (40). A subsequent HER2-CART trial in HER2+ sarcomas instead utilized the FRP5 scFV, omitted lymphodepletion, and selected a lower starting dose of $10^4/m^2$. There were no dose-limiting toxicities, but also no CART expansion. Doses greater than $10^6/m^2$ were associated with greater persistence. 4/17 evaluable patients had stable disease and 1 patient had a PR after a second CART infusion (41). To improve CART expansion and persistence, an ongoing phase I HER2-CART trial (NCT00902044) has incorporated lymphodepletion and HER2-CART doses up to $10^8/m^2$. Thus far two CRs have been reported (42, 43).

Clinical experience with CART targeting the diaganglioside GD2, which is highly expressed on osteosarcoma, neuroblastoma, and many central nervous system (CNS) tumors, also suggests that lymphodepletion and adequate cell dose are important for CART expansion and persistence. A phase 1 trial utilizing first-generation GD2-CART without lymphodepletion in neuroblastoma demonstrated safety and clinical activity with 3 CRs, but showed limited expansion and persistence (44, 45). A subsequent trial (NCT02107963) utilized a third-generation GD2-CART with lymphodepletion, and demonstrated good expansion (46). A phase I study of a third-generation GD2-CART with or without lymphodepletion in relapsed/refractory neuroblastoma showed increased CART expansion following lymphodepletion (47). A phase I trial utilizing escalating doses of a second-generation GD2-CART with lymphodepletion of varying intensity in relapsed/refractory neuroblastoma showed regression of soft tissue and bone marrow disease following CART doses of at least $10^8/m^2$ (48). GD2-CART have shown promising clinical activity in a phase 1 trial in H3K27M+ diffuse midline gliomas, which are universally fatal malignancies (NCT04196413) (49). Based on preclinical data suggesting that incorporation of IL-15 into CART further enhances persistence and cytotoxicity (50, 51), ongoing trials are utilizing GD2-CART and GD2-CAR-NKT cells engineered to express IL-15 (NCT03721068, NCT03294954).

The checkpoint molecule B7-H3(CD276) is another CART target of interest given its high expression on multiple solid tumor types. Preclinical studies have demonstrated encouraging activity of B7-H3-CART in various xenograft models (52–54). Clinical trials utilizing B7-H3-CART are underway in pediatric

and adult solid tumors (NCT04897321, NCT04483778, NCT04432649, NCT05211557, NCT04670068) and CNS tumors (NCT04185038, NCT04385173, NCT04077866).

TRuC™-T-Cell Based ACT

TCR fusion constructs (TRuCs) also enable HLA-independent cell killing. In contrast to CART, which incorporate only the intracellular signaling domain of the CD3ζ chain, TRuCs involve fusion of the scFv to the N-terminus of any of the other five subunits in the TCR complex. TRuCs are incorporated into the TCR on translation, engage the TCR complex upon activation, and are efficacious in solid tumor xenograft models (55). Anti-mesothelin TRuCs are being studied in a phase 1/2 clinical trial (NCT03907852) with preliminary evidence of activity, with 3/7 patients (2 with mesothelioma, 1 with ovarian cancer) achieving a PR (56).

NK-CELLS

NK-cells are innate immune cells and protect against infections and cancer (57, 58). Efforts to harness NK-cell biology for ACT in cancer treatment has gained considerable interest as an alternative to T-cell based immunotherapeutics. NK-cells possess qualities which may allow them to overcome the hostile TME (58–60). While T-cells recognize unique tumor antigens, NK-cell-mediated cytotoxicity depends on the sum of activating and inhibitory signals, including tumor cell lack of MHC class 1 expression or antibody-dependent cell-mediated cytotoxicity (61). Furthermore, NK-cells can produce inflammatory cytokines such as IFNγ and TNFα which can activate CD8+ TILs and enhance their cytotoxicity (62).

These properties allow NK-cells to be engineered or manipulated *via* different mechanisms from T-cell-centric immunotherapies. Examples include the administration of agonist cytokines or engineering NK-cells which constitutively secrete these cytokines (63). Others have proposed NK-cells which constitutively secrete chemotactic factors to recruit cytotoxic lymphocytes to the TME (64). Tri-specific NK-cell engagers (TriKEs) have been proposed to confer tumor-specificity to NK-cells and enhance NK-cell activation by engaging stimulatory receptors such as the IL-15 receptor (65, 66). Additionally, CAR NK-cells (CAR-NK) designed from stem cell progenitors represent another way to generate tumor-specific NK-cells. Attractively, CAR-NK may be less toxic and could be produced at lower cost than CART (67). Recent experience with CD19-CAR-NK in B-cell malignancies provides proof-of-concept that this strategy can be safely and effectively utilized and with potential for persistence (68). Barriers remain to production and monitoring of persistence of these cells, but additional alterations to the NK-cell product and manufacturing strategies have been proposed to mitigate these issues. Finally, NK-cells also express immune checkpoint molecules such as PD-1, and either combination with ICIs or intrinsic downregulation of these checkpoint molecules have been proposed as mechanisms to further enhance the efficacy of NK-cell-based approaches (69, 70).

MYELOID CELL THERAPIES

Myeloid cells readily infiltrate primary tumors and metastases. Harnessing this property for ACT shows promise in the treatment of solid malignancies (71). Myeloid cells are highly plastic and may acquire a wide spectrum of immune-stimulatory or immune-suppressive phenotypes in response to the local milieu. Tumor associated macrophages (TAMs) are polarized to an anti-tumor M1 phenotype in response to pro-inflammatory factors such as IFNγ, GM-CSF and lipopolysaccharide. M1 TAMs promote Th1 responses, phagocytosis of tumor cells, and antigen presentation. Tumor-associated cytokines such as IL-10, IL-4, IL-13 and TGF-β promote polarization towards an immunosuppressive M2 phenotype. M2 TAMs promote tumor progression through mechanisms including angiogenesis, extracellular matrix (ECM) remodeling and regulatory T-cell recruitment (72). This M1/M2 classification is an oversimplification, however induction of an M1-like, anti-tumor phenotype is important for the success of myeloid-based ACT. The first myeloid-based ACT utilized macrophages polarized to the M1 phenotype *ex vivo* with IFNγ. Clinical trials showed limited efficacy, but these therapies were generally well-tolerated (73–75).

Subsequent work has focused on engineering myeloid cells towards a more potent and durable anti-tumor phenotype. Anti-HER2 CAR-macrophages (CARM) reduced tumor growth and prolonged survival while reprogramming the immune-suppressive TME in xenograft models (76). A first-in-human trial evaluating CARM is now underway in HER2-overexpressing solid tumors (NCT04660929). Preclinical work has shown that myeloid cells can also be used to deliver cargo to the TME. Administration of myeloid cells genetically engineered to express IL-12, a potent anti-tumor cytokine, resulted in durable cures in a syngeneic model of embryonal rhabdomyosarcoma through activation of T-cell responses in the tumor and metastatic microenvironment (77).

CHALLENGES IN SOLID TUMOR ACT

Significant remaining challenges for optimization of solid tumor ACT are outlined in this section. Additionally, we will summarize proposed strategies to overcome these challenges (**Figure 2**).

Selection of antigens such as GD2 (78) and CTAs (79), which are expressed on numerous solid tumors, leverages the possibility that a single ACT could be active across multiple histologies. However, few antigens are tumor-specific. Thus, identifying a target antigen which will allow tumor clearance without unacceptable normal tissue toxicity (on-target/off-tumor effect) is problematic. In addition to selecting the proper target antigen, low antigen density and antigen downregulation within heterogeneous solid TMEs have emerged as additional barriers to ACT (80, 81).

ACT trafficking is also challenging in solid tumors. Trafficking can be inhibited by physical barriers, loss of MHC class 1 expression, repellent cytokine gradients, expression of inhibitory ligands such as PD-L1, and abnormal tumor vasculature (82). CNS tumors are further shielded by the

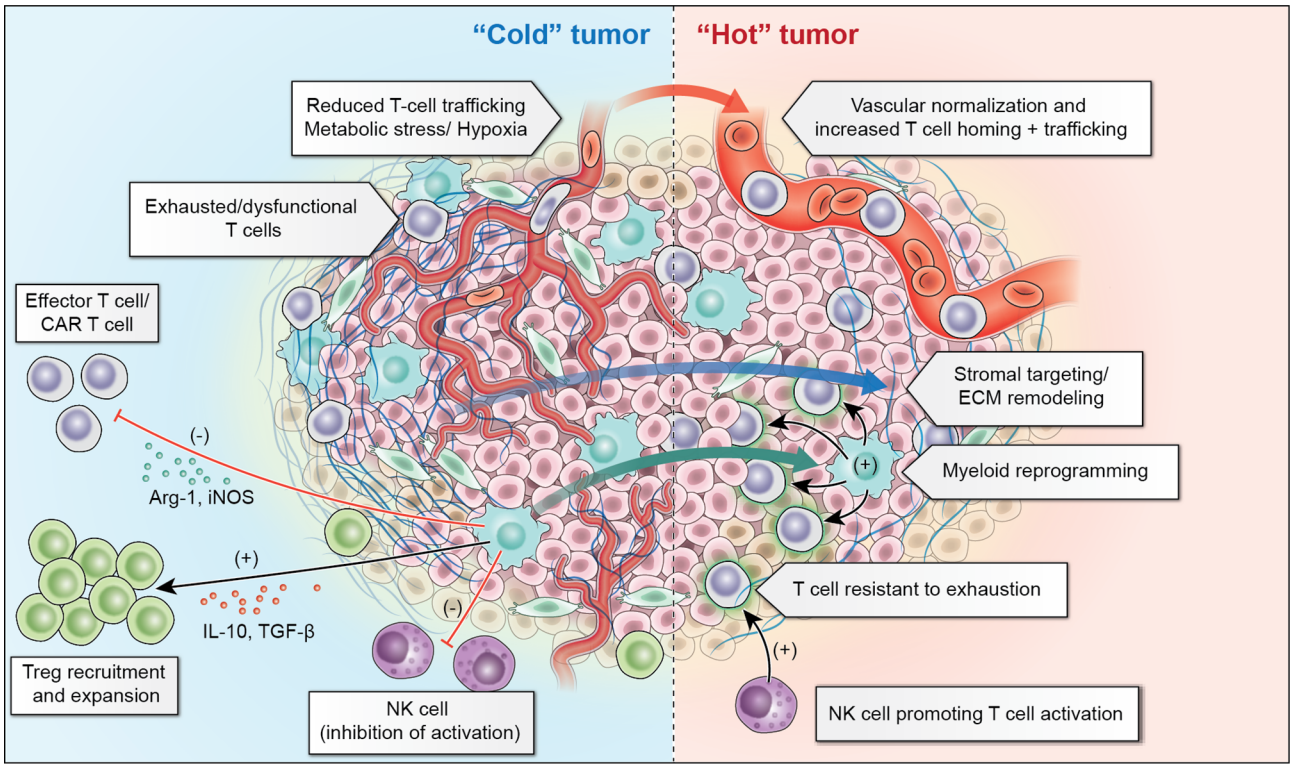

FIGURE 2 | "Cold" solid tumors present a number of challenges within their tumor microenvironment including reduced trafficking related to abnormal tumor vasculature and resident inhibitory myeloid cells which recruit regulatory T cells (Treg) and lead to exhaustion of T-cells and NK-cells. Adoptive cellular therapies aim to overcome these challenges through vascular normalization and extracellular matrix (ECM) remodeling to promote improved trafficking, as well as myeloid cell reprogramming to diminish the inhibitory contribution of these cells. Additionally, T-cells which are resistant to inhibition or "armored" T-cells, or NK-cells which can augment T-cell responses may make it possible to overcome the inhibitory tumor microenvironment.

blood-brain barrier (83). If ACTs cannot traffic to the tumor and engage their target antigen, they fail to be activated and expand, leading to rapid loss of ACT.

Finally, the TME present in many solid tumors is hostile to the ACT. Tumors recruit immunosuppressive TAMs and myeloid-derived suppressor cells (MDSCs) (84) which express inhibitory molecules such as PD-L1 (84), secrete inactivating cytokines such as IL-10 (85), and promote a hypoxic TME (86) which can thwart ACT cytotoxicity. These tumor-sustaining programs promote rapid and irreversible ACT exhaustion, inhibit expansion, and result in failure of tumor clearance. Further ACT engineering or combination with agents to allow ACTs to overcome these challenges, will be necessary for ACT optimization in solid tumors.

DISCUSSION: OVERCOMING THE IMMUNE-SUPPRESSIVE TME IN ACT FOR SOLID TUMORS

Aberrant tumor vasculature and ECM deposition impede ACT trafficking. Regional ACT administration is one strategy to overcome this hurdle. A recent phase I trial demonstrated that intrapleural administration of mesothelin-CART combined with pembrolizumab was safe and feasible, and showed potential efficacy with 2 patients demonstrating metabolic CR on PET scan (87). Intraventricular CART administration for both primary brain tumors and CNS metastases is also under evaluation in early-phase clinical trials and in preclinical models (88, 89) (NCT04196413). Additionally, methods to disrupt the blood-brain barrier to allow trafficking of ACT to CNS tumors, such as focused ultrasound (90) or other mechanical or pharmacological methods (91) have been piloted.

Further genetic modification of ACTs to overcome and leverage features of the hostile TME is currently being explored. Many solid tumor types recruit TAMs by producing chemokines such as CXCL8 and CXCL2. Preclinical data suggest that chemokine secretion can be leveraged to enhance CART trafficking by engineering CART to express chemokine receptors. For example, CXCR2-modified GPC3-CART had improved trafficking in a hepatocellular carcinoma model (92), while a CXCR1/2-modified CD70-CART enhanced CART trafficking and efficacy in murine GBM, ovarian cancer and pancreatic cancer models (93). Many groups have also sought to generate a more "fit" ACT through enhanced cytokine secretion

[thoroughly reviewed by Bell and Gottschalk (94)]. Additional modifications include creation of ACT which is resistant to exhaustion [e.g. DNA methyltransferase 3 alpha knock-out (95) or PD-1 deletion (96)] or tuning ACT to be effective despite low antigen density [e.g. c-Jun overexpression (97, 98)]. These modifications of ACTs are now entering clinical trials (e.g. TGF-βR knockout CART NCT04976218).

Tumor-associated vasculature is characterized by pericyte loss, resulting in leakiness and adhesion molecule down-regulation impairing T-cell migration into the tumor (99). VEGF inhibitors, which promote vascular normalization, may enhance CD8+ T-cell infiltration into tumors (100). Anti-VEGF agents have shown synergy with ICIs in select solid malignancies, resulting in FDA approval of these combinations in hepatocellular carcinoma and renal cell carcinoma (101). Preclinical studies suggest that antiangiogenics can also improve ACT trafficking (102, 103). Combining ACTs with antiangiogenics warrants further study in clinical trials.

ECM-remodeling agents may enhance the ability of ACTs to infiltrate tumors. In gastric cancer models, hyaluronic acid reduced mesothelin-CART infiltration, however these CART had superior efficacy when combined with infusion of a secreted form of the human hyaluronidase PH20 (104). CART engineered to express heparinase, which degrades heparan sulfate proteoglycans, showed superior anti-tumor activity and were associated with increased T-cell infiltration in preclinical models (105).

The solid TME contributes to T-cell exhaustion *via* multiple mechanisms, including repeated TCR stimulation and metabolic stress, thereby reducing the ACT efficacy. Engineering CART to reduce tonic signaling through incorporation of the 4-1BB costimulatory domain vs CD28 costimulatory domain showed reduction in CART exhaustion and enhanced persistence and efficacy in preclinical studies (106). Induction of transient rest periods in CART, such as by dasatinib utilization, has shown exhaustion reversal and improved efficacy (107). A dasatinib-containing culture platform is being used to manufacture GD2-CART in ongoing clinical trials (NCT04539366, NCT04196413). CART combination with ICIs is also under evaluation in clinical trials (108).

The ability of myeloid cells to orchestrate immune responses in the TME makes them an attractive therapeutic target. Low-dose chemotherapy has shown reduction of tumor MDSCs (109–111). MDSC differentiation with ATRA reduced their immune-suppressive function and enhanced efficacy of GD2-CART in preclinical models (112). In a pilot trial studying ipilimumab vs ipilimumab combined with ATRA, patients receiving ATRA had fewer circulating MDSCs (113). Inhibiting myeloid cell trafficking through CSF1R inhibition is another potential avenue to reduce myeloid cell immune-suppression in the TME. CSF1R-targeting agents are generally well-tolerated in the clinic, and the multi-TKI CSF1R inhibitor Pexidartinib is FDA-approved to treat tenosynovial giant-cell tumor (114, 115). Clinical trials studying CSF1R inhibitors with ICIs are underway (NCT02777710, NCT02829723, NCT03502330, NCT04848116, NCT02526017).

CONCLUSION

While ACT has yet to yield the transformative results in solid tumors that CART have shown for hematologic malignancies, evidence exists that some patients with solid tumors may respond to ACT. T-cells, NK-cells, and myeloid cells have each been engineered to target these tumors, and each have advantages and unique challenges. Further engineering ACTs to overcome tumor immune resistance mechanisms and better understanding how to combine with TME-modifying agents will be critical to expanding the number of patients with solid tumors who may derive therapeutic benefit.

AUTHOR CONTRIBUTIONS

JL, KW, and JG wrote the first version of the manuscript. NS provided critical feedback and additions. All authors contributed to the final version of the manuscript.

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REVIEW
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Cancer Therapy With TCR-Engineered T Cells: Current Strategies, Challenges, and Prospects

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To redirect T cells against tumor cells, T cells can be engineered *ex vivo* to express cancer-antigen specific T cell receptors (TCRs), generating products known as TCR-engineered T cells (TCR T). Unlike chimeric antigen receptors (CARs), TCRs recognize HLA-presented peptides derived from proteins of all cellular compartments. The use of TCR T cells for adoptive cellular therapies (ACT) has gained increased attention, especially as efforts to treat solid cancers with ACTs have intensified. In this review, we describe the differing mechanisms of T cell antigen recognition and signal transduction mediated through CARs and TCRs. We describe the classes of cancer antigens recognized by current TCR T therapies and discuss both classical and emerging pre-clinical strategies for antigen-specific TCR discovery, enhancement, and validation. Finally, we review the current landscape of clinical trials for TCR T therapy and discuss what these current results indicate for the development of future engineered TCR approaches.

Keywords: T cell receptor, TCR, chimeric antigen receptor, CAR, TCR-engineered T cells, TCR T, adoptive cell therapy

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INTRODUCTION

The past decades have seen rapid advancements in our understanding of the mechanisms underlying the antitumor function of immune cells, and as such adoptive cell therapy (ACT) strategies have emerged as a major platform of cancer therapeutics. A milestone in ACT was the success of tumor infiltrating lymphocyte (TIL) therapy for metastatic melanoma beginning in the 1980s (1). While TIL therapy remains an important ACT modality, the manufacture of TIL products is logistically challenging. ACT efforts have thus largely transitioned towards strategies to engineer peripheral blood T cells with receptors that confer desired antigen specificity. These predominantly include chimeric antigen receptor T cell (CAR T) and T cell receptor engineered T cell (TCR T) therapies. Due to the remarkable efficacy of CAR T therapies in treating B cell malignancies (2), interest in CAR T therapy has eclipsed that of TCR T therapy. However, TCR T therapy is gaining interest as CAR T trials have so far failed to elicit satisfactory responses in the treatment of solid cancers (2), and many believe TCRs may be better suited for the treatment of solid

cancers (3). Indeed, exciting clinical results are now emerging that demonstrate safety and efficacy of TCR T therapies in both hematological and solid cancers. In this review we describe the biology of TCRs and tumor antigen targets and discuss state of the art techniques for TCR discovery and preclinical assessment. Finally, we describe the current landscape of TCR T trials and the challenges that remain.

BIOLOGY OF TCRs AND TUMOR ANTIGEN TARGETS

Redirecting T Cell Specificity Through Genetic Engineering

Conventional T cells recognize MHC-presented antigens through their T cell receptor (TCR), a disulfide-linked heterodimer comprised of an α and β chain. To form a functional receptor, TCR α/β heterodimers further complex with CD3ϵ/γ/δ/ζ subunits (4–7). TCRs recognize enzymatically cleaved peptides that are presented at the cell surface by MHC molecules (pMHC). In humans, antigen-presenting MHC alleles are broadly classified as HLA class I (A, B, or C) or HLA class II (DR, DP, or DQ), which predominantly present cytosolic or extracellular derived peptides, respectively (4). The coreceptors CD8 and CD4 enhance TCR antigen sensitivity through interaction with MHC class I or II molecules, respectively (8). TCR binding to cognate pMHC leads to the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in intracellular regions of the CD3 subunits (5, 6), which results in T cell activation and initiation of effector functions including proliferation, cytokine secretion, and cytolysis *via* secretion of perforin and granzyme (**Figure 1**). In TCR T therapy, T cells are edited to express TCR α and β chains that confer a desired specificity. Here, introduced TCR α and β chains dimerize and complex with endogenous CD3 components to form a functional TCR that redirects T cell specificity towards an antigen of interest.

Another common method for redirecting T cell specificity is through the genetic transfer of chimeric antigen receptors

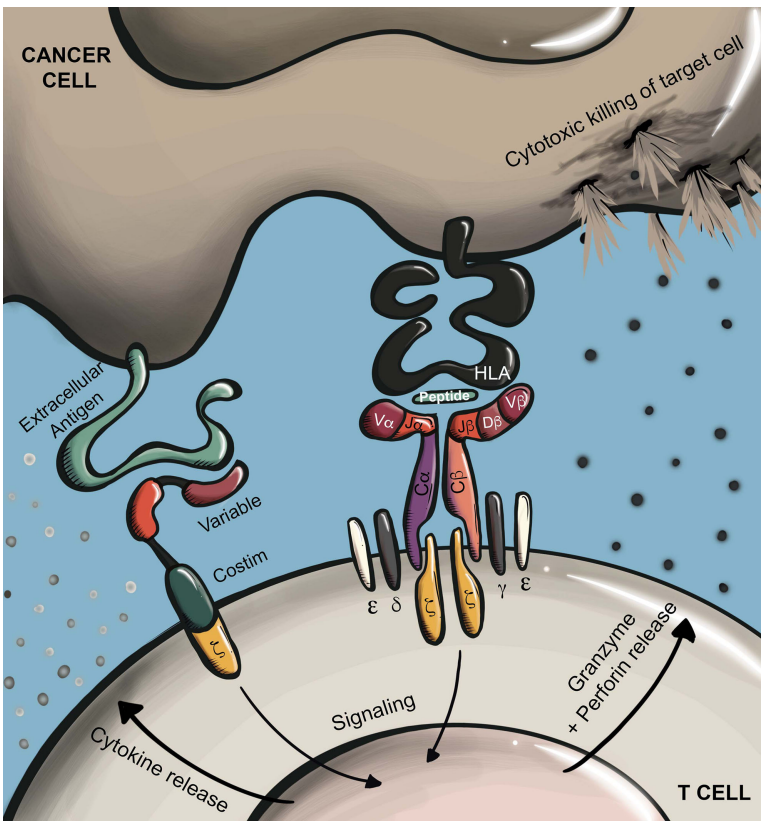


FIGURE 1 | Antigen recognition by CARs and TCRs. CARs recognize surface proteins typically through an antibody-derived scFv recognition domain. Antigen recognition leads to T cell activation *via* phosphorylation of ITAMs in a conjugated intracellular CD3ζ domain. In the case of later generation CARs, ligand binding also leads to additional stimulation of conjugated costimulatory receptors (e.g. CD28, 4-1BB). TCRs recognize HLA-presented peptides which may be derived from any cellular compartment. Antigen recognition by TCRs leads to T cell activation through phosphorylation of ITAMs in the associated CD3ϵ/γ/δ/ζ subunits. Depending on T cell subtype, T cell activation through either receptor type will trigger effector functions including proliferation, cytokine secretion, and target cell killing through directed secretion of perforin and granzyme.

(CARs), which are broadly comprised of an extracellular-facing antigen-binding domain linked to an intracellular immune cell activation signaling domain. Most often, CARs recognize antigen through the single-chain variable fragment (scFv) of an antibody. In a typical CAR design, the antigen-binding scFv is linked *via* a hinge, or spacer, region to a transmembrane domain that is further conjugated to an intracellular CD3ζ signaling domain. In this manner, antigen binding by the scFv drives CD3ζ phosphorylation and downstream T cell activation. Later generation CARs include the addition of intracellular costimulatory domains such as CD28 and 4-1BB, which further improve CAR T function and persistence (**Figure 1**) (9).

TCRs vs CARs

While TCRs recognize antigens in the context of HLA presentation, CARs recognize natively folded proteins at the cell surface. Therefore, CARs overcome clinical limitations imposed by the HLA-restriction of TCRs. HLA encoding genes are the most polymorphic in the human genome, with over 20,000 HLA-class I alleles identified to date (10). Therefore, unlike CAR T therapy, patients selected for TCR T therapy must express not only the targeted antigen, but also the corresponding antigen-restricting HLA allele. For this reason, TCR T therapies typically utilize TCRs that are restricted to relatively common HLA alleles, such as HLA-A*02:01, which is present in about 47.8% and 16.8% of Caucasian and African American populations in the United States, respectively (11).

As CARs and TCRs utilize differing signaling mechanisms, they exhibit several important differences in their functional response to antigen stimulation. While TCRs can elicit a cytotoxic response to as few as a single pMHC molecule, CARs typically require thousands of target surface molecules to mediate an effective response (12–14). A consequence of the reduced antigen sensitivity of CARs is seen in patients with B cell malignancies who initially respond to CAR T therapy but subsequently relapse with progression of antigen-low cancer cells (15). Upon stimulation, CARs mediate supraphysiologic T cell activation, leading to enhanced cytokine secretion. For this reason, CAR T cells are more likely to cause cytokine release syndrome (CRS) in patients as compared to TCR T cells; however, recent advancements in treatment have made CRS generally clinically manageable (16, 17).

Tumor antigens recognized by CARs must be located at the surface of cancer cells. Conversely, TCRs recognize HLA presented peptides that may be derived from any cellular compartment. As transmembrane proteins constitute only an estimated 14-26% of the proteome (18–20), CAR-targetable antigens are considerably more limited. However, the repertoire of CAR-targetable antigens is extended to some degree by the ability of CARs to recognize not only protein antigens, but also other molecules like glycoproteins and glycolipids (21). This difference in repertoire of potential targetable antigens has significant implications for CAR T and TCR T therapies, which must aggressively target tumor cells while avoiding toxicity directed towards healthy tissue. Indeed, expression of target antigen on normal cells can lead to T cell-mediated destruction of healthy tissue, known as 'on-target off-tumor' toxicity (22). Therefore, the degree to which a target antigen is exclusively expressed by cancer cells is an important factor. So far, the primary success of CAR T therapy has been in the treatment of B cell malignancies targeting CD19, an antigen expressed ubiquitously on malignant and healthy B cells. While CD19-directed CAR T therapy leads to ablation of both malignant and healthy B cells, such on-target off-tumor toxicity is clinically manageable through replacement antibody therapy (22). However, in the case of many other types of cancer, including almost all solid cancers, such T cell-mediated ablation of healthy organ tissue is not clinically manageable, and thus target antigens with exclusivity of expression in cancer cells are best. Currently described tumor antigens with the greatest specificity of expression in cancer cells are predominantly intracellular derived antigens, accessible to TCRs but not CARs (23). Therefore, TCR T therapies may have an advantage over CAR T therapies in the ability to aggressively target cancer cells while minimizing toxicity.

TCR Targeted Tumor Antigens

Significant progress has been made in identifying the precise cancer antigens that mediate immune rejection. While the nomenclature describing tumor antigens varies, widely studied classes of tumor antigens include tumor-associated antigens (TAAs), cancer-germline antigens (CGAs), and tumor-specific antigens (TSAs) (24–26) (**Figure 2**).

TAAs

TAAs are expressed by tumor cells but are also expressed in at least some healthy tissue. As a result, therapies targeting TAAs must contend with potential T cell mediated on-target off-tumor toxicity. TAAs are further classified as differentiation antigens or overexpressed antigens.

Differentiation antigens are expressed by cancer cells as well as normal cells of the same tissue origin. Melanoma differentiation antigens were among the first discovered tumor antigens and include the widely studied melanoma-associated antigen recognized by T cells (MART-1) (27) and glycoprotein 100 (gp100) (28). Among tumor antigens, differentiation antigens typically pose the greatest risk for on-target off-tumor toxicity. As discussed in further detail in a later section, clinical experience has demonstrated that targeting differentiation antigens is likely only clinically appropriate when antigen expression is restricted to dispensable healthy tissue, such as CD19-expressing B cells. Likely for this reason, only one TCR T clinical trial targeting a solid cancer differentiation antigen has been initiated since 2012 (**Supplementary Table 1**).

Overexpressed antigens are expressed at high levels in cancer cells but are minimally expressed in healthy cells. While targeting such antigens continues to pose a risk for on-target off-tumor toxicity, the differential expression between cancer and normal cells allows for the possibility of achieving a therapeutic window by which adoptively transferred T cells may destroy high-antigen expressing cancer cells with minimal destruction of low-antigen expressing healthy tissue. An example of a widely studied overexpressed antigen is Wilms' Tumor Antigen 1 (WT1), a transcription factor with 10- to 1000- fold higher expression in leukemic cells as compared to normal cells (29, 30).

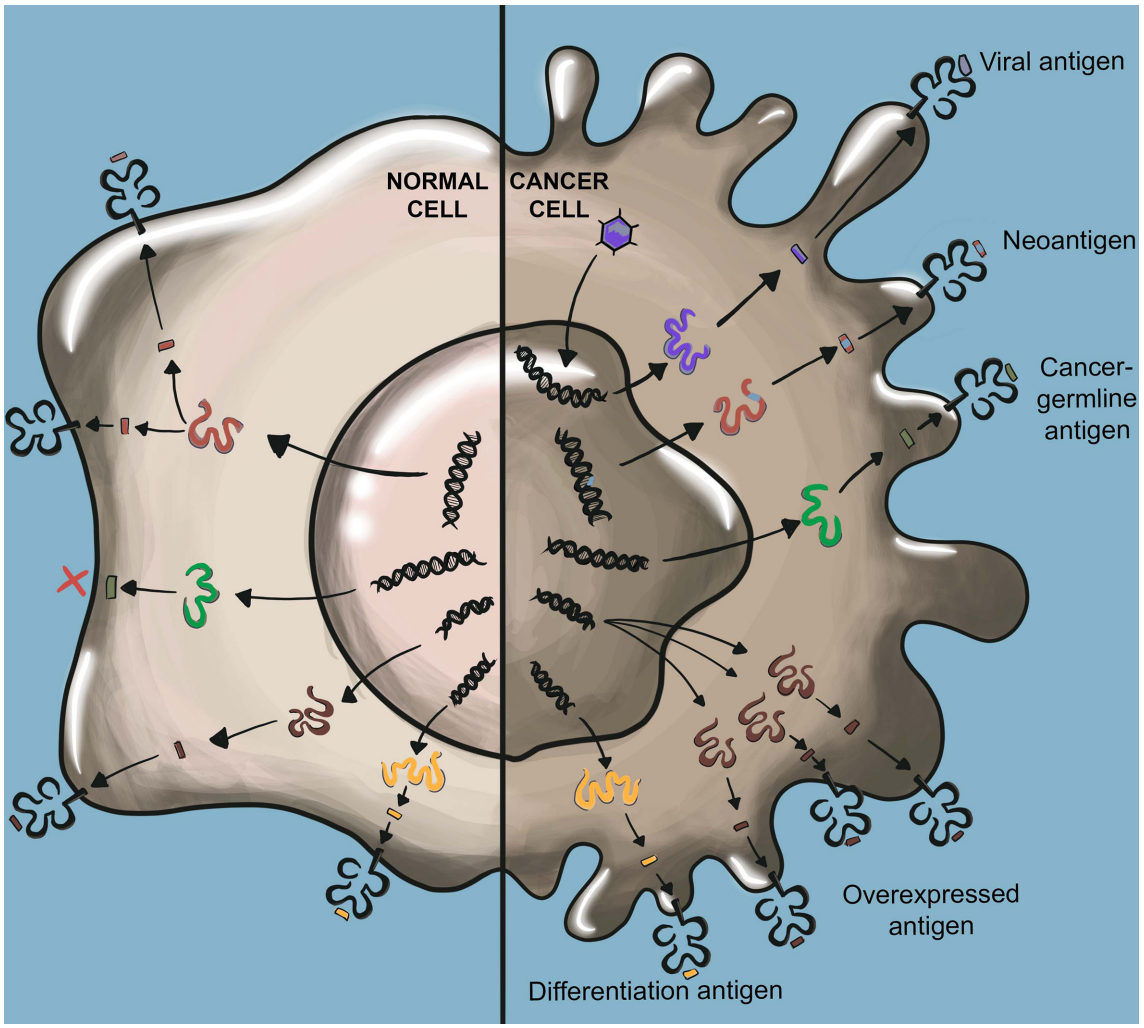


FIGURE 2 | TCR-recognized tumor antigens. Viral antigens result from viral oncogenes which are not present in normal cells. Neoantigens arise from somatic mutations not found in normal cells. Viral antigens and neoantigens are collectively referred to as tumor-specific antigens (TSAs). Cancer germline antigens (CGAs) are derived from proteins that are normally only expressed in germ cells such as testis which lack HLA class I expression. Overexpressed antigens arise from proteins highly overexpressed in cancer tissue as compared to normal tissue. Cancer differentiation antigens are expressed by cancer cells and their expression is otherwise limited to only the normal cells of the same tissue origin as the cancer. Overexpressed antigens and cancer differentiation antigens are collectively referred to as tumor-associated antigens (TAAs).

CGAs

CGAs are aberrantly expressed in cancer cells while their expression in normal tissue is restricted to germline cells, such as those of testis, which lack HLA-class I expression, thus greatly reducing the risk for on-target off-tumor toxicity. As such, CGAs are currently among the most aggressively pursued targets for TCR-based immunotherapies. Examples of CGAs with high clinical significance include NY-ESO-1 and MAGE-A4, which are detected with high levels of expression in various solid and hematological cancers (31–35). However, several studies have reported that CGAs are heterogeneously expressed within tumors, which could limit potential therapeutic efficacy when targeting a single CGA (36).

TSAs

TSAs are genetically encoded in cancer cells but are not present in the genome of any normal cells. TSAs are further classified as viral antigens or neoantigens.

Many human cancers are caused by viral infections, such as human papillomavirus (HPV) (37), hepatitis B virus (HBV) (38), and Epstein-Barr virus (EBV) (39, 40). In many cases, virus-driven cancers are mediated by the expression of viral oncogenes that drive cellular transformation and cancer progression (41–43). As viral oncogenes are often homogenously expressed in virus-driven cancers, and their expression is nearly absent in normal cells, they represent highly attractive tumor antigen targets. Specific examples of clinically relevant viral antigens

include the HPV viral oncogenes E6 and E7, which are expressed in several types of epithelial carcinoma (37, 44, 45), and the EBV viral oncogenes LMP1 and LMP2, which are expressed in several solid and hematological cancers (40, 46–49).

Genomic instability is a cardinal feature of cancer (50), which results in the accumulation of many tumor-specific mutations. Some of these mutations will give rise to new proteins, or neoantigens. As neoantigens are expressed exclusively by cancer cells, these serve as attractive targets for ACT that would pose essentially no risk for on-target off-tumor toxicity. However, a challenge is that the vast majority of cancer mutations are so-called bystander mutations, which do not enhance the fitness of the cancer cell. Such random, non-selected mutations are typically heterogeneously expressed and are unlikely to be shared across patients, rendering them ineffective antigen targets. Conversely, a small fraction of cancer mutations improve cellular fitness and directly promote cancer progression, which are known as driver mutations. These mutations may be expressed homogenously by cancer cells and shared among patients within particular cancer types (51–54). If immunogenic and restricted to a common HLA, such driver mutations give rise to so called 'public neoantigens' (55–57). While few public neoantigens have been discovered so far, these highly selective targets are of significant clinical interest. Examples of currently described public neoantigens include KRAS G12D/G12V, collectively found in 60-70% of pancreatic adenocarcinomas and 20-30% of colorectal cancers (58), and PIK3CA H1047L, detected in about 5% of metastatic breast cancers (59, 60).

STRATEGIES FOR THE ISOLATION AND TRANSGENIC EXPRESSION OF ANTIGEN-SPECIFIC TCRs

Enrichment of Antigen-Specific T Cells

V(D)J recombination of TCRs during thymic development results in a tremendous diversity of TCR sequences within the human T cell repertoire. It is estimated that in an average adult human, there are approximately 4×10^{11} total circulating T cells and an estimated 10^{10} unique T cell clonotypes (61). Thus, for the vast majority of T cell clones with specificity towards non-viral antigens, the clonal frequency in peripheral blood is far below what is needed to perform the various manipulations required to isolate antigen-specific TCRs given current technologies. Therefore, TCR isolation efforts generally begin with a method that allows for enrichment of T cells with the desired antigen specificity. The following section describes several common T cell enrichment methods employed for TCR discovery.

Expansion of Tumor Infiltrating Lymphocytes

In certain types of solid cancers there is often a large presence of tumor infiltrating lymphocytes (TILs) (62). Compared to peripheral blood T cells, T cells within the tumor tissue are often enriched in clones with tumor-antigen specificity. Several groups have used expanded TILs as sources for discovery of tumor specific TCRs (63–67). Of note, for decades *ex vivo* expanded TILs have in themselves served as an effective ATC for several types of solid cancer (66–70). The TIL therapy lifileucel from Iovance has demonstrated strong efficacy in clinical trials and the company plans to submit for FDA approval (71).

Vaccination

T cells with specificity towards antigens of interest can be selectively expanded *in vivo* through vaccination strategies. A common approach is to vaccinate human HLA transgenic mice with an antigen of interest, which can result in robust enrichment of antigen-specific T cells harvested from the lymph nodes and spleen (46, 58, 72). In select cases, the peripheral blood of patients participating in cancer vaccine trials has been used as a source of antigen-enriched T cells for TCR discovery (73, 74).

Selective *In Vitro* Expansion of Peripheral Blood T Cells

Several strategies have been developed to stimulate peripheral blood T cells *in vitro* in an antigen-specific manner, driving the selective expansion of T cells with a desired specificity. Early pioneering work in this regard performed *in vitro* stimulations of peripheral blood T cells to preferentially expand virus-specific T cells (75–78). These stimulation methods have since been used to expand T cells enriched in specificity for TAAs (79–81) and neoantigens (60, 82, 83). These approaches typically stimulate T cells *via* autologous antigen presenting cells (APCs), usually dendritic cells (DCs), pulsed with the antigens of interest in the form of exogenous peptide or through cDNA/RNA delivery (60, 80, 82–84). In the case of patient-derived peripheral blood, several studies have shown that initial selection of PD-1+ and/or antigen-experienced (CD45RO+CD62L+, CD45RO+CD62L-, or CD45RO-CD62L-) T cells can further enhance *in vitro* enrichment of tumor-specific T cells (82, 83, 85–87).

To overcome the requirements of generating autologous mature DCs for antigen stimulation, several groups have developed so called artificial antigen presenting cells (aAPCs). One common aAPC system uses the myelogenous leukemia cell line K562, which is negative for HLA-A, B, and DR. This cell line serves as a modular aAPC through the stable transduction of various HLA alleles and costimulatory molecules. Other cell-free aAPC systems have been developed that conjugate HLA and costimulatory molecules onto beads and nanoparticles (88–90).

Isolation of Antigen-Specific T Cells

After obtaining polyclonal T cell products that are enriched for T cells with specificities of interest, it is necessary to isolate the antigen-specific T cells from the bulk T cell population.

Approaches in this regard typically involve stimulating T cells with the cognate antigen of interest, and then isolating antigen-responsive T cells based on increased expression of known T cell activation-associated molecules. This includes antibody staining of transmembrane proteins that are transiently upregulated following T cell stimulation (e.g., 4-1BB and OX40 in CD8+ and CD4+ T cells, respectively), allowing for isolation of these cells by FAC sorting or magnetic bead separation (82, 83). Another approach is IFN-γ-capture, whereby antigen stimulated T cells are identified and captured based on

production of IFN-γ, which is rapidly secreted by antigen-stimulated CD8+ and Th1 CD4+ T cells. In certain cases, staining with peptide-HLA multimers followed by FAC sorting or magnetic bead separation is an efficient method to identify and isolate antigen specific T cells (63). However, this requires upfront knowledge of an antigen restricting HLA and minimal epitope. Although the repertoire of HLA multimer reagents is expanding, these reagents remain limited to relatively common HLA alleles.

TCR Sequencing

TCR α and β chains of T cells of interest are then cloned from cDNA through PCR amplification. However, a unique challenge is that their 5' regions are highly variable. To overcome this, one of two PCR variations are typically employed, 5' RACE or multiplex PCR (66, 67, 73, 79, 82, 91). The fact that TCR specificity is encoded by regions of two separate genes imposes a unique challenge for determining functional TCR sequences from a population of T cells; that is, once T cells are lysed for RNA extraction, the TCR α and β transcripts from each T cell clone intermix, making it ambiguous as to which TCR α sequence pairs with which TCR β sequence. Therefore, prior to sequencing the functional TCR α and β chain transcripts of antigen-specific T cells, it is typically required to first separate individual T cell clones. While by no means exhaustive, we outline here several classical and emerging strategies to isolate T cell clones for TCR sequencing.

Limiting Dilution

A classical method for obtaining T cell clones is the outgrowth of T cell clones in individual wells. In the limiting dilution method, T cells are diluted to obtain a cell concentration allowing for approximately one cell to be deposited into each well of a 96-well plate. An alternative method is to FACs sort the T cell population to deliver a single cell into each well. The goal is to obtain expanded clonal populations of the T cells of interest, which can then be additionally screened for antigen-specificity and sequenced *via* Sanger sequencing (27, 46, 72, 73, 84, 92–97).

Single Cell RT-PCR

Several studies have obviated the need to expand T cell clones following antigen-specific T cell separation by instead performing single cell RT-PCR to amplify TCR α and β chains. In such methods, single T cells are FAC sorted into wells containing RT-PCR reaction buffer, and from a single cell RT-PCR is performed and the TCR α and β chains are PCR amplified (66, 79, 82, 83, 91, 98). This method reduces the time and labor required for expansion of individual T cell clones; however, a downside to this approach is that confirmatory assays to assess antigen specificity cannot be performed on the T cell clones prior to sequencing.

Single-Cell RNA Sequencing

Single-cell RNA sequencing (scRNAseq) is a rapidly advancing technology that has emerged as a uniquely effective platform for TCR discovery, as it allows for single-cell assessment of cellular gene expression as well as the sequence of gene transcripts. As such, several recent studies have successfully used this platform for TCR discovery by stimulating T cells with antigens of interest and then performing scRNAseq. This allowed the researchers to identify antigen specific T cells through their increased expression of effector cytokines such as IFN-γ, TNF-α, and/or IL-2, and from this same data set the researchers then obtained the sequences of transcripts for the TCR α and β chains from the activated cells (60, 65, 99).

PRECLINICAL ASSESSMENT OF CANDIDATE TCRs

TCR discovery efforts often yield sequences of several TCRs with a desired antigen specificity. How does one select an optimal TCR from this list of candidates? And once a lead TCR is selected, what preclinical evaluations can be performed that may predict the likelihood of clinical success? The following section describes several TCR features that are commonly evaluated in preclinical TCR studies.

HLA Restriction

Often the first characteristic of an isolated TCR that must be determined is its HLA restriction **(Figure 3).** Not only is understanding the HLA restriction of a TCR necessary to identify patients that may respond to a TCR T therapy, it is also needed to perform many of the experiments for preclinical assessment. In the case of several TCR discovery approaches, knowledge of HLA restriction is already incorporated into the pipeline, such as in the case of vaccination of HLA-transgenic mice or the use of HLA tetramers to select T cells, in which case the HLA restriction of resulting TCRs will be near certain. However, in TCR discovery approaches that do not incorporate *a priori* knowledge of HLA restriction, such as through stimulating with autologous DCs, HLA restriction must be determined experimentally. A commonly used approach is to deliver individual cloned HLA alleles into the non-human primate COS-7 cell line, which possesses antigen processing and presentation capabilities but does not express potentially confounding endogenous human HLA alleles. The COS-7 cells are then induced to express one of the HLA candidates and the antigen of interest through delivery of cDNA/RNA or peptide loading, and cocultured with TCR T cells. Here, the antigen restricting HLA is evident as the HLA that elicits a TCR T cell response, observed through functional responses such as cytokine secretion and/or 4-1BB/OX40 upregulation (58, 66, 82, 83, 87, 93, 100–104).

TCR Affinity/Avidity

The successful interaction between a TCR and the appropriate pMHC complex is a critical component of effective antitumor immune responses. TCR affinity and avidity describe the binding and kinetic interactions between the TCR and the pMHC (105). Affinity plays a central role in TCR sensitivity and specificity, and refers to the physical strength of the interaction (105–107). Affinity is quantified *via* surface plasmon resonance (SPR), a 3D interaction that measures binding in terms of an association rate (K_{on}) and a dissociation rate (K_{off}) (105, 107, 108). Together, K_{off} and K_{on} make up the binding constant (K_D), where $K_D = k_{off}/$

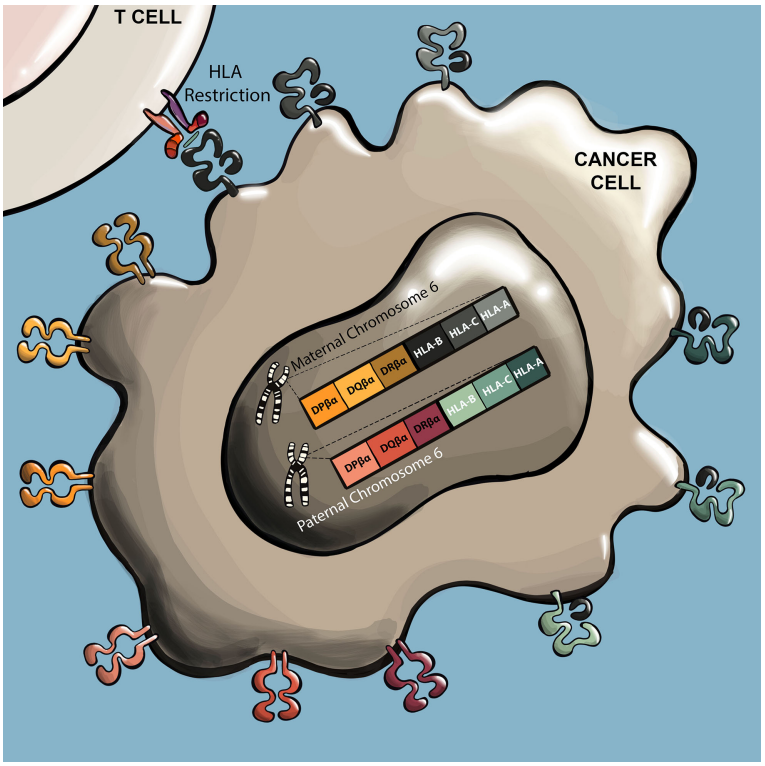


FIGURE 3 | TCRs recognize antigens presented by specific HLA alleles. TCR antigens are predominantly presented by six HLA genes. These include genes for HLA class I (A, B, and C), and class II (DR, DP, and DQ). These HLA genes are highly polymorphic, with many allele variants in the human population. Humans inherit one set of each gene from each parent, and human cells can therefore express up to twelve different HLA presenting alleles. For a given TCR, the specific HLA allele that presents the cognate peptide is referred to as the 'restricting HLA' of the TCR.

k_{on} (105, 107). High affinity TCRs recognize lower levels of antigen, do not require the CD8 coreceptor, and can enable CD4+ T cells to recognize and lyse tumor cells in an MHC class I-dependent manner (106, 109).

TCR avidity usually correlates with affinity, and refers to the combined effect of multiple TCR-pMHC interactions, coreceptors (CD8), TCR density, and T cell functional status (105, 110). Different aspects of avidity (i.e. structural or TCR avidity) can be measured *via* staining with pMHC monomers or multimers with defined valency (105). TCR affinity, avidity, and the various kinetic constants all contribute directly/indirectly to functional avidity, which describes how well T cells expressing a specific TCR respond to decreasing abundance of peptide, and is sometimes referred to as antigen sensitivity (105, 108, 111). Assessments of TCR functional avidity typically include measurement of TCR T cell cytokine secretion or cytolytic function in response to target cells that are pulsed with titering concentrations of peptide.

Antigen Processing by the Standard or Immunoproteasome

HLA class I epitopes are peptide fragments, typically 8-12 amino acids in length (112, 113), generated through processing of ubiquitinated proteins by the proteasome. The proteasome is a large protein complex responsible for the degradation of endogenous proteins that have been damaged or are not needed by the cell and have been tagged by ubiquitin conjugation. The subunits β1, β2, and β5 of the proteasome's 20S catalytic core are associated with the three major catalytic activities of the proteasome. While proteasomes that incorporate subunits β1, β2, and β5 are referred to as the 'standard proteasome', hematopoietic cells and cells stimulated with certain inflammatory cytokines (e.g., INF-γ, IFN-α, IFN-β, and TNF-α) alternatively express β1i, β2i, and β5i subunits that displace β1, β2, and β5 subunits in the proteasome, forming an isoform termed the 'immunoproteasome'. The immunoproteasome displays several biochemical differences that influence peptide cleavage activity. This results in the immunoproteasome producing peptide products with enhanced immunogenicity compared to the standard proteasome, as these immunoproteasome-generated peptides are more likely to contain C-terminal hydrophobic residues, which are associated with more efficient HLA-class I binding (24, 114). In addition, there are 'intermediate proteasomes' that contain a mixture of standard and immunoproteasome subunits, specifically substituting only β5i or β1i plus β5i and result in a peptide repertoire similar to that produced by the immunoproteasome, but includes additional unique peptide products (24, 115).

Several tumor antigens have now been characterized as being produced by the standard, intermediate, and/or immunoproteasomes (101, 115–118). Because the dendritic cells used to enrich tumor specific T cells express predominantly intermediate and immunoproteasomes (115), it is likely that most preclinical TCRs will recognize antigens produced by immunoproteasome and/or intermediate proteasome. These cognate peptides may or may not be additionally produced by the standard proteasome. The proteasomal requirements of a cognate peptide can be determined in several ways. Many cell lines predominantly express the standard proteasome, but will upregulate immunoproteasome subunits in response to IFN-γ. Therefore, the response of TCR T cells can be compared against antigen expressing cell lines with or without pretreatment with IFN-γ (73, 101, 116, 117). Peptide proteasomal requirements have also been determined with further resolution by testing T cell responses against antigen expressing 293 cells with or without overexpression of specific inducible proteasomal β subunits (101, 115–117, 119). There is a growing appreciation of the importance of proteasomal processing dynamics in immunotherapies such as immune checkpoint blockade (120) and TCR T therapy (121). In cases in which a therapeutic TCR recognizes a peptide processed exclusively by the immunoproteasome, it may be useful to select patients whose tumors have confirmed expression of immunoproteasome subunits. As downregulation of immunoproteasome subunits has now been observed in some cancer types (122, 123), it is likely ideal for a therapeutic TCR to recognize an antigen that is generated by both the standard and immunoproteasome.

Safety Assessment of Lead TCRs

Of major importance is the identification of potential safety concerns of lead TCRs, as previous clinical trials have observed severe cases of both on-target off-tumor toxicities (124–126) and off-target toxicities (127, 128). This section describes state-of-the-art techniques to assess the safety of preclinical TCRs and their cognate-antigen targets.

In cases in which a candidate TCR targets a novel or putative TAA or CGA epitope, it is imperative to preclinically assess its pattern of expression and HLA-presentation in healthy tissue. The importance of such validation was made clear by the fatal neurotoxicity that occurred in two patients following administration of T cells expressing an affinity enhanced TCR recognizing an epitope shared by MAGE-A3 and MAGE-A12. Autopsy performed on these patients revealed infiltration of CD3+CD8+ T cells in the brain. Further investigation identified unexpected expression of MAGE-A12 in a subset of neurons in the human brain (129). Several strategies are now available for preclinical assessment of expression profiles of putative CGA or TAA targets that should be employed. Kunert et al. provide suggested strategies based on their experience of assessing the expression profile of a MAGE-C2 derived epitope that is now being targeted clinically (NCT04729543). As an early step in the assessment of putative TAAs or CGAs, the authors suggest consulting online databases such as The Human Protein Atlas (proteinatlas.org) and the CTdatabase (cta.lncc.br) (130), which compile extensive data throughout literature concerning RNA and protein expression of many genes in both healthy and cancerous tissue (131). An additional tool that has recently emerged for the validation and/or discovery of CGAs and TAAs is the HLA Ligand Atlas (hla-ligand-atlas.org), an open-source, community resource of comprehensive human HLA ligandome data collected originally from 29 distinct non-malignant tissues derived from 21 individuals (113). To experimentally and independently evaluate antigen expression in healthy tissue, Kunert et al. suggest performing qPCR on commercially available cDNA libraries derived from a wide array of healthy tissues types, and if possible, further evaluating protein expression by performing IHC on a panel of healthy tissue types (131).

In addition to establishing the safety of the cognate-antigen target, it is of critical importance to investigate potential off-target reactivities of candidate TCRs. The clinical importance of such investigation was highlighted by the deaths of two patients resulting from off-target reactivity of an affinity-enhanced MAGE-A3-specific TCR towards a Titin-derived epitope expressed in cardiomyocytes (127, 128). Several preclinical strategies are now commonly employed by investigators to identify possible off-target reactivities of candidate TCRs. Several groups have identified specific amino acids within the cognate peptide that are necessary for TCR recognition. This is accomplished by mutating each residue within the cognate peptide and identifying the mutant versions unable to elicit a T cell response. The investigators then searched for all other human peptides containing an identical or similar amino acid motif through the use of webtools such as BLAST and ScanProsite, and then assessed whether these structurally similar peptides elicited a response by the candidate TCR (60, 63, 131–134). In cases in which one or several off-target peptides were identified, the researchers further investigated the immunogenicity of these peptides by determining TCR T cell response at titering concentrations (63, 131, 134) or determining if the off-target peptide is actually capable of being naturally processed (132, 133). While this approach is highly valuable for identifying cross-reactive peptides that are structurally similar to the cognate peptide, it would not identify structurally dissimilar peptides that mediate cross-reactivity (135).

Several groups have also assessed potential alloreactivity of therapeutic TCRs by performing functional assays in which TCR T cells are cultured with many different lymphoblastoid cell lines (LCLs) expressing various HLA alleles (131–133). The utility of this approach is highlighted by Sanderson et al., who identified that a lead HLA-A*02:01 restricted MAGE-A4 specific TCR mediated an alloresponse to HLA-A*02:05, indicating that patients that express HLA-A*02:05 should be excluded from treatments using this TCR (133).

ENGINEERING STRATEGIES TO IMPROVE TCR SAFETY AND EFFICACY

Promoting Proper Pairing of TCR α/β Chains

TCR α/β chains form heterodimers largely through interactions within TCR constant regions. A challenge facing

TCR T applications is that the endogenous TCR α/β chains expressed by conventional T cells can pair with introduced TCR α/β chains. Several consequences arise from such TCR mispairing. Firstly, TCR mispairing reduces the surface expression of introduced TCRs, as a significant fraction of the introduced TCR α/β chains will participate in non-productive mispairings with endogenous TCR α/β chains. Furthermore, mispaired TCRs compete with the engineered TCR heterodimer for association with limiting CD3 components (136). A second consequence of TCR mispairing is the production of brand new TCRs that have not undergone thymic selection, and which may have unexpected specificity for autoantigens. Indeed, TCR mispairing was shown to cause lethal graft-versus-host-disease (GVHD) in mice (137), and led to the formation of alloreactive and autoreactive human T cells *in vitro* (138). However, incidence of GVHD has not been observed in human TCR T clinical trials to date, including early TCR T trials utilizing unmodified human TCRs (139). To avoid issues associated with TCR mispairing, the vast majority of TCR T applications now use at least one strategy to reduce TCR mispairing (**Figure 4**).

Murinization

Extending on the serendipitous observation that human T cells exhibited greater biological activity when engineered with a murine-derived TCR as compared to human-derived TCRs, Cohen et al. demonstrated that murine TCR α/β chains preferentially dimerize with each other in the presence of endogenous human TCR α/β chains. The investigators further demonstrated that preferential pairing of introduced TCRs is also achieved when the constant regions of human TCRs are replaced with murine constant regions (140). A concern of using engineered TCRs with murine constant regions is that the foreign murine sequences may elicit an immune response in patients, as has been observed in other cell therapy trials utilizing foreign proteins such as green fluorescent protein (141, 142) and the HyTK suicide gene (143). One study identified anti-murine TCR antibodies in the post-treatment sera of 6/26 patients treated with TCR T cells expressing fully murine TCRs. However, epitope mapping revealed that the antibodies were specific for the variable regions of the TCRs, not the constant regions (144). In a separate TCR T trail utilizing a human TCR with murinized constant regions, anti-TCR serum antibodies were not detected in any of the 11 patients screened (44). Together, these clinical findings suggest that murine TCR constant regions have low or negligible immunogenicity. Nonetheless, strategies have also been developed to partially murinize TCRs by substituting specific murine amino acid sequences (145, 146).

Additional Disulfide Bond

Endogenous TCR α/β chains form a disulfide bond between TCR α constant region (Cα) residue 94 and TCR β constant region (Cβ) residue 130 (147). The proper pairing of introduced TCRs can be improved by introducing a second stabilizing disulfide bond through cysteine substitutions at Cα residue 48 and Cβ residue 57, which increases interchain binding affinity of introduced TCR α/β chains while decreasing binding affinity with endogenous TCR α/β chains (148, 149).

Transmembrane Hydrophobic Substitutions

The endogenous TCR α chain has a relatively low stability, which can be increased by substituting leucine and valine residues within the Cα transmembrane region. TCR α chains containing these stabilizing mutations, termed α-LVL, demonstrate increased TCR surface expression and biological activity. While this strategy promotes pairing of an introduced TCR by stabilizing the TCR α chain, the TCR β chain remains unmodified and thus susceptible to mispairing. However, this can be addressed by incorporating the α-LVL substitutions into murinized TCRs, the combination of which can synergistically enhance TCR expression and biological activity (150).

Domain Swapping/Conjugation

Through TCR crystal structure analysis, Voss et al. identified several amino acids mediating TCR α/β dimerization. By swapping two such interacting residues, a Cα glycine and Cβ arginine, the authors generated mutant TCR α/β chains with a similar propensity for dimerizing with each other, but with a significantly reduced propensity to bind with unmodified endogenous TCR α/β chains (151). Bethune et al. employed a similar strategy when they designed TCRs with large regions of Cα and Cβ segments exchanged, referred to as $dsTCR_c$. Interestingly, mispairing of introduced $dsTCR_c$ α/β chains with endogenous α/β chains was completely undetectable (152). Other examples of TCR domain swapping/conjugation strategies include swapping with γδ TCR constant regions (152), replacing regions with CD3ζ (153, 154) or CD28/CD3ϵ (155), or conjugation to leucine zipper dimerization motifs (156, 157).

Single-Chain TCRs

To combine the antigen recognition properties of a TCR α/β heterodimer into a single chain, several groups have developed so-called three-domain single-chain TCRs (scTCR), which are composed of Vα/Vβ regions fused by a short peptide linker and conjugated to a Cβ domain (153, 158, 159). To mediate signal transduction, three-domain scTCRs are typically further conjugated to CD3ζ (153, 158, 160). Zhang et al. compared the function of nine three-domain scTCR constructs conjugated to CD3ζ with or without conjugation to the additional stimulatory domains CD28 and Lck. Although the addition of both CD28 and Lck improved scTCR function, none of the scTCR constructs performed as well as native TCRs in terms of functional avidity (160). scTCR constructs utilizing CD3ζ transmembrane and signaling domains function independently of the CD3 complex (160), which theoretically allows for higher surface expression to be achieved with scTCRs than with native TCRs, as scTCRs are not limited by the abundance of CD3 components. The CD3-independence of scTCRs may also be beneficial in applications where it is desirable to maintain levels of endogenous TCR expression. However, scTCRs utilize signaling mechanisms distinct from those of CD3-dependent native TCRs, which may partially explain the reduced functional avidity of scTCRs (160). To generate scTCRs that preserve CD3-dependence, Voss

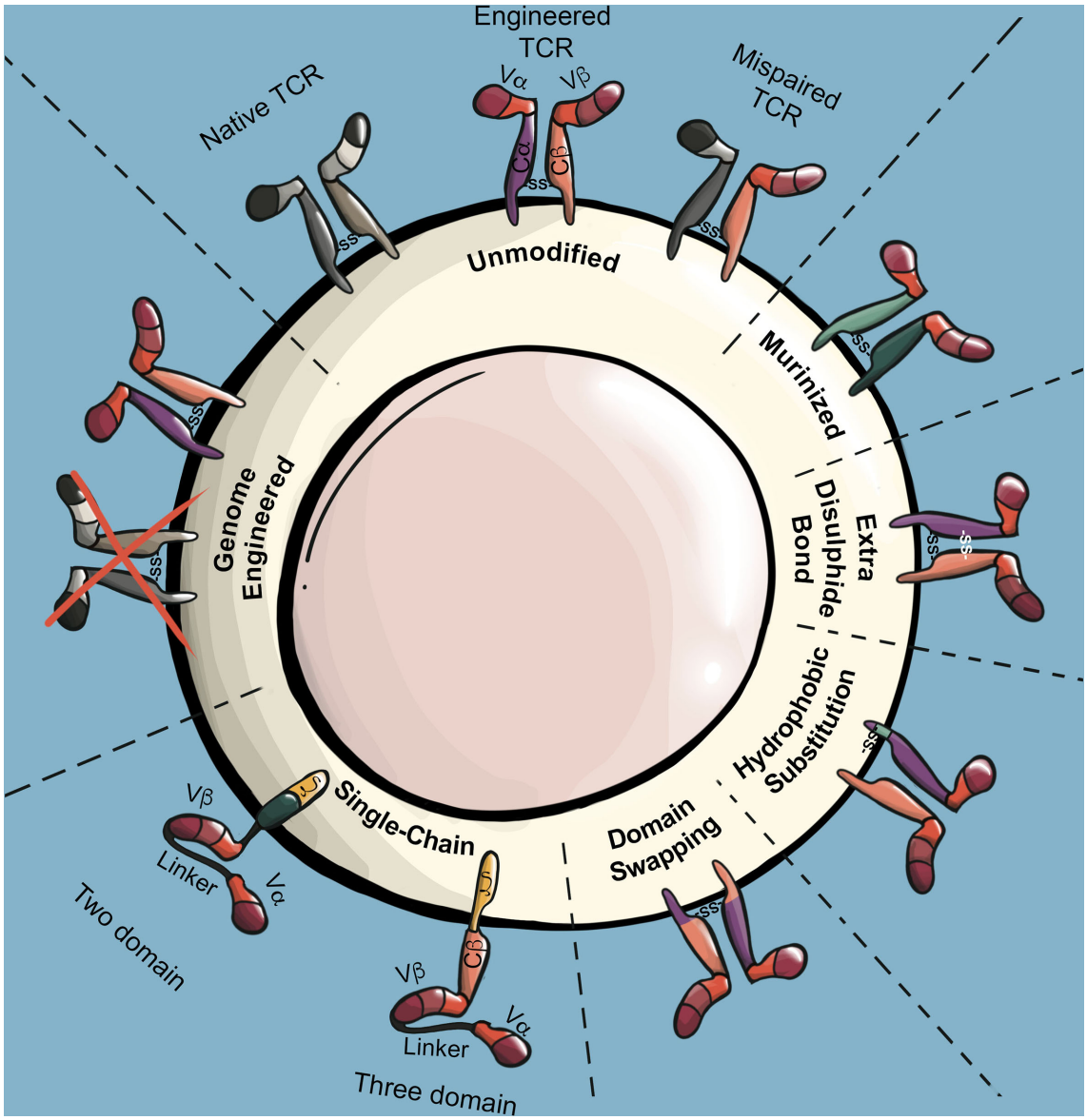


FIGURE 4 | TCR modifications to prevent mispairing and maximize surface expression. Illustration of mispairing between endogenous TCR and engineered TCR. Murinized TCRs replace the human TCR constant regions with those of a mouse TCR constant region. The addition of an extra disulfide bond in the TCR constant region through cysteine substitutions stabilizes interchain binding affinity of engineered TCR α/β chains while reducing their binding affinity with endogenous TCR α/β chains. Stability of the engineered TCR α chain can be increased through select hydrophobic substitutions in its transmembrane region. Domain swapped TCRs invert large or specific segments of the engineered TCR α/β constant regions, which reduces propensity of engineered TCRs to mispair. Single-chain TCRs (scTCR) encode TCR antigen recognition and signaling domains into a single chain. Three-domain and two-domain scTCRs differ by the inclusion or absence of the TCR β constant region, respectively. Genome engineering strategies utilize RNA interference or endonuclease technologies to reduce or ablate endogenous TCR expression.

et al. designed a system whereby three-domain TCRs without CD3ζ conjugation were coexpressed with a Cα domain. In this manner, the three domain scTCR dimerizes with the coexpressed Cα domain, presenting at the cell surface in a four-domain structure similar to that of a native TCR heterodimer. Intriguingly, these scTCR/Cα constructs have similar functional avidities as native TCRs (161). However, Aggen et al. demonstrated that three-domain scTCRs continue to mispair to some extent with endogenous TCR α chains due to the presence of the Cβ domain. To generate a scTCR system that completely eliminates mispairing, the group generated two-domain scTCRs, which utilize stabilizing Vα/Vβ mutations to obviate the need for a Cβ domain (162). To mediate signaling, two-domain scTCRs are conjugated to intracellular signaling domains such as CD3ζ and CD28. Intriguingly, CD3ζ/CD28 containing two-domain scTCRs are essentially CARs that utilize

a Vα/Vβ antigen recognition domain. As such, two-domain scTCRs display features typical of CARs including CD3-independent signaling and decreased sensitivity to low antigen density. However, unlike typical CARs, two-domain scTCRs are still dependent on HLA presentation (14).

Genome Engineering Strategies

Rather than modifying the introduced TCR, other strategies address mispairing through knock-down or knock-out of the endogenous TCR. Provasi et al. combined the use of zinc-finger nucleases (ZFN) to knock-out endogenous *TRAC* and *TRBC* genes with lenti-viral delivery of a WT-1 specific TCR. Here, the authors described an elegant, although relatively extensive manufacture system utilizing sequential rounds of *TRAC/TRBC* disruption, magnetic bead separation, and TCR α/β chain delivery. This resulted in a TCR T product with enhanced expression of the introduced TCR and a complete absence of endogenous TCR α/β chains (163). In a recent first-in-human trial, Stadtmauer et al. employed multiplexed CRISPR/Cas9 editing to disrupt T cell *TRAC*, *TRBC*, and *PDCD1* genes in combination with lenti-viral delivery of a NY-ESO-1 specific TCR. In the four patient-derived products described in this report, disruption of *TRAC* and *TRBC* was achieved in an average of 45% and 15% of cells, respectively. However, as *TRAC*/*TRBC* edited T cells were not selected prior to lenti-viral transduction with the NY-ESO-1 TCR, a significant fraction of the TCR-engineered T cells likely continued to express endogenous TCR α/β chains (164). Several groups have also developed virus-free systems to deliver TCRs and/or disrupt endogenous *TRAC*/*TRBC* genes, which may also aid in improving TCR T clinical cost and feasibility. Davo et al. electroporated T cells with Dicer-substrate small interfering RNAs (DsiRNA) targeting the endogenous *TRAC* and *TRBC* loci, achieving an approximately 6-fold and 3-fold reduction in expression of *TRAC* and *TRBC*, respectively. The authors then electroporated the T cells with a codon optimized WT1-specific TCR that isn't recognized by the DsiRNA. This resulted in T cell products with relatively high engineered TCR expression (60.2% tetramer+) with no observable TCR mispairing. However, as transgene expression in this system is transient, this would likely necessitate multiple infusions in a clinical setting (165). Roth et al. used CRISPR/Cas9 editing to mediate targeted insertion of TCR α and β variable regions into the first exon of the *TRAC* and *TRBC* loci, respectively. This mediated the combined effect of disrupting the endogenous TCR α/β chains, while placing expression of the introduced TCR under physiologic control. A potential challenge of this approach is the relatively low editing efficiency, with about 3% of cells expressing the introduced TCR, which could therefore necessitate sorting and/or extended selective expansion (166).

Affinity/Avidity Enhancement

Given that affinity plays a central role in TCR function, the manufacturing of high-affinity TCRs is an attractive method to improve the efficacy of TCR T therapies. Naturally occurring TCRs, including those that recognize self/tumor antigens, have relatively low affinities as a result of negative selection (105, 167). Methods to improve TCR affinity focus on the introduction of amino acid sequence (AAS) variations into the TCR complementarity-determining regions (CDRs). For example, single and dual AAS substitutions that enhanced the functions of TCRs specific for NY-ESO-1 (1G4) and MART-1 (DMF4, DMF5) were generated through overlapping PCR (168). These TCRs had affinities in the low μM and even nM range, which surpass the affinity of most naturally occurring TCRs, which range from 1-100 μM (106, 110). Yeast and bacteriophage display are additional powerful, high-throughput tools that can generate TCRs with affinities in the pM range (109, 167, 169). While these techniques are effective at identifying high affinity TCR variants, higher affinity has been associated with increased cross-reactivity (106, 109, 168). TCRs with affinities greater than the normal range (1-100 μM) are more likely to demonstrate cross-reactivity to similar or completely different peptides (109, 135, 170). In various studies of high affinity TCRs, increasing the affinity within the nM and pM range resulted in recognition of control antigen and antigen negative target cells (109, 168). Efforts to improve the affinity of TCRs should thus proceed with caution and thorough evaluation as these high affinity TCRs could have detrimental effects when used as patient therapies.

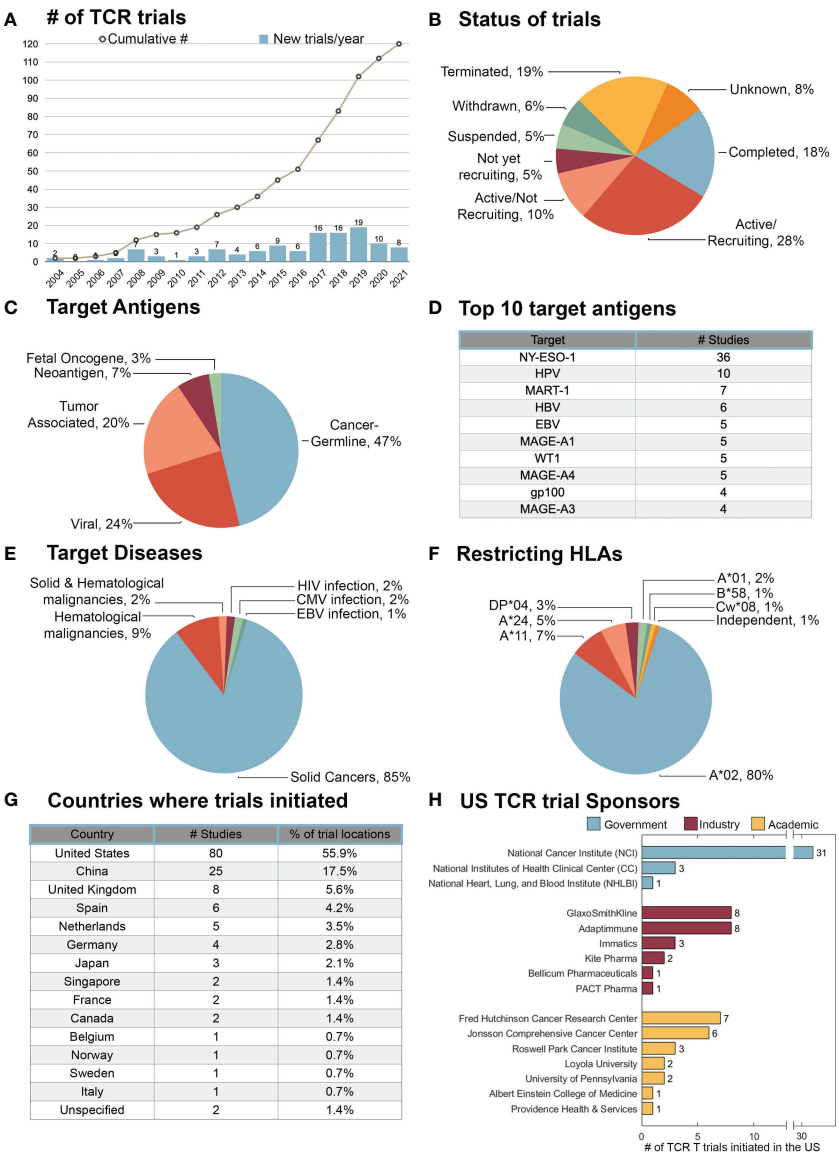
Recent work in TCR affinity maturation has focused on incorporating a more thorough assessment of the structure of the TCR and how it interacts with the target pMHC. Hellman et al. utilized a structure-guided design that incorporated both positive and negative designs (106, 170). In other words, they utilized mutations that either enhanced or weakened the interaction of the TCR with the MHC protein. These mutations redistributed the binding free energy in a way that forces the TCR-pMHC interaction to rely more on the presence of the correct target peptide, leaving less flexibility for off-target peptides. In the MART-1 specific DMF5 TCR, these structure-guided modifications decreased cross-reactivity to MART-1 homologs and eliminated cross-recognition of a selection of divergent peptides. Structure-guided approaches, therefore, have the potential to improve ACT while minimizing the risk of off-target toxicities.

CLINICAL LANDSCAPE OF TCR T THERAPIES

Trends in TCR T Trials

As of October 3rd, 2021, the search term "TCR" (and synonyms "T Cell Receptor" and "T Cell Antigen Receptor") in clinicaltrials.gov yielded 538 interventional trials. Through manual inspection of these trials, 119 were identified to include the adoptive transfer of TCR T cells. One TCR T trial that did not include these search terms (NCT04044768) was also identified and included in this analysis (**Supplementary Table 1**). The first TCR T trial was initiated in 2004 by Steven Rosenberg at the National Institutes of Health (NIH) targeting the melanoma differentiation antigen gp100 (NCT00085462). Since then, the number of new TCR T trials initiated has steadily increased, with a particular acceleration between the years of 2017 – 2019, in

which 51 new TCR T trials were initiated (**Figure 5A**). Of the TCR T trials to-date, 53% have been in phase I, 24% in phase I/II, and 22% in phase II. To date, no phase III TCR T trials have been initiated (**Supplementary Table 1**). The status of these TCR T trials was assessed as of November 3rd, 2021 (**Figure 5B**). There were 118 antigens targeted in the 116 TCR T trials with specified targets. Of the targeted antigens, the majority are CGAs (47%), followed by viral antigens (24%), tumor-associated antigens (21%), neoantigens (7%), and fetal oncogenes (3%) (**Figure 5C**). The CGA NY-ESO-1 is by far the most targeted antigen in TCR T trials to-date (36 trials). Although not first targeted until 2014, HPV now constitutes the second most common TCR T target (10 trials). While the melanoma differentiation antigen MART-1 is the third most targeted antigen in TCR T trials (7 trials), these largely constitute early TCR T trials, as MART-1 targeted-trials have not been initiated since 2012 (**Figure 5D**). The majority of the TCR T trials to date have been for the treatment of solid cancers (85%), followed by hematological malignancies (9%), and trials targeting both solid and hematological cancers (2%). A small subset of TCR T trials



Target	# Studies
NY-ESO-1	36
HPV	10
MART-1	7
HBV	6
EBV	5
MAGE-A1	5
WT1	5
MAGE-A4	5
gp100	4
MAGE-A3	4

Country	# Studies	% of trial locations
United States	80	55.9%
China	25	17.5%
United Kingdom	8	5.6%
Spain	6	4.2%
Netherlands	5	3.5%
Germany	4	2.8%
Japan	3	2.1%
Singapore	2	1.4%
France	2	1.4%
Canada	2	1.4%
Belgium	1	0.7%
Norway	1	0.7%
Sweden	1	0.7%
Italy	1	0.7%
Unspecified	2	1.4%

FIGURE 5 | Trends in TCR T trials initiated thus far. TCR T trials registered in clicaltrials.gov were assessed as of October 3rd 2021. **(A)** The number of new TCR T trials initiated each year and the cumulative number of registered TCR T trials by year. **(B)** Clinical status of the 120 TCR T trials. **(C)** Classifications of 118 tumor antigen targets in 116 TCR T trials with specified target antigens. **(D)** Ten most common targets in TCR T trials. **(E)** Diseases targeted in TCR T trials. **(F)** Frequency of 111 target antigen-restricting HLAs in 100 TCR T trials that specified HLA restriction. **(G)** Locations where TCR T trials have been conducted by country. **(H)** Primary sponsors of the 80 TCR T trials conducted in the United States.

have been for the treatment of HIV, CMV, or EBV infections (4%) (**Figure 5E**). More information about the precise disease targets of these TCR T trials can be found in **Supplementary Table 1**. Of the 100 trials with specified HLA-restrictions, 111 restricting-HLA alleles were listed, as some trials included multiple antigen targets and HLA restrictions. HLA-A*02 was by far the most common restricting HLA allele (80%), followed by HLA-A*11 (7%) and HLA-A*24 (5%). HLA class II restricted antigens were targeted in 3 trials, all of which were restricted by HLA-DP*04 (3%) (**Figure 5F**). The majority of TCR T trial locations were in the United States (56%), followed by China (18%), and the United Kingdom (6%) (**Figure 5G**). Among the 80 TCR T trials occurring in the United States, 44% were sponsored by the NIH, 28% by academic institutions, and 29% by industry. The support of industry in TCR T, which accelerated around 2017 (**Supplementary Table 1**), will likely aid in the development of later phase TCR T trials. The NCI has been by far the most active individual institution in United States TCR T trials, sponsoring 31 trials to date. Among academic institutions, the Fred Hutchingson Cancer Research Center has sponsored the most trials (7 trials), followed by the Johnson Comprehensive Cancer Center (6 trials). Among TCR T trials sponsored by pharmaceutical companies, Adaptimmune and GlaxoSmithKline have sponsored the most trials (8 trials each) (**Figure 5H**).

Assessment of Safety and Efficacy of TCR T Trials to Date

There is a quickly expanding body of literature detailing the results of TCR T trials. Clinical results encompassing over twenty-five TCR T trials are detailed in **Table 1**. This section discusses broad findings that have emerged from these early phase trials, particularly relating to TCR T safety and efficacy. Of note, many TCR T clinical protocols include lymphodepleting regimens prior to TCR T infusion, which has been demonstrated in early ACT trials to improve T cell engraftment and persistence. Many studies also include systemic administration of IL-2 following TCR T infusion to support T cell activity and persistence. Both interventions consistently induce various toxicities that, while undesirable, are generally clinically manageable. Detailed description of the impact of these interventions is beyond the scope of this review, but is extensively reviewed elsewhere (189–191). As such, discussion of toxicities observed in TCR T trials will focus on those mediated directly by the infused T cells. Finally, it is worth noting that to date all TCR T trials have been early phase and almost exclusively treating patients with highly advanced, treatment refractory disease.

Trials Targeting Cancer Differentiation Antigens: Evidence of Efficacy and On-Target Off-Tumor Toxicity

In one of the earliest TCR T trials, patients with metastatic melanoma were treated with autologous T cells transduced with a TCR (DMF4) recognizing the melanoma differentiation antigen MART-1. The objective response rate in these patients was relatively modest, with 2/17 (12%) patients achieving durable partial responses. No TCR T induced toxicity was observed (171). In a later related study, patients were treated with TCR T cells that expressed a higher affinity MART-1 specific TCR (DMF5). Compared to the prior trial using the lower affinity DMF4 receptor, this trial observed enhanced efficacy, with objective responses observed in 6/20 (30%) patients. However, the increased biological activity mediated by the DMF5 TCR was also associated with the emergence of on-target off-tumor destruction of melanocytes, leading to widespread erythematous skin rash (14 pts), uveitis (12 pts), and hearing loss (10 pts). Similar results were observed in patients treated with T cells expressing a high affinity mouse-derived TCR targeting the melanoma differentiation antigen gp100 (126). In a later study, three patients with metastatic colorectal cancer were treated with an affinity-enhanced TCR recognizing the cancer differentiation antigen carcinoembryonic antigen (CEA). While a partial response was observed in 1/3 (33%) patients, all three patients presented with severe transient colitis as a result of on-target off-tumor destruction of CEA-expressing colonic mucosa (124). Ultimately, these studies demonstrated that while TCR T therapies targeting cancer differentiation antigens can mediate objective clinical responses, they are often associated with potentially dangerous on-target off-tumor toxicities. Likely owing to this, few TCR T trials targeting cancer differentiation antigens have been initiated in the past decade (**Supplementary Table 1**).

Trials Targeting NY-ESO-1: A Safe and Effective Target

The CGA NY-ESO-1 has been the most widely targeted antigen in TCR T trials, and several groups have now published promising results from trials targeting this antigen. An early landmark trial treating patients with refractory melanoma or synovial sarcoma with TCR T cells expressing an affinity-enhanced NY-ESO-1 specific TCR reported objective responses in 11/20 (55%) melanoma patients, including four durable complete responses, and 11/18 (61%) synovial sarcoma patients, including one durable complete response. No TCR T associated toxicities were observed (174). Later, a large study of synovial sarcoma patients treated with TCR T cells expressing an affinity enhanced NY-ESO-1 specific TCR (SPEAR T cells) observed clinical responses in 15/42 patients (36%), including one complete response, and 24/42 patients presenting with stable disease. These patients were also divided into treatment cohorts based on magnitude of NY-ESO-1 tumor expression and the lymphodepleting regimen they received. The greatest clinical responses were observed in a cohort of twelve patients (cohort 1) whose tumor expressed +2 or +3 NY-ESO-1 staining by immunohistochemistry in ≥50% of cells and who received a relatively intensive lymphodepleting regimen. Here, clinical responses were observed in 6/12 (50%) patients, including one complete response, and a median duration of response of 30.9 weeks (179). Of these twelve patients, five experienced cytokine release syndrome of grades 1 (2 pts), 2 (1 pt), or 3 (2 pts) (192). Several other studies reporting TCR T trials targeting NY-ESO-1 to treat various cancer types have also observed clinical responses

TABLE 1 | Available TCR T trial results.

Ref	Year	Trial	Phase	Sponsor	Target	HLA	Construct Details	Disease	# Pts	Pre. Cond.	Combination	Responses	TCR T Induced Toxicity
(124)	2011	NCT00923806	I/II	NCI	CEA	A*02:01	affinity enhanced TCR	colorectal cancer	3 pts	Cy + Flu	IL-2	**1/3, 33.3% (ORR/PR)**	Severe colitis (3 pts)
(126)	2009	NCT00509496	II	NCI	gp100	A*02:01		melanoma	16 pts	Cy + Flu	IL-2	**3/16, 18.8% (ORR)** 1/16 (CR), 2/16 (PR)	Skin toxicity (15 pts), Uveitis (4 pts), Hearing loss (5 pts)
(171)	2006	Not Specified (NS)	NS	NS	MART-1	A*02:01	DMF4 TCR	melanoma	17 pts	Cy + Flu	IL-2 Peptide vaccine	**2/17, 12% (ORR/PR)** 1/17 (MR)	None
(126)	2009	NCT00509288	II	NCI	MART-1	A*02:01	DMF5 TCR	melanoma	20 pts	Cy + Flu	IL-2	**6/20, 30% (ORR/PR)**	Skin toxicity (14 pts), Uveitis (12 pts), Hearing loss (10 pts)
(172)	2014	NCT00910650	II	JCCC	MART-1	A*02:01	DMF5 TCR	melanoma	10 pts	Cy + Flu	IL-2 Peptide-pulsed DC vaccine	**0/14, 0% (ORR)** 7/14 (SD) *ORR assessed at Day 90*	Erythematous skin rash (3 pts), Acute respiratory distress (2 pts)
(173)	2018	NCT01586403	I	Loyola University	tyrosinase	A*02	TIL1383I TCR	melanoma	3 pts	Cy + Flu	IL-2	**1/3, 33% (ORR/PR)**	Vitiligo (2 pts)
(174)	2015	NCT00670748	II	NCI	NY-ESO-1	A*02:01	1G4-a95:LY TCR (affinity enhanced)	melanoma synovial sarcoma	38 pts	Cy + Flu	IL-2 ± AVIPOX- ESO vaccine	**22/38, 58% (ORR)** 5/38 CR, 17/38 PR	None
(175)	2019	NCT02366546	I	Mie University	NY-ESO-1	A*02:01 A*02:06	affinity enhanced TCR siRNA *TRAC/TRBC*	various solid tumors	9 pts	Cy	None	**3/9, 33.3% (ORR/PR)**	CRS (3 pts)
(176)	2019	NCT02070406 NCT01697527	I	JCCC	NY-ESO-1	A*02:01	1G4-a95:LY TCR (affinity enhanced)	various solid tumors	10 pts	Cy + Flu	IL-2, DC-peptide vaccine, ± ipilimumab	**2/10, 20% (ORR)** 1/10 (CR), 1/10 (PR), *4/10 (tR)*	None
(177)	2019	NCT02869217	Ib	University Health Network	NY-ESO-1	A*02:01 A*02:06	TBII-1301 (MS3II-NY-ESO1-SiTCR)	various solid tumors	9 pts	Cy	None	**2/9, 22.2% (ORR/PR)** 5/9 (SD), *1 pending*	CRS grade 1-2 (5 pts)
(178)	2019	NCT01352286	I/IIa	GlaxoSmithKline	NY-ESO-1/LAGE-1	A*02:01	NY-ESO-1 SPEAR T cells (NY-ESOc259 TCR)	multiple myeloma (Post-HSCT)	25 pts	melphalan	lenalidomide	**21/25, 84% (ORR)** 2/25 (sCR), 1/25 (CR), 13/25 (VGPR), 5/25 (PR), 4/25 (SD)	GVHD (6 pts). *Likely not related to the engineered TCR.*
(179)	2019	NCT01343043	I/II	GlaxoSmithKline	NY-ESO-1	A*02	NY-ESO-1 SPEAR T cells	synovial sarcoma	42 pts	Cy ± Flu as per cohort	None	**15/42, 35.7% (ORR)** 1/42 (CR), 14/42 (PR), 24/42 (SD)	CRS grades 1 (2 pts), 2 (1 pts), and 3 (2 pts)
(164)	2020	NCT03399448	I	University of Pennsylvania	NY-ESO-1	A*02:01	8F TCR, CRISPR KO *TRAC/TRBC/PDCD1*	multiple myeloma, MRCLS	3 pts	Cy + Flu	None	**0/3, 0% (ORR)** 2/3 (SD)	None
(128)	2013	NCT01350401 NCT01352286	I/II	Adaptimmune GlaxoSmithKline	MAGE-A3	A*01	MAGE-A3a3a TCR (affinity enhanced)	melanoma, myeloma (Post-ASCT)	2 pts	Cy (melanoma pts)	None	NA	Off-target toxicity in cardiac tissue leading to 2 pt deaths
(129)	2013	NCT01273181	I/II	NCI	MAGE-A3	A*02:01	affinity enhanced TCR	various solid tumors	9 pts	Cy + Flu	IL-2	**5/9, 55.6% (ORR)** 1/9 (CR), 4/9 (PR)	Neurological toxicity (3 pts) 2 pt deaths.
(194)	2017	NCT02111850	I/II	NCI	MAGE-A3	DPB1*04:01		various solid tumors	17 pts	Cy + Flu	IL-2	**4/17, 23.5% (ORR)** 1/17 (CR), 3/17 (PR)	Fever (10 pts), Elevated ALT, AST, and creatinine (2 pts)
(181)	2015	UMIN000002395	I	Mie University	MAGE-A4	A*24:02		esophageal cancer	10 pts	None	Peptide vaccine	**0/10, 0% (ORR/PR)** 3/10 SD	None
(182)	2020	NCT03132922	I	Adaptimmune	MAGE-A4	A*02	ADP-A2M4 SPEAR T cells	various solid tumors	34 pts	Cy + Flu	None	**7/28, 25% (ORR/PR)** 11/28 (SD)	2 pt related deaths (aplastic anemia and CVA), not likely off target toxicity
(183)	2020	NCT04044859	I	Adaptimmune	MAGE-A4	A*02	ADP-A2M4CD8 SPEAR T cells	various solid tumors	5 pts	Cy + Flu	None	**2/5, 40% (ORR/PR)** 3/5 (SD)	No DLTs or SAEs
(184)	2021	NCT04044768	II	Adaptimmune	MAGE-A4	A*02	ADP-A2M4 SPEAR T cells	synovial sarcoma, MRCLS	37 pts	Cy + Flu	None	**13/33, 39% (ORR)** 11/33 (PR), 2/33 (CR), 15/33 (SD)	CRS grades 1-2 (21 pts), 3 (1 pt)
(185)	2018	NCT02989064 NCT02592577	I	Adaptimmune	MAGE-A10	A*02	MAGE-A10c796 TCR (affinity enhanced)	various solid tumors	8 pts	Cy ± Flu	None	**0/8, 0% (ORR/PR)**	CRS (1 pt), Increase in serum amylase (1 pt)
(186)	2019	NCT02280811	I/II	NCI	E6	A*02:01	E6 TCR	HPV-associated solid cancers	12 pts	Cy + Flu	IL-2	**2/12, 16.7% (ORR/PR)** 4/12 (SD)	None
(44)	2021	NCT02858310	I	NCI	E7	A*02:01	E7 TCR	HPV-associated carcinomas	12 pts	Cy + Flu	IL-2	**6/12, 50% (ORR/PR)** 4/12 (SD)	1 DLT, not likely off target toxicity
(187)	2017	UMIN000011519	I	Several sponsors	WT1	A*24:02	TAK-1 TCR, siTCR	AML, MDS	8 pts	None	Peptide vaccine	Transient decrease of blasts in BM in 2 pts. SD in 1 pt.	No adverse events greater than grade 3
(188)	2019	NCT01640301	I/II	Fred Hutchinson CRC	WT1	A*02:01	TCR_{C4}, Allo EBV-specific T cells	AML (Post-HCT)	12 pts	None	IL-2	100% RFS vs 54% in comparative group	GVHD in several patients, not likely caused by TCR T cells, but rather HCT.

NA, Not Applicable. Bolded values highlight the overall response rates of the trials.

without instances of on- or off- target toxicity attributed to TCR T cells (164, 176–178, 193). Together, these studies demonstrate that NY-ESO-1 targeting TCR T therapies are safe and capable of eliciting potent antitumor responses.

Trials Targeting MAGE-A Family Antigens: Early Toxicity and Recent Success

The MAGE-A family of proteins have also served as highly attractive CGA targets, and results from several TCR T trials targeting members of this family are now available. Unfortunately, two early reports of trials targeting MAGE-A3 described fatal TCR T mediated toxicity. In one study, two patients treated with TCR T cells expressing an affinity enhanced MAGE-A3 specific TCR died of cardiac toxicity following TCR T infusion. *Post hoc* investigation into the cause of these fatalities revealed cross-reactivity of the affinity enhanced TCR towards Titin expressed in cardiomyocytes (127, 128). This study emphasized the need for extensive preclinical investigation into potential off-target reactivities of lead TCRs. In a study published the same year, nine patients with metastatic cancer were treated with TCR T cells expressing an affinity enhanced TCR recognizing a similar MAGE-A3 and MAGE-A12 epitope. Clinical responses were achieved in 5/9 (56%) of patients, including a durable complete response in one patient. However, three patients experienced severe neurotoxicity following TCR T cell infusion, with two patients dying as a result. *Post hoc* analysis identified unexpected expression of MAGE-A12 in a subset of neurons in the brain, and the observed toxicity was thus presumed to be due to on-target off-tumor recognition of MAGE-A12 the brain (129). This study demonstrated the need for extensive preclinical characterization of cognate antigen targets in healthy tissue. A later study targeted MAGE-A3 with a high affinity HLA-DPB1*04:01 restricted TCR derived from a T regulatory cell. Objective responses were achieved in 4/17 (24%) of patients, with one patient achieving a durable complete response. Following TCR T treatment, one patient experienced grade 4 toxicities including increased ALT, AST, and creatinine, and eventually developed respiratory failure requiring hospitalization. A second patient experienced grade 3 toxicities of increased ALT, AST, and creatinine lasting two days. The cause of these toxicities was not described (194). Two recent reports of phase I trials of TCR T cells expressing an affinity-enhanced TCR specific for MAGE-A4 (ADP-A2M4 SPEAR T cells) to treat various solid cancers observed antitumor responses without evidence of serious TCR T mediated toxicity (182, 183). This year, Adaptimmune reported results of a phase II trial of ADP-A2M4 SPEAR T cells (now afamitresgene autoleucel) to treat patients with synovial sarcoma or myxoid/round cell liposarcoma (MRCLS). Here, objective responses were observed in 13/33 (39.4%) patients, including two durable complete responses, and disease control was achieved in 28/33 (84.8%) patients. Grades 1-2 CRS were observed in 21 patients and grade 3 CRS was observed in one patient. Based on this data, the company plans to file for afamitresgene autoleucel approval next year (184). In summary, results of early TCR T trials targeting MAGE-A3 demonstrated the need for more extensive preclinical testing of both on-target and off-target effects of lead TCRs and enhanced preclinical safety assessment strategies have emerged as a result of these studies. However, recent trials of Adaptimmune's MAGE-A4-targeting afamitresgene autoleucel have demonstrated strong efficacy treating solid cancers in the absence of major TCR T cell mediated toxicity and may be nearing FDA approval.

Emerging Targets

Recent results have demonstrated efficacy of TCR T cells targeting HPV antigens in patients with various HPV-associated solid cancers. Relatively modest efficacy was achieved in a study treating twelve patients with TCR T cells expressing an E6-specific TCR, with objective responses observed in 2/12 (17%) patients, with no dose-limiting toxicities (186). In a related study using a higher affinity TCR recognizing E7, objective responses were achieved in 6/12 (50%) patients, with one dose-limiting toxicity that was presumably unrelated to TCR T mediated toxicity (44). However, the duration of clinical responses observed in both trials were relatively short (2 – 9 months). Recent studies have also demonstrated safety and efficacy of TCR T trials targeting the cancer overexpression antigen WT1. In one study, twelve AML patients at high risk for relapse following hematopoietic cell transplant (HCT) were treated with allogenic EBV-specific T cells engineered to express a WT1 specific TCR. Relapse free survival was achieved in 100% of the TCR T treated patients, as compared to 54% relapse free survival post-HCT in a comparative group of eighty-eight AML patients at similar risk of relapse. Nine patients exhibited grade 1-2 GVHD following TCR T infusion, with one patient developing grade 3 acute GVHD. However, the GVDH was determined to be most likely caused by the use of allogenic T cells rather than the introduced TCR (188).

Experiences of TCR T *vs* CAR T Trials to Treat Solid Tumors to Date

Recent years have seen the emergence of clinical trials reporting on the safety and efficacy of TCR T therapeutics in the context of both hematological malignancies and solid tumors (**Table 1**). Several TCR T trials focused on the latter have achieved improved therapeutic outcomes as compared to those achieved in CAR T trials (195), and it now appears that TCR T therapeutics are closer to receiving FDA approval for the treatment of solid cancers. A possible explanation for this disparity lies in the biological differences between TCRs and CARs (**Figure 1**), including 1) the ability of TCRs to recognize HLA-presented antigens derived from any cellular compartment including high specificity antigens such as CGAs and viral antigens, 2) TCRs are considerably more sensitive to low concentrations of a target antigen as compared to CARs, particularly in the case of affinity-enhanced TCRs (12–14), and 3) unlike CAR T cells, engineered TCRs do not drive ligand-independent tonic signaling (196, 197), making them better equipped to maintain function *in vivo*. Ultimately, the aforementioned factors are speculated to play a role in the comparatively improved performance of TCR T therapeutics

targeting solid cancers, however, both fields are evolving rapidly with new antigen discoveries and further genetic engineering of the T cells, which should lead to improved efficacy in the upcoming solid tumor CAR T trials.

Remaining Challenges

Intense efforts have been made to understand and address factors that limit the efficacy and clinical applicability of T cell-based ACTs, including TCR T therapy. This section describes select challenges currently facing TCR T therapy and the strategies available to meet them.

Manufacturing Cost and Complexity

TCR T products typically require 1-3 weeks of manufacture and involve several relatively complex processing steps that must be performed in highly regulated good manufacturing process (GMP) facilities. Despite the relative complexity, current TCR T manufacturing processes generally result in high rates of successful product manufacture. However, the cost of these T cell products is high. This is demonstrated by the high costs of FDA approved CAR T therapies, which utilize similar manufacturing processes. For example, single infusions of axicabtagene ciloleucel or tisagenlecleucel cost $373,000 and $475,000, respectively (198). In response to this, a strategy under development by many groups is the use allogeneic, or off-the-shelf, TCR T and CAR T products. However, a major challenge facing this approach is the likelihood of GVHD mediated by allogeneic T cells. Strategies to mediate GVHD of allogeneic T cell products include the use of oligoclonal virus-specific T cells with tightly restricted antigen specificities (188, 199), invariant T cell subsets such as γδ T cells (200) and iNKT cells (201), or *TRAC/TRBC* disrupted T cells (202). Another strategy to reduce the manufacturing cost of both TCR T and CAR T products is the use of non-viral gene delivery methods such as RNA electroporation (203, 204), transposons (205–207), and CRISPR/Cas9 (166).

T Cell Persistence

The ability of infused T cells to persist within the patient is an important factor mediating ACT antitumor efficacy (208). As such, intense efforts have been made to develop strategies that improve T cell persistence. Perhaps the most common strategy is to administer non-myeloablative lymphodepleting chemotherapy prior to T cell infusion, which alleviates competition of infused T cells with endogenous T cells for homeostatic cytokines such as IL-7 and IL-15, among other likely mechanisms (189). Several studies in mice have demonstrated that less differentiated T cell subsets (e.g., central memory and stem cell memory), have improved *in vivo* engraftment and persistence compared to highly differentiated T cells subsets (e.g., effector memory and terminally differentiated effectors) (209–211). As such, several strategies are now commonly employed to preserve less differentiated T cell subsets during ACT manufacture, including reduced expansion times and use of less differentiation-inducing cytokines (181, 212, 213). Some TCR T trials have further extended this approach by selecting specific T cell differentiation subsets prior to infusion (NCT02062359, NCT02408016). Another factor that limits T cell persistence is the paucity of costimulatory ligands within the tumor microenvironment. Several early TCR T trials performed coadministration of vaccines in efforts to provide TCR stimulation and costimulation *in vivo*. However, several of these studies failed to observe increased TCR T efficacy mediated by combination with vaccination (174, 176, 180). Genetic strategies to improve T cell costimulation include modified TCRs incorporating costimulatory domains (155) and coexpression of costim-only CARs (214) or domain swapped inhibitory receptors (215). Finally, several genetic strategies are also being explored to maintain T cell homeostatic cytokine signaling, including auto-secreting IL-15 or IL-12 elements (216–219) and a constitutively active IL-7 receptor (220).

Immunosuppressive Tumor Microenvironment

Another obstacle faced by adoptively transferred T cells is the immunosuppressive tumor microenvironment (TME). The TME supports tumor survival by recruiting immunosuppressive cell types, including myeloid derived suppressor cells (MDSCs), regulatory T cells (Tregs), and tumor-associated macrophages (TAMs) (221). Several pre-clinical studies of ACT are working to incorporate strategies to enable T cells to function in the hostile TME, including cotreatment with immune checkpoint blockade (222, 223), and genetic incorporation of inverted cytokine receptors (224) or MDSC targeting costimulatory receptors (214). A thorough understanding of the TME and its effect on T cells is necessary for the future success of ACT for solid cancers.

Tumor Intrinsic Escape Mechanisms

TCR T efficacy is influenced by the heterogeneity of cognate-antigen expression by tumor cells. This is especially the case when targeting CGAs, such as NY-ESO-1 and MAGE-A family proteins, which are often heterogeneously expressed by tumors (36). Nonetheless, complete responses have been observed in patients treated with TCR T cells targeting NY-ESO-1 and MAGE-A family proteins (**Table 1**). This may be partially explained by a phenomenon known as epitope spreading, whereby the immune response mounted by the infused TCR T cells leads to priming and activation of endogenous T cells to other non-cognate tumor antigens. Indeed, epitope spreading has been observed in humans following vaccination (225) and ACT (226, 227). Several genetic engineering strategies have now been developed to promote epitope spreading, including constitutively expressing inflammatory cytokines IL-12 (217, 218) and IL-18 (228), CD40L (229), or the DC growth factor FLT3 (230). Another approach to address antigen heterogeneity is to genetically encode specificity towards multiple antigens. While several multitargeting approaches have been developed in the CAR T realm (231), far less progress has been made in the development of multitargeting TCR T therapies to date.

TCR T therapies are also susceptible to tumor cell escape through perturbations in APM pathways. Downregulation of HLA-class I and other APM components such as TAP1 has been observed in many cancer types (232, 233). The impact of these escape mechanisms has been clearly demonstrated in TCR T and TIL trials where mutations in tumor APM components resulted

in tumor escape and cancer progression (44, 67). These studies highlight an urgent need for strategies to address such tumor intrinsic mechanisms of escape, especially as we see further improvements of the antitumor efficacy of TCR T therapeutics. In cases of tumor downregulation of APM pathways, expression of these components may be recovered through administration of interferon (234) or epigenetic modifying drugs (235). Addressing cases where APM components are lost through hardwired genetic mutations is considerably more challenging. One strategy that has been investigated in mice is *in situ* gene delivery of *β2M* with an adenoviral vector (236). However, this approach delivered adenovirus into relatively small tumors (7 – 10 mm in diameter) by intratumoral injection, therefore it is unclear if this approach will be effective in patients whose tumors are large, inaccessible, and/or dispersed. Therefore, patients with hardwired loss of APM components will likely need to be treated with HLA-independent therapies such as CAR T. Interestingly, HLA-independent TCRs are also currently under development. These TCRs bind natively folded surface proteins similar to CARs, but possess binding affinities within the range of pMHC-TCR interactions (237).

CONCLUSIONS

Immunotherapies have swiftly risen to become one of the major pillars in cancer treatment. Among them, TCR-engineered T cell therapies are a rapidly growing, active, and evolving field. Since the first report of redirected T cell specificity through TCR transfer in 1986 (238), tremendous progress has been made in TCR T therapies and their applications. Emerging technologies and enhanced strategies have made TCR discovery efforts considerably more time and cost effective. This will allow for more groups to become involved in TCR discovery and will ultimately lead to an increase in TCRs targeting new tumor antigens and restricted to a broader range of HLAs. Several clinical findings from early TCR T trials have shaped the past decade of TCR T development. Toxicities observed in early trials have led to improved preclinical safety assessments of TCRs and a transition towards antigen targets with increased tumor specificity. Since 2015 there has been an influx of results of TCR T trials treating various solid cancers and hematological malignancies. Several of these trials have demonstrated impressive clinical responses in the absence of serious toxicities, and it now seems that the first approval of TCR T therapies for solid cancer may be around the corner. These early results give reason for optimism in the continued development of TCR T therapies for cancer.

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PS and LK contributed to the development, writing, and review of the article. VH contributed to the figures and review of the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: PS and VH have submitted a patent application for TCR discovery.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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GMP-Compliant Manufacturing of TRUCKs: CAR T Cells targeting GD_2 and Releasing Inducible IL-18

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Chimeric antigen receptor (CAR)-engineered T cells can be highly effective in the treatment of hematological malignancies, but mostly fail in the treatment of solid tumors. Thus, approaches using 4th advanced CAR T cells secreting immunomodulatory cytokines upon CAR signaling, known as TRUCKs ("*T cells redirected for universal cytokine-mediated killing*"), are currently under investigation. Based on our previous development and validation of automated and closed processing for GMP-compliant manufacturing of CAR T cells, we here present the proof of feasibility for translation of this method to TRUCKs. We generated IL-18-secreting TRUCKs targeting the tumor antigen GD_2 using the CliniMACS Prodigy® system using a recently described "all-in-one" lentiviral vector combining constitutive anti-GD_2 CAR expression and inducible IL-18. Starting with 0.84 x 10^8 and 0.91 x 10^8 T cells after enrichment of $CD4^+$ and $CD8^+$ we reached 68.3-fold and 71.4-fold T cell expansion rates, respectively, in two independent runs. Transduction efficiencies of 77.7% and 55.1% was obtained, and yields of 4.5 x 10^9 and 3.6 x 10^9 engineered T cells from the two donors, respectively, within 12 days. Preclinical characterization demonstrated antigen-specific GD_2-CAR mediated activation after co-cultivation with GD_2-expressing target cells. The functional capacities of the clinical-scale manufactured TRUCKs were similar to TRUCKs generated in laboratory-scale and were not impeded by cryopreservation. IL-18 TRUCKs were activated in an antigen-specific manner by co-cultivation with GD_2-expressing target cells indicated by an increased expression of activation markers (e.g. CD25, CD69) on both $CD4^+$ and $CD8^+$ T cells and an enhanced release of pro-inflammatory cytokines and cytolytic mediators (e.g. IL-2,

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granzyme B, IFN-γ, perforin, TNF-α). Manufactured TRUCKs showed a specific cytotoxicity towards GD_2-expressing target cells indicated by lactate dehydrogenase (LDH) release, a decrease of target cell numbers, microscopic detection of cytotoxic clusters and detachment of target cells in real-time impedance measurements (xCELLigence). Following antigen-specific CAR activation of TRUCKs, CAR-triggered release IL-18 was induced, and the cytokine was biologically active, as demonstrated in migration assays revealing specific attraction of monocytes and NK cells by supernatants of TRUCKs co-cultured with GD_2-expressing target cells. In conclusion, GMP-compliant manufacturing of TRUCKs is feasible and delivers high quality T cell products.

Keywords: TRUCK, IL-18, GD2-CAR, Prodigy, GMP, 4th generation CAR

INTRODUCTION

One of the most significant recent developments in cancer therapy is the CAR T cell technology. To enable and improve CAR T cell proliferation, anti-tumor activity, and *in vivo* persistence, advanced generations of CARs have been developed (1). A promising strategy to target solid tumors with their phenotypic heterogeneity has led to the fourth generation of CARs also known as TRUCKs or armored CARs (2, 3). TRUCKs are CAR T cells that release a transgenic protein upon CAR engagement of cognate antigen and signaling. TRUCKs are thereby used as "living factories" to produce and deposit substances with anti-tumor activity in the targeted tissue. These factors include cytokines, such as IL (interleukin)-12 and IL-18, but also enzymes and costimulatory ligands augmenting T cell activation. Innate immune cells are attracted and activated by IL-12 or IL-18 (4) to eliminate antigen-low expressing or antigen-negative cancer cells within the tumor (2). CAR T cells engineered with inducible IL-18 release improve T cell effector functions towards superior activity against pancreatic and lung tumors in mice that were refractory to CAR T cells without cytokines (5, 6). In this study we focused on the manufacturing of TRUCKs targeting disialoganglioside GD_2. Physiological expression of GD_2 is restricted to low densities on neurons, skin melanocytes and peripheral pain fibers (7). GD_2 is highly and consistently expressed in childhood cancer neuroblastoma and can be found on the cell surface of other solid cancer entities including breast cancer (8), osteosarcoma (9), melanoma (10), glioblastoma (11), small cell lung cancer (12), retinoblastoma (13), soft tissue sarcoma (14) and Ewing sarcoma (15, 16).

GD_2 therefore is a promising target for redirected immunotherapy. Initial GD_2-CAR T cell clinical studies targeting neuroblastoma by first to third generation CAR T cells showed moderate or transient anti-tumor responses but failed to produce sustained remissions, emphasizing the need to modulate the T cell response (17–19). With the increasing expectation of GD_2 as a broad target for CAR T cell therapy and the expected benefit in applying TRUCKs with transgenic IL-18 release, there is a need to manufacture such cellular medicinal products in a safe, validated and reproducible fashion. CAR T cell manufacturing for clinical use is a complex process and places high standard demands on safety, quality and efficacy. Chemistry, Manufacturing and Controls (CMC) even in the preclinical phase of drug development includes significant quality attributes and critical process parameters, including cell composition and transduction efficiency, assessment of potency, product sterility, process validation, stability and production at multiple manufacturing sites (20). The CliniMACS Prodigy® (Miltenyi Biotec B.V. & Co. KG) allows *ex vivo* magnetic bead-based cell separation followed by activation, transduction, expansion, final formulation and sampling of T cells in one device leading to robust, reproducible and automated, supervised cost-effective manufacturing processes [Process of CAR T cell Therapy in Europe EHA Guidance Document, 2019 (21)]. The feasibility of the T cell transduction (TCT) process for use in automated and closed GMP-compliant manufacturing of CAR T cells on the CliniMACS Prodigy® platform, as shown by us and others (22–25) is here extended to the manufacturing of IL-18 TRUCKs targeting GD_2 as an example. Preclinical characterization showed equivalent quality and function of the final clinical-scale products compared to manually produced IL-18 TRUCKs in laboratory-scale.

MATERIALS AND METHODS

Human Sample Materials

For the manufacturing of TRUCKs in a clinical-scale process (n=2) on the CliniMACS Prodigy® (Miltenyi Biotec, Bergisch Gladbach, Germany) and for the manufacturing of laboratory-scale TRUCKs (n=3) lymphapheresis products from two healthy donors (D1 and D2) were obtained from the Institute for Transfusion Medicine of Hannover Medical School (MHH) after donors' written informed consent. According to standard donation requirements, the donors had no signs of acute infection and no previous history of blood transfusion.

IL-18 TRUCK Construct and Production of Lentiviral Supernatants

The generation of the lentiviral IL-18 TRUCK SIN vector was described previously (26). In brief, the lentiviral 3rd generation

SIN vector pCCL.PPT.NFATsyn.hIL18.PGK.GD2CAR.PRE was used. The human codon-usage optimized ORF second-generation CAR containing scFv (14.G2a), human IgG1- hinge, CD28 transmembrane, CD137 (4-1BB), and CD3ζ signaling domains was flanked by restriction enzymes AgeI and SalI and cloned into the lentiviral "all-in-one" SIN vector driven by an hPGK promoter (27). A schematic map of the construct is presented in **Supplement Figure S8**. The construct was confirmed by sequencing (Microsynth SeqLab, Germany). Lentiviral vector particles were generated as described previously (26, 28). Briefly, 5×10^6 293T cells were used for calcium phosphate transfection in the presence of 25 µM chloroquine. For transfection, the following plasmids were used: lentiviral vector plasmid (10 µg), pcDNA3.HIV-1.GP.4 × CTE (lentiviral gag/pol) (12 µg) (29), pRSV-Rev (5 µg; kindly provided by T. Hope, Northwestern University Chicago, IL), and VSVg-encoding pMD.G (1.5 µg) (30). For better standardization, pcDNA3.HIV-1.GP.4×CTE, pRSV-Rev and pMD.G were produced and purified by PlasmidFactory (Bielefeld, Germany).After 36 h and 48 h of transfection, supernatants were harvested and concentrated *via* ultracentrifugation at 4°C and 13,238× g or 82,740× g for 16 h or 2 h, respectively. The particles were resuspended in TexMACS™ GMP medium. Lentiviral supernatant was titrated in HT1080 fibroblasts *via* spinoculation-mediated transduction, i. e. 1×10^5 cells were seeded, the supernatant containing viral particles and 4 µg/ml protamine sulfate (Sigma-Aldrich, St. Louis, USA) were added and cells were centrifuged (1 h, 800× g, 37°C). Three days post transduction, transduction efficiency was determined *via* flow cytometric staining of GD_2-CAR expression and functional viral vector titers were calculated from samples with GD_2-CAR expression percentages of ≤ 30% to avoid cells with multiple integrations (26, 31).

GMP-Compliant Manufacturing of IL-18 TRUCKs Targeting GD_2 With CliniMACS Prodigy® (Clinical-Scale Process)

GMP-compliant manufacturing of IL-18 TRUCKs was performed using the CliniMACS Prodigy® platform, which allows for automated cell processing in a closed system controlled by operating software version V1.3 and process software for T cell transduction (TCT) version V2.0 (released). For overview of clinical-scale process see **Figure 1**. Within the scope of the automatically running process, the input of different variable process parameters like time points of transduction, media exchange, culture wash, harvesting and volume of media exchange is possible. Buffer, media, starting cell material and vector were connected directly to TS520 *via* sterile tubing welder device (TSCDII Terumo BCT).

The applied materials were either medicinal products with a marketing approval (HSA, PEI.H.03272.01.1), GMP-grade reagents and tubing sets from Miltenyi Biotec (designed following the recommendations of USP <1043> on ancillary materials and/or compliant with the requirements laid down in the Ph. Eur. Chapter 5.2.12, where applicable), or approved medical devices (DMSO, Composol, SSP+, transfer bags, connections, syringes). The pool-Human Serum P-HS/Tü was purchased from the Centre for Clinical Transfusion Medicine Tübingen/ZKT and certified as suitable for manufacturing of pharmaceutical products. The single non-regulated reagent was the vector, designed and produced at the Institute of Experimental Hematology, Hannover Medical School, Division of Hematology/Oncology. Detailed information regarding the materials was recorded, including the supplier, lot number, and expiration date. Starting material for manufacturing of IL-18 TRUCKs were CD3+ T cells derived from a non-mobilized lymphapheresis. Cell processing started within 24 h after product collection with immunomagnetic enrichment of 1×10^9 CD4+ and CD8+ T cells using CliniMACS® CD4 Reagent, CliniMACS® CD8 Reagent and CliniMACS® PBS/EDTA buffer supplemented with human serum albumin (HSA, Human albumin 200 g/l Baxalta, Shire Deutschland GmbH, Berlin, Germany) to a final concentration of 0.5%. For cultivation, the basal TexMACS™ GMP medium was supplemented with 12.5 ng/ml MACS GMP Recombinant Human IL-7, 12.5 ng/ml MACS GMP Recombinant Human IL-15 and 3% heat-inactivated human AB serum (pool-human serum P-HS/Tü, Centre for Clinical Transfusion Medicine Tübingen/ZKT, Germany) until day 5. T cells were activated for 72 h (day 0 to day 3) with CD3/CD28 MACS GMP T Cell TransAct Beads. On day 1 of culture transduction took place by adding 10 ml lentiviral vector with a multiplicity of infection (MOI) of 29 D1 and 10 D2 in total volume of 100 ml. At day 3, CD3/CD28 T cell TransAct beads and non-bound vector were washed out (culture wash) and culture volume increased to 200 ml. The culture was fed every 12 – 24 h after day 5 of culture. Hereby the concentration of AB serum in the culture was reduced continuously using supplemented TexMACS™ GMP medium without AB serum for further medium exchange. On day of harvest (day 12), cells were formulated in Composol PS (Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) with 2.86% (w/v) HSA (D1) or SSP+ (D2) (Maco Pharma International GmbH, Langen, Germany) with 3.33% HSA for later cryopreservation. During cultivation, the temperature and atmosphere was maintained at 37°C with 5% CO2. After 3 days of static culture shaking modus was activated (culture agitation) enabling high cell concentrations in the limited volume of the CentriCult Chamber.

Monitoring of Culture and In-Process Controls

Total cell number and viability was analyzed by flow cytometric analysis as described below. The glucose concentration of the cell-free culture supernatant was determined with the blood glucose meter Accu-Chek® Aviva (Roche, Mannheim, Germany). For analysis of pH during cell cultivation pH-indicator strips MColorpHast™ pH 6.5 - 10.0 (Merck Millipore, Darmstadt, Germany) were used.

Flow Cytometric Characterization of IL-18 TRUCKs Manufactured in Clinical-Scale Process

Flow cytometric analysis was performed by using anti-human monoclonal antibodies. Cellular composition antibody table is

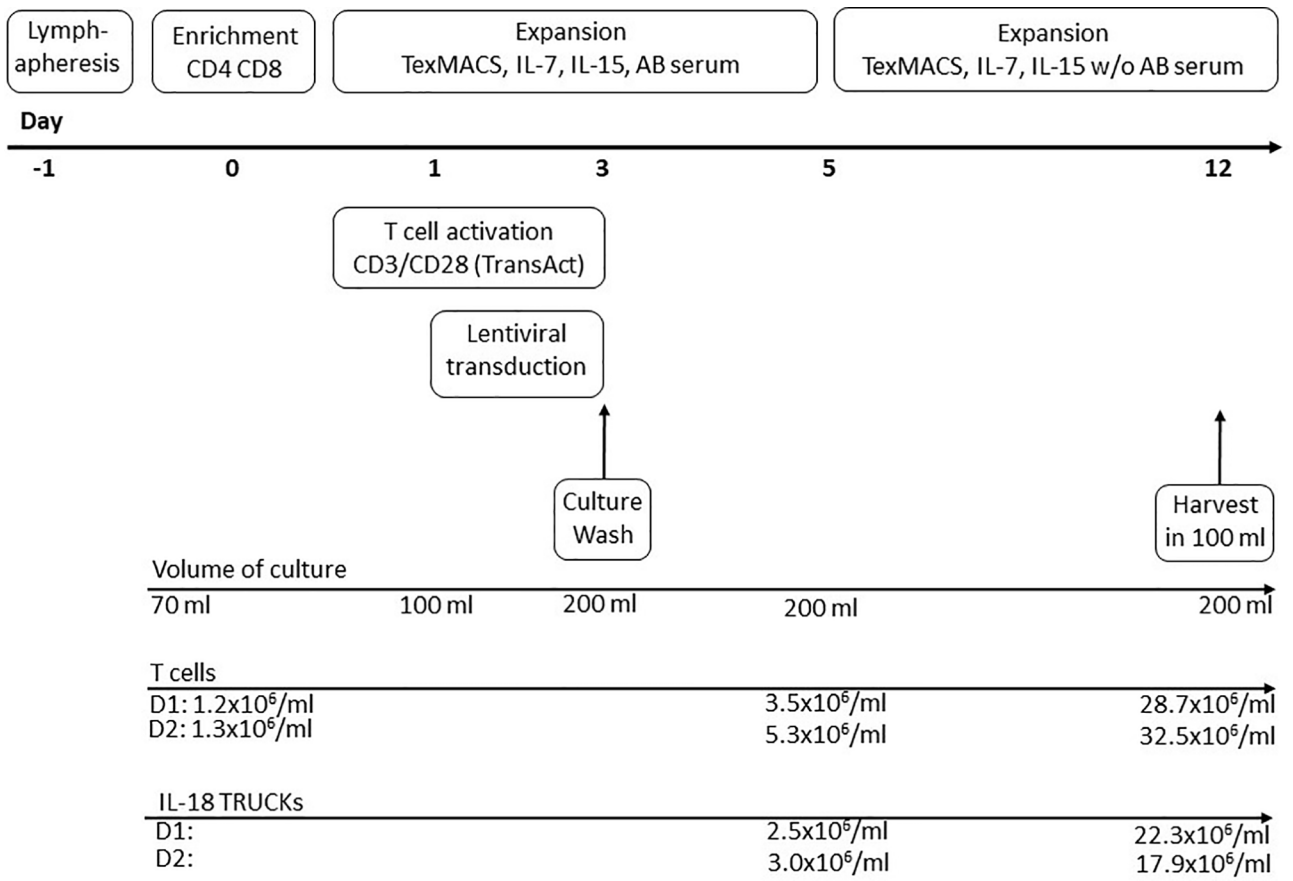

FIGURE 1 | Schematic diagram of producing IL-18 TRUCKs targeting GD_2 utilizing the CliniMACS Prodigy® automated cell processor (clinical-scale process). The entire 12-day process from lymphapheresis material to formulated final products was performed using the CliniMACS Prodigy®. **Day -1**: Overnight storage of the lymphapheresis at 4°C. **Day 0**: Immunomagnetic enrichment of $CD4^+$ and $CD8^+$ cells. Start of T cell activation with CD3/CD28 TransAct Beads (day 0-3) and cultivation in TexMACS™ basal medium supplemented with IL-7, IL-15 and human AB serum until day 5. **Day 1-3**: Lentiviral transduction. **Day 3**: Culture wash and start of culture agitation. **Day 5**: Switch to TexMACS™ basal medium supplemented with IL-7, IL-15 without human AB serum for following media exchanges. **Day 12**: Wash of cells and harvest in 100 ml formulation buffer for following cryopreservation. During T cell expansion several feeding steps by medium exchange were included in the activity matrix.

shown in **Supplementary Table 1**. Transduction efficiency was determined by GD_2-CAR detection with the antibody Ganglidiomab, which was conjugated with Phycoerythrin (PE) by Miltenyi Biotec, herein after referred to as Ganglidiomab-PE. The antigen-specific single-chain variable fragment (scFv) of the GD_2-CAR is derived from the anti-GD_2 antibody 14G2a (32, 33). Ganglidiomab is a monoclonal anti-idiotype antibody to 14G2a and therefore allows for a direct detection of the GD_2-CAR on cells. Subsequently antibody staining (10 min at RT) cells were incubated (10 min at RT) with freshly prepared red blood cell lysis solution (Miltenyi Biotec). T cell phenotype antibody panel is shown in **Supplementary Table 1**. After antibody staining (10 min at RT in PBS supplemented with 4% FCS) cells were washed and resuspended with PBS supplemented with 4% FCS (both Merck, Darmstadt, Germany). Prior to flow cytometric analysis 7-AAD and Flow-Count Fluorospheres (both Beckman Coulter) were added to the samples for dead cell discrimination and single platform cell quantification, respectively. Flow cytometric analysis was performed with the Navios flow cytometer (Navios 3L 10C, Software 1.3, Beckman Coulter). For gating strategies see **Supplement Figures S6, 7**.

Cell Lines and Cell Culture

The cell lines 293T, HT1080, HT1080-GD_2 and SH-SY5Y were cultivated as recently described (26). NK-92 cells (human natural killer lymphoma #ACC 488; DSMZ, Braunschweig, Germany) were cultivated in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% FBS, 2 mM L-glutamine (c.c.pro, Oberdorla, Germany) and 400 IU/mL human IL-2 (Proleukin S, Novartis Pharma GmbH, Nürnberg, Germany). THP-1 (human acute monocytic leukemia, #ACC 16; DSMZ, Braunschweig, Germany) cells were cultivated in RPMI 1640 medium with 2 mM L-glutamine, 2-mercaptoethanol to a final concentration of 0.05 mM, 10% (v/v) heat-inactivated fetal bovine serum (HI-FBS), and 50 IU/ml penicillin and 50 µg/ml streptomycin. All cells were tested for mycoplasma

contamination on a regular basis using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland) according to the manufacturer's protocol.

Laboratory Manufacturing of IL-18 TRUCKs Targeting GD_2 (Laboratory-Scale Process)

$CD4^+$ and $CD8^+$ T cells isolated by the CliniMACS Prodigy® (see clinical-scale manufacturing) were transduced and expanded as previously described (34). Briefly, they were activated with anti-CD3/CD28 beads (Thermo Fisher Scientific, Waltham, MA, USA) at a ratio of 1:1 in TexMACS™ (Miltenyi Biotec) with 3% human serum (c.c.pro, Oberdorla, Germany) supplemented with 12.5 ng/ml IL-7 and IL-15 (PeproTech, Rocky Hill, NJ, USA). On the following day, T cells were either left untransduced or transduced with lentiviral particles by spinoculation using an MOI of 7 and addition of 5 µg/ml Polybrene Infection/Transfection Reagent (Merck Millipore, Burlington, MA, USA). The anti-CD3/CD28 beads were removed on the following day and cells were further cultivated in TexMACS™ medium supplemented with 3% human serum, 12.5 ng/ml IL-7 and IL-15 and splitting 1:2 every 2 - 3 days for a total expansion time of 12 days.

Cryopreservation and Thawing of IL-18 TRUCKs Targeting GD_2

Untransduced and transduced T cells were cryopreserved in Composol® PS Fresenius Kabi, Bad Homburg, Germany/2.86% (w/v) HSA (Biotest, Dreieich, Germany) (D1) and SSP+ (Maco Pharma International GmbH, Langen, Germany) supplemented with 3.33% (w/v) HSA (D2), respectively after manufacturing by adjusting the cell counts and addition of DMSO (CryoSure-DMSO, USP grade; WAK Chemie, Steinbach, Germany) to a final concentration of 10% (v/v). After cryopreservation in a < -80°C freezer overnight, the cells were stored in the vapor phase above liquid nitrogen at < -140°C. For T cell phenotype analysis cells were thawed in RPMI 1640 medium with 20% (v/v) FCS (both Merck, Darmstadt, Germany) and rested for 1 h in RPMI 1640 with 10% FCS (37°C, 5% (v/v) CO_2) before flow cytometric analysis. For functional analysis of the cryopreserved cells, they were thawed and seeded in TexMACS™ medium in a cell density of 2.5 x 10^6 cells/ml and rested overnight.

Co-Culture of Laboratory- and Clinical-Scale IL-18 TRUCKs With Target Cells

Directly after expansion or after cryopreservation and thawing (cryo), functionality of the laboratory- and clinical-scale IL-18 TRUCKs in comparison to untransduced T cells was assessed by co-culturing them with target cells. For flow cytometry and soluble mediator measurements, 5 x 10^4 target cells were seeded in 800 µl of their respective culture medium, which was removed after 4 - 24 h followed by addition of effector cells according to the specified effector-to-target (E:T) ratio in 800 µl CTL medium. For cytotoxicity, microscopy and intracellular cytokine assessment, 2 x 10^4 target cells were seeded in 200µl and co-cultured with effector cells accordingly.

Flow Cytometry of Laboratory-Scale Experiments

The antibodies used for flow cytometric analysis are listed in **Supplement Table S1**. The transduction efficiency was analyzed with the Ganglidiomab-PE mAb. Intracellular staining of TNF-α was performed by using the IntraPrep Permeabilization Reagent (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. Samples were read on a BD FACSCanto™ Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). To determine cytotoxicity of engineered T cells, target cells were gated as $CD3^-$ cells. Percentage of killed cells was normalized to untransduced cell co-cultures using the following formula:

$$\text{CD3}^- \text{ cell killing} = 100\,\% - \frac{\text{CD3}^- \textit{ cell} \text{ frequency (co-culture)}}{\text{CD3}^- \textit{ cell} \text{ frequency (respoective co-culture with untransduced T cells)}}$$

Multiplex Cytokine Analysis

Cytokine concentrations in the supernatant were determined using a customized LEGENDplex™ Multi-Analyte Flow Assay (BioLegend, San Diego, CA, USA), which allowed for the detection of human IL-2, IL-4, IL-10, IL-18, granzyme B, perforin, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. Samples were analyzed with LEGENDplex v8.0 software (BioLegend, San Diego, CA, USA).

Determination of Cytotoxicity by LDH Assay

The release of lactate dehydrogenase (LDH) into the cell culture supernatant was assessed by using the Cytotoxicity Detection Kit (Roche, Basel, Switzerland). Cells lysed by adding Triton X-100 (Merck, Darmstadt, Germany) to a final concentration of 1% to all control wells served as maximum controls. Absorbance was assessed at a wavelength of 490 nm with a reference wavelength of 690 nm on a Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA). LDH release (%) was calculated according to the manufacturer's protocol.

Microscopy

Transmitted-light microscope images of co-cultures of target and effector cells were taken with an Olympus IX81 microscope combined with a digital B/W camera using 10x objective lenses and analyzed with Xcellence Pro image software (all from Olympus, Hamburg, Germany). Representative pictures are shown.

XCelligence

Target cell killing by cryopreserved and thawed TRUCKs was furthermore determined with the XCelligence RTCA S16 Real Time Cell Analyzer and using E-Plates 16 PET (both ACEA Biosciences, San Diego, CA, USA). Background impedance of all wells was assessed with cell culture medium measurements. Afterwards, target cells were seeded in an amount of 1 x 10^5 cells (SH-SY5Y) or 2 x 10^4 cells (HT1080, HT1080-GD_2) in 200 µl of their respective culture medium and adhesion was checked by measurements every 30 min. T cells were added after target cell adhesion shown by a constant impedance after 19 – 25 h. For

this, 150 µl medium were carefully removed and replaced by T cells in the specified E:T-ratios in 150 µl CTL medium. Impedance was measured every 30 min. Cell indices were normalized to the respective indices after T cell addition.

Cell Migration Assay

To test the chemo-attractive potential of supernatants that were derived from co-culture experiments of primary human T cells transduced with the IL-18 TRUCK vector and GD_2^+ target cells, migration assays with THP-1 or NK-92 cells were performed using a Boyden chamber (NeuroProbe, Gaithersburg, MD, USA) as recently described (26). The cell number was calculated using an Olympus IX71 microscope and imageJ 1.53k software. To normalize results from different plates, cell numbers of migrated cells towards untransduced T cells as background migration were subtracted from all values for each plate.

Isolation of Genomic DNA and Determination of the VCN by qPCR

Genomic DNA was isolated from 1 x 10^6 transduced or untransduced cells (-20°C frozen cell pellets) with the QIAamp® DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The determination of the VCN by qPCR was performed as recently described (35).

Statistics

Statistical analysis was performed with GraphPad Prism V.9.1.2 using the Kruskal-Wallis and uncorrected Dunn's test. Only mean ranks of preselected data sets were compared: large-scale TRUCKs cultured alone or co-cultured with different target cells, large-scale TRUCKs co-cultured with different target cells immediately after generation (indicated by black asterisks) or cryopreservation (indicated by grey asterisks), large-scale TRUCKs co-cultured with different target cells in comparison to the same co-cultures with all laboratory-scale manufactured cells. Significant differences are shown (*$p \leq 0.05$, **$p \leq 0.01$).

RESULTS

GMP-Compliant Manufacturing Process of IL-18 TRUCKs Targeting GD_2 Using Automated Cell Processing in a Closed System

To assess feasibility, we performed two complete GMP-compliant processes (D1 and D2 with two individual donor lymphocyte products) to manufacture IL-18 TRUCKs targeting GD_2 using an automated TCT protocol for CliniMACS Prodigy® as shown in **Figure 1**.

Recovery and Purity of $CD4^+$ and $CD8^+$ Cells Enriched in an Automated Process

Starting with the lymphapheresis, 1.14 x 10^9 D1 and 1.17 x 10^9 D2, respectively, $CD4^+$ and $CD8^+$ cells (20.6% D1 and 46.1% D2 of unstimulated short time lymphapheresis) were applied for enrichment. An amount of 0.67 x 10^9 D1 and 0.88 x 10^9 D2 $CD4^+$ and $CD8^+$ cells were obtained representing a recovery of 58.8% D1 and 75.2% D2. Thereby, the isolation of $CD4^+$ cells with a recovery of 63.9% D1 and 84.04% D2 was more effective than enrichment of $CD8^+$ cells with 46.8% D1 and 61.7% D2 recovery, respectively (**Figure 2A**). The achieved T cell purities and cell compositions are shown in **Figure 2B**. Due to the CD8 based isolation procedure, NK as well as NKT cells were not completely depleted releasing 7.6% D1 and 4.4% D2 contaminating NKT cells in the final cell product (**Figure 2B**). The CD4/CD8 ratio pre-enrichment was 2.1 D1 and 1.6 D2 and post-enrichment 2.9 D1 and 2.2 D2 reflecting a superior recovery of $CD4^+$ cells (**Figure 2D**).

Expanded IL-18 TRUCKs Targeting GD_2 Display a High Purity and Favorable Phenotype

In accordance with the TCT protocol, the cell expansion started with 0.84 x 10^8 D1 and 0.9 x 10^8 D2 viable T cells (corresponding 1.18 x 10^8 D1 and 1.0 x 10^8 D2 viable WBC) after enrichment of $CD4^+$ and $CD8^+$ cells. The expansion rate during the 12-day manufacturing process was 68.3-fold D1 and 71.4-fold D2 for T cells. Viability of the cells was >96% during the whole process until final harvest (**Figure 2C**). Cell concentration increased in a constant culture volume from 3.8 x 10^6/ml D1 on day 5 and 5.5 x 10^6/ml D2 to 31.2 x 10^6/ml D1 and 34.2 x 10^6/ml D2 in the final products (**Figure S1A**). Monitoring of cell culture condition revealed a pH of 7.0 - 7.1 and a glucose level above the critical level of 100 mg/dl. (**Figure S1B**). T cell purity and cell composition of the final products is shown in **Figure 2B**. During expansion, the CD4/CD8 ratio increased in the first run from 2.9 to 3.3 D1 but decreased slightly in the second run from 2.2 to 2.0 D2 (**Figure 2D**). The proportion of T cell phenotypes during *ex vivo* cell expansion was analyzed by comparing T cell phenotypes in the initial product post $CD4^+$ and $CD8^+$ enrichment at process day 0 and in the final product at process day 12 as shown in **Figure 2F**.

Transduction of T Cells With the "All-In-One" Lentiviral Vector Is Highly Efficient

The recently described "all-in-one" lentiviral vector (26) encoding constitutive GD_2 CAR and inducible IL-18 expression was used for cell transduction on day 1 of the manufacturing process with a multiplicity of infection (MOI) of 29 for D1 and 10 for D2 derived T cells. The percentage of transduced $CD3^+$ cells in the final product, was 74.9% D1 and 52.2% D2, while $CD4^+$ cells exhibited a higher transduction efficiency (80.6% D1 and 65.8% D2) compared to $CD8^+$ cells (57.2% D1 and 26.5% D2) (**Figure 2E**). The VCN determined by qPCR of genomic DNA in the final products were 2.6 D1 and 2.4 D2 copies/cell, respectively.

Preclinical *In Vitro* Characterization Using Clinical- and Laboratory-Scale Manufactured TRUCKs

IL-18 TRUCKs targeting GD_2 manufactured using the CliniMACS Prodigy® (referred to as clinical-scale TRUCKs)

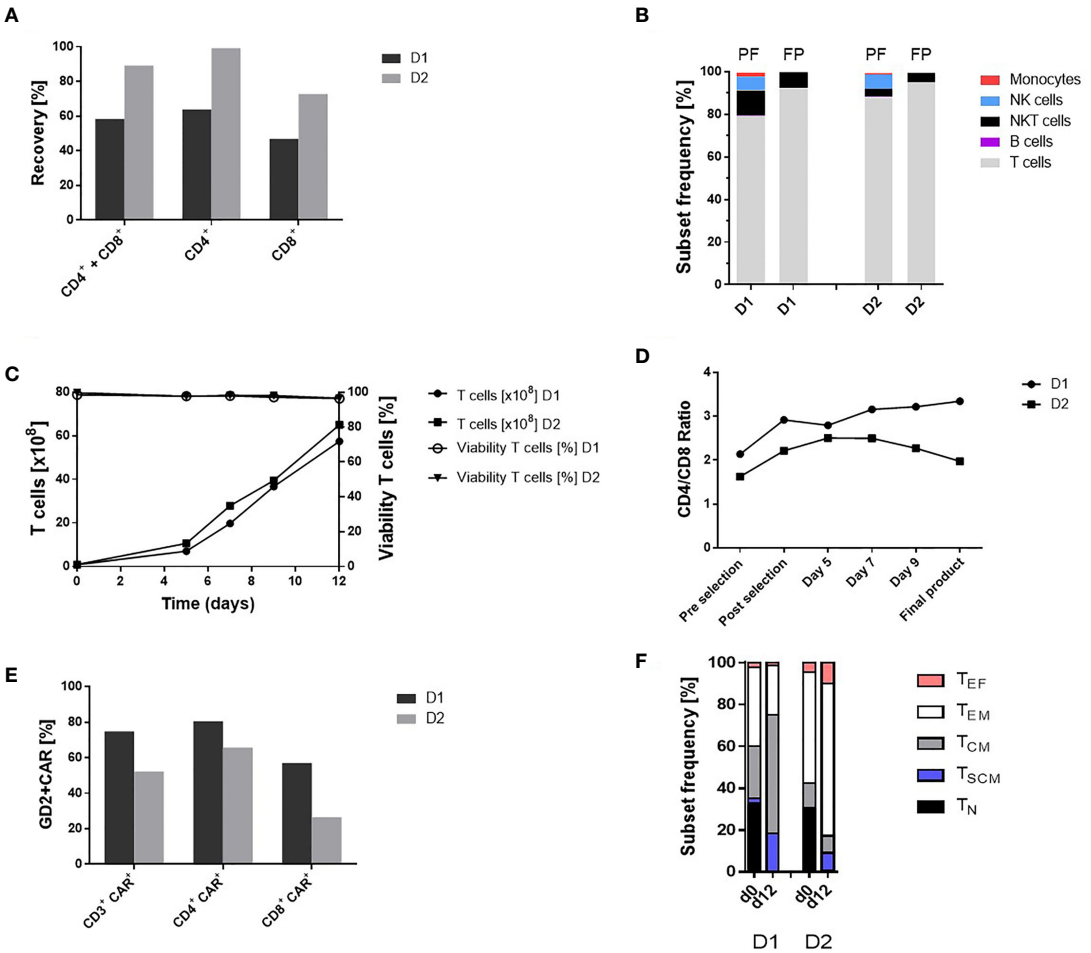

FIGURE 2 | Automated GMP-compliant manufacturing of IL-18 TRUCKs targeting GD_2 using the CliniMACS Prodigy® (clinical-scale process). **(A)** Recovery (cell population in percent of cell population pre-enrichment) of $CD4^+$ and $CD8^+$ cells after enrichment. **(B)** Cell composition after enrichment of $CD4^+$ and $CD8^+$ cells and in the final products. The achieved T cell purity in the positive fraction (PF) was 79.1% D1 and 87.7% D2 with contaminating $CD56^+CD3^+$ NKT cells (12.0% D1 and 3.8% D2), $CD56^+CD3^-$ NK cells (6.4% D1 and 6.8% D2), $CD14^+$ monocytes (1.9% D1 and 0.6% D2) and $CD20^+$ B cells (0.2% D1 and 0.5% D2). Cell composition of the final products (FP) with a purity of 92.1% D1 and 95.0% D2 T cells ($CD3^+/CD56^-$) and impurities with NKT cells (7.6% D1 and 4.4% D2) as well as residual 0.1% D1 and 0.2% D2 NK cells. Residual B cells (0.05% D2) were detected but no monocytes in the final products. **(C)** Expansion and viability of T cells ($CD3^+$ $CD56^-$) during 12-day manufacturing. **(D)** CD4/CD8 ratio during cultivation. The CD4/CD8 ratio changed during the manufacturing process (post-enrichment) from 2.9 to 3.3 D1 and 2.2 to 2.0 D2 in the final products. **(E)** Transduction rate of $CD3^+$ cells and $CD4^+$ $CD8^+$ subtypes in the final products. **(F)** T cell phenotypic analysis of the starting material on day 0 (lymphapheresis) and in the final products on day 12. The analyses were performed by flow cytometry based on the expression of CD45RO, CCR7 and CD95 among viable $CD3^+$ cells to define naïve (T_N: $CD45RO^-$, $CCR7^+$, $CD95^-$), stem-cell memory (T_{SCM}: $CD45RO^-$, $CCR7^+$, $CD95^+$), central memory (T_{CM}: $CD45RO^+$, $CCR7^+$ $CD95^+$), effector memory (T_{EM}: $CD45RO^+$, $CCR7^-$ $CD95^+$) and effector (T_{EF}: $CD45RO^-$, $CCR7^-$ $CD95^+$) T cell subsets. T_N (33.1% D1 and 30.0% D2), T_{CM} (25.1% and 11.8% D2) and T_{EM} (37.6% D1 and 53.0% D2) cells were present in the initial product with differences in the T cell subsets (T_{SCM}: 2.2% D1 and 0.62% D2, T_{EF}: 2.1% D1 and 4.62% D2). In contrast, the final TRUCK products harbored cells with less differentiated memory phenotypes with a T_{CM} (56.9% D1 and 8.17% D2) and T_{SCM} (18.3% D1 and 8.62% D2) cells, T_{EM} (23.6% D1 and 72.74% D2) and T_{EF} (1.2% D1 and 9.9% D2) cells and the decrease of T_N (<0.1% D1 and 0.54% D2) cells For cell composition, cells **(B, D)** were gated as viable $CD45^+$ cells using lineage-specific markers: T cells ($CD3^+$ $CD56^-$), monocytes ($CD14^+$), NK cells ($CD56^+$ $CD16^+$), NKT cells ($CD56^+$ $CD3^+$), B cells ($CD20^+$). n = 2.

were compared to the respective TRUCKs generated under laboratory conditions (referred to as laboratory-scale TRUCKs). These cells were manufactured manually as previously described (34) using the same isolated $CD4^+$ and $CD8^+$ T cell starting population, lentiviral vector, expansion media, cytokines, and experimental timing. Untransduced T cells as well as GD_2 TRUCKs with inducible EGFP expression (referred to as EGFP-TRUCKs) were generated in laboratory scale and served as controls. From a starting fraction of 0.6 x 10^6 cells, laboratory-scale TRUCKs expanded to a cell number of 48 x 10^6 cells, which was slightly lower compared to untransduced T cells and EGFP-TRUCKs with final cell numbers of 53 x 10^6 and 54 x 10^6 cells, respectively (**Figure S2A**). Both, laboratory- and clinical-scale IL-18 TRUCKs, showed an enhanced frequency of $CD4^+$ T cells after expansion with CD4/CD8 ratios of 2.2 for clinical- and 2.1 for laboratory-scale TRUCKs, whereas it was

0.79 for untransduced T cells (**Figures 3A, B**). Utilizing the recently described "all-in-one" vector" (26), we obtained a high percentage of transduced cells with 65% and 85% under clinical scale as well as under laboratory conditions, respectively, with a higher frequency of transduced $CD4^+$ T cells than $CD8^+$ T cells (**Figures 3C, D**). Activation marker expression on clinical-scale TRUCKs, assessed after expansion of T cells with anti-CD3/CD28 stimulation and IL-7 and IL-15 supplementation, revealed CD25 and CD69 expression on $CD3^+$ T cells (45% and 24%, respectively), moderate CD154 expression on $CD4^+$ cells (13%) and absent CD137 expression on $CD8^+$ cells (**Figures 3E–H**); similar data were obtained for laboratory-scale cells. Clinical use of modified cells often requires cryopreservation to allow for centralized manufacturing and administration flexibility (36). To assess the feasibility of cryopreservation of the obtained T cell product, the manufactured T cells were also characterized after freezing and thawing. Cryopreservation of clinical-scale IL-18 TRUCKs did not significantly change their activation state, CD4/CD8 ratios and frequency of transduced cells (**Figures 3A, B, D–H**).

Clinical-Scale-Manufactured IL-18 TRUCKs Targeting GD_2 Specifically Respond to GD_2^+ Target Cells With an Increase of Activation Marker Expression

We tested the ability of the generated TRUCKs to recognize and react towards GD_2^+ target cells. Manufactured cells were co-cultivated either with GD_2^- HT1080, HT1080 cells expressing GD_2 or with the GD_2^+ neuroblastoma cell line SH-SY5Y as target cells and examined for activation marker expression. Clinical-scale IL-18 TRUCKs targeting GD_2 responded with increased expression of CD25 and CD69 by 50 – 64% and 35 – 45%, respectively, on all $CD3^+$ T cells to both GD_2-expressing target cells, whereas no specific response towards unmodified HT1080 cells was detected (**Figures 4B, C** and **S3A–D**). The slightly lower activation response towards SH-SY5Y in comparison to HT1080-GD_2 coincides with the lower GD_2 expression levels (26). Clinical-scale compared to laboratory-scale TRUCKs exhibited a similar activation marker increase for all markers and target cells, and also cryopreserved TRUCKs did not show significantly different responses regarding expression of CD137 on $CD8^+$ and CD154 on $CD4^+$ T cells, the same effects were observed, indicating cells of the $CD4^+$ and $CD8^+$ populations in manufactured IL-18 TRUCKs were specifically and effectively activated in response to GD_2^+ target cells (**Figures 4A, D, E** and **S3E–H**). Assessment of respective activation marker expression levels as mean fluorescence intensity (MFI) or in further effector-to-target (E:T) ratios confirmed the observed results (**Figures S3A–H**).

Clinical-Scale-Manufactured IL-18 TRUCKs Targeting GD_2 Increased Release of Cytokines Upon Target Recognition

The release of cytokines by manufactured cells into the cell co-culture supernatants upon target contact was assessed. In co-cultures with HT1080-GD_2 cells and, to a lower extent also with SH-SY5Y cells, the release of several cytokines and cytolytic factors was upregulated in clinical-scale IL-18 TRUCKs compared to background secretion: IL-2 (108 and 25-fold), IL-4 (39 and 23-fold), IL-10 (5.8 and 2.2-fold), interferon (IFN)-γ (70 and 49-fold), granzyme B (290 and 150-fold), perforin (3.7 and 2.7-fold) and tumor necrosis factor (TNF)-α (430 and 380-fold; all in an E:T ratio of 8:1) (**Figures 5** and **S4**). Thereby, antigen-induced cytokine release by clinical-scale TRUCKs was similar compared to laboratory-scale TRUCKs and, furthermore, cryopreservation of cells did not significantly change the secretion pattern. A higher expression of TNF-α, by IL-18 TRUCKs upon specific target contact was confirmed by intracellular staining (**Figure S3I**).

Clinical-Scale-Manufactured IL-18 TRUCKs Targeting GD_2 Specifically Eliminate GD_2^+ Target Cells

Finally, the killing capacity of manufactured IL-18 TRUCKs targeting GD_2 was determined by flow cytometric analysis of target cells in the co-cultures. Relative to co-cultures of untransduced T cells with HT1080-GD_2 and SH-SY5Y, clinical-scale TRUCKs eliminated 69 - 88% of HT1080-GD_2 and 65 - 86% of SH-SY5Y cells at different E:T ratios, whereas the frequency of HT1080 in co-cultures with the clinical-scale TRUCKs was on the level of untransduced T cells or laboratory-scale manufactured cells, likely representing expected allo-reactivity (**Figure 6A**). Compared to TRUCKs generated in the laboratory-scale, clinical-scale-manufactured TRUCKs exhibited a similar cytotoxic ability to eliminate GD_2^+ target cells, which was moreover not impeded by cryopreservation. The release of lactate dehydrogenase (LDH) into the supernatant as parameter for cytolysis confirmed the result; cytotoxicity was enhanced in co-cultures of both IL-18 TRUCKs with HT1080-GD_2 cells (18-23% and 0-17% for large- and laboratory-scale TRUCKs, respectively) compared to the respective co-cultures with unmodified HT1080 cells, in which LDH was not released above background of cells cultured alone (**Figure S5A**). Target cell death by LDH release in co-cultures of clinical-scale TRUCKs with SH-SY5Y was also enhanced (6-16%) and similar to the cytotoxicity by laboratory-scale TRUCKs (2-8%). LDH measurements in the co-cultures with cryopreserved T cells revealed a similar cytotoxic capability towards GD_2^+ target cells. Real-time measurement of target cell viability confirmed these results. After addition of both TRUCKs to adherent SH-SY5Y cells, the cell index was rapidly reduced, resulting in almost complete absence of adherent target cells after co-cultivation with laboratory- or clinical-scale TRUCKs for 60 h in different ratios (**Figures 6B, C**). Transmitted-light microscopy visualized the process of target cell elimination. In co-cultures of HT1080 with all T cell products, cells are distributed equally, and the target cells stayed viable in all E:T ratios (**Figure 6D**). In co-cultures of both TRUCKs with HT1080-GD_2 or SH-SY5Y, the target cells were diminished or even absent after 72 h and T cells formed large clusters around the target cells. For thawed TRUCKs, the clusters tended to be even larger and already appeared at low E:T ratios in co-cultures with SH-SY5Y cells (**Figure S5B**).

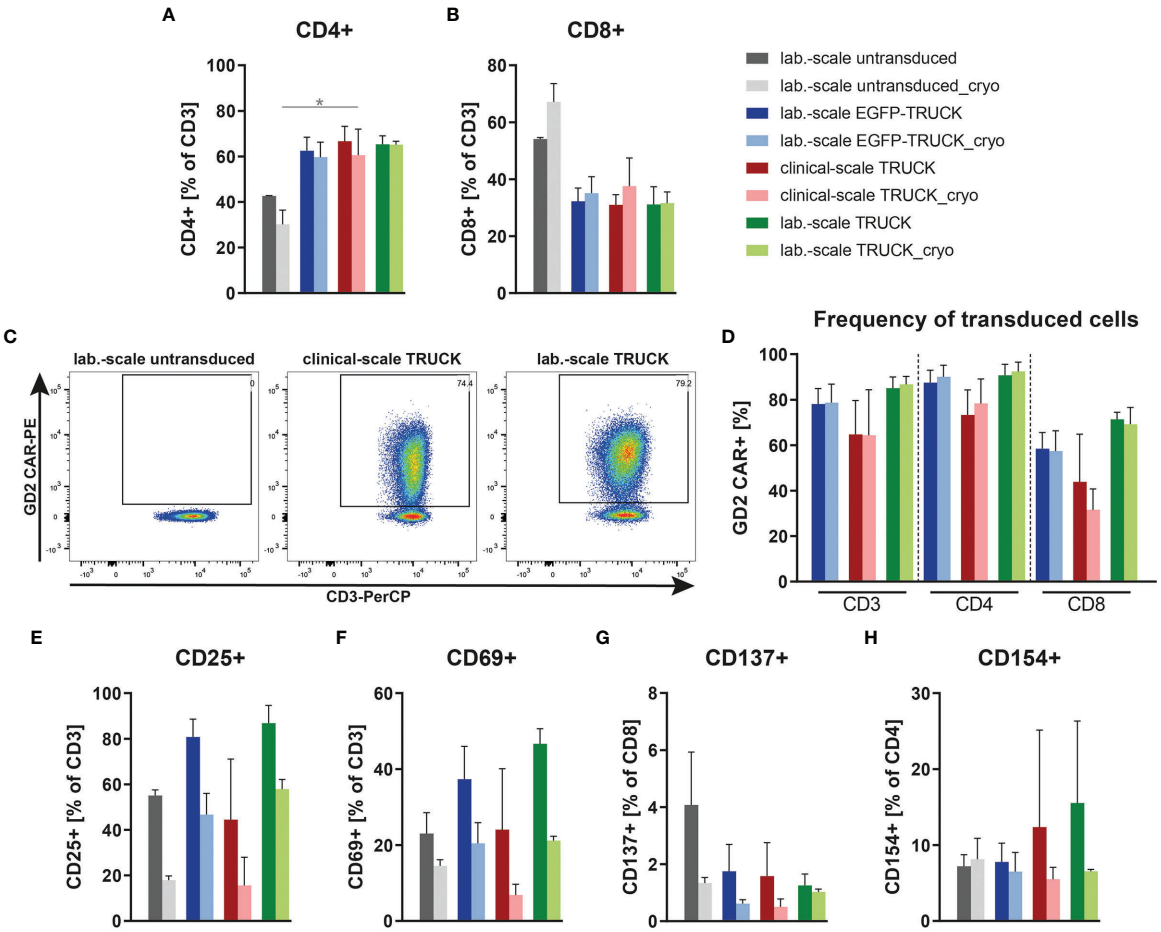

FIGURE 3 | Clinical-scale-manufactured IL-18 TRUCKs targeting GD_2 are transduced and pre-activated at similar efficacies compared to laboratory-scale-manufactured TRUCKs. IL-18 TRUCKs targeting GD_2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK; n=2) or under laboratory conditions (lab.-scale TRUCK; n=3). Untransduced T cells (lab.-scale untransduced; n=3) as well as GD_2 TRUCKs with inducible EGFP expression (EGFP-TRUCK; n=3) served as control. The manufactured cells were either characterized directly after the generation process (d12) or after cryopreservation and thawing (cryo). **(A, B)** Frequency of **(A)** $CD4^+$ and **(B)** $CD8^+$ T cells in the final cell product. **(C, D)** Percentage of CAR^+ cells of **(C)** $CD3^+$ cells shown as representative plots and **(D)** $CD3^+$, $CD4^+$ and $CD8^+$ cells as assessed by staining of the scFv-domain of TRUCKs with a Ganglidiomab antibody after expansion. **(E–H)** Expression of the activation markers **(E)** CD25 on $CD3^+$, **(F)** CD69 on $CD3^+$, **(G)** CD137 on $CD8^+$ and **(H)** CD154 on $CD4^+$ T cells. **(A, B, D–H)** Data are shown as mean ± SD. Statistical differences of large-scale TRUCKs directly after generation or cryopreservation as well as in comparison to laboratory-scale manufactured cells were assessed by Kruskal-Wallis and Dunn's test, significant differences are shown (*$p \leq 0.05$).

Clinical-Scale-Manufactured IL-18 TRUCKs Targeting GD_2 Released IL-18 in a Target-Specific Manner Leading to Innate Immune Cell Attraction

To address the ability of the generated TRUCKs to selectively release IL-18 following CAR engagement of GD_2 target antigen, the cytokine was measured in the co-culture supernatants. Importantly, the release of IL-18 into the cell culture supernatant by freshly-generated or cryopreserved clinical-scale TRUCKs was induced in a target-specific manner up to 41 pg/ml upon HT1080-GD_2 and 18 pg/ml upon SH-SY5Y encounter (**Figure 7A**). To assess the effect of anti-GD_2 IL-18 TRUCK-induced cytokines with respect to the recruitment of innate immune cells, we used a modified Boyden chamber assay to compare the migration potential of cell supernatants collected from co-culture experiments of IL-18 TRUCKs vs. untransduced T cells and GD_2^+ target cells (HT1080-GD_2 and SH-SY5Y) to promote the recruitment of monocytes (THP-1 cells) and NK cells (NK-92 cells). The capacity of IL-18 containing supernatants to recruit innate immune cells was shown by positive Giemsa staining of the transwell membrane through which migrating cells were recruited (**Figure 7B**). Giemsa staining of migrated cells revealed that increased numbers of the monocytic cells THP-1 as well as NK-92 cells were recruited by supernatants collected from IL-18 TRUCKs co-cultured with HT1080-GD_2 or SH-SY5Y target cells compared to

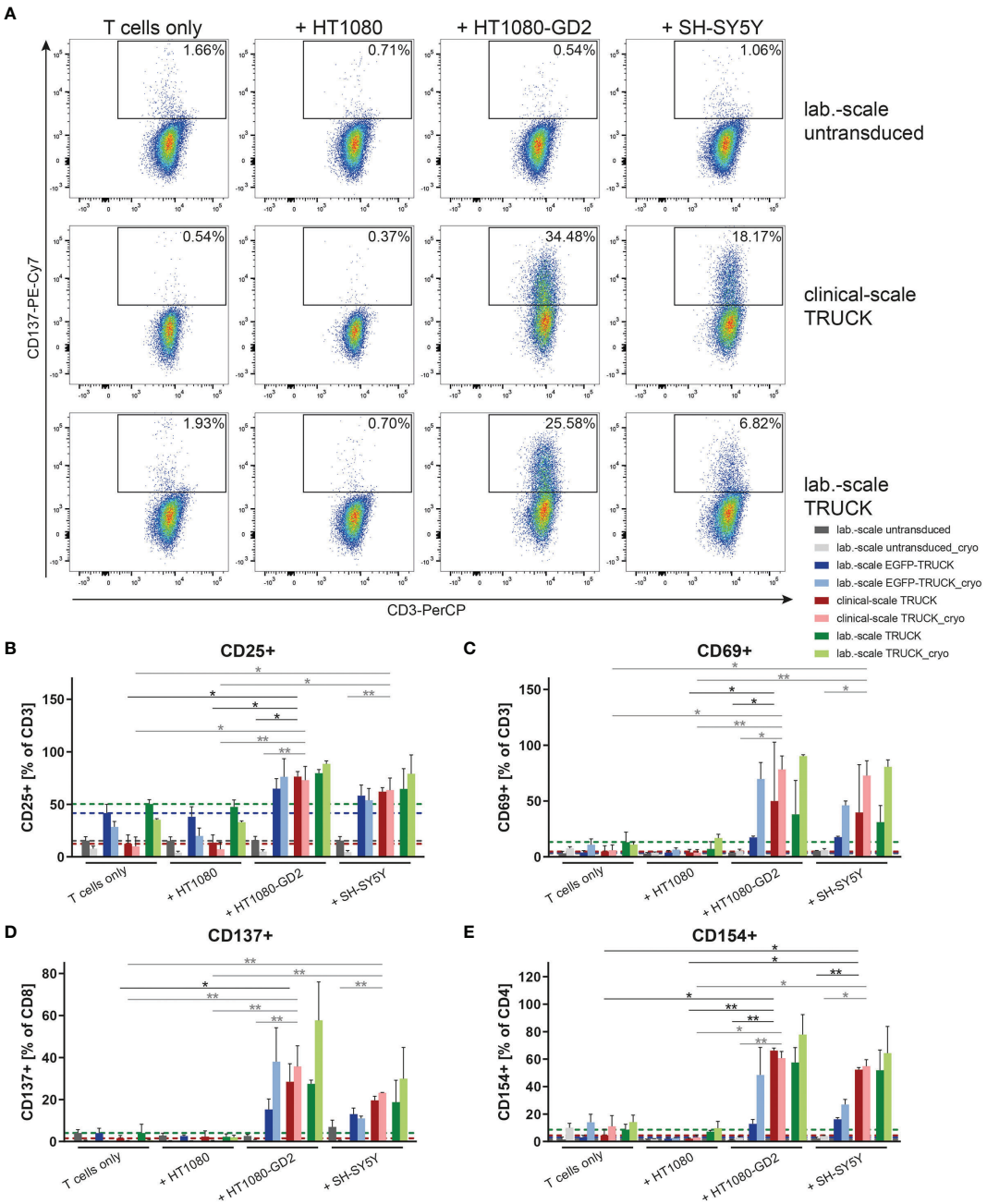

FIGURE 4 | Clinical-scale-manufactured IL-18 TRUCKs targeting GD_2 specifically respond to GD_2^+ target cells with an increase of activation marker expression. IL-18 TRUCKs targeting GD_2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK; n=2) or under laboratory conditions (lab.-scale TRUCK; n=3). Untransduced T cells (lab.-scale untransduced; n=3) as well as GD_2 TRUCKs with inducible EGFP expression (EGFP-TRUCK; n=3) served as control. The manufactured cells were tested for GD_2-CAR-mediated activation either directly after the generation process (d12) or after cryopreservation and thawing (cryo) by co-cultivation with the indicated target cells for 48h in an effector-to-target (E:T) ratio of 4:1 or cultivation alone (T cells only). **(A–E)** Frequency of **(A)** $CD137^+$ of $CD8^+$ as representative plots**, (B)** $CD25^+$ of $CD3^+$, **(C)** $CD69^+$ of $CD3^+$, **(D)** $CD137^+$ of $CD8^+$ and **(E)** $CD154^+$ of $CD4^+$ T cells as determined by flow cytometry. **(B–E)** A dashed line indicates background levels of the respective expression by untransduced T cells (grey), EGFP-TRUCKs (blue), as well as clinical-scale (red) and laboratory-scale (green) TRUCKs cultured alone. Data is shown as mean ± SD. Statistical differences of clinical-scale TRUCKs co-cultured with different target cells after generation or cryopreservation as well as in comparison to laboratory-scale manufactured cells were assessed by Kruskal-Wallis and Dunn's test, significant differences are shown (*$p \leq 0.05$, **$p \leq 0.01$).

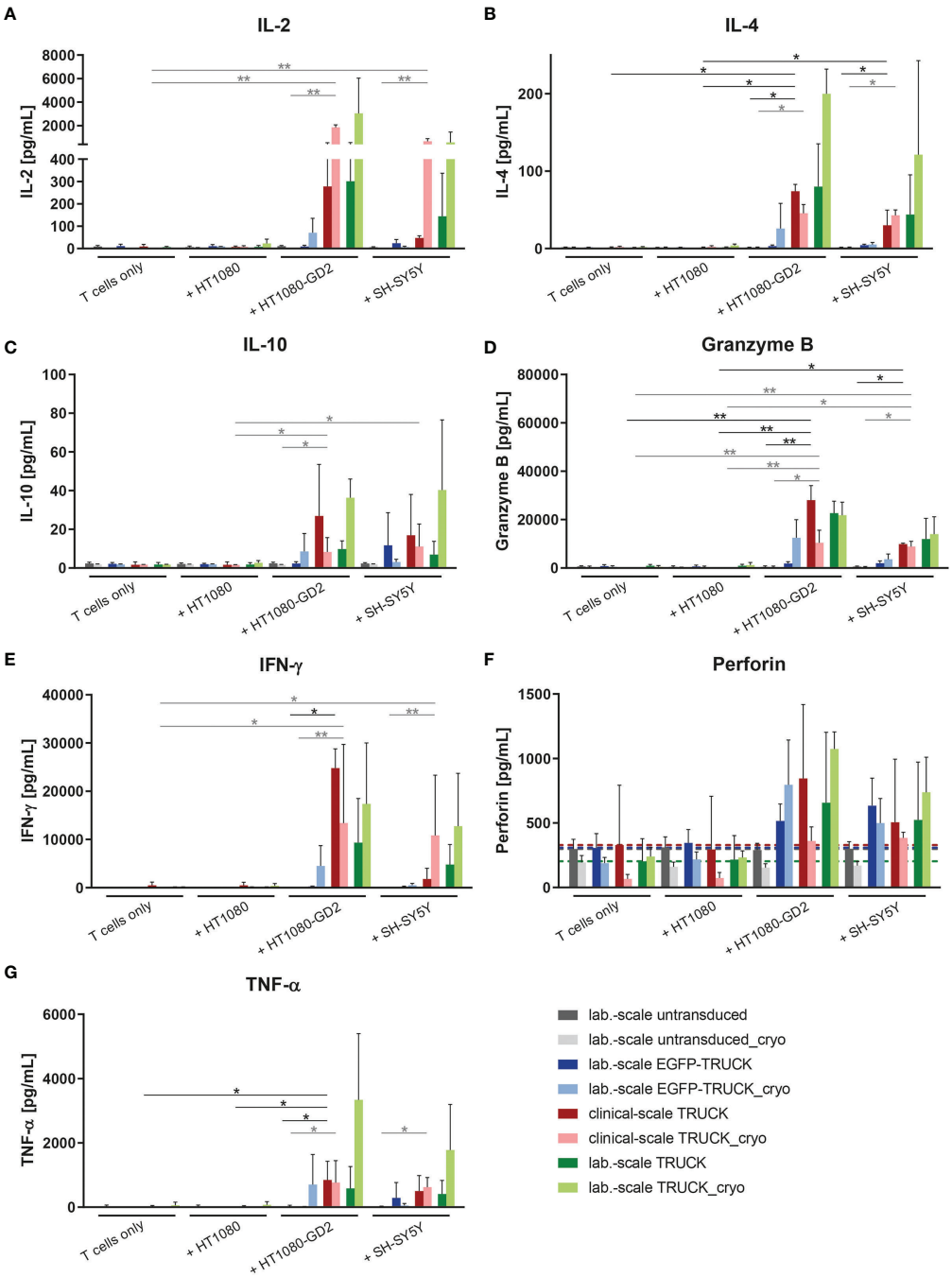

FIGURE 5 | Clinical-scale-manufactured IL-18 TRUCKs targeting GD_2 specifically react to target recognition with increased release of soluble mediators. IL-18 TRUCKs targeting GD_2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK; n=2) or under laboratory conditions (lab.-scale TRUCK; n=3). Untransduced T cells (lab.-scale untransduced; n=3) as well as GD_2 TRUCKs with inducible EGFP expression (EGFP-TRUCK; n=3) served as control. The manufactured cells were tested for functionality either directly after the generation process (d12) or after cryopreservation and thawing (cryo) by co-cultivation with the indicated target cells in an effector-to-target (E:T) ratio of 8:1 or cultivation of T cells only. The concentration of released cytokines **(A)** IL-2, **(B)** IL-4, **(C)** IL-10, **(D)** granzyme B, **(E)** IFN-γ, **(F)** perforin, and **(G)** TNF-α in the cell culture supernatants after 48 h was assessed by LEGENDPlex™. **(F)** A dashed line indicates background levels of the respective cytokine release by untransduced T cells (grey), EGFP-TRUCKs (blue), as well as clinical-scale (red) and laboratory-scale (green) TRUCKs cultured alone. **(A–G)** Data are shown as mean ± SD. Statistical differences of clinical-scale TRUCKs co-cultured with different target cells after generation or cryopreservation as well as in comparison to all laboratory-scale manufactured cells were assessed by Kruskal-Wallis and Dunn's test, significant differences are shown (*$p \leq 0.05$, **$p \leq 0.01$).

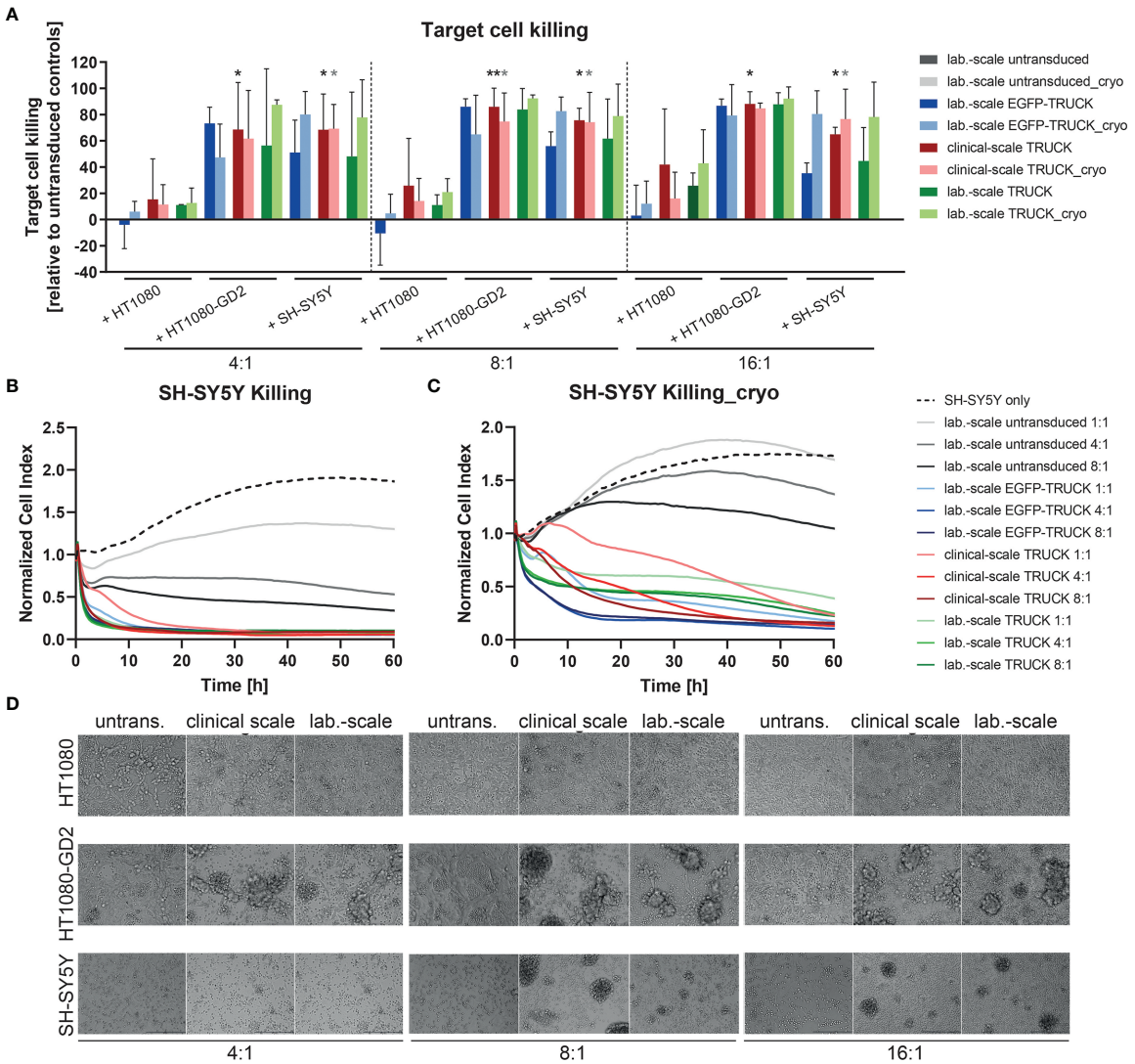

FIGURE 6 | Clinical-scale-manufactured IL-18 TRUCKs targeting GD_2 specifically eliminate GD_2^+ target cells. IL-18 TRUCKs targeting GD_2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK; n=2) or under laboratory conditions (lab.-scale TRUCK; n=3). Untransduced T cells (lab.-scale untransduced; n=3) as well as GD_2 TRUCKs with inducible EGFP expression (EGFP-TRUCK; n=3) served as control. The manufactured cells were tested for cytotoxicity either directly after the generation process (d12) or after cryopreservation and thawing (cryo) by co-cultivation with the indicated target cells and in the indicated effector-to-target (E:T) ratios. **(A)** Target cell killing after 48h was measured by flow cytometry as percentage of killed $CD3^-$ cells relative to those eliminated in co-cultures with untransduced T cells. Data is shown as mean ± SD. Statistical differences of large-scale TRUCKs co-cultured with different target cells directly after generation or cryopreservation as well as in comparison to all laboratory-scale manufactured cells were assessed by Kruskal-Wallis and Dunn's test, whereby only significant differences are shown (*$p \leq 0.05$, **$p \leq 0.01$). **(B, C)** Killing of SH-SY5Y cells by the generated cells **(B)** directly after their generation (here: lab.-scale untransduced n=2, clinical-scale TRUCK n=1) or **(C)** after cryopreservation and thawing was determined with the XCelligence Real-Time Cell Analyzer. Cell indices were normalized to the respective indices after T cell addition. Data is shown as mean. **(D)** Representative transmitted-light microscope images of co-cultures of fresh effector cells with target cells taken after 48 h by an Olympus IX81 microscope combined with a digital B/W camera using 10x objective lenses.

untransduced T cells, EGFP-TRUCKs or upon co-culture with GD_2^- target cells, likely as a result of induced IL-18 cytokine secretion (**Figures 7C, D**). Taken together, supernatants from TRUCKs releasing IL-18 in an inducible manner by CAR activation upon antigen recognition exhibit an innate immune cell recruitment potential *in vitro* indicating IL-18 biological activity.

DISCUSSION

In general, CAR and TRUCK vector design is a critical aspect for the generation of engineered T cells. Especially, the costimulatory domains within the CAR might be of high interest to improve CAR T-cell activity and long-lasting persistence with reduced T cell exhaustion, also with respect to the targeted tumor antigen

(37). TRUCKs additionally combine the redirected CAR T cell attack with the on-site release of a biologically active protein while avoiding its systemic toxicity, thereby holding promise to modulate the environment of the targeted solid tumor. The cytokine of choice within the TRUCK concept should be chosen with respect to the desired immune response within the tumor microenvironment (TME). The IL-18 cytokine creates a proinflammatory environment, recruits bystander effector cells to the tumor site and enhances cytolytic activity (4–6). Therefore, IL-18 was an attractive cytokine for the development of the "all-in-one" lentiviral vector combining constitutive anti-GD_2 CAR expression and inducible IL-18 (26) as well as for the automated and closed processing for GMP-compliant manufacturing process for CAR T cells (24, 25).

TRUCKs combine the redirected CAR T cell attack with the on-site release of a biologically active protein while avoiding its systemic toxicity, thereby holding promise to modulate the environment of the targeted solid tumor. Further development of the strategy is based on design and broad *in vitro* characterization of the "all-in-one" lentiviral vector combining constitutive anti-GD_2 CAR expression and inducible IL-18 (26) as well as on an automated and closed processing for GMP-compliant manufacturing process for CAR T cells (24, 25). We present the proof of feasibility for translation of the method to activate and expand IL-18 TRUCKs targeting GD_2 for clinical application.

Our protocol is optimized to produce CAR-engineered T cells in clinically sufficient numbers under GMP-compliance using the CliniMACS Prodigy® platform that integrates different steps of manufacturing including cell isolation, activation, transduction, cell washing, cultivation and formulation of the final product in a single process and thus minimizes variability emanating from various manual work steps. The fully integrated modular system allows for flexibility and standardized procedure at the same time, which is the key for the production of personalized cell products of various kinds. Multiple steps are required to produce gene modified effector cells starting with enrichment of $CD4^+$ and $CD8^+$ T cell subset followed by activation, transduction and expansion of effector cells. $CD4^+$ and $CD8^+$ enrichment is regarded as a safety procedure to decrease blast counts in the culture (38). $CD4^+$ and $CD8^+$ enrichment also decreases contaminating cells such as monocytes, which inhibit CAR T cell expansion (39). The primary objective was the feasibility of cell production for 3 dose levels (e. g. 5×10^5, 1×10^6, and 3×10^6 anti-GD_2 IL-18 TRUCKs/kg). For enrichment of $CD4^+$ and $CD8^+$ cells the TCT process is limited to a maximum of 3×10^9 target cells. This means for our two processes that only a part (20.6% D1 and 46.1% D2) of the lymphapheresis from a healthy donor was used for enrichment. Likewise, only part of enriched cells (12.5% D1 and 10.0% D2) could be applied for activation and expansion. Due to the limited culture volume and growth area, it is recommended to start with 1×10^8 cells. Any remainder may be frozen as backup for the patient.

After $CD4^+$ and $CD8^+$ enrichment we found high T cell purities with low contaminating cell populations of $CD8^+$ NK and NKT cells which are not removed during the $CD4^+$ and $CD8^+$ enrichment step also described by other groups (23, 38). With a T cell expansion rate of 68.3-fold D1 and 71.4-fold D2 and a transduction rate of 77.7% D1 and 55.1% D2 we reached sufficient cell doses of 4.8×10^9 D1 and 3.7×10^9 D2 IL-18 TRUCKs. This would allow for the application of 60.0×10^6 cells/kg respectively 46.0×10^6 cells/kg IL-18 TRUCKs targeting GD_2 to a recipient weighing 80 kg even in a multi-dose base. We and others show the eligibility of the T cell transduction (TCT) process developed by Miltenyi Biotec with fixed process parts (enrichment, activation and transduction) as well as the possibility of variable culture set up by modular programming of the activity matrix (22–25, 38, 40–43).

Safety aspects in the clinical use of Advanced Therapy Medicinal Products (ATMPs) have high priority. Lentiviral vectors are known to have a lower risk for mutational oncogenesis than γ-retroviral vectors (38). The European Medicines Agency (28) reflection paper (EMA/CAT/190186/2012) on the management of clinical risks deriving from insertional mutagenesis highlighted the VCN as a risk factor for oncogenesis and recommended risk assessment and management of the integration copy numbers, integration profile and sites in cellular products. The IL-18 TRUCK final products contained 2.6 D1 and 2.4 D2 copies/cell, respectively, which is below 5 copies/cell that is considered to be safe (44). The transduction rate of 74.9% D1 and 52.2% D2 in the final products was higher than reported by other groups using CliniMACS Prodigy® for production of CAR T cells (22, 23, 25, 38, 40–43, 45). Transduction efficiency was higher for $CD4^+$ compared to $CD8^+$ T cells as also shown by previous reports (25, 43).

To prolong the *in vivo* persistence of CAR T cells in patients, enrichment of cell products with less differentiated T cell subsets such as central memory (T_{CM}) or stem cell memory T (T_{SCM}) T cells is thought to be crucial. These subpopulations have gained substantial attention, as the adoptive transfer of even low numbers of T cells from these subsets can reconstitute robust and long-term maintained immune responses (46, 47). IL-18 TRUCK final product T cells from Donor 1 showed a T_{cm}/T_{scm} phenotype while Donor 2 showed predominantly a T_{EM} phenotype. CD3/CD28 activation and culture of naïve T (48) cells in presence of IL-7 and IL-15 promotes the acquisition of T_{CM} or T_{SCM} phenotypes (22, 23, 38, 49). This is in line with previous reports (23, 43). In addition, whereas T cells expressing CARs with CD28 domains predominantly differentiate into effector memory T (T_{EM}) cells, *in vitro* expansion of 4-1BB-containing CAR T cells as the TRUCKs used here produces a higher proportion of T_{CM} cells (50). We also demonstrate, that the TRUCK manufacturing process did not lead to unspecific secretion of IL-18 even after T cell activation with TransAct (CD3/CD28) and cytokines (IL-7 and IL-15).

In vitro characterization of the obtained engineered T cell product revealed an upregulation of activation markers (CD25, CD69, CD137 and CD154) on both $CD4^+$ and $CD8^+$ T cells and a variety of pro-inflammatory cytokines and cytotoxic mediators (IL-2, IL-4, granzyme B, perforin, IFN-γ, TNF-α) upon specific recognition of GD_2-expressing target cells, but not after co-cultivation with a control cell line lacking the target. In addition, TRUCKs induced release of the engineered cytokine

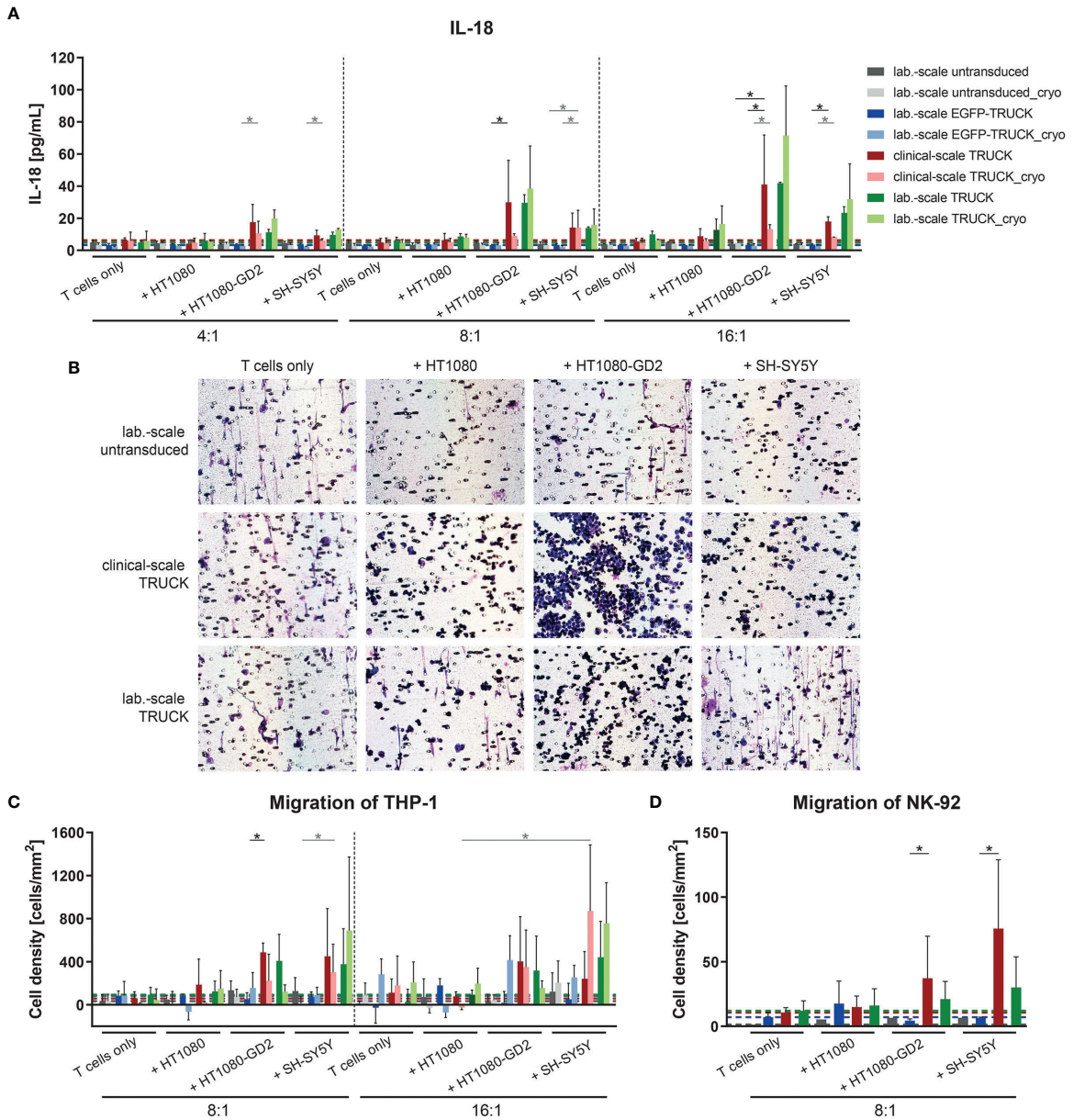

FIGURE 7 | Clinical-scale-manufactured IL-18 TRUCKs targeting GD_2 released IL-18 in a target-specific manner leading to innate immune cell attraction. IL-18 TRUCKs targeting GD_2 were generated using the CliniMACS Prodigy® (clinical-scale TRUCK; n=2) or under laboratory conditions (lab.-scale TRUCK; n=3). Untransduced T cells (lab-scale untransduced; n=3) as well as GD_2 TRUCKs with inducible EGFP expression (EGFP-TRUCK; n=3 [except for D, in which n=1]) served as control. The manufactured cells were tested for functionality either directly after the generation process (d12) or after cryopreservation and thawing (cryo) by co-cultivation with the indicated target cells in the indicated effector-to-target (E:T) ratios or cultivation alone (T cells only). **(A)** The concentration of released cytokines in the cell culture supernatants after 48h was assessed by LEGENDPlex™. The chemoattractive potential of IL-18 released by TRUCKs upon target recognition in terms of the migration of **(B, C)** THP-1 and **(D)** NK-92 cells was assessed. Supernatant of engineered T cells cultured alone (T cells only) or together with the indicated target cells for 48 h was placed in a Boyden chamber, covered with an 8 µm polycarbonate membrane and incubated for another 4h. Medium served as the control supernatant. Cells migrated through the membranes were Giemsa stained. **(B)** Representative pictures of Giemsa stained THP-1 cells (violet) on the bottom of the membrane. **(C, D)** The number of cells that migrated through the membrane was determined. To normalize results from different plates, cell numbers of migrated cells towards untransduced T cells only were subtracted from all values. **(A, C, D)** Data is shown as mean ± SD. A dashed line indicates background levels of the respective cytokine release by untransduced T cells (grey), EGFP-TRUCKs (blue), as well as clinical-scale (red) and laboratory-scale (green) TRUCKs cultured alone. Statistical differences of large-scale TRUCKs co-cultured with different target cells after generation or cryopreservation as well as in comparison to laboratory-scale manufactured cells were assessed by Kruskal-Wallis and Dunn's test, significant differences are shown (*$p \leq 0.05$).

IL-18 in an antigen-dependent manner and mediated a very low background secretion upon co-cultivation with target-negative cells or spontaneous release without target cells. The risk of toxicity for IL-18 TRUCKs is different to IL-12 TRUCKs that revealed severe toxicity of T cells engineered with inducible IL-12 in a melanoma mouse model due to off-target cytokine secretion (4). However, other studies with IL-12 TRUCKs reported a safe administration into mice and such toxicities were not observed for IL-18 TRUCKs (2, 3, 5, 51). GMP-compliant manufactured IL-18 TRUCKs targeting GD_2 showed a high cytotoxic capability and were able to eliminate tumor cells while forming clusters around target cells. IL-18 TRUCKs show higher toxicities against high GD_2-expressing target cells confirming the *in vitro* studies by Wiebel et al. (52).

To accurately quantify and confirm cytotoxicity towards target cells, we combined different methodologies including the label-free, real-time monitoring by impedance measurements, flow cytometric analysis allowing for concurrent phenotypic evaluation of TRUCKs, and detection of pro-inflammatory cytokines as indirect and cytotoxic mediators as direct parameter of cell lysis in the co-culture. Kiesgen (53) *et al.* comprehensively compare the power and limitation of different cytotoxicity assays and emphasize that especially impedance-based assays display a superior sensitivity and signal-to-background ratio over the "gold standard" ^{51}chromium-release assay making it possible to evaluate even low E:T ratios as most appropriate to resemble physiological conditions. Such low E:T ratios would be interesting to evaluate in further experiments, since the manufactured TRUCKs exhibited rapid elimination of SH-SY5Y cells in the lowest E:T ratio of 1:1. Moreover, *in vitro* tests using repeated antigen stimulation or inclusion of immunosuppressive factors present in the TME are increasingly being used and could give an insight about persistence and exhaustion level of the generated TRUCKs upon high antigen stress.

We show similar manufacturing of clinical-scale and laboratory-scaled IL-18 TRUCKs concerning transduction and amplification efficiency and cellular functionality. After cryopreservation of the T cell products, the specificity and cytotoxicity of TRUCKs was maintained. Attempts to treat solid tumors with redirected T cells have largely failed so far, with very few patients responding and with only transient and partial tumor regression (17, 18, 48, 54–57). The poor clinical outcome is thought to be due at least in part to an unfavorable environment in the tumor tissue that suppresses CAR T cell responses. TRUCK-secreted cytokine IL-18 led to increased recruitment of monocytes and NK cells in an *in vitro* cell migration assay. This may contribute to reprogramming the tumor stroma towards a more favorable environment for CAR T cell function, thereby enhancing their efficacy in the treatment of solid tumors. Furthermore, IL-18 was shown to polarize TRUCKs towards more potent pro-inflammatory effector cells that do not drive into functional exhaustion in the long term (5).

In conclusion, GMP-compliant manufacturing of IL-18 TRUCKs targeting GD_2 using the automated closed CliniMACS Prodigy® system is feasible and enables the manufacturing of a sufficient number of cells for clinical application. The automatic mode of operation improves standardization and robustness of the manufacturing process. This benefits the manufacturing at different sites for an academia-initiated multicenter trial. The smooth adaption of the process established and validated for the manufacturing of CAR T cells to generate IL-18 TRUCKs encourages the translation of the procedure to other cells and targets.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

For this study the donors gave their written informed consent and approval was granted by the corresponding ethics committee (No. 2829-2015). The study was approved by the ethics committee of Hannover Medical School (2519-2014, 2830-2015, 3639-2017).

AUTHOR CONTRIBUTIONS

Conceptualization: RE, WG, BE-V, AD, UK, KZ, and ASc. Methodology: AD, WG, RE, MM, AM-E, CK, ASt, KZ, and ASc. Formal analysis: WG, AD, RE, AM-E, MM, CK, ASt, KZ, and KA. Resources: HL, NS, and CR. Writing original draft application: WG, AD, RE, and AM-E. Writing review and editing: all authors. Supervision: HA, LA, TM, RB, LG, ASc, BE-V, and UK. Funding acquisition: BE-V, HA, TM, RB, ASc, UK, and CR. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.839783/full#supplementary-material

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Conflict of Interest: HA, ASc, and KZ have submitted a patent application describing the "all-in-one" TRUCK vector technology. The University Medicine of Greifswald licensed Ganglidiomab to AnYxis Immuno-oncology for commercialization.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Leukemia's Next Top Model? Syngeneic Models to Advance Adoptive Cellular Therapy

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In recent years, there has been an emphasis on harnessing the immune system for therapeutic interventions. Adoptive cell therapies (ACT) have emerged as an effective option for B-cell derived hematological malignancies. Despite remarkable successes with ACT, immune dysregulation and the leukemia microenvironment can critically alter clinical responses. Therefore, preclinical modeling can contribute to the advancement of ACT for leukemias. Human xenografts, the current mainstay of ACT *in vivo* models, cannot evaluate the impact of the immunosuppressive leukemia microenvironment on adoptively transferred cells. Syngeneic mouse models utilize murine tumor models and implant them into immunocompetent mice. This provides an alternative model, reducing the need for complicated breeding strategies while maintaining a matched immune system, stromal compartment, and leukemia burden. Syngeneic models that evaluate ACT have analyzed the complexity of cytotoxic T lymphocytes, T cell receptor transgenics, and chimeric antigen receptors. This review examines the immunosuppressive features of the leukemia microenvironment, discusses how preclinical modeling helps predict ACT associated toxicities and dysfunction, and explores publications that have employed syngeneic modeling in ACT studies for the improvement of therapy for leukemias.

Keywords: adoptive cell immunotherapy, leukemia, syngeneic animal model, leukemia microenvironment, cell therapy

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Abbreviations: ALL, Acute lymphocytic leukemia; AML, Acute myeloid leukemia;ACT, Adoptive cell therapy; αβ, Alpha-beta; CARs, Chimeric antigen receptors; CLL, Chronic lymphocytic leukemia; CML, Chronic myeloid leukemia; CTL, Cytotoxic T-lymphocyte; DC, Dendritic cell; FMuLV, Friend murine leukemia virus; FBL-3, Friend virus-induced erythroleukemia; γδ, Gamma-delta; GEMM, Genetically engineered mouse model; HSC, Hematopoietic stem cell; HLH, Hemophagocytic lymphohistiocytosis; HGF, Hepatocyte growth factor; IDO, Indoleamine 2,3-dioxygenase; IL, Interleukin; LSC, Leukemia stem cell; MSCs, Mesenchymal stromal cells; mCD19-CAR, Murine CD19-CAR; MDSC, Myeloid derived suppressor cells; NK, Natural killer; PDX, Patient derived xenograft; scFv, Single chain variable fragment; TCRs, T cell receptors; Tregs, T regulatory cells; TGF- β, Transforming growth factor β; TIL, Tumor-infiltrating lymphocyte; TME, Tumor microenvironment; LSTRA, Virally-induced syngeneic leukemia.

INTRODUCTION

Adoptive cell therapy (ACT) is the expansion and infusion of immune cells, including natural killer (NK) cells, gamma-delta (γδ) T cells, and alpha-beta (αβ) T cells, into patients for therapeutic benefit. Advancements in the field of ACT have resulted in engineered cellular products that express performance-enhancing receptors, such as cytokine receptors, T cell receptors (TCRs), or chimeric antigen receptors (CARs). Overall, the innovation of genetically engineered immune cells in the ACT setting has resulted in improved outcomes, especially for patients with B-cell derived hematologic malignancies (1, 2). However, challenges remain like antigen selection and overcoming an immunosuppressive microenvironment (3–5).

Despite promising preclinical ACT data, patients can fail to respond to treatment once a strategy is translated into the clinic. One of the many factors contributing to failed ACT is a highly immunosuppressive tumor microenvironment (TME), causing adoptively transferred cells to become dysfunctional or exhausted (6). Structural components, soluble factors, and immune cells found within the leukemic TME contribute to a hostile environment in the bone marrow niche, which poses a threat to adoptively transferred cells. Leukemic blasts can reprogram both the structural components of the microenvironment and the function of immune cell populations, allowing for a more favorable environment for leukemia progression (7).

These immune interactions are understudied in preclinical ACT models. The most frequently used preclinical model to determine ACT anti-leukemia activity is a xenograft model. Xenograft models assess human cellular products against human cells, making it feasible to study multiple human-derived cell lines with different genetic drivers of leukemogenesis. However, xenografts lack a functional immune system and tumor heterogeneity found in leukemia patients preventing them from having the necessary rigor to predict clinical responses (8, 9). Therefore, there is room for improvement in preclinical modeling to achieve continued development of effective immunotherapies for leukemias.

Syngeneic models encompass allografts of mouse tumors in immunocompetent mice. This allows for evaluation of toxicities, including on-target/off-tumor side effects, and the immunosuppressive microenvironments (8). Immunocompetent models have not been readily adapted to ACT studies, in part, because of the difficulty to isolate and expand murine immune cells, lack of homology between targeted proteins, and cross-reactivity of CAR T cells' single chain variable fragments (scFv). Despite inherent hurdles to establishing syngeneic models, they provide an avenue to ensure the optimization of ACT against hematological malignancies. In this review, we evaluate the role of the bone marrow niche and leukemia microenvironment on leukemogenesis, assess available models to test ACT, and discuss literature that utilizes syngeneic modeling to evaluate ACT.

BONE MARROW NICHE/LEUKEMIA MICROENVIRONMENT

The bone marrow microenvironment is essential for the pathogenesis and progression of leukemias (10, 11). The niche provides a physical sanctuary site for developing rare populations of leukemic cells and harbors an immunosuppressive environment that downregulates the natural immune surveillance required to eliminate tumor cells successfully. When choosing a model of ACT, it is essential to consider structural and immune components within each *in vivo* model (**Figure 1**). As investigators design new cellular immunotherapies, there has been an emphasis on understanding the impact the TME will have on therapeutic success.

There are two major patterns in leukemia relapse: i) the initial clone gains mutations; or ii) a subclone survives initial treatments (12). In most cases the relapse clone is characterized as a leukemia stem cell (LSC) (13). LSCs have distinct properties from bulk leukemic cells, such as limitless self-renewal and initiation of leukemia. LSCs are inherently less susceptible to traditional chemotherapeutics and can escape immune surveillance (14).

Structurally, the bone marrow contains two anatomically different hematopoietic stem cell (HSC) niches, known as the central and endosteal, that are crucial for the production and maintenance of healthy HSCs (15, 16). Within these compartments, HSCs are regulated by endothelial, osteoblastic, and stromal cell components, specifically mesenchymal stromal cells (MSCs) (17, 18). MSCs are multipotent cells that make up most of the structural components of the bone marrow stroma. In a leukemic state, MSCs play a large role in the leukemia pathogenesis through two major mechanisms: 1) providing physical protection of leukemic cells and 2) reprogramming of bone marrow niche (14, 19). Thus, MSCs and the bone marrow stroma play an important role in leukemia progression and relapse but are not commonly considered in most models of ACT.

Soluble factors, such as cytokines, chemokines, and enzymes, are important components of the TME that suppress the endogenous immune response and support leukemia progression. Compared to the healthy bone marrow landscape, leukemia cytokine signatures show an increase in transforming growth factor β (TGF-β) and hepatocyte growth factor (HGF) levels (20). These two factors help mediate T cell suppression and reduce expression of NK cells (20, 21). Increases in anti-inflammatory cytokine, interleukin (IL)-10, are observed in a variety of leukemia models, and often limit ACT functionality (22). Chemokines play an important role in both trafficking of leukemic cells and cellular immunotherapies. The CXCL12-CXCR4 chemokine pathway, is involved in the homing of HSCs within the bone marrow (23). CXCL12 secreted by the bone marrow along with the upregulated expression of CXCR4 on leukemia blasts increases the homing of tumor cells to the bone marrow (24). Once the leukemic blasts are within the bone marrow niche, they are protected structurally, capable of secreting anti-inflammatory soluble factors, and dysregulating immune cell populations.

In addition, concentrations of certain enzymes within the bone marrow contribute to leukemia progression (23, 24). Blasts mediate expression of arginase II, promoting a low arginine microenvironment. The limited arginine drives monocytes to a suppressive phenotype while suppressing T cell expansion (5). Indoleamine 2,3-dioxygnenase (IDO) is also released by blasts, which converts CD4+ T cells into T regulatory cells (Tregs), thus enhancing the suppressive capacity of the microenvironment (5).

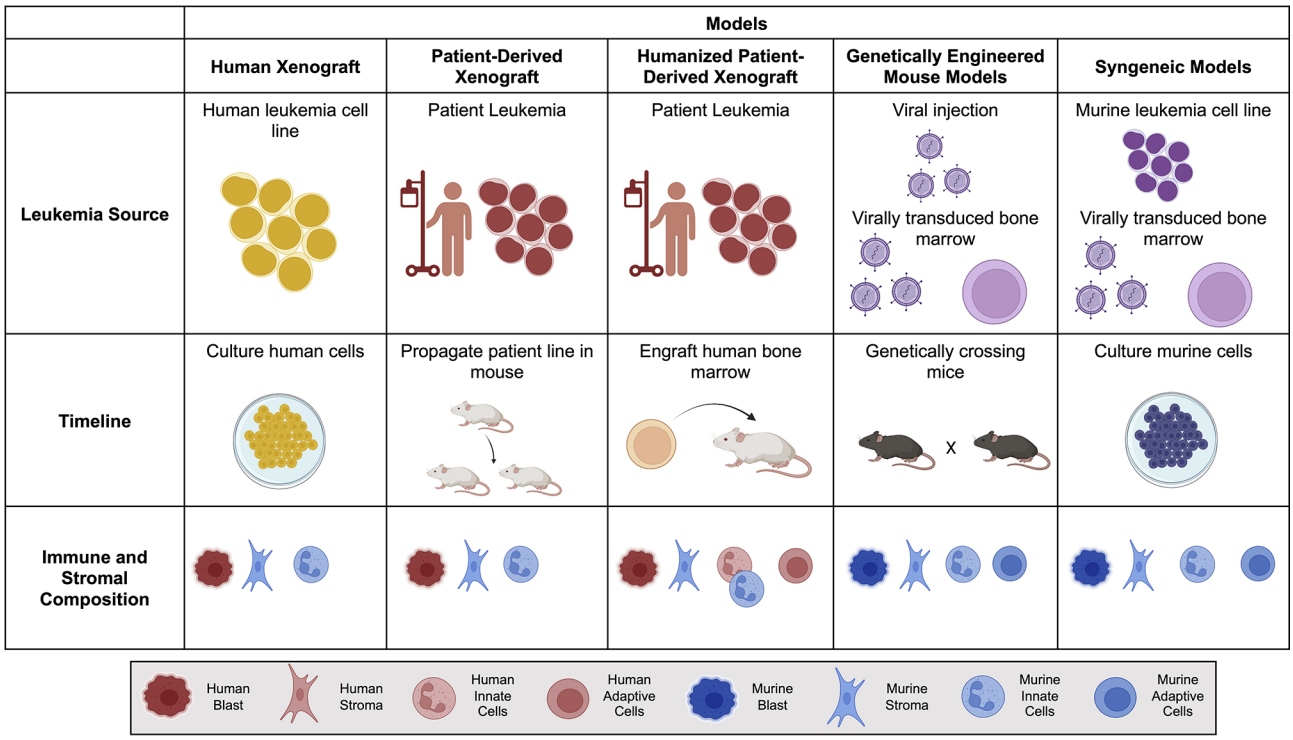

FIGURE 1 | Comparison of mouse models for Adoptive Cell Therapy (ACT). Brief overview of the leukemia source, timeline to propagate and test tumor model, and immune and stromal composition of each mouse model. Human is denoted by warm colors (yellow, orange, red) and murine is denoted by cool colors (blue and purple). Created with BioRender.com.

Leukemia-induced remodeling of the TME alters the structural and chemical components within the bone marrow and influences immune cell populations. The leukemia microenvironment comprises innate (dendritic cells [DC] and macrophages) and adaptive (myeloid derived suppressor cells [MDSCs], Tregs, and NK cells) immune cells. Leukemic cells hinder the maturation of DCs, often promoting immune tolerance and thus inducing the development of Tregs (25). TME-associated macrophages can be inhibitory or stimulatory, but their inhibitory function diminishes the anti-tumor activity of adoptively transferred cells within the TME (26). MDSCs arise from myeloid progenitors and are a subset of immature myeloid cells that lead to NK-cell dysfunction and recruitment of Tregs, among other immunosuppressive cells (27, 28). They are difficult to model and contribute to the failure of many AML therapeutic interventions, making them a potential therapeutic target.

The bone marrow microenvironment plays an aggressive role in leukemia progression, highlighting the importance of using preclinical models to evaluate interactions between the host immune system, leukemic TME, and adoptively transferred cells. However, there is limited work analyzing the contribution of the immune system and microenvironment on effective cellular therapies for leukemia. The advancement of these interventions relies on the active exploration and adaptation of preclinical modeling, and especially in the syngeneic context.

PRECLINICAL LEUKEMIA MODELING FOR ACT

Xenograft Models

The initial development of ACTs for leukemias has been aided by using human xenografts. These models have allowed functional evaluation of human cell therapy products against human cell lines or patient tumors (patient-derived xenograft- PDX). In addition, they have facilitated high throughput screening of many ACT interventions. PDXs provide a heterogenous leukemia model but lack a comprehensive and intact immune system required to adequately study ACT interventions. In addition, human T cells can recognize mouse xenoantigens in this setting, increasing the risk of graft-versus-host disease. Alternatives to human xenografts include using humanized PDXs, genetically engineered mouse models (GEMMs), and syngeneic mouse models (8, 9).

Humanized Models

PDXs are the best option to increase the heterogeneity of leukemic burden. The major difference between a humanized or non-humanized PDX model is the reconstitution of human immune cells in the immunocompromised mouse (29). Immune reconstitution is not maintained for long periods due to the high turnover of bone marrow cells and decreased engraftment of human cells within a mouse (29, 30). There is no guarantee that each immune population will reconstitute within the mouse,

leading to differences in the humanized immune system and an inaccurate or incomplete representation of an immune system (30). In humanized models, there is an added challenge of patient leukemia cells engrafting within the same timeframe of complete immune cell reconstitution. Humanized PDXs are time-consuming to establish with a low yield of implantation and are not reliable when screening multiple interventions in a timely manner.

Genetically Engineered Mouse Models

GEMMS are unique murine models which genetically manipulate the somatic activation of oncogenes or inactivation of tumor suppressors to elicit *de novo* tumor development (31). Leukemias that arise from these genetic alterations typically mimic histological and molecular features of human disease. An advantage to this model is the maturation of leukemia cells within an immunocompetent host. This allows researchers to analyze scenarios of immune pressure on leukemia development which can then result in genomic instability. While this strengthens the tumor heterogeneity within the system, murine leukemia can mature to express unique tumor-associated antigens between mice with the same genetic manipulation (9). This makes studies utilizing GEMMs challenging to reproduce because of their genetic drift within "equivalent" models. Because ACT, such as CAR T cells, rely on targeting a tumor antigen on the surface of leukemia cells, GEMMs do not provide the consistency to measure antigen specificity. Also, without knowing the surface proteins on each mouse in a GEMM experiment it is difficult to study immune escape mechanisms, such as lineage switch or antigen down-regulation (9). Additionally, these systems can be unpredictable with variable latencies and penetrance.

Syngeneic Mouse Models

Syngeneic models use murine cell lines or virally-transduced murine HSCs to express genes of interest (i.e., oncogene amplification, knock out tumor suppressors, overexpress fusion proteins) that result in leukemia initiation (32). They do not require the complex breeding necessary in GEMMs but do not have the advantage of leukemic development within the native immune system. They offer a rigorous option to test cellular immunotherapies due to their rapid growth, reproducibility, intact immune system, and hostile leukemia microenvironment. However, they can lack heterogeneity and there are few readily available leukemia options (9).

To bypass the lack of immune system in human leukemia models, the complicated breeding of GEMMs, and the difficulty in generating relevant syngeneic leukemia tumor models, researchers have expressed human antigens on readily available murine ALL tumor cells to test CAR T cells (8, 9, 32). However, this is confounded by the potential of the mouse's endogenous immune response to recognize the human antigen, making it difficult to discern the cause of the autoimmune response (8, 9, 32). It is noteworthy that GEMMs and syngeneic models utilize mouse biology to draw conclusions on human therapeutic interventions. In addition, they are difficult to adapt to replicate cell-based immunotherapy for hematologic malignancies successfully. Despite this, given the significant contributions the immune system and TME have on leukemia progression and relapse, it is essential to accurately mimic these systems for successful ACT evaluation.

SYNGENEIC MODELS EVALUATING ACT FOR HEME MALIGNANCIES

There are limited syngeneic leukemia models that have been used for ACT evaluation. While several murine B-ALL models exist, the C1498 cell line has served as one of the only commercially available murine AML cell lines and has been sparingly used in ACT research (33–35). The studies presented below highlight their utility, allowing for toxicity evaluation as well as the careful mechanistic dissection of ACT.

Syngeneic Models Evaluating Cytotoxic T-Lymphocyte (CTL) or Transgenic TCR Responses

As early as 1981, investigators used *in vitro* sensitization or immunization to generate murine lymphocytes specific to murine tumor antigens. Cheever et al. isolated splenocytes from BALB/c mice and cultured them *in vitro* in the presence of virally-induced syngeneic leukemia (LSTRA), an ascitic lymphoma originally induced in newborn BALB/c mice with Moloney leukemia virus. This exposure was meant to sensitize the murine lymphocytes to LSTRA (36). They did not find any direct evidence supporting a link between *in vitro* culture of lymphoid cells with LSTRA and increased antitumor activity *in vivo*. However, they observed that depletion of the T cell population diminished antitumor effectors, demonstrating the importance of T cells in antitumor activity (36). Subsequently, another syngeneic leukemia model expanded tumor-specific T cells from spleens of FBL-3 (friend virus-induced erythroleukemia) *ex vivo*. Although they determined that immunized mice responded to antigen *in vivo*, they also observed that antigen naive T cells extracted from mice became dependent on IL-2, limiting the therapeutic potential of the T cells. They overcame this hurdle by exposing the extracted T cells to anti-CD3 antibody and IL-2 (37). These studies were instrumental in the advancement of tumor-infiltrating lymphocyte (TIL) therapy tumor models.

Several syngeneic systems have been used to assess leukemia-specific immune responses. Mumprecht et al., for example evaluated responses to 2 different CML models including a chronic (BCR/ABL) and a blast crisis CML (BCR/ABL-NUP98/HOXA9) model that had been previously described (38, 39). Mumprecht et al. determined that mice that received a lower tumor burden and had disease elimination developed a LCMV-gp33- specific CTL response, while mice that had CML progression lacked persistence of CTLs (40). In a follow up study, they determined that elimination of CD8+ T cells in a CML model led to disease progression and that IL-7 secreted by CML helped maintain a CTL response, leading to stable disease as it is characteristic of chronic phase CML (41). This data highlights the importance of syngeneic modeling cellular immunotherapies to pursue effective non-cellular therapy-based combinations.

Zhou et al. used C57BL/6 bearing C1498 murine AML to evaluate the impact of Tregs on adoptively transferred tumor-reactive CTLs (35). They showed that anti-AML reactive CTLs had potent antitumor activity *in vitro* but not *in vivo*, due to the presence of tumor-localized Tregs. To bypass this hurdle, they pretreated tumor bearing mice with IL-2 diphtheria toxin restoring CTL proliferation and effect.

Syngeneic leukemia models have also been utilized to evaluate responses and the biology of transgenic TCRs, highlighting their versatility to investigate the effects of ACT. One group of investigators evaluated long-lasting antitumor activity of CD8+ T cells specific to the gag epitope of an oncogenic Friend murine leukemia virus (FMuLV) model (42, 43) and confirmed leukemia control after injection of T cells expressing a transgenic TCR. Other investigators have used a syngeneic C1498 model to better understand mechanisms of immune evasion using TCR transgenic mice (34).

Syngeneic Models for CAR T Cell-Based Immunotherapy

CD19-CAR T cells have improved outcomes for patients with relapsed/refractory B cell malignancies. However, xenograft mouse models used to test the CD19-CAR are limited in determining how T cell function is affected by Tregs, possible off-target/on-tumor activity of the CAR, and possible immune rejection of adoptively transferred T cells. With that in mind, Cheadle et al. designed a first generation murine CD19-CAR (mCD19-CAR) which allowed for temporary tumor regression in an A20 murine lymphoma model. Importantly, mCD19-CAR infusion did not result in any overt toxicities (44). Kochenderfer et al. subsequently generated a second generation CAR that achieved reduction in lymphoma burden, albeit with limited CAR T cell persistence (45). This corresponds with comparisons between first- and second-generation CAR constructs in humans and reiterates the importance of a costimulatory domain for enhanced antitumor activity.

Davila et al. subsequently tested this mCD19-CAR in a Eμ-ALL01 B-ALL model, a leukemia with similar genetic and cellular characteristics as adult human B-ALL (46). In this study, they were able to prove that mCD19-CAR T cells recognize and kill Eμ-ALL01 leukemia cells. They also noted that CD8+ mCD19-CAR T cells allowed for long-term tumor control. Most importantly, the established syngeneic model allowed them to dissect the effects of lymphodepletion and T cell dose on the effector function of CAR T cells.

In addition, B-ALL models have been used to further investigate complications stemming from ACT. One group used a E2aPBX murine pre-B ALL model to study the function granule-mediated cytotoxicity in anti-mCD19-CAR T cell efficacy (47). Researchers knocked out perforin from mCD19-CAR T cells and discovered perforin was not required for cytotoxicity and when tested *in vivo*, perforin knockout CD19-CAR T cells produced more proinflammatory cytokines than WT counterparts (47). This led to the mice developing hemophagocytic lymphohistiocytosis (HLH)-like toxicities.

Furthermore, Jacoby et al. demonstrated lineage switch after mCD19-CAR T cell therapy, evaluating late relapses in 2 different B-ALL models (E2a.PBX and Eμ-RET) (4). They demonstrated that Eμ-RET leukemia did not show lineage switch upon relapse after mCD19-CAR T cell treatment. However, mice bearing E2a:PBX exposed to mCD19CAR T cells underwent lineage switch upon relapse, showing downregulation of Pax5 and Ebf1. They could recapitulate this lineage switch by deletion of Pax5 or Ebf1. This study further demonstrated the utility of syngeneic models in the quest to optimize CAR T cell therapy for hematological malignancies.

CONCLUSION

Developing effective ACT for leukemias still poses several challenges requiring a better understanding of both the adoptively infused cells and the TME. Although clinical trials provide the ultimate test for ACT, murine models can be a powerful tool to gain insight. One of the largest drawbacks of current preclinical modeling of leukemia targeted ACTs, is that it heavily relies on xenografts which lack a representative immune system and TME. Syngeneic models offer an alternative to better evaluate these factors. However, the availability of certain leukemia syngeneic models, such as AML, are still limited and establishing new systems can often be time consuming and unreliable (8, 9, 32). Additionally, it is not always possible to adapt human-target ACT towards respective murine antigen counterparts. For example, the evaluation of CAR T cell therapies is limited by finding an antigen recognition domain (i.e. scFv) that recognizes the corresponding cell surface murine antigen. In addition, trafficking of adoptively transferred cells to the TME can greatly affect the efficacy of treatment (48). Thus, several factors that impact homing, such as target antigen expression, immune cell populations (25–28, 48), and chemokine production (23) are important to recognize and incorporate into preclinical modeling. Syngeneic models provide these factors and allow for a better understanding of immune cell trafficking to the tumor site. Nevertheless, the field of syngeneic experimentation has adapted to include additional genetic modifications on cellular products such as cytokine receptors on mCAR T cells (49). The analysis of ACT therapies in syngeneic models can aid in answering critical questions and warrants further exploration and development.

AUTHOR CONTRIBUTIONS

JZ, SM, and MV conceptualized the manuscript and provided content. All authors reviewed, edited, and approved the final manuscript.

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Emerging Strategies in TCR-Engineered T Cells

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Immunotherapy of cancer has made tremendous progress in recent years, as demonstrated by the remarkable clinical responses obtained from adoptive cell transfer (ACT) of patient-derived tumor infiltrating lymphocytes, chimeric antigen receptor (CAR)-modified T cells (CAR-T) and T cell receptor (TCR)-engineered T cells (TCR-T). TCR-T uses specific TCRS optimized for tumor engagement and can recognize epitopes derived from both cell-surface and intracellular targets, including tumor-associated antigens, cancer germline antigens, viral oncoproteins, and tumor-specific neoantigens (neoAgs) that are largely sequestered in the cytoplasm and nucleus of tumor cells. Moreover, as TCRS are naturally developed for sensitive antigen detection, they are able to recognize epitopes at far lower concentrations than required for CAR-T activation. Therefore, TCR-T holds great promise for the treatment of human cancers. In this focused review, we summarize basic, translational, and clinical insights into the challenges and opportunities of TCR-T. We review emerging strategies used in current ACT, point out limitations, and propose possible solutions. We highlight the importance of targeting tumor-specific neoAgs and outline a strategy of combining neoAg vaccines, checkpoint blockade therapy, and adoptive transfer of neoAg-specific TCR-T to produce a truly tumor-specific therapy, which is able to penetrate into solid tumors and resist the immunosuppressive tumor microenvironment. We believe such a combination approach should lead to a significant improvement in cancer immunotherapies, especially for solid tumors, and may provide a general strategy for the eradication of multiple cancers.

Keywords: T cell receptor, genetic engineering, cancer immunotherapy, TCR-engineering, new strategies

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Abbreviations: CAR, Chimeric antigen receptor; CDR3, Complementarity-determining region 3; Cα: Constant alpha; Cβ: Constant beta; CRISPR, Clustered regularly interspaced short palindromic repeat; CRS: Cytokine release syndrome; CSR, Chimeric switch receptor; CTL, Cytotoxic T-Lymphocyte; CTLA4: Cytotoxic T-Lymphocyte Associated Antigen 4; EBV, Epstein-Barr Virus; GvHD, Graft-vs-host disease; HLA, Human leukocyte antigen; IFNγ, Interferon gamma; IL2, Interleukin 2; LCK, Lymphocyte-specific protein tyrosine kinase; mCα, Murine Constant alpha; MDSC, Myeloid-derived suppressor cells; MHC, Major histocompatibility complex; PBMC, Peripheral blood mononuclear cell; PLK, Polylinker; Sc, Single chain; Sc-TCR, Single-chain T-cell receptor; siRNA, Small interfering RNA; sgRNA, Single guide RNA; TAA, Tumor associated antigen; TALENs, Transcription activator-like effector nucleases; Tcm, Central memory T cells; TCR, T-cell receptor; TGF-β; Transforming growth factor-β; TNFα, Tumor necrosis factor-alpha; Treg, Regulatory T cells; TRAC, T cell receptor constant-alpha; TRBC, T cell receptor constant-beta; Tscm, Stem cell memory T cells; WT1, Wilms' tumor 1.

INTRODUCTION

Immunotherapy of cancer based on adoptive cell transfer (ACT) of T lymphocytes can be classified into three approaches. The first, tumor-infiltrating lymphocyte (TIL) therapy, harvests naturally occurring T cells that have already penetrated patient tumors, expands them *ex vivo*, and then re-infuses them into patients (1, 2). However, it is often very difficult to isolate tumor-specific TILs, which are not present in all patients or may generate too few cells for therapeutic efficacy.

The second approach, chimeric antigen receptor (CAR)-modified T cells (CAR-T), bypasses this problem by directly engineering T cells with known tumor-specific CARs. CARs are fusion molecules that link a single-chain antibody with T-cell activation signaling domains such as CD28-CD3ζ (3) or 4-1BB-CD3ζ (4). When a CAR is transduced into human T cells, the antibody fragment is expressed on the surface of the engineered T cells to recognize a tumor antigen expressed on tumor cells, while the CD28-CD3ζ or 4-1BB-CD3ζ domain delivers a stimulatory signal once the antibody binds to a tumor antigen, activating CAR-T cells to attack the tumor.

CAR-T cells are not restricted by MHC molecules, and thus one CAR-T construct can be used to treat any patient regardless of genetic background. Currently, the most widely used and successful CAR is the CD19-CAR, which recognizes the CD19 molecule expressed on the surface of B cells, thus can eliminate some B-cell-derived leukemias and lymphomas (5, 6), including complete response in nearly 90% of B-cell leukemia patients (5). However, antibody-based CARs can only recognize antigens expressed on the cell surface, and not intracellular antigens, limiting the number of targets and potential tumor types addressable by CAR-T therapy.

The third approach, T cell receptor (TCR)-engineered T cells (TCR-T), uses TCRS as found on native T cells to confer specificity, instead of antibody-based CARs. TCRS can be isolated from tumor-reactive T cells and further modified for enhanced expression and functions. TCRS can recognize both cell-surface and intracellular targets, these include neoantigens (neoAgs) that arise from mutations and are specific to tumor cells. The disadvantage of using TCR-T is that TCRS are restricted by MHC molecules, thus any given TCR can only be used to treat patients with the corresponding MHC genetic background. In the following sections, we describe the settings in which TCR-T may prove most effective.

TCR-T IMMUNOTHERAPY FOR CANCER AS A COMPLEMENT TO CAR-T

CAR-T has been most successful in hematological malignancies (7–10), with FDA-approved therapies targeting CD19 (Kymriah, Yescarta, Tecartus, Breyanzi) and BCMA (Abecma) as of December 2021. CD19-CAR-T can achieve complete response in nearly 90% of B cell leukemia patients, although 50% of patients may nonetheless relapse (11). One of the major reasons for this relapse is loss of surface expression of CD19 from tumor cells (12), thus evading recognition by CD19-CAR-T cells. These patients may no longer respond to CD19-CAR-T, although targeting different antigens may still be viable, in which case TCR-T may be used as a late-line option. For example, a recent study reported transfer of WT1-TCR-engineered donor T cells into AML patients at high risk of relapse following allogeneic stem cell transplantation, 12/12 treated patients achieved relapse-free survival (13), compared to 54% in a concurrent group of 88 similar high-risk patients, and WT1-TCR-T cells also showed prolonged persistence and maintenance of antigen-specific polyfunctional activity.

The greater opportunity for TCR-T may exist in solid tumors, where CAR-T has been less effective (14–16). The mechanisms behind these limitations are poorly understood and under active investigation. CAR-T recognition is limited to surface antigens, and moreover CAR-T activation requires higher concentration of target antigens (17, 18). This lower sensitivity helps avoid damage to normal tissues with low antigen expression (19), but conversely may be unsuitable for tumors with similarly low tumor antigen expression. For example, CAR-T specific for anaplastic lymphoma kinase (ALK) showed variable efficacy towards different cell lines depending on expression level of ALK (17). Recently, a study investigated B cell malignancies with up to 33-fold lower CD20 expression than healthy B cells – below the concentration required to activate CAR-T – but found that CD20-specific TCR-T clones with high avidity were able to overcome self-tolerance and eliminate these tumor cells (20). It is estimated that CAR-T cells need in the order of hundreds of target molecules to be activated (17, 18, 21), whereas TCR-T can be activated by a single target molecule (22).

Initial clinical studies of TCR-T in solid tumors have shown promising results (23, 24). An affinity-enhanced NYESO1-TCR achieved 45-55% clinical response rate in metastatic melanoma patients (25, 26), and 50-61% clinical response rate in metastatic synovial sarcoma patients (25–27). The same NYESO1-TCR achieved 80% clinical response rate in multiple myeloma patients without apparent side effects, including 70% complete response rate with median progression-free survival of 19 months (28). Recently, a phase 1 trial of TCR-T targeting HPV-16 E7 in metastatic HPV-associated epithelial cancers achieved 50% clinical response rate (6/12 patients), including 4/8 patients refractory to PD-1 blockade (29).

In recent years, neoAgs have been discovered as a class of immunogenic tumor-specific antigens that are derived from tumor-specific mutations of self-proteins (30–32) or from tumor-causing oncogenic viral proteins (33) in the estimated 15% of human cancers attributed to viruses (34). T cells specific for neoAgs and viral proteins would not have undergone central thymic tolerance selection, making it possible to isolate high-avidity T cell clones against these targets. These antigens are rarely expressed on the cell surface, and represent a therapeutic opportunity in solid tumors using TCR-T that CAR-T may be unable to match (35).

Despite these promising results (24–27, 29), several hurdles remain to be overcome to realize the true promise of TCR-T immunotherapy. In early clinical trials, some non-responder

patients lacked *in vivo* persistence of the infused T cells (36, 37), suggesting that the transferred TCR-T cells need additional support to enhance their *in vivo* survival. Some patients with late relapse showed no evidence of T cell infiltration in the tumor, and moreover infused TCR-T cells face a hostile immunosuppressive tumor microenvironment (TME). In the following sections, we discuss current and future engineering strategies to address these challenges to deliver effective and long-lasting tumor control to a broad range of cancer patients.

OVERCOMING THE CHALLENGES OF TCR-T CANCER IMMUNOTHERAPY

Enhancing TCR Expression and Function Through Reducing TCR Mis-Pairing

TCR-T therapy relies on mRNA or viral transduction of tumor-reactive TCR genes to redirect T cell specificity towards tumor cells. However, using either mRNAs or randomly integrating viruses to deliver the exogenous TCR, leaves the endogenous TCR genes intact. Therefore, this could potentially result in some degree of mis-pairing between the introduced and endogenous TCR chains (38–40). Mis-pairing poses certain safety risks, as T cells expressing mis-paired TCRS may be auto-reactive against the patient's MHC molecules. Indeed, a murine study showed that TCR mis-pairing in the context of adoptive transfer of TCR-gene-modified T cells combined with increased conditioning resulted in graft-versus-host disease (GvHD) and serious animal death (41). Similarly, an *in vitro* study of human EBV-transformed lymphoblastoid cell lines showed that mis-paired TCRS drove potentially dangerous off-target toxicity (42).

Several strategies have been explored to prevent TCR mis-pairing (43, 44). The interaction between TCRα and TCRβ chains is largely governed by the invariant Cα/Cβ-interface (45), enabling modification of this region to prevent pairing with endogenous TCRS. Reciprocal "knob-hole" amino acid changes in the center of the TCR C domains led to preferential pairing of the modified chains while disfavoring combinations with native TCR chains (46). Introduction of an additional inter-chain disulfide bond within the TCR Cα/Cβ-interface (47) also enhanced the pairing of the modified chains whilst reducing the efficiency of pairing with wild-type chains (48, 49). This preferential pairing of cysteine-modified TCR chains has accounted for improved TCR gene expression and enhanced antitumor activity of transduced T cells (50). Replacing the human TCR constant domains with whole (49, 51, 52) or partial (53, 54) murine sequences represents an alternative strategy to reduce unwanted mis-pairing, and can also increase the expression level of the introduced TCR genes (51). The enhanced expression of the human/murine hybrid TCR in human T cells may be partly due to the greater binding capacity of the murine TCR constant domains to human CD3 molecules when compared with human TCR constant domains (51). Finally, instead of using murine sequences, exchanging the human TCR constant domains Cα and Cβ with each other (domain swapping), or replacing Cα and Cβ with the corresponding γδ TCR constant domains, could also generate functional TCRS with reduced mis-pairing and improved safety profile (55). However, it is important to note that the individual TCR subfamily V-domains and even the antigen-binding CDR3 α/β-loops may also contribute to the interaction of TCR α and β chains (56, 57), and hence manipulation of the TCR constant domains can only partially reduce the frequency of mis-pairing, rather than eliminate the risk completely.

Another common approach to reduce mis-pairing is to generate a so-called single-chain TCR (Sc-TCR) by covalently linking the Vα and Vβ domains with a polylinker (PLK), resulting in a single polypeptide which will in theory inhibit mis-pairing through steric hindrance (58). T-cell-activation signaling upon antigen encounter is provided by fusion of CD3ζ onto the Cβ-chain within the Sc-TCR. Using this approach, Sebestyen et al. showed preferential pairing between CD3ζ-modified TCR α and β chains while reducing mis-pairing with unmodified TCR chains (59). To develop this concept further, Aggen et al. replaced the constant domains of the Sc-TCR construct with a CD28 or 4-1BB together with CD3ζ or LCK signaling domains (60). Although this strategy was able to reduce mis-pairing, activation of these T cells upon antigen encounter no longer followed natural TCR signaling pathways, but rather that of conventional CARs. Because CAR signaling is less efficient than that of the TCR complex (23, 61), Voss et al. developed an alternative Sc-TCR scaffold Vα-PLK-Vβ-Cβ plus Cα (62), relying on assembly with the native CD3 complex for more physiologic T-cell signaling. To stabilize the structure of the Sc-TCR, we introduced an extra new disulfide bond between the Vα and the polylinker, which strengthens the interaction between the Vα and Vβ domains, favoring surface expression of the Sc-TCR, while also greatly reduced TCR mis-pairing (63). One of the potential drawbacks of using this technology is that not all of the TCRS can form a stable Sc-TCR. According to our experience, some of the weak TCRS can not be expressed on the surface of T cells as a Sc-TCR due to the weak interaction between the Vα and Vβ domains. With such weak TCR, genetic engineering of certain frame work regions of the TCR may be able to help to resolve the problem (64).

Aside from TCR protein design, another way of reducing TCR mis-pairing and its related side effects is to knock out the endogenous TCRS *via* genetic engineering, which also reduces competition for CD3 binding from endogenous TCRS (65). Several strategies have been explored to achieve this goal, including the use of siRNAs (66, 67), zinc-finger nucleases (68), transcription activator-like effector nucleases (TALENs) (69, 70), and CRISPR/Cas9 technologies (71, 72). As the CRISPR/Cas9 has several advantages, including (i) simple and highly efficient editing, (ii) rapid and affordable manufacturing, (iii) versatile multiplex genome editing through simultaneously targeting several genes, and (iv) user-friendly and easily deliverable. Therefore, this CRISPR/Cas9 system holds great promise and may lead the way for future genetic engineering of T cells for cancer immunotherapy (73). The feasibility of genome editing using CRISPR/Cas9 targeting the TRAC and TRBC loci was recently demonstrated in primary T cells (71, 74).

Using multiplex genome editing, the beta-2 microglobulin class I MHC and PD-1 genes can also be disrupted alongside the TCR α and β genes (75, 76). Removal of endogenous TCR and class I MHC eliminates allogeneic antigen recognition and reduces risk of GvHD and donor T cell rejection, generating allogeneic 'universal' T cells that can be infused in any recipient (77, 78), as opposed to autologous T cells that can only be re-infused into the donor patient. The immune checkpoint gene PD-1 is removed to enhance T cell activity, for which reason the T cell suppressor LAG-3 has also been knocked out to improve antitumor activity *in vitro* and in murine xenografts (79). We anticipate that other immune inhibitory receptors such as TGFβ receptors can also be disrupted to generate universal CAR-T and TCR-T cells with enhanced resistance to the inhibitory TME.

TCR-T cells have been generated by TCR transduction of T cells in which the endogenous TCR α chain (80), β chain (81), or both α and β chains (82) were removed using CRISPR/Cas9, or by orthotopic replacement of TCR-αβ chains with tumor reactive TCRS using CRISPR/Cas9 (83, 84), resulting in engineered TCR-T cells with enhanced TCR expression and prolonged control of tumor growth in preclinical murine models. However, for clinical use, the potential off-target toxicity of the CRISPR/Cas9 technology has to be taken into considerations (85). A recent study showed that DNA breaks introduced by sgRNA/Cas9 can lead to on-target mutagenesis, such as large deletions and genomic rearrangements at the targeted sites in mouse embryonic stem cells and in a human differentiated cell line (86). Therefore, strategies for improving safety of the CRISPR/Cas9 technology need to be put in place. To reduce the off-target toxicity, high-fidelity CRISPR–Cas9 nuclease has been developed (87), and a recent review has provided many strategies about how to refine the CRISPR/Cas9 technology for clinical applications (88). Recently, a phase 1 clinical trial was designed to test multiplex CRISPR-Cas9 gene editing of T cells from patients with advanced, refractory cancer (89), in which endogenous TCR α and β chains were removed to prevent mis-pairing, and PD-1 removed to avoid T cell exhaustion. The NYESO1-TCR engineered T cells persisted for up to 9 months and trafficked to tumor sites, demonstrating proof-of-concept for multiplex CRISPR gene editing in cell therapy. Another study applied CRISPR-edited T cells in patients with refractory non-small-cell lung cancer also concluded that clinical application of CRISPR–Cas9 gene-edited T cells is generally safe and feasible (90).

To achieve the best results of maximally reducing mis-pairing and enhancing expression and function of the tumor-specific TCR, we have recently combined multiple strategies by knocking out endogenous TCR using CRISPR/Cas9 together with transduction of a single-chain EBV-specific TCR (EBV-Sc-TCR) (91). This almost eliminated mis-pairing between the introduced EBV-Sc-TCR and endogenous TCR chains, and we further enhanced tumor-specific TCR expression, functional avidity, and IL-2 production by introducing an extra intra-chain disulfide bond between the Vα and the poly-linker (91).

Enhancing Persistence and Anti-Tumor Functions of the Genetically Engineered T Cells

T cell persistence is a fundamental requisite for durable immunosurveillance, as many clinical trials revealed that most non-responder patients showed no *in vivo* persistence of the infused tumor specific T cells (36, 37), and in contrast, patients who achieved complete response or relapse-free survival and tumor control showed robust proliferative capacity and long-term persistence of engineered T cells (13, 27). To maintain persistence of the transferred T cells, a variety of cytokines have been coadministered to support T cell survival and expansion. The standard ACT regimen comprises lymphodepletion with cytotoxic agents, including cyclophosphamide and fludarabine, followed by administration of recombinant human IL-2 after T cell transfer (92). Systemic delivery of IL-2, is known to expand T cells while maintaining functional activity (93), has achieved durable regression in some metastatic melanoma and renal cancer patients (94), is approved by the FDA, and is used in both CAR-T and TCR-T immunotherapy of cancers today. However, there is evidence suggests that IL-2 may preferentially expand $CD4^+$ regulatory T (Treg) cells rather than tumor-killing $CD8^+$ cytotoxic T cells (CTLs) (95, 96). Therefore, recent attention has focused on modifying the IL-2 molecule to preferentially bind and activate $CD8^+$ CTLs over Treg cells (97). For example, a half-life-extended super mutant IL-2 conjugated to a tumor-targeting antibody allowed more efficient CTL stimulation and expansion in the TME, resulting in significantly improved complete response rate and lower tumor relapse *in vivo* (97).

IL-7, is a hematopoietic cytokine regulating multiple aspects of T cell biology (98), is essential for T cell survival and homeostatic proliferation, and promotes the survival of naïve and memory T cells by upregulating the antiapoptotic molecule Bcl-2 (99, 100). IL-7 supplementation improved the persistence and efficacy of transferred T cells, supporting its usage as an adjuvant for adoptive immunotherapy (101). When IL-7 was co-expressed in NKG2D-based CAR-T cells, it enhanced CAR-T persistence and expansion while inhibiting apoptosis and exhaustion (102). Similarly, IL-7 co-expression in GPC3-CAR-T cells improved CAR-T efficacy toward liver cancer (103).

IL-12 is a major contributor to effective anti-tumor immune responses (104), stimulating the effector functions of activated T cells and NK cells *via* induction of cytotoxic enzymes such as perforin and cytokines such as IFN-γ (104, 105). Cytotoxic enzymes can mediate direct killing of tumor cells (106), while production of IFNγ from NK cells as well as $CD4^+$ and $CD8^+$ T cells inhibits tumor growth (107, 108). IL-12 further modifies the TME through inhibition of Tregs (107), upregulating MHC class I presentation on tumor cells (109, 110), and converting immunosuppressive M2 macrophages into activated antitumor M1 macrophages (111). IL-12 also prevents the activation-induced cell death of naïve $CD8^+$ T cells, favoring their survival and differentiation towards the effector phenotype to sustain anti-tumor activity against mouse models of melanoma (112).

These studies demonstrate that IL-12 is not only required for the activation of anti-tumor cytotoxic immune responses, but also directly relieves immune suppression (107). However, systemic administration of IL-12 is very toxic (113), severely limiting its utility in clinical applications. To minimize systemic exposure and potential toxicity while maintaining the beneficial effects of IL-12, several strategies have been explored, for example local delivery (114, 115), encapsulating IL-12 with nanoparticles or heparin (116, 117). Alternatively, deleting the N-terminal signal peptide of IL-12 or tethering IL-12 to the surface of TCR-engineered T cells *via* a membrane anchor prevents secretion (118, 119), thereby attenuating toxicity while improving antitumor efficacy. These treatment strategies may have broad applications to cellular therapy with TILs, CAR-T, and TCR-T cells. A recent multi-center phase 1 trial used a chemically activatable IL-12 gene delivered into the tumor site, where IL-12 expression triggered by the drug veledimex achieved conversion of an immunologically "cold" TME to an inflamed "hot" TME with increased influx of IFN-γ–producing T cells (120). IL-18 is another cytokine that shares biological effects with IL-12 but with reduced toxicity (121). Recent studies exploring IL-18 in the place of IL-12 suggest that CAR-T cells engineered to secrete IL-18 enhances CAR-T cell survival and antitumor activity both *in vitro* and *in vivo* by producing IFN-γ and several other cytokines, stimulating expansion of human $CD4^+$ cells as well as activating the endogenous immune system in immunocompetent mice (122, 123).

IL-15 is known to stimulate the generation of stem cell memory T cells (Tscm) with potential to sustain durable T cell responses (124). Unlike IL-2, IL-15 does not bind to the IL-2Rα chain, and thus does not stimulate Tregs and may have a more selective effect. When compared with IL2, IL15 tend to enhance CAR-T cell antitumor activity by preserving their Tscm phenotype (125). A comparison of CAR-T cell expansion in the presence of IL-2, IL-15, or a combination of IL-15/IL-7, revealed that IL-15 best enhances CAR-T persistence and function in a mouse model of multiple myeloma (126). Preclinical observations strongly support the antitumor activity of IL-15 mediated by $CD8^+$ T cells (127), and IL-15 co-expression in CD19-CAR-T not only revealed a strong killing effect against leukemia cells, but most of the persistent T cells were phenotypically consistent with Tscm that drive long-term persistence (128).

To improve IL-15 half-life and effectiveness *in vivo*, IL-15 was associated with IL-15-receptor-α to form a pre-bound IL-15/IL-15Rα dimer, which showed stronger antitumor activity than IL-15 monomer (129). Recently, subcutaneous injection of recombinant human IL15 was tested in patients with advanced solid tumors, although the treatment produced substantial increases in circulating NK and $CD8^+$ T cells, nonetheless, no objective responses were observed (130). However, when IL-15/IL-15Rα sushi-domain was co-expressed on CD5-specific CAR-T cells, and tested in a patient with relapsed T-lymphoblastic lymphoma with CNS infiltration, a rapid ablation of the CNS lymphoblasts to undetectable levels within 4 weeks and disease remission was observed (131). To address IL-15-induced immune checkpoint activation, IL-15 can also be combined with anti-PD-(L)1 and anti-CTLA-4 antibodies (132).

IL-21 is a newly discovered member of the common γ-chain family of cytokines. Like IL-12 and IL-15, and in contrast to IL-2, IL-21 does not stimulate Tregs, instead, it inhibits Treg expansion through suppression of Foxp3, thus favoring the enrichment of antigen-specific $CD8^+$ T cells (133). IL-21 facilitates the maturation and enhances the cytotoxicity of $CD8^+$ T cells and NK cells, and promotes the differentiation of memory $CD8^+$ T cells (134, 135). IL-21 synergizes when combined with IL-12 to further inhibit Tregs (136), and synergizes when combined with IL-15 to expand CD28-expressing antigen-specific $CD8^+$ T cells (137, 138). Utilizing these characteristics, IL-21 performed much better than IL-2 or IL-15 during *in vitro* generation of antigen-specific $CD8^+$ CTL and in an *in vivo* murine model of cancer immunotherapy (139, 140). In murine tumor models, intratumoral injection of IL-21 strongly inhibited tumor growth and increased the frequency of tumor-infiltrating $CD8^+$ T cells and mice survival (141). In a phase 1/2 trial, 4 out of 4 leukemia patients who received WT1-specific CTL generated in the presence of IL-21 demonstrated both relapse-free survival without GvHD and did not need further anti-leukemic treatment (142).

To reduce the toxicity and increase the half-life of IL-21, IL-21 has been conjugated to tumor-targeting antibodies such as anti-EGFR antibody (143), selectively expanding functional CTLs while restricting exhausted T cells in the TME. IL-21 upregulates perforin and granzyme expression in memory and effector $CD8^+$ T cells (144), thus augments the antitumor activity of $CD8^+$ T cells (145), consistent with the requirement of IL-21 for the long-term maintenance and function of $CD8^+$ T cells (146). IL-21 fused to anti-PD-1 antibody stimulated generation of Tscm with enhanced cell proliferation and tumor-specific $CD8^+$ T cells, outperforming anti-PD-1 antibody and IL-21 infused as separate treatments (147). These results demonstrated that IL-21 can be used alone or in combination with other cytokines to produce tumor-specific T cells with a memory phenotype, with enhanced persistence, proliferative capacity, and antitumor efficacy for adoptive cancer immunotherapies.

Endogenous immune cells can act as a "sink" for administered cytokines (148), thus the use of a lymphodepleting conditioning regimen prior to ACT helps to spare the limited cytokines for the transferred T cells. Moreover, conditioning can also eliminate immunosuppressive Tregs and MDSCs (149), further supporting the engraftment and expansion of engineered T cells and improving therapy persistence and efficacy (150, 151).

Finally, purposeful selection of T cell sub-populations is another way to enhance persistence and functionality of the adoptively transferred T cells. Less differentiated T cells such as Tscm and central memory (Tcm) cells are more effective than effector T cells when transferred into tumor-bearing mice (152), thus CAR modification of naïve T cells can generate antigen-specific Tscm and Tcm cells with long *in vivo* persistence which mediated robust, long-lasting antitumor responses (153, 154). To preserve this early differentiated T cell population, tumor-specific CTLs can be stimulated by a combination of IL-21 and

anti-CD3/anti-CD28 antibody-conjugated microbeads or nanomatrices (155, 156). Addition of IL-21 alone or in combination with other cytokines (such as IL-7 and IL-15) into the expansion culture, can further support the enrichment and expansion of Tscm cells with superior antitumor activity (157), consistent with recent clinical data that WT1-TCR engineered T cells generated in the presence of IL-21 showed long-lasting persistence with superior anti-leukemia activity in humans (13, 142).

Enhancing the Homing and Penetration of Engineered T Cells Into Solid Tumors

To achieve tumor eradication, cancer-specific CTLs need to migrate and penetrate into solid tumors (158), driven by interactions between tumor-secreted chemokines and chemokine receptors expressed on CTLs (159–163). This process is rate-limited when CTLs express chemokine receptors at low density or that do not match the specific chemokines secreted by the tumors (164, 165). This creates an opportunity to engineer tumor-specific T cells' chemokine receptors to match known chemokines or cytokines abundant in the TME, with encouraging results for enhanced CTL homing and antitumor efficacy.

The chemokines CCL2, CCL7, and CCL8 are expressed in many cancer types as well as cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), MDSCs, and mesenchymal stem cells found in the TME, which support the tumor growth and metastasis (164). All three of these chemokines are ligands for the CCR2 receptor, thus when CCR2b was transduced together with a CAR specific for GD2 into human T cells, these modified T cells showed enhanced trafficking with >10-fold improved homing to CCL2-secreting neuroblastoma, and significantly enhanced activity against neuroblastoma xenografts *in vivo* (166). The same approach, resulted in 12.5-fold increase in infiltration of CAR-T specific for mesothelin into established mouse tumors, and significantly enhanced antitumor activity and tumor eradication (167). When CCR2 was transduced together with a TCR specific for WT1 into $CD3^+$ human T cells, double gene-modified $CD3^+$ T cells demonstrated CCL2-tropic tumor trafficking and potentiated antitumor activity against WT1-expressing LK79 lung cancer cells both *in vitro* and *in vivo* (168). Similarly, transduction of CCR2 into TCR-T cells specific for the SV40 large T antigen, enhanced recruitment into CCL2-expressing metastatic prostate adenocarcinoma, and improved *in vivo* antitumor effect (169).

Multiple chemokine ligands for the CXCR2 receptor are expressed in many tumors (170), and also promote tumor initiation, proliferation, migration, metastasis, and immune invasion. Thus, CXCR2 has been explored intensively for cancer immunotherapy. Human hepatocellular carcinoma (HCC) tumor tissues and cell lines express several chemokine ligands for CXCR2, however, both human peripheral T cells and TILs of HCC lack expression of CXCR2. In a recent study (171), Liu et al. transduced human T cells with a GPC3-CAR together with CXCR2; compared with CAR-T cells without CXCR2, these cells exhibited identical cytotoxicity but significantly increased migration *in vitro*, as well as accelerated *in vivo* trafficking and tumor-specific accumulation in a xenograft tumor model. Similarly, CXCR2 enhanced trafficking and *in vivo* antitumor efficacy of CAR-T cells specific for integrin αvβ6 in advanced pancreatic and ovarian tumor xenograft models (172). In the TCR-T field, when CXCR2 was transduced into pmel-1 TCR transgenic T cells (173), or MAGE-A3-specific TCR-engineered T cells (174), the CXCR2-TCR-T cells showed increased *in vivo* homing, enhanced tumor infiltration, and preferential accumulation in tumor sites in mice, with enhanced survival and tumor regression compared with mice receiving control TCR-T cells. These results indicate that introduction of the CXCR2 gene into tumor-specific T cells can enhance their homing and localization to tumors and improve antitumor immune responses. CXCR2 has also been used to enhance the migration and homing of NK cells to CXCL5-expressing renal cell carcinomas (175). Recently, Jin et al. used radiation therapy to induce tumor secretion of IL-8 (CXCL8), and found that CD70-CAR-engineered T cells expressing either of the IL-8 receptors CXCR1 or CXCR2, showed enhanced migration and persistence, leading to complete tumor regression and immunologic memory in models of aggressive tumors, including glioblastoma, ovarian, and pancreatic cancers (176). Like radiation therapy, chemotherapy may also induce chemokine secretion from tumor cells, resulting in increased homing and infiltration of adoptively transferred T cells (177). These studies indicate that genetic engineering of tumor-specific T cells with chemokine receptors can be combined with conventional radiation and chemotherapy to enhance antitumor efficacy. CXCR1 has also been used to enhance migration and tumor infiltration of NK cells modified with a CAR specific for NKG2D (178).

Other chemokine receptors used in this way include CCR4 and CXCR4. Similarly, coexpression of CCR4 enhanced migration of CD30-specific CAR-T cells in response to CCL17 secreted by Hodgkin's lymphoma in murine xenografts (179). CXCR4 has also been explored as a means of recruiting T cells into the bone marrow, whose microenvironment is suggested to improve memory T cell formation and self-renewal. Khan (180) et al. overexpressed CXCR4 in $CD8^+$ T cells, observing enhanced migration toward CXCL12-expressing cells in the bone marrow, with enhanced memory differentiation, expansion, persistence, and antitumor function of adoptively transferred T cells. CXCR4 also enhanced migration of NK cells to bone marrow as a means of targeting bone-marrow-resident tumor cells such as leukemia (181). CXCR4-modified CAR-NK cells also significantly improved survival and tumor regression of mice bearing glioblastoma (182).

Aside from engineering T cells with chemokine receptors, chemokines can be directly introduced into tumors to enhance T cell recruitment. For example, intratumoral injection of CXCL2 plasmid DNA combined with inactivated Sendai virus envelope, suppressed the growth of murine breast cancers and inhibited lung metastasis through recruitment of CTLs and neutrophils, further enhanced with anti-PD-1 antibodies to inhibit T cell

exhaustion (183). Chemokines can also be introduced into tumors through chemokine-armed oncolytic viruses, which simultaneously, replicate within and directly kill tumor cells to amplify antitumor efficacy (184). T cells can also be engineered to express both chemokines and cytokines to improve antitumor efficacy. For example, transduction of CAR-T cells to express IL-7 and the chemokine CCL-19, not only enhanced T cell survival, infiltration and accumulation in the tumor, but also achieved complete regression of pre-established solid tumors and prolonged mouse survival (185). When the chemokine CCL21 and IL7 was transduced into CAR-T cells, it significantly improved survival and infiltration of both CAR-T and dendritic cells in the tumor, leading to complete tumor remission (186).

Overcoming the Immunosuppressive Tumor Micro-Environment

Infiltration of genetically engineered T cells into the tumor is only the first step in fighting cancers. Tumor cells inhabit a heterogeneous microenvironment of infiltrating and resident host cells, secreted factors and extracellular matrix (187). Infiltrated cells include immune cells, such as T cells (TILs and Tregs), macrophages (M1 and M2), and MDSCs, and secreted factors include the immunosuppressive cytokines IL-10 and TGFβ. The TME also includes stromal cells such as CAFs and TAMs. These components can mutually interact to induce a supportive milieu for malignant cell growth, migration, and metastasis, that evades the immune system and tumor-specific CTLs (188, 189).

Most tumor stromal cells in the TME express the immunosuppressive checkpoint ligand PD-L1 (190–192), which can interact with PD-1 expressed on T cells, resulting in inhibition of antitumor function and exhaustion of adoptively transferred TILs (193), CAR-T (194) and TCR-T (195). This effect can be relieved *via* checkpoint blockade with anti-PD-1 (196–199) and anti-PD-L1 (200, 201) antibodies. CTLA-4 expressed on activated T cells have a similar effect, as CTLA-4 binds to CD80/86 on antigen-presenting cells with higher affinity in competition with the T cell costimulation molecule CD28, dampening antitumor immunity (202). Anti-CTLA4 antibodies both block the interaction between CTLA4 and CD80/86, and can also deplete Tregs (203), thus facilitate the costimulation and expansion of tumor-specific CTL with improved clinical benefits (204, 205).

Checkpoint inhibitors alone induce a response rate of approximately 20% of patients in one meta-analysis (206), and some responding patients will develop resistance (207). One important resistance mechanism is the upregulation of PD-L1 expression on tumor cells treated with immunotherapy, resulting in T cell exhaustion and relapse (207, 208). Immune checkpoint blockade is also associated with significant and in some cases life-threatening toxicity (209). An alternative approach to eliminating the immunosuppressive effect of PD-1 on tumor-specific CTLs uses CRISPR/Cas9 technology to remove PD-1 from CAR-T (210), and TCR-T cells (89). It is possible to go beyond PD-1-deletion by introducing a chimeric switch receptor (CSR) consisting of a PD-1 extracellular domain (PD1ex) and CD28 intracellular domain (CD28in). When this PD-1:CD28 CSR was transduced together with a CAR (211, 212) or TCR (213, 214) into T cells, the engineered CTLs still interact with PD-L1 on tumor cells, but delivers a costimulation signal *via* CD28 rather than an inhibitory signal. CAR-T cells generated using this strategy show increased cytokine production (211), enhanced killing ability, and increase in central memory T cells (212). Similarly,this PD-1:CD28 CSR enhanced TCR-T cells to increase cytokine production and cell proliferation *in vitro* and *in vivo* (213), and prevented PD-L1 upregulation and Th2 polarization in the TME (214). CSR TCR-T cells also synergized with anti-PD-L1 antibody to secrete more IFNγ compared with control TCR-T (214). Recently, this strategy has begun to be used in the clinic. In a CD-19 CAR-T study of relapsed/refractory diffuse large B-cell lymphoma (215), 6 patients who progressed following CD19-CAR-T therapy, were given CAR-T cells engineered with CSR PD1ex-CD28in, of which 3/6 patients achieved complete remission, and 1/6 achieved partial response. In another study, in relapsed/refractory PD-L1$^+$ B-cell lymphoma (216), CSR-engineered CAR-T cells targeting CD19 showed superior T-cell proliferation, cytokine production, and cancer cell killing *in vitro* and *in vivo*. Among 17 treated adult patients, 10 patients had objective response (58.8%), including 7 with complete remission (41.2%). In both trials no severe neurologic toxicity or cytokine release syndrome was observed. Endogenous PD-1 was not depleted in these trials, thus we anticipate additional opportunity to enhance antitumor activity by combining CSR-engineered T cells with PD-1 knockout. The same CD28 CSR approach has also been applied to the immune checkpoint molecules TIGIT (T cell immunoreceptor with Ig and ITIM domains) and CTLA-4. Co-transduction of a TIGIT : CD28 CSR together with a tumor-specific TCR or CAR into human T-cells, drove enhanced cytokine production and superior anti-tumor function in a xenograft model of established human melanoma tumors (217). Transduction of a CTLA-4:CD28 CSR into tumor-specific T cells, resulted in elevated IFN-γ and IL-2 production and enhanced antitumor effect without systemic autoimmunity (218). A recent study engineered a CTLA4:CD28-CD3z CSR with the intracellular domains of both CD28 and CD3z, demonstrating increased cytokine production and cytotoxicity *in vitro* and in xenograft models (219). These engineered CAR-T cells were found to accumulate in tumors and to target MDSCs without severe GvHD or CRS (219).

Among the multiple immunosuppressive factors secreted within the TME, TGF-β plays a central role driving tumor signaling, remodeling, and metabolism (220). TGF-β is produced by many cell types including tumor cells, stromal cells and Tregs (221), and stimulates autocrine and paracrine signaling to promote angiogenesis (222), suppress CD8$^+$ and T_h1 anti-tumor responses (223), and induce epithelial-to−mesenchymal transition of neoplastic cells and thus facilitate tumor invasion (224). Recent clinical data associated patient non-responders to checkpoint blockade with TGF-β signaling (225). Therefore, blocking TGF-β signaling in the TME could

potentiate antitumor responses. Indeed, one possible mechanism of anti-CTLA4 antibody therapy is the depletion of immunosuppressive TGF-β-producing Treg cells (203), thus facilitating the costimulation and expansion of tumor-specific CTL with improved clinical benefit (204, 205). To further improve on the efficacy of checkpoint inhibitor antibodies, bifunctional antibody-ligand traps comprising an antibody targeting CTLA-4 or PD-L1 fused to a TGFβ receptor II ectodomain (TGFβRIIecd) have been generated (226), in which TGFβRIIecd sequesters TGF-β secreted in the TME, while the checkpoint inhibitor antibody depletes Tregs and facilitates the CTL costimulation. This dual strategy may be more effective against cancers that are resistant to immune checkpoint inhibitors alone.

As an alternative to TGF-β sequestration, tumor-reactive T cells can be transduced with a dominant-negative TGFβ receptor-II (dnTGFβRII), generating TGF-β-resistant antitumor T cells (227). TCR-T cells expressing dnTGFβRII demonstrated complete tumor regression and prolonged survival in a mouse model of advanced and invasive prostate carcinoma. In a recent clinical study, patients with relapsed Hodgkin lymphoma were treated with EBV-specific T cells engineered to express dnTGFβRII, and 4/8 patients showed an objective clinical response (228), demonstrating that TGFβ-resistant tumor-specific T cells can persist safely in patients and potentially enhance the efficacy of T cell immunotherapy. To take advantage of the high concentration of TGF-β in the TME, a recent study fused the TGF-β receptor II (TGFβRII) extracellular domain to the intracellular domain of 4-1BB to convert the immunosuppressive effect of TGF-β into an immunostimulatory signal (229). The same cells were also transduced with a CAR-CD3ζ targeting the prostate-specific antigen and an inverted cytokine receptor consisting of the IL-4R extracellular domain fused to the IL-7R intracellular domain. Coexpression of these 3 transgenic receptors generated an additive effect with improved expansion, persistence, tumor lysis, and selective antitumor activity *in vivo*. Transduction of the TGFβRII-41BB CSR together with a NYESO1-specific TCR promoted abundant effector cytokine production in T cells, resulting in markedly enhanced tumor clearance in an *in vivo* solid tumor model (230). Like TGF-β, the Fas ligand-mediated T cell death signal that is highly expressed in the TME can also be converted into pro-survival signal *via* CSR, by fusing the Fas extracellular domain with the 4-1BB intracellular domain (231), resulting in engineered T cells with increased pro-survival signaling, proliferation, antitumor function, and enhanced *in vivo* efficacy against leukemia and pancreatic cancer mouse models. These studies clearly demonstrate the potential for using CSRs to convert the TME's immunosuppressive signals into immunostimulatory signals in engineered antitumor T cells.

Enhancing Tumor-Specific Killing by Targeting Neoantigens

Over the past decade, many tumor-associated antigens (TAAs) have been discovered and investigated as targets for cancer immunotherapy. These include the cancer testis antigens (e.g., New York esophageal squamous cell carcinoma 1 (NY-ESO)-1) (25), melanoma-associated antigen (MAGE-A3 (174), MAGE-A4) (232), differentiation antigens (e.g., melanoma antigen recognized by T-cells 1 (MART-1) (233), tyrosinase/gp100 (234), overexpressed oncogenes (e.g., Wilms' Tumor antigen 1 (WT1) (13, 235, 236), surviving (237), tumor suppressor genes (e.g., TP53) (238); and TAAs that are organ-specific (e.g., prostate-specific antigens) (239) or cell-type-specific antigens that are transiently expressed during differentiation (e.g., terminal deoxynucleotidyl transferase) (240), or normally expressed only during embryonic development (e.g., carcinoembryonic antigen) (241). While many of these have advanced to the clinic, the residual expression of many TAAs in normal tissues often leads to toxicity from TAA-targeted therapy (234, 242–244). There is therefore a need to target truly tumor-specific antigens. This is the basis for targeting antigens from oncogenic viruses, such as HPV (245), EBV (246) and HBV (247), which can potentially eradicate virus-induced cancer cells (248, 249).

While most cancers are not viral in origin, they share a hallmark of genomic instability (250), which often leads to the occurrence of a large number of mutations. Mutant amino acid coding sequences can be expressed, processed, and presented on the surface of tumor cells as cancer-specific neoAgs, and subsequently recognized by T cells. CTLs targeting neoAgs are less likely to react against normal tissues or face immune tolerance. Indeed, evidence from treatment of cancers with checkpoint blockade suggests that tumors with higher mutational burden are likely to respond to immunotherapy (251, 252). In a study of 266 cancer patients, responders to checkpoint blockade therapy more often had tumors harboring TILs (so-called 'hot' tumors), while non-responders had tumors with few TILs ('cold' tumors) (253). It is thought that tumors harboring more mutations generate more neoAgs, which can be recognized by neoAg-specific TILs (254). These TILs are frequently suppressed in the TME by immune checkpoint molecules such as CTLA-4 and PD-1/PD-L1, but can be reactivated following checkpoint blockade and thus able to induce tumor regression. As a result, cancers with high mutational load, such as melanoma and lung cancer, are more susceptible to checkpoint blockade therapies. There is also evidence that checkpoint blockade not only increases the number but also enhances the antitumor activity of neoAg-specific TILs (255).

Combining these clinical data, we hypothesize that immunotherapy through checkpoint blockade could be further augmented with neoantigen vaccines. Such vaccines could stimulate and amplify neoAg-specific TILs, which are released and reactivated upon checkpoint blockade to destroy tumor cells. Indeed, a personalized RNA-based vaccine was recently used to treat stage III and IV melanoma patients (256). All 13 patients developed T cell responses against multiple neo-epitopes, and each patient developed T cells against at least three mutations. Vaccination reduced rate of metastases and sustained progression-free survival in 8 patients. Notably, 1 patient showed complete response when the vaccination was

combined with PD-1 blockade therapy. In another study, 4/6 neoAg-vaccinated patients showed no tumor recurrence at 25 months after treatment, while 2/6 patients with recurrent disease subsequently showed complete tumor regression following treatment with anti-PD-1 therapy (257). A phase Ib trial combining a personalized neoAg vaccine with PD-1 blockade found durable, neoAg-specific $CD4^+$ and $CD8^+$ T cell responses in all 82 treated patients, and T cells migrated to metastatic tumors and mediated tumor cell killing (258). Many clinical studies suggest that neoAg-specific T cells are the main mediators of tumor destruction in patients who responded to checkpoint blockade therapy (252, 259) or adoptive T cell transfer (260, 261). Both $CD8^+$ and $CD4^+$ neoAg-specific T cells contribute to lasting tumor clearance (262–265), and work continues to develop strategies to promote maximal cytotoxic T cell responses (266).

SUMMARY AND FUTURE PERSPECTIVE

The power of the human immune system in fighting cancer has been demonstrated by the adoptive transfer of TILs (1, 2) and CAR-T therapy for hematological malignancies (5, 6). However, the promise of adoptive cell therapy for solid tumors has not yet been fully realized (14, 15). TCR-T therapy holds a number of advantages over alternative strategies. TCRS can recognize epitopes derived from both surface and intracellular proteins, enabling detection of a much broader range of targets compared to CAR-T, including TAAs, cancer germline antigens, viral oncoproteins, and neoAgs. Moreover, TCRS have naturally developed to sensitively detect low epitope concentrations, down to as little as a single molecule. Recent clinical research on TCR-T has produced meaningful responses in a variety of cancers (24–27), and in some cases durable and curative responses in solid tumor patients (29, 262–265). However, as many of these studies mainly targeted TAAs, we envisage targeting tumor-specific neoAgs to produce more profound antitumor immune responses. To achieve the goal of complete eradication of solid tumors, several aspects need to be considered:

1. Targeting tumor-specific antigens, combining neoAg vaccines, checkpoint blockade therapy, and adoptive transfer of genetically engineered neoAg-specific T cells (**Figure 1**). While targeting neoAgs can achieve complete tumor regressions in some settings, combination strategies are likely to expand their utility and increase response rates. We propose to extend the concept of combining neoAg vaccines and checkpoint blockade therapy, together with the adoptive transfer of neoAg-specific T cells as a generalizable therapeutic strategy. This starts with high-throughput screening of large patient cohorts – encompassing multiple tumor types – to identify and collect a library of patient-derived neoAgs. This will enable vaccination using combinations of unique and shared cancer-specific neoAgs, to be tested in conjunction with checkpoint blockade therapies. In a second step, we propose to isolate neoAg-specific CTLs from responding patients, especially the CTLs specific for neoAgs representing driver mutations, to construct a TCR library that can be used to generate cell therapies for patients where vaccination is not effective (267). Challenges faced in this step include the limited availablity of patient derived materials and the low frequencies of neoAg-specific T cells from patients. To overcome these limitations, Stronen et al. developed a strategy for the induction and isolation of neoAg reactive T cells from healthy donor T cell repertoires (268), which contain higher frequency of neoAg reactive T cells that are not affected by the patient's TME. Recently, this strategy has been optimized by Ali et al. into a standard protocol which will facilitate the isolation of neoAg specific T cells for cancer immunotherapy (269).
2. Eliminating TCR mis-pairing. Although neoAgs represent ideal targets for cancer immunotherapy, nonetheless, they are frequently ignored by patient's TILs (268). Under such situation, adoptive transfer of high avidity neoAg-specific TCR-T cells will be a valuable supplementation to the neoAg vaccine and check point blockade therapies. However, mis-pairing of introduced TCR with endogenous TCRS could potentially cause auto-reactivity against patient's MHC molecules, thus thoughtful innovations in TCR engineering technology could be incorporated. In this regard, genetic engineering of the TCR constant domains can be combined with design of a single-chain TCR, and the CRISPR/Cas9 genome editing can be used to orthotopically replace the endogenous TCR with tumor reactive TCR (83, 84). Through applying these recent innovative technologies in T cell engineering, TCR mis-pairing can be eliminated, while generating antitumor T cells with enhanced TCR expression and functions.
3. Maintaining long-lasting immunosurveillance against tumors and keeping patients in relapse-free survival. To achieve this goal, a fundamental requisite is persistence of genetically engineered T cells after ACT. Provision of cytokines can play important roles in supporting T cell survival and functions (99, 100, 137, 138), but is often associated with severe cytotoxicity if delivered systemically (113). Therefore, controlled and targeted delivery of cytokines through genetic engineering of tumor-specific T cells (116–119), can not only support T cell survival and generate long-term memory T cells (124, 138), but can also modify the TME to create an inflammatory environment (109, 111), and maintain a "hot" tumor milieu that self-sustains the antitumor immune responses (120).
4. Facilitating migration and penetration of genetically engineered T cells into the solid tumor (158). Genetic engineering of tumor-specific T cells with chemokine receptors that match chemokines secreted by the TME can be adopted to recruit T cells to the tumor sites (168, 174). For enhanced antitumor effect, chemokines and cytokines can be combined (185, 186), and introduced through oncolytic viruses or vaccine adjuvants (159, 184). Radiation and chemotherapy can further augment ACT by stimulating chemokine secretion from tumor cells, increasing homing

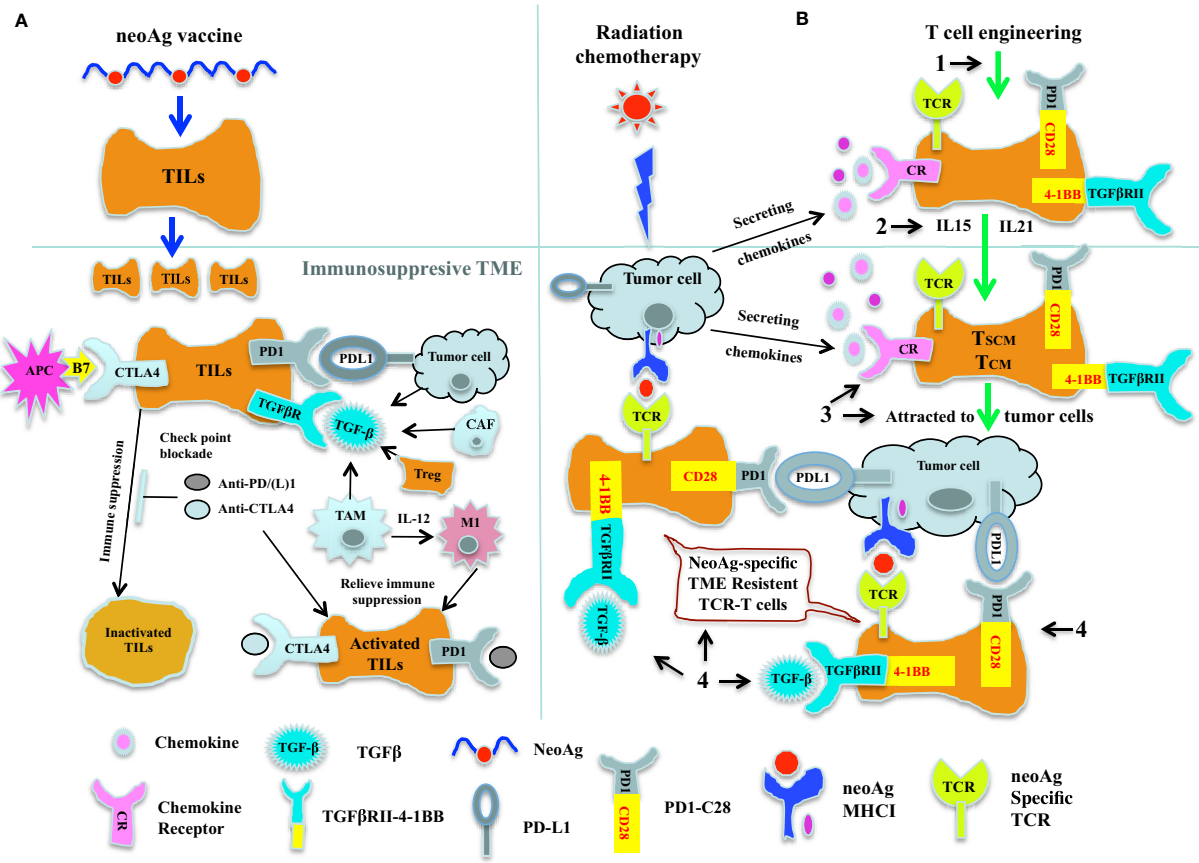

FIGURE 1 | Cancer immunotherapy by combining neoAg vaccine and chekpoint blockade therapy together with adoptive transfer of neoAg-specific TCR-T. **(A)** Combination of neoAg vaccine and chekpoint blockade therapy. NeoAg vaccines can be used to stimulate and expand tumor reactive CTLs in the circulation system. When these expanded CTLs come into the TME, they will be inactivated by the check point molecules such as PDL1 and CTLA4, or by the immunosuppressive cytokine such as TGF-β (left). Using check point blockade therapies such as anti-PD(L)1 or anti-CTLA4 to block the check point interactions (middle) or using immunomodulatory cytokines such as IL-12 (right) to convert the tumor associated macrophage M2 into activated antitumor M1, these neoAg vaccine expanded CTLs can be reactivated and attack the tumor. **(B)** Genetically engineered TCR-T cells as a complement to neoAg vaccine and chekpoint blockade therapy. Radiation and chemotherapy can induce secretion of chemokines, which could potentially attract the tumor reactive CTLs to the tumor site. But more than offten, tumor reactive CTLs from patients are either too rare or with low avidity, thus could not control the tumor growth, as reflected by the fact that only a proportion of patients responded to neoAg vaccines or check point blockade therapies. Therefore, genetic engineering strategies could be used to complement the neoAg vaccines and check point blockade therapies. (1). By transducing patient's T cells with neoAg-specific TCR, we could obtain truly tumor specific T cells. (2). Expanding these neoAg-specific TCR-T cells with IL-15 or IL-21, we could potentially acquire T cells with early differentiated phenotype of Tscm and Tcm. (3). By introducing chemokine receptor genes into these TCR modified T cells, these TCR-T cells could be attracted to the tumor site. (4). By expressing chimeric switch receptor (CSR) on these neoAg-specific TCR-T cells, the immunosuppressive effect of certain immune suppression factors such as PD-L1 or TGF-β within the TME could be potentially converted into immunostimulatory signals inside these TCR-T cells. Thus, with these innovative engineering strategies, we could not only obtain sufficient numbers of high avidity, early differentiated long lasting tumor reactive TCR-T cells, but these T cells could also be attracted and infiltrate into the solid tumor, and within the TME, these TCR-T cells would have the ability not only to resist the the immunosuppressive effect of the TME, but could also get stimulated and further expanded by neoAg vaccine and check point blockade therapies, and finally achieve the ultimate goal of destroying the tumor.

and infiltration of adoptively transferred T cells into solid tumors with enhanced antitumor activity (176, 177).

5. Overcoming the immunosuppressive effects of the hostile TME and fully realizing the antitumor potential of engineered T cells. The TME represents a formidable hostile environment for antitumor T cells and favors tumor growth, metastasis, and immune evasion. The field has made advances in blocking the immunosuppressive factors of the TME (206), and developed innovative genetic engineering strategies to convert immunosuppressive ligands/factors into immunostimulatory signals (211–214, 229). These strategies can not only remodel the immunosuppressive network within the TME (230), and convert 'cold' tumors lacking TILs into 'hot' tumors with genetically engineered T cells, but can also enhance T cell co-stimulation and survival, and produce TME-resistant antitumor T cells (214, 231, 270). These TME-resistant T cells can be further expanded by neoAg vaccines, and their antitumor activity can be further enhanced by the checkpoint blockade therapies, and potentially lead to complete tumor eradication.

With the fast development and innovation of genetic engineering technologies, incorporating the aspects summarized above into the TCR-T therapeutics development, TCR-engineered T cells can be made truly tumor-specific and have the ability to migrate and penetrate into solid tumors, and become TME-resistant. TCR-T has potential to become a powerful tool for fighting cancers, especially solid tumors where other approaches have been less effective. By combining neoAg vaccines, checkpoint blockade therapy, and the adoptive transfer of neoAg-specific TCR-engineered T cells, we believe such a combination approach could lead to significant improvement in cancer immunotherapies, and this approach is scalable across different tumor types, and may provide a general strategy for the eradication of multiple cancers.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Design and Evaluation of TIM-3-CD28 Checkpoint Fusion Proteins to Improve Anti-CD19 CAR T-Cell Function

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Therapeutic targeting of inhibitory checkpoint molecules in combination with chimeric antigen receptor (CAR) T cells is currently investigated in a variety of clinical studies for treatment of hematologic and solid malignancies. However, the impact of co-inhibitory axes and their therapeutic implication remains understudied for the majority of acute leukemias due to their low immunogenicity/mutational load. The inhibitory exhaustion molecule TIM-3 is an important marker for the interaction of T cells with leukemic cells. Moreover, inhibitory signals from malignant cells could be transformed into stimulatory signals by synthetic fusion molecules with extracellular inhibitory receptors fused to an intracellular stimulatory domain. Here, we designed a variety of different TIM-3-CD28 fusion proteins to turn inhibitory signals derived by TIM-3 engagement into T-cell activation through CD28. In the absence of anti-CD19 CAR, two TIM-3-CD28 fusion receptors with large parts of CD28 showed strongest responses in terms of cytokine secretion and proliferation upon stimulation with anti-CD3 antibodies compared to controls. We then combined these two novel TIM-3-CD28 fusion proteins with first- and second-generation anti-CD19 CAR T cells and found that the fusion receptor can increase proliferation, activation, and cytotoxic capacity of conventional anti-CD19 CAR T cells. These additionally armed CAR T cells showed excellent effector function. In terms of safety considerations, the fusion receptors showed exclusively increased cytokine release, when the CAR target CD19 was present. We conclude that combining checkpoint fusion proteins with anti-CD19 CARs has the potential to increase T-cell proliferation capacity with the intention to overcome inhibitory signals during the response against malignant cells.

Keywords: CAR T cells, checkpoint fusion proteins, pediatric leukemia, acute lymphoblastic leukemia (ALL), TIM-3, CD19, CD28

INTRODUCTION

Adoptive cell therapy using chimeric antigen receptor-(CAR-) modified T cells has induced high initial response rates in patients with acute lymphoblastic leukemia and B-cell lymphoma (1–3). These encouraging clinical studies led to approval of multiple CD19-targeting CAR products in the last couple of years (4–6). However, not all patients benefit from CAR T-cell treatment, and 40–60% experience relapse in the course of their disease (3, 5). Moreover, treatment of solid tumors with CAR T cells has not been broadly effective to date. Major causes for nonresponse and relapse are insufficient CAR T-cell expansion and loss of CAR T-cell persistence as well as mutation or downregulation of the target antigen (7–9). The ability to escape the attack of the immune system is a very particular characteristic of malignant tumors. In order to do so, tumors can utilize and redirect immune checkpoint axes, which are physiologically used to balance T-cell responses between activation and inhibition in order to allow sufficient control of infections while preventing autoimmunity (10). Immune checkpoint blockade has been used to reactivate and redirect antitumor T cells and is currently investigated as single therapy and in combination with anti-CD19 and other CAR specificities (11).

TIM-3 (T-cell immunoglobulin and mucin domain-containing protein 3) is a type I transmembrane protein that belongs to the TIM family of proteins (12). It is expressed on not only activated T cells but also other immune cell types such as natural killer (NK) cells, myeloid cells, and regulatory T cells (Tregs). Interestingly, unlike other checkpoint receptors such as PD-1 (programmed cell death protein 1), TIM-3 has no known inhibitory signaling motifs in the intracellular domain, but five tyrosines that seem to interact with BAT3 and Fyn (13, 14). Upon binding of TIM-3 to its ligands, the tyrosines get phosphorylated, BAT3 gets released from the complex, and TIM-3 starts to inhibit the T-cell activation. Known ligands of TIM-3 include galectin-9, HMGB1 (high mobility group box protein 1), phosphatidylserine, and CEACAM1 (CEA cell adhesion molecule 1) (15–17). While galectin-9 and HMGB1 are soluble ligands that can be secreted by a variety of different cell types, phosphatidylserine expression is induced on apoptotic cells. The most recently discovered ligand, CEACAM1, is a membrane protein expressed on T cells, but also other immune cells and tumor cells such as melanoma. Increased expression of TIM-3 on T cells has been associated with terminal differentiation and dysfunction (18). In a previous work by our group, we identified TIM-3 expression on bone marrow T cells as a marker of dismal prognosis in pediatric ALL patients and showed that TIM-3 overexpression can inhibit antileukemic T-cell responses mediated by Blinatumomab (19). While TIM-3 blockade is currently investigated in multiple clinical trials, mostly in combination with PD-(L)1 blockade, the exact mechanism of TIM-3 blockade is not known yet as it might interfere with multiple cell types.

The combination of immune checkpoint blockade with genetically modified T cells has shown promising results in early clinical trials. However, as the CAR but not the therapeutic antibody is tumor-specific, checkpoint blockade can lead to systemic side effects (20). In order to specifically block inhibitory checkpoint axes only on tumor-specific T cells, checkpoint fusion proteins were developed. These fusion proteins usually consist of the extracellular domains of the inhibitory molecule (e.g., PD-1) fused to stimulatory intracellular domains (e.g., CD28) to redirect inhibitory signals toward T-cell stimulation. In the last couple of years, PD-1-CD28, TIM-3-CD28, and CD200R-CD28 fusion receptors were described (21–26). Here, we describe a systematic evaluation of TIM-3-CD28 fusion protein designs to specifically overcome inhibitory signals with the potential to increase CAR T-cell functionality and persistence.

MATERIALS AND METHODS

Generation of CAR T Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Biocoll separation solution (Biochrom). Next, T cells were purified using CD4/8 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. T cells were cultured in TexMACS GMP media (Miltenyi Biotec) plus 2.5% human AB serum (kindly provided by Prof. Ramin Lotfi, University Hospital Ulm, Institute for Transfusion Medicine and German Red Cross Blood Services Baden-Wuerttemberg-Hessen, Institute for Clinical Transfusion Medicine and Immunogenetics, Ulm) supplemented with 12.5 ng/ml IL-7 and IL-15 (human, premium grade, Miltenyi Biotec). T cells were activated using T Cell TransAct, human (Miltenyi Biotec) per the manufacturer's recommendation.

Retroviral particles were generated using producer cells (293Vec-RD114) kindly provided by BioVec Pharma. Supernatant was frozen and stored at -80°C.

T cells were washed and transduced two days after activation. Twenty-four-well plates were coated with 2.5ug RetroNectin Reagent (Takara) followed by a 30-min blocking step (2% Albumin Fraction V, Sigma-Aldrich) and one wash step (1:40 dilution of HEPES 1M (Biochrom) in PBS). The viral supernatant was centrifuged on the coated wells (3,000g, 90 min, 32°C) and discarded afterward. T cells were added and centrifuged 450g for 10 min at 32°C. T cells were washed 48 h after transduction and put back into T-cell media, now containing 6 U/ml IL-2 in addition to IL-7/-15. Cellular composition and T-cell phenotype were analyzed by flow cytometry before cells were frozen on day 12 after transduction. Transduction rates were analyzed by flow cytometric staining of c-myc-FITC (Miltenyi Biotec) and TIM-3-BV421 (Biolegend). For assays, transduction rates were adjusted to the construct with the lowest transduction rate by adding untransduced T cells. Effector cell count refers to the number of CAR/fusion receptor-positive T cells. CD19- and/or CEACAM1-transduced K562 cells were used as target cells unless otherwise stated. Multiple T-cell transductions were not performed; T cells were only transduced once in the process.

Proliferation Assay

T cells were labeled with a CellTrace Violet (CTV) Cell Proliferation Kit (ThermoFisher Scientific) according to the

manufacturer's instruction. Labeled T cells were co-cultured with target cells at a 1:1 effector-to-target (E:T) ratio for 72 h. Percent proliferating cells and absolute cell counts were analyzed using a MACSQuant Analyzer 10 (Miltenyi Biotec).

Cytotoxicity Assay

After NK cell depletion using CD56 MicroBeads according to the manufacturer's instruction (Miltenyi Biotec), T cells were co-cultured with CTV-labeled target cells (ThermoFisher Scientific) at different E:T ratios. The absolute number of remaining target cells was evaluated after 48 h using a MACSQuant Analyzer 10 (Miltenyi Biotec) to calculate the killing capacity of CAR T cells.

Intracellular Cytokine Stain (ICS)

T cells and target cells were co-cultured for 6 h. Two hours after stimulation, 10 ug/ml Brefeldin A (Sigma-Aldrich) was added. Cells were washed and stained after the indicated time. Intracellular cytokine stain for IFN-γ-PE (BD), TNF-α-PacificBlue (Biolegend), and IL-2-APC (BD) was performed using the FIX & PERM cell Fixation and Permeabilization kit (ThermoFisher Scientific) according to the supplier's information. Intracellular cytokine stains were analyzed using a MACSQuant Analyzer 10 (Miltenyi Biotec).

Surface Marker Stain

Activation markers were analyzed by flow cytometry 14 h after starting the co-culture of T cells with target cells at a 1:1 E:T ratio. Anti-CD25-PE, anti-CD69-PE-Vio770, anti-CD137-APC, anti-CD8-APC-Vio770, anti-CD4-VioGreen, and anti-c-myc-FITC (all Miltenyi Biotec) and TIM-3-BV421 (Biolegend) were used. Surface marker stains were analyzed using a MACSQuant Analyzer 10 (Miltenyi Biotec).

CD3 Coating Assays

CD3 coating assays were performed as previously described (21). Briefly, 96-well plates were coated with CD3 monoclonal antibody (HIT3a, ThermoFisher Scientific). Anti-CD3 of 2ug/ml or 0.25ug/ml anti-CD3 were used for ICS or proliferation assays, respectively. Ligands galectin-9 and HMGB1 were added at 200 ng/well. Fusion receptor-positive T cells (0.1e6) were added per well, and proliferation/cytokine stains were performed after 72 and 6 h, respectively.

Cell Lines

Cell lines were regularly tested for the absence of contamination/mycoplasma and STR-typed. Cell lines were cultured in RPMI (Biochrom) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 1% penicillin/streptomycin (Gibco, ThermoFisher Scientific), and 1% L-glutamine (Gibco, ThermoFisher Scientific).

Correlation Analysis (RNA-seq)

Correlation analysis was done using publicly available RNA-seq datasets and the online platform by H.E. Miller, correlationAnalyzeR, (2021), GitHub repository, https://github.com/Bishop-Laboratory/correlationAnalyzeR.

Statistics

Statistics were performed using GraphPad Prism. Statistical significance was calculated using t-test or one-way ANOVA as outlined in the figure legends. P values: * <0.05, ** <0.01, *** <0.001, **** <0.0001. Mean plus standard error mean is shown unless stated otherwise.

RESULTS

Systematic Design of TIM-3-CD28 Fusion Proteins

We first analyzed TIM-3 expression on anti-CD19 CAR T cells and found a rapid induction of TIM-3 expression already after a single stimulation with $CD19^+$ target cells (**Figure 1A**). When analyzing the target cells for the expression of the membrane-bound ligand of TIM-3, CEACAM1, we observed that many leukemia and lymphoma cell lines upregulate CEACAM1 when exposed to Th1 cytokines IFN-γ and TNF-α (**Figure 1B**). As T cells can potentially also express CEACAM1 and contribute to CAR T-cell inhibition through TIM-3, we next checked correlation analyses of publicly available RNA-seq data (**Figure 1C**). We found that in healthy immune cell datasets, TIM-3 (HAVCR2) and CEACAM1 expression levels are inversely correlated hinting toward the fact that TIM-3 and CEACAM1 are usually not expressed simultaneously. In contrast, in immune cancer datasets, TIM-3 and CEACAM1 expressions are strongly correlated. As we did not observe TIM-3 expression on leukemic cell lines (**Supplementary Figure 1A**), we hypothesize that high TIM-3 levels on T cells (or other immune cells) in cancer might correlate with CEACAM1 expression on target or T cells. To transform TIM-3-mediated inhibition into CD28-based co-stimulation (**Figure 1D**), we generated a variety of different TIM-3-CD28 fusion receptors (**Figure 1E**). While fusion receptor 1 (TIM-3/28-1) had a CD8 transmembrane domain, in analogy to CARs, the other fusion receptors 2–6 were comprised of either the TIM-3 or the CD28 transmembrane domain. TIM-3/28-6 had the largest portions of CD28 as it had been shown before that the cysteine in amino acid position 141 of CD28 can increase signaling through the receptor (23). We first retrovirally transduced only the fusion receptors without a CAR into primary human T cells to check expression levels and basic functionality. Transduction rates were analyzed by flow cytometric staining for TIM-3 (**Figure 1F**). While the CD8-containing fusion receptor TIM-3/28-1 showed decreased transduction rates, receptors 2–6 showed robust transduction

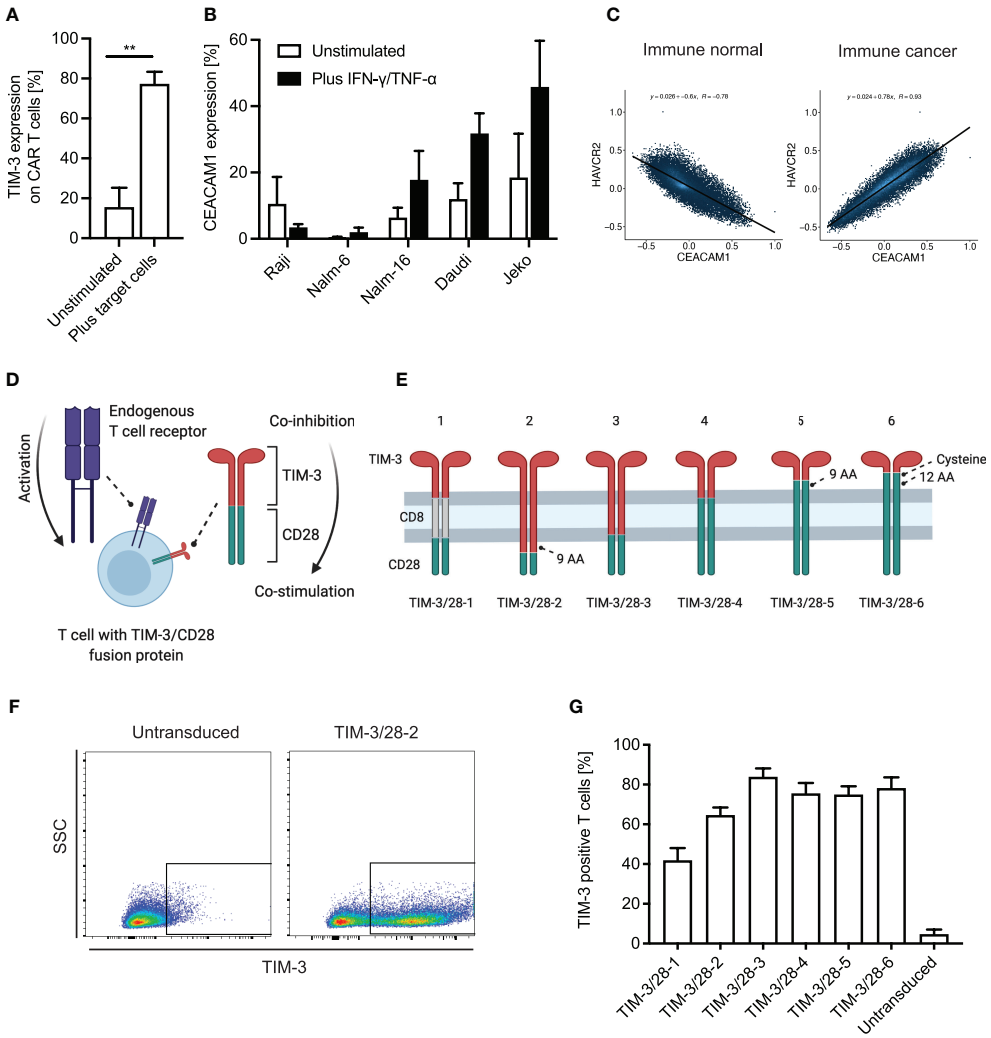

FIGURE 1 | TIM-3 and CEACAM1 expression on T cells/leukemic cells and design of TIM-3-CD28 fusion proteins. **(A)** T cells were retrovirally transduced with an anti-CD19 CAR and co-cultured with $CD19^+$ target cells (K562 cells transduced with CD19) for 48 h. TIM-3 expression on CAR T cells was analyzed by flow cytometry. N = 4 individual donors; unpaired t-test was performed. Data are representative of four independent experiments ** < 0.01. **(B)** Leukemia and lymphoma cell lines were either left unstimulated or stimulated with 100 ng/ml IFN-γ and 10 ng/ml TNF-α. CEACAM1 expression was analyzed by flow cytometry. N ≥ 3; unpaired t-test was performed. Data are representative of three independent experiments. **(C)** Correlation of CEACAM1 and TIM-3 (HAVCR2) expression in publicly available datasets was evaluated using the online tool Correlation AnalyzeR (H.E. Miller, correlationAnalyzeR, (2021), GitHub repository, https://github.com/Bishop-Laboratory/correlationAnalyzeR). **(D)** Schematic illustration of a T cell with its endogenous TCR and the TIM-3-CD28 fusion protein that is intended to turn co-inhibition into co-stimulation. **(E)** Schematic illustrations of the six different fusion proteins designed for this study. **(F)** Exemplary flow plot showing transduction of TIM-3/28-2 into primary human T cells as analyzed by TIM-3 expression in flow cytometry. **(G)** Transduction rates as analyzed by flow cytometric staining of TIM-3. N ≥ 3 individual donors. Data are representative of three independent experiments. AA, amino acid; SSC, side scatter. Schematic illustrations created using biorender.com.

rates of >60% (**Figure 1G**). When analyzing the geometric mean fluorescence intensity, we observed differences between the TIM-3-CD28 fusion proteins hinting at a different number of molecules per cell based on the type of construct used (**Supplementary Figure 1B**). The transduced T cells consisted mainly of $CD8^+$ T cells 12 days after transduction, and the distribution of cell types was not significantly different between the fusion receptors or untransduced T cells (**Supplementary Figure 1C**). The T-cell phenotype was slightly switched toward more effector memory T cells in constructs 2–6 compared to untransduced T cells or T cells transduced with construct 1 (**Supplementary Figure 1D**). Differences in viability throughout the culture were not observed (**Supplementary Figure 1E**). Expansion rates after transduction were comparable (**Supplementary Figure 1F**) with a slight advantage for untransduced T cells.

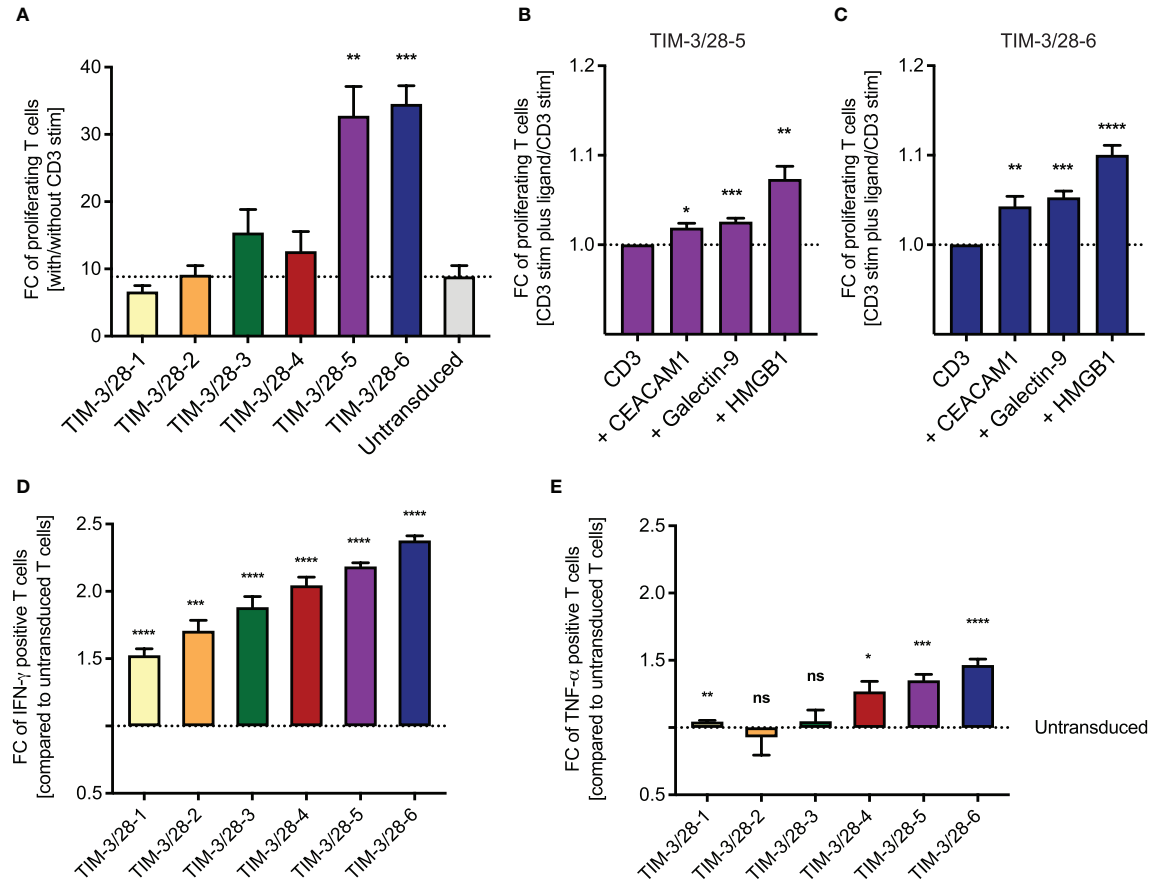

FIGURE 2 | Choosing the best TIM-3-CD28 fusion receptor based on proliferation and cytokine release. **(A)** Fusion receptor-transduced T cells were cultured on anti-CD3-coated plates. Percent proliferating T cells was evaluated by CTV staining and the fold change with/without CD3 stim calculated for each construct. To evaluate the impact of ligand addition, the fold change of proliferating T cells was calculated on CD3 stimulation plus ligand vs minus ligand for TIM-3/28-5 **(B)** and TIM-3/28-6 **(C)**. Fold change of IFN-γ **(D)** and TNF-α **(E)** positive T cells compared to untransduced T cells was analyzed by intracellular cytokine stain for IFN-γ or TNF-α with/without CD3 stimulation. FC, fold change; stim, stimulation. Dotted line represents fold change of untransduced T cells **(A, D, E)** or CD3 stimulation only **(B, C)**. Experiments were performed in two individual donors and technical duplicates. Data are representative of two independent experiments. Unpaired t-test was performed to determine significance. Physiologic expression levels of TIM-3 ligands are shown in **Supplementary Figure 3**. * <0.05, ** <0.01, *** <0.001, **** <0.0001, ns, not significant.

TIM-3-CD28-Fusion Proteins With Parts of CD28 for the Hinge Region Exhibit Largest Proliferation Potential and Cytokine Release

We next tested the different fusion receptor T cells in response to CD3 stimulus and observed that the fusion proteins TIM-3-CD28-5 and TIM-3-CD28-6 showed the highest fold change of proliferating cells (with vs. without CD3 stimulation) as analyzed by CTV staining (**Figure 2A**). Background proliferation without CD3 stimulation was below 20% at that timepoint for all constructs (**Supplementary Figure 2A**). This effect was amplified in the presence of the mostly membrane-bound ligand CEACAM1 and the soluble ligands galectin-9 and HMGB1 (**Figures 2B, C**). When analyzing the cells by intracellular cytokine staining of IFN-γ and TNF-α, we found that all fusion proteins can enhance IFN-γ release while only some of them show an effect in TNF-α secretion (**Figures 2D, E**). TIM-3-CD28-5 and -6 were identified as fusion receptors with highest levels of cytokine release. Background cytokine secretion without CD3 stimulation was below 10% for all constructs (**Supplementary Figures 2B, C**). Cytokine release could only be amplified by the addition of HMGB1 to the culture; the other ligands did not lead to significant changes in cytokine levels tested on the two best-performing constructs (**Supplementary Figures 2D–G**). For reference, physiologic expression levels of TIM-3 ligands were extracted from publicly available RNA-seq datasets and are shown in **Supplementary Figure 3**.

Generation of Anti-CD19 CAR T Cells With TIM-3-CD28 Fusion Proteins

As TIM-3-CD28-5 and -6 were the fusion receptor designs with the highest proliferation and cytokine release, we tested these two constructs in combination with the first- and second-generation CAR T cells. Thus, we created multicistronic constructs with an

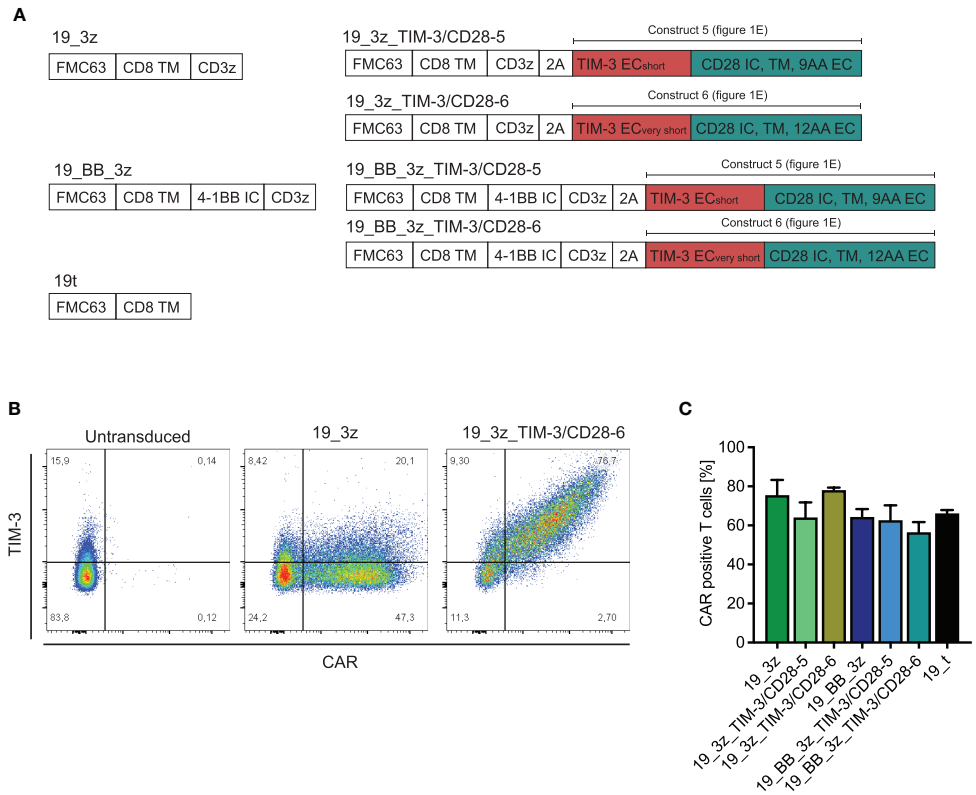

FIGURE 3 | Transduction of primary human T cells with anti-CD19 CARs in addition to TIM-3-CD28 fusion receptors. **(A)** Schematic illustration of transduced CAR T-cell constructs with/without fusion receptors and control 19t. **(B)** Exemplary flow plot showing CAR (myc)/TIM-3 stain in 19_3z CARs with and without fusion protein. **(C)** Transduction rates of CARs with/without fusion proteins as determined by flow cytometric stain for myc. N = 2 individual donors. Data are representative of two independent experiments.

FMC63-based anti-CD19 CAR and the fusion receptor separated by a 2A cleavage site as depicted in **Figure 3A**. We included a myc tag into the CAR construct for better detection in flow cytometry. Primary human T cells were transduced with the six different CAR constructs plus a truncated anti-CD19 CAR (19t) control, which is lacking intracellular signaling domains. Coexpression of the fusion receptor and CAR was confirmed by flow cytometry (**Figure 3B**). All fusion receptor–CAR combinations showed comparable transduction rates of around 60% (**Figure 3C**) and no difference in viability throughout the culturing process (**Supplementary Figures 4A, B**).

TIM-3-CD28 Fusion Proteins Can Increase CAR T-Cell Functionality

First, we tested whether the fusion proteins can increase the functionality of first-generation CAR T cells as a model system for a suboptimal (and thus optimizable) CAR setting. As expected, we observed a slight decrease in killing capacity of conventional first-generation CARs without fusion protein when the cells got co-cultured with $CD19^+/CEACAM1^+$ compared to CD19+/CEACAM1- target cells (**Supplementary Figure 5A**). Next, we co-cultured first-generation CAR T cells with or without the fusion receptor with $CD19^+/CEACAM1^+$ target cells. Indeed, the addition of the fusion receptor significantly increased the killing capacity of conventional CAR T cells (**Figure 4A**). The same trends were observed after co-culture with $CD19^+/CEACAM^-$ target cells (**Supplementary Figure 5B**). When analyzing the proliferative capacity of the fusion receptor first-generation CAR T cells, we interestingly observed an increased frequency of proliferating fusion protein CARs compared to conventional CARs without the addition of target cells (**Figure 4B**). The same trend was observed when analyzing absolute CAR T-cell counts (**Supplementary Figure 5C**). Looking back at the behavior of the cells during the culturing process, we confirmed this finding as the fusion receptors showed higher proliferative capacity in the absence of target cells (**Figure 4C**). As this result might raise concerns of limited target specificity and potential off-target side effects, we next measured the cytokine release of the fusion protein CAR T cells both in the absence and presence of target cells (**Figure 4D**). Reassuringly, we did not see cytokine release of the fusion protein CAR T cells in the absence of targets, while in the presence of targets, they were able to increase the cytokine release beyond levels detected by conventional first-generation CARs. Moreover, CAR T cells with TIM-3-CD28 fusion protein showed higher levels of CD25 compared to conventional CARs

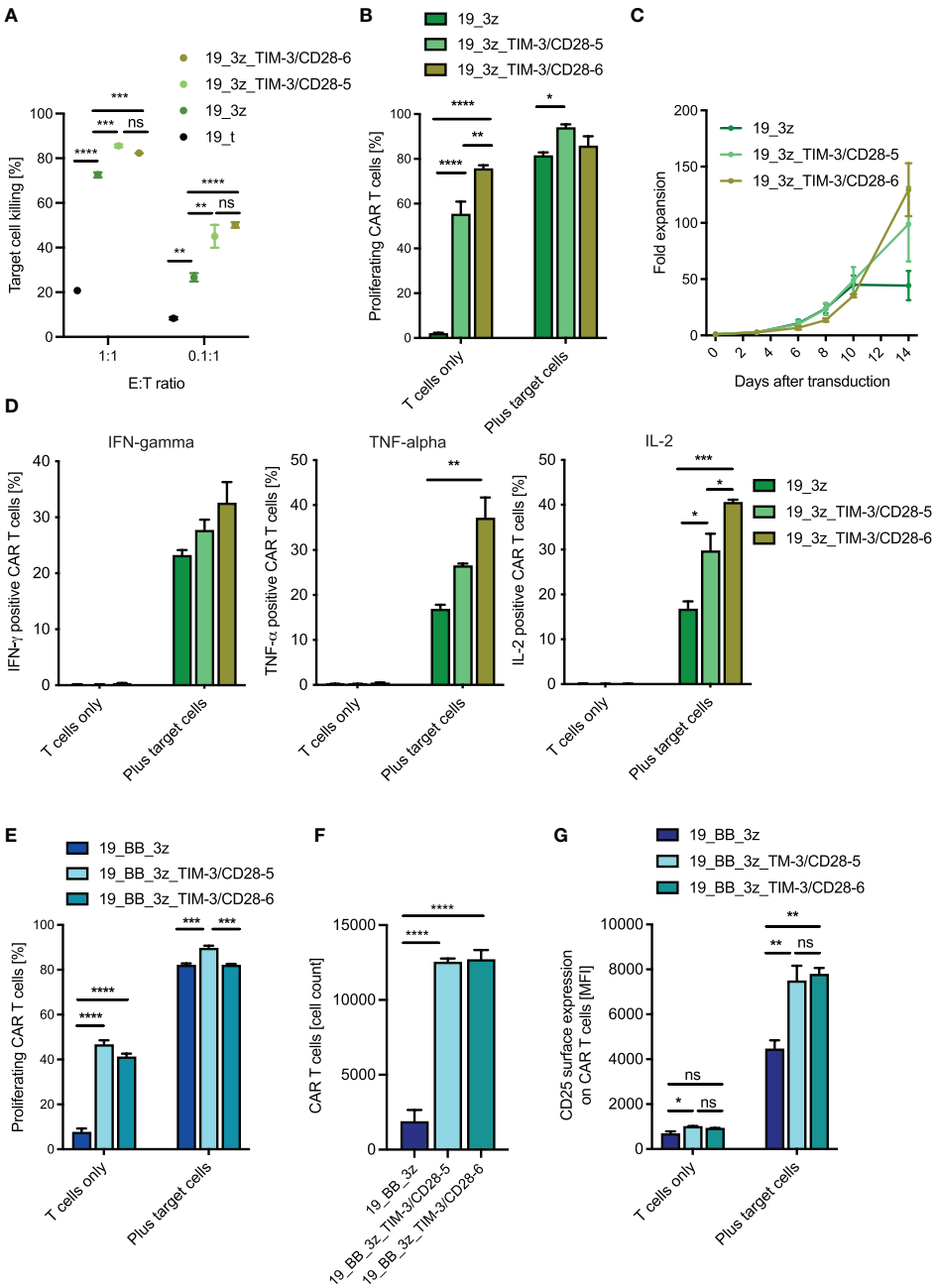

FIGURE 4 | Functionality of anti-CD19 CAR T cells with TIM-3-CD28 fusion proteins. **(A)** Killing of CD19$^+$/CEACAM$^+$ K562 target cells by first-generation anti-CD19 CAR T cells with/without fusion proteins was calculated after 48 h of co-culture. N = 1 individual donor in technical duplicates for 1:1 E:T ratio and n = 2 individual donors in technical duplicates for 0.1:1 E:T ratio. One-way ANOVA was performed to determine statistical significance. **(B)** First-generation anti-CD19 CAR T cells were co-cultured with target cells (CD19$^+$/CEACAM$^+$ K562) for 72 h, and percent proliferating cells were analyzed by flow cytometry (CTV). N = 2 individual donors in technical duplicates. One-way ANOVA was performed to determine statistical significance. **(C)** Fold expansion of different CAR T-cell constructs throughout the culture process. Cell count was normalized on the day of transduction. N = 2 individual donors. **(D)** First-generation anti-CD19 CAR T cells with/without fusion proteins were co-cultured with target cells (CD19$^+$/CEACAM$^+$ K562), and cytokine production was analyzed by intracellular cytokine stain for IFN-γ, TNF-α, and IL-2 6 h after the start of the co-culture. **(E)** Second-generation CAR T cells with/without fusion protein were co-cultured with target cells (CD19$^+$/CEACAM$^+$ K562), and proliferative potential both in terms of percent proliferating cells **(E)** and absolute CAR cell count **(F)** were analyzed after 72 h. N = 2 individual donors in technical duplicates. One-way ANOVA was performed to determine statistical significance. **(G)** CD25 surface expression was evaluated by flow cytometry 14 h after target cell contact (CD19$^+$/CEACAM$^+$ K562). N = 2 individual donors, each in technical duplicates. One-way ANOVA was performed to determine statistical significance. Data are representative of two independent experiments **(B–G)**. E:T ratio, effector-to-target ratio; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; IL-2, interleukin-2. * <0.05, ** <0.01, *** <0.001, **** <0.0001, ns, not significant.

(**Supplementary Figure 5D**), potentially making them an interesting modification in situations of IL-2 competition seen in the presence of regulatory T cells. Surprisingly, we observed higher levels of early differentiation marker CD62L on fusion protein CAR T cells (**Supplementary Figure 5E**), which translated to decreased percentages of terminally differentiated effector cells and higher percentages of early differentiated stem cell-like memory (Tscm) and central memory (Tcm) cells in fusion protein CARs (**Supplementary Figure 5F**). We next investigated second-generation CAR T cells with 4-1BB-based co-stimulation. Again, we observed that the killing capacity of conventional second-generation CARs is slightly decreased when the target cells express CEACAM1$^+$ (**Supplementary Figure 6A**). When combining TIM-3-CD28 fusion proteins with conventional second-generation CAR T cells, we saw comparable trends to the combination with first-generation CAR T cells. While the fusion proteins were not able to increase short-term (48 h) killing capacity of second-generation CARs (**Supplementary Figure 6B**), fusion receptor CAR T cells showed higher proliferative potential in the absence of target cells. After the addition of targets, the percent proliferating cells were potentially maxed out at around 80% (**Figure 4E**). The trend of increased T-cell numbers without the addition of targets was again seen in the second-generation CARs (although not significant) when looking at the growth curves (**Supplementary Figure 6C**) and confirmed by analyzing the CAR T-cell counts during the proliferation assay (**Figure 4F**). Surprisingly, the percentage of cytokine-secreting T cells was decreased in fusion receptor second-generation CAR T cells (**Supplementary Figure 6D**). Consistent with the findings in first-generation CARs, we again observed increased levels of CD25 expression (**Figure 4G**) and decreased levels of late-effector phenotype (**Supplementary Figure 6E**) in fusion protein CAR T cells. In summary, TIM-3-CD28 fusion receptor CAR T cells can improve conventional CAR T cells in certain situations. Despite decreased percent cytokine secretion in second-generation CAR T cells, short-term killing is not decreased and TIM-3-CD28 fusion proteins can mediate higher CAR numbers, increased proliferative potential, CD25 expression, and earlier differentiation states of CAR T cells.

DISCUSSION

Despite high initial response rates in B-cell precursor leukemia and lymphoma, anti-CD19 CAR T-cell therapy can lack long-term efficacy due to multiple factors including limited CAR T-cell proliferation and persistence (3, 27). In recent work, we and others showed that TIM-3 expression on T cells can limit antileukemic T-cell responses both in terms of cytokine release and proliferation (19, 28). Here, we found that TIM-3 gets upregulated on conventional anti-CD19 CAR T cells after a single stimulation with target cells potentially to prevent excessive stimulation. On the other hand, we identified substantial upregulation of the TIM-3 ligand CEACAM1 on leukemic cell lines upon simulation of a Th1 attack. In publicly available RNA-seq data, a correlation in immune cancer between TIM-3 and CEACAM1 expression is seen, which is consistent with reports by other groups that have shown an overexpression of both TIM-3 and CEACAM1 on tumor-infiltrating T cells in a variety of different tumors (29). While the exact impact of CEACAM1 expression and the expression of the other TIM-3 ligands in childhood leukemia is unknown, our group has recently shown that the three protein-based ligands are detectable on RNA level and identified high TIM-3 expression on bone marrow T cells as a prediction marker of dismal prognosis hinting to an important role of the inhibitory TIM-3 axis in ALL (19). We thus decided to generate TIM-3-CD28 fusion receptors to turn TIM-3-mediated inhibition into CD28-based stimulation. For fusion receptor TIM-3-CD28-1, we decided to test a CD8 transmembrane domain as this domain has been used in CAR T cells and shows good surface expression. However, in our experiments, transduction with TIM-3-CD28-1 yielded the lowest transduction rates and geometric mean fluorescent intensity. This is in line with a report by Schlenker et al. (30) who tested PD-1-CD28 fusion proteins and showed that their CD8 transmembrane design led to lowest percent PD-1$^+$ cells. The other five TIM-3-CD28 fusion proteins tested here comprised of different portions of TIM-3 and CD28 proteins. As recent reports of CD200R-CD28 fusion receptors have indicated that using larger parts of CD28 including the membrane-proximal extracellular cysteine in amino acid position 141 is superior to other designs (23), we tested TIM-3-CD28 receptors with large CD28 fragments and very short TIM-3 parts, too. To ensure a physiologic distance in the immune synapse between the artificial TIM-3 and its binding partners, we kept the total number of extracellular amino acids stable. In analogy to previous reports of PD-1-CD28 fusion proteins (21), we next tested the activation and proliferation potential of the different fusion receptors by stimulating the T cells with CD3 antibody. While TIM-3-CD28-1 and -2 did not show increased proliferative potential, the two receptors with the largest CD28 parts exerted the highest fold change in proliferation when the percent proliferating cells before/after target cell addition were compared. While the background proliferation (without CD3 stimulation) was <20% for all constructs, it was the lowest for TIM-3-CD28-5 and -6, which contributed to the increased fold change. However, we chose fold change as a readout because the aim was to identify the fusion protein with the highest dynamic range (low background proliferation, strong response to CD3 stimulation). As expected, the proliferative effect was potentiated by adding the soluble form of the different protein-based TIM-3 ligands to the culture. The impact of CEACAM1 addition was rather minimal compared to the other ligands. There are two potential explanations for this finding: 1) We added the soluble version of CEACAM1, and the impact of soluble CEACAM1 on TIM-3 signaling in T cells is not well understood yet. 2) The activated T cells themselves most likely expressed CEACAM1 on the surface, which would dilute the effect of adding additional CEACAM1 to the culture. Increases in proliferative capacity and FC of cytokine release compared to untransduced T cells were observed even without the addition of the ligands. This further underlined the possibility that the activated or bystander T cells could upregulate or secrete the respective ligands. As TIM-3-CD28-5 and -6 also showed the

highest dynamic range in cytokine release, we decided to follow up on these two receptors and cloned them into multicistronic constructs in combination with first- and second-generation anti-CD19 CARs. We decided to pair the CD28-based fusion proteins with 4-1BB-mediated costimulation for the second-generation CAR to investigate potential synergistic effects of CD28 and 4-1BB. While the addition of the fusion proteins to first-generation CARs showed slightly increased cytotoxicity against $CD19^+$/$CEACAM1^+$ target cells, the fusion receptors were not able to increase killing beyond the level of second-generation CARs. Notably, short-term killing assays (48 h) represent prompt effector function, while the expected advantage of the TIM-3-CD28 fusion receptor is pointing toward longevity of T cells. Further *in vivo* studies to evaluate the long-term proliferative and killing capacity would be helpful to analyze the full therapeutic potential of the switch receptors. The strength of the TIM-3-CD28 fusion receptors described here appears to be mostly in terms of proliferation and increased CAR T-cell numbers. Interestingly, without restimulation of the cells, we observed a stronger proliferative advantage of the fusion receptors when paired with a CAR (**Figure 4C**; **Supplementary Figure 6C**) compared to fusion receptors that were not combined with a CAR (**Supplementary Figure 1F**). Reasons for that are speculative but could include differences in soluble or membrane-bound ligand levels, a small amount of tonic signaling through the CAR, or remaining small numbers of $CD19^+$ cells in the culture that could provide some background activation to the CAR T cells. While the fusion receptors led to higher CAR numbers and increased proliferation even in the absence of target cells, we did not detect substantial cytokine release without target cell presence. However, the addition of these receptors into CAR T cells might require additional safety considerations, such as suicide switches or synthetic circuits. Further studies are needed to understand the proliferative behavior of TIM-3-CD28 fusion receptors. To clearly dissect the role of the different domains in activating the fusion receptor, mutations could be introduced into the CD28 signaling or the TIM-3/ligand binding domain to disrupt the signal and investigate the specificity and signal transduction of the receptor. Moreover, transcriptional/gene set enrichment analyses will describe the proliferative phenotype and ensure that the increase in proliferation does not lead to long-term dysfunction/exhaustion in the fusion receptor CAR T cells. Overexpressing TIM-3-CD28 fusion proteins together with second-generation CAR T cells led to decreased percentages of IL-2 releasing cells. However, this effect might be outweighed by the increased overall number of CAR T cells with a fusion receptor. The slightly increased percentage of fusion protein positive cells expressing CD62L as a marker of early T-cell differentiation states might create an additional benefit. An interesting finding was the increased levels of CD25 that might render fusion protein CAR T cells more effective in situations of competition for IL-2 or presence of Tregs. Moreover, increased levels of CD25 might have contributed to the proliferative phenotype of the fusion protein CAR T cells. Although they were cultured with minimal levels of exogenous IL-2, the bystander/surrounding CAR T cells most likely produced IL-2, which might have led to a competitive advantage of the fusion protein CAR T cells. In analogy to reports of PD-1-CD28 fusion proteins that seem to increase functionality of low avidity TCRs rather than high avidity TCRs (30), the TIM-3-CD28 fusion receptors might work best in a more challenging setting of, e.g., lower CAR affinity, tonic signaling, or in a solid tumor microenvironment with a high expression of multiple TIM-3 ligands. Genetically modified CAR T cells in combination with checkpoint fusion receptors are a promising treatment alternative to systemic combinations of checkpoint inhibitors together with CAR T cells. While checkpoint inhibitors can cause severe systemic side effects and usually have to be administered multiple times, the fusion receptors will only be expressed specifically on CAR T cells when both the CAR construct and fusion receptor are introduced into the T cell using a polycistronic construct. The rationale is that CAR T cells with a fusion receptor can persist after a single infusion and will not have side effects beyond the known CAR-related complications as shown in recent CAR T-cell trials with PD-1-CD28 fusion proteins (31). Last year, Zhao et al. (26) published a TIM-3-CD28 fusion protein that uses the transmembrane and intracellular domain of CD28 and the extracellular domain of TIM-3 and is thus similar to TIM-3/CD28-4 from our study. Their design mediated increased persistence and antitumor efficacy when combined with a second-generation anti-CD19 CAR. Differences between the two studies include the CAR design as well as the transduction method and the culturing of the cells. While their protocol uses 50 U/ml IL-2, our culturing protocol uses lower levels of IL-2 (6 U/ml) in combination with IL-7 and IL-15, which might have contributed to differences in the two studies. Because Oda etal. (23) had recently shown that using larger parts of CD28 for fusion proteins can be beneficial due to a potential cysteine bond in the extracellular part of CD28, we decided to analyze different portions of CD28 systematically. Our study underlines the finding that including larger parts of CD28 into fusion protein designs might offer possibilities to expand the proliferative potential even further. Thus, the present systematic characterization of TIM-3-CD28 fusion receptors can lay the groundwork for future investigations of these receptors in CAR settings other than the clinically used second-generation anti-CD19 CARs. Further analysis of TIM-3-CD28 fusion proteins could include combination with other CAR specificities, other target cell lines with different expression levels/secretion of the TIM-3 ligands, blocking experiments, as well as co-culture with primary B-precursor blasts. Subsequent evaluation in suitable *in vivo* models (e.g., conventional xenograft or patient-derived xenografts) may reveal additional potential of TIM-3-CD28 fusion proteins.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission bei der Medizinischen Fakultät der LMU München. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The approach of the study was set up by TF, FB and SK. Experimental design was done by FB and DS. Fusion proteins were designed by FB, TF and SK. EO, FB, JM, AA, SW, DS, TW, NH and TK performed experiments. FB and TF wrote the manuscript. The manuscript was reviewed by all co-authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.845499/full#supplementary-material

Supplementary Figure 1 | Expression levels of TIM-3, cellular composition, and quality control. **(A)** Leukemia and lymphoma cell lines were stimulated with 100 ng/ml IFN-γ and 10 ng/ml TNF-α for 48 h, and TIM-3 expression was evaluated by flow cytometry with/without stimulation. Experiment was performed in technical triplicates. **(B)** GeoMean fluorescent intensity of TIM-3 on T cells transduced with the different fusion proteins as determined by flow cytometry. N ≥ 3 individual donors. Cellular composition **(C)** and phenotype **(D)** of the T-cell culture 12 days after transduction were analyzed by flow cytometric staining for CD3, CD4, CD8, CD56, c-myc, CD14, and CD19 **(C)** and CD62L, CD45RO, and CD95 **(D)**. N ≥ 3 individual donors. Viability **(E)** and expansion rate **(F)** of transduced T cells were evaluated by trypan blue stain/cell count throughout the culture period. N ≥ 3 individual donors. Data are representative of at least three independent experiments **(B–F)**. Teff, effector T cells; Tem, effector memory T cells; Tcm, central memory T cells; Tscm, stem cell-like memory T cells; Tn, naïve T cells.

Supplementary Figure 2 | Proliferation and cytokine release of fusion protein-transduced T cells. **(A)** Fusion receptor-transduced T cells were cultured without prior anti-CD3 coating. Percent proliferating T cells was evaluated by CTV staining. **(B, C)** Background cytokine release of fusion receptor T cells without prior anti-CD3 stimulation. **(D–G)** Fusion protein-transduced T cells were cultured with/without anti-CD3 and the soluble ligands galectin-9 and HMGB1. Cytokine release was determined by intracellular cytokine stain for IFN-γ **(D, E)** and TNF-α **(F, G)**. Experiments were performed in two individual donors and technical duplicates. Data are representative of two independent experiments. Unpaired t-test was performed to determine significance **(D–G)**. FC, fold change; ns, not significant. Physiologic expression levels of TIM-3 ligands are shown in **Supplementary Figure 3**.

Supplementary Figure 3 | Expression levels of TIM-3 ligands. Physiologic expression levels of TIM-3 ligands CEACAM1 **(A)**, Galectin-9 **(B)**, and HMGB1 **(C)** were extracted from publicly available RNA-seq data (DICE dataset, https://dice-database.org/).

Supplementary Figure 4 | Viability of first- and second-generation CAR T cells. Viability of first-generation **(A)** and second-generation **(B)** CAR T cells with/without fusion proteins was analyzed by trypan blue staining throughout the culture period. N = 2 individual donors. Data are representative of two independent experiments.

Supplementary Figure 5 | Efficacy of first-generation CAR T cells with/without fusion proteins. **(A)** First-generation CAR T cells were co-cultured with CEACAM1$^+$ or CEACAM1$^-$ target cells for 48 h. Cytotoxicity analysis revealed slightly reduced killing capacity against the CEACAM-expressing cell line. N = 2 donors in technical triplicates. **(B)** First-generation CAR T cells with/without TIM-3-CD28 fusion proteins were co-cultured with CEACAM1- target cells, and cytotoxicity was evaluated 48 h later. N = 2 individual donors in technical duplicates. **(C)** First-generation CAR T cells with/without TIM-3-CD28 fusion proteins were co-cultured with CEACAM1+ target cells, and changes in absolute CAR T-cell count were detected by flow cytometry after 72 h. N = 2 individual donors in technical replicates. One-way ANOVA was performed to determine statistical significance. **(D)** CD25 surface expression was evaluated by flow cytometry on first-generation CAR T cells with/without fusion proteins +/- target cells. N = 2 individual donors in technical duplicates. One-way ANOVA was performed to determine statistical significance. **(E)** CD62L expression on first-generation CAR T cells with/without fusion proteins 12 days after transduction. N = 1 donor in technical duplicates. **(F)** T-cell subpopulation of first-generation CAR T cells with/without fusion proteins was analyzed by flow cytometry staining for CD62L, CD45RO, and CD95 on day 12 after transduction. N = 1 donor in technical duplicates. Data are representative of two independent experiments **(A–D)** and of one experiment **(E, F)**, respectively. Teff, effector T cells; Tem, effector memory T cells; Tcm, central memory T cells; Tscm, stem cell-like memory T cells; Tn, naïve T cells; E:T, effector-to-target ratio.

Supplementary Figure 6 | Efficacy of second-generation CAR T cells with/without fusion proteins. **(A)** Second-generation CAR T cells were co-cultured with CEACAM1$^+$ or CEACAM1$^-$ target cells for 48 h. Cytotoxicity analysis revealed slightly reduced killing capacity against the CECAM1-expressing cell line. N = 2 donors in technical triplicates. Data are representative of two independent experiments. **(B)** Killing capacity of second-generation CAR T cells with/without TIM-3-CD28 fusion proteins was analyzed 48 h after starting the co-culture with

$CD19^+/CEACAM1^-$ or $CEACAM^+$ target cells. N = 1 individual donor in technical duplicates for the 1:1 E/T ratio in the $CEACAM^+$ condition and n = 2 individual donors in technical duplicates for all other conditions. Data are representative of at least one independent experiment. One-way ANOVA was performed to determine statistical significance. **(C)** Fold expansion (relative to cell count on day of transduction) was calculated for second-generation CARs +/- fusion protein throughout the culture process. N = 2 individual donors. **(D)** Intracellular cytokine stain was performed after co-culturing second-generation CAR T cells with/without fusion protein with target cells. **(E)** T cell subpopulation of second-generation CAR T cells with/without fusion proteins was analyzed by flow cytometry staining for CD62L, CD45RO, and CD95 on day 12 after transduction. Data are representative of two independent experiments **(C, D)** and of one experiment **(E)**, respectively. Teff, effector T cells; Tem, effector memory T cells; Tcm, central memory T cells; Tscm, stem cell-like memory T cells; Tn, naïve T cells; E:T, effector-to-target ratio; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; IL-2, interleukin-2.

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REVIEW
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Impact of Manufacturing Procedures on CAR T Cell Functionality

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The field of chimeric antigen receptor (CAR) modified T cell therapy has rapidly expanded in the past few decades. As of today, there are six CAR T cell products that have been approved by the FDA: KYMRIAH (tisagenlecleucel, CD19 CAR T cells), YESCARTA (axicabtagene ciloleucel, CD19 CAR T cells), TECARTUS (brexucabtagene autoleucel, CD19 CAR T cells), BREYANZI (lisocabtagene maraleucel, CD19 CAR T cells), ABECMA (idecabtagene vicleucel, BCMA CAR T cells) and CARVYKTI (ciltacabtagene autoleucel, BCMA CAR T cells). With this clinical success, CAR T cell therapy has become one of the most promising treatment options to combat cancers. Current research efforts focus on further potentiating its efficacy in non-responding patients and solid tumor settings. To achieve this, recent evidence suggested that, apart from developing next-generation CAR T cells with additional genetic modifications, *ex vivo* culture conditions could significantly impact CAR T cell functionality – an often overlooked aspect during clinical translation. In this review, we focus on the *ex vivo* manufacturing process for CAR T cells and discuss how it impacts CAR T cell function.

Keywords: CAR T cell, *ex vivo* expansion, culture media, serum, cytokines, pharmacological inhibitor, manufacturing time, cryopreservation

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INTRODUCTION

With the promising clinical success of CD19 CAR T cell therapy for B-cell lineage malignancies (1–4), there have been more and more publications focusing on ways to enhance CAR T cell function by complex genetic engineering (5–7). However, less attention has been paid to culture methods for the *ex vivo* maintenance of therapeutic T cells, a necessary step to generate CAR T cells for both preclinical research and clinical implementation, and their effects on the quality of cell products. The general procedure for manufacturing CAR T cell begins with isolation of peripheral blood mononuclear cells (PBMCs). Next, PBMCs or T cells that have been further enriched from PBMCs are stimulated with antibody-coated beads (e.g. Dynabeads) or plate-bound antibodies to induce T cell activation and then genetically modified using lentiviral vectors (8, 9), gamma-retroviral vectors (10, 11) or other delivery methods (12, 13) to express the cell surface CAR molecule. Subsequently, these engineered T cells are expanded in culture to reach the required cell numbers for either experimental testing or clinical treatment. Importantly, *ex vivo* culture conditions are completely different from the homeostatic environment *in vivo*, warranting detailed investigations on the impact of each manufacturing step on T cell quality. For instance,

reagents used for *ex vivo* CAR T cell expansion, including media, sera, cytokines, and additional medium supplements, can collectively mount a significant impact on CAR T cell function. Additionally, the duration of CAR T cell expansion before cryopreservation can also affect the overall potency of CAR T cells. As the effects of different T cell enrichment/stimulation methods and gene delivery procedures have been extensively reviewed elsewhere (14), here we will focus on the impact of culture conditions, summarize and discuss how each component/step affects CAR T cell function.

IMPACT OF CULTURE MEDIA

Selection of media is one of the first considerations for *ex vivo* CAR T cell expansion. Historically, RPMI-1640 medium has been widely used for T cell manufacturing. It is however unclear if this is the best choice for generating clinical-grade CAR T cells. Currently, there are a variety of media available in the market, all designed to support optimal T cell expansion. It is therefore important to compare and choose the right medium, with the first and foremost objective being sufficient T cell expansion that meets the required cell doses for treatment.

Sato et al. compared expansion of OKT3-stimulated PBMCs in different media (RPMI-1640, AIM-V and Optimizer) supplemented with low concentrations of autologous serum (1-8%) in the presence of IL-2 (175 IU/mL) (15). The 7-day fold expansion of T cells in each medium was 52.7 ± 13.1 (RPMI-1640), 25.9 ± 16.8 (AIM-V) and 55.5 ± 20.6 (Optimizer). However, fold expansion on day 12 was similar between Optimizer (1180 ± 51) and AIM-V (1110 ± 71) but lower in RPMI-1640 (not defined). Of note, serum concentration was 8% at the beginning of culture and was diluted every 2-3 days when new media were replenished, with the resulting serum concentration reaching 1% on day 7 and maintaining at that level. These results demonstrated that T cell expansion in RPMI-1640 is highly dependent on serum concentrations.

In another study, Lu et al. compared three serum-free media (SFM): Optimizer, X-VIVO15 and TexMACS, for *ex vivo* OKT3-stimulated T cell expansion in the presence of IL-2 (300 IU/mL) for 6 days (16). Using SFM is ideal for clinical manufacturing of CAR T cell products, since it prevents or reduces the risk of inconsistent CAR T cell functionality resulting from lot-to-lot variations of serum quality (17–19). Among the three SFM tested, Optimizer resulted in the greatest number of stimulated T cells. However, the overall cell expansion in all SFM conditions was significantly lower than that in the AIM-V medium supplemented with 5% human serum (HS), which was their institutional standard for CAR T cell manufacture.

Finally, Xu et al. performed a comprehensive medium comparison study evaluating the growth of anti-CD3/CD28-activated T cells in various medium formulations, either with or without serum addition (20). Here, they compared RPMI-1640 (+10%FBS), IMDM (+10%FBS), AIM-V (+10% human AB serum), Optimizer, X-VIVO 15, and StemSpan SFEM, all supplemented with exogenous IL-2 (1,000 IU/mL). Over the course of a 10-day *ex vivo* cell expansion period, the authors found that activated T cells maintained in the Optimizer medium achieved the highest T cell number. In addition, they characterized T cell memory phenotypes at the end of expansion, but did not observe any statistical difference among various culture conditions. It is important to point out that this finding is contradictory to the one reported by Lu et al. where AIM-V (+5% HS) outperformed Optimizer in terms of cell expansion. This could be in part due to the difference in the cytokine concentrations and/or sources of sera used by those two groups. Although future studies from independent groups with side-by-side comparisons are still required, these aforementioned three studies suggest that the choice of media has a significant impact on *ex vivo* expansion of OKT3- or OKT3/CD28-stimulated T cells used for CAR T cell generation.

In addition to cell expansion, an equally important goal is to generate CAR T cell products with the highest function possible. Medvec et al. compared CAR T cell function after growing them in different media (21) and reported that in the absence of serum supplementation, a chemically defined medium, 1B2H, supported *ex vivo* T cell proliferation to a similar level with that of the X-VIVO15 SFM, with selective expansion of T cells exhibiting a more differentiated phenotype (CCR7-CD27-). Anti-CD19 CAR T cells expanded in 1B2H showed potent *in vivo* anti-tumor activity with improved T cell persistence compared to CAR T cells expanded in X-VIVO15, even though CAR T cells maintained in X-VIVO15 contained slightly higher percentages of T cells with a less differentiated phenotype at the end of *ex vivo* expansion. However, these results conflict with a number of published studies demonstrating the importance of maintaining a less differentiated T cell phenotype for prolonged *in vivo* T cell persistence in adoptive T cell therapies (22–26). Although the authors did not elucidate the underlying mechanism of 1B2H-induced enhancement of *in vivo* anti-tumor activity, their data indicated that 1B2H might have the potential to improve expansion of CAR T cells generated from cancer patients whose T cells are prone to poor proliferation with a more differentiated phenotype. Notably, the authors also evaluated the effect of HS in 1B2H which will be discussed in the following section. Above results are summarized in **Table 1**.

IMPACT OF SERA AND SERUM SUBSTITUTES

While the cell therapy field is shifting towards SFM, most current clinical CAR T cell manufacturing protocols still utilize sera to support *ex vivo* T cell growth (16, 27). Currently, fetal bovine serum (FBS) and HS have been widely used for CAR T cell manufacture. Since the use of FBS may be immunogeneic and has the potential to transmit non-human pathogen(s), human-derived supplements are preferable for clinical application. In addition to HS, multiple serum substitutes derived from human blood have been tested in CAR T cell manufacture. For example, Ghassemi et al. investigated the effect of Physiologix XF (Phx), a concentrated extract from human transfusion grade whole blood

TABLE 1 | Impact of media on cell expansion.

Activation method	Cytokine	Medium	Serum	Expansion period	Cell expansion		Ref
Plate-bound OKT3 (3.3 μg/mL)	IL-2 (175 IU/mL)	RPMI-1640	autologous serum (8% gradually reduced to 1%)	7 and 12 days	52.7±13.1	Lowest (not specified)	(15)
		AIM-V			25.9±16.8	1110±71	
		Optimizer			55.5±20.6	1180±51	
Soluble OKT3 (50ng/mL)	IL-2 (300 IU/mL)	Optimizer X-VIVO15 TexMACS	(–)	6 days	AIM-V(5%HS) > Optimizer > X-VIVO15 > TexMACS		(16)
		AIM-V	5% HS				
Dynabeads Human T-Activator CD3/CD28	IL-2 (1000 IU/mL)	RPMI-1640	10% FBS	10 days	$272.0 \pm 42.3 \times 10^5$		(20)
		IMDM			$344.0 \pm 87.0 \times 10^5$		
		AIM-V	10% AB serum		$426.0 \pm 46.6 \times 10^5$		
		Optimizer	(–)		$549.0 \pm 82.7 \times 10^5$		
		X-VIVO15			$374.0 \pm 98.0 \times 10^5$		
		StemSpan SFEM			$110.6 \pm 23.4 \times 10^5$		
Dynabeads Human T-Expander CD3/CD28	Not specified	RPMI-1640 AIM-V	(-)	15 days	1B2H ≈ X-VIVO 15 >>> AIM-V > RPMI-1640		(21)
		X-VIVO15					
		1B2H (+ glucose & galactose)					

fractions as a serum replacement, on CAR T cell expansion and function (28). They used different media, namely RPMI-1640, X-VIVO15 and Optimizer, and compared Phx (2%)-supplemented media with serum-containing media (10%FBS + RPMI1640, 5% HS + X-VIVO15, 5%HS + Optimizer). Phx-supplemented media exhibited comparable T cell expansion to serum-containing media, with the exception of the 10%FBS + RPMI condition that led to the greatest T cell expansion after 9-11 days of culture. The authors further analyzed the metabolites contained in Phx and HS and found modestly elevated levels of carnosine along with several monosaccharide derivatives in Phx compared to HS. Supplying carnosine to HS-containing media enhanced the expression of genes delivered by lentiviral vectors and shifted the metabolic profile of activated T cells from a glycolytic state to an oxidative one, which has been shown to correlate with superior anti-tumor function (29–32). Although the authors did not show the metabolic profile of CAR T cells expanded in Phx-supplemented Optimizer, in a mouse xenograft model of neuroblastoma, GD2 CAR T cells cultured under this condition exhibited more potent tumor control compared to T cells expanded in HS-containing Optimizer.

Our group has also explored the effect of three different types of sera: FBS, human AB serum and human platelet lysate (HPL), on CAR T cell function during *ex vivo* expansion (33). There was no difference in CAR T cell expansion when 10% of each serum was supplemented to the base medium (1:1 mixture of RMPI-1640 and Click's medium), however, when serum concentrations were titrated down to 5% and 2.5%, FBS was unable to support CAR T cell expansion while similar levels of robust expansion were achieved across the HPL conditions regardless of concentrations. Strikingly, CAR T cells expanded in the HPL-supplemented medium maintained a large fraction of less differentiated T cells (naïve and central memory) compared to those cultured with other sera assessed by both cell surface phenotypes and gene expression signatures. As a result, HPL-cultured CAR T cells showed potent *in vivo* anti-tumor activity in both hematological (B cell leukemia treated with CD19 CAR) and solid tumor (pancreatic adenocarcinoma treated with PSCA CAR) models with prolonged T cell persistence. Another benefit of choosing HPL for CAR T cell manufacture is its lot-to-lot consistency, as demonstrated by Canestrari et al. in a study where they compared cytokine levels in ten different lots of HPL (34).

Because serum composition is very complex, it is hard to pinpoint which factor(s) in HPL is responsible for its superior performance. Nonetheless, we were able to show that transforming growth factor beta 1 (TGFβ1), which plays an important role in memory T cell pool formation (35, 36), is elevated in HPL compared to human AB serum by human proteomic analysis (34). Indeed, supplementing TGFβ1 into the FBS-containing medium greatly increased the percentage of CAR T cells with a less differentiated phenotype during *ex vivo* expansion, consistent with another independent report (37). However, not surprisingly, since TGFβ1 is a potent immunosuppressive cytokine, the anti-tumor effect of TGFβ1-exposed CAR T cells was strongly inhibited in both *in vitro* and *in vivo* experiments, suggesting that there are multiple cytokines/proteins in HPL contributing to enhanced CAR T cell function.

It is worth noting that although a number of publications, including aforementioned ones, have shown the benefit of serum supplementation, Medvec et al. reported negative effects resulting from serum addition under certain culture conditions (14), in a study described in the previous section. When comparing the effects

of a chemically defined medium 1B2H and X-VIVO15 SFM, they found that CD19 CAR T cells showed significantly lower percentages of TNFα (+) and IL-2 (+) cells upon K562-CD19 stimulation when they were expanded in the presence of HS compared to those without HS. However, this was only observed when CAR T cells were generated from healthy donors rather than multiple myeloma patient samples. Despite this inferior cytokine secretion profile, 1B2H (serum-free)-expanded CAR T cells showed the most potent *in vivo* anti-tumor effects when compared to 1B2H (HS), X-VIVO15 (HS) and X-VIVO15 (serum-free) conditions. Although the underlying mechanism is still unknown, these results suggested that depending on the choice of medium, serum components might have an undesireable impact on CAR T cell function. Above results are summarized in **Table 2**.

IMPACT OF EXOGENOUS CYTOKINES

The addition of exogenous cytokines in cell culture promotes CAR T cell expansion and alters T cell phenotype and function. IL-2 is the most common cytokine used to expand CAR T cells including commercial products, such as KYMRIAH and YESCARTA (38, 39). Yet, preclinical studies utilizing other common gamma-chain (γc) cytokines such as IL-7, IL-15, and IL-21 have led to more effective immunotherapies and clinical investigations are underway (14, 40, 41). Therefore, the choice of cytokine supplementation during CAR T cell manufacturing must be considered to achieve enhanced T cell effector function and persistence.

In many current protocols, peripheral blood lymphocytes that are redirected to tumors with CARs are expanded in IL-2. IL-2 is primarily secreted from activated T cells and plays a role in T cell proliferation, differentiation, and contraction through activation induced cell death (42). IL-2 has been shown to promote both Th1 and Th2 effector T cell differentiation while inhibiting Th17 polarization. It also results in the expansion of regulatory T cells which express the high affinity IL-2 receptor and are known to limit inflammatory responses and impede anti-tumor activity (43). In a study comparing the expansion and efficacy of CD19 CAR T cells grown in IL-2 versus IL-7/15, two other homeostatic cytokines, IL-2-cultured CAR T cells (hereafter IL-2 CAR T cells) highly expressed the CAR molecule on day three post transduction and expanded 100 folds during two weeks of culture (44). IL-2 CAR T cells were robust in killing tumor cells both *in vitro* and *in vivo*, but overtime CAR T cells expanded in IL-7/15 outperformed IL-2 CAR T cells with increased expansion and improved persistence. Furthermore, IL-2 CAR T cells contained more regulatory T cells and expressed higher levels of PD-1 after multiple rounds of antigen stimulation, leading the authors to suggest that IL-2 CAR T cells are more exhausted compared to IL-7/15-expanded ones. Similar results were also found by Xu and colleagues that among CAR T cells expanded with different common γc cytokines, IL-2-exposed CAR T cells exhibited the poorest anti-tumor function (45). This particular study comprehensively measured the effect of single cytokine supplement on an anti-Folate receptor alpha-CAR and determined that addition of IL-2, IL-7, or IL-15 resulted in the greatest fold expansion compared to other conditions including no cytokine, IL-18, and IL-21. However, IL-21-exposed CAR T cells exhibited the greatest expansion of less differentiated CAR T cells defined by the CD62L+CCR7+CD27+CD28+ phenotype and therefore prolonged persistence in *in vivo* models. The authors suggested that while IL-2 had been widely used in the generation of clinical-grade CAR T cells, it might not be the best condition, but rather IL-7- and IL-15-expanded CAR T cells exhibited better properties (expansion, cytotoxicity, cytokine secretion) before *in vivo* infusion and IL-15- and IL-21-cultured CAR T cells might be best suited for optimal *in vivo* activity (45).

IL-21 is also a member of the common γc cytokine family and has been shown to enrich a less differentiated phenotype of T cells (46). In combination with IL-2, IL-21 supplemented to CAR T cell culture media was found to increase CAR T cell proliferation, promote outgrowth of naïve and memory T cells, improve anti-tumor function and also increase the expression of CAR molecules on the surface of T cells transfected with the Sleeping Beauty transposon (47). The improved CAR expression with IL-21 supplement after lentiviral transduction was also observed in studies by Du et al., through dampened IFNγ expression (48).

TABLE 2 | Impact of sera on CAR T cell function.

Serum	Medium	Cell expansion	Outcomes	Ref
10% FBS 2% Phx	RPMI-1640	Greatest expansion in 10% FBS+RPMI-1640 compared to other conditions	5% HS vs 2% Phx in Optimizer • enhanced lentiviral-mediated gene expression in Phx • enhanced GD2 CAR T cell activity *in vivo* in Phx	(28)
5% HS 2% Phx	X-VIVO15			
5% HS 2% Phx	Optimizer			
10% FBS	RPMI-1640 + Click's medium	Comparable in 10% serum conditions (lower expansion in FBS if % serum is reduced)	• HPL-maintained T cells had a less-differentiated phenotype *in vitro* • HPL-cultured CAR T cells exhibited enhanced proliferation upon *in vitro* antigen stimulation • HPL-expanded CD19 CAR and PSCA CAR T cells exhibited superior *in vivo* anti-tumor effect with prolonged T cell persistence	(33)
10% AB serum				
10% HPL				
(–) 5% HS	X-VIVO 15	Comparable	CD19 CAR T cells cultured in 1B2H (no serum) showed better *in vivo* anti-tumor effect compared to the ones in 1B2H (5% HS)	(21)
(–) 5% HS	1B2H (+ glucose & galactose)			

While IL-21 alone did not result in robust CAR T cell proliferation (45), when used in combination with other cytokines such as IL-7 and IL-15, IL-21 improved CAR T cell effector function (48).

With the discovery that T cells exhibiting a less differentiated phenotype, such as stem-cell like memory cells (T_{SCM}), are correlated with improved clinical outcomes, many studies have worked to maintain this population in CAR T cell products (49). In an effort to continue the use of IL-2, Kaartinen and colleagues titrated the dose of IL-2 for CD19 CAR T cell expansion and found that low doses of IL-2 (5 U/ml) resulted in less differentiated T cells but at the cost of reduced expansion, similar to that of no cytokine supplementation, and less effector function (50). The authors did note that if *in vitro* expanded T_{SCM} cells exhibited similar homing features to their physiological counterparts, then those cells might not have a strong capacity to enter the periphery and reach the tumor sites, and thus might not be the best product for solid tumor treatment. However, studies to preserve minimally differentiated T cells in *ex vivo* expansion are of current interest due to their potentially enhanced anti-tumor activity *via* increased persistence. Ultimately the combination of cytokines such as IL-7 and IL-15 have shown promise to preserve CD45RA+CCR7+ and CD45RA-CCR7+ T cells with improved proliferation after antigen exposure (42, 51). Above results are summarized in **Table 3**.

Interestingly, besides the common γc cytokine family and IL-18, *ex vivo* expanded CAR T cells in the presence of TGFβ1 has also been shown to promote central memory T cell accumulation and BCMA-targeting CAR T cells exhibited improved anti-tumor activity when exposed to TGFβ1 (37).

IMPACT OF PHARMACOLOGICAL INHIBITORS

Standard *ex vivo* expansion procedures often inevitably accelerate terminal differentiation and senescence of T cells, since initial TCR stimulation, CAR tonic signaling, and cytokine signaling can all lead to activation of signaling transduction pathways that drive effector T cell differentiation. In addition, as most cancer patients have undergone multiple rounds of pre-treatment, their T cells often exhibit an exhausted and/or senescent phenotype, resulting in poor expansion and functionality of their CAR T cell products. Therefore, multiple studies supplemented T cell culture with pharmacological inhibitors that specifically suppressing cellular programs that drive T cell terminal differentiation, exhaustion, and/or senescence, in order to reinvigorate patients' T cells and improve CAR T cell fitness during manufacture (**Table 4**).

As integrated signals from CD3ζ, costimulatory molecules and cytokine receptors lead to activation of a vast signaling transduction network in T cells, the majority of studies targeted critical components of a chosen signaling pathway, in order to selectively block pathways that mediate effector T cell differentiation while sparing pathways that contribute to T cell activation, proliferation, and memory formation. The most extensively explored pathway to date is PI3K/AKT/mTOR, a pathway known to facilitate T cell proliferation and effector differentiation through promoting glycolytic metabolism and functional suppression of the transcription factor FOXO1 (60, 62). Two independent groups reported that compared to conventionally grown CD19 CAR T cells, the addition of AKT inhibitors during manufacture resulted in enrichment of CAR T cells with a CD62L-expressing central memory (T_{CM}) phenotype and enhanced anti-tumor efficacy in different xenograft models, without compromising cell yields (62, 63). Multiple other studies have targeted PI3K, a kinase upstream of AKT, and demonstrated that PI3K blockade helped a variety of CAR T cells maintain a less differentiated phenotype with enhanced *in vivo* persistence and anti-tumor efficacy (52–57). Of note, one study compared the effect of blocking AKT versus PI3Kδ (a subset of PI3Ks) using mesothelin-specific CAR T cells and found that PI3Kδ inhibition upregulated the stem cell memory transcription factor TCF7 more than AKT inhibition, translating to better *in vivo* anti-tumor efficacy (56). Another study compared the efficacy of inhibitors specific to different subsets of PI3Ks and found that PI3Kδ suppression resulted in better CAR T cell functionality compared to PI3Kγ blockade *in vitro* (55). Importantly, two studies also demonstrated the feasibility of utilizing PI3K blockade to generate CD19 CAR T cells with superior anti-tumor activity in xenograft models of chronic lymphocytic leukemia (CLL) and acute lymphocytic leukemia (ALL), using T cells derived from CLL patients (54, 57).

Apart from targeting the PI3K/AKT/mTOR pathway components, several studies took advantage of other signaling pathways involved in T cell activation. One such study used ibrutinib to inhibit interleukin-2-inducible T-cell kinase (ITK) signaling that is involved in T cell differentiation (58). This approach improved the overall quality of CD19 CAR T cells generated from CLL patients, in that the ibrutinib-treated CAR T cells expressed lower percentages of exhaustion markers including PD-1, TIM-3 and LAG-3, and had elevated TNFα and IFNγ production following target stimulation *in vitro* (58). Another group used sorted CD8+CD62L+CD45RA+ naïve precursor T cells as the starting material, genetically engineered and expanded them in the presence of a mixture of IL-7, IL-21 and glycogen synthase kinase 3β (GSK-3β) inhibitor TWS119 (59). As GSK-3β destabilizes β-catenin (61), an activator of transcription factors (e.g., LEF/TCF) that facilitate expression of genes responsible for memory T cell differentiation, addition of the GSK-3β inhibitor along with IL-7 and IL-21 enriched for CD19 CAR-expressing CD45RO-CCR7+CD45RA+CD62L+CD27+CD95+ T_{SCM} that showed enhanced metabolic fitness and long-lasting anti-tumor activity in a leukemia xenograft model. A third study identified that inhibition of p38 kinase, a key driver of T cell senescence (64, 65), led to an increased percentage of $CD62L^+$ human CD19 CAR T cells with elevated IFNγ production following *in vitro* stimulation (66). They also showed that murine CD19 CAR T cells cultured in the presence of the p38 inhibitor had augmented anti-tumor activity *in vivo* (66). Last but not least, it has been reported that reversible yet complete blockade of CAR tonic signaling during manufacture using dasatinib (67, 68), a Src-family kinase inhibitor suppressing the function of LCK/FYN (69) that transmits CAR activation signals from CD3ζ to downstream Syk-family kinases, resulted in GD2 CAR T cells enriched for the CD62L+CD45RO+ T_{CM} subset, with lower expression of exhaustion markers including PD-1, TIM3, and

TABLE 3 | Impact of exogenous cytokines on CAR T cell expansion and function.

CAR	Cytokine	Expansion	Phenotype	Notes	Ref
19BBz	IL-2 (200U/mL)	100-fold (2 weeks)	Promoted CD8+ Effector Memory (CD45RA-CD62L-, CD45RA+CD62L-)	- Treg expansion	(44)
	IL-7 (10ng/mL) +IL-15 (10ng/mL)	>200-fold (2 weeks)	Promoted CD8+ naïve (CD45RA+CD62L+) & central memory (CD45RA-CD62L+)	- ↓ apoptosis - ↑ engraftment - ↑ anti-tumor activity	
C4-27z & CD19-27z	IL-2 (10ng/mL)	150-fold (2 weeks)	Mature Effectors (CD45RA-CD62L- & CD45RA+CD62L-)	- ↓ apoptosis, - ↑TNFα	(45)
	IL-7 (10ng/mL)	150-fold (2 weeks)	Highest T_{SCM} population (CD45RA+CD62L+CD95+)	- ↓ apoptosis - ↑ proliferation capacity	
	IL-15 (10ng/mL)	150-fold (2 weeks)	Promote T_{CM} (CD45RA-CD62L+)	- ↓ apoptosis - ↑ anti-tumor activity *in vivo*	
	IL-18 (10ng/mL) IL-21 (10ng/mL)	30-fold (2 weeks) 50-fold (2 weeks)	Phenotype similar to no cytokine control Increased CD62L+	- ↑ Granzyme - ↑ *in vivo* T cell persistence - Preserved CAR expression	
CD19-CD28z	IL-2 0 U/mL	11-fold (10 days)	Early memory T cells (T_{SCM}) CD95+CD45RO–CD45RA+CD27+ present with low IL-2 and short expansion time	- More IL-2 decreased early memory T cells	(50)
	IL-2 5 U/mL	12-fold (10 days)			
	IL-2 20 U/mL	39-fold (10 days)			
	IL-2 100 U/mL	60-fold (10 days)			
	IL-2 300 U/mL	62-fold (10 days)			
CD19RCD28	IL-2 (50U/mL) +IL-21 (30ng/mL)	39.2-fold (14 days)	CD8+ memory and naïve cells	- ↑ CAR expression overtime - ↑ antitumor activity compared to IL-2 alone	(47)
Her.2 BBz	IL-2 (150U/mL)	Approx.100-fold (12 days)	IL-21 promoted naïve like and T_{CM}	-IL-21 ↑CAR expression by reducing IFNγ - IL-15 + IL-21 best combination for expansion and effector function	(48)
	IL-7 (10ng/mL)	Approx.20-fold (12 days)			
	IL-15 (10ng/mL)	Approx.70-fold (12 days)			
	IL-2 (150U/mL) +IL-21 (20ng/mL)	>100-fold (12 days)			
	IL-7 (10ng/mL) +IL-15 (10ng/mL)	Approx.70-fold (12 days)			
	IL-7 (10ng/mL) +IL-21 (20ng/mL)	Approx.30-fold (12 days)			
	IL-15 (10ng/mL) +IL-21 (20ng/mL)	>100-fold (12 days)			
	IL-7 (10ng/mL) +IL-15 (10ng/mL) +IL-21 (20ng/mL)	>100-fold (12 days)			

TABLE 4 | Summarization of studies on pharmacological inhibitors.

Pharmacological inhibitors	Cellular targets	CAR specificity	Ref
Akt inhibitor VIII (aka Akti-1/2; PubChem Compound Identification: 10196499)	AKT	CD19	(52, 53)
LY294002	PI3K	CD33	(54)
bb007	PI3K	BCMA	(55)
Idelalisib (aka CAL-101)	PI3Kδ	CD19	(56)
Idelalisib (aka CAL-101)	PI3Kδ	Mesothelin	(57, 58)
Umbralisib (aka TGR-1202)	PI3Kδ	Mesothelin	(57)
Eganelisib (aka IPI-549)	PI3Kγ	Mesothelin	(57)
Duvelisib	PI3Kδ/PI3Kγ	CD19	(59)
Ibrutinib	ITK	CD19	(60)
TWS119	GSK-3β	CD19	(61)

LAG-3, as well as augmented *in vivo* function in a xenograft tumor model, compared to non-treated CAR T cells (70).

Besides signaling cascade blockade, other efforts to maintain minimally differentiated CAR T cells include epigenetic and metabolic interventions. One example is the a bromodomain and extra-terminal motif (BET) protein inhibitor JQ-1, which enabled enrichment of human CD19 CAR T cells with T_{SCM} and T_{CM} phenotypes as well as superior *in vivo* persistence and anti-tumor effects (71). Notably, *ex vivo* treatment of JQ-1 also reinvigorated exhausted and dysfunctional CD19 CAR T cells sourced from non-responding CLL patients (72). Mechanistically, BET proteins represent a protein family responsible for epigenetic marker recognition and transcriptional factor recruitment. Suppression of the BET protein BRD4 downregulates BATF gene expression in $CD8^+$ T cells, thereby inhibiting T cell differentiation into the effector memory phenotype (71). Blocking BRD4 also leads to upregulation of CAR transgene expression and methylcytosine dioxygenase TET2 gene (73) downregulation, ultimately improving functionality of CAR T cells derived from non-responding CLL patients (72). In terms of metabolic regulation, one study used avasimibe to inhibit the function of ACAT1, a key cholesterol esterification enzyme that reduces the plasma membrane cholesterol level of CD8+ T cells, thereby decreasing the level of TCR clustering and signaling (74). Blocking ACAT1 resulted in enrichment of CD8+ CD19 CAR T cells with enhanced *in vitro* cytotoxicity (75).

In addition to enriching T cells with a desirable memory phenotype, recent development also utilized pharmacological inhibitors to maintain CAR T cells with other preferred functionality. Nian et al. (76) showed that tonic signaling of EpCAM-specific CAR T cells during *ex vivo* expansion resulted in hyperactivation of mTORC1, which downregulated CXCR4 and impaired the ability of CAR T cells to migrate to bone marrow. By using rapamycin to attenuate mTORC1 signaling during *ex vivo* expansion, the treated CAR T cells, compared to untreated CAR T cells, had elevated CXCR4 expression, enhanced bone marrow infiltration capacity, and augmented cytotoxicity against bone marrow resident leukemic cells in various xenograft models of human acute myeloid leukemia (AML). In addition, they also observed similar functional improvement using a CD33-specific CAR, indicating that this strategy might be generally applicable with different CAR constructs.

IMPACT OF CULTURE PERIOD

Currently, T cells for generating CAR T cell products are sourced from patients themselves. The total manufacturing time varies depending on how much starting material (PBMCs or isolated T cells) is available and how fast patient-derived T cells grow. In some cases, patients failed to respond to treatment due to rapid disease progression during the lengthy manufacturing period. Therefore, much effort is being made to reduce the manufacturing time. Moreover, studies also suggested that a shortened *ex vivo* culture period correlated with improved CAR T cell functionality. Ghassemi et al. (77) compared function of CD19 CAR T cells harvested after short- (day 3 or 5) and long-term (day 9) culture. The authors showed that short-manufactured CAR T cells exhibited more robust tumor control compared to long-manufactured ones in a mouse xenograft model. Other groups have observed comparable results in similar xenograft mouse models (78, 79) as well as clinical trials (78, 80). In reality, due to prior lymphodepleting cancer treatment, the short-term *ex vivo* expansion protocol may not be feasible for patients with reduced T cell counts and activity. Nevertheless, these results suggested that circumstances permitting, shortened manufacturing time should be opted for. It is however worth noting that although the above studies mentioned maintenance of a less-differentiated T cell phenotype in short-manufactured CAR T cells as a reason for functional enhancement, the surface makers they used to define memory populations including CCR7 and CD45RO will appear artificially high shortly after antigen stimulation, including *in vitro* CD3 stimulation (81–85). Therefore, it requires extra caution when interpreting memory phenotype data from highly activated CAR T cells that are produced in a short manufacture period.

IMPACT OF CRYOPRESERVATION

At the end of the manufacturing process, CAR T cells are often cryopreserved, allowing sufficient time to complete quality control tests and flexible scheduling for infusion into patients. As cryopreservation may affect the viability and functionality of T cells, several groups have looked into its effect on CAR T cell products. Lee et al. demonstrated that there was no difference between fresh and frozen/thawed CD20 CAR T cells in terms of phenotype, cytokine secretion and *in vivo* cytotoxicity (86). Of note, they used the Cryostor medium to freeze CAR T cells, stored in liquid nitrogen overnight, and then thawed the next day for experimental use. A similar preclinical observation was reported by Xu et al. where *in vivo* functionality of BCMA CAR T cells between fresh versus one-month cryopreservation (10%DMSO + 90% FBS) groups was comparable, with only a slight decrease of cytokine levels produced from frozen/thawed CAR T cells (87).

To investigate the effect of cryopreservation on CAR T cell products used in the clinic, Panch et al. (88) retrospectively evaluated data from a total of 158 frozen/thawed autologous CAR T cell lines and PBMCs that are used as starting material for CAR T cell generation (freezing medium containing 5% DMSO and 6%

pentastarch with 4% human serum albumin) across 6 single-center clinical trials. Overall, cryopreservation procedure did not affect clinical outcomes. In addition to patient samples, they also looked into frozen/thawed CAR T cells generated from healthy donors and demonstrated that these cells had early apoptotic cell surface markers and activation of apoptotic pathways, mitochondrial dysfunction, and cell cycle damage pathways (88). In another retrospective clinical study, Su et al. reported similar results where fresh and cryopreserved (Cryostor-CS10) CD19 CAR T cells produced comparable clinical outcomes (89). In contrast, Shah et al. observed that peak CAR T cell expansion levels and the overall response rate (ORR) were improved in patients who received fresh CD20/CD19 tandem bispecific CAR T cells compared to cryopreserved ones, indicating potential advantages of fresh products over frozen/thawed ones (90). Given the limited number of reports available, more future studies are needed to determine the impact of cryopreservation.

OTHER FACTORS THAT MAY IMPACT CAR T CELL FUNCTION

As described above, *ex vivo* CAR T cell manufacture involves multiple, albeit straightforward, steps and variable factors. Beyond those reviewed above, there are other factors that may affect CAR T cell function. During Current Good Manufacturing Practice (CGMP) manufacture, CAR T cells can be expanded in culture plates, flasks, bags or much larger culture vessels such as G-Rex devices and rocking bioreactors (e.g. WAVE bioreactors) to simplify the process. Furthermore, CliniMACS Prodigy system provides a fully-automated manufacture system to generate clinical-grade CAR T cells. Advantages and disadvantages of these devices have been summarized in detail by another review (91). Validation studies of CAR T cell manufacture in each system (92–99) to date mainly focused on cell yields. One should keep in mind that it is difficult to compare the performance of different devices due to their distinct characteristics, such as volume of medium added, being a closed or open (semi-open) system and "static vs moving" culture. Those factors further dictate the optimal cell seeding density, efficacy of gas exchange and degree of pH alteration in each device. Although a simplified and large scale closed system is preferable for clinical CAR T cell manufacture due to its consistency in product quality and reduced complexity as well as risk of contamination, most preclinical studies utilize culture plates and flasks. Therefore, it is important to consider the effect of different culture devices for CAR T cell manufacture. During clinical translation, standard operating procedures need to be tailored to individual devices in order to maximize CAR T cell potency.

DISCUSSION

Although extensive efforts have been spent on inventing genetic engineering methods to enhance CAR T cell potency, fewer studies to date focused on the optimization of *ex vivo* cell expansion conditions. However, as discussed in the current review, the choice of reagents for *ex vivo* CAR T cell expansion can drastically affect the quality of therapeutic T cells, emphasizing the need for more detailed investigations. In general, most studies have found that culture methods leading to preservation of less differentiated, less exhausted, and/or less glycolytic CAR T cells during *ex vivo* expansion yielded T cell products with higher anti-tumor potency *in vivo*. This is consistent with prior observations that enhanced glycolytic metabolism impaired long-term memory formation of CD8+ CAR T cells (29, 31) and that CAR T cells generated from T_N and T_{CM} populations showed superior *in vivo* performance compared to CAR T cells derived from T_{EM} cells (22–26). Further, clinical evidence indicated that CAR T cells in complete-responding patients were enriched in memory-related genes while those from non-responders upregulated transcriptional pathways associated with effector differentiation, glycolysis, exhaustion and apoptosis (30). These data suggested that CAR T cells with long-lived memory phenotypes and enhanced metabolic fitness are desirable for clinical use.

During clinical translation, while it might be pragmatically difficult to standardize culture conditions for CAR T cells across different institutions, careful considerations must be given to the manufacturing process design, in order to maximize the potency of each final product. It is important to note that our knowledge about the effect of manufacturing methods remains insufficient, in that the number of studies investigating this aspect is limited and that many of these efforts relied on CAR T cell expansion (product quantity) as the sole readout, while overlooking the importance of CAR T cell functionality (product quality). To fill this gap of knowledge, future studies, especially clinical trials, will need to systemically evaluate the impact of different *ex vivo* expansion methods during each manufacturing step on both the quantity and quality of therapeutic T cells. Furthermore, new types of media, sera and serum substitutes, as well as other reagents are expected to be developed in the future, which will provide combinatorial and synergistic effects to boost CAR T cell function. Combining the optimal manufacturing procedure with additional innovative genetic engineering approaches will allow us to achieve our ultimate goal of developing an effective CAR T cell therapy for cancer patients.

AUTHOR CONTRIBUTIONS

FM drafted the impact of pharmacological inhibitor section. MM drafted the impact of exogenous cytokine section. NW drafted all remaining sections. All coauthors reviewed and edited the final manuscript. All authors contributed to the article and approved the submitted version.

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Rapid Generation of TCR and CD8αβ Transgenic Virus Specific T Cells for Immunotherapy of Leukemia

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Background: Virus-specific T cells (VSTs) are an attractive cell therapy platform for the delivery of tumor-targeted transgenic receptors. However, manufacturing with conventional methods may require several weeks and intensive handling. Here we evaluated the feasibility and timelines when combining IFN-γ cytokine capture (CC) with retroviral transduction for the generation of T cell receptor (TCR) and CD8αβ (TCR8) transgenic VSTs to simultaneously target several viral and tumor antigens in a single product.

Methods: Healthy donor peripheral blood mononuclear cells were stimulated with cytomegalovirus (CMV) and Epstein-Barr-Virus (EBV) peptide mixtures derived from immunogenic viral proteins, followed by CC bead selection. After 3 days in culture, cells were transduced with a retroviral vector encoding four genes (a survivin-specific αβTCR and CD8αβ). TCR8-transgenic or control VSTs were expanded and characterized for their phenotype, specificity and anti-viral and anti-tumor functions.

Results: CC selected cells were efficiently transduced with TCR8. Average fold expansion was 269-fold in 10 days, and cells contained a high proportion of CD8+ T central memory cells. TCR8+ VSTs simultaneously expressed native anti-viral and transgenic anti-survivin TCRs on their cell surface. Both control and TCR8+ VSTs produced cytokines to and killed viral targets, while tumor targets were only recognized and killed by TCR8+ VSTs.

Conclusions: IFN-γ cytokine capture selects and activates CMV and EBV-specific memory precursor CD8+ T cells that can be efficiently gene-modified by retroviral transduction and rapidly *ex vivo* expanded. Our multi-specific T cells are polyfunctional and recognize and kill viral and leukemic targets expressing the cognate antigens.

Keywords: immunotherapy, virus-specific T cells, cytokine capture, transgenic TCR, transgenic CD8, engineered T cells, interferon-gamma

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INTRODUCTION

Adoptive transfer of virus specific T cells (VSTs) rapidly restores antiviral immunity and prevents or treats viral infections after allogeneic hematopoietic stem cell transplantation (HSCT) (1). VSTs are both safe and effective when manufactured from the original stem cell donor or from unrelated partially HLA matched third party healthy donors (1, 2), setting the stage for their use as a cellular therapy platform for the delivery of engineered receptors targeting tumor-associated antigens (recently reviewed in (3)). Indeed, leukemia targeted chimeric antigen receptors (CARs) such as CD19-CARs in B-cell acute lymphoblastic leukemia (B-ALL) or T cell receptors (TCRs) targeting Wilms Tumor 1 (WT1) or the minor histocompatibility antigen HA-1H in acute myeloid leukemia (AML) have been expressed in VSTs and infused to patients post-transplant (4–9). Safety and some efficacy was demonstrated with CD19-CAR-modified VSTs produced from the stem cell donor (4–6), while feasibility and efficacy with TCR-modified VSTs was variable among studies (7, 8).

Manufacturing of engineered VSTs is challenging and operator intensive. Certain steps are performed in open systems such as flow cytometry-based sorting (7), or require knowledge of the targeted epitope such as streptamer-selection (8). Other processes require live Epstein Barr Virus (EBV) for the generation of autologous lymphoblastoid cell lines (4–6), several types of viral vectors for the transduction of antigen presenting cells and transduction of VSTs (3, 6), and prolonged *ex vivo* culture over several weeks (4–7).

For broader applicability of such multi-antigen targeted therapies, the complexity of the production processes needs to be reduced (recently reviewed in (10)). Here, we investigated at small scale if Interferon-γ (IFN-γ) cytokine capture (CC) selected virus-specific memory T cells from healthy donors are sufficiently enriched and activated to directly proceed to retroviral transduction introducing an HLA-A*02:01 restricted survivin targeted TCR (11) in combination with CD8αβ (TCR8) to redirect VSTs to a broad tumor-associated antigen (12, 13). We have previously demonstrated that the incorporation of CD8αβ as a transgene restores anti-viral activity of TCR transgenic VSTs, and redirects CD4+ T cells to the class I restricted cognate antigen (12, 13). CC is attractive because it is compatible with fully closed production of VSTs independently of donor HLA and can select T cells with diverse TCR repertoires recognizing various immunogenic epitopes (14–16). Now we show that enrichment and activation of anti-viral memory T cells by CC followed by retroviral transduction reduces manufacturing time by 7-10 days, reduces the overall complexity of the process, and yields cells with simultaneous anti-viral and anti-tumor activity.

MATERIALS AND METHODS

Cell Lines

BV173 and K562 cell lines were obtained from the German Cell Culture Collection (DSMZ) or the American Type Culture Collection (ATCC), respectively, and maintained in complete RPMI 1640 media (Hyclone, Thermo Scientific) supplemented with 10% or 20% fetal bovine serum (FBS, Hyclone), 1% penicillin-streptomycin (Gibco) and 1% glutamax (Gibco) (13). 293T cells (ATCC) were maintained in complete IMDM media (Hyclone) containing 10% FBS, 1% penicillin-streptomycin, and 1% glutamax.

Healthy Donor Buffy Coats

Buffy coats from CMV seropositive de-identified healthy human volunteers were procured from the Gulf Coast Regional Blood Center (Houston, TX, USA). HLA-A2 status was determined by FACS analysis and HLA-A2 positive donors were selected for the experiments.

Generation of Retroviral Vectors and Supernatant

The design of the retroviral vector encoding the survivin-specific (s24) TCR and CD8αβ has been described previously (**Figure 1A**) (11–13). Retroviral supernatant was prepared by transient co-transfection of 293T cells with RD114 and Pegpam plasmids and the SFG vector containing the genes of interest (11).

Generation of Gene Modified Virus-Specific T Cells Using IFN-γ Cytokine Capture and Retroviral Transduction

PBMCs were isolated from buffy coats using density gradient centrifugation by Lymphoprep (Accurate Chemical and Scientific Corporation), resuspended in T cell complete media (1:1 mixture of RPMI 1640 and Click's media, Hyclone, supplemented with 10% FBS, Hyclone, 1% penicillin-streptomycin, 1% glutamax) and rested overnight at 37°C (10^7 PBMCs per 2 ml in complete media/well in 24-well plate). After incubation, PBMCs were stimulated with a mixture of CMV and EBV pepmixes (CMV pp65, CMV IE1, EBV LMP2, EBV BZLF1, EBV EBNA, 1 μg/ml, all from JPT Technologies) and HLA-A*02:01 restricted immunodominant peptides (CMV pp65 derived NLV: NLVPMVATV, immediate early EBV BRLF1-derived YVL: YVLDHLIVV, early EBV BMLF1-derived GLC, 1 μg/ml, all from Genemed Synthesis) for 6 h at 37°C. The combination of pepmixes and peptides was chosen based on antigen expression patterns of CMV and EBV infection in the post-transplant period (1). IFN-γ secreting cells were isolated using the IFN-γ secretion assay-cell enrichment kit (Miltenyi Biotech, #130-054-202) according to the manufacturer's recommendations (**Figure 1B**). IFN-γ secreting cells were plated in complete media with IL7 (10 ng/mL, R&D Systems), IL15 (10 ng/mL, R&D Systems) and IL21 (30 ng/mL, R&D Systems), and the irradiated IFN-γ-negative fraction (30 Gy) was used as a feeder layer (0.02-0.5 x10^6 IFN-γ captured cells per 0.5 x10^6 feeder cells per well in 24-well plate). After 3 days, IFN-γ cytokine captured VSTs were harvested and transduced with retroviral supernatant encoding the survivin-specific αβTCR and CD8αβ (TCR8, **Figure 1A**) on retronectin coated plates or exposed to retronectin coated plates without viral particles (for non-transduced VSTs). VSTs were expanded for 5-7 days in the

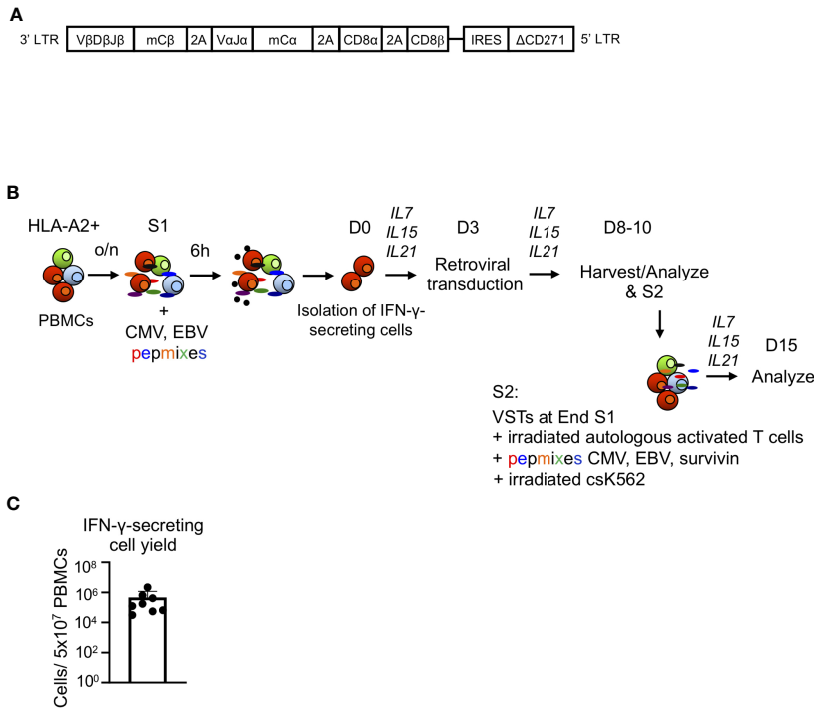

FIGURE 1 | Generation of TCR8 transgenic VSTs by IFN-γ cytokine capture and retroviral transduction. **(A)** Schematic of the retroviral vector containing the survivin-specific TCR with murine constant regions (mCα, mCβ), CD8αβ and a truncated selectable marker (ΔCD271). **(B)** Steps involved in the production of transgenic VSTs using IFN-γ CC, retroviral transduction and cell expansion. S1: first stimulation, S2: second stimulation (optional). **(C)** Total yield of IFN-γ captured cells from $5x10^7$ PBMCs after immunomagnetic separation, n=8 donors, mean ± SD.

presence of cytokines IL7 (10 ng/mL, R&D Systems), IL15 (10 ng/mL, R&D Systems) and IL21 (30 ng/mL, R&D Systems). A second stimulation (S2) was performed to evaluate the further expansion potential of the engineered VSTs. S2 was performed with CMV/EBV pepmix/peptide and HLA-A*02:01 restricted survivin peptide LMLGEFLKL (the cognate antigen of the transgenic TCR, Genemed synthesis) pulsed irradiated (40 Gy) autologous activated T cells (previously activated on OKT3/anti-CD28 coated plates) and irradiated (100 Gy) K562cs cells (K562 cells engineered to express CD80, CD83, CD86, 41BBL and CD32) at a ratio of 1:1:5 (**Figure 1B**) in G-Rex gas permeable culture devices (WilsonWolf, Saint Paul, USA) as previously described (17).

Immunophenotyping

For evaluation of cell surface marker expression, cells were stained with FITC-, phycoerythrin (PE-), allophycocyanin (APC), V450-, PerCP, APC-AF750 or Krome orange-conjugated antibodies (Abs) against CD4, CD8, CD45RO, CD62L, CCR7, CD45RA, CD56, TCRγδ, CD271, CD19, 7-AAD (BD Biosciences), murine TCRβ constant region (ebiosciences), NLV pentamer (Proimmune) or survivin LML dextramer (Immudex) for 30 min at 4°C. The degranulation assay was performed as described previously (13). Briefly, VSTs (10^6) were treated with golgi-plug/brefeldin A (Invitrogen) and CD107a/b VioBlue (BD Biosciences) followed by appropriate stimulations: CMV/EBV specific viral pepmixes or single peptide (pp65, IE1, LMP2, BZLF1, EBNA1, GLC, 1 μg/ml), survivin-specific LML peptide, viral pepmixes/peptide plus LML peptide, PMA (25 ng/ml)/Ionomycin (1 μg/ml) or control Influenza matrix protein GIL (GILGFVFTL, Genemed synthesis) peptide (negative control) for 4 h at 37°C. Intracellular staining (ICS) was performed using anti-human IFN-γ-FITC and TNF-α-PE (BD Biosciences) antibodies. The samples were acquired on a FACS Canto with BD FACSDiva software, and analysis was performed using FlowJo software (Tree Star Inc.).

IFN-γ ELISpot

For quantification of IFN-γ producing cells by ELISpot, VSTs (10^5 per well) were plated in triplicates and stimulated with individual pepmixes/peptides (1 μg/ml) or cell lines (BV173 or K562) at 1:1 ratio (10^5 cells per well) or media alone. Plates were incubated at 37°C/5% CO_2 over-night and developed. Spot Forming Units (SFUs) were enumerated by ZellNet.

Co-Culture Assay and Cytokine Detection

To determine the anti-tumor function, VSTs and BV173 cells were co-cultured at E:T ratio of 1:5 without exogenous cytokines. Supernatants from co-cultures were harvested after 24 h and were stored at -80°C for cytokine analysis. After 3 days, residual VSTs and tumor cells were enumerated using CountBright Beads (Life Technologies) and FACS analysis. Cytokines were

quantified in supernatants using the MILLIPLEX Human CD8+ T-cells Magnetic Beads Panel (EMD Millipore) and a Luminex 200 instrument (Luminex).

^{51}Chromium Release Assay

In vitro short-term cytotoxicity of VSTs was assessed using a standard ^{51}Cr-release assay as described previously (13). Briefly, autologous activated T cells (targets) were pulsed with the indicated peptides or pepmixes (1 μg/ml) and labeled with ^{51}Cr for 1 h. VSTs and target cells were incubated at various ratios for 4 h. For controls, target cells were incubated in media alone or with 1% triton-X 100 (Sigma-Aldrich) to measure the spontaneous and the maximum release, respectively. The mean percentage of specific lysis of triplicate wells was calculated as follows: [(test counts – spontaneous counts)/(maximum counts – spontaneous counts)] x100%.

Statistical Analysis

Descriptive statistics was used to summarize the data. Comparison between groups was made using student's t-test or One-Way ANOVA whichever was appropriate. GraphPad prism 6 (GraphPad software, Inc., La Jolla, CA) or higher was used for statistical analysis. P values <0.05 were considered statistically significant.

RESULTS

Rapid Generation of Transgenic VSTs by IFN-γ Cytokine Capture and Retroviral Transduction

To rapidly generate genetically engineered TCR8+ VSTs from healthy donors, we used the IFN-γ cytokine capture selection system to enrich for CMV and EBV-specific memory T cells upon peptide stimulation of peripheral blood mononuclear cells, followed by retroviral transduction. The scheme of the retroviral vector (**Figure 1A**) and the cell isolation, transduction and expansion process (**Figure 1B**) is shown in **Figure 1** and is fully Good Manufacturing Practice (GMP) laboratory compatible. The average yield of IFN-γ secreting VSTs from 50x10^6 PBMCs was 0.15x10^6 (range 3.13x10^4 – 2.2x10^6, n=8, **Figure 1C**). VSTs isolated by IFN-γ CC were sufficiently activated by the isolation to directly proceed to retroviral transduction on day 3 (TCR8+ vs NT VSTs, %mTCR+ cells; 66±9% vs 0.8±0.4%, p<0.0001, mean±SD, n=8) (**Figure 2A**). TCR8+ VSTs efficiently bound the survivin-specific dextramer compared to non-transduced (NT) VSTs (TCR8+ vs NT VSTs, %mTCR+Dex+; 42±7% vs 0.3±0.4%, p<0.0001, mean±SD, n=8, **Figure 2A**). Both, TCR8+ and NT VST lines were enriched in NLV+ cells (TCR8+ vs NT VSTs; 18±15.8% vs 28±27%, mean ±SD, p=ns, n=5), based on NLV-pentamer staining that is used to detect CMV specific cells within the product (**Figure 2B**). Non-transduced and TCR8+ VSTs expanded well with comparable fold expansions after first (S1) (NT: 294±267-fold, TCR8+: 269±285-fold) and second (S2) (NT: 6284±6646-fold, TCR8+: 5877±6682-fold) stimulation respectively (mean±SD, n=5, **Figure 2C**). Phenotypically, both NT and TCR8+ VSTs consisted predominantly of CD3+CD8+ T cells (NT: 95±3%, TCR8+: 93±3%, mean±SD, n=8) with low percentages of CD3+ CD4+ T cells (NT: 3±3%, TCR8+: 1±1%, mean±SD, n=8) (**Figure 2D**). We found a complete absence of NK cells (CD3-CD56+) and TCRγδ+ T cells in our products. However, TCR8+ VSTs contained slightly increased frequencies of CD3+CD56+ activated T cells as compared to NT VSTs (NT vs TCR8+: 0±0 vs 10±10%, p=ns, n=8) (**Figure 2E**). The memory subset distribution in the CD3+CD8+ compartment revealed high proportions of central memory T cells (T_{CM}) in both, NT and TCR8+ VSTs (NT vs TCR8+, naïve (T_N) 1.9±1.9% vs 2.5±2.7%, T_{CM}; 75±18% vs 81±12%, effector memory (T_{EM}) 22±18% vs 16± 11%, terminally differentiated (T_{EMRA}) 0.8±1.2% vs 0.7±0.9%, p=ns, n=6) (**Figure 2F**). Thus, we show that the combination of IFN-γ capture and retroviral transduction can rapidly generate engineered T cell products with simultaneous anti-viral and anti-tumor specificities that are highly enriched in CD8+ T_{CM} cells.

IFN-γ Capture TCR8+ VSTs React Against the Targeted Viral and Tumor Antigens

Next, we assessed antigen specific function of NT and TCR8+ VSTs by IFN-γ ELISPOT and intracellular cytokine staining (ICS). As expected, TCR8+ but not NT VSTs produced IFN-γ in response to the cognate survivin peptide (LML) or the HLA-A*02:01+survivin+ leukemia cell line BV173, targeted by the transgenic TCR (IFN-γ SFCs NT vs TCR8+ VSTs; LML: 7.0±3.7 vs 577±268, p=0.003; BV173: 71.5±122 vs 925±246 p<0.0001, n=6, mean±SD) (**Figure 3A**, top left). Importantly, both NT and TCR8+ VSTs showed comparable anti-viral reactivities against CMV (pp65 and IE1 pepmix) and EBV (LMP2, EBNA1 and BZLF1 pepmixes, GLC and YVL peptides) antigens, while a small but significant reduction in NLV reactivity was observed with TCR8+ VSTs (**Figure 3A**). These results were corroborated by ICS where we found similar degranulation (CD107a/b), IFN-γ and TNF-α levels in NT and TCR8+ VSTs in response to viral antigens (**Figure 3B**). Again, the survivin derived LML peptide was only recognized by TCR8+ but not NT VSTs. Thus, IFN-γ capture TCR8+ VSTs are specific for and reactive against both the targeted tumor and viral antigens, and anti-viral reactivities are not altered by the transduction and forced expression of TCR8.

IFN-γ capture TCR8+ VSTs Kill Viral and Tumor Targets *in vitro*

We next evaluated the cytotoxicity of NT and TCR8+ VSTs in co-cultures and in a 4-hour ^{51}Chromium-release assay. When we co-cultured NT or TCR8+ VSTs with HLA-A*02:01+survivin+ BV173 leukemia cells, we observed significant killing of target cells by TCR8+ but not NT VSTs (residual tumor cell count NT vs TCR8+: 2.3±0.6x10^6 vs 0.04±0.07x10^6, p=0.0004, mean±SD, n=6) (**Figure 4A**, left). No difference in the VST counts at the end of the co-cultures was seen (**Figure 4A**, right). We also analyzed cytokine secretion and lytic granules present in the co-

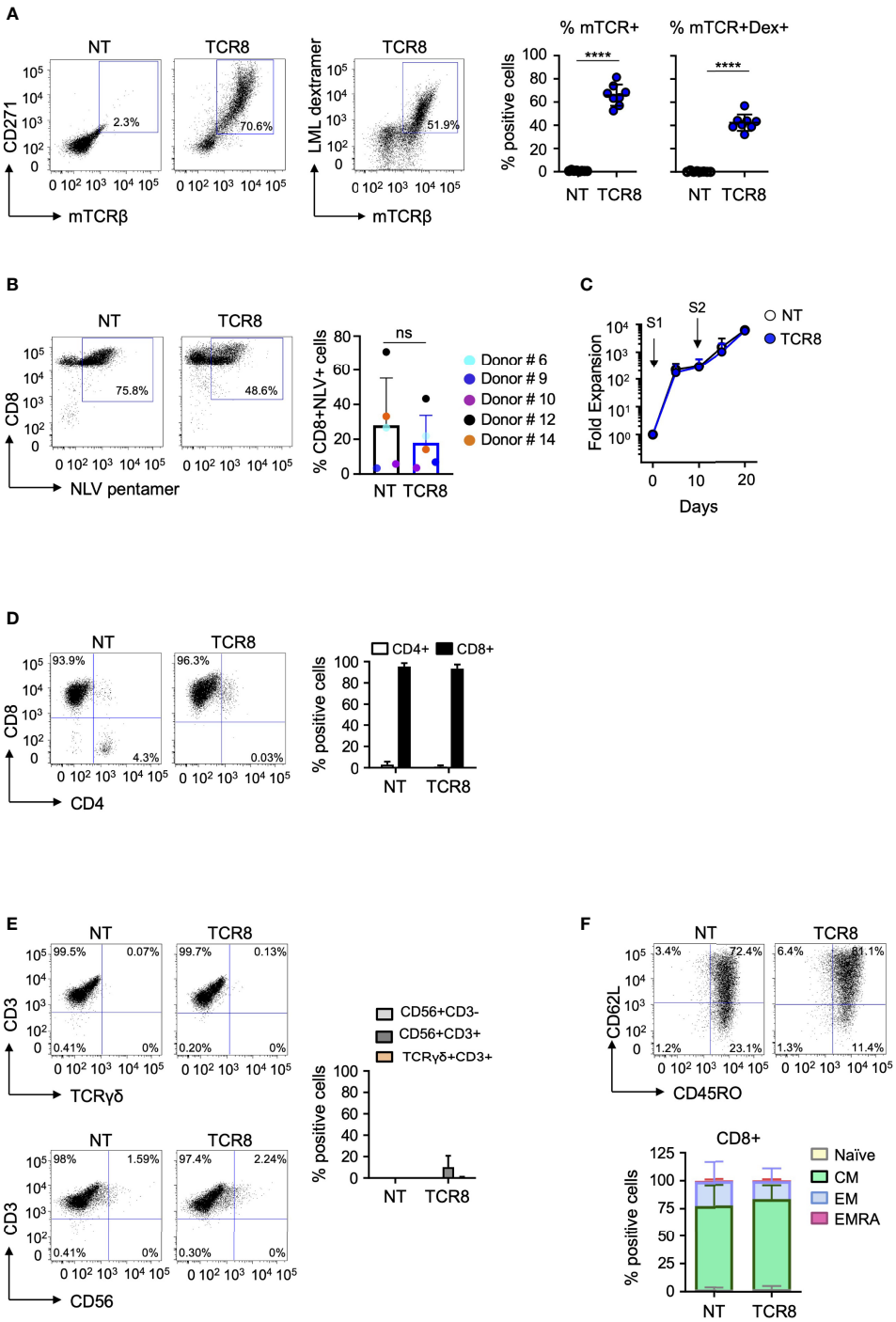

FIGURE 2 | Phenotypic characterization and fold expansion of TCR8+ VSTs. **(A)** Representative FACS plots (left) and summary (right) of transduction efficiencies (mTCRβ, CD271) and transgenic TCR specificity [LML dextramer (Dex)]. NT: non-transduced. NT vs TCR8+, mean ± SD, ****p <0.0001, n=8 in both. **(B)** Representative FACS plots (left) and summary (right) of CMV-specific T cells in NT and TCR8+ VSTs. NT vs TCR8+, mean ± SD, n=5, p=ns. Dot color indicates individual donors. **(C)** Fold expansion of NT (open circles) and TCR8+ (blue circles) VSTs after first (S1) and second (S2) stimulation. **(D)** Distribution of CD4+ and CD8+ T cells, representative FACS plots (left) and summary (right) (gated on total live cells), mean ± SD, n=5. **(E)** Analysis of NK cells (CD56+CD3-), activated T cells (CD56+CD3+), or TCRγδ T cells (TCRγδ+CD3+) in NT and TCR8+ VSTs. Representative FACS plots (left) and summary (right) (gated on total live cells), mean ± SD, n=5. **(F)** Representative FACS plot (top) and summary (bottom) (gated on live CD3+CD8+ T cells) of memory phenotype of VSTs: naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated (T_{EMRA}) subsets characterized in NT and TCR8+ VSTs based on CD45RO and CD62L staining, n=5, mean ± SD. ns, not significant.

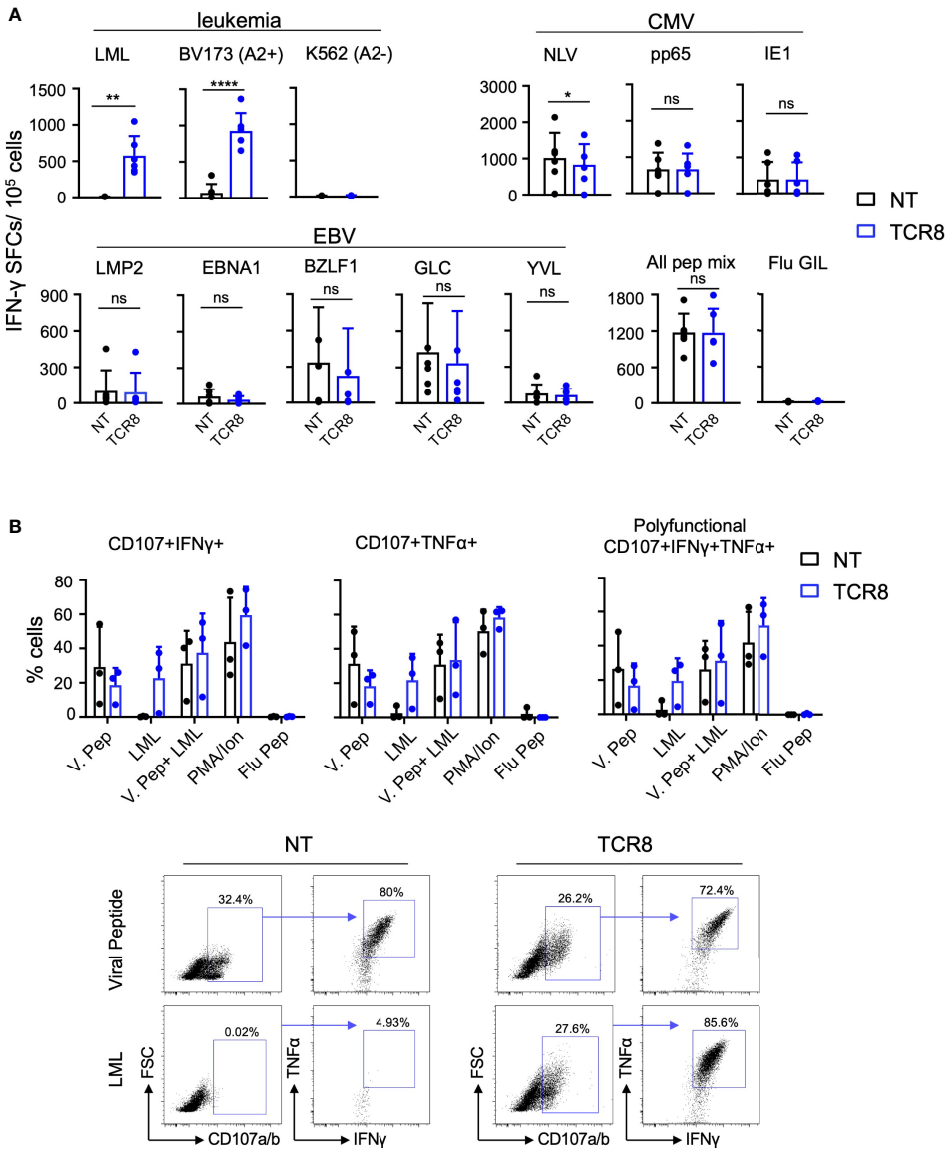

FIGURE 3 | TCR8+ VSTs are multi-antigen specific for survivin and viral (CMV and EBV) antigens. **(A)** NT or TCR8+ VSTs after S1 were stimulated with viral (CMV and EBV) pepmixes/peptides, survivin peptide, or leukemia cell lines (BV173: HLA-A2*02:01+survivin+ or K562: HLA-A2-) for 24 h and IFN-γ spot forming cells (SFC) were measured by ELISPOT, n=6, mean ± SD: NT vs TCR8+, LML peptide: **p=0.003, BV173: ****p<0.0001, NLV peptide: *p=0.035, pp65, IE1, LMP2, BZLF, EBNA1, GLC peptide, YVL peptide: mean ± SD, p=ns, n=6. **(B)** NT or TCR8+ VSTs were stimulated with viral pepmixes, LML peptide, viral pepmixes plus LML peptide, PMA/Ionomycin (25 ng/ml) or irrelevant influenza GIL peptide (negative control) for 4 h and stained for degranulation (CD107a/b) and intracellular IFN-γ and TNF-α followed by FACS analysis. The percentage of cells expressing CD107a/b+/IFNγ+ (top left), CD107a/b+/TNFα+ (top middle) and (CD107a/b+/IFNγ+/TNFα+ (polyfunctional, top right) are shown. Dots, mean ± SD, n=3 donors. Representative FACS plots are shown on the bottom (gated on total live cells, then gated on CD107a/b+ cells) for NT (left) and TCR8 (right). ns, not significant.

culture supernatant 24 hours after tumor challenge. TCR8+ VSTs produced significant amounts of T_H1 cytokines including IFN-γ, TNF-α and IL-10 (NT vs TCR8+ VSTs: IFN-γ; 0.9±1.0 vs 8.1±4.0 ng/ml, p=0.017, TNF-α; 0.02±0.01 vs 1.2±0.9 ng/ml, p=0.017, IL-10; 0.3±0.2 vs 2.8±2.1 ng/ml, p=0.028, mean±SD, n=6) and cytolytic granules (GZMB NT vs TCR8+: 2.7±1.5 vs 6.8±4.0 ng/ml, p=0.02, mean±SD, n=6) (**Figure 4B**). We detected GZMA and perforin release into the supernatant of NT VSTs even though no cytotoxicity was observed. In a 4-hour ^{51}Chromium-release cytotoxicity assay, we found that activated autologous T cells pulsed with viral peptides were efficiently lysed by both, NT and TCR8+ VSTs at various E:T ratios, while un-pulsed targets were not killed (**Figure 4C**, mean ±SD, n=3 donors, each plated in technical triplicates). HLA-

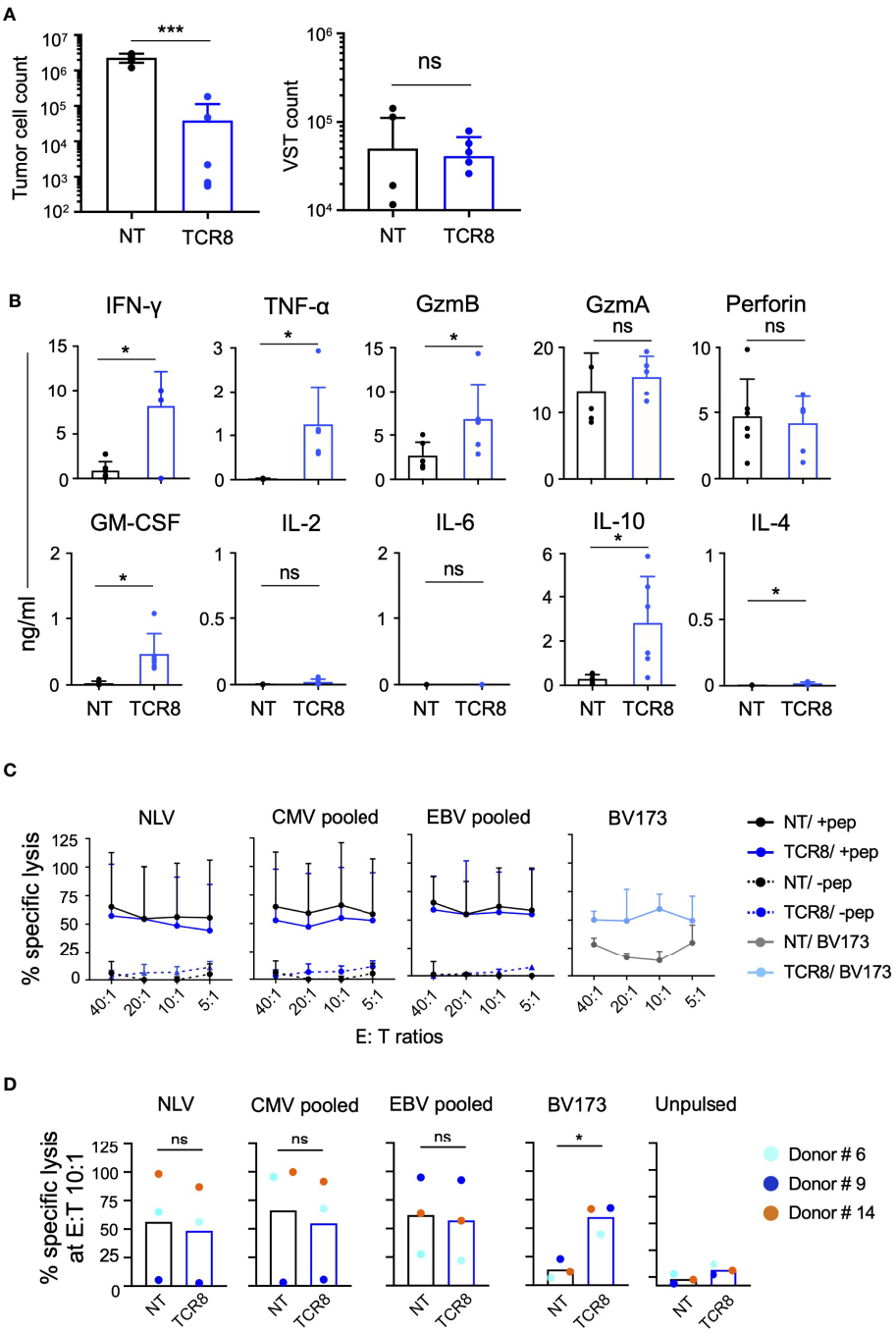

FIGURE 4 | TCR8+ VSTs are cytotoxic *in vitro* against the cognate viral and tumor targets. **(A)** Co-culture of NT or TCR8+ VSTs with BV173 leukemia cells (HLA-A2*02:01+survivin+); E:T ratio 1:5. Residual BV173 cells (left) or VSTs (right) quantified by FACS on day 3, NT vs TCR8+, mean ± SD, Tumor cells: ***p=0.0004; VSTs: p=ns; n=6. **(B)** Cytokine quantification in supernatants after 24 hours of coculture, NT vs TCR8+, mean ± SD, IFN-γ, TNF-α, Granzyme (Gzm) B, GM-CSF, IL-10 and IL-4: *p<0.05, n=6. **(C)** Percent specific lysis of peptide/pepmix pulsed or unpulsed activated autologous T cells or BV173 cells by NT or TCR8+ VSTs in a 4-hour ^{51}Cr-release assay at E:T ratios of 40:1, 20:1, 10:1, 5:1, mean ± SD, n=4. **(D)** Summary of percent specific lysis at E:T 10:1 ratio depicting individual donors (colored dots). NT vs TCR8+, mean ± SD, NLV peptide, CMV pooled, EBV pooled: p=ns; BV173 cells: *p=0.01, n=3, paired t-test. ns, not significant.

A*02:01+survivin+ BV173 leukemia cells were only killed by TCR8+ but not NT VSTs. Donor heterogeneity with regards to CMV and/or EBV reactivity was high, as illustrated in **Figure 4D**. For example, anti-viral specificity from donor #9 was almost exclusively directed against EBV and not against CMV, while the other two evaluated donors showed simultaneous responses against both viruses. The anti-leukemic activity conferred by the transgenic TCR was much more

consistent across donors. Thus, we demonstrate that TCR8+ VSTs generated with our approach are cytotoxic and functional against both viral and tumor targets.

DISCUSSION

Here we present an approach for the rapid generation of engineered human T cells with simultaneous anti-viral and anti-tumor activity (**Figure 5**). With the IFN-γ cytokine capture system we efficiently enriched and activated anti-viral memory CD8+ T cells that were directly amenable for retroviral transduction, significantly reducing the complexity of manufacturing compared to previously established processes. Transgenic co-expression of the CD8αβ co-receptor with the tumor targeted TCR ensured sufficient co-receptor availability for both endogenous anti-viral and transgenic anti-tumor TCRs. T cells generated with our approach efficiently recognized and killed both viral and tumor targets.

Our approach has significant advantages over other established processes for the production of engineered VSTs and allows considering moving to a semi-automated closed process. The most important advantages are that (1) no live virus is necessary for the production of lymphoblastoid cell lines, (2) viral antigen presentation and T cell activation is achieved with peptide pulsing of peripheral blood mononuclear cells and no additional generation of antigen presenting cells is necessary, (3) a one-step procedure is sufficient for T cell selection and activation that allows to directly proceed to gene-modification after a short culture period, (4) selection is performed with magnetic columns and does not require flow cytometry-based sorting, and importantly (5) the approach significantly reduces manufacturing time as well as the number of manipulations needed for product generation. IFN-γ captured VSTs can be potentially modified using non-viral gene delivery systems such as transposons or CRISPR/Cas9, that are more versatile and cost-effective (18). In addition, we plan to upscale the approach and reduce the need for open manufacturing steps. For example, cytokine capture as well as gene modification and cell expansion could be adapted to the capabilities of an automated closed manufacturing platform such as for example the CliniMACS Prodigy system (16, 19).

Nevertheless, we also identified some disadvantages, which include in our hands (1) the almost exclusive enrichment for CD8+ T cells, and (2) a high donor variability in T cell yield after the CC selection procedure. In fact, virus-specific CD4+ T cells play a key role in the development of long-lasting antiviral immunity by potentiating cytotoxic CD8+ T cell responses, by providing help to B cells for efficient and long-lasting antibody responses, and by direct cytotoxic effects (20–22). Upon adoptive transfer of VSTs to immunocompromised patients after HSCT, the CD4+ T cell compartment was instrumental for the development of long-lasting viral control (23). The lack of CD4+ T cell enrichment in our study may be due to the fact that our stimulation with the viral pepmixes was performed over 6 hours only, compared to previous literature where the stimulation lasted 16 hours (14, 24), a factor that needs to be evaluated in the future. The high variability in viral antigen-specific cell frequency is consistent with previous observations (14, 16, 24) and confirms the fact that circulating anti-viral memory T cell frequency varies over a broad range in different individuals.

VSTs are an interesting cell therapy platform for the development of allogeneic off-the shelf engineered T cell therapies. Several academic clinical trials have demonstrated safety and efficacy in controlling viral infections in immunocompromised patients after solid organ transplant or allogeneic HSCT with the infusion of third-party donor derived banked VSTs in partially HLA matched settings (25–30). Third party VST cell therapy is now on the way to commercialization. Because VSTs express a viral antigen restricted TCR repertoire, they did not produce significant graft-versus host disease in infused patients across studies. However, *in vivo* persistence was shorter when compared to VSTs derived from HLA matched donors [recently reviewed in (3)] indicating significant rejection by host T or NK cells. Recently, additional engineering strategies have been developed to confer resistance to rejection to the gene-modified VSTs (31, 32) which further enhances potential future applicability as a more general cell therapy platform.

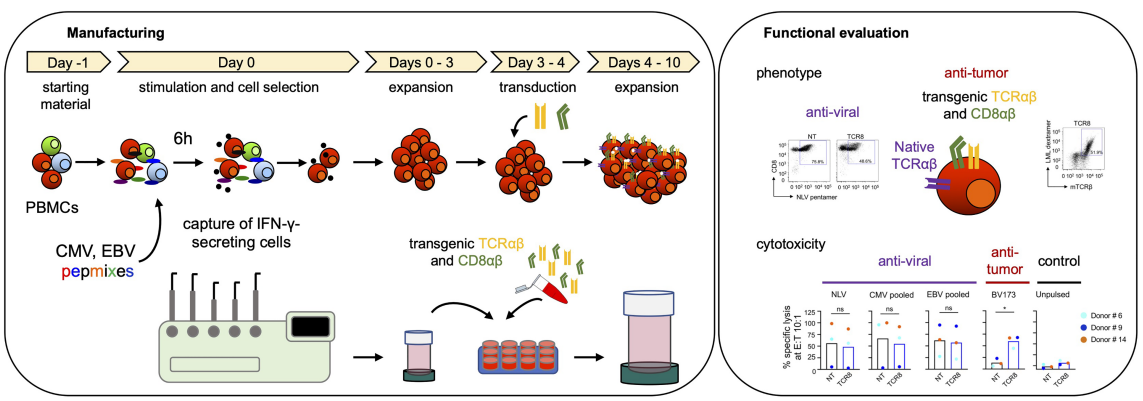

FIGURE 5 | Schematic overview of transgenic virus-specific T cell manufacturing and functional evaluation.

In summary, we show that manufacturing of gene-engineered VSTs can be simplified and shortened by combining IFN-γ cytokine capture and retroviral transduction. Our process is scalable, amenable to the use of non-viral gene delivery systems, and yields highly multifunctional T cells with both anti-viral and anti-tumor activity. Clinical translation of our approach can be envisioned in a clinical trial with the goal to prevent or treat viral infection and malignant relapse in patients after allogeneic stem cell transplant.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

GB designed research, performed experiments, analyzed, and interpreted results and wrote the manuscript. CA designed research, supervised the entire study, analyzed and interpreted results and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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CAR-T Therapy for Pediatric High-Grade Gliomas: Peculiarities, Current Investigations and Future Strategies

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High-Grade Gliomas (HGG) are among the deadliest malignant tumors of central nervous system (CNS) in pediatrics. Despite aggressive multimodal treatment - including surgical resection, radiotherapy and chemotherapy - long-term prognosis of patients remains dismal with a 5-year survival rate less than 20%. Increased understanding of genetic and epigenetic features of pediatric HGGs (pHGGs) revealed important differences with adult gliomas, which need to be considered in order to identify innovative and more effective therapeutic approaches. Immunotherapy is based on different techniques aimed to redirect the patient own immune system to fight specifically cancer cells. In particular, T-lymphocytes can be genetically modified to express chimeric proteins, known as chimeric antigen receptors (CARs), targeting selected tumor-associated antigens (TAA). Disialoganglioside GD2 (GD-2) and B7-H3 are highly expressed on pHGGs and have been evaluated as possible targets in pediatric clinical trials, in addition to the antigens common to adult glioblastoma – such as interleukin-13 receptor alpha 2 (IL-13α2), human epidermal growth factor receptor 2 (HER-2) and erythropoietin-producing human hepatocellular carcinoma A2 receptor (EphA2). CAR-T therapy has shown promise in preclinical model of pHGGs but failed to achieve the same success obtained for hematological malignancies. Several limitations, including the immunosuppressive tumor microenvironment (TME), the heterogeneity in target antigen expression and the difficulty of accessing the tumor site, impair the efficacy of T-cells. pHGGs display an immunologically cold TME with poor T-cell infiltration and scarce immune surveillance. The secretion of immunosuppressive cytokines (TGF-β, IL-10) and the presence of immune-suppressive cells – like tumor-associated macrophages/microglia (TAMs) and myeloid-derived suppressor cells (MDSCs) - limit the effectiveness of immune system to eradicate tumor cells. Innovative immunotherapeutic strategies are necessary to overcome these hurdles and improve ability of T-cells to eradicate tumor. In this review we describe the distinguishing features of HGGs of the pediatric population and of their TME, with a focus on the most promising CAR-T therapies overcoming these hurdles.

Keywords: high-grade gliomas (HGG), CAR-T therapies, immunotherapy, tumor microenvironment (TME), next generation CAR-T cells

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PEDIATRIC HIGH-GRADE GLIOMAS (PHGGS)

Pediatric HGGs are among the most common malignant brain tumors in pediatrics and represent the leading cause of cancer related death in childhood (1). Traditionally, pediatric and adult gliomas were commonly classified according to the WHO grading, with HGGs including WHO grade III and IV aggressive tumors (2). Over the last years, key differences in epigenomic and genetic features between pHGGs and their adult counterparts emerged, despite analogies in aggressiveness and histology (3). In 2021 WHO Classification of CNS tumors, pediatric gliomas are differentiated from adults– on the heels of 2016 WHO classification – and the term "glioblastoma" is abandoned in pediatric oncologic setting (4). Four groups of pHGGs are now described: *Diffuse midline glioma, H3 K27-altered*; *Diffuse hemispheric glioma, H3 G34-mutant*; *Diffuse pediatric-type high-grade glioma, H3-wildtype and IDH-wildtype*; and *Infant-type hemispheric glioma* (**Table 1**). *Diffuse midline glioma, H3 K27-altered* had already been included in previous classification but the term "altered" aims at including other mechanisms besides previously reported H3-K27 mutations. This group encompasses DIPG, one of the most aggressive types of pHGG. *Diffuse hemispheric gliomas, H3 G34-mutant* typically arise in cerebral hemispheres and are characterized by a G34R/V substitution of histone H3 due to a mutation of H3F3A gene (5). *Diffuse pediatric-type high-grade glioma, H3-wildtype and IDH-wildtype* encompass a heterogeneous group of pHGGs not showing either H3 or IDH mutations. Finally, *Infant-type hemispheric glioma* include HGGs occurring in infant and newborns. This latter includes 3 subgroups distinguished by molecular features: subgroup 1 involves alterations in one of the genes *ALK, ROS1, NTRK1/2/3*, or *MET;* subgroup 2, RAS/MAPK pathway alterations and hemispheric localization; subgroup 3 refers to tumors with RAS/MAPK mutations arising in midline structures (6–8). Molecular characterization of the first subgroup of this class promoted investigation of target therapies, such as Larotrectinib for NTRK-fusion positive pHGGs (NCT02576431).

Even though our knowledge on biological and molecular features of pHGGs largely increased during the past years, therapeutic approaches remain limited and ineffective. Current multimodal treatments encompass surgery, radiotherapy and chemotherapy, reaching 5-year survival rate less than 20% (9). New therapeutic strategies are necessary and novel immunotherapies hold great promise for possible effective treatment.

CAR-T CELLS

The principle of immunotherapy relies on restoring the physiological ability of immune system to recognize and eliminate tumor cells. This goal can be achieved through a wide variety of approaches and, so far, development of chimeric antigen receptor (CAR) expressing T cells is one of the ultimate advances in this field. CAR-T cells are T lymphocytes genetically modified by either viral vectors (retroviral or lentiviral) or by non-viral approaches (sleeping beauty transposition) to express a chimeric construct deriving from the fusion of the variable portions of a monoclonal antibody single chain to the signal transduction domains of the CD3 z chain (10). This structure combines the specificity of MHC-independent antibody recognition with the anti-tumor potential of T lymphocytes, thus allowing to transfer any antigenic specificity to T cells. In order to potentiate the antitumor efficacy of these constructs, second-generation CAR-T cells have been created by the inclusion of one costimulatory domain, such as the CD28, 41BB or OX40 molecules, resulting in a higher capacity for cytokine production, a greater expansion and a longer persistence (11). Subsequently, the combination of two signal domains into third-generation CAR-T constructs showed a further increase in the activation profile (12). CAR-T therapy has given outstanding results against several B-cell malignancies and myeloma, in both adults and children. Currently, the clinical trials reported so far on CAR-T cells directed towards the CD19 antigen, widely expressed by acute lymphoblastic leukemia cells (ALL), has documented a strong tumor activity (13, 14) even in patients highly resistant to conventional treatments or relapsed after allogeneic transplantation, obtaining CR rates of approximately 80%. Unfortunately, the results obtained so far with CAR-T cells in solid tumors have been less effective and fewer clinical trials or case reports have been reported in the literature. Several limits can hinder the development of CAR-T in solid tumors, including: the difficulty of finding a suitable target antigen (the so-called "antigen dilemma"), the strongly immunosuppressive TME, the limited persistence *in vivo* and finally, the insufficient T

TABLE 1 | Pediatric-type High Grade Gliomas according to new 2021 WHO CNS classification.

Group	Molecular Features	Localization	References
Diffuse midline glioma, H3 K27-altered	H3 K27 mutations, EZHIP overexpression, other H3 K27 alterations.	Thalamus, brainstem, spinal cord	(4)
Diffuse hemispheric glioma, H3 G34-mutant	H3 G34 mutations.	Cerebral hemispheres	(4, 5)
Diffuse pediatric-type high-grade glioma, H3-wildtype and IDH-wildtype	Wild-type H3 and IDH gene families.	Cerebral hemispheres and midline structures	(4)
Infant-type hemispheric glioma.	Subgroup 1: RTK- driven Fusion genes involving ALK, ROS1, NRK and MET	Cerebral hemispheres	(4, 6–8)
	Subgroup 2: RAS/MAPK pathway mutations	Cerebral hemispheres	
	Subgroup 3: RAS/MAPK pathway mutations	Thalamus, brainstem, spinal cord	

cell trafficking and homing to tumors and the limited persistence of exhausted T cells.

ACTUAL AND PROMISING TARGET ANTIGENS IN PHGGS

The first antigens proposed for targeting pHGGs derive from studies conducted on adult gliomas, in particular HER-2, EphA2 and IL-13Rα2 (15).

HER-2 is a tyrosine kinase receptor overexpressed in HGGs, whose levels of expression correlate to poor outcome not only in GBM but also in other pediatric tumors, such as medulloblastoma (16, 17). HER-2 is detected at low levels in normal, healthy brain whereas is overexpressed on CNS cancer stem cells, making it an effective and safe target for the treatment of HGGs (18). Monoclonal antibodies (moAbs) recognizing HER-2 showed extraordinary results in the treatment of breast cancer, but the presence of blood brain barrier (BBB) limits their use for brain tumors. Differently from moAbs, T-cells can cross the BBB and traffic to brain tissue and cerebrospinal fluid from blood flow to recognize tumor cells, as demonstrated by clinical response of melanoma brain metastasis after intravenous infusion of adoptive tumor-infiltrating lymphocytes and by the detection of CD19-CAR-T in cerebrospinal fluids of patients with ALL (19, 20). Indeed, Ahmed et al. tested the safety of intravenous injection of virus-specific (VS) CAR-T cells directed to HER-2 in a phase I clinical trial on patients affected by high grade gliomas: with 17 patients treated, including 7 children <18 years, the approach resulted to be safe, without any dose-limiting toxicity (DLT) reported (21). Interestingly, one of the adolescent patients with unresectable right thalamic HGG showed the reduction of 30% of the longest tumor diameter lasted for 9.2 months and an Overall Survival (OS) of 34.2 months after two infusions of CAR-T cells (21). In order to target CNS tumors, CAR-T can be delivered locally in tumor resection cavity or in the ventricular system with improved tumor control, even at lower doses, and reduced systemic circulation and toxicity, as recently highlighted by Theruvat et al. and Donovan et al. (22–24). Currently, two clinical trials are evaluating intracranial injection of Anti-HER-2 CAR-T for treatment of pHGGs (NCT02442297; NCT03500991) (**Table 2**). Preliminary results of the phase 1 trial "BrainChild-01" showed that repetitive intracranial infusion of HER-2 CAR-T on a cohort of pediatric and young adult patients with CNS tumors is safe, well tolerated, and able to induce immune response (18). Two other clinical trials, namely "Brainchild-02" and "BrainChild-03", are evaluating the effectiveness of locoregional infusion of CAR-T cells targeting EGFR and B7-H3, respectively (NCT03638167 NCT04185038).

EphA2 is a tyrosine kinase receptor involved in oncogenic pathways in several tumors, including breast cancer, lung cancer and HGG (25). High expression of EphA2 has been correlated to worse clinical outcomes in adult HGG and recently the same association was confirmed in pediatric HGGs (26). Preclinical studies reported a promising antitumoral activity of EphA2-redirected CAR-T-cells for the treatment of HGG. Recently, results of the first-in-human trial of intravenous administration EphA2-CAR-T in patients with high grade gliomas showed safety and transient clinical responses (27). However, the trial enrolled patients >18 years, therefore the efficacy of EphA2- CAR-T in pediatric cohorts remains to be investigated.

TABLE 2 | Ongoing clinical trials evaluating CAR-T therapies for pHGGs.

Official title	Antigenic target	Administration	Responsible party	NCT
T Cells Expressing HER2-specific Chimeric Antigen Receptors (CAR) for Patients With HER2-Positive CNS Tumors (iCAR)	HER-2	Locoregional delivery	Nabil Ahmed, Baylor College of Medicine	02442297
HER2-specific CAR-T Cell Locoregional Immunotherapy for HER2-positive Recurrent/Refractory Pediatric CNS Tumors	HER-2	Locoregional delivery	Julie Park, Seattle Children's Hospital	03500991
Genetically Modified T-cells in Treating Patients With Recurrent or Refractory Malignant Glioma	IL13Rα2	Locoregional delivery	City of Hope Medical Center	02208362
EGFR806-specific CAR-T Cell Locoregional Immunotherapy for EGFR-positive Recurrent or Refractory Pediatric CNS Tumors	EGFR	Locoregional delivery	Julie Park, Seattle Children's Hospital	03638167
GD2 CAR-T Cells in Diffuse Intrinsic Pontine Gliomas (DIPG) & Spinal Diffuse Midline Glioma(DMG)	GD-2	Intravenous injection after Lymphodepletion with Cyclophosphamide/Fludarabine Chemotherapy	Crystal Mackall, Stanford University	04196413
C7R-GD2.CAR-T Cells for Patients With GD2-expressing Brain Tumors (GAIL-B)	GD-2	Intravenous injection Lymphodepletion with Cyclophosphamide/Fludarabine Chemotherapy	Bilal Omer, Baylor College of Medicine	04099797
Study of B7-H3-Specific CAR-T Cell Locoregional Immunotherapy for Diffuse Intrinsic Pontine Glioma/Diffuse Midline Glioma and Recurrent or Refractory Pediatric Central Nervous System Tumors	B7-H3	Locoregional delivery	Julie Park, Seattle Children's Hospital	04185038
B7-H3-Specific Chimeric Antigen Receptor Autologous T-Cell Therapy for Pediatric Patients With Solid Tumors (3CAR)	B7-H3	Intravenous injection Lymphodepletion with Cyclophosphamide/Fludarabine Chemotherapy	St. Jude Children's Research Hospital	04897321

IL13Rα2 is a monomeric, high-affinity, IL13 receptor, detected at high levels in more than 50% of HGGs, whose overexpression is associated with poor outcomes (28). Brown et al. started a clinical trial to evaluate the efficacy of intracranial infusion of IL13Rα2-CAR-T in adult and pediatric patients with HGGs (NCT02208362). The first published results show safety of the therapeutic approach and documented a complete radiographic response in a patient for up to 7.5 months (29).

Recently, both GD2 and B7-H3 were found to be highly expressed in pediatric diffuse midline glioma (DIPG). Interestingly, Haydar et al. established a hierarchy of antigens expressions in pediatric brain tumors showing that, despite a high heterogeneity, GD2 and B7-H3 maintain the highest expression as compared to IL-13Rα2, HER2 and EphA2 (30). These data suggest the importance of focusing on these targets for pHGGs rather than antigens mostly relevant in adult gliomas.

GD2 is involved in mechanisms of cell growth, motility and invasiveness of several pediatric tumors like neuroblastoma, Ewing's Sarcoma and pHGGs (31). After the promising results of clinical trials using several generations of GD2-CAR-T cells to treat patients affected by neuroblastoma, first, and other $GD2^+$ solid tumors, after, (NCT03373097; NCT04099797) this approach is currently under preclinical and clinical evaluation also for pHGG (32, 33); NCT05298995). In particular, Mount and Majzner et al. demonstrated uniform and high levels of GD2 on H3-K27 diffuse midline glioma cells and developed a second-generation GD2 CAR construct which showed efficient anti-tumor activity in DMG PDXs models (34), leading to the activation of a phase I clinical trial, currently enrolling pediatric patients. They published the results of the first 4 patients with H3K27-altered diffuse midline glioma (DMG) treated in the trial and showed that the toxicity profile strongly depends on the tumor location and is manageable and reversible with intensive supportive care (35). In addition, reduction of neurological deficits and radiological improvement after CAR-T administration were reported. In particular, in one of the treated patients, affected by spinal cord DMG, a reduction of 90% of the tumor volume after the first intravenous administration and a further reduction of neoplasm dimensions of 80% after a second intraventricular injection was observed (34). At Bambino Gesù Children's Hospital we are currently activating a phase I clinical trial to test the safety and, preliminarily, efficacy of third-generation (incorporating 4.1BB and CD28 costimulatory domains) GD2-CAR T cells, infused i.v., for the treatment of pediatric patients affected by CNS tumors (36); NCT05298995). In particular, in view of the correlation between tumor location and toxicity, the study has an innovative design of sequential enrollment of patients into 3 different arms, the first being represented by medulloblastoma and other embryonal tumor (arm A), the second by hemispheric HGG (arm B) and the third by tumors with midline location, at higher risk of severe toxicity, namely thalamic HGG, DMG, DIPG (arm C).

B7-H3 is a transmembrane protein with important immune inhibition functions, belonging to the B7 family of immune checkpoint proteins, and is overexpressed in several pediatric malignancies - including pHGGs (37). Majzner et al. tested the efficacy of B7-H3 CAR-T in xenografts models of several different paediatric solid tumors, including osteosarcoma, medulloblastoma and Ewing's sarcoma) (38). Interestingly, they showed a potent antitumor activity, strictly dependent on the antigen density on tumors, which resulted to be aberrantly high compared to healthy tissues (38). The results is extremely important, considering the wide expression of the antigen in normal tissues. Recently, phase 1 clinical trials exploring the safety of either loco-regional or intravenous infusion of B7-H3 CAR-T cells for the treatment of paediatric patients with solid tumors, including pHGGs, started (NCT04185038; NCT04897321).

As already mentioned, the success of CAR-T cell therapy relies on the choice of target antigens highly expressed on tumor cells, amongst several factors. However, the recognition of a single antigen is limited by the possible occurrence of the so-called "antigen escape" phenomenon: either the down-regulation of the target antigen or the selection of already negative subclones by the pressure of the treatment, in highly heterogeneous tumors, can represent the cause of subsequent tumor recurrence. This issue is particularly relevant in the setting of pHGG which are extremely heterogeneous and often characterized by subpopulations of tumor cells with different antigen expression. For this reason, CAR-T cells able to recognize multiple antigens on target cells by incorporating 2 antigen targeting domains within one CAR construct have been developed. For HGG, a tandem CAR (TanCAR) targeting simultaneously HER-2 and IL-13Rα2 was designed (39). TanCAR-T cells showed an elevated capacity to lyse glioma cells *in vitro* and *in vivo* and to prevent tumor recurrence in the animal model. Moreover, CAR-T cells targeting 3 glioblastoma associated antigens (HER2, IL13Rα2, and EphA2) were also developed and showed even more effectiveness as compared to the bispecific CAR-T (40). Another interesting strategy to overcome antigenic escape in HGG is the combination of CAR-T cells with Bi-specific T-cell engagers (BiTEs) targeting different antigens. Choi et al. developed EGFRvIII-redirected CAR-T cells secreting EGFR-BiTEs in the tumor site (41). This strategy was tested in mouse models and resulted capable of dulling the antigenic loss effects. Moreover,-since systemic administration of EGFR-BiTE could induce off-tumor toxicities - the local release by these CAR-T cells reduces the risk of cytotoxic T-cells activity on healthy tissues expressing EGFR.

TUMOR MICROENVIRONMENT IN ADULT AND PEDIATRIC HIGH-GRADE GLIOMA

The complex intra- and inter-tumor heterogeneity of the tumor microenvironment (TME) plays a crucial role in mediating tumor progression and resistance to therapy (42). Several different cells, including tumor-associated glioma stem cells (GSC), stromal cells (resident brain glial cells, oligodendrocytes, astrocytes, ependymal cells, microglia) and infiltrating immune cells, are key regulators of growth and vascularization of HGGs (43, 44).

In adult HGGs, TME presents a wide range of immune suppressive mechanisms preventing tumor recognition and

eradication by the innate and adaptive immune system. The presence of immuno-suppressive cytokines (TGF-β, IL-10), chemokines, and regulatory immune-suppressive cells, such as tumor-associated macrophages/microglia (GAMs) and myeloid-derived suppressor cells (MDSCs), limits the effectiveness of current therapies and are briefly review below (45–47).

GSCs are characterized by their self-renewal properties and play a key role in drug escape mechanisms, for example driving radiation-induced DNA methylation changes after radiotherapy (48) GSCs can promote a microinvasion of healthy tissues in areas that support their proliferation – such as subventricular, perinecrotic and perivascular areas (49) - evading therapeutic agents and contributing to recurrence of disease. Recently, a mouse model of disease derived by orthotopic transplantation of GSCs of pHGGs samples was developed (50). Methylation, histology and clinical outcomes resembled accurately the patients' tumors of origin, indicating the ability of GSCs to differentiate in tumor cells. One of the most innovative approaches targeting GSCs niche in pHGGs is represented by immunovirotherapy (51). Friedman et al. observed the ability of oncolytic HSV-1 to kill tumor cells and CD133+ GSCs in preclinical models (52). Results from a phase 1 clinical trial on the use of oncolytic HSV1 in patients with pHGGs indicated the safety of this approach (53). Combining T-cells based therapies with this kind of modern immunological approaches affecting GSCs could significantly improve tumor control.

GAMs represent an important component of the glioma microenvironment (54) constituting the main proportion of infiltrating cells in adult and pediatric HGGs (i.e., glioblastoma and DIPG) (55, 56). Inside the tumor, GAMs usually acquire a specific phenotype of activation that favors tumor growth, angiogenesis and promotes the invasion of normal brain parenchyma. Most of the macrophages recruited into TME, polarize toward an M2-like phenotype and exhibit suppression of the proliferation and functionality of tumor-infiltrating T cells through the production of anti-inflammatory cytokines (57). In adult HGG, GAMs have been extensively described as pivotal drivers of progression and survival of malignant cells in the TME (58). Hence, several anti-GAMs aproaches are currently under evaluation for treatment of adult GBM. Recently, Lin et al. studied TME and GAMs features specifically in DIPG (59). Although DIPG tumor cells produce Colony Stimulating Factor 1 (CSF1), a cytokine associated with the M2 pro-tumorigenic phenotype, DIPG-associated macrophages do not seem to have the characteristic of macrophages of type 2 (56, 60). Transcriptome analysis have shown that GAMs from DIPG express lower levels of some inflammatory cytokines and chemokines (IL6, IL1A, IL1B, CCL3, CCL4) compared to adult HGG-associated macrophages. Moreover, Engler et al. observed a negative correlation between levels of GAMs accumulation and survival rate in adult HGGs but not in pediatric tumors, highlighting the marked biological differences between the pediatric and adult counterpart (59). In addition, the analysis of lymphocyte infiltrate in primary DIPG tissues reveals the lack of tumor-infiltrating lymphocytes (TILs), defining it as a immunologically "cold" tumor (56). For this reason, adoptive immunotherapies (e.g. CAR-T cells) leading to the transferal of T-cells in the TME represents a promising strategy to induce a strong inflammatory and antitumor response.

MDSCs are involved in immune suppression in several types of cancers, including HGG (61). Although only few studies focus on their role specifically in pHGGs, recently Mueller et al. reported a correlation between high levels of circulating MDSCs and poor prognosis in patients with DIPG, suggesting a role in immunosuppression and tumor escape mechanisms in pHGGs as well (62). Moreover, MDSCs showed to impair efficacy of immunotherapy in other pediatric tumors such as neuroblastoma, representing a relevant target to improve efficacy of modern CAR-T cells therapies (63).

Successful immune escape of tumor cells includes also the production of soluble factors in the microenvironment by tumor cells (TGF-β, LDH5), the induction of co-inhibitory molecules (PD-1, LAG-3 and TIM-3) and the release of immunosuppressive factors (CSF-1, VEGF, PGE2, NO, Arg I, IDO and Gal-1) (64). In adult HGG the production of lactate dehydrogenase isoform 5 (LDH5) and TGF-β impairs NK cells cytotoxic function, usually relevant for the elimination of glioma cells (65). Although in adult HGGs there is a strong infiltration of NK and myeloid cells into the tumor, a similar finding in pediatric brain tumors has not been reported (66).

NEXT GENERATION CAR-T CELLS AND COMBINED STRATEGIES

Solid tumor, including pHGG, showed important mechanisms of resistance to current CAR-T cell therapies. Important obstacles to CAR-T efficacy is the presence of immunosuppressive factors in TME – including, but not limited to, TGF-β, PD-1 or CTLA4 mediated signals (67, 68) – which prevent CAR-T cells expansion and finally induce their exhaustion (69). For this reason, strategies able to circumvent these barriers are needed to improve the effectiveness of CAR-T therapies for pHGGs.

Recently, CAR-T cells releasing transgenic cytokines after activation at tumor site have been developed. This approach was conceived to overcome the insufficient production of pro-inflammatory cytokines by T cells accumulated in the tumor environment. In details, T-cells redirected for universal cytokine-mediated killing (TRUCKs) cells are fourth generation CAR-T cells, armed with immune stimulatory cytokines that improve CAR-T cell expansion and persistence (70) **(Figure 1)**. In details, CAR-T cells can be engineered with inducible expression cassette for the cytokine of interest, including IL-7, CCL-19, IL-15, IL-18, and IL-1 (71–73). In particular, transgenic expression of IL-15 could be an appealing strategy to enhance CAR-T cell effector function in HGGs patients, thanks to the well-known ability of this cytokine to induce a more memory stem cell-like phenotype of transduced T cells (73). The group of Krenciute et al. for example, showed an increased persistence and a greater antitumor activity of IL-13Rα2-CAR-T cells expressing IL-15 constitutively as compared to conventional IL-13Rα2-CAR-T cells (74, 75). Also, anti-GD2 TRUCKs secreting IL-12 or IL-18

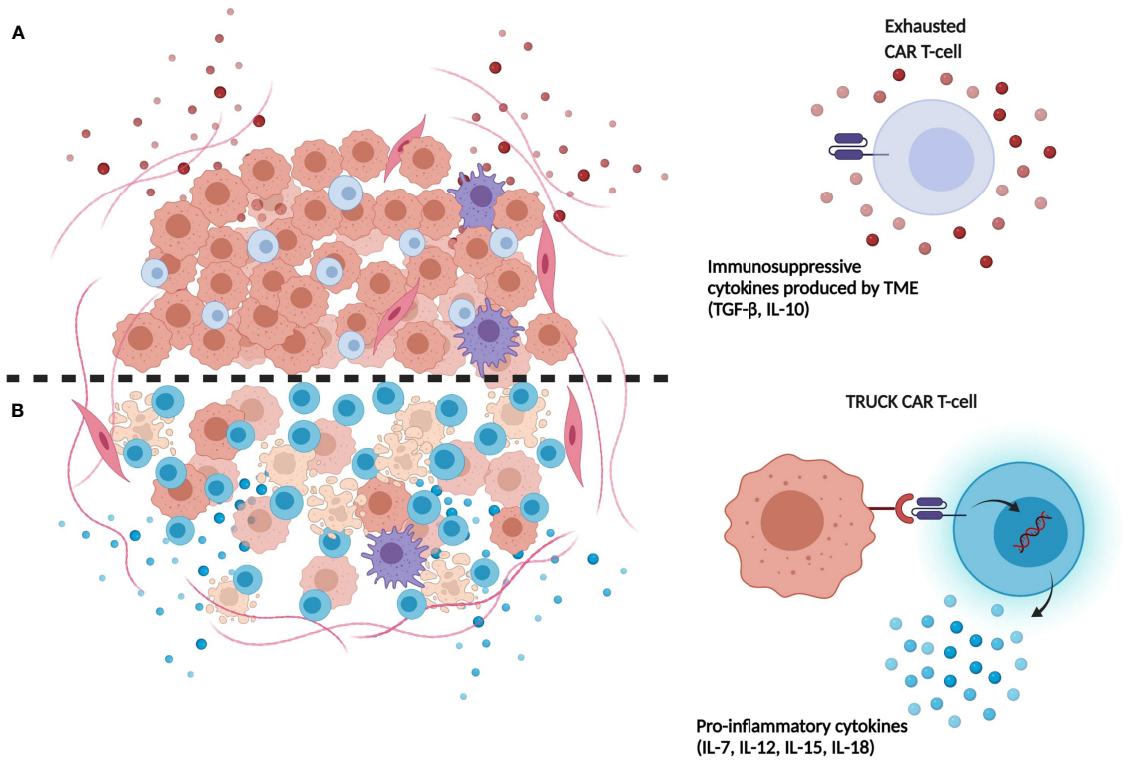

FIGURE 1 | Limitations of first-generation CAR-T-cells compared to next generation TRUCK CAR-T cells. **(A)** Immunosuppressive cytokines (e.g. TGF-β, IL-10) released in TME by tumor cells induce repression and exhaustion of CAR-T cells. **(B)** TRUCK CAR-T cells release transgenic immunostimulatory cytokines which promote their resistance and expansion in tumor site, contrasting TME immunosuppression mechanisms. (Illustration created with BioRender.com).

after activation have been developed, showing improved T-cells activation and increased monocyte recruitment after *in vitro* migration assay (76). On one hand, cytokines release following CAR-T-cells activation results in improved CAR-T cell persistence and stronger antitumor activity, both *in vitro* and *in vivo*. On the other hand, continuous release of secreted cytokine could cause toxicities, limiting the clinical therapeutic window of these CAR-T cells (74). This limitation could be overcome by the development of CAR-T cells expressing constitutively active cytokine receptors, such as IL-2, IL-7, and IL-15 receptors, able to activate the relative intracellular axis, instead of releasing the soluble cytokines. In particular, the expression of constitutively signaling IL-7 receptor (C7R) on a EphA2-CAR-T produced promising results in terms of proliferation, survival and antitumoral activity of CAR-T cells both *in vitro* and in orthotopic xenograft models of HGG (77).

Another major obstacle provided by the TME, to the activation and expansion of CAR-T cells is represented by the widely expressed immune checkpoint receptors – such as PD-1, CTLA-4, TIM-3, and LAG-3. Conventional immune checkpoint inhibitors (ICIs) blocking CTLA-4 (i.e. ipilimumab) or PD-1 (i.e. nivolumab) have shown great success in some solid tumors, including non-small cell lung cancer and metastatic melanoma, but not in HGGs, probably owing to the negligible infiltration of effector T cells in these tumors and to the low mutational burden of pHGGs, leading to few immunogenic tumor neoantigens (78–80). Nevertheless, combined with CART cells, ICIs might improve the ability of the transgenic T cells to exert their antitumor activity, overcoming the exhaustion induced by the TME (81). In pre-clinical models of glioma, checkpoint blockade has been studied as an adjuvant to improve the efficacy of CAR-T therapy. For example, combination of HER-2 redirected CAR-T cells with anti-PD1 antibody induced enhancement of CAR-T cells activity against HGG cells *in vitro* (82). Furthermore, Song et al. reported the ability of anti-PD1 antibodies to improve EGFRvIII-CAR-T cells antitumoral effects in a mouse model of HGG (83), suggesting that PD1 blockade might represent an effective strategy. Based on these promising pre-clinical studies, two phase I clinical trials are currently investigating 2nd generation CAR-T cells in combination with pembrolizumab or nivolumab for the treatment of adult patients with HGG (NCT03726515; NCT04003649).

Interestingly, the role of some intracellular signaling pathways in the activity of CAR-T cells has been investigated, unveiling new potential approaches to improve CAR-T cells efficacy. For example, the diacylglycerol kinase (DGK), a physiologic negative regulator of the signal transduction of the T-cell receptor (TCR), is able to negatively regulate CAR-T cell activation (84). Therefore, the knockout of DGK can induce an improvement of the anti-tumor cytotoxicity of CAR-T cells. In a mouse glioma model, EGFRvIII-CAR-T cells lacking DGK

revealed elevated effector function of the transgenic CAR-T cells, with increased antitumor activity and tumor infiltration (85). Moreover, recently, we reported that the co-administration of linsitinib – a dual IGF1R/IR inhibitor – is able to improve GD2-CAR-T antitumor activity and increase tumor cell death of primary cells of H3K27 diffuse midline glioma, both *in vitro* and *in vivo* (86). These results support the hypothesis that the use of combinatorial approaches might potentiate the efficacy of CAR-T cells for the treatment of pHGGs.

Another promising and sophisticated immunotherapy approach is represented by oncolytic viruses (OV): genetically modified viral agents able to replicate in tumor cells with a negligible replication ability in non-neoplastic cells (87). OV antitumoral activity is based on two mechanisms: i) induction of direct lysis of tumor cells through infection and replication; ii) stimulation of effector function and antitumor activity of the T cells in TME. The latter mechanism as shown to be extremely relevant, if not even the most relevant, in the antitumor activity of OV and represents the rational for combining OV and CAR-T cells for the treatment of solid tumors, with the aim to increase trafficking and antitumoral cytotoxicity of T-cells in TME (88). In particular, Huang et al. studied a preclinical model of the combination of anti-B7-H3 CAR-T cells with and IL-7-loaded oncolytic adenovirus (oAD-IL7) for the treatment of HGG (89). The combined strategy promoted T-cells proliferation and antitumoral activity *in vitro* and reduced mortality in the xenograft mouse models (89). Conversely, there are some controversial results showing the inefficacy of OVs and CAR-T cells combination for HGGs. Recently, it was observed that the pro-inflammatory activity of an OV (VSVmIFNβ) can impair EGFRvIII CAR-T cells cytotoxicity against HGG cells (90). The reason of these unexpected results may lie on the complexity of inflammation mechanisms occurring in TME, which need to be better understood in order to develop precise, effective, and safe combination strategies involving CAR-T cells. Moreover, the group of Park et al. developed an interesting combined strategy exploiting OVs to induce the expression of the CAR target in infected tumor cells, hence increasing CAR-T cells antitumoral activity (91). In details, in this approach an engineered OV delivers a transgene leading to the expression of a truncated form of CD19 (CD19t) on the neoplastic cells, promoting the cytotoxic activity of CD19-CAR-T cells.

Lastly, reduced T-Cell trafficking and homing in the TME was underlined as one of the major obstacles of CAR-T therapies. In particular, reduced production of chemokines and modification of Extra-Cellular Matrix (ECM) are involved in hindering the migration of T-cell to the tumor site (92). Interestingly, Jin et al. developed CAR-T cells expressing chemokines receptors (CXCR1 and CXCR2) to improve intratumoral trafficking (93). The results observed in xenograft models of HGG confirmed the efficacy of this approach, unveiling the importance of increased T-cells homing to improve CAR-T therapies efficacy. Moreover, the CNS location adds a relevant and peculiar obstacle to the migration of CAR T cells to tumor: the presence of the BBB, a permeability barrier characterized by the connection, through tight junctions, of endothelial cells with the luminal and abluminal membranes lining the capillaries of the brain. Despite the documented ability of i.v. administered CAR T-cells to cross the BBB, as already mentioned, targeted delivery of T cells at the level of the CNS is an attractive option to reduce systemic toxicity and increase CAR T-cell concentration at tumor site. Indeed, as mentioned above, a superior efficacy with reduced toxicity of intraventricular/intrathecal administration of CAR-T cells was already shown (23, 29) and the strategy represents a valid and promising approach to circumvent the BBB obstacle.

Despite all the presented approaches show great potential to improve CAR-T cell function and safety in preclinical models, their use in clinical setting is limited at present. The results of future clinical trials will shed new lights on potential and limitations of these highly innovative approaches.

CONCLUSIONS AND FUTURE STRATEGIES

Conventional therapies, radiation and chemotherapy are not sufficient for achieving a sustained disease remission in patients affected by HGG, both in adult that pediatric patients, and new therapeutic strategies are necessary. Immunotherapy is a new therapeutic approach that harnesses the inherent activity of the immune system to control and eliminate malignant cells. To date, CAR-T cell therapy has shown promise in early clinical trials in HGG patients but could not achieve the same sustained success observed in hematological malignancies. Several hurdles, including the immunosuppressive TME, the heterogeneity in target antigen expression and the difficulty of accessing the tumor site, impair the antitumor efficacy of CAR-T cells. Several CAR-T antigenic targets have been considered so far, and recently GD2 and B7-H3 look very promising for pediatric tumors. However, heterogeneity of expression is a limiting factor for single antigen-redirected CAR-T in solid tumors. For this reason, considering innovative strategies such as next generation CAR-T cells or combinatory approaches with other immunotherapy agents (e.g. BiTEs, Oncolytic viruses and ICIs) could improve tumor control. "Next generation" or multivalent CAR-T have been developed and might have a large impact on treatment of pHGG, improving efficacy of T cells therapies and overcoming obstacles of TME.

AUTHOR CONTRIBUTIONS

LA, GC, and FB conceived the article. LA and GC compiled the review and prepared the draft of the manuscript. FB, AM, AC, and GB critically reviewed the manuscript. FB edited the manuscript. All authors contributed to the article and approved the submitted version.

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The Past, Present, and Future of Non-Viral CAR T Cells

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Adoptive transfer of chimeric antigen receptor (CAR) T lymphocytes is a powerful technology that has revolutionized the way we conceive immunotherapy. The impressive clinical results of complete and prolonged response in refractory and relapsed diseases have shifted the landscape of treatment for hematological malignancies, particularly those of lymphoid origin, and opens up new possibilities for the treatment of solid neoplasms. However, the widening use of cell therapy is hampered by the accessibility to viral vectors that are commonly used for T cell transfection. In the era of messenger RNA (mRNA) vaccines and CRISPR/Cas (clustered regularly interspaced short palindromic repeat–CRISPR-associated) precise genome editing, novel and virus-free methods for T cell engineering are emerging as a more versatile, flexible, and sustainable alternative for next-generation CAR T cell manufacturing. Here, we discuss how the use of non-viral vectors can address some of the limitations of the viral methods of gene transfer and allow us to deliver genetic information in a stable, effective and straightforward manner. In particular, we address the main transposon systems such as Sleeping Beauty (SB) and piggyBac (PB), the utilization of mRNA, and innovative approaches of nanotechnology like Lipid-based and Polymer-based DNA nanocarriers and nanovectors. We also describe the most relevant preclinical data that have recently led to the use of non-viral gene therapy in emerging clinical trials, and the related safety and efficacy aspects. We will also provide practical considerations for future trials to enable successful and safe cell therapy with non-viral methods for CAR T cell generation.

Keywords: non-viral vectors, chimeric antigen receptor (CAR T), gene therapy, immunotherapy, adoptive cell transfer, cancer therapy, transposons, mRNA

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1 INTRODUCTION

1.1 The Rise of CAR T Immunotherapy in Hematological Malignancies

Chimeric antigen receptor (CAR) T cell therapy represents a revolutionary therapeutic reality. To unleash T cells against cancer, an artificial receptor has been generated fusing the antigen-binding domain of a monoclonal antibody with a T-cell receptor (TCR)-derived signaling domain, including costimulatory components (1, 2). CAR-mediated recognition of a tumor-associated antigen triggers

the activation of engineered T cells that consequently exert a response, characterized by potent cytotoxicity, cytokine secretion, and proliferation. The possibility of combining T cell lymphocyte effector functions with antibody specificity in a single component is appealing because it allows for a T cell-mediated immune response against the tumor in a major histocompatibility complex (MHC)-unrestricted manner. This strategy eliminates the need of designing different receptors according to the Human Leukocyte Antigen (HLA) haplotypes, as in the case of tumor specific TCR gene transfer. This type of immunotherapy is a multi-step process. Immune cells, typically of autologous origin, are collected, modified in specialized laboratories, and then infused into the patient undergoing lymphodepleting therapy to increase engraftment. The early concept formulated more than 30 years ago went through an extensive series of costimulatory design optimizations, which coupled the CD3-ζ domain with CD28 or 4-1BB costimulation, leading to impressive clinical results in patients with high-risk hematological malignancies (3–8).

To date, the clinical application that has determined the success of CAR T cells has been conducted mainly targeting the CD19 and CD22 molecules in B cell-Acute Lymphoblastic Leukemia (B-ALL) and B cell lymphoma (9–12) and against B-Cell Maturation Antigen (BCMA) for multiple myeloma (13, 14). In B-ALL, adoptive immunotherapy with CAR T cells achieved more than 80% complete response (CR) in the early stages of treatment and a sustained response through the establishment of immunological memory with 12-month event-free survival rates of 50% (3, 15). In diffuse large B-cell lymphoma (DLBCL), the CR was between 40 and 60% in multiple studies with different CAR T cell products while 12-months progression-free survival (PFS) was 40% (10, 11, 16, 17). The results in Mantle Cell Lymphoma and Relapsed/Refractory (R/R) Follicular Lymphoma are even more encouraging with a CR of 67% and 80%, and a PFS of 61% and 74%, respectively (18, 19). Finally, in Multiple Myeloma (MM), the CR is 33% while the PFS is 8.8 months (20). In light of these data (21), the U.S Food and Drug Administration (FDA) has approved five CAR T cell therapies, Abecma (idecabtagene vicleucel), Breyanzi (lisocabtagene maraleucel), Kymriah (tisagenlecleucel), Tecartus (brexucabtagene autoleucel), Yescarta (axicabtagene ciloleucel) as of January 2022. All of them are also authorized in Europe. Besides these five, the FDA recently approved ciltacabtagene autoleucel (CARVYKTI, cilta-cel, Janssen and Johnson & Johnson), a CAR T product direct against BCMA. Furthermore, China's National Medical Products Administration (NMPA) recently approved the autologous anti-CD19 CAR T cell product, relmacabtagene autoleucel, that was established based on a process platform of Juno Therapeutics. Progress has been made to implement CAR T cell therapies in Australia, China, Japan, Switzerland, Singapore and Canada and the approval status worldwide is summarized in **Table 1** and **Figure 1**. Kymriah and Yescarta have been commercially available since 2017 and 2018, respectively, and have been infused into nearly half a million patients worldwide.

1.2 Need for a More Flexible System to Allow Future CAR T Cell Engineering

Having shown such high therapeutic efficacy in hematological malignancies, the field is moving surprisingly fast, facing new challenges, and approaching other kinds of applications. Despite the high CR rate in hematological malignancies, patients with a high tumor burden and characterized by a history of multiple prior lines of therapy often do not respond. In some cases, the achieved responses do not last long. Relapses are mainly associated with loss of functional CAR T cells or the appearance of relapses in which the antigen recognized by the

TABLE 1 | Commercial CAR T products and their indication and availability worldwide.

Active substance	Name	Indications	Manufacturer	Approvals	Target	Costimulatory domain
tisagenlecleucel	Kymriah	Pediatric and young adult R/R acute lymphoblastic leukemia; Adult R/R DLBCL; R/R follicular lymphoma	Novartis	FDA, EMA, Health Canada, Swissmedic, Japan's MHLW, Singapore's HSA, Australian TGA, UK's NICE	CD19	CD137
axicabtagene ciloleucel	Yescarta	R/R large B-cell lymphoma (DLBCL, PMBCL, high grade B-cell lymphoma, DLBCL arising from FL)	Kite Pharma and Gilead	FDA, EMA, Health Canada, Swissmedic, Japan's MHLW, China's NMPA, Australian TGA, UK's NICE	CD19	CD28
brexucabtagene autoleucel	Tecartus	Mantle cell lymphoma; Adult lymphoblastic leukemia	Kite Pharma and Gilead	FDA, EMA, Swissmedic, UK's NICE, Health Canada	CD19	CD28
lisocabtagene maraleuecel	Breyanzi	R/R large B-cell lymphoma	BMS and Juno Therapeutics	FDA, Japan's MHLW, EMA	CD19	CD137
idecabtagene vicleucel	Abecma	Multiple myeloma	BMS and Bluebird Bio	FDA, EMA, Health Canada, Swissmedic, Japan	BCMA	CD137
ciltacabtagene autoleucel	CARVYKTI	Multiple myeloma	Janssen and Johnson & Johnson	FDA	BCMA	CD137
relmacabtagene autoleucel	Carteyva	R/R large B-cell lymphoma	JW Therapeutics	China's NMPA	CD19	CD137

MHLW, Ministry of Health, Labor and Welfare; HAS, Health Sciences Authority; TGA, Therapeutic Goods Administration; NMPA, National Medical Products Administration; NICE The National Institute for Health and Care Excellence.

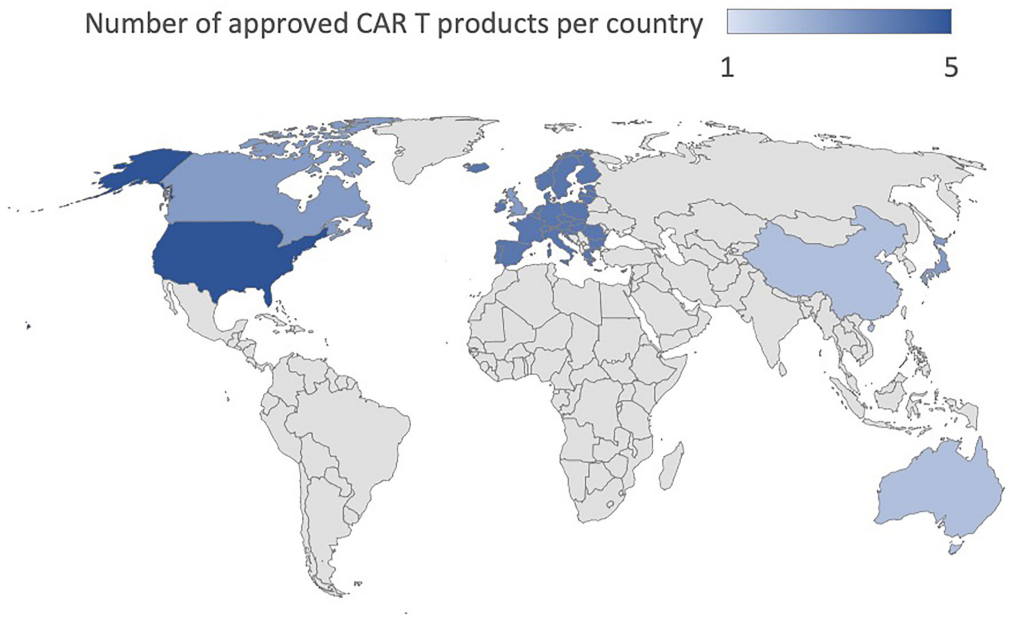

FIGURE 1 | Worldwide approval status of CAR T cell drugs.

CAR has a decreased expression or is completely absent, as in the case of CD19-negative relapses. For solid tumors, treatment with CAR T cells have not yet proven to be efficacious and it remains a challenge as of today. Few antigens with restricted expression to solid cancer and non-vital organs have been identified so far. Homing to the tumor is a critical aspect because T cells must migrate from the bloodstream through the endothelial cells that make up the tumor vasculature. In addition, the tumor microenvironment often has an immunosuppressive and hypoxic environment that impacts on T cell persistence by inducing a hypofunctional state. Unfortunately, clinical studies in solid tumors demonstrate a severely limited response. With this in mind, it is increasingly becoming urgent to combine CAR weapons with multiple targeting options and different functionalities, i.e., *de novo* cytokine production, activating signaling molecules and pro-inflammatory ligands, checkpoint blockages, increased trafficking with chemokines, receptors and extracellular matrix degrading enzyme, safety switches. Some of the issues associated to the current CAR T cell design that we need to face in the future are summarized in (22).

Addressing such challenges requires the development of approaches that move beyond single-target immunotherapy towards a building-block concept à la Lego or Minecraft, the popular video game that allows for endless combination opportunity. T cells can be modified to express CARs with different specificities and can therefore be equipped to improved efficacy, safety, and applicability. So far, the way most CAR T cell therapies approved or investigated in clinical trials are produced is utilizing viral vectors, particularly gammaretroviral and lentiviral vectors. Viral vectors are standardized systems with efficient gene transfer and a long-term history of application that demonstrates safety in the context of adoptive T cell therapy (23). However, the ability of viral vectors to transduce long gene cassettes is constrained by the capsid dimension. Viral capsids are about 100 nm in diameter and often cannot fit more than 8-9 kb (24). The use of two separate vectors for delivery of two different transgenes is often not efficient. Furthermore, viral production for the clinical application is a process that generally takes two-three weeks and is performed under good manufacturing practice (GMP) conditions in biosafety level 2 (BSL2) facilities, needing trained staff resources. The resulting high costs, limited number of available manufacturing facilities globally, and lot size limitations complicate their accessibility. This complexity along with the need for personalized treatment ultimately impacts the final price of CAR T product, which is particularly high and can reach up to $475,000 per person to which must be added the cost of hospitalization, and follow-up visits. Currently, CAR T therapies are often recommended for late-stage patients who have exhausted all other treatment options. Given recent data supporting the advancement of CAR T cell therapy to earlier lines of treatment (25, 26), it appears to be increasingly important to implement reductions in current spending.

For the future to come, genetic engineering technologies must address issues such as logistical complexities impacting on costs and availability, cargo limitations, and flexibility. We therefore need to take measures to mitigate these challenges and start using more versatile and flexible technologies to make CAR T cells capable of migrating to the tumor site, recognizing heterogeneous tumors, and surviving in hostile environments. To support the adoption of future CAR T cell therapies, non-viral vectors have been proposed, validated preclinically in their ability to generate functional CAR T cells, and more recently applied in pioneer clinical trials. Non-viral gene transfer allows

for an easier manufacturing process with lower costs of goods and rapid availability and may have less constraint on cargo capacity.

1.3 Stable Gene Transfer: Viral Transduction vs. Non-Viral Transfection

Gene therapy is an advanced medicine application in which the delivery of genetic material into cells is exploited to confer additional or restore impaired features to treat patients with a wide range of diseases, including genetic disorders, cancer, infectious and immunologic diseases, resulting in long-term therapeutic effects. Gene transfer can be classified as stable or transient depending on whether the genetic material is integrated into the host cell genome. To achieve stable gene transfer, integration of an expression cassette consisting of promoter, leader, transgene and transcriptional termination and polyadenylation sequences is needed. When there is integration into the genome, the transgene is stably expressed, and expression of the inserted gene persists in daughter cells resulting from cell division. In contrast, in the absence of integration, as for mRNA and plasmid vectors, expression will be lost as cells divide. Adoptive cell therapy with CAR T cells utilize *ex vivo* gene therapy that predominantly uses stable gene transfer. Gene transfer can be achieved through the process of viral transduction that utilizes the inherent ability of viruses and viral vectors to introduce genetic material into a variety of cell types. Alternatively, introduction of naked nucleic acids, including supercoiled DNA, messenger RNA (mRNA), small interfering RNA (siRNA), and guide RNA (gRNA), can be achieved by non-viral transfection. Transfection relies on the formation of transient pores in the cell membrane or, alternatively, endocytosis through the use of different chemical or physical techniques, such as electroporation, liposomes, and nanoparticles.

1.3.1 Viral Transduction

In the case of retroviral vectors, transduction requires the formation of infectious particles containing the transfer plasmid encoding the transgene flanked by Long Terminal Repeat (LTRs) and including the ψ (psi) encapsidation signal. Generally, infectious particles are generated by introducing the necessary viral sequences, i.e. *gal-pol, rev, env* coding sequences into the producer cell line by means of separated plasmids. Separation of the sequences required for virus formation allows the generation of a replication-deficient virus that is capable of infecting mammalian cells and integrate the genetic materials into the cellular genome but does not retain the natural ability to generate new viruses. Integration of DNA into the genome allows stable transduction of the T cell clone and its lineage, leading to long-term expression of the transgene in cells capable of long-term survival, and thus making CAR T cells living drugs.

The mechanism of integration of the cassette into the genome relies on the action of reverse transcriptase and integrase, encoded by the *pol* gene. Gammaretroviral vectors derived from Moloney murine leukemia virus (MLV) vectors integrate preferentially near transcription start sites (TSS) and in transcriptional regulatory regions, whereas Human Immunodeficiency Virus (HIV)-derived lentiviral vectors have a bias towards transcriptionally active regions (27–29). Integration of a transgene into the genome carries with it the risk of insertional oncogenesis, which is closely related to the propensity of each vector for a particular integration profile. In the case of gammaretroviral vectors there is thus a higher likelihood of inducing aberrant gene expression, which can result in the activation of oncogenes, whereas lentiviruses potentially have a greater risk of disrupting gene expression or leading to the expression of gene fragments that could theoretically lead to tumor-suppressor gene inactivation. However, this is particularly relevant in gene therapy applied to hematopoietic stem and progenitor cells (HSPC) (30, 31), while T cells have been considered to have a low susceptibility to transformation. Indeed, long-term safety has been demonstrated after viral transfection (23). No T cell transformation has been observed even in cases of gammaretroviral vector insertion into an oncogene, such as *Cbl*, and destruction of a tumor suppressor gene such as Tet Methylcytosine dioxygenase 2 (*TET2*) by lentiviral vector integration, as has been reported in patients treated with anti-CD19 and anti-CD22 CAR T cells (32, 33).

The two vectors also differ in the mode of infection, which also has practical implications. Gammaretroviral vectors can only infect cells with active cell division, whereas lentiviral vectors are able to transduce non-dividing as well as the dividing cells, but most current protocols activate T cells prior to transduction (34). In addition, lentiviral genomes are more complex than those of gammaretroviruses, making LV production more complicated. Both viral vectors suffer from a number of disadvantages as gene transfer vectors, including i. limited insert size, ii. difficulty in producing high titers of stable vector particles, iii. potential generation of replication competent retroviruses/lentiviruses (RCR/RCL) during production, and iiii. *in vivo* recombination with sequences from other viruses, such as post HIV infection (35, 36). The generation of RCR/RCL *in vitro* or *in vivo* is currently only a theoretical risk, as there have been no cases of recombination in cellular products or in patients treated with *ex vivo* gene therapy to date. Finally, viral vectors have an intrinsic risk of immunogenicity, caused by humoral and cellular immune response towards vector-encoded epitopes, which might limit the efficacy and persistence of transduced cells (37).

1.3.2 Non-Viral Transfection

Stable gene transfer delivery can also be achieved by using the non-viral integrative vectors represented by transposons. In this case, integration is obtained by means of transposase, an enzyme that binds to sequences in the genome called transposons and catalyzes their movement by a cut-and-paste or a replicative transposition mechanism. The existence of mobile sequences in the genome was originally discovered by the Nobel Prize-winning geneticist Barbara McClintock in the 1940s while studying kernel color variability in maize (38). The repositioning of genes encoding for pigments resulted in a variety of coloration patterns. The "jumping genes" in maize were then called transposable elements (TE), or transposons, and

we now know that they are quite abundant in the genome, constituting more than 80% of the maize genome and about 40% of the human's one, meaning that around 40% of the human genome has undergone the process of transposition over the course of human evolution (39). Transposition is known to cause genetic diversity and adaptability, such as color change in maize or antibiotic resistance in bacteria. This is likely the reason why genes encoding for transposases are widely distributed in the genome of most organisms (40).This class of genes belongs to the superfamily of polynucleotidyl transferases that comprises RNase H, Recombination-activating gene (RAG) proteins, and retroviral integrases. Indeed, RAG enzymes have been proposed to originate from TEs and have a pretty similar mechanistic features (41), that allow them to alter gene structure as in V(D) J rearrangements.

TEs are divided into two classes of TE, retrotransposons and DNA transposons. Retrotransposons move through a copy-and-paste mechanism using an RNA intermediate, represent the most frequent class of transposons in the human genome, and comprise Long Terminal Repeat (LTR) transposons, long interspersed nuclear elements (LINEs), and short interspersed nuclear elements (SINEs) (42). DNA transposable elements move through a DNA intermediate *via* a cut-and-paste mechanism and are the ones used in gene transfer applications. Most DNA transposon families have an element encoding a transposase gene flanked by inverted terminal repeats (ITRs). Transposase recognizes and binds elements incorporated into ITRs, catalyzes the excision of the transposon element from its original position, and integrates it into another position in the genome. The DNA sequence is inserted without the need for sequence homology. Transposon-based vector systems have been generated by splitting the transposase and the ITRs into two components, so that the transgene cassette lies between the two ITRs in a transposon vector. Throughout the next section, we will focus on the different transposons available for clinical applications, with an emphasis on the most widely used transposon systems, Sleeping Beauty (SB) and piggyBac (PB). Transposase is delivered in a 'trans' configuration to better control the system and avoid residual expression, which could then potentially lead to remobilization of the transposon into other genomic compartments which is currently the most prominent safety concern of this type of vector. One of the possibilities to deliver transposon and transposase to generate CAR T cells is through the use of a dual plasmid system, one for the gene of interest and the other for the enzyme, by electroporation of primary T cells, but has some limitations that can be solved by using mRNA and DNA vectors with decreased size compared to conventional plasmids. Anyway, these two-component vector systems are less complex than viral vectors and relies on relatively low costs of goods. Plasmids can be produced in very large quantities, so that the estimated costs are 5 to 10 times lower than the viral process (43). Compared to other vectors, they have a larger cargo size which is particularly relevant for future multi-targeting applications. The integration profile of transposon vectors depends on the transposase used, with some showing a bias towards specific regions, as in viral vectors, while others demonstrate a close-to-random and safer profile with no preference, and we will see how relevant this is in the next subsection. Unlike viral vectors, transfection with transposon systems works well in both pre-activated and resting primary T cells, leading to transgene expression even in naïve cells (44). The ability of transposons to transfect non-dividing and naïve cells might be exploited to increase the persistence of CAR T cells *in vivo*. In contrast to viral systems, which have weak preferences at the site of integration in terms of DNA sequence recognition, transposons recognize a consensus sequence (45).

Non-viral transposon vectors prove versatility, low immunogenicity, and ease of production. However, they are often associated with lower transfection efficiencies than viruses. This may be in part due to the toxicity associated with electroporation in the presence of DNA. Though, there could also be reasons related to the type of gene material, viral material being generally more efficient than plasmid DNA, and the integration pattern itself. Finally, the possibility of transposon vectors interacting with endogenous human DNA and protein sequences is a theoretical safety concern. Fortunately, mammals do not contain transposon DNA sequences sufficiently similar to be cleaved by SB transposase, and there is no human protein sufficiently similar to SB transposase to re-mobilize a SB vector integrated into the genome. Instead, the human *PGBD5* gene, apparently derived from PB transposases, has been shown to encode for a transposase capable of mobilizing insect PB transposons in human cell cultures (46). It remains unclear whether cross-reactions between the endogenous human transposase and the PB transposon vector can occur and undermine the genomic integrity of the transduced cells, raising a potential risk in the context of using this vector for genetic engineering (47).

Non-viral delivery of mRNA allows for transient transfection and is generally achieved by electroporation or nanoparticles (48). Once into the cell and without the need to reach the cell nucleus, the mRNA is translated into the encoded protein that can be stabilized when prolonged expression is needed and is generally lost after 2-4 cell divisions, which is why this technique is particularly suited to applications using non-proliferating cells. Along with safety, the main advantage of this approach is the availability of protocols for clinical translation of mRNA strategies, thanks in part to SARS-CoV-2 vaccine research. As of March 30, 2022, 64,4% of the worldwide population (49, 50), including the authors of this paper, have received at least one dose of Covid-19 vaccine and most of them thanks to advancements in nucleic acid delivery protocols. The lack of integration avoids the risk of genotoxicity associated with integrating vectors and transient expression safeguards against long-term toxicities, making this strategy a good approach to test the safety of first-in-human CARs, targeting molecules with expression in healthy tissues. The drawback of using transient gene transfer is the short-term potency that is counterproductive in strategies such as CAR T cell immunotherapy whose benefits are mainly associated with rapid *in vivo* expansion and generation of T cell memory and immunosurveillance.

Conversely, mRNA delivery is a versatile, flexible, and safe means for all technologies involving a hit-and-run mechanism that requires only transient expression, such as for nuclease complex in gene editing, epitopes in vaccination, and transposase in stable nonviral gene transfer.

2 PURPOSE OF THE REVIEW: NON-VIRAL APPROACHES FOR T CELL ENGINEERING

Despite great enthusiasm followed the approval by FDA of CAR T cell products on the market, confounding challenges persist for products based on *ex vivo* lentiviral or gammaretroviral transduction. Since their introduction, transposon-based platforms seem to represent a feasible, cheaper and useful alternative to mediate gene transfer, and pioneer clinical studies are currently emerging. We aim here to explore the world of non-viral vectors navigating through their advantages and drawbacks. In the next paragraphs, we will be reviewing preclinical and clinical applications of the SB and PB transposons, the utilization of the mRNA, and the modalities to deliver non-viral vector into the cellular nucleus, such as electroporation and nanocarriers. We will then be discussing the critical aspects related to the safety and efficacy, with the intention to provide practical considerations for exploiting these tools in future clinical studies. Finally, we provide our vision for future gene therapy with the advent of novel challenges, such as multi-targeting design, but also innovative tools, including DNA nanovectors and improved gene-editing technologies. From this perspective, technologies such as CRISPR/Cas9 are expanding the possibilities available in the field of adoptive T cell therapy as reviewed in (51). Their application in combination with viral techniques falls beyond the scope of this review, whereas we will discuss the virus-free CRISPR-Cas9 approach in the session related to future directions. We are encouraged by the prospect of non-viral vectors simplifying the CAR T supply chain, making it less expensive, safer, and efficacious.

3 SLEEPING BEAUTY

3.1 Vector Design and Delivery

Awakened after a long evolutionary "sleep", SB was reconstructed from inactive transposon sequences present in fish genomes, becoming the first transposon to show activity in vertebrate cells (52), thereby leading new horizons for gene therapy [reviewed in (53–61)]. Based on classical Tc1/mariner DNA Class II TE, these "jumping" units are able to translocate from one genomic position to another through a cut-and-paste mechanism (62). The SB vector is constituted by two functional components: the transposon DNA, which carries the gene of interest flanked by ITRs, and the SB transposase, which recognizes the ITR sequences and mobilizes the transgene from the donor DNA to an acceptor site inside the genome (63, 64).

During the years, many attempts have been made to improve the design of the SB vectors, leading to the generation of several variants. Regarding the original transposon vector, referred to as pT, the modification of nucleotide residues within the ITR sequences by means of mutations, additions or deletions have give rise to in improved versions such as pT2, pT3, pT2B, and, lastly, pT4 (65, 66), which has an optimized donor vector architecture. Similarly, transposase has also been extensively optimized to increase the transposition efficiency. The first SB10 transposase has passed through different mutagenized hyperactive versions including the second-generation SB11 transposase, approximately threefold more active than the first-generation SB transposase, to the more recent SB100X, holding 100-fold increase activity than the first-generation enzyme (67). The hyperactive SB100X system has shown to allow for efficient and stable gene transfer in various cell types, including primary human T cells (68), in a non-homologous recombination restriction manner. Identification of the crystal structure of the transposase catalytic domain has recently allowed the design of hyperactive transposase variants, including the SB transposase mutant (I212S), named hySB100X, which has 30% higher transposition activity than SB100X (69). The enzyme can also be modified to catalyze the excision but not the genomic re-introduction, leading to extrachromosomal circles similar to the excision circles formed during the process of VDJ recombination. The exc+/int- mutant can be exploited for transient transgenesis, e.g. to remove reprogramming factors after generation of pluripotent stem cells (69). Similar to what have been implemented for the CRISPR/Cas9 systems, attempts have been made to deliver the SB100X as a protein. However, the SB transposases showed intrinsic protein instability, associated with low solubility as well as aggregating properties. For this reason, efforts to improve their chemical properties have led to the generation of a new highly soluble variant (hsSB), including the C176S and I212S substitutions, which has shown high self-penetrating properties (70). The efficiency of this new type of SB transposase was tested in human and mammalian cells such as stem cells, both of embryonic and hematopoietic origin, induced pluripotent stem cells (iPSCs), and primary cells such as human T cells. hsSB was able to generate anti-CD19 CAR T cells, even though with a lower transduction efficiency, displaying antitumor activity analogous to CAR T cells engineered with viral vectors in xenograft mice (71).

The integration of the excised transposon takes place in a close-to-random manner inside the genome when the transposase finds a target site characterized by a TA dinucleotide (72) as illustrated in **Figure 2**. When the transposase recognizes the ITR sequences flanking the SB donor transposon and binds them, it induces double-stranded breaks through the formation of a synaptic complex. The resulting excision site is rapidly repaired by host non-homologous end joining (NHEJ) and the terminal sequences of the SB transposon that are formed after the cleavage generate a

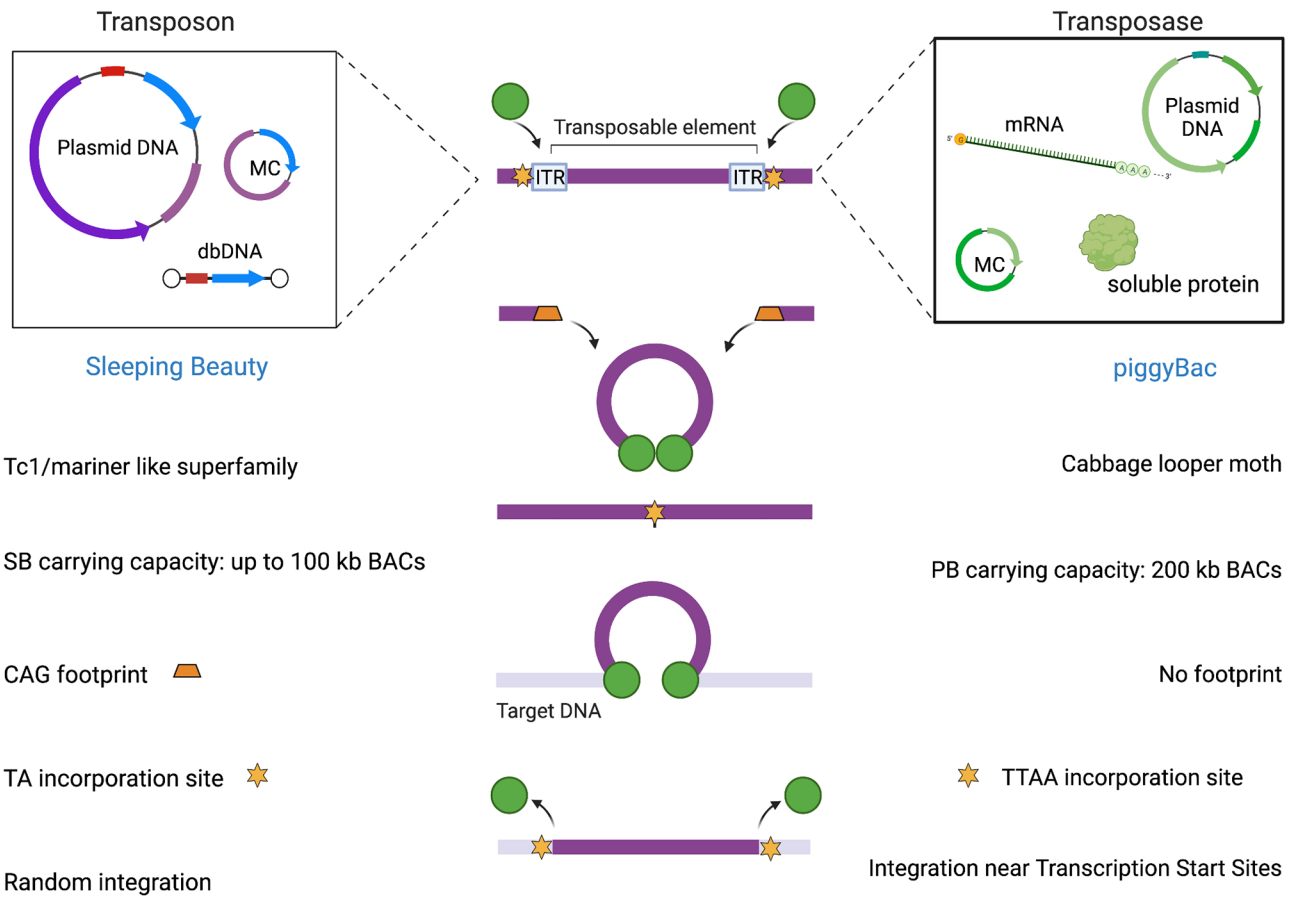

FIGURE 2 | SB and PB mediated integration.

characteristic footprint in the donor DNA. At this point, the transposon-transposase complex is free to find an appropriate target site in the genome and integrate inside, leading to target site duplication flanking the integrated element (73).

The peculiar integration bias has been deeply investigated through genome-wide integration analyses comparing SB with PB and Tol2 (74). The target site selection of SB, PB, MLV-derived gammaretroviral and HIV-derived lentiviral systems was compared in primary human CD4+ T cells. SB transposons demonstrated to have the highest probability to target safe harbors thanks to its unbiased, close-to-random integration profile as compared to other methods which instead showed a bias for transcriptional start sites, CpG islands and DNaseI hypersensitive sites (45). Therefore, the mechanistic features of SB make it a vehicle with a favorable risk-benefit assessment (45).

A big advantage of this type of strategy compared to viral systems is the greater cargo capacity, though there is an inverse correlation between the size of the insert and the efficiency of the transposition mechanism (75, 76). Optimal cargo size is under 6 kb but the sandwich version, comprising two complete transposon units flanking the cargo in an inverted orientation, favors an increase in load up to 11 kb thereby extending cloning capability of the SB based vectors (77, 78). Moreover, when combined with bacterial artificial chromosome (BACs), SB was shown to deliver transgenes up to 100 kb at reasonable efficiencies in human embryonic stem (ES) cells (79, 80).

Many attempts have been made to manage toxicity caused by the electroporation process. Since the damage is dependent on the amount of DNA delivered and the magnitude of voltage pulses in the electroporation process, the toxicity can be relieved by reducing the size of the SB vector and delivering the transposase in other forms than DNA plasmid. In this context, recent applications foresee the use of the SB transposase in the form of mRNA or recombinant protein and a minicircle vector (MC) encoding the transposon (71, 81). The transposase mRNA results in increased biosafety due to the fact that mRNA does not run the risk of chromosomal integration and allows transient expression of the enzyme. This aspect will be dealt with in more detail in paragraph 5.2. MCs are produced from plasmids through site-specific recombination to eliminate bacterial origin of replication and antibiotic resistance genes and retain exclusively the transgene with its promoter. The presence of antibiotic resistance gene in plasmids as a selection marker represent a safety concern for the risk of horizontal dissemination into pathogenic bacteria. MCs have a

better transposition rate with a gene transfer 5-fold higher compared to conventional plasmids and additionally a lower toxicity (82). The use of bacteria plasmids can trigger an immune response by the host caused by the activation *via* Toll-like receptors by the unmethylated CpG motif in the bacterial DNA sequence. The lack of bacterial sequences inside MCs favors their lower immunogenicity (83). Unfortunately, there are currently no commercial large-scale GMP producers of MCs, hindering commercialization.

3.2 Preclinical Evidence

The ability of SB vectors to provide long-term expression *in vivo* has been demonstrated in multiple preclinical studies spanning a wide range of fields, ranging from the application in cancer to multiple diseases, reviewed by Hodge and colleagues (84).

The first representative studies that demonstrated the feasibility of SB technology to generate anti-CD19 CAR T cells from peripheral blood or cord blood were reported by Dr. Huang at the University of Minnesota, and Dr. Cooper at the MD Anderson Cancer Center (MDACC, Houston, TX, USA) (85, 86). They showed that SB transposase can be delivered either as plasmid DNA or mRNA in combination with a CAR-encoding transposon plasmid into T cells by electroporation to produce functional anti-CD19 CAR T cells. To achieve a high transduction of the final cell product, electroporated T cells were expanded by multiple stimulations with CD19+ artificial antigen-presenting cells (APCs), resulting in rapid outgrowth of CAR expressing T cells.

Our group developed a clinical-grade protocol to engineered T cells differentiated towards memory T cells with a CD8+CD56+ phenotype *in vitro*, namely cytokine-induced killer (CIK) cells with different CAR molecules (87), including the anti-CD19 CAR, anti-CD123 CAR, anti-BAFFR CAR, and anti-CD33 CAR. We transfected CIK cells with the SB11 transposase and the pT vector (44, 87). The choice of the T cell population was based on the high safety profile with minimal occurrence of graft-versus-host disease (GvHD) (88), that allows the use of donor-derived cells in clinical trials (89–91). In order to mitigate cell damage induced by electroporation, we developed an improved platform for SB-mediated engineering by stimulating electroporated T cells with irradiated autologous PBMCs as feeder cells. A single stimulation step allowed us to achieve a sufficient number of CAR T cells for clinical applications and up to 80% transgene expression in CIK cells as well as conventional T cells (44, 87). The key benefit of our methods is the limited manipulation, avoiding multiple stimulations. We confirmed the close-to-random distribution of integrations in engineered CAR T cells and the absence of integration near cancer related genes (87). Adoptive transfer of anti-CD19 CAR or anti-CD123 CAR lymphocytes led to a significant anti-tumor response in B-ALL and acute myeloid leukemia (AML) disseminated disease models, respectively. The preclinical evaluation phase demonstrated the possibility of generating with this platform CAR T cells characterized by a dose-dependent therapeutic effect in patient-derived xenograft models, in the absence of toxicity, through a robust and reproducible production process (44). Recently, the platform was applied to generate anti-CD33 CAR T cells by using the hyperactive SB100X transposase and the pT4 vector, which showed improved transduction efficiency compared to SB11 and pT systems and *in vivo* activity toward chemotherapy resistant/residual AML cells (92).

Another example of enhanced SB-mediated engineering is the one proposed by Monjezi et al. using SB100X and a pT2-based MCs. The author stimulated T cells with anti-CD3/CD28 beads before electroporation and the resulting transduction efficiency was about 30%. Prior to functional testing, EGFRt-positive CAR T cells were purified and expanded with irradiated CD19+ feeder cells. The resulting anti-CD19 CAR T cells have potent anti-tumor responses and was shown to be equally functional as anti-CD19 CAR T cells prepared by lentiviral transduction *in vitro* and *in vivo* (81).

In recent years, more and more functional advancements are taking place to reduce *ex vivo* manipulation of CAR T cells in order to preserve their persistence and anti-tumor activity (93). One of these efforts is represented by the study of Chicaybam and collaborators, demonstrating the possibility of generating SB-engineered anti-CD19 CAR T cells in 8 days activating with Transact (Miltenyi Biotec) after electroporation. The resulting cell populations exhibit robust antileukemic activity both *in vitro* and *in vivo* associated with a central memory phenotype (94). The same group demonstrated that CAR T cells can be generated by SB and used without the need of stimulation and expansion. Similar *in vivo* activity was demonstrated by CAR T cells injected 24 hours after electroporation and cells expanded with anti-CD3/CD28 coated beads for 8 days (95). This point-of-care technology can even be optimized by co-expression of a safety switch and a membrane-bound version of interleukin-15 (mbIL15) to enhance safety and *in vivo* persistence and demonstrated anti-tumor activity against CD19+ tumors and prolonged T cell survival in mouse models (96).

This approach has been utilized for the development of UltraCAR T platform based on the use of the non-viral system to deliver multiple genes by SB vectors. Using this platform, Chan et al., developed autologous cells co-expressing a CD33 CAR and mbIL15 (PRGN-3006) for the treatment of r/r AML and high-risk myelodysplastic syndrome (MDS). This platform shortened the manufacturing process and allows infusion of the product the day after transduction, obviating the need for *ex vivo* cells expansion. In preclinical validation, *in vivo* administration of a single dose of PRGN-3006 UltraCAR T cells significantly improved the overall survival of AML-bearing mice compared to CAR T cells lacking mbIL15 (97). The same group developed a PRGN-3007 UltraCAR T co-expressing mbIL15, a CAR specific for receptor tyrosine kinase-like orphan receptor 1 (ROR1) that is frequently overexpressed in hematological and solid tumors, a safety switch, and a novel mechanism for intrinsic blockade of PD-1 gene expression. Notably, preclinical data demonstrated the safety and the improved anti-tumor activity of PRGN-3007 compared with the control ROR1 CAR T (98).

The optimized donor-vector architecture of the pT4 vector coupled to the use of the hyperactive SB100X allows the

generation CAR T cells engineered with bicistronic vectors. Using this platform, CAR T cells were incorporated with the inducible Caspase 9 (iC9) safety switch and showed anti-leukemic activity in mouse models and were as efficient as CAR T cells generated with a LV vector (99). Furthermore, this system was used to combine the expression of anti-CD33 CAR and the chemokine receptor CXCR4 to increase CARCIK cell homing to the bone marrow niche (100).

Recent evidence reveals the suitability of the SB vector to enable engineering of primary natural killer (NK) cells with anti-CD19 CAR, which showed a safe genomic integration profile and antitumor activity *in vivo* (101). Manufacturing protocols associated with preclinical studies employing SB in the context of CAR T cells are summarized in **Table 2**.

3.3 Clinical Applications

Following promising results obtained in the preclinical phase, the group of Cooper et al. contributed to the clinical debut of SB-engineered anti-CD19 CAR T cells and provided proof of concept of the convenience of SB transposition for CAR T cell engineering. Two pilot clinical trials (NCT00968760, NCT01497184) confirm the safety of SB-engineered anti-CD19 CAR T cells in 26 patients with B-ALL and non-Hodgkin's Lymphoma as adjuvant therapy after autologous or allogeneic hematopoietic stem cells transplant (HSCT) (103). Cell product manufacturing included T cell nucleofection with the transposase SB11 plasmid and pT2 vector encoding a second generation anti-CD19 CAR with CD28 as a costimulatory agent and *ex vivo* propagation for approximately 28 days with multiple stimulations using artificial APCs and cytokines. Patients were subsequently enrolled in a long-term follow-up study lasting up to 15 years and the persistence of genetically modified T cells was monitored annually using droplet digital polymerase chain reaction (ddPCR) and flow cytometry. Limited expansion and absence of B-cell aplasia were reported. However, CAR+ T cells were detected up to 4 years after infusion in autologous HSCT recipients and 2 years in allogeneic HSCT recipients (104).

TABLE 2 | Manufacturing protocols associated with preclinical studies employing SB in the context of CAR T cells.

Background	Description	Vector	Electroporation	Stimulation	Transduction and Yield	Reference
B-ALL and AML	Anti-CD19 CAR-T cells showed proof-of-concept tumor eradication in B-ALL xenograft models; anti-CD123 CAR T cells controlled KG-1 AML in xenograft models	CD19CAR/pT MNDU3 transposon (15 µg) + pCMV-SB11 transposase (5 µg)	10^7 peripheral blood mononuclear cells (PBMCs) using 4D-Nucleofector system (Program EO-115) with P3 Primary Cell 4D-Nucleofector X kit (Lonza)	Autologous PBMCs irradiated with 60Gy γ-rays are added after electroporation and OKT-3 is added at day 1. IL-2 is added weekly.	CAR expression: 75.6% for CD123.CAR and 80% for CD19.CAR Large scale T cell expansion: 23-fold in 18-21 days CAR T cell expansion: from 60×10^6 to 8.6×10^8	Magnani et al; 2018 (44)
AML	Anti-CD33 CAR-T cells showed delaying AML progression in patient-derived xenograft models	CD33CAR/pT transposon (15 µg) + SB100X-pT4 transposase (0.5 µg)	As Magnani et al.;2016 (87)	As Magnani et al.;2016 (87)	CD33CAR expression: 63.7%T cell expansion: 38.8-fold after 3 weeks.	Rotiroti et al.;2020 (92)
Glioma	Production of EGFRvIII CART cells in two weeks showed superior therapeutic efficacy in mice bearing established orthotopic gliomas	EGFRvIIICAR pT/neo transposon (10 µg) + pCMV-SB11 transposase (5 µg)	20 × 10^6 PBMCs or T cells using Amaxa Nucleofector 2B (Lonza) with program U-014 in T-cell electroporation buffer (Lonza)	CAR T cells were stimulated with 100 Gy-irradiated EGFRvIII+ K562 ells in the presence of IL-21. After7 days, T cells were restimulated in the presence of IL-2 and IL-21.	CAR T cell (back calculated inferred numerical expansion): From 10^6 to around 5×10^7 in two weeks and 10^9 in 30 daysEGFRvIII CAR expression: around 90%	Caruso et al.;2019 (102)
CD19+ B-cell malignancies	Anti-CD19 CAR-T cells generated in 8 days showed effective antitumor response in mice xenografted with RS4;11 or Nalm-6 B-cell leukemias	pT3 19BBz CAR transposon (20 µg) + pCMV-SB100x transposase (1 µg)	10^7 PBMC using Amaxa Nucleofector 2B with program U-14	After electroporation cells are cultured with IL-2 and, 2h later, are activated with T Cell TransAct (Miltenyi Biotec)	Absolute number of T-cell expansion: from 10^7 to 3.6×10^7 after 8 days of cultureCAR expression range: 20.4%–37.3%	Chicaybam et al.;2020 (94)
Lymphoma	anti-CD19 CAR T cells engineered with MC SB vectors eradicated lymphoma cells in Raji xenograft model	pT2 CAR EGFRt MC DNA (1 µg) +pCMV-SB100X MC (1:1 ratio) or pCMV-SB100X mRNA (1:4 ratio)	T cells are activated with anti-CD3/CD28 beads (Thermo Fisher Scientific) and on day 2 electroporated (1×10^6 T cells) using 4D-Nucleofector	After electroporation T cells are propagated with IL-2. Prior to functional testing, EGFRt-positive T cells are enriched and expanded with irradiated CD19+ feeder cells for at least 7 days	CAR T cell expansion: From 1×10^6 to around 20×10^6 (MC-MC) and 12×10^6 (MC-mRNA) in 2 weeksCAR expression: 49.8% for MC-MC and about 40% for MC-mRNA on day14	Monetzi et al;2017 (81)

Since long manufacturing processes and multiple stimulations are known to impair T cell fitness, resulting in decreased efficacy *in vivo*, in collaboration with Ziopharm Oncology (Boston, MA), a second-generation approach was developed by reducing to 2 weeks the time require for coculture with feeder cells. A clinical trial was designed (NCT02529813) in which CAR T cells have been infused in combination with a Fludarabine- and Cyclophosphamide-based lymphodepletion regimen in adult and pediatric patients with active CD19+ malignancies (105). This trial aim is to provide data supporting a 3rd-generation point-of-care trial to very rapidly manufacture (< 2 days) anti-CD19 CAR T cells in absence of feeder cells (96).

Thanks to the in-house establishment of a clinical-grade platform to obtain non-viral CAR T cells in about 20 days (44), we designed a multicenter phase I/II trial in B-ALL patients relapsed after allogeneic HSCT (NCT03389035). Donor-derived anti-CD19 CAR T cells were generated by electroporation with the SB11 transposase-encoding plasmid and a transposon expressing a third-generation CAR and differentiation into CIK cells (CARCIK-CD19). Cells were manufactured from 50 mL of peripheral blood from the allo-transplant donor. A total of 21 patients, 4 children and 17 adults were lymphodepleted and treated with a single infusion of CARCIK-CD19 product. In most patients, potent CAR T cell expansion and long-term persistence were achieved, which was associated with anti-leukemic activity and induction of a sustained response. Moreover, integration site analysis performed on patients' peripheral blood demonstrated that SB integration pattern, with absence of preference for transcriptional start sites and promoters, is maintained after infusion. High polyclonal marking and population diversity confirmed the positive safety profile of the SB technology (43). Cytokine release syndrome (CRS) was observed in six patients and neurotoxicity in two patients while acute GvHD was never observed (106). As a reinforcement of the previously implemented study, a new trial in our centers that involves re-treatment of patients has recently begun and patient enrollment is currently underway.

Besides the reported trials, CAR T studies using the SB platform are currently underway in the USA and Europe.

Given the promising preclinical data of UltraCAR T cells, two clinical trials have been launched. Specifically, a Phase 1/1b first-in-human dose escalation/dose expansion study (NCT03927261) is evaluating the safety of PRGN-3006 UltraCAR T co-expressing an anti-CD33 CAR and mbIL15 in adult patients with r/r AML, hypomethylating agents (HMA) failure, high risk MDS and chronic myelomonocytic leukemia (CMML). Preliminary data showed that PRGN-3006 infusion was well tolerated and achieved a 50% response rate in patients treated with lymphodepletion, associated with CAR T cell expansion and persistence (107).

A second study (NCT03907527), evaluating the safety of PRGN-3005 UltraCAR T cells co-expressing an anti-MUC16 CAR, mbIL15 and a kill switch in the treatment of patients with platinum resistant ovarian cancer patients is ongoing (108).

The CARAMBA trial has been recently launched as a joint effort supported by an EU Horizon grant and is using mRNA encoding the hyperactive SB100X transposases in conjunction with CAR transposon supplied as an MC vector. In this Phase I/II clinical trial autologous anti-slam family member 7 (SLAMF7) CAR T cells are being used against MM to investigate the feasibility, safety, and anti-myeloma efficacy (109).

4 PIGGYBAC

4.1 Vector Design

PB was originally isolated from the cabbage looper moth *Trichoplusia ni* over 30 years ago and has been optimized over the years (110). As with the SB vector, the PB system is constituted by the PB transposase (PBase), in the form of mRNA or DNA, and a separate transfer plasmid carrying the desired genetic cargo (111). It belongs to the class of DNA transposases and to improve its transposition efficiency, the transposase has been optimized through random mutations resulting in useful variants such as the hyperactive version of PBase (hyPBase) (112). Another interesting variant is the excision competent/integration defective (exc+int-) PB transposase that allows transient transgenesis, by enabling excision in the absence of re-integration into the host genome. One potential application of exc+int- PBase would be the transient introduction of transcription factors for transgene-free iPSC production, the same as for the exc+/int- mutant of SB100X. The exc+int- PBase can be fused to zinc finger proteins binding to safe harbors to favor integration into to specific genomic regions (113).

The design of PB transposon vectors is characterized by a single open-reading frame (ORF) flanked by ITRs that in PB are characteristically asymmetric. The transposase recognizes ITRs flanking the transposon and catalyzes transgene excision and integration into genomic DNA by a cut-and-paste mechanism. Specifically, the transposition involves a series of hydrolysis and transesterification reactions with the generation of a DNA intermediate in which DNA hairpins provide exonuclease protection for the transposon ends. One of the peculiar features of PB is its specificity towards TTAA sites for integration in contrast to SB's preference for TA dinucleotides. Although PB can integrate into any TTAA target site, the epigenetic status may affect integration site preference of PB transposons. Another attractive characteristic of the PB transposase is the lack of a DNA footprint after its excision (see **Figure 2**). In contrast to the mobilization of other conventional DNA transposons like SB, which are associated with NHEJ of the donor DNA (114), PB does not require DNA synthesis. Indeed, as long as an active transposase persists in the cell, integrated transposons can be remobilized to new sites. In the event that the transposon has integrated into a gene, the footprints created at the excision site could produce undesirable mutations of the gene in which they were left. This feature is an advantage of the PB system from the safety perspective.

PB has a higher transposition activity for transposon mobilization than SB in mammalian cells (115), a larger cargo capacity (up to 14 kb) than viral vectors, and allows multiple transgene delivery through the design of multicistronic cassettes (116). Moreover, like gammaretrovirus, PB showed a preference for integration near TSSs, CpG islands and DNaseI hypersensitive sites, the consequence being that the risk of gene dysregulation is increased (73). Furthermore, analyzing integration sites occupied under the selective pressure provided in insertional mutagenesis (IM) screens, it has been demonstrated that PB compared to SB, is more prone to association with oncogenes (117).

Recently, the discovery in human genome of the human piggyBac transposable element derived 5 (PGBD5) has raised possible safety concerns in PB-gene transfer application (46, 118). Indeed, the presence of PGBD5 could allow the remobilization of PB transposons in human cells with a higher risk of genes dysregulation. With respect to this aspect, however, there is still considerable uncertainty. Beckermann T. M. et al., observed that transposition activity is probably restricted within species to cognate ITR sequences and in particular, PGBD5 appeared in their study, unable to bind, excise or integrate PB transposon in human cells (119).

Therefore, PB has a series of useful characteristics for genetic engineering: i. a higher transposition activity than SB, ii. its precise excision from an insertion site, restoring the site to its pre-transposon state without DNA footprint, iii. its wide range of applications such as mutagenesis, introduction of reprogramming factors to generate iPSCs, and gene transfer. For the purpose of the review, we focus on the application of PB transposon system in the CAR T production.

4.2 Preclinical Evidence

One of the most challenging issues using transposons is the toxicity of the transduction procedure. In particular, electroporation in the presence of exogenous DNA is toxic and decreases cell survival to less than 40% after 24h from transfection. To improve the efficiency of PB transfection, different approaches have been tested such as the addition of survival-promoting cytokines such as IL-7 or IL-15 that increase the frequency of gene expression and the ability of the transduced cells to expand. Alternatively, T cell expansion was stimulated by the use of feeder cells represented by autologous PBMCs or other sources such as the K562 cells, modified to express costimulatory molecules. Therefore, the quality of the final CAR T product depends on several factors that go from the construct characteristics (such as cargo size, costimulatory domains, spacers) to the manufacturing platform. Many preclinical studies exploited PB as a tool to generate CAR T cells for hematological malignancies and solid tumors.

One of the first pieces of evidence of the potential for the PB platform to stably transfect human T cells in cancer therapy were reported by Nakazawa Y. et al. They obtained stable gene expression in about 20% of primary T cells without selection, improved to 40% with the addition of IL-15 (120). In a subsequent study by the same group, a significant increase in CAR expression was achieved using irradiated activated T cells as feeders and alternative means of TCR stimulation using viral antigens instead of anti-CD3/CD28 mAbs. Efficiency was further improved by reducing the size of the CAR cassette with the elimination of the long IgG1.CH2CH3 spacer (121). With this approach, PB-generated anti-CD19 CAR T were used to treat B-ALL cells in the central nervous system (CNS) in a xenograft mouse model comparing intra-venous and intra-cerebroventricular delivery. Direct CNS delivery of CAR T cells resulted in eradication of B-ALL from the CNS without fatal adverse events, proving the activity of PB-generated CAR T cell *in vivo* and suggesting this strategy as a possible therapeutic approach for isolated or advanced CNS disease (122).

The use of feeders to support generation of CAR T electroporated with non-viral transposons appears to be useful also for PB. Similar to what we first described for generating CAR T cells with SB (123), irradiated autologous PBMCs have been used to efficiently produce CAR T cells (124). Although the generation of anti-CD19 CAR T cells with the PB transposon system was demonstrated to be efficient using feeders, first attempts showed poor *in vivo* activity due to the interactions between the CAR spacer and Fc gamma receptor-expressing cells. Optimization of the construct led to the generation of an anti-CD19 CAR lacking the spacer IgG1 Fc region which demonstrated superior efficacy in a murine B-ALL xenograft model. Moreover, the inclusion of 4-1BB costimulatory domain had greater efficacy *in vitro* and *in vivo* at lower CAR T cell doses than those with a CD28 costimulatory domain (111).

Most of the manufacturing protocols for viral CAR T production activate T cells with anti-CD3/CD28 stimulation and the addition of IL-2 during culture. This system may have some limitations when applied to cells electroporated with transposons, such as the expansion of non-transduced T cells and the enrichment of terminal effector T cells at the expense of the immature stages. An alternative approach is the activation of the CAR T receptor by its cognate ligand or specific anti-CAR antibody in the presence of IL-4 and IL-7, which led to selected expansion of functional anti-CD19 CAR T cells, resulting in 90% of CAR positive cells. Moreover, the addition of IL-21 to the IL-4 and IL-7 mixture improves the immunophenotype of CAR T cells with more represented immature stages with less expression of exhaustion molecules such as PD-1, LAG-3, and TIM-3 (125).

CAR-mediated stimulation is often required to obtain sufficient numbers of CAR+ cells. For instance, none of the previously reported methods, including HER2-expressing tumor cells, irradiated activated feeder T cells with anti-CD3/CD28 antibodies, and autologous irradiated PBMCs alone, was able to improve the expansion of anti-HER2 CAR T cells modified with PB. Conversely, stimulation with autologous PBMCs engineered with HER2 and costimulatory molecules such as CD80 and 4-1BBL enhanced the expansion of anti-HER2 CAR T cells modified with PB. At the end of the expansion, the cellular product was enriched in CAR T stem cell memory-like cells and exerts anti-leukemic activity *in vitro* and *in vivo* (126).

Another manufacturing platform developed to reduce T cell exhaustion applied PBMCs pulsed with a pool of viral peptides

and IL-7 and IL-15 in the first week, followed by stimulation on anti-CD3 or anti-CD28 mAbs-coated plates. With this protocol, in the setting of neuroblastoma, functional anti-GD2 CAR T cells were associated to low expression of PD-1 and improved naïve/stem cell memory phenotype. In addition, the authors suggested a possible synergistic effect of PB anti-GD2 CAR T cells and MEK inhibitors (i.e. trametinib) regardless of the mutation status of the MAPK pathway in tumor cells with an enhanced efficacy of CAR T therapy in the setting of neuroblastoma (127).

CAR T cells directed against granulocyte-macrophage colony-stimulating factor receptor (hGMR or CD116) generated with PB were used in a non-human primate (NHP) model to evaluate their safety (128). To generate cynomolgus macaque CAR T cells, electroporated PBMCs were cultured in the presence of human IL-15 and IL-7 with the addition of immature dendritic cells, derived from autologous cynomolgus PBMCs using human IL-4 and GM-CSF.

The anti-hGMR CAR T design has been further optimized by substitution of the antigen-binding domain with a mutated GM-CSF and CH2CH3 hinge with a G4S spacer and an improved anti-tumor activity against CD116+ AML was demonstrated both *in vivo* and *in vitro* (129).

Another issue with classic transposon transduction protocols is their reliance on bacteria for the production of plasmid vectors. To avoid undesired qualities of bacterial plasmids, including activation of host immune responses, antibiotic resistance, and endotoxins, CAR+ T cells were produced by co-electroporation of a linear DNA transposon and mRNA encoding the PB transposase, reaching a transfection efficiency of 60% and a vector copy number (VCN) of less than 3 copies of transgene per transduced cell. The linear vector was prepared enzymatically *in vitro* by PCR whereas mRNA was obtained through *in vitro* transcription. Electroporated cells were cultivated in presence of IL-4, IL-7, and IL-21 and maintained an early memory immunophenotype at the end of the differentiation (125). Similarly, the possibility to include the gene of interest flanked by ITRs in doggybone DNA vectors (dbDNA) was investigated. dbDNA are synthetic, linear, covalently closed DNA vectors that can be inexpensively and rapidly produced *in vitro* at large scale in a bacteria-free system from the parent plasmid. Unlike open-ended linear DNA which had a propensity for integration, dbDNA with their covalently closed ends has a lower tendency to integrate with a reduced risk of undesirable genomic integration of PB transposase. Using two linear dbDNAs containing PB transposase and the anti-CD19 CAR cassette incorporating 200bp sequences flanking the ITRs, respectively, it was possible to produce CAR T cells *in vitro* (130). The manufacturing protocols for PB-generated CAR T cells are summarized in **Table 3**.

4.3 Clinical Applications

Growing preclinical data supporting the feasibility and safety of the PB-based platform for CAR T manufacturing have allowed this system to enter clinical trials. The Australian CARTELL trial is a phase-I study (ACTRN12617001579381) to investigate the efficacy and safety of donor-derived anti-CD19 CAR T cells obtained through the PB transposon system in patients with relapsed and refractory CD19+ B-cell malignancies after HLA-matched sibling HSCT. Early results suggested activity similar to that of anti-CD19 CAR T cells generated with viral vectors with a high response rate. However, two of 10 treated patients developed malignant CAR 19 T cell tumor, one of whom died of disease-related complications while the other patient was successfully treated (132). Malignant cells showed high transgene copy number (24 copies) in the first reported patient, CAR overexpression, alteration in genomic copy number variation, and insertion into the *BACH2* and *FYN* genes. It is not yet clear which event caused the CAR T cell transformation, but it is widely accepted that the probability of insertional oncogenesis increases as the transgene copy number increases, which is why a limit of 5 VCNs is normally required by the regulatory authorities. Furthermore, it is likely that the numerous genomic deletions and insertions observed may have been driven by the use of a single high voltage pulse or excessive transposase activity. Insertional mutagenesis may also have contributed to the process of transformation. Both patients who developed lymphoma have an intronic integration in the *BACH2* gene, whose expression is therefore downregulated. BACH2 is a DNA-binding and transcription-regulating protein that plays a key immunoregulatory role and has been previously associated with cutaneous T cells lymphomas (133). *BACH2* is one of the genes most frequently targeted by HIV-1 insertion but HIV integration into *BACH2* has never been associated with insertional mutagenesis (134). Hence for now there is no clear evidence of the contribution of these integrations to transformation.

Two phase I studies conducted respectively in Japan (UMIN Clinical Trials registry ID: UMIN000030984) and China (clinicaltrials.gov ID: NCT04289220) are investigating the feasibility and safety of anti-CD19 CAR T cells manufactured with the PB system. In the Japanese study, three patients with R/R B-ALL were infused with 1x10^5 autologous anti-CD19 CAR T cells per kilogram after lymphodepletion in cohort 1. All patients previously received HSCT. Interestingly, administration of T cells produced by PB was safe and none of the patients showed dose-limiting-toxicities so far. One patients showed a B-cell aplasia lasting 9 months (135).

Results from a phase I trial using PB-generated anti-EGFR CAR T in R/R advanced non-small cell lung carcinoma (NSCLC) were recently published. Nine patients were treated with anti-EGFR CAR T cells, without grade 4 adverse events. Despite most patients showing the presence of circulating CAR T cells, only one patient showed a partial response while the other patients had persistent disease or progressed (NCT03182816) (136).

Although the results of transposon-engineered CAR T cells in clinical trials are preliminary, several early signs of clinical efficacy are emerging. A step forward has been made with anti-BCMA CAR T cells (P-BCMA-101) engineered through the PB platform for patients with R/R MM. To improve transposition, the manufacturing process was changed during the study to include the use of nanoplasmids that allow for the reduction of backbone

TABLE 3 | Manufacturing protocols associated with preclinical studies employing PB in the context of CAR T cells.

Background	Description	Vectors	Electroporation	Stimulation	Transduction and yield	Reference
PB transposon platform	Optimization of PB transposon platform for T-cells engineering using GFP as reporter	different quantities of pIRII-eGFP and pCMV-PB transposase	5x10^6 PBMCs using Nucleofector Device (Lonza, program U-014) with the human T-cell Nucleofector Kit	stimulation with CD3/CD28 mAbs and cytokines (IL-2, IL-15, IL-7, IL-4); transgenic T cells were selected on day 8 and expanded with feeder cells (autologous PBMCs or modified K562 cells)	Optimal results were obtained with 5µg of transposon and transposase with a transfection efficiency of 20%, improved to 30-40% with addition of IL-15.	Nakazawa et al., 2009 (120)
B-ALL	anti-CD19 CAR-T cells lacking the spacer IgG1 Fc region demonstrated superior efficacy in murine B-ALL xenograft models	pVAX1PB (5µg) + pVAX1SPBase (5µg)	4x10^6 PBMCs using Neon Electroporator with single pulse, 20 ms and 2400 V	Electroporated cells were cultivated in presence of IL-15 and stimulated with irradiated autologous PBMCs on D1 and after every 7 days.	Expansion: 100-fold after 22 days CAR expression: from 35% to 97%, depending on the construct	Bishop D. C. et al., 2018 (111)
CD19+ B-cell malignancies	Anti-CD19 CAR T cells manufactured in the presence of IL-4, IL-7 and IL-21 showed effective cytotoxic activity *in vitro*	5 µg (2:1 mixture of PB transposon vector and pCMV-PB hyperactive-transposase)	4x10^6 PBMCs using Neon electroporator inbuffer T (1x20 ms/2300V)	Electroporated cells were stimulated the day after in the presence of IL-4, IL-7 and IL-21 (stimulation by CD19 expressed on the surface of B cells in PBMC)	CAR expression: 90% in the presence of IL-4, IL-7 and IL-21. 30% when stimulated with anti-CD3/CD28 mAbs Expansion: from 4x10^6 to about 30-40×10^6 in 17 days and 100-120×10^6 in 24 days	P. Ptackova et al., 2018 (131)
CD19+ B-cell malignancies	anti-CD19 CAR T cells generated with co-electroporation of linear DNA transposon and mRNA encoding transposase showed lytic activity *in vitro*	pPB DNA linear transposon produced by PCR (3-0,3µg) + hyPBase mRNA transposase (12 µg) with 3′-O-Me-m7G (5′) PPP(5′) G RNA cap structure	1x10^7 PBMCs electroporated as in Ptackova et al., 2018 (131)	stimulation with TransAct reagent the day after electroporation and expansion for 21 days in the presence of IL-4, IL-7, and IL-21	CAR expression: 60-70% after 14-21 day of expansion Expansion: from 1x10^7 to 1x10^7 in 14 days and 1x10^8 in 21 days	I. Kastankova et al., 2021 (125)
Neuroblastoma	Anti-GD2 CAR T cells manufactured using autologous PBMCs pulsed with a pool of viral peptides showed effective antitumor response in xenograft model when combined with MEK inhibitor	pIRII-GD2-28Z CAR plasmid (7.5µg) + pCMV-PB transposase plasmid (7. 5µg)	2x10^7 PBMCs using 4D-Nucleofector and the P3 Primary Cell 4D-Nucleofector X kit, program FI-115 [See Morita D. et al., 2018 (121)]	stimulation with 5x10^6 autologous PBMCs pulsed with MACS PepTivator (AdV5 Hexon, HCMVpp65, EBNA-1, and BZLF), IL-7 and IL-15. Transfer to anti-CD3 or anti-CD28 mAb-coated plates on day 7 and expansion in G-Rex 6 Multi-Well Cell Culture Plates (Wilson Wolf Corporation, New Brighton) on day 9	CAR expression: 44% ± 6% at day 14 after transfection.	Tomida A. et al., 2021 (127)
HER2 positive solid tumor	HER2-CAR-T cells showed the ability to control Her2-positive tumor in mice	pIRII-HER2-28z plasmid (5µg) + pCMV-PB transposase plasmid (7.5µg)	20x10^6 PBMCs using 4D-Nucleofector and the P3 Primary Cell 4D-Nucleofector X kit, program FI-115 or the MaxCyte ATX protocol RTC 14-3.	Electroporated cells were stimuled with PBMC, electroporated with plasmid encoding tHER2, CD80 and 4-1BBL and UV-inactivated, on day 1 and cultivated in presence of IL-7 and IL-15 for 14 days	Expansion: 8 ± 1 fold CAR CAR expression: 60% ± 9% at day 14.	Nakamura K. Et al, 2021 (126)

size and bring ITRs closer. The cellular product showed a high composition of T stem cell memory (TSCM). Ninety patients have been treated with P-BCMA-101 and early results showed an overall response rate (ORR) 57% in the initial dose escalation and 73% in combination with Rituximab with remarkably low toxicity (clinicaltrials.gov ID: NCT03288493) (137).

5 mRNA

RNA has emerged as a versatile therapeutic reagent (138, 139). Seminal work by Malone more than 30 years ago demonstrated that RNA mixed with lipids can be absorbed by human cells and translate protein from it (140). Malone postulated in 1988

that if cells can create proteins from external mRNA then it might be possible to "treat mRNA as a drug". Since then, RNA has been used in other ways such as to restore functional expression of a mutated gene, knock out genes to silence expression (141, 142), modify cell phenotypes or to encode antigens. Here we focus on the use of RNA to modify leukocytes (143) to achieve temporary or long-term expression of CAR receptors in T cells.

5.1 Vector Design

Successful protein expression from RNA depends on its stability and translational efficiency. Those features are determined by cis-acting elements such as a 5' cap structure, polyA tail, and the composition of the coding sequence as well as untranslated regions that might be present on 5' and 3' ends of the molecule. These cis-acting elements in the RNA conspire with trans-acting cellular factors leading to translation and protein production.

The sequence of the RNA cassette can be encoded by a linearized DNA plasmid or by a PCR fragment that contains an RNA polymerase binding site or promoter, such as the bacteriophage T7 RNA polymerase to initiate the transcription reaction. Tools are available for coding sequence optimization including codon optimization for changing synonymous codons for enhanced expression in target tissues or cells, reducing secondary structures of the RNA that lower translation levels (144) or modification of the coding sequence itself to express more active isoforms (145, 146). More recently, producing RNA molecules with modified ribonucleosides such as pseudouridine has demonstrated improved translational capacity as well as diminished immunogenicity by decreased stimulation of Toll-like receptors (TLRs). This makes it particularly useful for work with immune cells, such as T cells, that express TLRs or as a vaccine (147). The first-in-man data for the use of RNA with modified 1-methyl pseudouridine became widely available with the advancement of mRNA Covid-19 vaccines (148, 149).

The cap structure at the 5' end of the RNA molecule, required for translation, can be incorporated in a co-transcriptional manner, using the m7G(5')pppG cap analog (150), Anti-Reverser Cap Analog (ARCA) (151), by enzymatic methods such as recombinant vaccinia virus capping enzyme (152, 153) or by a more advanced technology called CleanCap® which results in a natural structure of the RNA cap (type 1 cap).

A PolyA tail can be encoded in a transcription template containing a stretch of 64T nucleotides at the 3' end of the molecule (146). Some RNA molecules (145) require longer polyA tails for efficient expression and biological activity. However, the longer polyA tail cannot be encoded in the plasmid template due to the instability of long homopolymeric stretches in plasmid DNA (154). A longer polyA tail can be added using enzymatic polyadenylation. We and others reported that a longer poly(A) tail of 120 A residues as opposed to the more conventional poly (A) tail of 64 bases achieves higher protein expression levels (145, 155).

Additionally, both the 5' and 3' ends of an RNA molecule can be further modified with flanking untranslated regions (UTRs) to enhance translation (155–161). **Figure 3** presents the design of a mRNA vector.

5.2 Preclinical Evidence

RNA lends itself to versatile transfection methods with cells including electroporation (162), cationic lipids (163), and cationic polymers (164). mRNA has been used in a number of *in vitro* and *in vivo* preclinical studies to introduce CARs into T cells for testing in model systems for hematological tumors chronic lymphocytic leukemia (CLL), AML, ALL and solid tumors. Cytotoxicity and tumor growth inhibition was demonstrated in these models (165, 166). While mRNA-based therapies were shown to have reduced off target effects, lower toxicity and alleviate integration-associated safety concerns, the transient nature of protein expression was also a disadvantage in these applications. CAR constructs introduced into T cells with RNA was shown *in vitro* to last for 7 days (167) in absence of proliferation, and would limit the ability for the functionality of modified cells to persist.

A different approach to genetic modification of lymphocytes is to deliver a transgene of interest in the form of DNA together with RNA encoding a transposase enzyme. The first reported demonstration of successful gene transfer using an mRNA-encoded transposase was in the SB11 system (168). It was shown that SB11 transposase RNA stabilized with 5' and 3' untranslated sequences of the *Xenopus laevis* beta-globin gene successfully integrated a puromycin resistance gene from a pT2/PGK-Puro DNA plasmid in HT1080 human epithelial cells *in vitro*. The transposition efficiency as measured by puromycin resistance was greater using SB11 encoded by DNA compared RNA. The number of puromycin-resistant colonies per 10^6 cells plated was greatest with SB11 DNA under a UbC promotor (40X increase over a no SB11 control) followed by SB11 DNA under a PGK promotor (23X) and SB11 RNA using the PGK promotor (9X).

There are several advantages to encoding transposase enzymes in the form of mRNA when co-transduced into target cells along with a DNA vector encoding the gene of interest (168–170). One of the benefits of mRNA-based expression of a transposase is that its narrow window of transposase expression reduces the rate of secondary transposition events, which are caused by re-excision and re-integration of the transposon (171). Work conducted with a hyperactive form of Sleeping beauty, SB100X RNA, generated evidence that transposase can be used to efficiently integrate CARs into genomes of human T cells. An advantage of this approach is that the ratio of SB mRNA and DNA CAR construct can be precisely titrated to achieve durable integration with a low number of integrations per genome (68). The use of mRNA was also shown to allow for a transient, dose-controlled expression of SB100X in the absence of cytotoxic effects in various cell types (172). Another form of transposase, PB delivered in the form of mRNA was also shown to genetically modify HeLa cells when co-transfected along with a DNA plasmid encoding the neomycin resistance gene (170). Similar to the outcome in the SB system, using an RNA-encoded transposase, the PB transposase RNA was less efficient by a

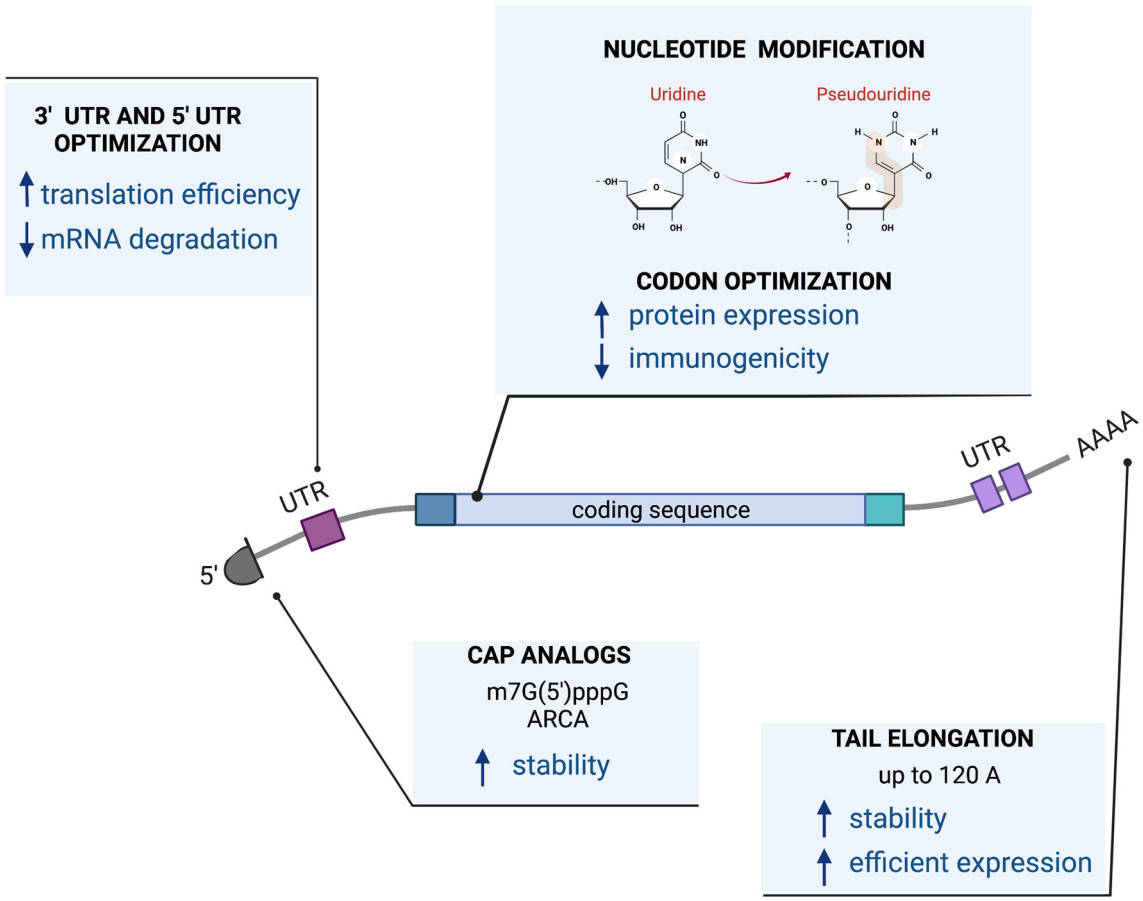

FIGURE 3 | mRNA vector.

significant margin at transposition compared to PBase encoded by DNA.

It is important to note that the use of RNA to encode transposases needs to be optimized for that specific system. It is not possible to compare results from independently published studies due asynchronous variables such as different transposase species, capping methodologies, poly-A tail lengths, cis-acting untranslated sequences, and transfection methods. To dampen the risk of insertional mutagenesis associated to genetic modification of cells *via* chromosomal integration, the choice of the vector system should be taken in account to avoid insertion into proto-oncogenes or transcription start sites that can lead to unintended transformation *via* insertional mutagenesis. We opted for SB system on the basis of the safer, more random integration pattern, as it does not demonstrate preferences to insert the transgene in active genes or TSSs thereby lowering the probability of integration in oncogenic genes. As previously highlighted in 4.3 paragraph, it was recently reported that 2 of 10 patients treated with anti-CD19 CAR T generated with the PB transposase system developed CAR-expressing CD4+ T cell lymphoma (132). To our knowledge, there have been no reports of insertional mutagenesis using the SB11 or SB100X transposase systems.

5.3 Clinical Applications

Potential safety advantages of transient CAR expression from mRNA may offer lower toxicity in both hematologic and solid tumor settings, especially outside of B-cell malignancies where off-tumor on-target collateral damage to healthy cells is a concern. Early phase clinical studies were conducted in hematological malignancies targeting CD123 and CD19 and in solid tumors targeting mesothelin and c-Met [reviewed in **Table 3** (165)].

While the studies report to be safe and generally lacking serious adverse events, one common denominator was the requirement for repeated dosing with 3-6 high doses. The need for multiple infusions of high doses of mRNA CAR T cells is most likely related to the lack of genetically modified cell persistence and aims to increase the duration of *in vivo* activity in these patients but repeat dosing may lead to other complications. Maus et al. reported a case of severe anaphylactic shock in a patient due to repeated doses of mesothelin-targeted CAR T cells, probably due to the murine

origin of the single-chain fragment variable (173). In addition, difficulties in producing enough mRNA CD123 CAR T product to sustain multiple doses have been reported, with only 60% of the planned T cell doses being able to be successfully produced, questioning about the feasibility of obtaining such a large starting material from patients. Given the lack of anti-leukemic efficacy, the trial was terminated, raising concerns about the efficacy of transient approaches to eradicate proliferating diseases. However, the study was able to confirm the safety of the approach, and thus proceed to clinical trials with a stable transfer approach using CAR T cells transduced with lentivirus (174).

6 ELECTROPORATION, HYBRID VIRAL-TRANSPOSON VECTORS AND NANOCARRIERS

6.1 Electroporation

Transfection or electroporation methods are typically used to deliver the mRNA and transposon vectors into cells. Indeed, nucleic acids are not able to penetrate spontaneously in target cells as viral vectors do through infection. To facilitate nucleic acid entry into cells, cells suspended in an electroporation cuvette are subjected to an electric field determined by a suitable electrical pulse. The process generate temporary pores in the cell membrane that allow vector penetration, and then seal up once the electric field is withdrawn. Once the nucleic acids enter the cell, they efficiently migrate into the nucleus. The electroporation efficiency depends on the voltage, the number of pulses, pulse width, and on the cell type and activation state. Furthermore, temperature, electroporation buffer, DNA and cell concentration influence the transduction efficiency. High-intensity pulses generally result in higher transduction efficiency but affect cellular viability. Small-scale electroporation can be achieved using Nucleofector 4D (Lonza, Basel, Switzerland) Neon (Thermo Fisher Scientific, Waltham) which use a cuvette and a pipette tip chamber, respectively. Other commercialized instruments are for example the Celetrix electroporation system (Celetrix, Manassas, VA, USA), and the BTX ECM 830 system (Harvard Bioscience, Hollistone USA). Commercially available electroporation devices for large-scale electroporation are Lonza LV unit and Maxcyte GTx (MaxCyte, Gaithersburg, MD, USA) platform. Lonza LV unit allows for closed electroporation of 1x10^7 to 1x10^9 cells. Maxcyte GTx device is a GMP-compliant, clinical-grade instrument and can electroporate up to 20x10^9 cells using flow electroporation technology.

6.2 Hybrid Viral-Transposon Vectors

Hybrid viral-transposon vector combine the entry properties of viral vectors with the integrative characteristic of transposons. This is particularly convenient when using recombinant adenovirus (Ad), a common vector due to its broad tropism, large carrying capacity, and optimal efficient transduction regardless the mitotic status of target cells. Ad has non-integrative features and thus results in transient transgene expression. The addition of integrative elements into the viral genome could overcome this limitation. A recombinant Ad vector containing a PB-transposon was shown to allow the integration of the transgene into the genome in presence of PB-transposase, included in the vector design or co-delivered. With these methods, stable expression of a reporter transgene was achieved in 20-40% of mouse liver cells after infusion and lasts for at least 5 months (175). Similarly, various groups have combined AAV vectors with the PB transposon system for *in vivo* delivery to correct several diseases such as diabetes type 1 (176), cystic fibrosis (177), and others. Recombinant adeno-associated viral vectors (rAAVs), Herpes simplex virus type-1 (HSV) vectors, baculovirus expression vectors (BEVs) have been tested with the SB transposon system (178).

6.3 *In Vivo* CAR T Cell Generation and Nanocarriers

As previously discussed, most protocols for adoptive T cell therapies require the collection of T cells and their *ex vivo* genetic manipulation. Patients are connected to an apheresis machine for several hours to extract T cells. Manufacturing involves activating and transducing purified T cells, expanding them *in vitro* for approximately 2 weeks and finally washing and concentrating them prior to administration. Often cells have to be cryopreserved in a central facility and transported to remote treatment centers. Quality controls on final product are mandatory for each batch. Manufacturing must be conducted under GMP conditions, and the entire process is expensive and needs specific resources, facilities and economic capital. In addition, because most CAR T products are currently obtained from an autologous source and thus from the patient's own cells, there are no economies of scale. In order to overcome the complexity of *ex vivo* manufacturing, *in vivo* CAR T cell generation is emerging as a new prospect and exploits the use of liposomal formulations, nanoparticles (NP), cell-penetrating peptides or advanced electroporation methods (179).

In this context, there are many promising attempts using nanocarriers composed of polymeric or lipid nanoparticles to produce CAR T cells directly from the patient's circulating T cells. Nanocarriers composed of biodegradable polymers are coated with ligands that targets them to specific cells and can encapsulate different substances such as drugs or non-viral transgenes. Smith and collaborators loaded nanoparticles with a PB transposon/transposase system encoding CAR (180). To ensure the specific delivery of the gene cargo to T cells, they coupled T cell targeting anti-CD3ef(ab')2 fragments to the surface of biodegradable poly (beta-amino ester) NPs. They co-encapsulated two PB plasmids, encoding a murine anti-CD19 (m194-1BBz) CAR and a hyperactive form of the PB transposase (iPB7), respectively, into the polymeric nanocarriers. DNA-carrying NPs were able to efficiently introduce the CAR genes into T cell nuclei, bind circulating T cells and cause tumor regression in mice with similar efficacies to adoptive T cell therapy. Although *in situ* programming of CAR T cells through injectable polymeric NPs is possible, this strategy has some limitations such as NP loading capacity which difficultly fits the large size of plasmids and the need to codelivery the

transposase vector. Moreover, as soon as NPs are infused, the small number of *in situ* transfected CAR T cells needs antigen drive expansion to show a visible anti-tumor activity. For these reasons, the same group evaluated the use of *in vitro* transcribed (IVT) mRNA encoding disease specific CAR or TCR encapsulated in poly (beta-amino ester) NPs, coupled to anti-CD8 antibody for specific T cell delivery. IVT-mRNA has the advantages of being directly translated into therapeutic proteins, without the need to enter the nucleus, improving transfection rates and avoiding uncontrolled insertional mutations and promoter dependence. Using this technology, circulating T cells have been reprogrammed with leukemia specific CAR and showed an anti-tumor efficacy when NPs were provided with repetitive infusion (181).

Building on the dazzling success of the application of lipid NPs encapsulated mRNA (LNP-mRNA) vaccines formulated against SARS-CoV-2, the study by Rurik et al. provides a great proof of concept on the possibility of producing CAR T *in vivo* for the treatment of cardiac injury (182). By employing CD5-targeted NPs, they succeeded in delivering the nanoparticles into T lymphocytes to generate CAR T *in vitro* and *in vivo*. *In vitro*, this strategy can drive the expression of an anti-FAP CAR efficiently (83% of cells expressing CAR measured by flow cytometry) and transiently, resulting in a dose-dependent killing activity similar to virally engineered cells. Administration of CD5-targeted LNP showed a reduction in fibrosis and restoration of cardiac function in a syngeneic model of cardiac injury, proving their ability to reprogram T cells *in vivo*. Although this platform is not suitable for diseases that require complete elimination of pathological cells, such as some forms of cancer, undoubtedly, for other applications, the ability to generate CAR T *in vivo* and the inherently transient nature of mRNA have the advantage of limiting toxicities, titrating doses, and offering an off-the-shelf process. **Figure 4** illustrates how non-viral CAR T cells can be generated through *in vivo* and *ex vivo* transfection.

7 NON-VIRAL CAR T CELL THERAPY: THE FUTURE

7.1 Nanovectors and Combination With Gene-Editing

To address future challenges, in addition to transposon platforms, an additional non-viral tool for gene engineering is represented by nanovectors whose use is gaining ground as a possible solution to overcome current barriers in gene delivery such as toxicity and low transfection efficiency. Among the latest advances in nanotechnology [reviewed in (183)], one of the most cutting-edge finding is reported by Bozza and collaborators, who developed a non-integrating DNA nanovector with the ability to generate CAR T cells that are active both *in vitro* and *in vivo*. This platform contains no viral components and is capable of replicating extra-chromosomally in the nucleus of dividing cells,

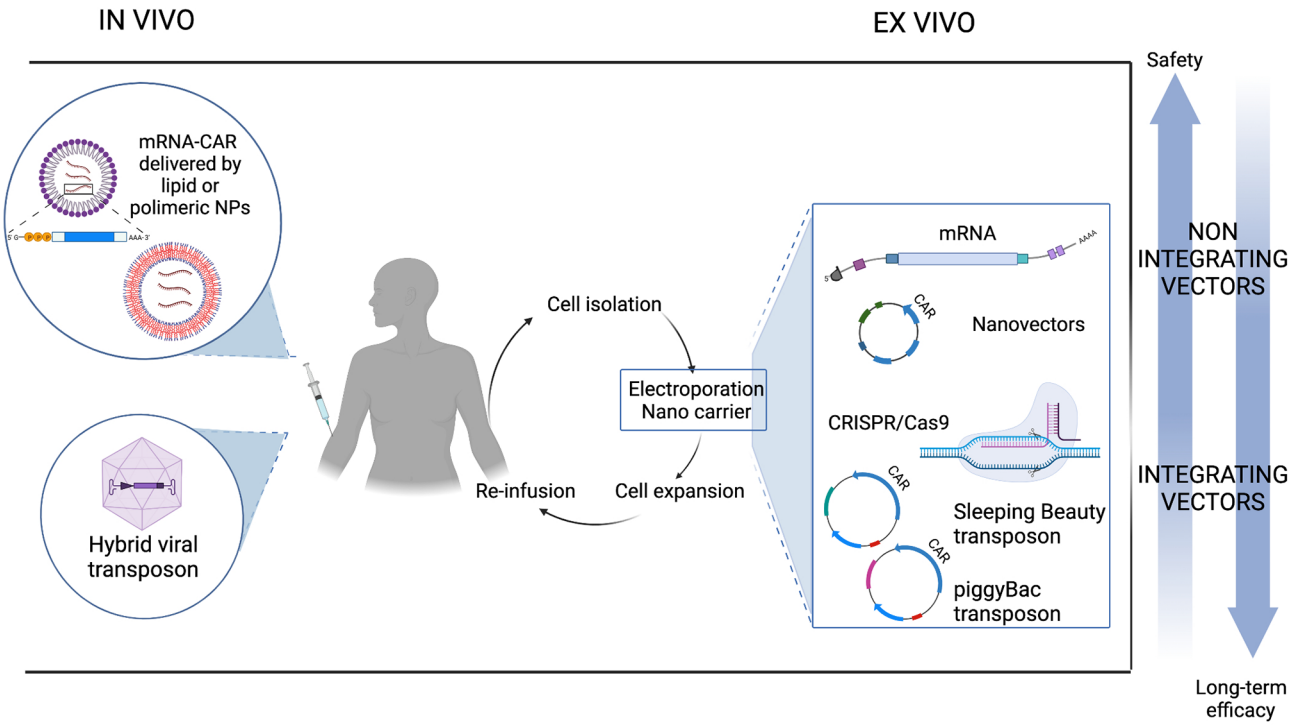

FIGURE 4 | Non-viral CAR T cell generation.

leading to persistent transgene expression, in the absence of integration and consequently genotoxicity. Moreover, it also shares all the advantages of non-viral vectors: it seems to be non-immunogenic, easy, simple, versatile and affordable to produce (184).

Also on the manufacturing front, progress is being made through the employment of biomaterials developed to improve isolation, activation, and genetic modifications of CAR T cells [reviewed in (185)]. One example is the use of a synthetic DNA aptamer and complementary reversal agent technology that permit isolation of label-free CD8+ T cells with high purity and yield from PBMCs. The great advantage of this method is represented by the possibility to isolate multiple distinct T cell populations in a single isolation step through aptamers with different specificities. Biomaterials, such as polymers and particles are also emerging to facilitate the T-cell activation process by eliminating the bead removal step, the first example of which is Miltenyi's T Cell TransAct. Another area in which substantial investment is underway is the ability to make the CAR T manufacturing process scalable, through first of all automation and the use of closed systems, but also the use of allogeneic products, as reviewed by Abou-el-Enein and colleagues (186). To this end, our lab has applied CAR T cells manufactured in-house from allo-transplant donors in patients previously transplanted with HSCT. The proven safety and therapeutic efficacy of this approach represents a proof of concept towards an off-the-shelf product, and in this perspective, we are currently working on the design of a study that will employ haploidentical allogeneic cells outside the transplantation context.

In the field of integrative non-viral approach, site-specific insertion approaches are undergoing rapid technological advances, thanks also to the ease of use of systems such as CRISPR/Cas9, which have also led to early clinical developments in the field of immunotherapy (187). Interestingly, it has recently been reported that electroporation of Ribonucleoprotein (RNP) and linear double-strand long DNA (>1kb) template reduces the toxicity of double-strand linear template, a finding that validates CRISPR/Cas9 as part of the state-of the-art in virus-free genome engineering technologies. Yet, in this early study, the integration efficiency was around 10% when applied to insertion loads of 1500 bp, as in the case of replacing the endogenous TCR with the antigen-specific TCR 1G4 NY-ESO-1 (188). Gene editing and targeted knock-in (KI) depend on the processes of host DNA double-strand break (DSB) repair and homology-directed repair (HDR), respectively, and HDR generally occurs with low frequency in primary cells and is restricted to small transgenes. Despite the low transfection efficiency due to the above intrinsic properties, there has been quite some progress for non-viral gene editing in adoptive T cell therapy, particularly in TCR engineering. Compared to the other technologies described above, gene editing combined with targeted KI allows TCR replacement with concomitant removal of the endogenous TCR, resulting in physiological expression of the transgenic TCR through the endogenous promoter. When combined with the elimination of both the α- and β-chains, it allows physiological TCR expression in absence of chain mispairing. Isolation of KI cells and *in vitro* expansion allow to reach highly purified cells to cope with the low efficiency of KI (189). In a direct comparison with conventional editing by viral transduction, orthotopic TCR replacement (OTR) using a library of 51 CMV-specific TCRs was characterized by more homogeneous and physiological TCR transcription, while surface expression by viral vectors was influenced by transgene copy number, leading to more variable TCR expression, with impact on *in vitro* and *in vivo* functionality (190).

Even under optimized condition, some cellular toxicity still limits the application of non-viral CRISPS/Cas9 and appears to depend on the electroporation of double strand DNA (dsDNA) and RNP aggregates into cells and the endogenous immune response triggered by innate DNA sensor protein pathway. To address the first challenge, Nguyen D. N. et al. used poly-L-glutamic acid, which physically disperse large RNP aggregates into smaller complexes. Additionally, they implemented the incorporation of a truncated Cas9 target sequence (tCTS) at the end of homology arms in order to facilitate the shuttling of the template into the nucleus. The combination of the two systems resulted in high KI efficiency of up to 50% in a wide set of primary human hematopoietic cells (191). To address the endogenous immune response, inhibition of the DNA sensor protein pathway was proposed in combination with insertion of a 2015 bp long CAR into the TRAC locus of human T cells *via* CRISPR/Cas9-mediated HDR. In conjunction with the poly-L-glutamic acid nanoparticle strategy, the use of DNA-sensor inhibitors and HDR enhancers achieved high editing efficiency with a insertion rate of 68% (192). Two other recent works have used the CRISPR/Cas9 platform to integrate a CAR with promising results. By targeting an anti-GD2 CAR into the first exon of TRAC locus, an average KI efficiency of 15% was obtained, improved up to 45% by increasing the length of the homology arms on both sides of the CAR with final DNA template size of 3,4 Kb and resulting in 34% CAR positive cells (193). Moreover, by using a hybrid single strand (ss) DNA HDR template incorporating CTS sites, increased KI was obtained, up to 40% efficiency in combination with small molecule inhibitors (194).

To provide DNA insertion in the absence of DSB and HDR, CRISPR/Cas9 has recently been combined with transposons to increase the efficiency of RNA-guided integration, using a transposase protein to catalyze the integration (195, 196). Recently, experiments have also been conducted to combine CRISPR/Cas9 with SB transposon. The transposase protein was fused to a catalytically inactive Cas9 for providing single guide RNA-dependent DNA insertion in the absence of DSB and HDR, leading to enrichment of integrations near the sgRNA targets (197). Recent results obtained with gene editing techniques demonstrate their suitability for future non-viral clinical applications. Approaches to identify and reduce the risk of genomic rearrangements and translocations may obviate concerns associated with the collateral damage of the technique and thus allow for greater development of potential clinical applications of gene editing in immunotherapy.

TABLE 4 | Summary of principal characteristics of methods in CAR T manufacturing.

	Characteristics and peculiarities	Pros/Cons	Technical requirement	Impact of costs
Viral vectors	Gammaretroviral vectors: Infection only in cycling cells Integration near TSS Lentiviral vectors: Infection in cycling/non cycling cells Integration in transcriptional regulatory region	Pros: Stable transduction Long term expression Cons: Limiting insert size Difficulty to scale synthesis up Risk of insertional oncogenesis More immunogenicity	Biosafety Level 2 Trained staff Cryopreserved facility	High costs Limited number of available manufacturing facilities globally, and lot size limitations
Sleeping Beauty	Tc1/mariner DNA Class II TE Cut-and-paste mechanism of insertion Transposon/transposase system Transfection in pre-activated and resting primary cells Close to random integration TA dinucleotide as target site CAG footprint	Pros: Easy to scale synthesis up Large cargo size (up to 100 kb BACs) Versatility Low immunogenicity Cons: Toxicity related to transduction procedure	Electroporation Cryopreserved facility	Relatively low cost Easier manufacturing process
Piggy Bac	PB superfamily DNA Class II TE Cut-and-paste mechanism of insertion Transposon/transposase system Transfection in pre-activated and resting primary cells Preference of integration near TSSs, CpG islands and DNase I hypersensitive sites TTAA dinucleotide as target site No footprint	Pros: Easy to scale synthesis up Large cargo size (200 kb BACs) Versatility Low immunogenicity Higher transposition activity Cons: High risk of gene dysregulation Toxicity related to transduction procedure	Electroporation Cryopreserved facility	Relatively low cost Easier manufacturing process
mRNA	Absence of integration Transient transfection	Pros: Availability of protocols for clinics Versatility and flexibility Safety transient expression (SB100X and PB transposase) Cons (mRNA encoding CAR): Short term potency Need for multiple doses	Electroporation Cation lipids Cationic polymers	High doses of mRNA CAR T are required to achieve efficacy
Nanotechnology	Nanocarriers or lipid nanoparticles coated with ligands ensure encapsulation of non-viral transgenes Ability to reprogram T cells *in vivo*	Pros: Low toxicity Off -the-shelf process Cons: Limited cargo capacity	Devices for scale up production Specialized staff	Costs of nanoparticles production and costs of encapsulated material

8 CONCLUSION AND PRACTICAL CONSIDERATIONS FOR FUTURE TRIALS

Successful CAR T cell therapies are so far associated with T cells engineered with viral vectors. Yet, the emergence of relapse, the complexity of the manufacturing process, and the application of these technologies to other diseases including solid tumors, require a more complex design and gene transfer technology that fits these challenges. So much progress is being made with non-viral technologies to fill these gaps, and it is certain that a great step forward will be taken soon.

Previously discussed non-viral manufacturing methods are briefly summarize in **Table 4**.

Non-viral CAR T cells engineered with transposon vectors have already shown some efficacy in early-stage clinical trials, but whether such systems may also show unexpected toxicities and comparable clinical outcomes remains to be seen in additional clinical studies. For first-in-human clinical trials, certain precautions have been implemented to ensure safety given the novelty of the gene transfer method. In our study (43), given the low-risk classification associated with non-viral vectors, physical containment of the vector and transfected cell product was managed at biosafety level 1. The duration of *in vitro* cell culture ensured the absence of plasmids at the end of culture. Whether the bacterial sequences below the limits of detection is sufficient to protect from an immune response by the host against bacterial sequences, however, is currently unknown. The mean transgene copy number was set at 5 as an upper limit, in order to limit the potential risk of genotoxicity. Others are instead using 8 as release criteria for transposon-generated CAR T cell products. Despite the almost random insertion profile of the transgene into the T cell genome, the study of integrations ensured the monitoring of the clonality of the CAR T population post-infusion. Based on this clinical experience, we therefore recommend the use of vectors and the development of manufacturing platforms designed to contain transposase

activity and to limit the number of integrations, and the implementation of a detailed follow-up plan for clonality monitoring by integration site analysis.

We believe that the main takeaway from the efforts described in this review is the possibility of using novel non-viral engineering approaches in future clinical trials. In addition to non-viral integrating methods, we think that the use of episomal nanovectors may be advantageous for the *ex vivo* production of safer transfected cells. In addition, viral-transposon hybrid vectors and mRNA delivered by polymeric and lipid nanoparticles represent technological platforms that can generate stable or transient CAR T cells *in vivo*. We are on a learning curve and looking forward to seeing the results of these technologies in future clinical trials.

AUTHORS CONTRIBUTIONS

CM conceptualized, wrote, and edited the manuscript. MP, AM, and IT wrote the manuscript. MP and AM designed the figures and prepared the tables. CN and AB edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Review: Sustainable Clinical Development of CAR-T Cells – Switching From Viral Transduction Towards CRISPR-Cas Gene Editing

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T cells modified for expression of Chimeric Antigen Receptors (CARs) were the first gene-modified cell products approved for use in cancer immunotherapy. CAR-T cells engineered with gammaretroviral or lentiviral vectors (RVs/LVs) targeting B-cell lymphomas and leukemias have shown excellent clinical efficacy and no malignant transformation due to insertional mutagenesis to date. Large-scale production of RVs/LVs under good-manufacturing practices for CAR-T cell manufacturing has soared in recent years. However, manufacturing of RVs/LVs remains complex and costly, representing a logistical bottleneck for CAR-T cell production. Emerging gene-editing technologies are fostering a new paradigm in synthetic biology for the engineering and production of CAR-T cells. Firstly, the generation of the modular reagents utilized for gene editing with the CRISPR-Cas systems can be scaled-up with high precision under good manufacturing practices, are interchangeable and can be more sustainable in the long-run through the lower material costs. Secondly, gene editing exploits the precise insertion of CARs into defined genomic loci and allows combinatorial gene knock-ins and knock-outs with exciting and dynamic perspectives for T cell engineering to improve their therapeutic efficacy. Thirdly, allogeneic edited CAR-effector cells could eventually become available as "off-the-shelf" products. This review addresses important points to consider regarding the *status quo*, pending needs and perspectives for the forthright evolution from the viral towards gene editing developments for CAR-T cells.

Keywords: CAR-T, lentiviral, retrovirus, gene editing, GMP, CRISPR-Cas, GMP, mouse models

INTRODUCTION

Retroviruses integrate into the genome, are able to effectively and persistently infect T cells, and are non-cytotoxic and poorly immunogenic. Their bio-engineered descendants, pseudotyped gammaretroviral and lentiviral vector systems (RVs/LVs), emerged more than three decades ago as useful tools for gene therapies using T cells and hematopoietic stem progenitor cells (HSPCs) for correction of defective genes and treatment of monogenic blood disorders and metabolic diseases (1). RVs and LVs are currently the mostly used gene delivery systems for manufacturing of T cells expressing chimeric antigen receptors (CARs). Nonetheless, there were several ups-and-downs on the path to clinical translation of these "living drugs" that can instruct the development of gene-edited CAR-T cells generated by non-viral materials and the use of site-specific gene transfer.

In 1990, the first clinical trial of gene-modified T cells used RV-mediated transfer of adenosine deaminase (ADA) for treatment of two children with severe combined immunodeficiency (ADA-SCID). The trial demonstrated engraftment, persistency and safety of the T cell gene therapy (2). Major improvements in efficacy and safety of multiple attenuated self-inactivating (SIN) RV/LV designs have significantly boosted the field of innate genetic defects corrected *via* gene therapy (3, 4). Thus, after more than two decades of clinical research and development, the European Commission granted market approval to GlaxoSmithKline (GSK) for *ex vivo* HSPC gene therapy for the treatment of ADA-SCID (5). The development of SIN viral designs drastically reduced the risks of insertional mutagenesis enabled better control of the transgene expression (6). These viral systems provided a robust insertion of a gene-of-interest (GOI), which was added to the genome of target cells (**Figure 1**).

Published clinical trial reports in 2011 and 2013 presented clinical objective responses against lymphoma and leukemia with CAR-T cells generated after SIN-LVs gene transfer (7, 8). To date, all CAR-T cell products approved by the United States Food and Drug Administration (FDA) and by the European Medicine Agency (EMA) for immunotherapy of lymphomas and/or leukemias are engineered *via* "add-on" transgenesis using SIN-LVs or SIN-RVs. These approved products target the B cell antigen CD19 including: LV-transduced CTL019 (KYMRIAH, Novartis Pharmaceuticals Corp) (8), RV-transduced KTE-C19 (YESCARTA, Kite Pharma, Inc., a Gilead Sciences Company) (9), RV-transduced brexucabtagene autoleucel (TECARTUS, Kite Pharma, Inc., a Gilead Sciences Company) (10) and LV-transduced liso-cell (BREYANZI, Juno Therapeutics, Inc., a Bristol-Myers Squibb Company) (11). LV-mediated gene delivery currently dominates CAR-T cell manufacturing. FDA/EMA-supported combination trials exploring alternative targets to CD19 (CD20, CD22, CD30, and the B cell maturation antigen, BCMA) are planned to improve efficacy in the CAR-eligible leukemia/lymphoma patient population (12). In addition, bi-specific CAR-T cells engineered with RV/LVs are in clinical testing (e.g. 2019-CD20-dual specific CAR-T cell product from Miltenyi Biomedicine) (13). In conclusion, SIN RVs/LVs have provided an important framework for the conception and clinical use of CAR-T cells as they are feasible and safe.

Alternative "add-on" transgenesis *via* DNA plasmid-based non-viral gene-modification technologies are being developed to replace viral systems in order to reduce the costs and facilitate the logistics of CAR-T cell manufacturing. CD19-specific CAR-T cells transfected with the Sleeping Beauty (SB) or *piggyBac* transposon showed exciting preclinical results (14) and promising results in early clinical trials (15). Sadly, unexpected and alarming insertional mutagenesis and T cell-lymphoma occurrences have been observed in some patients infused with CD19CAR-T cells produced with a highly active version of the *piggyBac* transposon system (16). Multiple transgene insertions and global transcriptional dysregulation through the strong promoters used are suspected to have caused the malignant transformation (15). Thus, additional preclinical studies and clinical monitoring efforts are urgently warranted for a better mechanistic understanding to prevent the onset and putative development of leukemias and lymphomas when using potentially pro-oncogenic transposon systems (17). Another pipeline in development is the transient transfection of T cells with mRNAs encoding CARs. Since the mRNA are degraded or diluted upon T cell expansion, the anti-tumor effect is predestined to be transient. The mRNA-CAR-T cell therapy would thus require repeated infusions, and it is yet not clear if this is a downside for this approach (18).

As an innovative alternative, clustered regularly interspaced short palindromic repeats (CRISPR) associated (Cas) 9 technology has emerged as a replacement for the "add-on" approaches with directed and precise T cell editing *via* "knock-in" (**Figure 1**). CRISPR-Cas allows the site-specific insertion of the CAR at potentially any point in the T cells genome, creating CAR-T cells with defined transgene copy numbers and predictable regulation of transgene expression. For example, the CAR transgene can be inserted within the early open reading frame of well-characterized genes, thereby disrupting the gene of interest ("knock-out") after "knock-in" of the CAR in a single genetic intervention. This technology is exceptionally useful to facilitate potent "off-the-shelf" CAR-T cells to reduce costs and avoid treatment delays in severely compromised patients.

Under the headings below, we explain how RVs/LVs became the current paradigm for gene modifications of CAR-T cells. We address some of the critical aspects regarding the development of gene-edited CAR-T cells to thrive as a program for the treatment of liquid and solid tumors. One important focus is on what was learned about the design, safety, manufacturing, upscaling, and quality control of CAR-T cell products generated with RV/LVs and the perspective for gene edited CAR-T cell products. In a next step, we extrapolate towards the need for new preclinical models, innovative design of clinical trials and monitoring of patients infused with allogeneic "off-the-shelf" gene-edited CAR-T cells.

PRINCIPLES AND USES OF RVs/LVs FOR CAR-T CELL ENGINEERING

Bioengineering of RV/LV systems for gene-modification of HSPC products has provided the fundamental know-how for subsequent

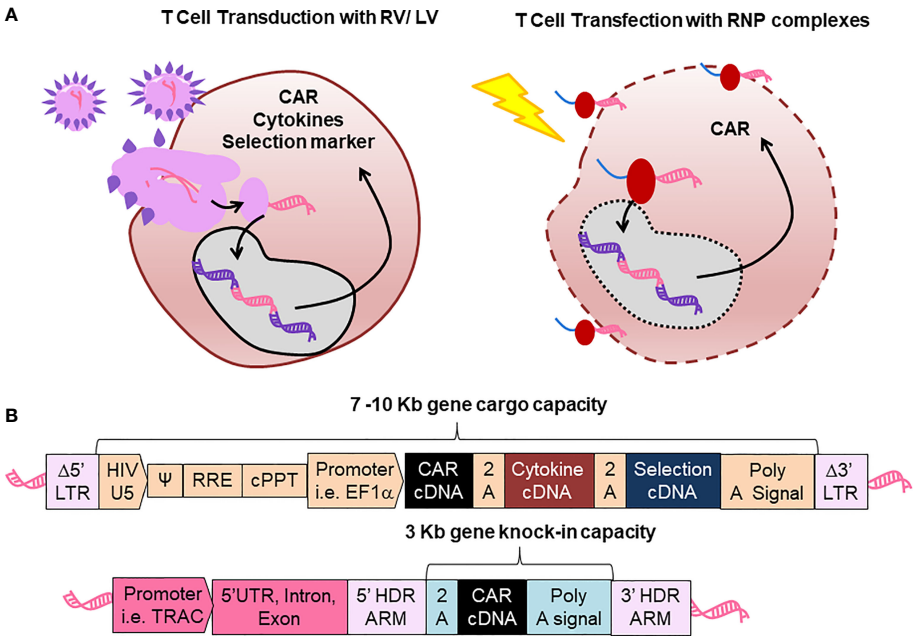

FIGURE 1 | Comparison between retroviral vector and lentiviral vector (RV/LV) gene delivery systems with CRISPR-Cas gene editing for production of chimeric antigen receptor (CAR)-T cells. **(A)** Scheme of T cell transduction with RV/LV (left) and cell transfection with ribonucleoprotein (RNP, Right). **(B)** Schematic representation of genetic structures. Upper structure: Displays an integrated prototypic LV gene transfer vector encoding a CAR, not to scale. LTR: Long terminal repeats; HIV: Human immune deficient virus U5: Untranslated region in the 5' side; Ψ: encapsidation signal; RRE, Rev responsive element; cPPT, polypurine tract; EF1α, Elongation factor 1 α. Lower structure: Represents a prototypic integrated CAR generated by gene editing. *TRAC*, Locus of T cell receptor alpha chain; HDR, Homology-directed recombination.

development of CAR-T cells. RVs/LVs have relied mostly on the third generation packaging system, whereby four plasmids are used for expression of the backbone vector, *gag/pol*, *rev* and *env* (19, 20). After infection of the activated and proliferating cells, the RNA genomes of RVs/LVs are converted through reverse transcription inside the cell into double-stranded DNAs capable of integrating into the chromatin (**Figure 1A**). Thus, the core of multicistronic RV/LV engineering is that a single vector will carry the combination of genes into the cells, however with quite unpredictable insertion patterns. An improvement was obtained with SIN mutations in the viral 3'LTR, disrupting the promoter/enhancer activity of the LTRs and enhancing the controlled expression through the internal promoter in the vector, and thereby minimizing the downstream expression of proto-oncogenes that could promote insertional mutagenesis (3). The design of RVs/LVs mostly include viral elements needed for packaging (parts of the LTRs, Ψ psi encapsidation signal), RNA reverse transcription (central polypurine tract, cPPT), internal non-methylated promoters (e.g. EF1-α), and the GOI (21) (**Figure 1B**). Since the gene cargo capacity of RVs/LVs spans from 7 to 10 Kb, additionally to the CAR (around 3 Kb), other transgenes can be combined as multicistrons interspaced with a 2A self-cleaving peptide or with internal ribosome entry site (IRES) elements.

As a result, there are numerous synthetic biology strategies relying on RV/LV systems to optimize the CAR gene-cargo, which include (i) tuning the affinity of the virally expressed CAR (s) to antigen(s) (22), (ii) use of different intracellular co-stimulatory domains in the CAR fusion protein such as CD28z, 4-1BB and other co-stimulation pathways directing the tonic power and/or persistency of T cell activation (23); (iii) metabolic editing to balance the oxidative phosphorylation and fatty acid oxidation or glycolysis during T cell activation (24); (iv) combinatorial co-expression of immune-stimulatory cytokines to improve the T cell persistency and function (25); and (v) inclusion of inducible on/off systems such as co-expression of suicide genes, surface markers that enable immune depletion, or combination of activation/inhibitory CARs in the same cell (22).

The clinical performance obtained for RV/LV-engineered CAR-T cells in the treatment of B cell malignancies has not yet been achieved in the treatment of solid tumors. The main difficulties encountered are the lack of exclusive tumor-specific antigens and the immunosuppressive nature of the tumor microenvironment (26). Although the challenges may rely rather on tumor-specific factors than the technology used for CAR gene-delivery, gene-editing may replace RVs/LVs for different approaches. For example, sophisticated tumor detection and targeting advances can be achieved by engineering T cells with CAR constructs expressed by RVs/LVs to function as comparative operators (27–29). Promising approaches based on the so-called TRUCK ("T cells redirected for antigen-unrestricted cytokine-initiated killing") strategy have recently emerged to increase the efficacy of CAR-T cells generated after RV transduction (30–32). TRUCKs combine the direct cytotoxic effect of the CAR-T cell on tumor cells with the immune modulating capacities of a pro-

inflammatory cytokine (33). In order to achieve therapeutic concentrations of a selected cytokine in tumors and surrounding tissue, the transgene of interest is inducibly released by tumor-specific CAR-T cells, thereby preventing systemic toxicity. The TRUCK concept is currently being explored using a panel of pro-inflammatory cytokines, including interleukin (IL)-12, IL-15, IL-18, IL-23, and combinations thereof (33).

LARGE-SCALE MANUFACTURING OF CLINICAL-GRADE RVs/LVs

The large-scale manufacturing of RVs was initially based on development of stable packaging cell lines. During the past two decades, with the advent of the third generation LV packaging systems, the field has largely explored transient transfection of different DNA plasmids into packaging cells (such as adherent or non-adherent HEK293T cells). Transient transfection for packaging of RVs/LVs became an important technology as it bypasses the need of selecting, expanding and characterizing different master packaging cell lines carrying different constructs. RVs/LVs obtained after transient transfection were validated for different types of clinical applications such as gene modification of HSPCs for correction of defective genes (1), for harnessing dendritic cells for active immunotherapy against cancer (34, 35) and, more prominently, for gene modification of T cells for different types of adoptive immunotherapies (21).

The large-scale bioprocessing of LVs has in recent years adopted the use of bioreactors for perfusion transfection and culture of adherent and suspension cells. Several advances were obtained with the downstream processing of the viral particles with purification technologies (such as tangential flow filtration) (36, 37). Quality control (QC) of SIN-LVs is well established and includes: Vector identity (qPCR), Vector concentration/titer (ELISA), Vector functional titer (flow cytometry), residual plasmid DNA (VSV-G DNA qPCR), Residual host DNA (antigen-specific qPCR), detection of replication competent lentivirus (RCL), quantification of residual Benzonase (ELISA), total protein measurements (protein assay), microbiological control (bacteria and fungi assay), detection of endotoxin (LAL assay) as well as volume, pH and appearance (36).

Several obstacles still limit the applicability of large scale use of clinical LVs for medical care. The high costs of LVs for production of T cell therapies, is an important bottle-neck contributing to exorbitant costs of the cell products for a single treatment course (currently >300.000 US dollars in the United States) (37). Further, due to the currently limited manufacturing capacity for LVs, the commercially available CAR-T cell therapies are only regarded as a second-, third- or fourth-line therapeutic option for patients failing to respond to, or have relapsed after conventional therapies (37).

CLINICAL MANUFACTURING OF CAR-T CELLS GENERATED WITH LVs/RVs

A major challenge for academic institutions, such that CAR-T cells become a standard clinical strategy, is to scale out the GMP-compliant manufacturing (38–41). The entire manufacturing process for semi-automated or automated processes requires 12 days (range 6-22 days) (39, 42). The subsequent procedures include T cell activation, gene transduction, expansion and often cryopreservation after the final formulation. First, T cells, selected (e.g. $CD4^+$ and $CD8^+$) or not, are commonly activated with agonistic anti-CD3 and anti-CD28 antibodies and expanded in the presence of stimulatory cytokines (mostly IL-7, IL-15 and/or IL-2) for 1-2 days (38). Afterwards, the viral vectors are added to the cell culture system, often in the presence of cationic adjuvants to enhance the transduction efficacy. Prior to large-scale CAR-T cell manufacturing, pilot lots are tested with different vector dosages to yield a satisfactory multiplicity of infection (M.O.I.), i.e., resulting in 5 or less viral copies per cell. After transduction, CAR-T cells are expanded in culture with cytokines for additional 5-10 days. Optimized GMP protocols using RVs/LVs have resulted in high gene delivery efficacy, resulting a range of 25-80% CAR-positive T cells including both $CD4^+CD3^+$ and $CD8^+CD3^+$ CAR-T cells after transduction and expansion. Since LV gene transfer is usually robust in actively replicating T cells, manual manufacturing methods can be efficiently replaced with closed automated systems (42, 43). Importantly, digitally controlled automated manufacturing systems can potentially improve the practicability and lower the costs associated with clean rooms and highly trained personnel for production of CAR-T cells for a broader patient usage (41). Thus, in sum, although the upstream production and testing of clinical grade RVs/LVs still remains complex and expensive, the downstream T cell transduction procedures are relatively straightforward, particularly with the launching of powerful automated cell manufacturing systems allowing consistent gene transfer efficacy, cell recovery and viability (**Table 1**).

TRANSGENE "KNOCK-IN" WITH CRISPR-Cas GENE EDITING

The 2020 Nobel Prize for Chemistry was awarded to Jennifer Doudna and Emmanuelle Charpentier, eight years after their original publication describing how the CRISPR RNAs (crRNAs) can guide recombinant Cas9 enzymes to recognize, bind and cut a DNA sequence of interest *in vitro* (44). They elucidated how a mature crRNA base-paired to trans-activating crRNA (tracrRNA) was able to form a duplex RNA structure, which guides the CRISPR-associated *Streptococcus thermophilus* and *Streptococcus pyogenes* (Sp)Cas9 proteins to the target DNA where it then introduces double-stranded (ds) breaks. They also demonstrated that dual-tracrRNA:crRNA when engineered as a single RNA chimera could also direct sequence-specific Cas9 dsDNA cleavage (44). The high flexibility and efficacy of the RNA-guided nuclease CRISPR represents a disruptive technology which has opened several doors for synthetic biology and cell therapies.

The use of a programmable nuclease to precisely edit DNA at specific loci was then used by Eyquem et al. to replace the endogenous T cell receptor (TCR) alpha chain with a CAR. They combined transfection of anti-CD3/CD28-stimulated T cells

TABLE 1 | Comparison of technical ease, elements needed, procedures, and efficacies between retroviral vector and lentiviral vector (RV/LV) gene delivery systems with CRISPR-Cas gene editing for production of chimeric antigen receptor (CAR)-T cells.

	RV/LV	CRISPR-Cas RNP
Generation of Gene transfer system	Viral packaging and purification, customized,complex, costly	Highly adaptable and modular, RNA/ DNA synthesis and recombinant protein, simple
QC of gene transfer system	Complex molecular biology and virology, biochemical, biological tests	Simple biochemical synthesis and biochemical tests
PBMC/T cell activation	1-2 days	1-3 days
T cell modification	Virus plus adjuvant, overnight incubation	Several reagents, electroporation and resting
T cell expansion	>1000 fold relative to input	Up to 200 fold relative to input
Insertion in genome	Mostly random and in pro oncogenic hotspots	Targeted to specific loci but off sites possible
Multicistronic gene transfer	Feasible within gene cargo capacity	Remains to be optimized
Production of HLA-KO Allogenic CAR-T cells	Feasible with shRNAs or gRNAs expressed in viral vector, and with electroporation of mRNAs expressing TALENs	Feasible with gRNAs included in gene editing procedure

with Cas9-single guide (sg)RNA ribonucleoprotein (RNP) complexes followed by transduction with a recombinant adeno-associated virus serotype 6 (rAAV6) to deliver the DNA donor template and homology-directed DNA repair (HDR) arms for CAR integration into the first exon of TCR-α constant gene (*TRAC*) (45). They observed homogeneous CAR expression in human T cells and *TRAC*-integrated CAR-T cells therapeutically outperformed CAR-T cells generated *via* RV transduction in a preclinical mouse model of acute lymphoblastic leukemia. Improving the design of the CD19-CAR was shown to further increase the potency of *TRAC*-replaced CAR-T cells in leukemia and lymphoma models (46). Subsequently, these advances were adopted by other groups for use of CAR-T cells in the context of haploidentical stem cell transplantation (47).

Fully non-viral gene editing approaches with DNA templates for CAR/TCR knock-ins are rapidly emerging (48) (**Table 2**). Roth et al. demonstrated the use of virus-free knock-in to replace the endogenous TCR with an ectopic TCR targeting the NY-ESO-1 cancer antigen (54). Cas9 RNPs were co-electroporated with a blunt-ended dsDNA HDR template (HDRT) with homology arms designed to introduce the α and β chains of the TCR into the *TRAC* gene (54). The resulting TCR-engineered T cells specifically recognized NY-ESO-1 and killed tumor cells expressing NY-ESO-1 *in vitro* and *in vivo*. Interestingly, the gene edited T cells engineered with the CRISPR-Cas system mounted better antitumor immune responses in a mouse model than T cells gene modified with lentiviral vector expressing the same TCR, probably because they could be better self-regulated to avoid exhaustion.

Both the automated and large-scale chemical production of the gRNAs and novel enzymatic techniques to synthesize the HDRT have sky-rocketed in recent years. Although still costly at the clinical stage, a large set of CRISPR products are broadly available for basic research from multiple commercial vendors. The number of manufacturers that provide GMP services for Cas enzymes and customized gRNAs or DNA templates is starting to expand, and due to demand and competition, will likely become more affordable for clinical use in the years to come. Since these products are chemically defined, the quality control analyses will be mostly chemical/biochemical. Furthermore, CRISPR-Cas related reagents have extraordinary stability. Some studies have successfully lyophilized defined RNP/DNA composition, which could further improve roll-out of the technology (55).

Unlike RVs/LVs, the RNP complexes used for gene editing lack the machinery to cross the cellular membrane and reach the chromatin within the intra-nuclear space (**Table 1**). Most published protocols use electroporation as means to introduce the RNP into the cell with minimal toxicity to T cells (56, 57) (58). However, co-delivery of large dsDNA donor templates required for CAR knock-in induces significant dose-dependent toxicity (50, 54) (**Table 2**). Physical shear stress, DNA damage responses as well as innate immune responses to free cytosolic RNA or DNA, endanger cell viability, gene modification and ultimately a good recovery of CAR^+ viable T cells. In contrast to dsDNA, TCR-knock-in with ssDNA donor templates is less toxic, however with significantly reduced integration rates compared to dsDNA for pooled CAR knock-ins (49). Use of anionic adjuvants that disperse RNPs have been shown to reduce toxicity and increase efficacy of virus-free reprogramming with large dsDNA donor templates (55).

As a result of different optimization steps, in most publications on virus-free TCR/CAR knock-ins blunt-ended dsDNA or plasmids were used with the frequency of knock-in T cells reported in a range between 5-50% after 7-14 days of expansion (49, 50, 51, 52, 53). Based on experience of authors of this review, the number of recovered T cells 10 days after initiation of the editing procedure can reach 10-200 times the number of PBMCs used as input (51). Initial cell loss after electroporation and the modest expansion rate observed remain limiting factors warranting innovative technologies. These could include nanocarriers, liposome or virus-like particle-based delivery platforms for DNA and/or RNPs which circumvent electroporation procedures. Furthermore, synthetic DNA donor templates may be optimized or enhanced to decrease toxicity, increase efficacy, and reduce the risk for unintentional integration events.

In conclusion, the materials used for virus-free CRISPR-Cas editing are and will be easier to produce, store and distribute for clinical use than large-scale manufacturing of RVs/LVs. The current challenge is to further optimize and standardize the gene editing procedures to improve CAR T cell yields and manufacturing stability. Subsequently, virus-free knock-in methods should be adopted for automated manufacturing systems to accommodate future clinical scaling (41, 59).

TABLE 2 | Examples of prominent studies using CRISPR system for genetic modification of T cells to produce CAR-T cells.

Reference	Target Antigen and co-stimulation	Target Genetic Locus	Methods for Gene Editing	Frequency of CAR+ T Cells after Knock-in	Potency Assays *in vitro* and *in vivo*
Eyquem et al. Nature 2017 (45)	CD19 CD28 zeta	*TRAC*	sgRNA and Cas9 mRNA AAV-mediated HDR	Up to 40%(10e6 AAV dose)	*In vitro* culture with Nalm-6/fLuc/GFP or NIH-3T3/CD19
		B2M	sgRNA-Cas9 mRNA AAV-mediated HDR	14%	*In vivo* Nalm-6/fluc/GFP xenogra fted in NSG male mice
Feucht et al, Nature Medicine 2019 (46)	CD19 CD28 Zeta (+ITAM-mutated versions)	*TRAC*	sgRNA and Cas9 mRNA AAV-mediated HDR	60-75%	*In vitro* culture with Nalm-6/fLuc/GFP or NIH/3T3/CD19 *In vivo* Nalm-6/fLuc/GFP xenograftedin NSG male mice
Wiebking et al, Haematologica 2021 (47)	CD19 CD28 zeta	*TRAC*	sgRNA-Cas9RNP AAV-mediated HDR	>70%	*In vitro* co-culture cytotoxicity assays & cytokine production from supernatants (ELISA) *In vivo* Nalm-6/fLuc/GFP xenograft in NSG mice
Roth et al. Cell 2020 (49)	Different chimeric receptors (pool) + TCR e.g. TGFβR2-41BB	*TRAC*	SgRNA-Cas9 RNP dsDNA-mediated HDR	5-6%	*In vitro* expansion, co-culture killing assay and *in vivo* solid tumor A375 melanoma xenograft in NSG mice
Ode et al. Cancers 2020 (50)	IL13Rα2 CD28	*TRAC*	sgRNA-Cas9RNP dsDNA-mediated HDR	20% (but low expression level)	none
Kath et al, Biorxiv preprint 2021 (51) *In press Mol Ther Meth Clin Dev 2022*	CD19 CD28 zeta	*TRAC* *AAVS1*	sgRNA-Cas9RNP dsDNA-medai ted HDR sgRNA-Cas9RNP dsDNA-medai ted HDR	25-68%(enhanced by drug co-treatments) 10-15%	*In vitro* co-culture cytotoxicity assays & intracellular staining of effector cytokineproduction *In vivo* Nalm-6/fLuc/GFP xenograft in NRG mice
Muller et al. Frontiersin Immunology 2021 (52)	HLA-A2 CD28 zeta	*TRAC*	sgRNA-Cas9RNP dsDNA-mediated HDR	ca. 8-10% (increased during expansion up to 90%)	*In vitro* assays for Treg function (phenotyping, activation status, proliferation suppression) *In vivo* mouse model of GvHD and xenogeneic GvHD
Jing et al. Small Methods 2021 (53)	CD19 or CD19/CD22 CD28 mutZeta or Zeta	*TRAC*	sgRNA-Cas9 RNP Minicircle pDNA-mediated HDR sgRNA-Cas9 RNP AAV-mediated HDR	10-18% (with two Cas9-target sequences in donor template & recombinant Cyclin D protein) No details regarding KI rates	*In vitro* expansion, co-culture cytotoxicity assays. (Nalm-6/fLuc/GFP) *In vivo* Nalm-6/fLuc/GFP xenograft in NSG mice

CLINICAL QUALITY CONTROL AND *IN VITRO* POTENCY ANALYSES OF CAR-T CELLS

In process and end process QC of CAR-T cells gene-modified with RVs/LVs include tests for cell identification (T cell number, cell viability, phenotypic characterization, expression of CAR or other transgenes), impurity measurements and safety (sterility, mycoplasma, endotoxin). More comprehensively, fluorescent-activated cell sorting (FACS) analyses of cell count, cell composition and transduction rate are established using basic panels including staining for CD3/CD4/CD8/CD14/CD16 CD45/CD56. A viability dye, such as 7AAD, is used for exclusion of dead cells. The panel also includes antibodies binding to the extracellular domains of the CAR-specific detection antigens (i.e., binding to the single-chain fragment variable, or scFv) in order to quantify the CAR expression levels and to determine the frequency of CAR^+ cells within the T cell subpopulations (42). In addition, transduction efficiency can also be determined by quantitative PCR. Although highly unlikely due to the use of SIN vectors, testing for the presence of replication-competent RV/LV particles (replication-competent retrovirus (RCRs) or the counterpart RCLs) by quantitative polymerase chain reaction (qPCR) is mandatory. Besides the above described parameters, the DNA encoding the VSV-G viral envelope (that can be carried by the transduced cells) is quantified using real time qPCR (according to the European Pharmacopeia). In addition, *in vitro* potency assays are needed, such as co-culture of CAR-T cells and target cells and measurement of IFN-γ and other cytokines into the medium supernatant in response to T cell activation.

In addition to these validated batch-release QC parameters, several other optional analyses can be included as monitoring only for research purposes. In this respect, FACS-based multiparametric immunophenotyping is used in order to characterize cell subpopulations including fitness of the cells, naïve/effector and central memory T cells as well as expression of co-stimulatory and inhibitory checkpoint receptors. Quantification is done in both the final CAR-T cell product and in the peripheral blood of patients for immune monitoring of CAR-T cell persistence (60).

In contrast to the QC analyses of CAR-T cells generated *via* RV/LV transduction as described above, there is currently little clinical experience with CRISPR-Cas gene edited T cell products (61–64). Overall, gene-edited CAR-T cell products will require the same validated batch-release QC parameters as RV/LV-transduced CAR-T cell products. Clinical release criteria of CAR-T cells engineered by knock-in into the *TRAC* locus should be complemented by a stringent FACS assessment of residual T cells expressing TCR-α/β^{+} in the final product. Additionally, quantification of residual xenogeneic Cas9 protein may be performed to avoid immunogenicity risks during short-expansion protocols (65). In our experience, Cas9 is usually rapidly diluted and degraded after transfection in highly proliferating T cells within just a few days (66).

Preclinical assessments and monitoring for research differ dramatically for gene-edited CAR T cells: The current main safety concern of gene-edited CAR T cells is related to unintended consequences of the nuclease activity, including off-target editing and chromosomal aberrations such as large deletions or translocations. Therefore, preclinical QC must include careful selection and off-target profiling of the gRNA and respective Cas enzyme. Regulators commonly request a set of assays to identify potential off-targets in the genome, which can include *in silico* prediction with computational tools, but must also include unbiased experimental approaches (67), which have been reviewed extensively elsewhere (68). Subsequently, in depth analysis of putative off-target sites must be performed typically by next generation sequencing (NGS). Large on-target deletions as well as other chromosomal arrangements are usually not detected by amplicon-based sequencing of predicted off-targets (69, 70). As standard karyotyping may not have the necessary sensitivity to identify these aberrations, novel NGS-based approaches including CAST-seq, a sensitive assay for identification and quantification of unintended chromosomal rearrangements have been developed (71). Clonality analysis at the preclinical stage may inform on excessive outgrowth of cell clones harboring driver mutations. However, recent evidence from a clinical trial with multiplex-gene edited T cells reported that cells harboring translocations between the intended cut-sites were lost, indicating decreased cell fitness of the aberrant cells (61, 62). Of note, as random integrations of double-stranded DNA templates are rare, HDR-based gene insertion has significantly reduced risk for insertional mutagenesis over RV/LV (49). Past experience with *in vitro* assays for prediction of insertional genotoxicity was established for RV/LV systems and this knowledge can be applied to formally prove the safety of gene editing (72).

Exploiting endogenous transcription programs by gene editing knock-in can further circumvent the need for viral promoters or promoters that lead to supra-physiologic transcriptional activity and that can impact the expression of neighboring genes (73). Therefore, in order to predict and assess long-term safety of gene-edited CAR-T cells, forward-looking and validated assays that allow quantification of off-targets or translocations will be highly important. *In vitro* potency assays for gene-edited CAR-T cells can be largely adopted from previous experience listed above for CAR-T cells generated with RVs/LVs. Remarkably, analysis of cytokines released or cytotoxicity effects after co-culture of CAR-T cells generated by knock-in into *TRAC* with target cells may show increased antigen-specific reactivity, most likely because the TCR^{neg} CAR-T cell product lacks allo-reactive effects. This is an important finding, as TCR^{neg} CAR-T cells can be tested against panels of several patient-derived primary tumor cells. Allogeneic CAR-T cells could be recognized by the recipient patient's immune system which can limit their therapeutic efficacy by preventing cell persistence or reducing effector functions (74). Certain patient populations, including transplant recipients or heavily transfused patients, may already have allo-specific antibodies, which could inactivate off-the-shelf CAR-T cells. Careful matching of healthy-donor or additional genetic interventions may circumvent this problem. Standard assays to evaluate allogeneic cell compatibility including screening of patient serum for presence of antibodies recognizing the major histocompatibility complex (MHC) or other features of the allogeneic CAR-T cell product could be included to select a suitable gene-edited CAR-T cell product based on the patients given allo-sensitization (74).

PRECLINICAL MODELS FOR TESTING CAR-T CELLS AND OFF-THE-SHELF CAR-T CELLS

The *in vivo* response to CAR-T based immunotherapies varies due to substantial molecular heterogeneity and immune suppressive pathways of human tumors and the poorly understood mechanisms that determine CAR-T efficacy as well as to predict side effects (75). Nonetheless, preclinical mouse models used to demonstrate efficacy of CAR-T cells generated after RV/LV transduction were indispensable for their subsequent evaluation in clinical trials and ultimately for their clinical approval.

In general, the first proof-of-concept models use cell-line derived xenograft (CDX) tumor models. Cell lines are commercially available from repositories for comparative studies performed by different laboratories and some molecular pathways associated with cancer in the cell lines are well defined. For example, studies by Brentjens, Sadelain et al, second-generation CD19CAR-T cells (with the CD28zeta costimulatory domain) produced after RV transduction were validated *in vivo*, in SCID-Beige mice implanted intravenously with Nalm-6 cells expressing firefly luciferase (fLuc). The injected cells that develop into B-cell acute lymphoblastic leukemia (ALL) in mice and can be monitored by optical imaging analyses (76). In the Nalm-6 model, ALL disease is systemic with involvement of the bone marrow and central nervous system (76). Studies by Tsukahara et al. evaluated the accumulation of CD19-CAR RV-modified T-cells in Burkitt's lymphoma lesions that develop in lymph node structures after i.v. implantation with the cell line Raji/fLuc (77). The Nalm-6/fLuc and Raji/fLuc xenograft models are useful

models that are still commonly used for comparative evaluation of new designs of CD19 CAR-T cells targeting leukemias and lymphomas generated after viral gene delivery or by gene editing (54, 78).

However, immortalized cancer cell lines, either expanded *in vitro* or grown as xenograft tumor models, cannot reflect the real complexity of human tumors and only provide limited insights into human malignancies (79). The cell lines do not accurately reflect the primary tumor in gene expression and tissue composition as they have been cultivated in laboratories for many years or even decades (80). Therefore, preclinical studies on such lines are not sufficient to offer personalized and well-differentiated CAR-T cell immunotherapy in the future. As a dynamically emerging field, collections of primary tumors grafted into immunodeficient mice, patient-derived xenograft (PDX) mouse models. The mouse strain used for PDX-based studies is a very important determinant for the engraftment of cells for development of xenograft tumors. Several xenograft models are currently exploring non-obese diabetic (NOD)-scid mice or their derivatives because fewer human cells are phagocytosed by mouse macrophages (81). Further, a mutation in the interleukin 2 (IL-2) receptor common gamma chain *(Il2rγ)*, resulted in the NOD-*scid-IL2rγ*$^{(-/-)}$ (NSG) mouse strain lacking murine T and B cells and as well as natural killer (NK) cells (82). Thus, effective engraftment of different tumor cell lines in the NSG and in the related NOD/Shi-*scid IL2rγ*$^{(-/-)}$ (NOG) mouse strains has been adopted in several laboratories for evaluation of CAR-T cells produced after RV/LV transduction (79). Milone, June et al. initiated the innovative use of NSG mice implanted with primary ALL cells to test CD19CAR-T cells with the CD28 and/or 4-1BB intracellular domains generated by LV transduction (83). CD19CAR-T cells containing 4-1BB-ζ showed higher anti-leukemic efficacy compared to CD19CARs containing CD28-ζ signaling receptors and were significantly more persistent *in vivo* (83). Such mouse models using primary tumor samples reveal a more differentiated view on inter- and intra-tumor heterogeneity and more closely resemble the patient's tumor in terms of histopathologic and molecular properties, as well as response to selected therapy. In particular, solid tumor types such as lung cancer (80), breast cancer (84) and gastric cancer (85) associated with vascular, mesenchymal and inflammatory architecture can be better recapitulated *in vivo* with PDX-based xenograft models. These preclinical models reflecting tumor heterogeneity are key for obtaining an understanding of how this heterogeneity affects responses to CAR-T cell immunotherapy and how it may change during treatment both at the genomic and at the phenotypic levels (86–90).

However, although the abovementioned models are extremely useful, they have a major limitation. CDX and PDX models are primarily generated in immunodeficient mice. The absence of essential elements of the human immune system in these mice limits the significance of such models to investigate the role of the immune system and interactions with CAR-T cells in anti-tumor responses, safety and immune toxicity. Immunodeficient mice transplanted with human hematopoietic stem cells (HSCs) are considered "fully humanized" human immune system (HIS) models since, after several months, they reconstitute a humanized immune system. Human HSCs engraft in the bone marrow and then differentiate systemically into several types of human hematopoietic lineages, such as mature leukocytes and myeloid cells, despite the full mismatch between the human leukocyte antigens (HLAs) expressed on the human hematopoietic cells and the mouse MHC expressed on tissues. Humanized mice are new animal models designed to address some of these efficacy and safety risks associated with cytokine release syndrome, thereby making them an attractive alternative for preclinical immunotherapy research (79, 91).

Allogeneic gene-edited TCR^{neg} HLA-I^{neg} HLA-II^{neg} CAR-T cells I will require preclinical efficacy testing in mice expressing HLAs matched to the tumors. Further, since cells lacking expression of HLAs can be targeted by natural killer (NK) cells, humanized mouse models with NK cells and that simulate the tumor microenvironment will substantially facilitate basic and translational research on allogeneic gene-edited CAR-T cell-based immunotherapy (92, 93).

DESIGN OF CLINICAL TRIALS FOR TESTING ALLOGENEIC GENE-EDITED CAR-T CELLS

To date, several thousand patients have been treated or included in trials testing autologous RV/LV transduced CAR-T cells (94). Although allogeneic gene edited CAR-T cells may ease the procurement of CAR-T cells for patients in urgent need, the clinical trials will have to address several new aspects. For CAR-T cells produced after RV/LV transduction, the efficacy of the T cell therapy is associated with parameters such as disease indication, numbers of CAR-T cell product administered per kilogram (95). However, if the efficacy of the allogenic gene-edited CAR-T cells is substantially higher or lower, these associations would need to be re-evaluated. The major advantage of the allogeneic CAR-T cells for clinical study designs is that the product of one donor can be tested simultaneously in different subjects, which may result in more consistent data per donor-derived product. However, there may also be significant batch-to-batch product differences due to donor characteristics. Of note, one study could demonstrate that healthy donor-derived CD19CAR-T cells outperformed autologous leukemia patient-derived CAR-T cells in an *in vivo* xenograft model (96). Multiple reasons could explain the phenomenon: i) damage introduced by prior chemotherapy regimen, because patients were refractory to standard of care; ii) patient-intrinsic defects in effector immunity, which contributed to cancer development in the first place.

Importantly, clinical trials with autologous CAR-T cells produced after RV/LV transduction have established a clear toxicity profile, in particular cytokine release syndrome (CRS) and immune-effector cell associated neurotoxicity (ICANS) (97). With optimized clinical management standards, the rates of

severe CRS and ICANS were markedly reduced and the results have been crucial to further expand the extended clinical application of CAR-T cells, e.g. in an outpatient setting. Of note, the timing of these complications can vary substantially between different CAR-T products, even for the same target. For BCMA targeted CAR-T therapies, CRS occurred within 1-7 days after infusion (98, 99). Similarly, for CD19 CAR-T cell therapies, the rate of neurological complications showed striking differences between two different cell products (100, 101). Thus, allogeneic gene-edited CAR-T cells will need to be benchmarked against these clinical results for CRS and ICANS obtained with autologous CAR-T cells, particularly because new immune-toxicities may emerge.

Regarding geno-toxicity, CAR-T cells generated with RV/LV transduction have shown an excellent safety profile. However, a recently, a trial of HLA-matched allogeneic CAR-T cells generated with the *hyperPiggyBac* transposon system, two out of ten children developed CAR-T derived lymphomas (102). Detailed genetic investigations were performed on biopsy material from tumor cells to elucidate the underlying pathogenesis. A high frequencies of genomic integration sites were found (16). Notably, in both lymphoma cases, *BACH2*, a gene involved in regulation of T cell plasticity, was downregulated with integration sites found within the *BACH2* locus (102). Although the mechanism of gene delivery by *hyperPiggyBac* and non-viral gene editing are different, these occurrences provide a note of caution regarding genotoxicity, as some loci may be hot-spots for insertional mutagenesis *via* HDR mechanisms.

Graft-versus-host-disease (GvHD) is not an issue in autologous CAR-T trials, however, if TCRs remain intact in allogeneic CAR-T cells, GvHD could become an additional relevant toxicity. In this case, it may have a different clinical presentation compared to GvHD presentation after allogeneic stem cell transplantation. Biopsies in affected tissues could inform about relevant cellular infiltrates. Further, lymphodepletion regimens may have to be optimized to enable a high engraftment of allogeneic CAR-T cells.

In addition to response rate and progression-free survival as typical efficacy endpoints, CAR-T cell persistence and clonality are important parameters to assess in clinical trials. This is typically done by assessing the CAR on T cells by flow cytometry or PCR amplification of the corresponding gene insertion in peripheral blood mononuclear cells. In contrast to the early expansion phase, quantification may be hampered at later stages because of the detection limit of these assays, in particular when CAR-T cells migrate to tissue niches. There is much greater genetic diversity between host cells and gene-edited allogeneic CAR-T cells which may hamper their persistence, but this could also be exploited for detection purposes. In addition to analysis of the CAR, analysis of HLA chimerism could be performed. Although MHC mismatches can be potentially eliminated by the knock-out of HLA class I and II, minor histocompatibility complexes and other polymorphic proteins can still potentially lead to allo-sensitization and rejection of the gene–edited CAR-T cells (74).

While these new complexities and additional safety risks of allogeneic CAR-T cells must be acknowledged, there are also significant advantages: Allogeneic CAR-T cells may be produced in large batches from healthy donor apheresis products and be made available as "off-the-shelf" products. This will dramatically shorten the delay between the decision to initiate CAR-T therapy and the actual delivery of the treatment. Currently, it may take up to 3 months from obtaining a production slot, organizing the apheresis, and shipping to the cell manufacturing facility, receiving the product, and infusing into the patient. Allogenic CAR-T cells may be available within a few days or even hours if stored at the site of care. In addition, the production of several batches from a single apheresis may substantially lower the cost of this treatment modality and thus alleviate the financial burden of CAR-T therapy.

PAVING THE WAY FOR GENE EDITED CAR-T CELLS: OUTLOOK AND CONCLUDING REMARKS

When considering a switch towards more innovative gene delivery approaches, i.e. from RV/LV systems to gene-edited non-viral CAR-T cells, several challenges need to be addressed until their broad clinical application:

- CAR-T cell performance will depend on the nature and location of transgene insertion. Identification of the optimal locus to allow for reasonable CAR expression level and its physiological regulation is paramount. As cargo payloads for HDR at a single locus are limited to the DNA repair mode (i.e., HDR), compact CAR formats and multicistronic knock-ins may be a first step toward enhanced CAR-T cells. However, improving the respective genetic cargo capacity using novel gene editors (e.g. CRISPR-integrases) or enhancing our ability for multiple knock-ins in a single CAR-T cell product will be needed for certain indications (e.g. solid cancers).
- The quality and safety of gene-edited/knocked-in CAR-T cells will largely depend on the gene editors used and what loci are targeted. Careful designs of gRNAs and HDRTs must be performed to avoid off-target effects and prevent insertional mutagenesis.
- The feasibility for clinical use is presently still limited by the relatively low number of recovered gene edited CAR-T cells as discussed above. New manufacturing and downstream technologies are required to decrease toxicity during gene editing. These could include, improved physical transfection systems, novel chemical transfection agents (e.g. lipid-nanoparticles) or pharmacological strategies to lower the cytotoxic effects of DNA double strand breaks that occur in the editing process. Furthermore, automated cell production, efficient expansion of T cells with favorable differentiation state and viable cell banking (for off-the-shelf purposes) are needed for success at clinical stage.
- Ultimately, the clinical potency of gene edited CAR-T cells will be strongly correlated with their *in vivo* activation upon antigen detection and persistence for long-term antitumor surveillance.

Herein, deducting the optimal strategy to improve allogeneic CAR-T cell persistence in immunocompetent hosts will be key to success: Choosing the right tool for genetic engineering, establishing advanced host conditioning protocols and potentially adding HLA-matching procedures are possible ways forward. The prospect of future off-the-shelf products will also require solid logistics for manufacturing, cryopreservation and distribution.

- The development of predictive *in vitro* assays and humanized mouse systems must be further enforced by the community to benchmark antitumor efficacy and safety (e.g. CRS, GvHD) of novel gene-edited CAR-T cell candidates. Due to the abundance of potential strategies to enhance gene-edited CAR-T cells in the future, stable and reproducible models are paramount to prioritize them in the translational efforts and early clinical trials.

Following the philosopher George Santayna's wise words "those who cannot remember the past are condemned to repeat it", the vast amount of knowledge acquired with CAR-T cells produced with viral systems will have to be remembered so that we are not condemned to experience again the past issues and, instead, to forthrightly improve the efficacy, safety and availability of gene edited CAR-T cells.

AUTHOR CONTRIBUTIONS

RS created the first concept of the manuscript, created the figure and tables, organized and distributed the headings and wrote the introduction and headings 1, 2, 6, 8 and 5, and revised the final manuscript. DW wrote headings 4, 5, 7 and 8, completed table 2, and revised the final manuscript. MC wrote headings 1 and 6. CS, wrote heading 7. UK wrote headings 2 and 3, and revised the final manuscript. All authors agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: RS has filed a patent application for generation of CAR-T cells targeting lytic herpes infections and is a founding shareholder and scientific consultant of BioSyngen/Zelltechs Lpt Ltd. DW has filed multiple patent applications on CRISPR-Cas gene editing and adoptive T cell therapy. CS is consultant for Bristol Myers Squibb, Janssen and Novartis regarding CAR-T cell therapy and is participating in clinical CAR-T studies from Bristol Myers Squibb, Janssen, Novartis and Miltenyi Biotec and is cooperating with Miltenyi Biotec in the production of CAR-T cells. UK states that she is a consultant in immuno-oncology for AstraZeneca, Affimed, Glycostem, GammaDelta and Zelluna, and that she has collaborations with Novartis and Miltenyi Biotec regarding the production of CAR-T cells.

MC is co-inventor in granted and filed patents describing CAR-T cells with additional functions to counteract the tumor microenvironment.

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DNAM-1-chimeric receptor-engineered NK cells, combined with Nutlin-3a, more effectively fight neuroblastoma cells *in vitro*: a proof-of-concept study

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Adoptive transfer of engineered NK cells, one of clinical approaches to fight cancer, is gaining great interest in the last decade. However, the development of new strategies is needed to improve clinical efficacy and safety of NK cell-based immunotherapy. NK cell-mediated recognition and lysis of tumor cells are strictly dependent on the expression of ligands for NK cell-activating receptors NKG2D and DNAM-1 on tumor cells. Of note, the PVR/CD155 and Nectin-2/CD112 ligands for DNAM-1 are expressed primarily on solid tumor cells and poorly expressed in normal tissue cells. Here, we generated human NK cells expressing either the full length DNAM-1 receptor or three different DNAM-1-based chimeric receptor that provide the expression of DNAM-1 fused to a costimulatory molecule such as 2B4 and CD3ζ chain. Upon transfection into primary human NK cells isolated from healthy donors, we evaluated the surface expression of DNAM-1 and, as a functional readout, we assessed the extent of degranulation, cytotoxicity and the production of IFNγ and TNFα in response to human leukemic K562 cell line. In addition, we explored the effect of Nutlin-3a, a MDM2-targeting drug able of restoring p53 functions and known to have an immunomodulatory effect, on the degranulation of DNAM-1-engineered NK cells in response to human neuroblastoma (NB) LA-N-5 and SMS-KCNR cell lines. By comparing NK cells transfected with four different plasmid vectors and through blocking experiments, DNAM-1-CD3ζ-engineered NK cells showed the strongest response. Furthermore, both LA-N-5 and SMS-KCNR cells pretreated with Nutlin-3a were significantly more susceptible to DNAM-1-engineered NK cells

than NK cells transfected with the empty vector. Our results provide a proof-of-concept suggesting that the combined use of DNAM-1-chimeric receptor-engineered NK cells and Nutlin-3a may represent a novel therapeutic approach for the treatment of solid tumors, such as NB, carrying dysfunctional p53.

KEYWORDS

NK cells, immunotherapy combined therapy, activating receptor, chimeric receptor, adoptive transfer of NK and CAR-NK cells, immunomodulation

Introduction

NK cells are cytotoxic lymphocytes that participate in innate immune responses and recognize virus-infected and transformed cells without prior specific sensitization or fine specificity. Recognition by NK cells of tumor or infected cells is mediated by specific activating receptors (1) that include NKG2D and the accessory molecule DNAX (DNAM-1, CD226) (2). Ligands for the NKG2D receptor are cell stress-inducible molecules such as MICA, MICB (3), and a group of ULBPs (4), whereas ligands for the DNAM-1 receptor are Nectin-2 (CD112) and the poliovirus receptor (PVR, CD155 or nectin-like molecule) (5).

Ligands for the DNAM-1 receptor are expressed at high levels in response to cellular stress (6) in many tumor cell types, especially in solid tumors of epithelial and neuronal origin (7–11), and in virus-infected cells (2, 12), including those infected by SARS-CoV-2 (13). The expression of these ligands can vary, determining the extent to which tumor or infected cells are able to evade the NK cell-mediated immune response (2, 8, 14).

Due to the reduced expression of NK cell-activating receptors in patients with hematological (15–17) and solid tumors (18–20), and the poor infiltration and impaired functions of NK cells in tumor microenvironment (TME) (21), multiple strategies have been adopted to enhance NK cell-mediated anticancer functions. Several approaches aimed at increasing the expression for NK cell-activating receptors (22–24) and their ligands (25, 26), or suppressing NK cell-inhibitory receptors (27–29) are emerging in preclinical studies and several clinical trials (ClinicalTrials.gov and Supplementary Table S1). However, if advanced results have been obtained for hematopoietic tumors, in the context of solid tumors many efforts are still needed. Furthermore, adoptive transfer of *extra vivo* expanded and activated NK cells in autologous and allogeneic settings, in combination with monoclonal antibodies (mAbs) recognizing immune checkpoint molecules (30, 31), activating cytokines and immunomodulatory drugs (25, 32) or engineered for Chimeric Antigen Receptors (CARs) (33), emerges as one of the first-line anti-cancer cell immunotherapy strategies with an increasing number of therapeutic clinical successes [(34–36), ClinicalTrial.gov and Supplementary Table S1].

The effective activation of DNAM-1 ensures a proper signal to predispose NK cells to induce target cell lysis through cytotoxic granule exocytosis and cytokine production (37). For this reason and based on our previous studies (2, 25, 26, 38–41), here we engineered primary human NK cells to express the DNAM-1-chimeric receptor and explored their ability to recognize *in vitro* target cells including K562 and the LA-N-5 and SMS-KCNR NB cell lines. Human DNAM-1 is a type I transmembrane glycoprotein of ~65 kiloDalton (kDa) containing two Ig-like domains; it is composed of an 18 amino acid (aa) leader sequence, an extracellular domain of 230 aa with two Ig-like C2-set domains, a transmembrane domain of 28 aa and a cytoplasmic region of 60 aa containing two residues (Tyr322 and Ser329) involved in DNAM-1-ligand mediated signal transduction. To explore the efficacy of DNAM-1-chimeric receptor-engineered NK cells, we designed plasmid vectors containing the sequence expressing for DNAM-1 in frame with that for costimulatory molecules such as CD3ζ and 2B4. CD3ζ is a signal-transducing molecule that contains 3 immunoreceptor tyrosine-based activation motifs (ITAMs) and is linked to several activating receptors expressed on the surface of NK cells (42, 43). It provides ITAMs for phosphorylation and activation of T cells expressing CARs, often referred to as first-generation CARs. 2B4 is a member of the CD2 family and recruits SAP and Fyn through cytoplasmic tyrosine motifs. The costimulatory sequence of CD3ζ is an intra-cytoplasmic domain of 112 aa, while the intra-cytoplasmic domain of 2B4 is of 119 aa. We generated four constructs containing: i) the full-length (FL) DNAM-1 sequence, ii) the FL-DNAM-1 sequence in frame with the CD3ζ (52-164 aa) sequence, iii) the DNAM-1 (1-275 aa) sequence, missing the Tyr322 and Ser329 residues, in frame with the CD3ζ (52-164 aa) sequence, iv) the FL-DNAM-1 sequence in frame with both 2B4 (251-370 aa) and CD3ζ (52-164 aa) sequences.

In addition, the potential of NK cells engineered with DNAM-1-based constructs to recognize target cells was evaluated in combination with Nutlin-3a, a small-molecule

known to antagonize MDM2, thereby restoring p53 function (44) and, as we previously reported (26), having a strong immunomodulatory function in NB cells. The increased susceptibility of the NB LA-N-5 and SMS-KCNR cell lines after *in vitro* treatment with Nutlin-3a to DNAM-1-engineered NK cells provides a proof-of-concept to design an innovative immunotherapeutic protocol to be adopted for a novel NK cell-based clinical approach to treat solid tumors with dysfunctional p53.

Materials and methods

NB cell lines, NK cells and reagents

The following human cell lines were used in this study: human erythro-leukemia cell line K562 (ATCC); NB cell line LA-N-5 (the Leibniz-Institute DMSZ), NB cell line SMS-KCNR (Children's Oncology Group Cell Culture). The cell lines were characterized by i) authentication by PCR-single-locus-technology (Eurofins-Genomic, Ebersberg, Germany) according to the instructions of the manufacturer, ii) array CGH and iii) routinely tested to confirm the absence of Mycoplasma by Mycoplasma Detection kit (Venor-GeM Advance). Cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Thermo Fisher Scientific), 2 mM glutamine, 100 mg/ml penicillin and 50 mg/ml streptomycin (Euroclone S.p.A.).

Human NK cells were isolated from blood of healthy donors by the RosetteSep NK-cell enrichment mixture method kit (StemCell Technologies) and Ficoll-Paque Plus (Lympholyte Cedarlane) centrifugation. NK cells were routinely checked for $CD14^-$ $CD19^-$ $CD3^-$ $CD56^+$ immunophenotype and the expression of activating receptors NKG2D, DNAM-1, NKG2C, the maturation marker CD57, the inhibitory receptor NKG2A, immune checkpoint receptors TIGIT and PD-1 and a panel of inhibitory/activating KIRs such as KIR2DL1/2DS1, KIR2DL2/L3/S2, and KIR3DL1 by flow cytometry. The gate strategy adopted to analyze NK cells is shown in Supplementary Figure S1. NK cells with greater than 90% purity and positive for all four inhibitory receptors were suspended in NK MACS medium (Miltenyi Biotec) supplemented with NK MACS Supplement, AB serum and 500 IU/mL of recombinant human IL-2 (PeproTech). NK cells were cultured at 200 x 10^3 cells/well in 96-well round-bottom plates at 37°C, divided every three days, after a week transferred in cell culture flask T-25 at 2x10^6 cells/ml, and used up to 20 days after isolation for experiments. NK cells, expanded *in vitro* at a rate of 15 to 20 times at day 20, were transfected with DNAM-1-based vectors to obtain DNAM-1-engineered NK cells (as described below).

LA-N-5 and SMS-KCNR cells were cultured at 37°C in 6-well plates and, at 70% confluence, treated with Nutlin-3a (Cayman Chemical, dissolved in DMSO at 10 mmol/L) at 2 µmol/L or DMSO as control (0.2 µl/ml) for 48 hours. LA-N-5 and SMS-KCNR cells treated with Nutlin-3a or DMSO were tested for the expression of PVR/CD155 and Nectin-2/CD112 (26) and used as target cells in NK cell degranulation assay.

Antibodies and flow cytometry

The following antibodies for flow cytometry were used: anti-CD107a-APC (H4A3), anti-CD3-AF700 (UCHT1), anti-CD3-PE-CF594 (UCHT1), anti-CD56-PE-Cy7 (B159), anti-CD56-PerCP Cy5.5 (B159), anti-CD57-PE (NK-1), anti-CD45-PE-Cy5 (HI30), anti-NKG2D-BV605 (1D11), anti-NKG2D-PE-CF594 (1D11), anti-CD16-BV510 (3G8), anti-DNAM-1-BV786 (DX11), anti-PD-1-BV421 (MIH4), anti-IFNγ-BV650 (4S.B3), anti-CD14-BV605 (M5E2), anti-CD19-BV650 (SJ25C1) purchased from BD Biosciences; anti-DNAM-1-APC (11A8), anti-NKp46-PE-Cy7 (9E2), anti-TNFα-AF700 (Mab11), anti-CD96-APC (NK92.39) purchased from Biolegend; anti-NKp30-PE (Z25), anti-KIR2DL1/2DS1-PE Cy5.5 (EB6B), anti-KIR2DL2/L3/S2-PE (GL-183) purchased from Beckman Coulter; anti-NKG2A-AF700 (131411), anti-KIR3DL1-APC (DX9) purchased from R&D Systems; anti-NKG2A-FITC (REA110), anti-NKG2C-PE (REA205) purchased from Miltenyi; anti-TIGIT-APC (MBSA43) purchased from eBioscience. All these antibodies were used according to the manufacturers' protocol. Prior to surface staining, NK cells were pre-stained with Live/Dead™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen). Flow cytometry was performed by using FACSCantoTM II (BD Biosciences) or Cytoflex (Beckman Coulter) and analyzed by FlowJo Software.

Plasmids, DNAM-1-based constructs and NK cell transfection

Four synthetic genes were designed encoding human full length (FL) DNAM-1 and DNAM-1-based chimeric receptors: 1) FL-DNAM-1; 2) FL-DNAM-1 in frame with CD3ζ (52-164 aa); 3) DNAM-1 (1-275 aa, missing the Tyr322 and Ser329 residues) in frame with CD3ζ (52-164 aa); 4) FL-DNAM-1 in frame with both 2B4 (251-370 aa) and CD3ζ (52-164 aa). DNA sequences encoding the four constructs, optimized for human codon usage, were synthesized by Geneart, Thermofisher. The synthetic genes were then cloned into the expression vector pVJ under the control of the human CMV promoter. Aminoacid sequences of the four chimeric constructs are reported in Supplementary Material and Methods.

Human primary NK cells were *in vitro* expanded by NK MACS medium (Miltenyi Biotec) supplemented with NK MACS supplement and IL-2 and transfected by Amaxa™ P3 Primary Cell 4D-Nucleofector™ X Kit L (Lonza) through Nucleofector® Device (Lonza) with DNAM-1-based constructs (5µg) or the

empty vector as control, according to the manufacturing protocol. The pmaxGFP™ Vector provided by the Kit was used to transfect primary NK cells in order to evaluate the transfection efficacy (around 20%, according to the manufacturing protocol). At 24 hours after the transfection, DNAM-1-engineered NK cells were assessed for DNAM-1 surface expression, as well as other receptors, and used for experiments of degranulation, cytotoxicity and cytokine production assays by flow cytometry.

DNAM-1-engineered NK cell degranulation, cytotoxicity and cytokine production assays

The functions of DNAM-1-engineered NK cells were tested by degranulation, cytotoxicity and cytokine production assays. DNAM-1-engineered NK cells were co-cultured with K562, LA-N-5 or SMS-KCNR target cells at 1:1 ratio for 3 hours, in complete medium in the presence of anti-CD107a (diluted 1:100). During the last 2 hours, GolgiStop (BD Bioscence) was added at 1:500 dilution. Cells were firstly pre-stained with Live/Dead Kit (L/D), stained with anti-CD56, anti-CD16, anti-CD3, anti-CD14, anti-CD19, anti-CD45 and, then, the expression of CD107a was evaluated in the $CD14^-$ $CD19^-$ $CD3^-CD56^+$ $CD16^+$ $CD45^+$ subset by flow cytometry. For the blocking experiments, NK cells were pretreated for 20 min with 25 µg/mL of neutralizing anti-DNAM-1 (DX11, BD-Pharmingen) or anti-NKG2D (149810, R&D Systems) before co-culture with K562 target cells.

The DNAM-1 engineered NK cell cytotoxic activity was tested by a standard 4-hour ^{51}Cr-release assay. K562 cells were labelled with ^{51}Cr [Amersham International; 100µCi (3.7 MBq)/1 x 10^6 cells] and were co-cultured (5 x 10^3) with DNAM-1 engineered NK cells at different effector-target (E:T) cell ratios, in 96-well plates round bottom in triplicates, and incubated at 37°C. At 4 hours of incubation, 25µL supernatant were removed, and the ^{51}Cr release was measured with TopCount NXT beta detector (PerkinElmer Life Sciences). The percentage of specific lysis by counts per minute (cpm) was determined as follows: 100 x (mean cpm experimental release – mean cpm spontaneous release)/(mean cpm total release – mean cpm spontaneous release). Specific lysis was converted to lytic units (L.U.) calculated from the curve of the percentage lysis and defined as the number of NK cells required to produce 20% lysis of 10^6 target cells during the 4-hour incubation.

IFNγ and TNFα production assays were performed by co-culturing NK cells with K562 target cells at 1:1 ratio, in complete medium at 37°C for 12 hours. After 1 h, Brefeldin A (Sigma-Aldrich) 10 µg/ml was added to the co-culture. Cells were pre-stained with L/D, surface-stained as for the degranulation assay (as described above), fixed with 1% paraformaldehyde, permeabilized with Permeabilizing Solution (BD), stained with anti-IFNγ and -TNFα antibodies and analyzed in the $CD14^-$ $CD19^-$ $CD3^-CD56^+$ $CD45^+$ subset by flow cytometry.

Statistical analysis

For all data, statistical significance was evaluated with the non-parametric Mann-Whitney test. Normalized values were analyzed for correlation by the regression analysis using GraphPad software. *P* values not greater than 0.05 were considered to be statistically significant.

Results

Enhanced DNAM-1-engineered NK cell degranulation, cytotoxicity and cytokine production against K562 cells

First, we assessed whether transfection of NK cells with our four DNAM-1-based constructs [FL-DNAM-1, FL-DNAM-1-CD3ζ, DNAM-1 (1-275)-CD3ζ, FL-DNAM-1-2B4-CD3ζ] (Figure 1) could affect the surface expression of DNAM-1. NK cells engineered with all four DNAM-1-based constructs showed significantly higher levels of DNAM-1 expression than NK cells transfected with empty vector, as evaluated by flow cytometry (Figure 2). Furthermore, both FL-DNAM-1- and FL-DNAM-1-CD3ζ-engineered NK cells showed significantly higher levels of DNAM-1 expression than NK cells engineered with DNAM-1 (1-275)-CD3ζ and FL-DNAM-1-2B4-CD3ζ constructs (Figure 2B). In addition, the expression of activating receptors NKG2D, NKp30 (45) and NKp46 (46), the immune checkpoint molecules PD-1 and TIGIT (47), the inhibitory receptor CD96 which, together with TIGIT, is known to compete with DNAM-1 for binding to the same ligands (2), as well as the marker CD57 associated with terminal differentiation of NK cells (48) was unchanged in NK cells engineered with all four DNAM-1-based constructs (Figure 2C). These data suggest that in our model i) the intracellular domain of DNAM-1 stabilizes DNAM-1 surface expression; ii) the 2B4 sequence, in frame with DNAM-1 and CD3ζ sequences, partially destabilizes DNAM-1 expression levels; iii) the DNAM-1-based construct transfection does not affect the expression of other receptors.

Then, DNAM-1-engineered NK cells were analyzed for degranulation and cytotoxicity assays against K562 cells. As shown in Figures 3A, B, Supplementary Figure S2, the percentages of CD107a and cytotoxicity were significantly higher in NK cells transfected with all four DNAM-1 constructs than in those transfected with the empty vector. In addition, FL-DNAM-1-CD3ζ-engineered NK cells showed significantly higher CD107a expression and cytotoxicity compared to FL-DNAM-1-, DNAM-1 (1-275)-CD3ζ- and FL-DNAM-1-2B4-CD3ζ-engineered NK cells (Figures 3B,

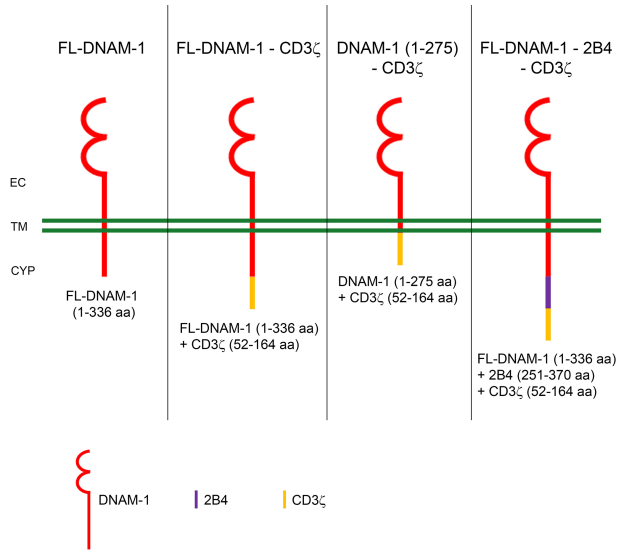

FIGURE 1
Schematic diagram of DNAM-1-based chimeric receptors. The amino acids (aa) sequences of DNAM-1 (red), CD3ζ (yellow) and 2B4 (blue) are shown below each diagram of DNAM-1-based chimeric receptors. EC, extracellular; TM, transmembrane; CYP, cytoplasm.

Supplementary Figure S2). These results suggest that in our model CD3ζ chain conferred a stronger signal to DNAM-1-engineered NK cells than to NK cells transfected with FL-DNAM-1 construct alone, leading to increased degranulation and cytotoxicity in response to a natural target such as K562 cells. However, it was less efficient in the absence of the DNAM-1 intracellular domain or in presence of 2B4 expression.

Furthermore, blocking experiments demonstrated that the chimeric DNAM-1 receptor was involved in the degranulation of NK cells engineered with DNAM-1-based constructs. Indeed, neutralization of DNAM-1 resulted in a drastic reduction of degranulation, with no differences between DNAM-1-engineered NK cells. In contrast, after neutralization of NKG2D, although resulting in a significant reduction of degranulation in all conditions, the differences between the engineered NK cells were comparable to those without neutralizing antibody, as a result of the remaining different contribution of DNAM-1 to the degranulation signal in each condition (Figures 3A, B).

In addition, DNAM-1-engineered NK cells were analyzed for the production of cytokines such as IFNγ and TNFα in response to K562 cells, by flow cytometry. As shown in Figures 3C, D, the percentage of both IFNγ and TNFα was significantly higher in NK cells transfected with FL-DNAM-1, FL-DNAM-1-CD3ζ and DNAM-1 (1-275)-CD3ζ constructs than in those transfected with the empty vector. In addition, FL-DNAM-1-CD3ζ-engineered NK cells showed significantly higher production of both cytokines IFNγ and TNFα compared to FL-DNAM-1-, DNAM-1 (1-275)-CD3ζ- and FL-DNAM-1-2B4-CD3ζ-engineered NK cells (Figure 3D). Therefore, the FL-DNAM-1-CD3ζ construct was shown to be the most effective to confer enhanced degranulation, cytotoxicity and production of both IFNγ and TNFα to NK cells in response to K562 cells.

Nutlin-3a enhances the susceptibility of LA-N-5 and SMS-KCNR cells to DNAM-1-engineered NK cells

Next, we evaluated the degranulation of DNAM-1-engineered NK cells in response to LA-N-5 and SMS-KCNR NB cells. In addition, we evaluated the combined effect of DNAM-1-engineered NK cells with Nutlin-3a, a drug that antagonizes with MDM2 and, consequently, restores the functions of p53 (44), which is known to act as a transcription factor for genes encoding ligands for NK cell activating receptors (49), including *PVR* for DNAM-1 receptor as we reported (26). To test the effect of Nutlin-3a treatment on the susceptibility of NB cells to DNAM-1-engineered NK cells, LA-N-5 and SMS-KCNR cells were treated with Nutlin-3a or DMSO as a control and used as target cells for DNAM-1-engineered NK cell degranulation assay. As shown in Figure 4, the CD107a percentage of NK cells transfected with the four DNAM-1 constructs in response to LA-N-5 or SMS-KCNR cells (treated with DMSO as control) was significantly higher than that of NK cells transfected with empty vector (Figure 4B). Furthermore, the degranulation of FL-DNAM-1-CD3ζ-, DNAM-1 (1-275)-CD3ζ– and FL-DNAM-1-2B4-CD3ζ–engineered NK cells in

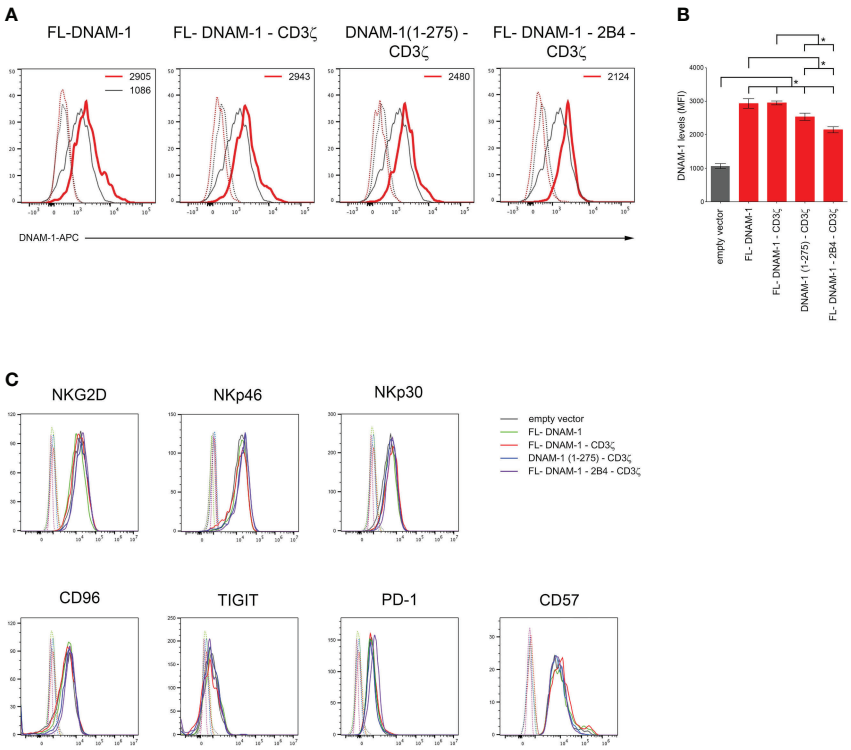

FIGURE 2
Enhanced surface expression of DNAM-1 receptor on DNAM-1-engineered NK cells. **(A)** Flow cytometry analysis of DNAM-1 surface expression in NK cells transfected with the indicated DNAM-1-based vectors (red) compared to that of NK cells transfected with empty vector (gray). Isotype-matched negative control antibody is displayed as gray and red dashed lines for NK cells transfected with empty vector and each indicated DNAM-1-based vector, respectively. A representative experiment of eight performed in NK cells isolated from independent healthy donors and transfected with DNAM-1-constructs is shown. **(B)** Summary of flow cytometry analyses performed in NK cells isolated by eight independent healthy donors and transfected with DNAM-1-based constructs. MFI, mean fluorescence intensity. Mean ± SD; *$p<0.05$; p value (two-tailed non-parametric Mann-Whitney test). **(C)** Flow cytometry analysis of NKG2D, NKp46, NKp30, CD96, TIGIT, PD-1 and CD57 surface expression in NK cells transfected with the indicated DNAM-1-based vectors. Isotype-matched negative control antibody is displayed as dashed lines for NK cells transfected with empty vector and each indicated DNAM-1-based vector. A representative experiment of seven performed in NK cells isolated from independent healthy donors and transfected with DNAM-1-based vectors is shown.

response to LA-N-5 or SMS-KCNR cells treated with DMSO was significantly higher than that of NK cells transfected with FL-DNAM-1 construct. Interestingly, the degranulation of FL-DNAM-1-CD3ζ-engineered NK cells was significantly higher than that of NK cells transfected with FL-DNAM-1-2B4-CD3ζ construct in response to both NB cell lines. These data confirm that CD3ζ chain conferred DNAM-1 constructs with a greater potential to mediate NK cell degranulation in response to NB cell lines such as LA-N-5 and SMS-KCNR cells, which was instead less efficient in the presence of 2B4. Furthermore, Nutlin-3a was able to significantly increase the susceptibility of both LA-N-5 and SMS-KCNR cells to NK cells under all conditions (Figure 4B), in agreement with our previous data (26). In response to LA-N-5 or SMS-KCNR cells treated with Nutlin-3a, a significant increase in degranulation was found in NK cells transfected with DNAM-1-based constructs compared to NK cells transfected with empty vector, reflecting the data in response to LA-N-5- or SMS-KCNR treated with DMSO (Figure 4B). Interestingly, in response to LA-N-5 or SMS-KCNR cells treated with Nutlin-3a, while a significant increase in degranulation was assessed in NK cells transfected with the three DNAM-1-CD3ζ-based constructs compared to NK cells transfected with the FL-DNAM-1 construct, no differences was revealed between NK cells transfected with the three DNAM-1-CD3ζ-based constructs (Figure 4B). These data suggest that Nutlin-3a increased the susceptibility of LA-N-5 and SMS-KCNR cells to NK cells transfected with DNAM-1-CD3ζ-based constructs regardless of the presence of the DNAM-1 intracellular domain or 2B4 sequences. Overall, these data indicated that in our model i) DNAM-1-engineered NK cells had an enhanced ability to recognize NB cell lines such as LA-N-5 and SMS-KCNR, ii) that the CD3ζ sequence in frame with DNAM-1 further enhanced this function, which was maintained by DNAM-1 intracellular domain, but did not require the 2B4 costimulatory sequence. Finally, Nutlin-3a treatment of NB LA-N-5 and SMS-KCNR cells maximized the degranulation of

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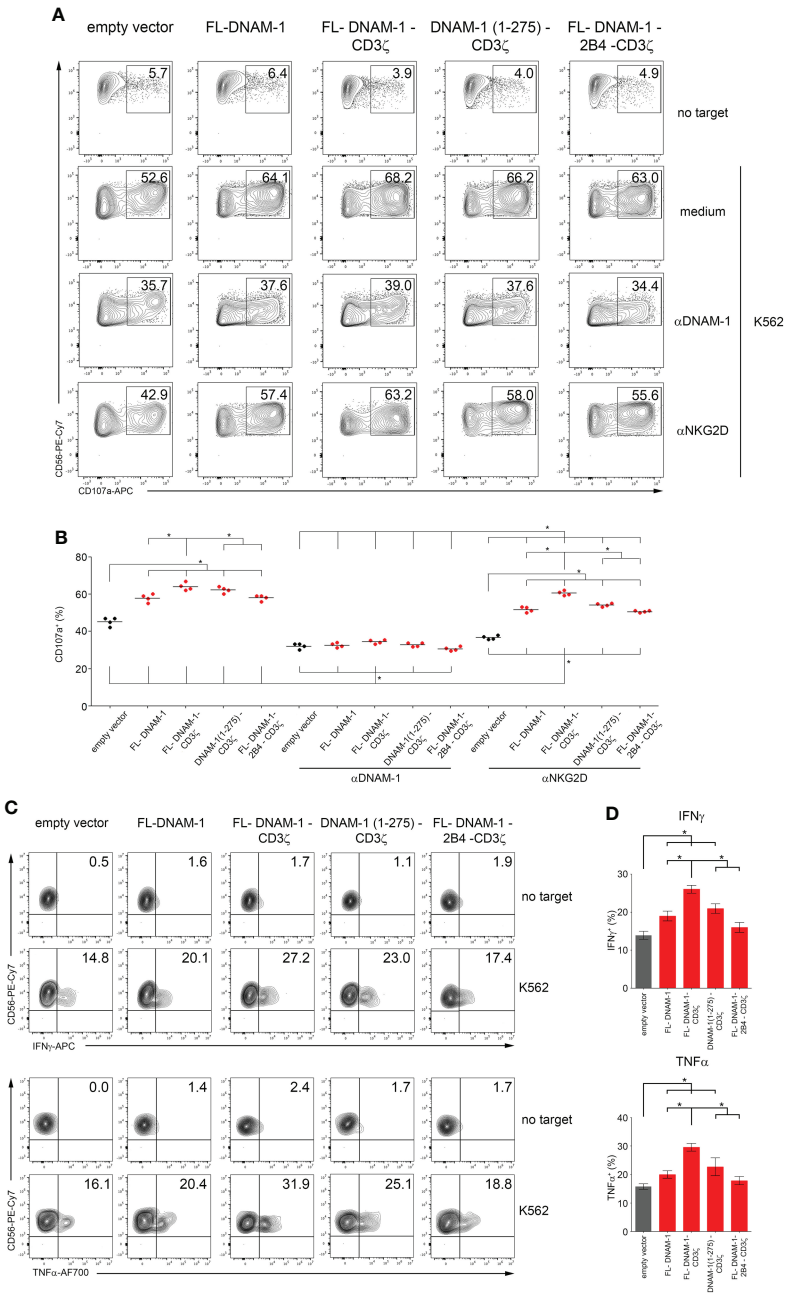

FIGURE 3
Enhanced degranulation and cytokine production of DNAM-1-engineered NK cells against K562. DNAM-1-engineered NK cells were tested for degranulation and cytokine production assays in response to K562. **(A)** Degranulation of NK cells engineered for the empty vector as control or for the four DNAM-1-based constructs as indicated, in the absence (medium) or presence of neutralizing anti-NKG2D or anti-DNAM-1 antibodies, measured as CD107a expression on co-culture with or without K562. The percentages of CD107a in NK cell subset are indicated in each plot. A representative experiment of four performed in NK cells isolated from independent healthy donors and transfected with DNAM-1-constructs is shown. **(B)** Summary of degranulation of NK cells isolated from four independent healthy donors, transfected with DNAM-1-constructs, in the absence or presence of neutralizing antibodies. Dots correspond to the percentage of $CD107a^+$ NK cells from each healthy donor transfected with the indicated DNAM-1-based constructs; horizontal bars indicate the mean; *$p<0.05$; p value (two-tailed nonparametric Mann-Whitney test). **(C)** DNAM-1-engineered NK cells production of IFNγ and TNFα in co-culture with or without K562. The percentages of IFNγ+ and TNFα+ in NK cell subset are indicated in each plot. A representative experiment of four performed in NK cells isolated from independent healthy donors and transfected with DNAM-1-constructs is shown. **(D)** Summary of cytokine production of NK cells isolated from four independent healthy donors and transfected with DNAM-1-based constructs. Mean ± SD; *$p<0.05$; p value (two-tailed non-parametric Mann-Whitney test).

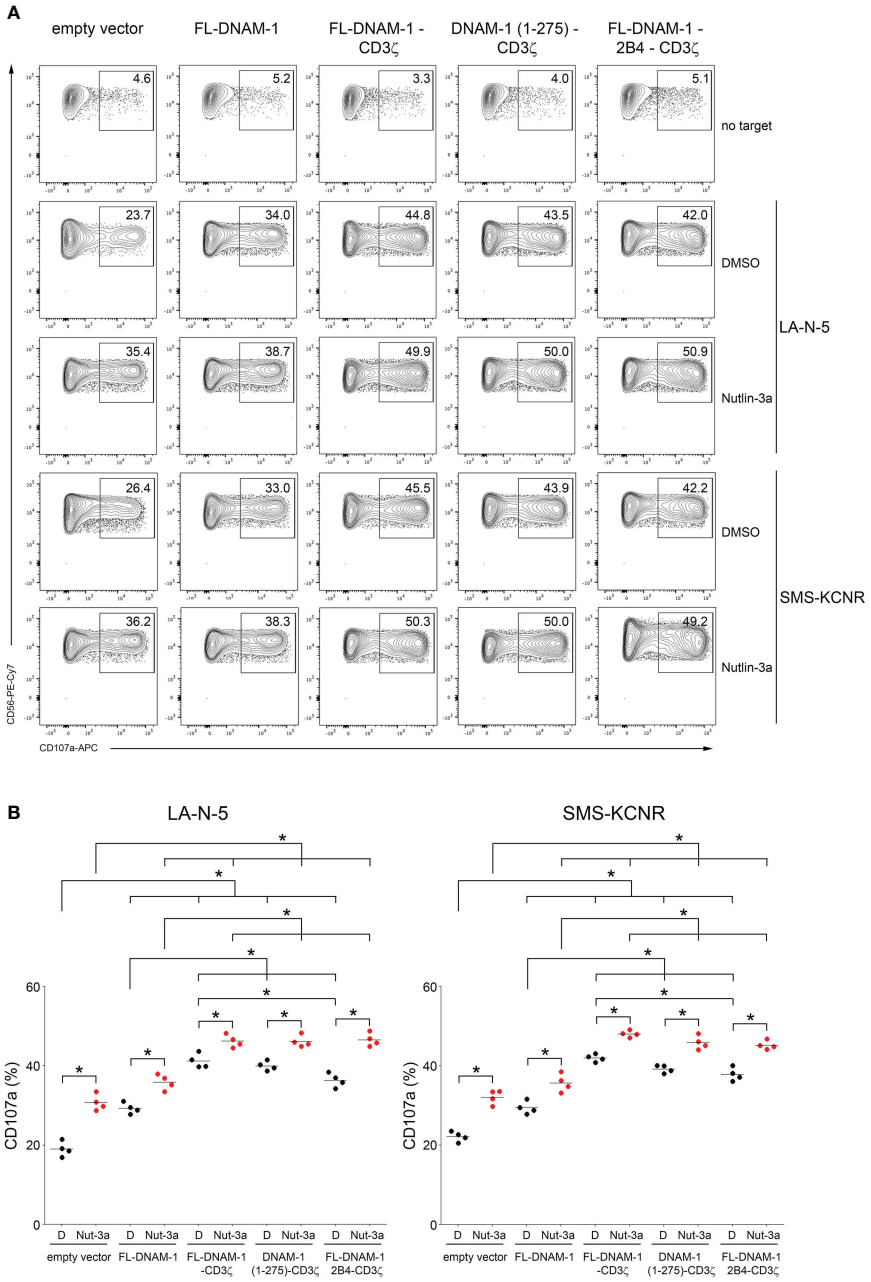

FIGURE 4
Nutlin-3a boosted the susceptibility of NB LA-N-5 and SMS-KCNR cells to DNAM-1-engineered NK cells. NB LA-N-5 and SMS-KCNR cell lines were left untreated (DMSO) or treated with Nutlin-3a at 2 µmol/L for 48 hours and used as targets for degranulation assay of DNAM-1-engineered NK cells. **(A)** Degranulation of NK cells, measured as CD107a expression upon stimulation with LA-N-5 or SMS-KCNR cells treated with DMSO or Nutlin-3a. The percentages of $CD107a^+$ DNAM-1-engineered NK cells are indicated in each plot. A representative experiment of four performed in NK cells isolated from independent healthy donors and transfected with DNAM-1-constructs is shown. **(B)** Summary of degranulation of NK cells isolated from four independent healthy donors and transfected with DNAM-1-constructs, in response to LA-N-5 or SMS-KCNR cells treated with DMSO **(D)** or Nutlin-3a (Nut-3a). Dots correspond to the percentage of $CD107a^+$ NK cells from each healthy donor transfected with each indicated DNAM-1-based constructs; horizontal bars indicate the mean; *$p<0.05$; p value (two-tailed non-parametric Mann-Whitney test).

DNAM-1-engineered NK cells, thus providing a proof-of-concept for which its administration, combined with adoptive transfer of DNAM-1-engineered NK cells, may prospectively represent a potential clinical approach.

Discussion

The treatment of solid tumors, including childhood cancers, still remains a major challenge for oncologists, despite significant advances with multimodal chemotherapy and radiation therapy regimens in combination with several recent immunotherapy approaches.

The efficacy of NK cells has been reported in several tumors as well as in viral infection (21, 50). Of note, the anticancer NK cell-mediated immune response in patients with malignancies is often impaired by the TME cells (e.g. macrophages, myeloid suppressor cells, and stromal/fibroblastic cells), which release inhibitory cytokines/factors (21). Recently, of great interest in the treatment of solid tumors is the adoptive transfer of *ex vivo* expanded and activated NK cells which, due to their peculiar innate characteristics, relatively short lifespan, low risk of hyperproliferation in infused patients, higher safety than infused T cells, and low cost, are an excellent "off-the-shelf" product that could be adopted for a new anticancer therapeutic strategy (51). Furthermore, current good manufacturing practice (GMP) protocols for NK cell adoptive transfer provide for the expansion of NK cells with high activation and low exhaustion status and with greater trafficking and killing performance (34). Thus, the use of adoptive transfer of NK cells appears to be a promising clinical approach to eliminate cancer cells by overcoming the limitations imposed by TME (52, 53).

Currently, several clinical trials indicate that NK cell-based immunotherapy, in combination with cytokines, mAbs, including those recognizing immune checkpoint molecules, is indeed an effective and safe anticancer treatment (21). Furthermore, in the last decade, the use of CARs-armed NK cells, which are thus enhanced in the recognition of specific molecules expressed on the surface of tumor cells, thereby mediating greater tumor eradication, is increasingly emerging in the clinical setting [(54, 55), ClinicalTrial.gov and Supplementary Table S1].

Optimizing CAR signal transduction by incorporating additional costimulatory domains is a focal point for improving their anticancer function (56, 57). In addition, identifying anticancer drugs that have additional advantages of immunomodulatory effects, such as induction of ligands for NK cell-activating receptors, in supporting the NK cell- and CAR-NK cell-based immunotherapy still remains challenging (25). Therefore, in the search for more efficient and less toxic therapeutic approaches, new strategies are needed to support and improve NK cell-based immunotherapy of cancer. In this context, the Nutlin-3a-mediated restoration of the p53 function (58), whose abnormality contributes to the severity of various forms of cancer (59), has been widely recognized as an effective and non-toxic therapeutic approach (26, 58, 60).

Here, our results represent a proof-of-concept aimed at designing a novel immunotherapy approach for solid tumors based on the use of DNAM-1-chimeric receptor-engineered NK cells in combination with Nutlin-3a. Our data refer to an *in vitro* exploration on cellular models of NB, a solid tumor known to have dysfunctional p53 (61) and to be able to evade NK cell-mediate immunosurveillance through down-regulation of ligands for NK cell activating-receptors (26, 38, 62, 63). Since the expression of ligands for NKG2D and DNAM-1 receptors is shared in many solid tumors (21, 25), this type of prospective therapeutic approach is expected to be effective not only for NB but also for a broad spectrum of solid tumors with p53 dysfunction, such as colorectal, breast, ovarian, lung and pancreatic cancers (64). Moreover, the DNAM-1 engineered NK cell-based approach should also be effective against infected cells, as the ligands PVR/CD155 and Nectin2/CD112 for DNAM-1 are mainly expressed, not only on solid tumor cells, but also on virus-infected cells (2, 5), including SARS-CoV-2 (13). Interestingly, these ligands are poorly expressed in normal tissues as reported on proteinatlas.org, thus assuming that the use of DNAM-1 engineered-NK cells may be safe.

The prospective success of DNAM-1-engineered NK cells is also supported by the encouraging results regarding to the use of NK cells engineered for different chimeric receptors such as anti-CD19, -PSMA, -5T4, -CD22, -BCMA, -ROBO1, NKG2D, NKG2D-ACE2, as reported in different clinical trials (ClinicalTrial.gov and Supplementary Table S1), and anti-GD2, -HER-2, -CS1, -CD20, -EGFR, -PSCA as reported in several preclinical studies (21, 55). Studies concerning the use of NKG2D-chimeric receptor engineered-NK cells, tested for the treatment of leukemia and solid tumors such as colorectal cancer, have shown promising results [(65, 66), ClinicalTrial.gov and Supplementary Table S1]. This latter type of chimeric receptor was designed to be expressed in concert with a molecule capable of ensuring signal transduction such as CD3ζ chain and a costimulatory molecule for NKG2D, such as DAP10 (65). As for DNAM-1-chimeric receptors, encouraging data have been reported on engineered T cells against melanoma (67) and the engineered-NK-92 cell line against hepatocellular cancer cells (68) and sarcoma (69).

Here, we generated four different DNAM-1-based expression vectors consisting of the sequence for DNAM-1 in frame with that for the CD3ζ chain and a costimulatory molecule such as 2B4. The choice of co-expressing DNAM-1 with the CD3ζ chain and the costimulatory molecule 2B4 depended on several experimental evidences: i) CD3ζ is a signal transduction molecule that contains three ITAMs associated with different activating receptors expressed on the

surface of NK cells (42, 43); it provides ITAMs for intracellular phosphorylation and, thus, activation of CAR-expressing T cells, often referred to as first-generation CARs (70); ii) 2B4 is a member of the CD2 family and recruits SAP and Fyn through tyrosine-based cytoplasmic motifs, thereby mediating its functional relationship with DNAM-1 (71).

We transfected primary human NK cells to obtain DNAM-1-engineered NK cells and performed functional assays against a natural NK cell-target such as K562 cell line and against NB cells such as LA-N-5 and SMS-KCNR cell lines, the latter treated or not with Nutlin-3a. When comparing the target cell recognition and cytokine production abilities of DNAM-1-engineered NK cells transfected with four different DNAM-1-based vectors, FL-DNAM-1-CD3ζ proved to be the best. These data indicated that optimal activation of the DNAM-1 chimeric receptor required the FL-DNAM-1 sequence, including the intra-cytoplasmic sequence. These results were in line with those obtained in the mouse model demonstrating that Tyr319 (Tyr322 in human) is critical for murine DNAM-1-mediated signalling and cytotoxicity (72). Furthermore, optimal activation of the DNAM-1 chimeric receptor required the in-frame sequence CD3ζ (52-164 aa). The cytoplasmic 2B4 domain (251-370 aa) appeared to impair activation, consistent with previous data reporting that anti-CD19-based CAR carrying the 2B4 sequence required both transmembrane and full cytoplasmic 2B4 domains to enhance activation of engineered primary NK cells (73); in contrast, CARs based on anti-CD5 (74) and -GPC3 (68) or -CD19 (75) and -mesothelin (76) carrying the intracytoplasmic 2B4 domain enhanced the activation of engineered NK-92 cell line or primary NK cells, respectively. Thus, both the type of CAR and the nature of NK cells would appear to affect the contribution of the 2B4 sequence to CAR performance differently. Furthermore, blocking experiments by using both anti-NKG2D and anti-DNAM-1 neutralizing antibodies demonstrated the involvement of chimeric DNAM-1 activating receptor in the recognition mediated by DNAM-1-engineered NK cells of K562 cells. Interestingly, Nutlin-3a rendered both LA-N-5 and SMS-KCNR NB cells significantly more susceptible to DNAM-1-engineered NK cells than NK cells transfected with empty vector. This indicated that a synergistic effect occurred between signals triggered by upregulated ligands for both NKG2D and DNAM-1 receptors through Nutlin-3a-mediated immunomodulation (26, 49) and DNAM-1 chimeric receptor. In addition, Nutlin-3a restored the differences between NK cells transfected with DNAM-1-CD3ζ-based constructs evaluated instead in response to DMSO-treated LA-N-5 and SMS-KCNR cells, indicating that it has an immunomodulatory effect that outperforms weaker NK cells transfected with DNAM-1-based constructs, such as DNAM-1 (1-275)-CD3ζ and FL-DNAM-1-2B4-CD3ζ constructs.

Of note, NK cells engineered for DNAM-1-chimeric receptor represent a further advantage in view of the fact that the protocol for *in vitro* expansion and activation of human NK cells, which we adopted, involved the culture of mature, educated and armed cells with the progressive elimination of uneducated hyporesponsive cells (77). On the other hand, the increased expression of the DNAM-1 chimeric-receptor on DNAM-1-engineered NK cells should be an additional advantage over the expression of CD96 and TIGIT, two inhibitory receptors that compete with DNAM-1 for binding to PVR and Nectin-2 or PVR, respectively (2).

However, translating this experimental design to the clinical setting requires many other considerations. Current methods for transfection of primary NK cells for clinical use, such as those with retroviral and lentiviral vectors (78), including pseudotyping with a modified baboon envelop glycoprotein (BaEV-gp) (79), pseudoviral particles (80) or mRNA electroporation (81), to obtain stable, high-efficient, and in large-number DNAM-1-engineered NK cells suitable for the clinical grade, should be applied and, therefore, further investigations are required. In addition, the nontoxic dose of Nutlin-3a used in this study, which showed immunomodulatory effect in both *in vitro* and *in vivo* NB models, as we previously reported (26), or that of its analogues, combined with DNAM-1-engineered NK cells, should be defined in the clinical setting based on the currently applied nontoxic doses of MDM2-targeting drugs reported in several clinical trials for the treatment of different forms of solid tumors (ClinicalTrial.gov and Supplementary Table S2).

In conclusion, our results provided a proof-of-concept that the use of FL-DNAM-1-CD3ζ engineered-NK cells in combination with immunomodulatory drugs, such as Nutlin-3a, could represent a novel immunotherapeutic approach to be employed for the treatment of solid tumors with dysfunctional p53. Furthermore, in view of the widely reported involvement of ligands for DNAM-1 in the immune response against cells infected with different types of viruses (2), including SARS-CoV-2 (13), these data suggest to extend the exploration of the use of DNAM-1-engineered NK cells in the context of viral infection.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for

participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

LC conceived the study and coordinated the project. LC, CF, MB and CP performed the experiments. LC, AV and FN designed DNAM-1-based constructs. LC, AV, NC, DF, PP, PR, and RB discussed the results and provided critical comments. LC wrote the manuscript. All authors critically revised and edited the paper. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

Authors AV and FN are employed by ReiThera Srl.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.886319/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Gating strategy used to analyse NK cell subsets by flow cytometry. *In vitro*-expanded and activated NK cells were checked for the expression of their specific markers at 0 (immediately after isolation from blood of healthy donors), 8, 15 days and before performing functional experiments (15-20 days) of *in vitro* culture in supplemented NK MACS medium. After gating singlet cells (side scatter-A versus side scatter-H), and alive cells (negative for Live/Dead staining), NK cells were identified by gating on $CD14^-$ $CD19^-$ $CD3^-$ subsets to evaluate in $CD56^+$ $CD16^+$ subset the expression of surface markers, as indicated. NK cells expressing NKG2A, KIR2DL1/2DS1, KIR2DL2/L3/S2 and KIR3DL1 were used for experiments. A representative staining of expanded and activated NK cells at 8 days of culture is shown. The expressions of CD3, CD56 and CD16 in cultured NK cells were compared to those in PBMCs isolated from the same healthy donors. The percentages of NK cells are shown in CD56 vs CD16 dot plots.

SUPPLEMENTARY FIGURE 2
The cytotoxic activity of DNAM-1-engineered NK cells was evaluated against K562 cells at the indicated effector:target (E:T) ratios by a standard 4-hour ^{51}Cr-release assay. A representative experiment out of three performed is shown. The specific lysis from cytotoxic activity in triplicates were converted to L.U. 20% (right panel). The dots in the curves (left panel) and bars in the histogram (right panel) indicate the mean ± SD of triplicates; *$p<0.05$; p value (two-tailed non-parametric Mann-Whitney test; in the left panel, *1, empty vector versus all four DNAM-1-based vectors; *2, FL-DNAM-1 vectors versus all three DNAM-1-CD3ζ-based vectors.

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CAR T cells targeting the ganglioside NGcGM3 control ovarian tumors in the absence of toxicity against healthy tissues

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Chimeric antigen receptor (CAR) T cells have emerged as a powerful immunotherapeutic tool against certain hematological malignancies but a significant proportion of patients either do not respond or they relapse, sometimes as a result of target antigen loss. Moreover, limited clinical benefit has been reported for CAR therapy against epithelial derived solid tumors. A major reason for this is the paucity of solid tumor antigens identified to date that are broadly, homogeneously and stably expressed but not found on healthy tissues. To address this, here we describe the development and evaluation of CAR T cells directed against N-glycoslylated ganglioside monosialic 3 (NGcGM3). NGcGM3 derives from the enzymatic hydroxylation of N-acetylneuraminic acid (NAc) GM3 (NAcGM3) and it is present on the surface of a range of cancers including ovarian, breast, melanoma and lymphoma. However, while NAcGM3 is found on healthy human cells, NGcGM3 is not due to the 7deletion of an exon in the gene encoding for the enzyme cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH). Indeed, unlike for most mammals, in humans NGcGM3 is considered a neoantigen as its presence on tumors is the result of metabolic incorporation from dietary sources. Here, we have generated 3 CARs comprising different single chain variable fragments (scFvs) originating from the well-characterized monoclonal antibody (mAb) 14F7. We show reactivity of the CAR T cells against a range of patient tumor fragments and we demonstrate control of $NGcGM3^+$ SKOV3 ovarian tumors in the absence of toxicity despite the expression of CMAH and presence of $NGcGM3^+$ on healthy tissues in NSG mice. Taken together, our data indicate clinical potential for 14F7-based CAR T cells against a range of cancers, both in terms of efficacy and of patient safety.

KEYWORDS

T cells, immunotherapy, tumors, chimeric antigen receptor (CAR), ganglioside

Introduction

While T cell receptors (TCRs) recognize peptide fragments displayed on antigen presenting cells in a major histocompatibility complex (MHC)-restricted manner, in principle CARs can be designed to target any cell surface expressed antigen, including a protein, carbohydrate, glycolipid or ganglioside (1). Indeed, CARs are synthetic modular receptors comprising an antigen binding ectodomain, usually a scFv, followed by a hinge, a transmembrane region, and endodomains needed for T cell activation (i.e., CD3ζ) and co-stimulation (usually derived from CD28 or/and 41BB). Modification to CAR components, such as binding affinity of the scFv, hinge length/flexibility, and choice of costimulatory endodomain(s) (2), can have a profound impact on effector function, tumor control, and risk of on-target but off-tumor toxicity of the engineered T cells. In addition, T cell state/phenotype (3) at the time of transfer, CAR density at the cell surface, and the number of CAR T cells engrafted in proportion to tumor burden and target antigen expression levels (4), can influence the risk of adverse patient reactions. In fact, most tumor antigens targeted by CARs are also found at varying levels on healthy tissues [reviewed in (5–7)].

Major continued investments in the development of CAR T cells for treating solid tumors are predicated on the unprecedented clinical success of CD19 directed CAR T cells of up to 70-90% complete, durable responses (including some that are curative) against acute and chronic leukemias (8–11). However, CD19 represents an ideal target for CAR therapy because it is largely restricted to B cells and it is typically homogeneously expressed. Moreover, the B cells themselves can provide costimulatory support (e.g. from CD80/86) to CAR T cells, and they are readily accessible (i.e., in the bloodstream and lymphatic system) rather than being sheltered within an oftentimes difficult to access and suppressive solid tumor microenvironment (TME, 5).

Important research efforts are underway to identify solid tumor antigens that are broadly, homogeneously, and stably expressed across multiple tumor types but absent from healthy tissues (i.e., a bona fide tumor antigen rather than a tumor *associated* antigen). A deletion variant of epidermal growth factor (EGFRvIII) is an example of a tumor-restricted target (it is a driver mutation in some forms of glioblastoma), but there is considerable intra- and intertumor heterogeneity (12, 13) and antigen loss has been reported in the clinic following CAR therapy (14). Recently, proof-of-principle for the development of CARs targeting the oncogenic immunopeptidome of neuroblastoma (so-called peptide-centric CARs) has been reported (15) but, although promising, clinical efficacy and safety, as well as applicability to other cancer-types, remains to be demonstrated (16).

Here, we sought to develop CARs directed against the ganglioside NGcGM3 which we propose is a promising target tumor antigen. Gangliosides have been implicated in tumor establishment and metastases as well as in immune suppression, and numerous studies indicate that NGcGM3 is a negative prognostic marker [reviewed in (17)]. Briefly, gangliosides are glycosphingolipids having at least one sialic acid linked on the sugar chain. The two major sialic acid variants in mammals are N-acetylneuraminic acid (NAc) and N-glycolyneuraminic acid (NGc), but the latter is not found in normal human tissues due to the deletion of an exon in the gene encoding for the enzyme CMAH needed for converting NAc to NGc. Although humans lack CMAH activity, NGcGM3 derived from dietary sources (e.g., meat and dairy products) has been detected in the plasma membranes of a broad range of cancer-types including ovarian, breast, lung, melanoma, prostate, neuroblastoma, sarcoma and lymphoma as a result of their higher metabolic rate (17) and upregulation of sialin, a sialic acid transporter, by hypoxia (18). In our study, we have generated CAR T cells targeting NGcGM3 with scFv derived from the well-characterised mAb 14F7 (19, 20) and achieve *in vitro* activity against SKOV3 ovarian tumor cells as well as a range of patient biopsies. In addition, we demonstrate robust control of NGcGM3$^+$ SKOV3 tumors in the absence of toxicity against healthy tissues.

Results

14F7-based CAR T cells demonstrate *in vitro* activity against SKOV3 tumor cells and a panel of patient derived tumor fragments

The IgG1 mAb 14F7 was originally generated by immunizing BALB/c mice with NGcGM3 conjugated to human very-low density lipoproteins in the presence of Freund's adjuvant (19, 20). We began our study by generating a panel of scFv-based CARs comprising the original murine variable heavy (V_H) domain of 14F7 and 3 previously described human variants of the variable light (V_L) domain (herein named 'human' (h) h1, h2 and h3) (21) in a pRRL based lentiviral vector. Briefly, the bicistronic lentiviral transfer vectors encode the human phosphoglycerate kinase (PGK) promoter, green fluorescent protein (GFP), a T2A sequence, and the human CD8 leader sequence followed by each of the CARs [scFv, hinge, transmembrane (TM) and intracellular (IC) domains derived from CD28 and CD3ζ, Figure 1A].

We efficiently transduced a Jurkat-NFAT-mCherry reporter cell line (Figure 1B) that we previously generated (22) to express each of the 3 CARs (Figure 1C). Because Jurkat cells are NGcGM3$^+$ *in vitro* [by NGc uptake from fetal bovine serum (FBS) in the culture media, Figure 1D], they quickly became activated (as evaluated by mCherry expression) following transduction to express the CARs (Figure 1E). As a control, the transduced reporter cells were activated for 48h with Phorbol 12-Myristate 13-Acetate/Ionomycin (PMA/Iono) (Supplementary Figure 1A).

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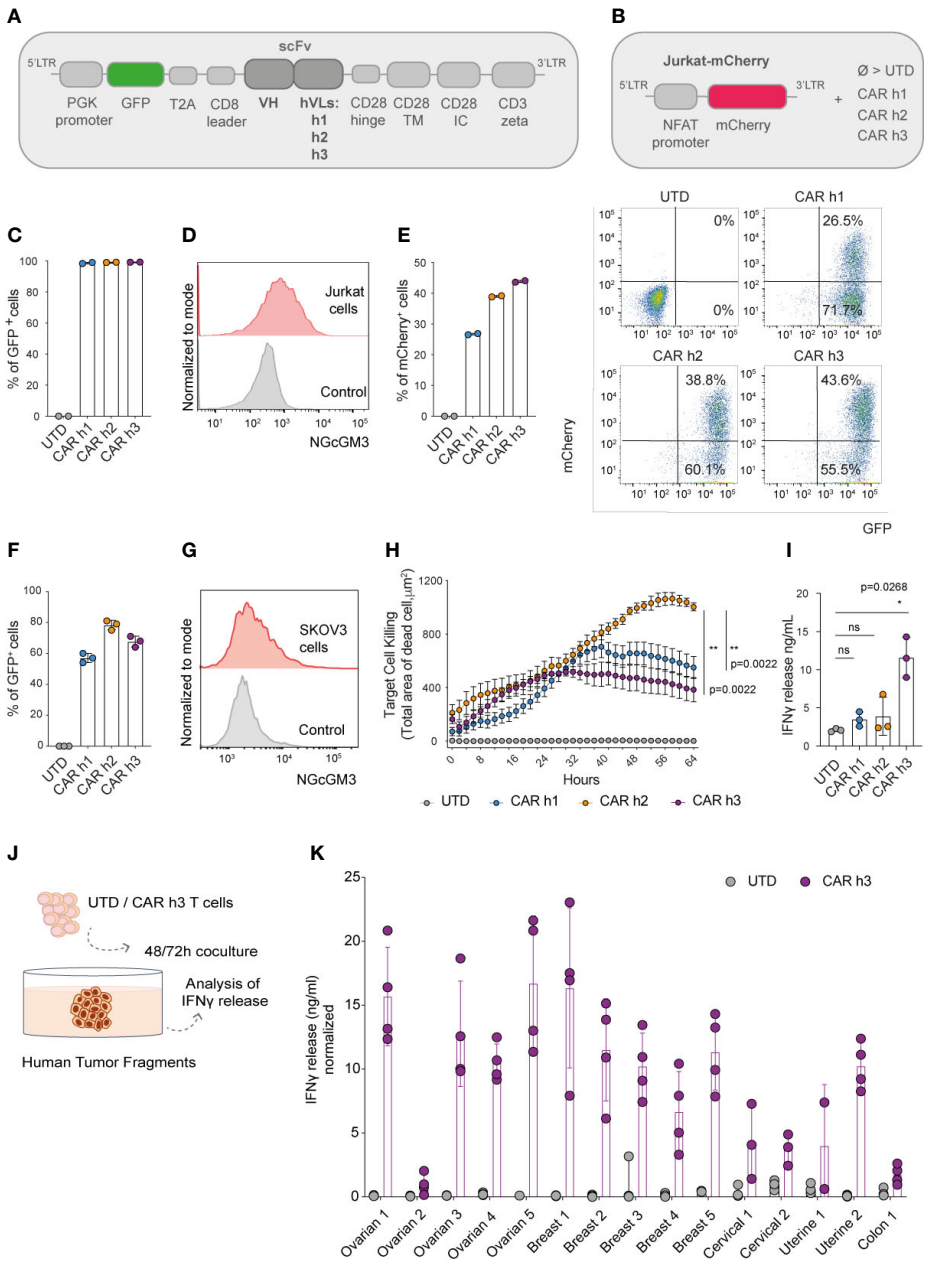

FIGURE 1
Anti-NGcGM3 CAR T cells demonstrate reactivity *in vitro* against NGcGM3$^+$ SKOV3 ovarian tumor cells and a panel of patient biopsies. **(A)** Schematic of lentiviral vectors encoding GFP and the different 14F7-based anti-NGcGM3 CARs. **(B)** Schematic of the vector used to generate Jurkat-mCherry reporter cells which were then transduced with the 3 CAR variants (CAR h1, h2 and h3) or not (UTD=untransduced) **(C)** Evaluation of CAR expression by transduced Jurkat-mCherry reporter cells as assessed by GFP expression measured by flow cytometric analysis. **(D)** NGcGM3 expression on Jurkat cell surfaces as assessed by flow cytometric analysis (top, in red) and secondary antibody staining alone control (bottom, in grey). **(E)** mCherry expression levels in transduced Jurkat-mCherry reporter cells at 48h (left) and representative dot plots of reporter gene and CAR expression (evaluated by GFP expression) (right). **(F)** Percent GFP expression by lentivirally transduced primary human T cells assessed by flow cytometry. **(G)** SKOV3 cell line NGcGM3 expression (in red) assessed by flowcytometry compared to control (secondary Ab alone, in grey). **(H)** Anti-NGcGM3 CAR T cell killing of SKOV3 tumor cells (calculated as dead cell count/µm^2) measured over days in an IncuCyte assay. **(I)** IFNγ production by anti-NGcGM3 CAR T cells upon 24h coculture with SKOV3 tumor cells. **(J)** Schematic of anti-NGcGM3 CAR untransduced (UTD) T cell coculture with patient tumor fragments. **(K)** IFNγ release in anti-NGcGM3 CAR T cell and tumor fragment coculture assays. Shown is average ± standard deviation (SD) **(F, I, K)** or standard error mean (sem) **(H)** of different cultures. Statistical analysis by unpaired, two-tailed Mann-Whitney test **(H)** and paired, two-tailed t test **(I)**. (**p< 0.01; *p < 0.05; ns, non-significant). All experiments were performed for a minimum of n=3 donors.

Having demonstrated the ability of the 3 different CARs to trigger mCherry expression in our reporter cell line, we subsequently efficiently transduced primary human T cells derived from the peripheral blood of healthy donors (Figure 1F). Coculture of the human CAR T cells with SKOV3 ovarian tumor cells which are NGcGM3$^+$ *in vitro* (Figure 1G) revealed highest target cell killing by CAR h2 engineered T cells as evaluated in an IncuCyte assay (Figure 1H), but highest IFNγ production by CAR h3 T cells (Figure 1I).

Finally, we sought to test the reactivity of anti-NGcGM3 CAR T cell against patient biopsies. We obtained a panel of ovarian, breast, cervical, uterine and colon tumor fragments, cancer-types previously shown to present NGcGM3 at their surface (17), and upon 48 or 72h coculture with CAR h3 T cells versus untransduced (UTD) T cells, we evaluated IFNγ production (Figure 1J). We observed varying levels of IFNγ release by the CAR h3 T cells in response to each of the tumor-types, but none by the UTD T cells (Figure 1K).

In summary, we built 3 different anti-NGcGM3 CARs and demonstrated *in vitro* reactivity of engineered T cells against the tumor cell line SKOV3 as well as a panel of patient biopsies. CAR h2 conferred the highest level of target cell killing and CAR h3 the highest level of IFNγ production by engineered human T cells *in vitro*.

14F7-based CAR T cells efficiently control the growth of NGcGM3$^+$ SKOV3 ovarian tumors upon adoptive cell transfer

The human ovarian SKOV3 cell line has been previously gene-modified to express CMAH (named SKOV3 CMAH) needed for the enzymatic hydroxylation of NAcGM3 to NGcGM3, and it has been shown that intraperitioneal administration of humanized 14F7 mAb efficiently controls SKOV3 CMAH growth *in vivo* (23). With the aim of evaluating our anti-NGcGM3 CAR T cells *in vivo*, we began by analysing NGcGM3 expression by wild type (wt) SKOV3 versus SKOV3 CMAH subcutaneous tumors *ex vivo* and confirmed elevated expression levels by the latter (Figure 2A).

For *in vivo* testing of the anti-NGcGM3 CAR T cells we subcutaneously engrafted NSG mice with SKOV3 CMAH cells which are able to convert NAcGM3 to NGcGM3 (schematic shown in Figure 2B). Because CMAH is expressed in murine cells we transferred the T cells by peritumoral injection to avoid systemic on-target but off-tumor toxicity, or/and sequestration of the CAR T cells in healthy tissues. We measured significant tumor control by CAR h1 and h3 T cells as compared to treatment with UTD T cells and saline alone (Figure 2C). Evaluation of tumors at the end of the study revealed a similar and significant reduction in weight (Figure 2D) and comparable T cell infiltration levels upon treatment with the 3 different CARs as compared to the controls (Figure 2E).

In two additional independent *in vivo* experiments with lower doses of CAR h3 T cells we demonstrated significant tumor control, a significant reduction in tumor weight at the end of the study, and confirmed significant CAR h3 T cell infiltration, as compared to UTD T cells (Supplementary Figures 1B–G). We further evaluated CAR h3 T cells *in vivo* against prostate (22Rv1) and Ewing's sarcoma (A673). While both of these cell lines uptake and present NGcGM3 *in vitro* (from FBS in the culture medium) (Figures 2F, G, top) we did not observe any tumor control *in vivo*. This is not unexpected because these human tumor cell lines do not express CMAH and the mice receive a vegetarian diet (i.e., there is not the possibility of NGcGM3 uptake by the tumors *in vivo*).

In summary, we demonstrated significant control of SKOV3 CMAH ovarian tumors upon adoptive transfer of 14F7-based anti-NGcGM3 CAR T cells, but not of 22Rv1 prostate nor A673 sarcoma tumors.

Anti-NGcGM3 CAR T cells do not cause toxicity against healthy tissues in NSG mice

NSG mice express the enzyme CMAH thus healthy murine cells can present enzymatically generated NGcGM3 in their outer membranes. However, while humans do not express CMAH, one cannot exclude the possibility of dietary uptake of NGcGM3 by normal tissues. Hence, we sought to evaluate the potential for anti-NGcGM3 CAR T cell toxicity against healthy tissues in NSG mice as a surrogate for potential toxicity against human tissues arising from dietary uptake of NGcGM3 (24).

We intravenously injected both female and male NSG mice with a high dose of anti-NGcGM3 CAR h3 T cells (10^7), as well as control GFP$^+$ T cells and saline, and carefully monitored them for 9 days (schematic in Figure 3A). We observed no weight loss of the mice (Supplementary Figure 2A) nor signs of distress. In addition, hematocrit analysis of the blood 8 days post-adoptive cell transfer (ACT) revealed no difference in the levels of white blood cells (WBC), red blood cells (RBC), platelets, or systemic hemoglobin (HGB) levels amongst the CAR T cell treated versus control mice (Figure 3B, left to right). Similarly, analysis of the sera indicated no signs of liver, pancreatic or kidney toxicity as there were no differences in alanine aminotransferase (ALT), lipase, and creatinine levels respectively, amongst the groups of mice (Figure 3C, left to right). At autopsy we observed no difference in liver or spleen weights for the CAR T cell treated versus control mice (Figure 3D, left and right). Flow cytometric analysis of 14F7 mAb stained single cell suspensions of organs revealed varying levels of NGcGM3 expression for the spleen, lungs (even though lower in comparison to SKOV3 CMAH tumor cells; Supplemental Figure 2B), liver, pancreas, ovary,

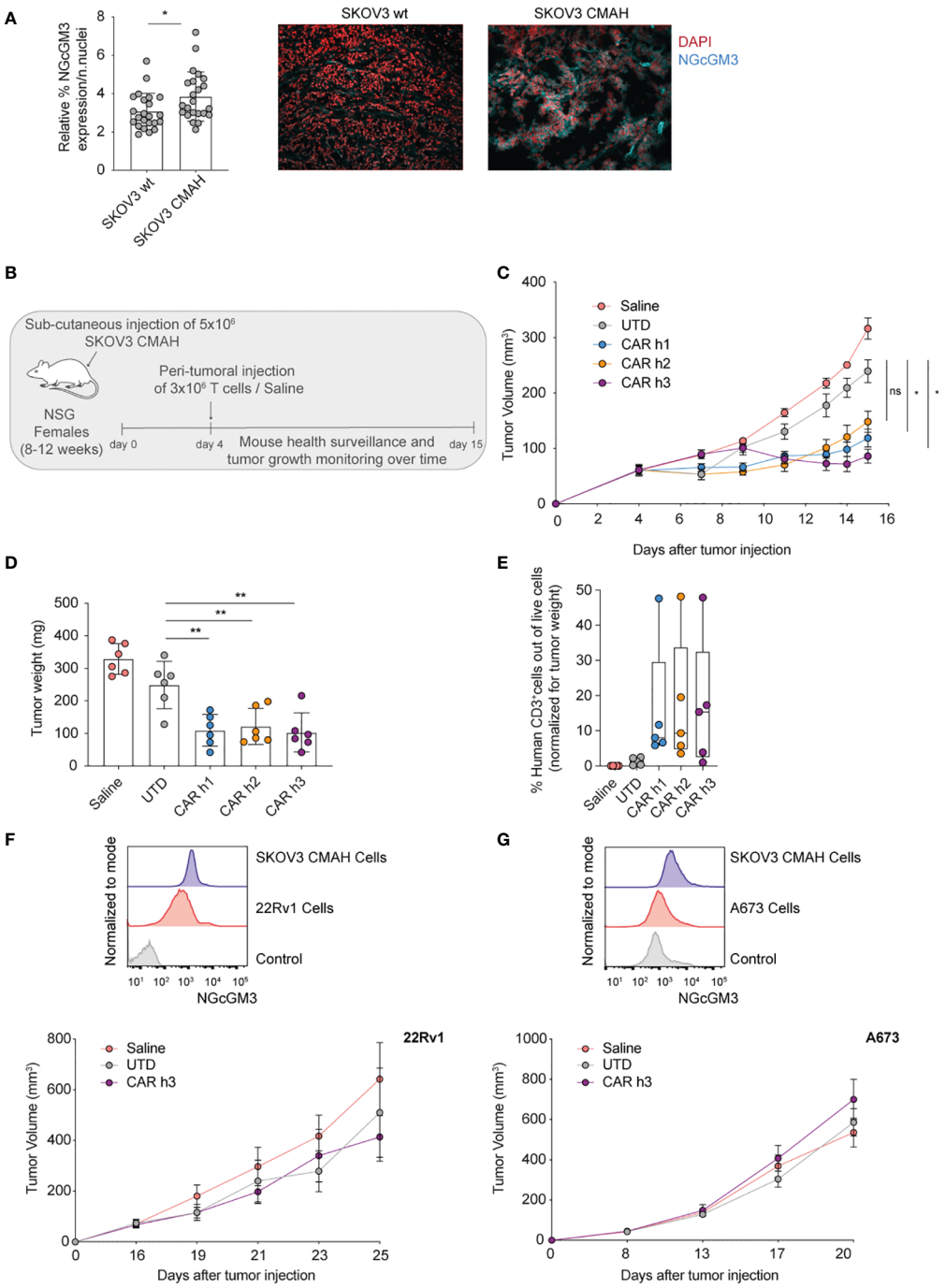

FIGURE 2
Anti-NGcGM3 CARs T cells efficiently control the *in vivo* tumor growth of an ovarian human tumor cell line expressing the target antigen. **(A)** Relative expression of NGcGM3 on SKOV3 wt versus SKOV3 CMAH subcutaneous tumor fragments (left). Representative immunofluorescence images (right); DAPI staining of nuclei in red, 14F7 mAb plus Alexafluor 647 labeled secondary Ab in blue. **(B)** Schematic of *in vivo* ACT study. **(C)** Tumor growth (SKOV CMAH) curves over days following subcutaneous injection. **(D)** Tumor weights (SKOV3 CMAH) at the end of the study (day 15). **(E)** Percentage of human $CD3^+$ T cells infiltrating tumors at the end of the study (normalized for tumor volume). **(F, G)** Relative expression levels of NGcGM3 on SKOV3 CMAH, 22Rv1 and A673 tumor cells assessed by flow cytometry (control = secondary Ab alone) (top). Tumor growth curves for 22Rv1 and A673 over days (in this ACT study the mice received $2x10^6$ CAR T or UTD cells by peritumoral injection at days 16 and 8, respectively, post tumor injection) (bottom). Shown is average ± SD **(A, D)** or ± sem **(C)** and (**F**, **G**, bottom panels) or box and whiskers (min to max) **(E)**. Statistical analysis by unpaired, two-tailed Mann-Whitney test **(A)** and paired, two-tailed t test **(D)**; two-way analysis of variance (ANOVA) with correction for multiple comparisons by *post hoc* Tukey's test **(C)**; unpaired, two-tailed t test **(D)**.(**p< 0.01; *p < 0.05; ns, non-significant).

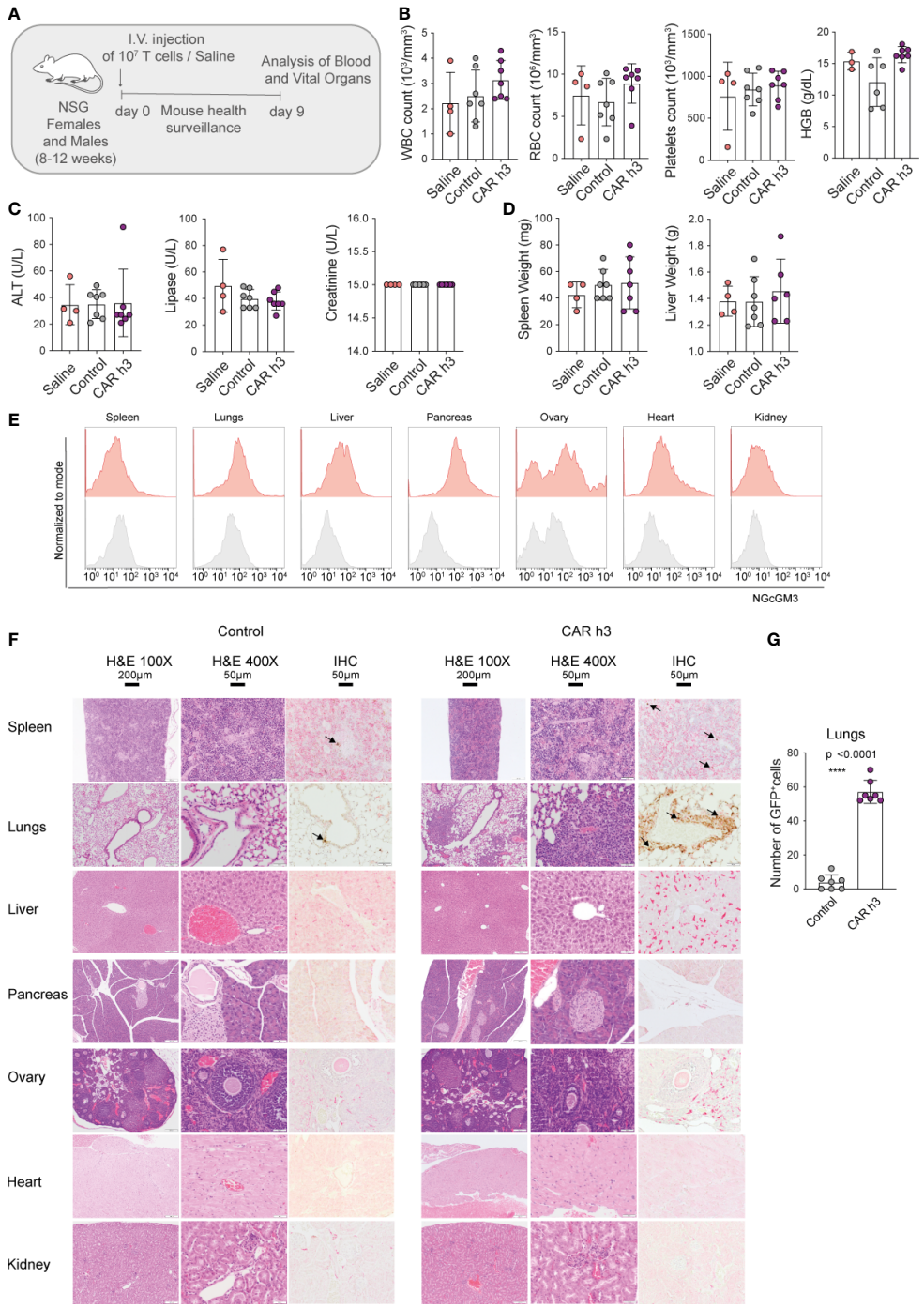

FIGURE 3
Anti-NGcGM3 CAR T cells do not cause toxicity in NSG mice. **(A)** Schematic of *in vivo* toxicity study. **(B)** Blood analysis to assess white blood cells (WBC), red blood cells (RBC), platelets counts and hemoglobin (HGB). **(C)** Measurement of alanine aminotransferase (ALT), lipase and creatinine in serum. **(D)** Spleen and liver weight upon necropsy. **(E)** NGcGM3 expression in different organs (spleen, lungs, liver, pancreas, ovary, heart, kidney). **(F)** Histopathology of organs from control (GFP transduced T cells) and anti-NGcGM3 CAR h3 T cell treated mice on day 9 post intravenous injection of transduced 10^7 T cells. H&E = hematoxylin and eosin staining at 100X and 400X magnification; IHC = immunohistochemistry to detect GFP^+ T cells (brown, indicated with arrow). **(G)** Quantification of GFP^+ T cells in the lungs. Shown is average ± SD **(B, C, D, G)**. Statistical analysis by unpaired, two-tailed t test **(G)**. (****p< 0.0001).

heart and kidney (Figure 3E, left to right), and substantially lower NGcGM3 levels on the brain and prostate (Supplementary Figure 2C, left and right).

Finally, by histopathology (blinded study by a trained pathologist) we observed no signs of toxicity by the anti-NGcGM3 CAR T cell treatment to the spleen, lungs, liver, pancreas, ovary, heart and kidney (Figure 3F, top to bottom), or to the brain and prostate (Supplementary Figure 2D). Constitutive GFP expression by both the control and CAR h3 T cells allowed for semi-quantitative analysis of the transferred T cells. We observed higher levels of CAR h3 T cells versus control T cells in the lungs (in the absence of any aberration of the vessels or alveolar walls; Figure 3F, G), in line with previous reports of activated T cell retention in the lungs upon intravenous transfer (25). Notably, anti-F4/80 staining of the different organ cross-sections revealed no differences in macrophage infiltration into organs (except for the lungs) for the CAR T cell versus control mice T cells treated (Supplementary Figure 2E), further indicative that no toxicity was caused by the treatment. In an independent ACT study, we confirmed the daily well-being of the mice upon high doses of anti-NGcGM3 CAR T cell transfer, and once again showed higher retention of both $CD8^+$ and $CD4^+$ CAR T cells in the lungs of mice (Supplementary Figure 2F).

In summary, despite the expression of CMAH in NSG mice, and the presence of NGcGM3 on most organs, transfer of high doses of CAR h3 T cells did not cause adverse reactions against healthy tissues.

Discussion

The unprecedented clinical success of CAR T cells against some advanced hematological malignancies has driven tremendous efforts to develop effective CAR therapies for treating epithelial derived solid tumors which represent the majority of cancers. Obstacles to solid tumor control by CAR T cells can be divided into 3 main categories: (i) insufficient CAR T cell homing and infiltration, (ii) barriers in the TME that are limiting to CAR T cell persistence and effector function, and, (iii) the paucity of target tumor antigens that are broadly, homogeneously and stably expressed but not found on healthy tissues. Here, we sought to address the identification of suitable solid tumor antigen targets for CAR therapy.

In our study, we explored NGcGM3 as a CAR target. NGcGM3 is a ganglioside that is not endogenously produced in humans due to the deletion of an exon in the gene encoding for the enzyme CMAH required for the conversion of NAc to NGc. However, as a result of dietary uptake by highly metabolic tumor cells, NGcGM3 has been identified as present on a range of human tumors, both epithelial cell derived and of hematological origin. NGcGM3 levels can range from moderate to intense depending on tumor-type and the patient [reviewed in (17)]. To build our CAR panel, we took advantage of the previously described murine mAb 14F7 which can exquisitely distinguish NGcGM3 from NAcGM3 *via* a subtle chemical modification of a CH_2OH group instead of CH_3 in the context of a trisaccharide. Notably, the anti-NGcGM3 mAb 14F7 labeled with (99m)Tc has been used to demonstrate clinical evidence of NGcGM3 expression in human breast cancer (26) and the GlycoVaxGM3 vaccine, a nanoparticulated product obtained through the insertion of NGcGM3 into the outer membrane protein complex of *N. meningitides*, has been tested in the clinic (27–29).

We successfully built second generation CARs comprising 3 different scFvs targeting NGcGM3. The scFvs that we employed comprise the original murine V_H of 14F7 linked to 3 different human V_L fragments previously identified by phage display light chain shuffling (21). All 3 of our CARs conferred *in vitro* reactivity of engineered human T cells against SKOV3 tumor cells. Moreover the best CAR candidate showed functional activity against a panel of human tumor fragments. In addition, we demonstrated *in vivo* tumor control of $NGcGM3^+$ SKOV3 ovarian tumors in the absence of any toxicity against healthy tissues in NSG mice, despite the observed presence of NGcGM3 across many organs. Notably, ovarian cancer is the 8^{th} most commonly diagnosed cancer in women globally, and it is the 4^{th} most common cause of cancer-related death in women in the developed world. Indeed, due to the lack of specific symptoms, nearly 75% of ovarian cancer patients are diagnosed at a late stage with widespread intra-abdominal disease (30) and an effective CAR therapy would thus represent an important medical breakthrough (31).

The mAb 14F7 has been extensively characterized with respect to its specific reactivity against NGcGM3 (17) but concerns have been raised about potential reactivity against healthy tissues in which NAcGM3 may be naturally present at high levels, as well as NGcGM3 from dietary sources. In our study, we detected NGcGM3 on human Jurkat (T cell leukemia), SKOV3 (ovarian), 22Rv1 (prostate) and A673 (Ewing's sarcoma) tumor cell lines *in vitro*, most probably acquired from the FBS [an abundant source of NGcGM3 (18, 32)] in the culture medium. Similarly, others have reported NGcGM3 on retinoblastoma (33) and epidermoid carcinoma (34) cell lines *in vitro*. However, *in vivo* we achieved tumor control of SKOV3 CMAH tumors (i.e., overexpressing the enzyme needed to generate NGc from NAc) but not of 22Rv1 or of A673 tumors. Indeed, because the NSG mice receive vegetarian nourishment there is no dietary source of NGcGM3 for the tumors to acquire. Notably, in our ACT study in which NSG mice received 10^7 anti-NGcGM3 CAR T cells there were no signs of toxicity identifiable in the blood or to any of the organs despite that they express NGcGM3. Taken together, these observations indicate that there is a minimum threshold of NGcGM3 that must be present for 14F7-based CAR T cell reactivity. If change to diet (i.e., high consumption of meat or dairy products) can increase anti-NGcGM3 CAR T cell responses

against tumors and/or lead to toxicity against healthy tissues has not been explored in this study but is relevant to their clinical translation (6).

As described above, we employed 14F7 derived scFvs comprising a murine V_H region and human V_L regions. Such murine/human scFv could potentially be immunogenic in humans resulting in unwanted depletion of the CAR T cells (35). The mAb 14F7 has been humanized (14F7hT) to reduce its immunogenicity (36), and the testing of fully humanized scFv variants for CAR therapy is warranted. Of course, any new scFv should be carefully evaluated for retained target specificity, as well as for propensity to aggregate at the T cell surface which can result in tonic signaling and T cell exhaustion (37, 38). Notably, 14F7hT has been demonstrated by others to exhibit significant antitumor effects in preclinical hematological tumor models [reviewed in (17)]. Indeed, anti-NGcGM3 CAR T cells offer the possibility of treating a range of solid and liquid tumors alike.

Another approach to improve the efficacy of anti-NGcGM3 CAR T cells is to co-engineer them with gene-cargo that can either directly support the fitness/function of the CAR T cells themselves or/and reprogram the TME to harness endogenous immunity (39). We have comprehensively demonstrated, for example, the numerous benefits of IL-15 coengineering of murine CAR T cells in a syngeneic melanoma tumor model (40). However, the impact of additional gene-cargo on target tumor antigen must be carefully evaluated. For example, although transgenic expression of IL-15 was shown to improve the antiglioma activity of IL-13alpha2 CAR T cells, antigen loss was reported (41).

Because of the broad expression of NGcGM3 by both solid and liquid tumors *via* dietary uptake in humans, there is also the potential for coadministration of anti-NGcGM3 CAR T cells with CAR T cells targeting a second antigen as a means of mitigating escape (42). Or, one could develop anti-NGcGM3 costimulatory CARs to enhance T cell receptor (TCR) based immunotherapy (43), or in the context of a parallel (p)CAR design (44). Finally, in recent years, several remote-control designs including ON-CARs (45), STOP-CARs (22), and OFF-CARs (46, 47) have been developed that could provide the means to more safely explore the translation of NGcGM3 redirected T cells to the clinic. Taken together, we conclude from our study and recent literature the strong clinical potential of NGcGM3 redirected CAR T cells for immunotherapy against a broad range of cancers.

Materials and methods

14F7-based CAR construction

Second generation self-inactivating (SIN) lentiviral expression vector pRRL containing single chain fragment variable specific for PSMA (22) was used as a starting construct for building the second generation antiNGcGM3 CARs. Three human variants of the V_L (7Ah, 8Bh and 7Bh, which we named CAR h1, CAR h2 and CAR h3), previously developed (21), were ordered as genestrings (GeneArt, Invitrogen) and cloned in the lentiviral vector using SpeI and SalI restriction site digestion in frame with CD28 derived hinge, TM, IC domains and a CD3ζ signaling endodomain, under the control of a human PGK promoter, in a bicistronic construct together with the gene reporter GFP. The two proteins are separated by T2A self cleaving peptide. For toxicity *in vivo* experiments, control T cells were transduced with a pRRL vector carrying GFP only to allow the *ex vivo* tracing of transferred cells.

Recombinant lentivirus production

All plasmids were purified using the HiPure Plasmid Filter Maxiprep Kit (Invitrogen, Thermo Fisher Scientific). High-titer replication-defective lentivirus were produced and concentrated for primary T cell transduction. Briefly, 24h before transfection, 293T human embryonic kidney (HEK) cells were seeded at 10^7 in T-150 tissue culture flasks. HEK cells were transfected with pVSV-G (VSV glycoprotein expression plasmid), R874 (Rev and Gag/Pol expression plasmid), and pRRL transgene plasmid using a mix of Turbofect (Thermo Fisher Scientific AG) and Optimem media (Invitrogen, Lifetechnologies). The viral supernatant was harvested at 48h post-transfection. Viral particles were concentrated for 2h at 24,000g at 4°C with a Beckman JS-24 rotor (Beckman Coulter) and resuspended in fresh culture media followed by immediate snap freezing in dry ice.

Human T cell transduction and expansion

Primary human T cells were isolated from the peripheral blood mononuclear cells (PBMCs) of healthy donors (HDs) prepared as buffy coats. All blood samples were collected with informed consent of the donors. Total PBMCs were obtained *via* Lymphoprep (Axonlab) separation solution using a standard protocol of centrifugation. $CD4^+$ and $CD8^+$ T cells were isolated using a negative selection kit coupled with magnetic beads separation (easySEP, Stemcell Technology). T cells were then cultured in complete media [RPMI 1640 with Glutamax, supplemented with 10% heat-inactivated FBS (Gibco), 100 µg/ml penicillin, 100 U/ml streptomycin sulfate (Invitrogen, Lifetechnologies)], and stimulated with anti-CD3 and anti-CD28 mAb coated beads (Lifetechnologies) in a ratio of 1:2, T cells: beads. Twelve to 24h after activation, T cells were transduced with lentivirus particles titrated by serial dilution in Jurkat cells. $CD4^+$ and $CD8^+$ T cells used for *in vitro* experiments

were mixed at a 1:1 ratio, activated, and transduced. For *in vivo* studies and *in vitro* coculture with tumor fragments, $CD4^+$ and $CD8^+$ T cells were activated, transduced separately and then mixed prior to the experiments at a 20%: 80%, $CD4^+$: $CD8^+$ ratio. Human recombinant IL-2 (h-IL-2; Peprotech) was added every other day to obtain a 50 IU/ml final concentration until 5 days post stimulation (day +5). At day +5, magnetic beads were removed and h-IL-2 was switched to h-IL-15 and h-IL-7, both at 10 ng/mL (Miltenyi Biotec GmbH). A cell density of 0.5-1 × 10^6 cells/ml was maintained for expansion. Rested engineered T cells were adjusted for identical transgene expression before all functional assays.

Cell lines

293T HEK, Jurkat, 22Rv1 and A673 cells were purchased from the ATCC. SKOV3 wt and SKOV3 CMAH were kindly provided by Dr. Kalet Leon (CIM, Cuba). The Jurkat-mCherry cell line generated in the lab was engineered to express a 6x NFAT-mCherry -reporter system such that upon activation the cells turn red. 293T HEK, 22Rv1, A673 and Jurkat cells were cultured in complete media. SKOV3 wt and SKOV3 CMAH were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mmol/l L-glutamine, and 100 μg/ml penicillin, 100 U/ml streptomycin. To select SKOV3 $CMAH^+$ cells, geneticin (Invitrogen G418, 1-2 mg/mL) was added to the culture medium.

Cytokine release assays

Cytokine release assays were performed by co-culture of $5x10^4$ T cells with $5x10^4$ target cells per well, in duplicate, in 96-well round bottom plates in a final volume of 200μl complete media. After 24h, co-culture supernatants were harvested and tested for presence of human IFN-γ using an ELISA Kit, according to the manufacturer's protocol (Biolegend). The reported values represent the mean of engineered T cells derived from three HDs. Patients derived tumor fragments were sectioned in 2-3 mm cubes and cocultured with T cells in a 96 well round bottom plate for 48-72h prior to supernatant collection and IFN-γ release analysis with ELISA (Biolegend).

Cytotoxicity assays

Cytotoxicity assays were performed using the Incucyte System (Essen Bioscience). Briefly, $1.25×10^4$ target cells were seeded 18h before the co-culture set up in flat bottom 96 well plates (Costar, Vitaris). The following day, rested T cells (no cytokine addition for 48h) were counted and seeded at $2.5x10^4$/well, at a ratio 1:2, target:T cells in complete media. No exogenous cytokine was added in the assay medium during the co-culture period. Cytotox Red reagent (Essen Bioscience) was added at a final concentration of 125nM in a total volume of 200μl. Images of total number of red cells/$μm^2$ were collected every 2h of the co-culture for a total of 3 days and were analyzed using the software provided by the Incucyte manufacturer. Data are expressed as mean of 3 different HDs +/- standard deviation.

Flow cytometric analysis

InfraRed live/dead was used for viability staining. All mAbs were purchased from BD Biosciences. Tumor cell surface expression of NGcGM3 was achieved by primary staining with 14F7 mAb (kindly provided by Dr. Kalet Leon, Cuba) and then secondary staining with Alexafluor 647 anti-mouse Fc mAb. (115-605-071, Jackson Immune research Laboratory) Acquisition and analysis were performed using a BD FACS LRSII with FACS DIVA software (BD Biosciences).

Immunohistochemistry

Wild type SKOV3 and SKOV3 CMAH subcutaneous tumors were cryopreserved in OCT compound prior to sectioning (Mouse Pathology Facility, University of Lausanne) and staining. The tumors sections were fixed with a solution of 10% NBF (Formalin solution neutral buffered, HT501128, Sigma), permeabilized with a solution of PBS 0.5% Triton (X100, Sigma), and the aspecific binding sites were blocked with a solution of PBS, 2% heat-inactivated FBS and 1% BSA. The samples were then incubated overnight with 10μg/ml 14F7 mAb. Upon extensive washes with PBS the tumor sections were incubated with secondary Ab anti- antigen binding fragment (Fab) labeled with Alexafluor 647 (115-606-072, Jackson Immune research Laboratory) for 1h at RT. The sections were further stained with DAPI (D9545, Sigma Aldrich) and the slides then analyzed with an Epifluorescence microscope.

Jurkat-NFAT-mCherry cell line transduction and reporter assays

Jurkat-NFAT-mCherry reporter cells previously developed in the lab (22) were transduced with lentivirus encoding both GFP and each of the different anti-NGcGM3 CARs. Briefly, $1x10^6$ cell/mL cells were seeded into 48-well plates in 500 μL/well and 50 μL of virus supernatant was mixed with protamine sulfate (P4020, Sigma Aldrich) for a final concentration of 10 μg/mL. After incubation for 24h at 37°C the cell media was refreshed and the cells were incubated for an additional 72h at 37°C before use. The transduced cells were cultured with the addition or not of PMA/Iono for 48h and analyzed by flow cytometry for mCherry expression (FL-2 channel) and GFP (FL-1 channel).

Mice and *in vivo* experiments

NOD SCID gamma knock-out (NSG) mice were bred and housed in a specific and opportunistic pathogen-free (SPF) animal facility at the Epalinges campus of the University of Lausanne. All experiments were conducted according to the Swiss Federal Veterinary Office guidelines and were approved by the Cantonal Veterinary Office. All cages housed 5 animals in an enriched environment providing free access to food and water. During experimentation, all animals were monitored at least every other day. Mice were euthanized upon meeting distress criteria and at end-point by carbon dioxide overdose. A total of $5x10^6$ SKOV3 wt or $5x10^6$ SKOV3 CMAH tumor cells were subcutaneously injected in flanks of mice (6-10 mice per group). Tumor volume was monitored by caliper measurements every other day starting from day 4 post injection. For ACT experiments, 2-10 $x10^6$ CAR^+ T cells ($CD8^{+:}$ $CD4^+$ = 80%: 20%) were peritumorally or intravenously injected and tumor volume was monitored over time as indicated. Tumor volume was determined with the calculation: volume in mm^3 = (length x $width^2$)/2, where length is the greatest longitudinal measurement and width is the greatest transverse measurement.

In vivo toxicity study

To evaluate *in vivo* toxicity of anti-NGcGM3 CAR T cells, NSG mice received an intravenous injection of 10^7 transduced T cells (control GFP, CAR). The mice were monitored daily for 9 days at which time point they were sacrificed and the organs collect (blood was sampled on day 8).

The blood was analyzed in a blinded manner for white blood cell (WBC) count, red blood cell (RBC) count, platelets and hemoglobin (HGB) concentration with the mythic18 Vet instrument according to the manufacturer's suggestions. The serum was analyzed for units per liter (U/L) of alanine aminotransferase (ALT), lipase, and creatinine at the Clinical Chemistry Laboratory at the Lausanne University Hospital (CHUV).

For Haematoxylin-Eosine (H&E) staining, 4µm paraffin sections were stained using a standard histology procedure to assess general morphology. For the double IHC F4/80 and GFP staining, the double chromogenic IHC assay was performed using the Ventana Discovery ULTRA automate (Roche Diagnostics, Rotkreuz, Switzerland). All steps were performed automatically with Ventana solutions. Primary mAbs were applied sequentially. First, dewaxed and rehydrated paraffin sections were incubated with a rat anti-F4/80 mAb (clone Cl:A3-1, Thermo Fisher MA191124, diluted 1:50), followed by a rat Immpress AP (Vector Laboratories) and revelation with the Discovery red chromogen. Next, a heat pretreatment was applied using the CC1 solution for 40 min at 95°C. Sections were subsequently incubated with a goat anti-GFP mAb (Abcam ab6673, diluted 1:400), followed by a goat Immpress HRP (Vector Laboratories) and revelation using the ChromoMap DAB chromogen. Sections were counterstained with Mayer hematoxyline (J.T. Baker) and permanently mounted with Pertex (Sakura). All H&E stainings were performed in a at the Histology Core Facility at the Swiss Federal Institute of Technology in Lausanne (EPFL). Slides were analyzed in a blinded manner by a trained pathologist at the same facility.

Statistical analysis

GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA) was used for statistical calculations. $P < 0.05$ was considered significant. Statistical analyses used include two-way ANOVA, unpaired two-tailed Mann-Whitney, and two-tailed paired and unpaired t tests, depending on the type of experiment and as indicated in the figure legends.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Swiss Cantonal Veterinary Office.

Author contributions

MI directed the study and GC provided expert advice. EC, GMPGA and MI planned experiments and interpreted results. EC and GMPGA performed experiments. EC, MI and GMPGA wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.951143/full#supplementary-material

SUPPLEMENTARY FIGURE 1

(A) Evaluation by flow cytometry of GFP and mCherry expression by Jurkat NFAT-mCherry reporter cells transduced or not with anti-NGcGM3 CARs and stimulated with phorbol myristate acetate and ionomycin (PMA/Iono). **(B)** SKOV3 CMAH tumor growth curves over days upon peritumoral injections of $2x10^6$ T cells (versus $3x10^6$ T cells in Figure 2B to test the activity and infiltration of a lower number of T cells) or saline. **(C)** Tumor weight at the end of the study. **(D)** Percentage of human $CD3^+$ T cells and **(E)** $CD3^+GFP^+$ cells infiltrating tumors at the end of the study (normalized for tumor volume). **(F)** SKOV3 CMAH tumor growth curves over days upon peritumoral injections of $2x10^6$ T cells or saline (independent repetition of B). **(G)** Tumor weight at the end of the study. Shown is average ± sem **(B, F)**, ± SD **(C, G)** or box and whiskers (min to max) **(D)**. Statistical analysis by two-way ANOVA **(B, F)** and unpaired two-tailed t test **(C, D, G)**. (****p< 0.0001, **p< 0.01; *p < 0.05).

SUPPLEMENTARY FIGURE 2

(A) Weight of female and male mice over time for *in vivo* toxicity study (treated as per Figure 3A). **(B)** Flow cytometric analysis to evaluate NGcGM3 expression by 14F7 mAb staining of dissociated lungs (in purple) and SKOV3 CMAH cells (in red) compared to control (secondary Ab alone, in grey). **(C)** Flow cytometric analysis to evaluate NGcGM3 expression by 14F7 mAb staining for different dissociated organs (in red) compared to control (secondary Ab alone, in grey). **(D)** Histopathology of the organs [treated as per Figure 3A): H&E = hematoxylin and eosin staining at 100X and 400X magnification; IHC = immunohistochemistry to detect GFP^+ T cells (brown, indicated with arrow)]. **(E)** Quantification of GFP^+ and $F4/80^+$ cells in different organs evaluated by IHC (treated as per Figure 3A). **(F)** Flowcytometric analysis of dissociated organs and peripheral blood (mice treated as per Figure 3A) to detect human $CD8^+$ **(left)** and $CD4^+$ **(right)** T cells. Shown is average ± sem **(A)**, ± SD **(E)**. Statistical analysis unpaired two-tailed t test **(E)**. (****p< 0.0001).

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Combinatorial suicide gene strategies for the safety of cell therapies

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Gene-modified cellular therapies carry inherent risks of severe and potentially fatal adverse events, including the expansion of alloreactive cells or malignant transformation due to insertional mutagenesis. Strategies to mitigate uncontrolled proliferation of gene-modified cells include co-transfection of a suicide gene, such as the inducible caspase 9 safety switch (ΔiC9). However, the activation of the ΔiC9 fails to completely eliminate all gene-modified cells. Therefore, we tested a two suicide gene system used independently or together, with the goal of complete cell elimination. The first approach combined the ΔiC9 with an inducible caspase 8, ΔiC8, which lacks the endogenous prodomain. The rationale was to use a second caspase with an alternative and complementary mechanism of action. Jurkat cells co-transduced to co-express the ΔiC8, activatable by a BB homodimerizer, and the ΔiC9 activatable by the rapamycin analog sirolimus were used in a model to estimate the degree of inducible cell elimination. We found that both agents could activate each caspase independently, with enhanced elimination with superior reduction in cell regrowth of gene-modified cells when both systems were activated simultaneously. A second approach was employed in parallel, combining the ΔiC9 with the RQR8 compact suicide gene. RQR8 incorporates a CD20 mimotope, targeted by the anti-CD20 monoclonal antibody rituxan, and the QBend10, a ΔCD34 selectable marker. Likewise, enhanced cell elimination with superior reduction in cell regrowth was observed when both systems were activated together. A dose-titration effect was also noted utilizing the BB homodimerizer, whereas sirolimus remained very potent at minimal concentrations. Further *in vivo* studies are needed to validate these novel combination systems, which may play a role in future cancer therapies or regenerative medicine.

KEYWORDS

inducible caspase 8, inducible caspase 9, suicide gene, safety switch, RQR8

Introduction

Given the recent surge of novel cellular therapies, there is an urgent need to develop strategies to mitigate untoward events of gene-modified cells. Chimeric antigen receptor (CAR) redirected T-cells, gene-modified hematopoietic stem cells (HSC), and inducible pluripotent stem cells (iPSC) are emerging treatments for a wide variety of malignant and non-malignant disorders (1–3). However, gene modifying therapies carry the intrinsic risks of excess proliferation (4, 5), and insertional mutagenesis (6–10). In the case of CAR-T specifically, off-target side effects could result in potentially fatal organ damage and death including immune effector cell-associated neurotoxicity syndrome (ICANS) and severe cytokine release syndrome (CRS) (11). Currently available treatments for CRS are limited to corticosteroids or anti-interleukin-6 receptor antibodies, which are associated with broad immunosuppression.

It is very challenging to predict the type or degree of the toxicities that may occur. For example, injection of even unmodified autologous HSC into the kidneys to treat renal failure resulted in angiomyeloproliferative lesions that required nephrectomy (12). Autologous stem cells derived from adipose tissue and injected intravitreally for macular degeneration were associated with worsening vision in three people, two of whom became legally blind (13). Gene-modified HSC infused into patients with monogenic disease (6, 7, 9, 10, 14), resulted in leukemia from insertional mutagenesis in several patients. This risk is possible also with the use of iPSC, *e.g.*, a patient developed glioneuronal multifocal brain cancer after the infusion of fetal donor-derived neuronal stem cells (4). These detrimental effects could be potentially alleviated by employing a cellular suicide gene strategy in which a gene is inserted into the therapeutic cell and can then be activated 'on demand', causing cell death. Suicide gene technologies can be broadly classified based upon their mechanism of action in (i) metabolic (gene-directed enzyme prodrug therapy, (ii) dimerization-induced apoptosis, and (iii) monoclonal antibody-mediated cytotoxicity. However, currently available suicide gene systems are not ideal.

Gene-directed enzyme prodrug therapy converts a nontoxic drug to a toxic drug in gene-modified cells, as with the human herpes-simplex-virus-thymidine-kinase (HSV-TK)/ganciclovir, which is the first invented suicide gene system. The HSV-TK system showed promise but has major limitations. The HSV-TK transgene used as the suicide gene is immunogenic, and its activation requires the administration of the therapeutic anti-viral agent ganciclovir.

Its immunogenicity could preclude persistence and activity of the infused therapeutic cells. In the setting of donor lymphocyte infusion post allogeneic HSCT this still resulted in anti-tumor effect, likely due to the slow kinetic of cell's elimination (15).

The requirement for ganciclovir may limit its use in patients with cytomegalovirus infection where this agent is a primary treatment option. In addition, ganciclovir has other off-target toxicities, including myelosuppression and renal dysfunction.

The ΔiC9 suicide gene is expectedly less immunogenic, and the activating agent is not a therapeutic drug. Triggering of the ΔiC9 suicide gene leads to the activation of multiple executioner caspases (caspases 3, 6, and 7) and is effective in inducing apoptotic cell death.

The ΔiC9 suicide gene is a chimeric protein composed of a drug-binding domain linked in frame with a component of the apoptotic pathway, allowing conditional dimerization and apoptosis of the transduced cells after administration of a non-therapeutic small molecule dimerizer, such as AP1903 or BB homodimerizer (15–19). Straathof et al. (19) and Tey et al. (20) validated the ΔiC9 construct for T cell applications, demonstrating optimal transduction efficiency, expansion, and elimination of ΔiC9 T cells with strong expression of the transgene (19–21). *ΔiC9* was cloned in-frame, using a *2A*-like sequence from Thosea asigna insect virus (22, 23), with a truncated CD19 domain (*ΔCD19*) serving as a selectable marker to ensure ≥90% purity (20, 24, 25).

The ΔiC9 suicide gene has been investigated in gene modified T cells after allogeneic HSCT (26), CAR T-cells (27), mesenchymal stromal cells (3), iPSC (28), iPSC derived T cells (1) and for cancer therapy (29).

In a phase I clinical trial using the ΔiC9system (21) recipients of CD34-selected haplo-HSCT for hematological malignancies received escalating doses ($1x10^6$-$1x10^7$ cells/kg) (20, 30) of ΔiC9-modified allo-depleted T cells. In patients with acute graft versus host disease (GVHD) administration of a single dose of 0.4 mg/kg AP1903 resulted in apoptosis of ≥90% of ΔiC9-modified T cells within 30 minutes, followed by a rapid (within 24 hours) and permanent abrogation of GVHD. Remarkably, residual ΔiC9-modified T cells were able to re-expand, contained pathogen-specific precursors, and had a polyclonal T cell receptor repertoire. Although the incomplete elimination can benefit microbiological diseases in the setting of allogeneic HSCT, since the elimination of the gene-modified cells is incomplete [~75-90% of gene-modified T-cells or iPSC (1, 28)], even after repeated (31) or higher doses (32).

Seminal work of Straathof et al. showed 99% elimination of primary T-cells *in vitro* and *in vivo* but only after selection for high transgene expression (19).

As such additional strategies are needed to ensure that the infused, genetically modified cells can be reliably and completely eliminated. Several issues need to be addressed, including (a) improving efficiency of cell killing, (b) predicting inter-individual variations in responsiveness and/or acquisition of resistance, and (c) titrating the therapeutic effect based upon the degree of toxicity., .

Ideally, the strategies would permit flexibility in which the cell therapy could be either downregulated to control moderate toxicities or eliminated completely in the case of severe toxicities.

Our previous finding that the proportion of cells that were not susceptible to ΔiC9-induced apoptosis had elevated levels of BCL-2 (32), prompted us to examine whether BCL-2-mediated pathways could interfere with the induction of apoptosis by the ΔiC9 pathways. We found that combined treatment with the BCL-2 inhibitor, ABT-199 and the BB homodimerizer resulted in the complete elimination of ΔiC9 cells *in vitro* (33). These results support the concept that targeting the mitochondrial apoptotic pathways might enhance the efficiency of ΔiC9.

We hypothesized that co-expression of an additional caspase molecule with an alternative (apoptotic mitochondrial pathway activation) and complementary mechanism of action (activation of the executioner caspase 3) would result in superior/complete gene-modified cell elimination. Since caspase 9 activity is inhibited by direct phosphorylation from mitogen proteins, including the Ser196 residue present in ΔiC9 (34), at a greater extent than what was reported for caspase 8 (35), concurrent inducible activations of caspase 8 would contribute to cell killing in a subset of cells in which CASP9 is inhibited (less likelihood of failure). In parallel, we also tested the combination of ΔiC9 and the RQR8 compact suicide gene activatable by rituxan (36).

Our studies provide fundamental information on the degree and mechanisms of a novel method of cellular regulation based on the ability to activate one or both arms of a combinatorial suicide gene strategy.

Materials and methods

Tumor cell lines

All cell lines were freshly acquired from the American Type Culture Collection *(ATCC)*. The Institutional Review Board of the University of Alabama for Human Use at Birmingham (Birmingham, AL) approved this study (IRB# 160516007). 293T HEK17 cells were maintained in culture with DMEM medium *(Thermo Fisher Scientific)* containing 10% fetal bovine serum (FBS) *(GE Healthcare Life Sciences)* and 2mM L-glutamine *(Thermo Fisher Scientific)*. Jurkat clone E6.1 cells were maintained in culture with RPMI 1640 *(GE Healthcare Life Sciences)*, supplemented with 10% FBS and 2 mM L-glutamine.

Transgene constructs

The nucleotide and amino acid sequence of the constructs employed in this study is reported in the supplementary materials. The *F36V (FKBP12 mutated)* inducible *caspase 9* retroviral vector with a *Δ*CD19 selectable marker was obtained from Baylor College of Medicine (MTA#8733). The *F36V* inducible caspase 8 (*caspase 8* without *CARD* domain) was cloned by PCR and ligated into the SFG vector (gift of Dr. J Maher, King's College of London) using the Infusion technique *(Takara)*. The SFG-RQR8-IRES-GFP vector was provided by Dr. M. Pule (University College of London) and combined with the inducible caspase 9 by Infusion technique to create SFG.iC9-RQR8-IRES-GFP. The lentiviral constructs *(Elafin alpha promoter, F36V-ΔiC8-T2A-RQR8, and Human PGK promoter FRB-L-FKBP-L-ΔiC9-T2A-ΔCD19)* were cloned by Vectorbuilder. The FKBP12-GGSGG-FKBP12-rapamycin binding domain (FRB)-SGGGSG domains were connected to caspase 9 without CARD (catalytic subunity). We used the same number of amino acid published by Stavrou et al. (37) with a slightly different linker sequence.

The integrity of cloning for all constructs used in this manuscript was confirmed by Sanger sequencing performed either by Vectorbuilder or by the Heflin Center for Human Genetics of the University of Alabama at Birmingham, using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit as per the manufacturer's instructions (Applied Biosystems). The sequencing products were run following standard protocols on an Applied Biosystems 3730 Genetic Analyzer with POP-7 polymer.

Transduction

For transduction, replication-incompetent retroviral or lentiviral supernatant was prepared by transfecting *293T* with DNA encoding our construct of interest, the *Peg-Pam-e* plasmid containing the sequence for *MoMLV gag-pol* (or PsPAX2, from Addgene) and the plasmid containing the sequence for the VSVG envelope (Addgene), as previously described (38). The lentiviral supernatant was manufactured by Vectorbuilder or in house using the LV-max third generation packaging system (VSVG envelope). Supernatant harvested at 48 or 72 hours post-transfection was used for transduction. Cells were gene-modified with 2mL of unconcentrated retroviral supernatant on retronectin coated plates for 3 days or overnight with lentiviral supernatant at an MOI of 10, based on ELISA titration, in the presence of Polybrene 16 ug/mL.

Phenotype

Monoclonal antibodies conjugated with a fluorescent marker were used for flow cytometry as indicated (*BD Biosciences and Invitrogen)*. Expression of the QBend10 selectable marker was assessed using a biotinylated anti-QBend10 monoclonal antibody (Invitrogen) followed by Streptavidine-APC (Biolegend). Cells were analyzed by a FACS Canto II *(BD Biosciences)* for fluorescence signals. For each sample, a minimum of 10,000 viable events were acquired and analyzed using the Kaluza software v.2.1 *(Beckman Coulter: Brea, CA)*.

Killing assay

We performed ***in vitro*** experiments to demonstrate drug elimination of suicide gene-modified cells through activation of the suicide gene of interest. The following drugs were applied at the indicated concentrations unless otherwise stated: non-therapeutic chemical inducer of dimerization, BB homodimerizer, [100nM] *(AP-20187, Clontech; Mountain View, CA)*, sirolimus 25 ng/mL, or rituxan 100 ug/mL in the presence of rabbit serum complement *(Innovative research, Novi, MI)*. Cells were incubated overnight with chemical inducer, except in experiments using rituxan, where the incubation time was 4 hours. After the appropriate treatment, cells were washed and stained for viability and apoptosis using the Annexin V/7AAD kits (BD Biosciences). We added 123e counting beads (Invitrogen) and acquired a constant number of beads for each experimental condition using the FACS Canto II. The degree of cellular's elimination was estimated using the following formula: [*100% - (%Viability treated/% Viability non-treated cells)]*. To confirm that killing was due to apoptosis, we performed some experiments after pretreatment with 20 uM of the pan-caspase inhibitor Z-VAD-FMK for 1 hour (BD Pharmingen).

Regrowth experiments

After the appropriate treatment, cells were washed and re-cultured for regrowth. This was followed by Annexin V/7-AAD staining *(BD Biosciences)* and FACS analysis, as previously indicated.

Mitochondrial dysfunction

Mytosox red (Invitrogen) was used to test for mitochondrial dysfunction. Cells were treated with 1 ul of a 5 uM working solution. After 10 minutes of incubation at 37° Ccells were washed three times in PBS by centrifugation, followed by flow cytometry acquisition and analysis on FACS Canto II.

Western blot

Western blot was performed on whole-cell lysates lysed with 1x lysis buffer (Phosphosolutions), NuPage (Invitrogen), a protease inhibitor (Bimake), 10 mM sodium fluoride and 20 mM beta-glycerophosphate (Fisher scientific). An equal number of cells was plated for each condition, and the cells were harvested after 3 hours of incubation. The lysates were separated by electrophoresis using a standard lab technique and transferred using the dry transfer iBlot2 *(Invitrogen)*. All primary antibodies (Cell Signaling or Proteintech for GAPDH) were used at 1:1000 dilution and incubated overnight at 4° C. All secondary horseradish peroxidase-conjugated antibodies (Cell signaling rabbit and Proteintech mouse) were used at 1:10,000 and incubated for 1 hour at room temperature. Signal development was performed using the ECL Select detection system (Amersham) and acquired on the G-box automated dark room (Syngene). PageRuler prestained protein ladder was used (Thermofisher).

Statistical analysis

All data are presented as the average± standard deviation (SD) or standard error of the mean (SEM) where indicated. Unpaired Student *t*-test was used to determine the statistical significance of differences between samples, and a (two-sided) *P*-value less than 0.05 was accepted as a statistically significant difference. Average ± standard error of the mean (SEM) is shown/reported for all the experiments unless otherwise indicated. Data were analyzed and plotted using GraphPad Prism.

Results

Generation of an inducible caspase 8

We generated an inducible caspase 8 (ΔiC8) construct activatable by the BB homodimerizer. ΔiC8 consists of the pro-domains of caspase 8 with or without the caspase activator recruitment domain in frame with the F36V drug-binding domain (Figure 1A). After transducing Jurkat cells with replication-incompetent retroviral supernatant, triggering of the inducible caspase 8 after administration of the BB homodimerizer resulted in appreciable cell killing (~20%, not shown); such killing was increased after removal of the endogenous caspase activator recruitment domain (Figure 1B, and Suppl. Table 1), as previously published (39). Considering these results and that the caspase 8 is upstream of the apoptotic pathway activating both the extrinsic and intrinsic cascade, it was chosen for further evaluation.

Co-activation of inducible caspase 8 and 9 activated by a homodimerizer

To assess whether co-activation of ΔiC8 and ΔiC9 would result in superior cell elimination compared with the activation of each caspase alone, we performed co-transduction experiments in Jurkat cells transduced with replication-incompetent retroviral supernatant encoding each caspase separately or in combination. ΔiC8 was co-expressed with a

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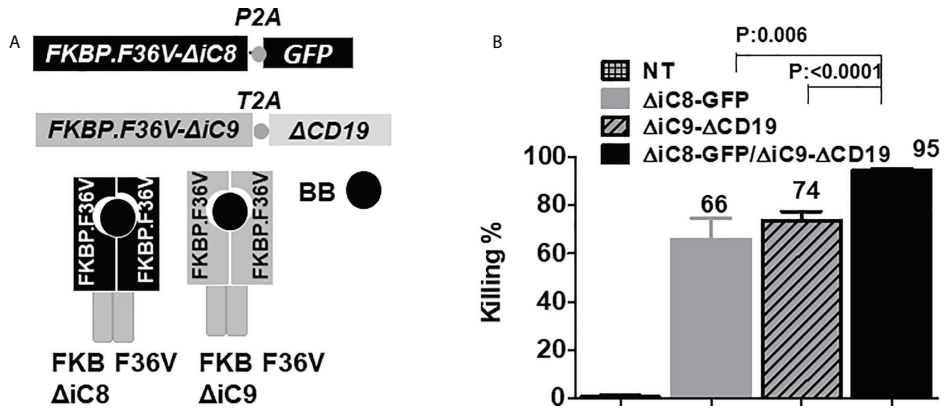

FIGURE 1
(A) *F36V ΔiC8* and *F36V ΔiC9* retroviral constructs and proteins diagram, activatable by a BB homodimerizer. **(B)** Inducible elimination of ΔiC8-GFP, ΔiC9-ΔCD19, and ΔiC8+ΔiC9 co-expressing Jurkat cells (N=5-7 experiments) after overnight exposure to the BB homodimerizer at a concentration of 100 nM.

GFP selectable marker, and ΔiC9 with a truncated CD19 selectable marker. After overnight exposure, we found that the BB homodimerizer at a concentration of 100nM resulted in the elimination of 66 ± 8.5% ΔiC8-GFP expressing cells, 74 ± 3.5% ΔiC9-ΔCD19 expressing cells, and 95 ± 0.7% ΔiC8+ΔiC9 co-expressing cells, with a statistically significant difference as compared with each caspase alone (P=0.006, or <0.0001, respectively). As a control, exposure of non-transduced (NT) Jurkat to the BB homodimerizer resulted in only 0.7 ± 0.7% killing. N=5-7 experiments (Figure 1B).

Co-activation of inducible caspase 8 and 9 activated by a homodimerizer and a rapalog analog

To activate each caspase with an independent agent, we cloned the FKBP-linker-FRB-linker heterodimerization domain before the inducible caspase 9, enabling dimerization after exposure to the commercially available rapamycin analog sirolimus. Administration of sirolimus results in binding of the pockets with heterodimerization of the FKBP12-rapamycin binding domain (FRB) fragment of mammalian target of rapamycin (mTOR) with FKBP12 and activation of the caspase 9 pathway.

To make our system applicable to different cell types, we used a lentiviral vector system, and either the EF1alpha promoter for the ΔiC8 or the human PGK promoter for the ΔiC9. In those constructs, a truncated human CD19 molecule was again co-expressed with ΔiC9 as a selectable marker. In contrast, for ΔiC8, we used the RQR8 compact selectable marker, which can be detected using the QBend10 CD34 antibody.

Jurkat cells were gene-modified with replication-incompetent lentiviral supernatant encoding each construct alone or together as co-transduction. We used an MOI of 10 based on ELISA titration. Jurkat cells gene-modified with the ΔiC8-RQR8 encoding supernatant had 91 ± 1.7% expression of QBEND10. Jurkat cells gene-modified with the Rapa.ΔiC9-ΔCD19 encoding supernatant had 83 ± 1% expression of ΔCD19. Jurkat cells were gene-modified with supernatant encoding the ΔiC8-RQR8, and the Rapa.ΔiC9-ΔCD19 had 56 ± 4% co-expression of QBend10 and ΔCD19. Figure 2A shows dot plots from a representative experiment.

Cells were not enriched for transgene expression using selection because (i) the expression of the selectable marker was robust, and (ii) non-gene-modified cells act as an internal control after killing. Cells were able to expand in culture (Figure 2B) despite the constitutive expression of a suicide gene, and in this limited observation gene-modified cells expanded at a higher rate as compared with non-transduced cells. In standard killing experiments, we used the BB homodimerizer at a concentration of 100 nM, which was previously demonstrated as a plateau concentration *in vitro*, and readily achievable in patients after therapeutic dosing. We used sirolimus at the therapeutic concentration of 25 ng/mL. We observed ~20% background cell elimination in non-transduced cells treated with the BB homodimerizer or sirolimus. Activation of each construct alone resulted in elimination through apoptosis of a significant number of gene-modified cells (≥80%). Notably, there was no interference between the two systems (Figures 3A, B).

For the cells co-transduced with both constructs, the percentage of cell killing was statistically higher (P=0.04) for the inducible ΔiC8 (97 ± 1%) than Rapa.ΔiC9 (86 ± 6%).

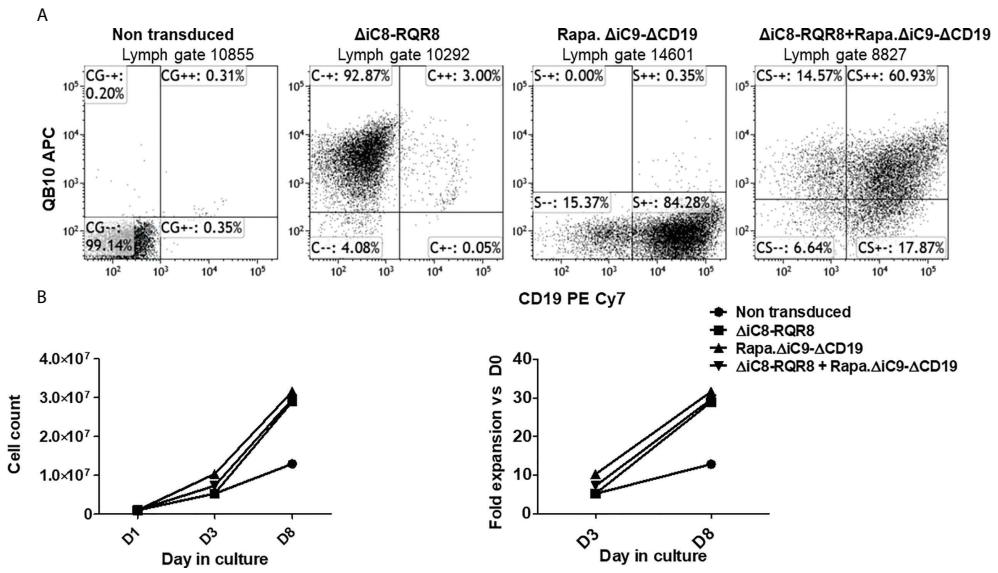

FIGURE 2
(A) Representative flow cytometry expression of the selectable marker for Jurkat non-transduced or gene-modified with (*F36V).ΔiC8-RQR8*, Rapa.*ΔiC9-ΔCD19* or co-transduced. **(B)** (left) Average with SD of cell count and (right) fold expansion of non-transduced or suicide gene-modified cells (one experiment in duplicate).

Notably, the cell-killing observed using a single agent in cells co-transduced with both constructs was comparable to cells transduced with a single construct. The combination of the two suicide genes resulted in higher elimination of cells (99 ± 0.5%), which was statistically significant as compared with sirolimus alone (P=0.02), and biologically relevant as compared with BB homodimerizer alone (Figures 3A, B and Suppl. Table 2). For regrowth experiments, cells were recultured an additional 14 days, then recounted. We found that the number of ΔiC8-RQR8/Rapa.ΔiC9 suicide gene-modified cells regrowing in the condition treated with both agents was statistically significantly lower and at the background level in all experiments. The average cell count and standard error for the untreated condition, was 2.4e6 ± 3.2e4, for the BB treated condition was 1.5e6 ± 1.2e4, for the sirolimus treated condition was 1e6± 2.3e4, and for the BB/sirolimus treated condition, 6.5e4 ± 3.1e3 (Figure 5B). The superior reduction in cell regrowth after activating both systems is potentially linked to preventing resistance mechanisms (Figure 5B).

Cell elimination was preceded by the cleavage of the effector of the apoptosis Poly (ADP-ribose) polymerase (PARP). Consistent with the lack of interference between the two systems, as shown in the cell elimination assay, only the specific activation of each construct with the appropriate agent resulted in cleavage of PARP (Figure 4A).

Mitochondrial dysfunction was assessed using the MytoSox Red flow cytometry method (Figure 5A). Interestingly, the magnitude of mitochondrial dysfunction was higher with sirolimus-treated cells. The cell elimination was reversed by pre-treatment with a pan-caspases inhibitor (Figure 4B).

We report the expression of the selectable marker on day 1 and on day 14 after the killing assay and reculturing the cells *in vitro* from one representative experiment (Figure S1).

Titration experiments showed that the BB homodimerizer offered the flexibility of titration in doses potentially achievable in the clinical setting. Sirolimus remained very potent at minimal concentrations unachievable *in vivo* (Figure 5C).

Co-expression of inducible caspase 9 and the RQR8 compact suicide gene

In parallel, we investigated if expressing two suicide genes with a different mechanism of action would lead to the complete elimination of gene-modified cells. We generated a construct co-expressing the ΔiC9, the RQR8 compact suicide gene, and a GFP selectable marker. In addition to the QBend10 as a selectable marker, the RQR8 compact suicide gene contains a CD20 mimotope that the CD20 antibody that rituxan can target, resulting in complement and antibody-dependent cytotoxicity, Figure 6A. We gene-modified Jurkat cells to express the RQR8-GFP transgene alone or with the ΔiC9 in a single construct (ΔiC9-RQR8-GFP).

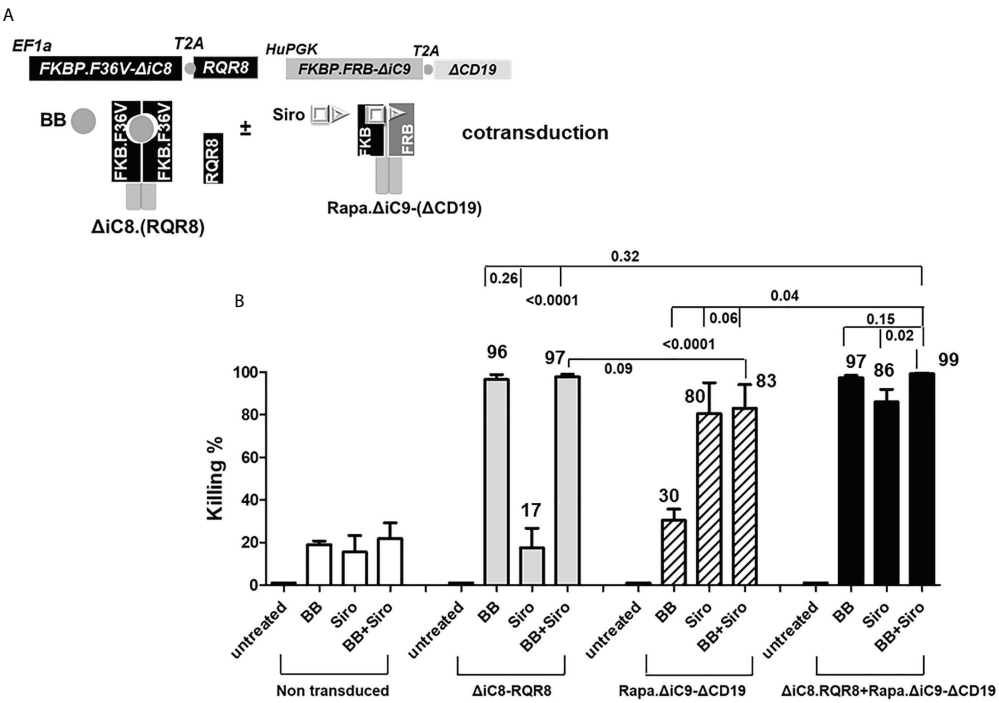

FIGURE 3
(A) *ΔiC8-RQR8* (Elafin1a promoter), Rapa.*ΔiC9-ΔCD19* (Human-PGK promoter) lentiviral constructs, and proteins diagram; *ΔiC8 and* Rapa.*ΔiC9* are activatable by the BB homodimerizer or sirolimus, respectively. **(B)** Average with SD of the percentage of killing in non-transduced Jurkat cells, or Jurkat cells gene-modified to express ΔiC8-RQR8, Rapa.ΔiC9-ΔCD19, or both (N: 3-7 experiments) after overnight exposure to the BB homodimerizer at 100nM, sirolimus (siro) 25 ng/mL or both.

We assessed the co-expression of the GFP and the RQR8 by flow cytometry. Non-transduced cells had 0.13% ± 0 co-expression, RQR8-GFP 81% ± 11, and ΔiC9-RQR8-GFP 82% ± 2.8 (N: 2), as shown in Figure 6B and Suppl. Table 3.

The ΔiC9 suicide gene alone resulted in 98% cell elimination after applying the BB dimerizer. As expected, the killing with rituxan or the BB dimerizer of non-transduced cells was negligible. Rituxan alone resulted in 80 ± 11% elimination of gene-modified cells, whereas sequential administration of the BB dimerizer followed by rituxan resulted in 98 ± 1% elimination (P=0.06). The co-activation of the ΔiC9 and RQR8 suicide genes improved killing compared to that achieved with activation of the RQR8 suicide gene alone with rituxan (Figures 6C, D), with complete elimination of the gene-modified cells with no regrowth (not shown).

Discussion

Our studies provide information on the activity of two suicide gene systems expressed in combination for the safety of cellular therapeutics. Our first system is based on an inducible caspase 8, activatable with a BB homodimerizer, and an inducible caspase 9, activatable after the administration of sirolimus

The second system is based on an inducible caspase 9 activatable by the BB homodimerizer and the RQR8 compact suicide gene, targeted by an anti-CD20 monoclonal antibody, such as rituxan, Both the RQR8 and the Rapamycin-activatable caspase 9 have been investigated *in vitro* and *in vivo* in mice in combination with CAR T-cells targeting CD19 (36, 37) and proved effective in inducing cell elimination. We observed a superior cell killing after using the homodimerizer to activate either caspase 8 or caspase 9, compared with the activation of Rapa.ΔiC9 with sirolimus. Differences in vector design, including length and structure of the linker sequence separating the two domains of the Rapa.ΔiC9, may account for such differences as previously reported (37). Our experiments estimated the degree of cell killing for each system independently or in combination, achieving a more comprehensive cell elimination when activating both arms of either system. The superior reduction in cell regrowth after activating both systems is potentially linked to preventing resistance mechanisms. The results add to the literature on combination suicide gene systems. Shah et al. (40) published on a mifepristone-induced gene expression of inducible caspase 3 and inducible caspase 9 activatable by the BB homodimerizer *in vitro* and a novel mice model. However, in this study, a quantification of cell killing with each suicide gene alone or in combination is missing. Fang

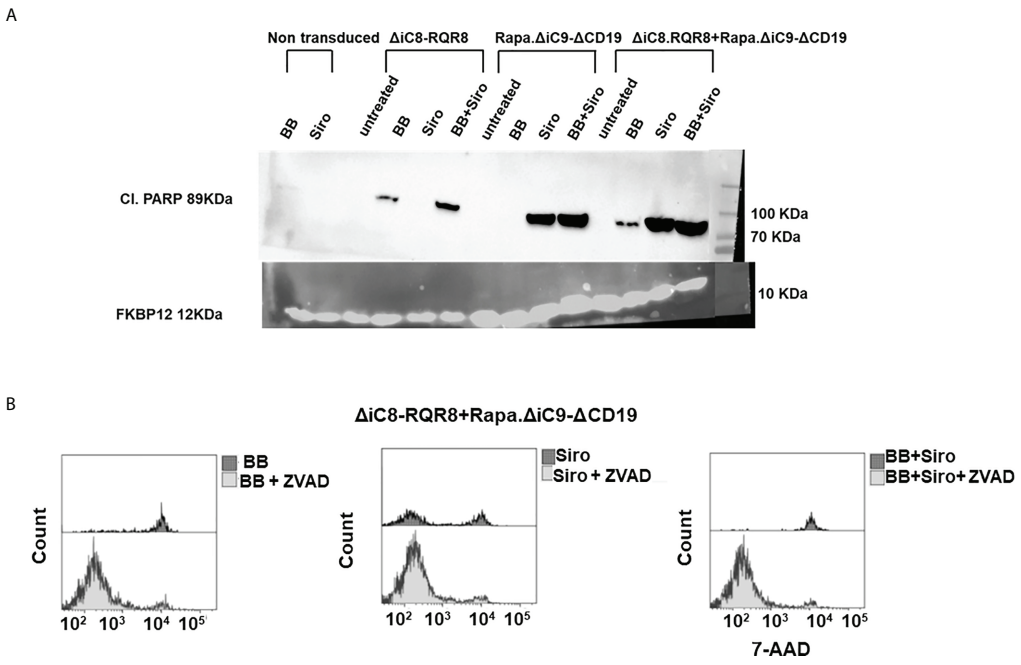

FIGURE 4
(A) Western blot for cleavage of the effector of the apoptosis Poly (ADP-ribose) polymerase (PARP), or FKBP12 endogenous control. **(B)** Histogram depicting viable ΔiC8-RQR8/Rapa.ΔiC9-ΔCD19 Jurkat cells after administering the BB homodimerizer, sirolimus (siro) or both in the absence or presence of the ZVAD fmk pan-caspases inhibitor.

et al. (41) developed a reversibly immortalized hepatic progenitor cell line for regenerative medicine, where the removal of the SV40T gene was guaranteed through HSV-TK/GCV selection. In contrast, cell elimination was performed under the control of a single cytosine deamines/5-fluoruracil suicide gene system. In addition to the limitation of using metabolic suicide gene systems, it is unclear whether cells could regrow after activating the suicide gene. Martin et al. (42) published a study on improving the safety of iPSC using genome-edited orthogonal safeguard. They used targeted integration to express an inducible caspase 9 activated by the BB homodimerizer at the end of the NANOG gene using a 2A sequence, showing the ability to prevent or ablate teratomas. They integrated a second suicide gene after the ACTB gene using a 2A sequence to eliminate differentiated cells. After observing that the HSV-TK system was slow and preferentially killing proliferating cells, they investigated using an inducible caspase 9 activated by the AC heterodimerizer. While this system is similar to a sirolimus-induced safety switch, the AC heterodimerizer has never been investigated in patients and is not available for clinical infusion. This study supports using two safety switches, albeit here each for a different cellular differentiation state. Based on the risk of incomplete cell elimination with a single suicide gene, a double system for each differentiation state is more ideal. Since selectable markers are useful for cell selection or tracking, incorporating RQR8 would also grant an additional safety measure. Additionally, using two different caspases would reduce the risk of gene recombination.

It is crucial to perform safety studies to elucidate the effect on gene-modified cells, such as the propensity for insertional mutagenesis and tumorigenesis in stem cell products. Albeit chromosomal position effects are less likely to silence two suicide switches provided on two independent vectors, we plan to compare them with a single construct to assess if it can already circumvent this effect in case of high transgene copy numbers, as previously reported (19).

The inducible Caspase 9 (43) and the RQR8 suicide gene are under active clinical investigation in CAR-T clinical trials. In a case report, activating the inducible caspase 9 with the BB homodimerizer rimiducid resulted in the resolution of ICANS that was resistant to the administration of tocilizumab and corticosteroids (27). In a subsequent small clinical trial in nine patients, the infusion of iC9-CAR-T targeting CD19 proved safe and effective in controlling leukemia. However, none of the patients met the eligibility criteria to activate the suicide gene (43). The authors are also investigating if a lower dose of rimiducid would ameliorate the CRS/ICANS without eliminating the infused CAR-T cells (43).

One alternative approach would be to combine a suicide genes with other strategies to mitigate side effects from the infused cells, activating the suicide gene only as a last resort.

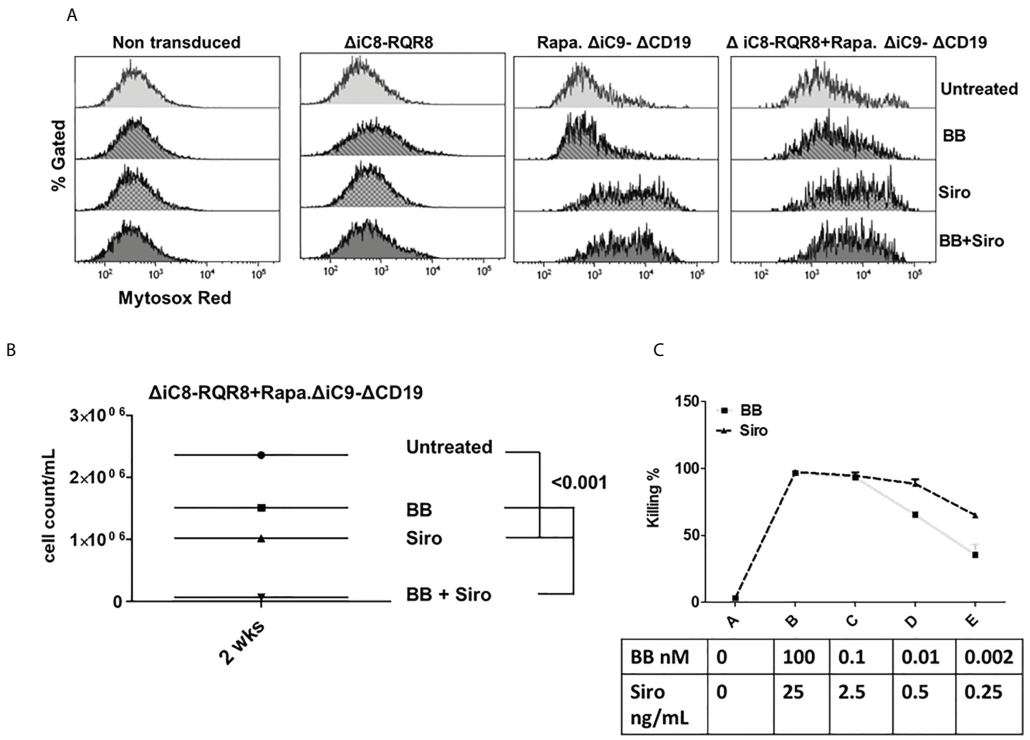

FIGURE 5
(A) Mitochondrial dysfunction assessed with the MytoSox Red flow cytometry method on ΔiC8-RQR8, Rapa.ΔiC9a-ΔCD19, or F36VΔiC8/RapaΔiC9 co-expressing cells treated with the BB homodimerizer, sirolimus (siro) or both. **(B)**: Day 14 analysis of regrowth (N: 3) of ΔiC8-RQR8/Rapa.ΔiC9 suicide gene-modified Jurkat cells treated with the BB homodimerizer, sirolimus (siro) or both. **(C)** Titration of ΔiC8-RQR8/Rapa.ΔiC9 suicide gene-modified Jurkat cells with decreasing concentration of BB homodimerizer or sirolimus.

Wiebking et al. published on a transgene-free safety switch in cell lines, pluripotent cells and primary human T-cells. Using genome editing methods, they disrupted uridine monophosphate synthetase in the pyrimidine *de novo* synthesis pathway making proliferation dependent on external uridine and enabling to control cell growth by modulating the uridine supply, both *in vitro* and *in vivo* in a murine model. Additionally, disrupting this pathway created resistance to 5-fluoroorotic acid, enabling positive selection of the gene edited cells (44).

Other strategies are more specific for CAR T-cells include logic-gated CAR T-cells with functional operations triggered by two input signals. For example, it is possible to increase the specificity by either requiring recognition of two antigens on the cell's surface (bispecific CAR) or the absence of an antigen (inhibitory CAR). An additional approach involves the use of a modular CAR which is split into two interactive parts, the signaling module on T cells and the switching module is administered separately to recognize the target antigen. The use of T-cells electroporated with mRNA encoding a CAR molecule granted transient CAR expression, providing an additional method for finer spatial and temporal control. Majzner et al. (45) found that change in signaling domains or the hinge-transmembrane domain region can alter activity against low vs. high antigen tumors to open a therapeutic window that could prevent possible on-target off-tumor toxicity.

A regulatable elimination of cellular therapeutics is also relevant in allogeneic HSCT. It is well accepted that allogeneic T-cells mediate both GVHD and a graft-versus-tumor effect (46). In the setting of GVHD, it was hypothesized that high levels of transcription of the ΔiC9 transgene caused by T-cell-receptor activation in alloreactive T-cells explained the selective elimination of these cells by the dimerizer (21), and it is important to experiment if a low dose of dimerizer would control of graft-versus-host disease while maintaining a few alloreactive cells potentially granting a graft-versus-tumor effect.

While the incomplete elimination proved of some benefit to control microbiological disease after allogeneic HSCT (21), it is necessary to completely eliminate the infused gene-modified cells for severe toxicities. The reason why some cells survived after activation of the inducible caspase 9 remains elusive. Hypotheses include the survival of cells with a low level of transgene expression or with higher expression of anti-apoptotic molecules. Chang *et al.* reported that the elimination of ΔiC9

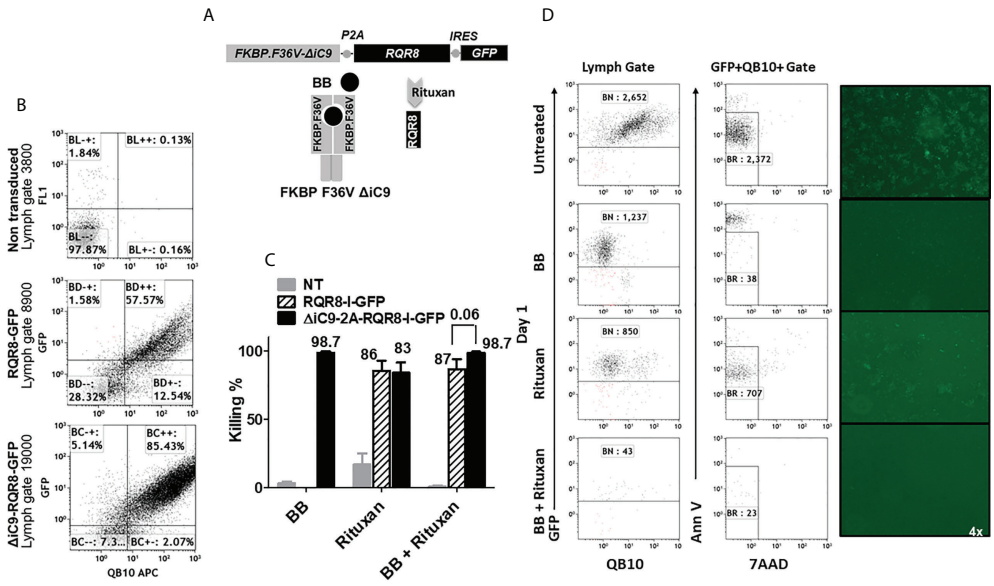

FIGURE 6
(A) Retroviral transgenic construct and protein diagram of *ΔiC9-RQR8-GFP* activatable with the BB homodimerizer and the anti-CD20 monoclonal antibody rituxan. 6**(B)** Dot-plots of QBend10/GFP expression from a representative experiment. **(C)** Summary of N: 3-5 killing experiments of Jurkat non-transduced, expressing the RQR8 compact suicide gene or the iC9 and the RQR8 suicide gene in a single construct treated with the BB homodimerizer overnight [100nM], or rituxan [100 ug/mL] for four hours in the presence of rabbit serum complement. **(D)**. Dot-plot and fluorescent images from one representative killing assay.

gene-modified cells is determined by a minimum expression threshold of the transgene in activated T-cells, which is dependent on T-cell receptor activation state of the T-cells, as well as *cis*-acting influences by host promoters on the proviral transgene (47).

Our approaches also offer a model to study a specific inducible cell death pathway by inducing caspase 8 or 9 in this case. Building on this, we are also investigating the combination of an inducible caspase with inducible strategies to inactivate anti-apoptotic molecules (e.g. BCL-2) or to induce an additional pro-apoptotic molecule.

Furthermore, the results of our study have applications beyond CAR T-cells and support the development of a cellular safety switch for genetically modified stem cells and other iPSC-derived progeny for cancer or regenerative medicine. There is growing interest in generating off-the-shelf T-cell therapy products for treating solid cancer or hematologic malignancies, which is important for patients with quantitative or qualitative T-cells defects (48). Additionally, applying gene-editing techniques aimed at knocking out the endogenous T-cell receptor (49, 50) and/or human leukocyte antigens (HLA) molecules (51) is essential to reduce alloreactivity and immune response, enabling the infusion of cellular therapeutics across HLA barriers. However, with the concern of insertional mutagenesis, the incorporation of a suicide gene is ideal. Examples of insertional mutagenesis and malignant transformation were reported in gene-modified HSC using gamma retroviral vectors in clinical trials for severe combined immune deficiency (6–8), X-linked chronic granulomatous disease (52), and Wiskott-Aldrich syndrome (10). Clonal dominance has also been reported from a clinical trial in beta-thalassemia using a lentiviral vector (9, 14). Modifications of the lentiviral vector (incorporation of insulator sequences) have reduced some complications, and this approach is currently under clinical investigation in patients with hemoglobinopathies (NCT01745120 and NCT02140554), and sickle cell disease (NCT04293185). Integration hotspots have been identified in stem cell products of some patients without transformation events.

In conclusion, we performed *in vitro* validation of two inducible suicide gene combinations (caspase 8: caspase 9 and caspase 9:RQR8). We showed that gene-modifying cells with two suicide gene constructs and a selectable marker is feasible. We also demonstrate that the two systems can be activated independently to control the cells of interest, with superior cell killing with superior reduction in cell regrowth compared to single suicide gene systems. While results need to be confirmed in other cell types, especially primary cells, they provide a framework for enhancing the safety of cellular therapeutics, facilitating the translation of novel gene therapy strategies in the pre-clinical and clinical setting.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Author contributions

CF and LS performed cloning and experiments, drafted the manuscript, edited, and reviewed the final version of the manuscript. MA-O, MA-Z, and KP performed cloning and experiments, edited, and reviewed the final version of the manuscript. KM, contributed to vector's cloning, edited, and reviewed the final version of the manuscript. MA, DS, RB, and FG, concept design, monitoring, reviewed data, and reviewed the final version of the manuscript. AD conceived the idea, designed the experiments, supervised research, analyzed the results, reviewed, and edited the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.975233/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Expression of the selectable marker on days 1 and 14 after killing from one representative experiment using Jurkat expressing ΔiC8-RQR8, Rapa.ΔiC9- ΔCD19 or both, treated with the BB homodimerizer [100nM], sirolimus (siro) [25 ng/mL] or both.

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