Supplemental Material

Title: Antibody modified T cells: CARs Take the Front Seat for Hematologic Malignancies

Authors: Marcela V. Maus,1,2 Stephan A. Grupp,1,3 David L. Porter,1,2 and Carl H. June1,4

Affiliations:
1Abramson Cancer Center and the 2Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA
3Children’s Hospital of Philadelphia, Philadelphia, PA
4Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA

Corresponding Authors:
Marcela Maus and Carl June
3400 Civic Center Boulevard, 8th Floor
Philadelphia, PA. 19104-5156
marcela.maus@uphs.upenn.edu
cjune@exchange.upenn.edu
Supplemental Text

As noted in the main text, there are currently 14 publications reporting clinical trials of CAR-T cells in hematologic malignancies. All but one of these focuses on B cell malignancies by targeting CD19 or CD20; the other has focused on AML by targeting Lewis Y antigen. Each group has designed slightly different protocols, and they vary with regard to design of the CAR, expression of the CAR on the T cells, T cell culture conditions, lymphodepleting strategy, cytokine support for the infused T cells, disease targeted, and timing of CAR T cell infusion with regard to standard therapy such as bone marrow transplantation. The details of trials conducted at the Fred Hutchinson Cancer Research Center, City of Hope, Baylor College of Medicine, MD Anderson Cancer Center, National Cancer Institute, Memorial Sloan-Kettering Cancer Center, the University of Pennsylvania and the University of Melbourne are found below.

Fred Hutchinson Cancer Research Center (FHCRC)
The Seattle group was the first to publish clinical trial results with CAR T cells in hematologic malignancies 1. In their initial trial, a first-generation CAR directed to CD20 with an IgG1 hinge and CD4 transmembrane domain and incorporating a neomycin selection gene under a separate promoter was electroporated into autologous PBMC that had been activated with the anti-CD3 antibody OKT3 and IL-2. The preparation of the CAR-modified T cell products involved 2-4 months of culture and expansion with neomycin selection. Nine patients were enrolled, but 2 did not have successful product manufacturing and only 7 were treated. In the first 3 patients, modified CARs were detectable by PCR for 5-21 days. In the last 4 patients, who had also received subcutaneous IL-2, modified T cells were detectable for 5-9 weeks. Four of the 7 patients had stable disease, 1 had a partial response for 3 months, and 2 of the 7 were in complete remission before T cell infusion, and remained without evidence of disease for 3 months and 13 months, respectively. No humoral or cellular immune responses to the CARs were found by a series of in vitro assays. A second study was reported four years later 2 with a third generation CAR incorporating the additional signaling domains of CD28 and 4-1BB. The same type of in vitro expansion was used. A CAR- T cell product was adequately manufactured for 3 of the 4 patients; in all cases, expression of the CAR on the manufactured cell product was so low that it could only be detected by PCR, and was
undetectable by flow cytometry or western blot. Modified cells were detected by PCR in the blood, in the range of 1-3.2%, and lasted 9 months in one patient and 12 months in two other patients. One of the three patients had a delayed partial response 3.5 months after the therapy, but then developed progressive disease. The other two patients had no evaluable disease but no disease progression. An excisional biopsy performed in one patient demonstrated that the modified T cells did traffic to the lymph nodes. In hindsight, we learned a great deal from these trials. Low-level expression of the CAR may not be effective, and this is not necessarily overcome by additional signaling domains or by lymphodepletion or systemic cytokine support. Second, prolonged culture conditions increase manufacturing failures.

City of Hope/FHCRC
The City of Hope and the FHCRC have published on two trials with hematologic malignancies. In one trial, a first generation CAR composed of anti-CD20 fused to IgG1 hinge and CD4 transmembrane domains was introduced into T cells via electroporation following OKT3 and IL-2 stimulation. The T cell product underwent repetitive stimulation with EBV-transformed irradiated feeder cells and repeated selection on neomycin, similarly to the other Seattle trials described above. However, in this case, a release criterion for the product was detectable expression of the CAR by Western blot and flow cytometry. Five patients were enrolled, but only 2 were treated due to manufacturing difficulties. In a parallel trial with a similar first generation CAR directed against CD19 and with hygromycin resistance and HSV-tk suicide genes under control of a separate promoter, the starting population consisted of CD8+ T cell clones that were infused into 2 patients with follicular lymphoma following 5 days of fludarabine. The patients also received low-dose subcutaneous IL-2 after the CAR T cell infusion. Of the four patients reported, one had detectable persistence of the transgene one week after the $10^8/m^2$ dose; transferred cells were detectable by PCR one week after the $10^9/m^2$ dose in two of seven administrations of that cell dose, but none were detected after administration of the higher dose of $2\times10^9/m^2$. Cell persistence was measured by real time quantitative PCR, and expressed as a percentage of CAR+ cells in PBMC, reaching a maximum of 1.8%. The authors postulated that there was rejection of the modified T cells; indeed, one subject developed a cellular immune response to the neomycin resistance gene product,
and two more had cellular immune responses to an undefined antigen epitope of the CAR T cell product. No humoral immune responses were detected.

City of Hope has since changed their gene-delivery method to be based on a 3rd generation self-inactivating lentiviral vector, and now has a number of other CAR trials in hematologic malignancies. These studies focus on second generation CD19-directed CARs with a potential suicide mechanism by incorporating a gene derived from a truncated form of EGFR that can be targeted with cetuximab.

**Baylor College of Medicine**

The Baylor group was the first to report an intra-patient competitive repopulation study design and the use of allogeneic donor-derived virus-specific T cells. In 2011, Savoldo et al. reported on a 6-patient trial where they infused each patient with two different CAR T cell products, directly comparing identical scFv’s directed to CD19 with identical hinge domains but one was first generation (CD3zeta) and the other was a second generation CAR with CD28 costimulation. T cells were generated by stimulating PBMC with OKT3 and IL-2, followed by retroviral transduction on day 3 and a short in vitro expansion for 6-18 days. Although no tumor regressions were observed in these patients with non-Hodgkin’s lymphoma, there was a clear difference between the two CARs in that the second generation CAR exhibited *in vivo* expansion and persistence for at least 4-6 weeks, whereas the first generation CAR had no in vivo expansion and was undetectable at 6 weeks. Both CD4 and CD8 T cells contributed to the in vivo expansion. Still, the relative expansion and persistence was modest. Three patients were retreated, and had the same pattern of persistence, indicating that rejection of the CAR T cells was not likely to be the reason for their disappearance.

The Baylor group has also made significant inroads in generating virus-specific gene-modified T cell products; the rationale for choosing virus-specific T cells is elegant in that the T cells will theoretically receive continued antigen stimulation through their TCR, and there is the added potential benefit of controlling viremia in the post-allogeneic transplant setting. Pre-clinical data generating tri-virus specific T cells from peripheral blood and from cord blood units were impressive. This group was the first to publish the use of donor-derived CD19-CAR virus-specific T cells in a cohort of eight patients. The
CAR was a second-generation CAR directed to CD19 and incorporating CD28 and CD3zeta signaling domains; the T cell products were 20-48% CAR+ by flow cytometry, indicating efficient transduction and expression of the CAR. The culture process was complex and prolonged as the total culture time was approximately 5-6 weeks. Because of the potential risk of allogeneic GvHD, no lymphodepleting chemotherapy was administered, and patients were treated with a dose escalation scheme based on the total number of T cells, ranging from 1.5x10^7/m^2 to 1.2x10^8/m^2. Six patients had relapsed 4 months to 13 years after allogeneic transplant. Two patients were in remission 3 or 8 months after their allogeneic transplant, but were at high risk for relapse. CAR T cells were detected at low levels for a median of 8 weeks in the blood by PCR. Two of the six patients with relapsed disease had a response: one with B-ALL, and one with B-CLL. Three patients had a viral reactivation; two of these were EBV, which were associated with an increase in the CAR signal, and one with adenovirus, which was not associated with an increase in the CAR signal. One issue with the use of virus-specific CTL is the difficulty in interpreting the cause and effect of the observations; if there is viremia, does that mean the infused T cells are not controlling the virus, or does the viremia help to expand the T cells?

Encouragingly, there was no evidence of GvHD or cytokine release syndrome. Four of the 8 patients had a decrease in B cell counts, which was an expected on-target toxicity, and is likely an indicator of CAR T cell function and persistence in vivo. There are several other ongoing trials of CAR therapies in hematologic malignancies at Baylor. Three trials of CD19-directed CAR T cells are focused on evaluating the effect of ipilimumab administered after CAR T cells in patients with CLL and NHL. A study comparing bulk T cells to EBV-specific CTLs transduced with a CD19 second generation CAR enrolled 4 patients but has not yet been reported. Baylor also has open protocols with CARs directed to kappa light chain in CLL, NHL, and myeloma, and CARs directed to CD30 for Hodgkin’s and NHL, either in EBV-specific CTLs or bulk T cells (Table 2). These CARs are all CD28-based second generation CARs introduced by retroviral transduction.
**MD Anderson Cancer Center**

Laurence Cooper has combined two platform technologies to facilitate manufacturing of CAR-modified T cells; one is to use the Sleeping Beauty transposase system to introduce the CAR gene product into T cells derived from peripheral blood or cord blood, and another is to use cell-based artificial APCs to expand the CAR-modified T cells. Sleeping beauty, like integrating retrovirus and lentivirus, is still subject to the potential concern of mutagenesis and oncogenesis, however the cost of goods is considerably less than with live viral vectors. There are currently 4 clinical trials targeting CD19 with this platform, including autologous T cells after chemotherapy or autologous HSCT, and cord-blood or donor-derived T cells combined with allogeneic transplant (Table 2). Results of these trials have not yet been reported, but preliminary data presented at a recent RAC meeting indicated that manufacturing was feasible and that both autologous and donor-derived CAR-modified T cells were detectable at low levels by flow cytometry in peripheral blood several months after infusion, indicating engraftment and continued expression of the CAR in the modified T cells.

**National Cancer Institute (NCI)**

The NCI group was the first to publish a 32-week-long partial remission and B cell aplasia after infusion of CD19-directed CAR T cells. In the case report, a single patient with follicular lymphoma was treated with high-dose cyclophosphamide (60 mg/kg x 2 days) followed by fludarabine (25 mg/m² x 5 days) and sequential doses of 1x10⁸ transduced cells followed by 3x10⁸ transduced cells and intravenous IL-2 every 8 hours for 8 doses. The T cell product was manufactured by stimulating PBMC with OKT3 and IL-2 followed by retroviral transduction of the CD28-based second generation CAR, followed by a rapid expansion protocol which includes irradiated allogeneic feeder cells from pooled donor PBMC; the total culture time was approximately 24 days. The patient developed B cell aplasia that lasted 39 weeks, and CAR transgene was detected in blood by PCR for 27 weeks; an impressive partial remission was noted. However, 7 months after the first infusion, he developed progressive disease and was retreated; after re-treatment, he had a partial remission that lasted 18 months, reported in. In the follow-up publication on this trial, eight patients were reported: 3 with follicular lymphoma, 4
with CLL, and 1 with splenic marginal zone lymphoma. There were 6 objective responses with one complete remission in the 7 evaluable patients. Four of the 8 treated patients had a B cell aplasia for at least 6 months. The T cell product had detectable CAR by flow cytometry, and T cells were detectable by flow cytometry and PCR in peripheral blood and bone marrow samples, indicating engraftment and surface expression of the CAR. The most prominent toxicities experienced by patients on this trial were transient fevers, hypotension, fatigue, renal failure and obtundation, generally peaking during the first 8 days. Many of these toxicities can be observed with the high-dose IL-2 these patients received, but the clinical toxicity and new elevations in serum IFN γ and TNF α that occurred 4 days after the last dose of IL-2 in several patients were likely related to de novo cytokine release syndrome (CRS) related to CAR T cells. The authors concluded that the strong lymphodepleting regimen, in combination with the CD28-based second generation CAR used in this protocol contributed to the improved in vivo persistence and efficacy observed compared to previous protocols.

The most recent clinical publication 12 from the NCI group in this field is a 10-patient trial of donor-derived CD19-directed CAR T cells infused in patients with relapsed CD19+ malignancy after allogeneic stem cell transplant from either a sibling matched related or matched unrelated donor (MUD). In this trial, donor-derived PBMC were stimulated with OKT3 and IL-2, transduced with the same retrovirus encoding the CD19-directed CD28-CD3ζeta CAR, and cultured for 8 days, which was much shorter culture time than the previous 24-day culture. Ten patients who had relapsed and tolerated at least one prior standard donor lymphocyte infusion (DLI) without GvHD were treated. In this trial, no conditioning or lymphodepleting regimens were used because of the concern for GvHD. Two patients had progressive disease, six patients had stable disease, one patient had a partial response, and one patient had a complete remission. Three of the patients had B cell aplasia, including 2 with stable disease and the patient with a complete remission. In 8 of the 10 patients, CAR+ T cells were detectable in blood 5 days after infusion, increased rapidly to peak at 7-14 days after infusion, but did not persist at detectable levels for more than one month in any patient. The patients who had had a MUD transplant had the highest peak levels of CAR+ T cells and the most severe cytokine-release syndrome.
In a pediatric trial testing the same CAR vector, Crystal Mackall and Daniel Lee are expanding T cells with anti-CD3/28 beads, giving lymphodepleting chemotherapy with fludarabine and cyclophosphamide, and administering 1x10^6/kg or 3x10^6/kg cells with no exogenous IL-2. Thus far, they have observed a 67% CR rate overall, with a 75% CR rate in ALL. Interestingly, they have noted anti-tumor effects even with doses of much less than 1x10^6 cells/kg, and have noted clearance of cerebrospinal fluid involved by ALL; all patients have had B cell recovery, and several have undergone or are preparing for an allogeneic transplant.

**Memorial Sloan-Kettering Cancer Center (MSKCC)**

The MSKCC group utilizes a retroviral construct with a CD19-directed scFv and a CD28-based second generation CAR. T cells are selected and stimulated with anti-CD3/28 beads, followed by transduction on day 2 and culture for a total of approximately 16 days. Here, the first cohort of three CLL patients was treated with 1.2-3x10^7 CAR+ cells/kg, and the first patient in the second cohort was treated at the same dose but after moderate lymphodepletion with a 1.5 gm/m^2 dose of cyclophosphamide. This patient unfortunately developed a syndrome consistent with sepsis and died soon after CAR T cell infusion; in retrospect, the investigators found that cytokine elevations were present before T cell infusion. Following this serious adverse event, the next four patients were treated at a dose-de-escalation of 0.4 – 1x10^7 CAR+ T cells/kg, split over 2 days. In the ALL protocol, subjects were treated with 3 gm/m^2 of cyclophosphamide followed by a split-dose infusion of 3x10^6 CAR+ cells/kg. In this report, none of the 8 CLL patients treated exhibited a complete or durable partial remission, but the one ALL patient treated had a persistent B cell aplasia in the blood and bone marrow; the ALL patient was treated in complete remission, so no specific disease response was evaluable. CAR+T cells were detectable by PCR in the blood and bone marrow by IHC in 2 of the CLL patients for up to 8 weeks and the ALL patient for 6-8 weeks. Of note, the 8 patients with CLL had mostly CD4+ CAR T cell infusions, whereas the manufactured products for the ALL patients were more even mixtures of CD4+ and CD8+ CAR T cells. The authors concluded that CD19-targeted T cells were more likely to persist in the setting of a lower tumor burden, as seen in ALL rather than CLL.
In the second MSKCC paper, the investigators noted rapid and profound remissions in adult 5 patients with ALL. The trial had enrolled 14 patients; 8 products were manufactured, and 5 patients were treated with the same retrovirally-transduced, CD19-directed CD28-based second generation CAR. Patients were conditioned with 1.5 – 3 gm/m² of cyclophosphamide one day prior to infusion of 1.5 – 3x10⁶ CAR+ cells/kg. Of the 5 patients treated, two had active disease, with 63% or 70% blasts in the marrow at the time of infusion. Both patients had a rapid morphologic CR; one was negative for minimal residual disease (MRD) at day 8, and the other became negative for MRD at day 59, and relapsed at day 90. Two of the other patients had MRD before T cell infusion, and became negative for MRD after T cell infusion and went on to allogeneic transplant. One patient was negative for MRD before T cell infusion, and went on to receive an allogeneic transplant after T cell infusion. The authors found that the cytokine-mediated toxicities occurred 3-5 days after T cell infusion, appeared to correlate with the disease burden, and was adequately managed with corticosteroids. CAR T cells were detectable by flow cytometry and PCR at 1 week and remained detectable 3-8 weeks. The patient who relapsed had CD19+ blasts that were still sensitive to CAR T cell-mediated killing in vitro, but presumably relapsed due to the abrogation of the CAR T cells from steroids given to treat the CRS. All subjects also had recovery of B cell counts, indicating that CAR T cell effects were transient. The authors concluded that CAR T cells could be used as a ‘bridge to transplant’ in patients who could not achieve remission with chemotherapy.

**University of Pennsylvania**

We published two papers describing remissions, delayed tumor lysis syndrome and CRS, along with CAR T cell persistence that is ongoing at 3 years in two of three patients treated with CLL. The Penn CAR was different in design than all of the other second generation CARs, because it included 4-1BB costimulation rather than CD28 costimulation. The rationale for 4-1BB costimulation included our prior data showing that 4-1BB stimulation improved the growth of CD8 T cells by making them resistant to activation induced cell death, and pre-clinical data demonstrating improved survival and engraftment of T cells transduced with this vector in xenogeneic models. Lentiviral transduction was used to introduce the CARs, and this resulted in relatively high
expression on the T cells, which has been sustained for several years. CAR-T cells were manufactured with a relatively short time window of 10 days; T cells from leukapheresis products were selected and stimulated with anti-CD3/28 beads, transduced on days 0 and 1, and expanded for 10 days in culture prior to cryopreservation. Subjects were treated with lymphodepleting chemotherapy within one week before CAR T cell infusion; $1.4 \times 10^7$ to $5 \times 10^8$ CAR-T cells were given over 3 sequential days. All 3 patients had advanced, heavily pre-treated CLL with high leukemia cell burdens. Two achieved complete remission and one had a partial remission for 4 months. In each case there was robust expansion and persistence of the T cells detectable by flow cytometry and by PCR at 10,000 copies per microgram of DNA in PBMC at their peak, and all had persisting T cells for at least 6 months. A delayed tumor lysis syndrome was also observed coincident with T cell proliferation. The relative contributions of the T cell culture system, the 4-1BB costimulation, the lentiviral transduction, and the lymphodepleting regimen are unknown; however, the dose of CAR T cells did not seem to be a significant factor given the wide range of cell doses given to these patients. Interestingly, all 3 patients had a delayed CRS syndrome at 7-21 days after infusion that was associated with high levels of IL-6, IFN-γ, and several other cytokines, and coincided with the peak concentrations of CAR T cells in peripheral blood. Persistent B cell aplasia was observed in both patients who achieved CR.

Since the initial publication of these first 3 patients, over 30 patients with advanced, high-risk and heavily pre-treated CLL have been treated. Overall response rates are approximately 50%, with about half complete and half partial remissions. Notably, many of the patients with partial responses experienced complete clearance of CLL from the blood and marrow and have slowly improving adenopathy, converting to complete remission in some cases over many months. The numbers of patients treated remains small, but to date it has not been possible to identify pre-treatment characteristics that predict for response. Interestingly, complete responses have occurred in patients with mutated p53, and in patients who have progressed after prior ibrutinib therapy. The most consistent correlates of response have been the \textit{in vivo} expansion and persistence of the CAR T cells, beyond 3 years in some cases. Most responding patients develop a CRS at the time of T cell expansion.
In April 2013, two pediatric patients with B-ALL treated with the same Penn CAR T cell product were reported \(^{21}\); both patients had complete remissions, CRS, and B cell aplasia. Both patients had a dramatic expansion and persistence of CAR+ T cells: in one patient, \(>70\%\) of the T cells expressed the CAR by flow cytometry on day 10, and in the other, \(>33\%\) of the T cells expressed the CAR on day 9, although only the second patient received conditioning chemotherapy prior to T cell infusion. Both patients had dramatic elevations in IL-6 and IFN \(\gamma\), and a host of other cytokines. One patient had severe CRS with biochemical evidence of macrophage activation syndrome (MAS) \(^{22}\). MAS closely related to hemophagocytic lymphohistiocytosis syndrome, and biochemically is characterized by highly elevated serum ferritin and C-reactive protein levels \(^ {23,24}\). The syndrome rapidly improved after administration of IL-6 receptor blockade with tocilizumab. One patient has remained in a complete remission more than 18 months after the CAR T cell infusion, and has not received an allogeneic transplant. The second patient ultimately relapsed with CD19-negative ALL; this was the first report of target loss induced by the CD19 directed CAR therapy. Unexpectedly, CAR T cells were also found in the cerebrospinal fluid, though neither patient had prior evidence of CNS involvement with leukemia.

As of October 2013, 16 pediatric and 6 adults patients with relapsed or refractory ALL have been treated with CART19 yielding a complete response rate of \(>80\%\) (13/16 pediatric patients and 5/5 evaluable adult patients at day 28 after infusion. Eleven of the 13 pediatric patients and all 5 adults in CR were also MRD negative. Notably, many of the pediatric patients were treated for relapsed ALL after prior allogeneic SCT. All responding patients developed a CRS of varying severity, ranging from mild  (i.e. low grade temperatures) to severe  (i.e. hypotension requiring multiple vasoactive agents and ICU care). Several patients have been treated with tocilizumab, with prompt resolution of fevers and other manifestations of their CRS and MAS.

**University of Melbourne**

One group in Australia has reported on the persistence and efficacy of T cells retrovirally transduced with a CD28-based second generation CAR directed to the Lewis Y antigen in AML \(^ {25}\). The Lewis Y antigen is a difucosylated carbohydrate antigen that is
overexpressed in a wide range of malignancies including AML, but has only limited expression in normal tissues. The CAR is composed of an scFv directed to Lewis Y, a CD8 hinge region, and the CD28 transmembrane and intracellular domains fused to CD3zeta. T cell products are prepared by stimulating PBMC with OKT3 and IL-2 for 3-4 days, followed by retroviral transduction and 7 days of in vitro culture. CAR T cell products were infused into 4 patients after bone marrow recovery from a course of fludarabine and cyclophosphamide. Three of the patients received T cells with MRD by cytogenetics, and one had active leukemia with blasts in the blood and marrow. Two patients had stable disease, one had a transient decrease in blasts, and one had a cytogenetic remission for 5 months. Lewis Y antigen expression remained stable in cases of relapse, and CAR+ T cells were noted to traffic to both bone marrow and skin lesions in a patient with leukemia cutis.

References

