How do CARs work?
Early insights from recent clinical studies targeting CD19

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Abbreviations: B-ALL, B-cell acute lymphoblastic leukemia; BENDA, bendamustine; CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukemia; CTX, cyclophosphamide; EOP, end of production; FLU, fludarabine; IL-2, interleukin-2; MSKCC, Memorial Sloan-Kettering Cancer Center; NCI, National Cancer Institute; PENT, pentostatin; scFv, single-chain variable fragment; TCR, T-cell receptor; UPenn, University of Pennsylvania

Second-generation chimeric antigen receptors (CARs) are powerful tools to redirect antigen-specific T cells independently of HLA-restriction. Recent clinical studies evaluating CD19-targeted T cells in patients with B-cell malignancies demonstrate the potency of CAR-engineered T cells. With results from 28 subjects enrolled by five centers conducting studies in patients with chronic lymphocytic leukemia (CLL) or lymphoma, some insights into the parameters that determine T-cell function and clinical outcome of CAR-based approaches are emerging. These parameters involve CAR design, T-cell production methods, conditioning chemotherapy as well as patient selection. Here, we discuss the potential relevance of these findings and in particular the interplay between the adoptive transfer of T cells and pre-transfer patient conditioning.

Introduction

Chimeric antigen receptors (CARs) are emerging as powerful tools for reprogramming T-cell specificity and function.1–3 CARs are hybrid receptors comprising a ligand for a cell-surface molecule, most often consisting of a single-chain variable fragment (scFv) derived from a monoclonal antibody or an antigen-binding fragment (Fab) fused to signaling domains assembled to redirect T-cell function.4 Unlike transduced T cell receptors (TCRs), CARs endow T cells with a new specificity that is independent of HLA restriction and do so without competing with the endogenous TCR for the rate-limiting CD3 complex. First-generation CARs mediated limited T-cell activation, enabling cytotoxicity but only short-term T-cell expansion. Second-generation CARs, which combine activating and co-stimulatory signaling domains, enable improved cytokine secretion, T-cell expansion upon repeated antigen exposure and T-cell persistence.5,6 CARs have been generated against a large number of cell surface molecules,4 including CD19, HER2, GD2, prostate-specific membrane antigen (PSMA) and mesothelin, and many of them are presently under evaluation in over 30 Phase I clinical trials (www.clinicaltrials.gov). To date, the most promising clinical outcomes of this technology have been reported in patients treated with autologous CAR-modified T cells targeting CD19.6–10 CD19 is an attractive target for CAR-based therapy as it is expressed by most B-cell leukemias and lymphomas but not in tissues other than normal B lineage cells.11,12 In pre-clinical settings, CD19+ malignancies were the first cancers to be eliminated by CAR-engineered human T cells administered intravenously to systemic tumor-bearing mice.13 Successful B-cell tumor eradication was eventually obtained with different CD19-directed CARs,14–17 paving the way for multiple clinical studies and making the targeting of CD19 a paradigm for evaluating CAR technology.18 Here, we review and compare recently published results from clinical trials involving patients treated with CD19-targeted, CAR-modified T cells. These results identify at least some of the requirements for effective CAR therapy that should inform the design of future clinical studies.

Clinical Outcomes in the First Six Clinical Trials Targeting CD19 with CARs

The results of 6 clinical trials targeting CD19+ malignancies utilizing CAR-targeted autologous T cells have been recently reported.6–10,19,20 A total of 28 patients were treated, including 22 with chronic lymphocytic leukemia (CLL, Table 1). Jensen et al.19 reported of two patients with relapsed follicular lymphoma who were treated with multiple infusions of CD19-targeted clonal T cells. Both patients developed progressive disease (PD) within 6 mo after the last T-cell infusion.19 Savoldo et al.20 reported results from six patients with indolent or aggressive lymphomas, of whom two had stable disease (SD), the longest duration being 10 mo, while the other four developed PD. Kochenderfer et al.6 reported the first promising clinical outcome with CD19-targeted T-cell therapy in a patient with relapsed follicular lymphoma who achieved a partial response (PR) as well as B-cell aplasia, a surrogate marker for CAR-modified T-cell functionality in vivo. Three studies from the Abramson Family Cancer Research Institute at the University of Pennsylvania (UPenn),
CAR Design

CARs have considerably evolved over the past decade. First generation CARs, comprising an activation domain as the sole signaling component, effectively redirected cytotoxicity but showed major limitations in sustaining T-cell function. The introduction of dual-signaling receptors, combining activation and co-stimulatory signaling domains, paved the way for generating more potent and persisting immune responses. In an elegant side-by-side comparison, Savoldo et al. demonstrated the greater persistence of T cells expressing a CD28/CD3ζ-based CAR as compared with concomitantly administered CD3ζ CAR-transduced T cells, validating earlier comparisons of first and second generation CARs in mouse models.

Second generation CARs comprise the signaling domain of co-stimulatory receptors such as CD28, 4–1BB, OX-40, DAP10 and others, but have not been extensively compared with each other. In one study treating the CD19+ pre-B ALL cell line NALM-6 in SCID/beige mice, the CD28/CD3ζ construct outperformed a panel of other second generation CARs in terms of therapeutic efficacy. Milone et al., who also utilized a B-ALL model in NSG mice, found that CD28/CD3ζ-based CARs are similar in terms of therapeutic efficacy, but that the 4–1BB/CD3ζ CAR-transduced T cells exhibit greater accumulation over time, possibly due to antigen-independent T-cell proliferation and persistence. CAR comparisons in xenogenic mouse models are important to study the
UPenn utilizes a 4–1BB/CD3ζ motif. The NCI and UPenn groups selected the same scFv, which is different from that used at MSKCC. The three constructs thus differ in antigen recognition and/or signaling properties, but the degree to which these differences contribute to different outcomes needs to be analyzed in the context of other important parameters, as discussed below.

T-Cell Manufacture

There are important differences in the T-cell production processes employed at different centers (Table 2). T-cell doses were generally obtained within 10 d to 3 weeks of ex vivo culture, although some approaches required a longer culture time. All centers use anti-CD3 antibody stimulation for T-cell activation in combination with either anti-CD28 antibody co-stimulation or co-culture with peripheral blood mononuclear cells (PBMCs). Although one run at UPenn failed to reach the required cell dose, the limited amount of infused T cells was sufficient to achieve a ≥ 10 mo-long CR. The biology of CARs and guide therapeutic choices, but they are complex to interpret. First, the xenogenic nature of these models does not recapitulate all the cell interactions that affect T-cell function, trafficking and persistence. Second, all CARs of a given kind, e.g., CD28/CD3ζ CARs, are not equal (for example, some require more interleukin-2 stimulation than others). These considerations command caution in the comparison of CARs of different types (optimized CARs representative of their category should be used for valid comparisons). In this respect, third-generation CARs combining CD28 and 4–1BB co-stimulatory signals in addition to CD3ζ-mediated activation are even more complex. Third, the role of other components of the CAR should not be underestimated. Indeed, the nature of the scFv or Fab, the topology of the targeted epitope and its distance relative to the cell surface, as well as the affinity of CARs, represent additional variables that profoundly influence CAR function.

The CD19-targeting CARs tested in CLL patients in the aforementioned clinical trials are shown in Figure 2. The constructs used at the MSKCC and NCI were based on the same CD28/CD3ζ structure, whereas the construct employed at UPenn utilizes a 4–1BB/CD3ζ motif. The NCI and UPenn groups selected the same scFv, which is different from that used at MSKCC. The three constructs thus differ in antigen recognition and/or signaling properties, but the degree to which these differences contribute to different outcomes needs to be analyzed in the context of other important parameters, as discussed below.
determined how the distinct modes for gene transfer affect CAR expression by T cells over time, upon infusion into patients. The long-term impact of different gene transfer modalities still remains difficult to apprehend. Scholler et al.35 have recently demonstrated the presence of T cells harboring a gamma-retroviral encoded CD4/CD3ζ fusion receptor up to 7 y after infusion, the expression of which could be detected upon ex vivo T-cell activation. Burns et al.36 also reported that TCR transgenes are still expressed in patients with melanoma 2 to 10 mo post-infusion. The transduced vector was likewise detected for up to 9 y in patients treated with donor-derived Epstein-Barr virus-specific cytotoxic T lymphocytes (EBV-CTLs).3 Additional insights into the in vivo expression levels of CARs over time are needed to discern the impact of viral and non-viral vectors. Controlled studies evaluating the manufacturing process are required to determine the extent to which T-cell production conditions determine the clinical outcome of CAR-based immunotherapeutic strategies.

Tumor Burden, T-Cell Persistence and Clinical Response

In our initial studies at MSKCC,9 we noted an inverse correlation between a detectable persistence of CAR-modified T cells and disease burden at the time of T-cell infusion. Moving this analysis forward, we reviewed three patients treated at UPenn7,8 and note a similar inverse correlation between disease burden and the degree of clinical benefit (Table 1). While there is no uniform standard to measure tumor burden in patients with B-cell malignancies, June and colleagues’ estimated CLL tumor burden by taking into account circulating tumor cell counts, the amount of tumor cells in the bone marrow and peripheral tumor masses (i.e., lymph

Figure 2. Schematic diagram of chimeric antigen receptor (CARs) used to treat chronic lymphocytic leukemia (CLL) patients at MSKCC, NCI and UPenn. (A) 19–28z (MSKCC). (B) FMC63–28z (NCI). (C) 19–BB–ζ (UPenn). Groups at MSKCC and NCI utilized the CD28/ζ design described by Maher et al.5 The UPenn group used the 4–1BBζ design described by Imai et al.33 The MSKCC group used the SJ single-chain variable fragment (scFv)13 while researchers at NCI and UPenn used the FMC63 scFv.44 TM, transmembrane.
nodes infiltrated by tumor cells). Calculations made the additional assumption that $1 \times 10^{12}$ CLL tumor cells held an equivalent weight of 1 Kg. Utilizing this tumor burden calculation, the authors concluded that patient tumor loads in the bone marrow and blood ranged from $8.8 \times 10^{11}$ to $3.5 \times 10^{12}$ CLL tumor cells (Table 1). The degree of treatment response in this small sample size was inversely proportional to tumor burden, with the patient bearing the lowest tumor mass achieving the best clinical response (Table 1). Utilizing the same algorithm, we calculated tumor burden in our patients (Table 1). Acknowledging the caveat that tumor burdens are not uniformly measured at the same time point prior to CAR-modified T-cell infusion in the UPenn and MSKCC studies, we nonetheless found a similar inverse correlation between treatment response and initial tumor burden. Nevertheless, these studies indicate that large tumor burdens are not totally insensitive, and can even be eradicated by CAR-based therapy. Hence, while overall responses are greater when tumor burdens are smaller, tumor burden is not the sole predictor of response and should not be used to exclude patients from trials. Limited clinical data from the Baylor and NCI studies did not allow us to conduct similar retrospective analyses on these cohorts.

**Pre-infusion Chemotherapy: Tumor Reduction, Tumor Conditioning or Lymphodepletion?**

Most preclinical in vivo studies utilizing human CD19-targeted T cells that have been reported so far were conducted in immunocompromised mice bearing xenotransplanted human CD19+ tumors. Cell interactions that are closer to the physiological setting can be investigated in immunocompetent mice bearing syngeneic CD19+ tumor cells treated with syngeneic CD19-targeted T cells. These studies demonstrated that pre-cell infusion conditioning chemotherapy is required to enable meaningful antitumor responses by CAR-modified T cells. The results of these preclinical studies are consistent with those from clinical studies performed at the NCI in melanoma patients treated with autologous tumor infiltrating lymphocytes (TILs) expanded ex vivo.40 In the Baylor study, 20 patients were treated with CD19-targeted T cells in the absence of conditioning chemotherapy. In spite of interesting biological observations relative to T-cell persistence, the clinical outcomes of this study were poor. Of note, the design of the MSKCC study allowed for a direct comparison of CAR modified T-cell infusions given with and without conditioning chemotherapy. In particular, three patients were treated without prior conditioning chemotherapy, while a second cohort of patients was given cyclophosphamide 1.5 gm/m² before cell transfer. In this setting, conditioning chemotherapy enhanced both T-cell persistence and disease outcome.9

However, it is essential to note that all patients treated at MSKCC had previously received the conditioning agent (cyclophosphamide) in one or more cycles of unsuccessful conventional chemotherapy. Therefore, the conditioning regimen probably mediated a lymphodepleting effect but had marginal activity against cyclophosphamide-refractory tumor cells.9 In contrast to conditioning based on a chemotherapeutic agent to which the underlying tumor is refractory, patients treated at UPenn received a conditioning chemotherapeutic regimen containing agents with high antitumor efficacy.7, 8, 41 In fact, the eligibility criteria for the UPenn trial require that patients manifest either reduction or SD in response to the most recent cycle of chemotherapy. As a consequence, all patients treated at UPenn received effective second-line chemotherapy agents prior to CD19-targeted T cells.41, 42 One patient treated with bendamustine had previously been treated only with the antibody alemtuzumab, and therefore could arguably be deemed chemotherapy-naïve. Another patient was conditioned with

| Center       | T-cell activation | Gene delivery and expression methods | EOP T-cell phenotype | Range days in culture | Ref. |
|--------------|-------------------|--------------------------------------|----------------------|-----------------------|------|
| UPenn        | Anti-CD3/Anti-CD28 stimulation | Lentiviral vector (EF-1α promoter) | CD45RA+ (5–26%), CD62L+ (4–35%), CCR7+ (5–37%) | 10–14 | 7, 8 |
| NCI          | Anti-CD3 (OKT3) + autologous PBMCs | MSCV-Gammaretroviral vector | CD62L+ (9–78%), CCR7+ (1–36%) | 24 | 6, 10 |
| MSKCC        | Anti-CD3/Anti-CD28 stimulation | SFG-Gammaretroviral vector | CD28+ (43–94%), CD25+CD4+, FOXP3+ (0.6–2.4%) | 11–19 | 9 |
| Baylor       | Anti-CD3 (OKT3) | SFG-Gammaretroviral vector | CD45RA+ (0–15%), CD62L+ (15–90%), CCR7+ (0%) | 6–18 | 20 |
| City of Hope | Anti-CD3 (OKT3) + PBMCs/lymphoblastoid cell lines | Plasmid electroporation and hygromycin B selection | NA | $\geq 55$ | 19 |

EOP, end of production; NA, not available; PBMC, peripheral blood mononuclear cell.
cyclophosphamide in combination with pentostatin, a highly effective second-line agent for relapsed CLL patients⁴ⁱ that the patient had never received earlier. The patients treated at UPenn had advanced CLL tumors with TAP53 deletions,⁷,⁸ but the prognostic significance of this parameter is different depending on whether this deletion was present at diagnosis or relapse.⁴⁵

The first patient from the NCI who received CD19-targeted T cells was a highly pretreated patient with follicular lymphoma.⁶,¹⁰ Also in this setting, conditioning chemotherapy included a robust regimen combining high dose cyclophosphamide (2.5 mg/m² × 2 d) and fludarabine (25 mg/m² × 5 d), a drug that is efficient against low-grade B-cell malignancies to which the patient had never been exposed. In recently reported clinical data involving seven additional patients,¹⁰ prior chemotherapy regimens were not specified, limiting the analysis of tumor sensitivity to the conditioning therapy and subsequent clinical responses.

Based on these collective results, one can conclude that the nature of pre-infusion conditioning chemotherapy plays a critical role in the efficacy of targeted T-cell therapy. In contrast to the clinical outcomes observed at MSKCC in patients whose tumors were refractory to the conditioning regimen, the remarkable results obtained at NCI and UPenn⁶–⁸,¹⁰ were seen in the setting of a conditioning chemotherapy that probably resulted in lymphodepletion, in direct antitumor effects and possibly in other tumor modifications.

**Does T-Cell Dose Matter?**

Phase I clinical trials testing conventional drugs often adopt a dose escalation scheme to identify the maximal tolerated dose (MTD). However, in contrast to chemotherapeutic agents, infused modified T cells may undergo significant expansion under optimal conditions, as well as rapid disappearance under suboptimal conditions. Furthermore, the nature of the T cells that constitute the final T-cell product is likely to vary to considerable extents from one individual to another. One may therefore question whether a dose escalation paradigm in early studies with CAR-modified T cells is appropriate. The MSKCC studies⁹ followed this paradigm, while the UPenn study did not. Given the outcomes of CD19-targeted CAR modified T cells from various centers, we are now able to reflect upon this question in a more evidence-based manner. Cumulated data suggest that there is no correlation between T-cell dose and clinical response (Table 1). We noted optimal T-cell persistence and antitumor efficacy at the planned -1 T-cell dose (1 × 10³ 19–28z T cells/kg) as compared with patients treated with the initial dose level 1 (3 × 10³ 19–28z T cells/kg).² In the UPenn study, similarly dramatic clinical responses were noted in a patient infused with a standard dose of T cells as well as in another patient treated with an amount of T cells that was 2 logs lower.⁷,⁸ Further, in the NCI studies,⁷,¹⁰ two patients receiving a 10-fold higher T-cell dose than the patient who achieved a CR failed to exhibit as good an outcome. In summary, adoptive therapy with CD19-targeted T cells appears to be less dependent on T-cell dose than on other factors discussed above.

**Perspectives**

The introduction of second-generation CARs in the clinic is showing the first signs of success. The concept of T-cell potency, achieved through a combination of T-cell targeting and engineered co-stimulatory support, is supported by remarkable tumor regressions induced in patients with bulky disease. Yet, despite being born out of extensive preclinical molecular and animal modeling, how CARs work remains an enigma. Our early interim analysis of results obtained in 28 patients treated with CD19-targeted T cells at 5 centers places the spotlight not only on the CAR themselves but also on pre-infusion conditioning and individual patient characteristics. The impact of tumor burden and tumor chemosensitivity needs to be better defined. The importance of T-cell manufacturing, gene-transfer modality and T-cell subset composition of the infusion product are likewise important to evaluate. While mouse models can address these questions, at least in part, definitive answers are more likely to come from additional, well-designed clinical trials. The success of this research effort will benefit from inter-institutional collaborations to enable multi-center comparisons, accelerate patient enrollment and ensure an homogenous patient selection. Such concerted efforts will eventually lead to the optimal clinical exploitation of the CAR technology.

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