Clinical and immunological responses after CD30-specific chimeric antigen receptor–redirected lymphocytes

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BACKGROUND. Targeting CD30 with monoclonal antibodies in Hodgkin lymphoma (HL) and anaplastic large cell lymphoma (ALCL) has had profound clinical success. However, adverse events, mainly mediated by the toxin component of the conjugated antibodies, cause treatment discontinuation in many patients. Targeting CD30 with T cells expressing a CD30-specific chimeric antigen receptor (CAR) may reduce the side effects and augment antitumor activity.

METHODS. We conducted a phase I dose escalation study in which 9 patients with relapsed/refractory HL or ALCL were infused with autologous T cells that were gene-modified with a retroviral vector to express the CD30-specific CAR (CD30.CAR-Ts) encoding the CD28 costimulatory endodomain. Three dose levels, from 0.2 × 10^8 to 2 × 10^8 CD30.CAR-Ts/m2, were infused without a conditioning regimen. All other therapy for malignancy was discontinued at least 4 weeks before CD30.CAR-T infusion. Seven patients had previously experienced disease progression while being treated with brentuximab.

RESULTS. No toxicities attributable to CD30.CAR-Ts were observed. Of 7 patients with relapsed HL, 1 entered complete response (CR) lasting more than 2.5 years after the second infusion of CD30.CAR-Ts, 1 remained in continued CR for almost 2 years, and 3 had transient stable disease. Of 2 patients with ALCL, 1 had a CR that persisted 9 months after the fourth infusion of CD30.CAR-Ts. CD30.CAR-T expansion in peripheral blood peaked 1 week after infusion, and CD30.CAR-Ts remained detectable for over 6 weeks. Although CD30 may also be expressed by normal activated T cells, no patients developed impaired virus-specific immunity.

CONCLUSION. CD30.CAR-Ts are safe and can lead to clinical responses in patients with HL and ALCL, indicating that further assessment of this therapy is warranted.

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Introduction
Provision of antigen specificity to T cells by insertion of chimeric antigen receptor (CAR) molecules offers an effective therapeutic option for patients with B cell lymphoid malignancies (1–10). The selective expression of the CD19 antigen by normal and malignant B cells has fueled the development of CAR-T therapy, since the associated B cell aplasia is a manageable side effect if objective and sustained clinical responses are achieved (11–13). Similarly, the limited expression of CD30 by normal tissues and its consistent overexpression by Hodgkin lymphoma (HL) and anaplastic large cell lymphoma (ALCL) tumor cells (14) have encouraged the development of CD30-directed CAR-T cell (CD30.CAR-T) therapy. In addition, the observed lack of “on-target” toxicities with toxin-conjugated CD30-specific monoclonal antibodies (15), such as brentuximab, together with the promising outcomes produced by this drug (16–18), further boosted the interest in developing CD30.CAR-Ts to improve the rate of clinical responses.

The proof of concept that HL is susceptible to immune-mediated killing originates from the remarkable responses observed in patients receiving ex vivo expanded T cells targeting virus-associated antigens, such as Epstein-Barr virus (EBV) in EBV-associated HL and non-Hodgkin lymphoma (19–21). Targeting CD30 with CD30.CAR-Ts offers the opportunity to rapidly generate tumor-specific T cells in all patients with HL.

Conflict of interest: While this clinical trial was being conducted, the Center for Cell and Gene Therapy at Baylor College of Medicine had a Collaborative Research Agreement with Celgene Corp. and Bluebird Bio.

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and ALCL, independent of their association with EBV. In addition, tumor recognition by CD30.CAR-Ts is MHC-unrestricted and thus overcomes potential tumor escape secondary to HLA downregulation or defective antigen processing (1). Finally, CAR engineering couples antigen recognition with T cell costimulation, providing a major advantage compared with canonical TCR recognition, as tumor cells may lack the expression of costimulatory molecules. The recent success of PD1 blockade of CD4+ and CD8+ T cells, with an overall CD8+ T cell predominance when grown in IL-2 (61.6% ± 12.6%) compared with IL-7/IL-15 (42.3% ± 18.7%; P = 0.019, t test on log-transformed data) (Figure 2C). CAR expression was comparable (~89%; corresponding to mean transgene copy numbers of 71,140 ± 7,800 per 100 ng of DNA) in all manufactured products (Figure 2B). CD30.CAR-Ts were composed of more than 99% CD3+ T cells, and each contained a variable ratio of CD4+ and CD8+ T cells, with an overall CD8+ T cell predominance when grown in IL-2 (61.6% ± 12.6%) compared with IL-7/IL-15 (42.3% ± 18.7%; P = 0.01) (Figure 2C). The majority of CD30.CAR-Ts were CD45RO+ and only a small fraction expressed central memory–associated phenotypic markers such as CD62L and CCR7 (Supplemental Table 1), which may reflect the heavy preponderance of CD4+ T cells in CD30.CAR-T manufacture. CD30.CAR-T expansion and persistence. We gave CD30.CAR-Ts to each patient as a single administration over a 2- to 5-minute period. Molecular signals (genomic quantitative PCR (qPCR) or IL-2) of CD30.CAR-Ts were detected in the peripheral blood of all patients by 3 hours after infusion (94 ± 23 copies/μg) in all manufactured products (Figure 2B). CD30.CAR-Ts were manufactured using either IL-2 (11 products) or IL-7/IL-15 (11 products) within 15 ± 4 and 15 ± 2 days, respectively (Figure 2 and Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI94306DS1). By day 15 of culture, CD30.CAR-Ts grown in IL-7/IL-15 had greater expansion from baseline and higher final cell numbers (45 ± 13 and 1.2 × 10^9 ± 5.5 × 10^8, respectively) than those expanded in IL-2 (27.4 ± 13 and 6.5 × 10^8 ± 3.3 × 10^8, respectively; P = 0.019, t test on log-transformed data) (Figure 2A). CAR expression was comparable (~89%; corresponding to mean transgene copy numbers of 71,140 ± 7,800 per 100 ng of DNA) in all manufactured products (Figure 2B). CD30.CAR-Ts were composed of more than 99% CD3+ T cells, and each contained a variable ratio of CD4+ and CD8+ T cells, with an overall CD8+ T cell predominance when grown in IL-2 (61.6% ± 12.6%) compared with IL-7/IL-15 (42.3% ± 18.7%; P = 0.01) (Figure 2C). 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Clinical outcomes and correlative studies. We observed clinical responses in 3 of 9 patients (2 CR, 1 continued CR), as well as 3 patients with stable disease (Figure 5A). Patient 9 (ALK+ ALCL) had a dramatic response after the first CD30.CAR-T infusion (Figure 5B) apart from modest reduction in eosinophils (Figure 4A). B and T cell counts remained stable (Supplemental Figure 2, A and B).

Acute and long-term toxicities. CD30.CAR-T infusions were well tolerated at all dose levels and after 1 or more infusions (Table 1). None of the adverse events reported (most frequently fatigue, hyper- or hypokalemia, and transient elevation of aspartate aminotransferase [AST]) were considered related to CD30.CAR-T infusion. After infusion, no significant differences were observed in white blood cell counts (Supplemental Figure 2, A and B) from modest reduction in eosinophils (Figure 4A). B and T cell counts remained stable (Supplemental Figure 3, A and B). No patients developed symptoms consistent with cytokine release syndrome. Elevations of inflammatory cytokines, such as IL-6 and TNF-α, were observed, which generally coincided with the peak of CD30.CAR-T expansion, but the fold increases from baseline were modest (Figure 4B and Supplemental Figure 2C). Similarly, no significant changes were observed in plasma levels of IL-15, IL-7, or IL-2. As CD30 is expressed on activated T cells (28), we monitored T cell immunity to viral antigens in all patients before and after infusion of CD30.CAR-Ts. Confirming our preclinical assessment (26), CD30.CAR-Ts did not impair the frequency of T cells (CTLP) reactive to common viral pathogens such as EBV, CMV, adenovirus, or influenza (Figure 4C). No patients developed viral infections after receiving CD30.CAR-Ts.

### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>UPIN</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Previous therapies</th>
<th>Time from last chemotherapy treatment</th>
<th>DL</th>
<th>CAR+ cells in product (%)</th>
<th>CAR+ T cells/ m² infused</th>
<th>Number of infusions</th>
<th>Best response</th>
<th>Current outcome</th>
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<tbody>
<tr>
<td>1</td>
<td>31</td>
<td>M</td>
<td>NS HL</td>
<td>ABVD, ICE, GDP, XRT, HDT/ASCT, EBV-CTL, brentuximab, 20-anti-Tac, IC/methylprednisolone</td>
<td>3 mo</td>
<td>1</td>
<td>90</td>
<td>2 × 10⁶</td>
<td>2</td>
<td>SD × 8 wk</td>
<td>Alive with disease</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>M</td>
<td>Cutaneous ALK-ALL</td>
<td>Methotrexate, pralatrexate, bortezomib, everolimus, brentuximab</td>
<td>9 mo</td>
<td>1</td>
<td>90</td>
<td>2 × 10⁶</td>
<td>1</td>
<td>CR</td>
<td>Alive with disease</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>M</td>
<td>NS HL</td>
<td>ABVD, ESHAP, IGEVE, HDT/ASCT</td>
<td>15 mo</td>
<td>2</td>
<td>90</td>
<td>1 × 10⁴</td>
<td>2</td>
<td>SD × 6 wk</td>
<td>Dead from heart disease</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>M</td>
<td>NS HL</td>
<td>ABVD, ESHAP, IGEVE, rituximab, C-MOPP, brentuximab</td>
<td>6 mo</td>
<td>2</td>
<td>89</td>
<td>1 × 10⁴</td>
<td>2</td>
<td>SD × 6 wk</td>
<td>Alive with disease</td>
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<tr>
<td>5</td>
<td>20</td>
<td>M</td>
<td>NS HL</td>
<td>ABVE-PC, XRT, IGEVE, gemcitabine/brentuximab, HDT/ASCT, rituximab, prednisone</td>
<td>8 mo</td>
<td>3</td>
<td>90</td>
<td>2 × 10⁴</td>
<td>1</td>
<td>CR</td>
<td>Alive with disease</td>
</tr>
<tr>
<td>6</td>
<td>36</td>
<td>M</td>
<td>NS HL</td>
<td>ABVE, IE-brentuximab, R-DHAP, GND, vorinostat, sirolimus, bendamustine, HDT/ASCT</td>
<td>1 mo</td>
<td>3</td>
<td>90</td>
<td>2 × 10⁴</td>
<td>2</td>
<td>CR × 36+ mo</td>
<td>Alive, NED</td>
</tr>
<tr>
<td>7</td>
<td>65</td>
<td>F</td>
<td>DLBCL/MC HL</td>
<td>R-CHOP, ICE, HDT/ASCT, ABVD</td>
<td>1 mo</td>
<td>3</td>
<td>89</td>
<td>2 × 10⁴</td>
<td>1</td>
<td>CR × 24+ mo</td>
<td>Alive, NED</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>F</td>
<td>NS HL</td>
<td>ABVD, ICE, HDT/ASCT, brentuximab, GND, bendamustine</td>
<td>2 mo</td>
<td>3</td>
<td>85</td>
<td>2 × 10⁴</td>
<td>1</td>
<td>CR</td>
<td>Alive with disease</td>
</tr>
<tr>
<td>9</td>
<td>30</td>
<td>F</td>
<td>Systemic ALK-ALL</td>
<td>CHOP, brentuximab, crizotinib</td>
<td>8 mo</td>
<td>3</td>
<td>90</td>
<td>2 × 10⁴</td>
<td>4</td>
<td>CR × 9 mo</td>
<td>Alive with disease</td>
</tr>
</tbody>
</table>

ABVD, doxorubicin, bleomycin, vinblastine, dacarbazine; ABVE-PC, doxorubicin, bleomycin, vincristine, etoposide, prednisone, cyclophosphamide; CCR, continued complete response; (R)-CHOP, (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone; C-MOPP, cyclophosphamide, vincristine, procarbazine, prednisone; CR, complete response; DL, dose level; DLBCL, diffuse large B cell lymphoma; EBV-CTL, EBV-specific cytotoxic T lymphocytes; ESHAP, etoposide, methylprednisolone, cytarabine, cisplatin; GDP, gemcitabine, dexamethasone, cisplatin; GND, gemcitabine, navelbine, liposomal doxorubicin; HDT/ASCT, high-dose therapy followed by autologous stem cell transplant; I(C)(E), ifosfamide, (carboplatin), (etoposide); IGEV, ifosfamide, methylprednisolone, cytarabine, cisplatin; GDP, gemcitabine, dexamethasone, cisplatin; GND, gemcitabine, navelbine, liposomal doxorubicin; HDT/ASCT, high-dose therapy followed by autologous stem cell transplant; I(C)(E), ifosfamide, (carboplatin), (etoposide); IGEV, ifosfamide, gemcitabine, vinorelbine; MC HL, mixed-cellularity HL; NED, no evidence of disease; NR, no response; NS HL, nodular sclerosis HL; R-DHAP, rituximab, dexamethasone, cytarabine, cisplatin; SD, stable disease; UPIN, universal patient identifier number; XRT, radiation therapy.
and was deemed to achieve a CR after the fourth infusion that was maintained for 9 months. Before CD30.CAR-Ts, patient 6 had residual pelvic lymphadenopathy (standardized uptake value of 3.3, which was above the activity in the mediastinum blood pool) after high-dose therapy and autologous stem cell transplantation. Six weeks after the first infusion of CD30.CAR-Ts, a repeat PET scan showed resolution (no activity above mediastinum blood pool), which was also documented in a scan performed 20 weeks later; he has been in CR for more than 2½ years (Figure 5C). Patient 7 had CD30.CAR-T infusion while in CR after salvage chemotherapy given for relapse after autologous stem cell transplantation, and she has remained in continued complete response for more than 2 years. Patients 1, 3, and 4 (nodular sclerosis HL) had transient stable disease at the 6-week evaluations. The remaining 3 patients had progressive disease (Figure 5A). Objective clinical responses were mainly achieved in patients infused at the third dose level.

Although the 7 patients who received 2 or more CD30.CAR-T infusions had lower CAR-T expansion following the second or subsequent infusion than after the initial cell dose (Supplemental Figure 1, A and B), human anti-mouse antibody (HAMA) remained undetectable or below levels of biological activity (maximum level 12.5 U/ml, with a negative HAMA assay defined as ≤74 U/ml) (Figure 6A), even after multiple infusions. The amount of peripheral blood we were permitted to collect after CD30.CAR-T infusions was insufficient to directly explore the emergence of cellular immune responses to the CD30.CAR in vivo. However, we consistently failed to elicit cytotoxic T cell responses targeting the CD30.CAR by exposing healthy individual T cells to multiple rounds of CAR.CD30-T–loaded T cells in vitro (ref. 29 and Supplemental Figure 4). The frequency of CD4+FoxP3+ regulatory T cells (Tregs) in the peripheral blood was 7.2% ± 1.3% before infusion and did not significantly change after CD30.CAR-T infusions (6.3% ± 1.4% and 6.4% ± 0.9% 1 week and 2 weeks after infusion, respectively) (Figure 6B). No correlation was found between the frequency of Tregs in the peripheral blood, expansion and persistence of CD30.CAR-Ts, or clinical response (data not shown). However, the limited expansion of CAR-Ts in the circulation after subsequent infusions, combined with the generally poorer persistence with subsequent infusions, supports the possible development of an unidentified negative factor in the host.

Soluble CD30 (sCD30) is typically elevated in advanced HL (30). Although CD30.CAR-Ts are not blocked by sCD30 (26, 31), we found an inverse correlation (P = 0.0087) between sCD30 levels at the time of CD30.CAR-T infusion and the subsequent expansion of CD30.CAR-Ts (Figure 6C). Notably, in responding patients the peak of CD30.CAR-T molecular signals in peripheral blood coincided with a transient increase of sCD30 consistent with the release of sCD30 upon tumor destruction (ref. 30, Figure 6D, and Supplemental Figure 3C). Finally, numerous reports have described high expression...
in T or B cell counts, or impairment in the frequency of circulating virus-specific T cells, and infection episodes were not observed, indicating an overall good safety profile of CD30.CAR-Ts. A fraction of eosinophils has been reported to express CD30 (28), and we observed a transient decrease of these cells in the peripheral blood after CD30.CAR-T infusions. Since eosinophils may promote the growth of HRS cells, a similar reduction of these cells at the tumor site may be beneficial (32).

In our study, CD30.CAR-Ts were well tolerated, and CRs were observed at the highest dose of CD30.CAR-Ts and after 2 or more T cell doses. The highest degree of expansion of circulating CD30.CAR-Ts (area under the curve, Supplemental Figure 1E) occurred at the third dose level. Our study has the major strength of showing direct effects of CD30.CAR-Ts, in contrast to results from Wang et al. (25), who followed the more general practice of using lymphodepletion prior to CAR-T infusion (2, 3, 5, 6, 9, 13, 33–35). There are several other differences between our trial and the one described by Wang et al., including the method for transgene delivery (lentiviral versus γ-retroviral) and the costimulatory endodomains (4-1BB versus CD28).

Nonetheless, both lentiviral and γ-retroviral vectors, and 4-1BB and CD28 endodomains have been associated of PD-L1 by Reed-Sternberg cells (23), and infusion of blocking antibodies to PD1 has produced significant clinical responses in patients with the disease (22–24). We therefore evaluated the expression of the PD-L1 cognate receptor PD1 in infused CD30.CAR-Ts. Overall, PD1 was expressed by 33% ± 9% of infused CD30.CAR-Ts (Figure 6E), but no correlation was observed between PD1 expression and CD30.CAR-T expansion/persistence in vivo.

Discussion
We report here the outcome of 9 patients with relapsed/refractory EBV-negative HL and ALCL treated with autologous CD30.CAR-Ts in a dose-finding study in which T cells were infused in patients without a conditioning regimen. Promising antitumor activity was documented in both HL and ALCL with the achievement of CR.

CD30 is an attractive target for immunotherapy because it is highly expressed by Hodgkin Reed-Sternberg (HRS) cells and by the malignant cells of ALCL. However, CD30 is also transiently expressed by a small fraction of activated B and T cells (28), and thus concerns remain that effective CD30.CAR-Ts could further impair cell-mediated immunity in patients who are already immunodeficient. None of our patients experienced significant changes in T or B cell counts, or impairment in the frequency of circulating virus-specific T cells, and infection episodes were not observed, indicating an overall good safety profile of CD30.CAR-Ts. A fraction of eosinophils has been reported to express CD30 (28), and we observed a transient decrease of these cells in the peripheral blood after CD30.CAR-T infusions. Since eosinophils may promote the growth of HRS cells, a similar reduction of these cells at the tumor site may be beneficial (32).

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with responses using other CARs (2–9, 13, 33–35). Therefore, these structural differences may not themselves account for the observed differences in response. An alternative possibility is that the affinity of the scFv may play a role, since sCD30 is present in the plasma of patients with advanced/aggressive disease. While the scFv we used in our CAR was previously shown to be unaffected by sCD30 (26, 31), no information is available for the scFv used by Wang et al. Moreover, we observed our best responses in patients with low sCD30, suggesting that a lower burden of disease may be important. No sCD30 data are available for patients in the Wang et al. trial.

The correlation between the dose of CD30.CAR-Ts and the in vivo expansion we observed in this small cohort of patients suggests that host lymphodepletion before CAR-T infusion would be beneficial in further improving their expansion and their antitumor activity. Indeed, while CD19-specific CAR-Ts encounter B cell targets in the peripheral blood upon infusion and thus receive immediate and appropriate costimulation, the expansion of CD30.CAR-Ts in the absence of circulating target cells may require homeostatic cytokines like IL-15 that will be more available following lymphodepletion (36). At present, the optimal conditioning regimen is unknown (25) and needs to balance the requirement for sufficient lymphodepletion and disruption of the tumor environment, with the desire to prevent toxicity from the depletion regimen itself, or the subsequently greater CAR-T expansion. However, chemotherapy may also prove dispensable for HL, a disease subset characterized by intrinsic overall immune deficiency (37). Indeed, previous experience with adoptive T cell therapy in EBV-associated HL has shown that responses can be achieved with the adoptive transfer of EBV or LMP-CTLs even in the absence of chemotherapy conditioning (20, 21, 38). Finally, we cannot rule out the possibility that the incorporation of IL-7 and IL-15 in the manufacturing procedure contributes to improved CAR-T functions.

Figure 4. Immunological effects of infused CD30.CAR-Ts. (A) Decrease of circulating eosinophils in the peripheral blood after CD30.CAR-T infusions. Shown are means ± SEM (n = 9; paired t test). (B) Plasma levels of inflammatory cytokines (shown are IL-6 and TNF-α) modestly increased after CD30.CAR-T infusion. Each symbol denotes a different patient (legend shows universal patient identifier numbers [UPINs]), and the dashed line represents the mean value. (C) Frequency of T cells targeting viral antigens in the peripheral blood at different time points before and after CD30.CAR-T infusions as assessed by IFN-γ ELISPOT assays. SFCs, spot-forming cells.
failed to elicit any T cell responses against the CD30.CAR. The limited persistence of CD30.CAR-Ts may also be a consequence of the affinity of the CD30-specific scFv used to construct the CD30.CAR. CD30 is present in a soluble form in the plasma of HL patients with advanced/aggressive disease (30), and the affinity of scFv may prove critical in determining the overall avidity of the CD30.CAR-Ts for sCD30 as opposed to membrane-bound CD30. Extensive binding of sCD30 may render CD30.CAR-Ts more susceptible to sequestration by scavenger cells. Although the scFv derived from the HSR3 antibody used to generate our CD30.CAR has been previously shown to be unaffected by sCD30 (26, 31), we found an inverse correlation between sCD30 levels at the time of the CD30.CAR-T infusion and expansion of CD30.CAR-Ts. Finally, the specific design of the CD30.CAR we used may affect their persistence. Our CD30.CAR construct contains a long IgG1 spacer region that may bind to the Fc receptor of monocytes/macrophages, and also favor CD30.CAR-T sequestration (40). In view of the clinical responses achieved, it seems unlikely that these cells were totally eliminated, and the disappearance of CD30.CAR-T molecular signals may instead reflect relocation of CD30.CAR-Ts in peripheral tissues (25).

HRS cells often express high levels of PD-L1, and checkpoint blockade with nivolumab or pembrolizumab shows significant efficacy in HL (22–24). Indeed, nivolumab has been granted accelerated FDA approval for treatment of HL progressing after treatment with brentuximab vedotin and autologous stem cell transplant. Nevertheless, checkpoint blockade is expected to be ineffective in the absence of endogenous tumor-infiltrating lymphocytes recognizing tumor neoantigens, or in the event of MHC dysfunction (41). In these patients the adoptive transfer of CD30.CAR-Ts opens the opportunity to provide tumor-specific T cells and thereby increase the effectiveness of checkpoint blockade.

In summary, our study demonstrates the tolerability, safety, and potential efficacy of CD30.CAR-Ts in CD30+ lymphoid malignancies. Appropriate tumor reduction and lymphodepletion before CD30.CAR-T infusion should enhance their clinical activity without increasing toxicity. Future exploration of the synergy between CD30.CAR-Ts and PD1/PD-L1 blockade may also prove beneficial.

Methods

Clinical study. We conducted a phase I study (NCT01316146) designed to assess the feasibility and safety of infusing escalating doses of autologous, polyclonally activated, peripheral blood T cells that were genetically modified to express a CD30-specific CAR (CD30.CAR) (31) encoding the CD28 endodomain in patients with relapsed/refractory CD30+ lymphoproliferative disorders (HL and non-Hodgkin lymphoma). All patients with a diagnosis of HL had CD30 positivity determined by a pathologist according to her/his institutional hematopathology standard measured against positive and negative controls. In all those cases, the Reed-Sternberg cells were deemed CD30+ and CD15+ (in a membrane/Golgi pattern, when described). Patient 2 (ALK– ALCL) had malignant cells that were also reported by a pathologist to be positive for CD30, CD4, and epithelial membrane antigen (EMA). Patient 9 (ALK+ ALCL), who had the best response, had malignant cells that were described as having "strong membranous and cytoplasmic staining with Golgi accen-
brane and cytoplasmic domain of the CD28 costimulatory endodomain, and the ζ chain. The CAR cassette was then subcloned into the SFG retroviral backbone (26). We generated the clinical-grade packaging cell line using PG13 cells (gibbon ape leukemia virus pseudotyping packaging cell line; American Type Culture Collection CRL-10686) (7). We used the highest-titer clone to establish a master cell bank and released the clone for clinical use only after safety testing and vector sequencing. We stored the final viral supernatant at –80°C and tested it before clinical release.

Generation and transduction of CD30.CAR-Ts.

To generate CD30.CAR-Ts, we transduced PBMCs obtained by Ficoll density centrifugation of peripheral blood obtained by conventional phlebotomy 1–3 months before CD30.CAR-T administration. We activated PBMCs with OKT3 antibody (Janssen Biotech) or CD3 and CD28 antibodies (Miltenyi Biotec), and recombinant human IL-2 (IL-2, 100 U/ml; Prometheus), or IL-7 (10 ng/ml; R&D Systems) and IL-15 (5 ng/ml, R&D Systems), in 24-well plates precoated with a recombinant fibronectin fragment (FN CH-296, Retronectin Takara; Clontech). After transduction, T cells were expanded ex vivo in the presence of IL-2 (100 U/ml) or IL-7 (10 ng/ml) and IL-15 (5 ng/ml), added twice a week. The change in the manufacture process with IL-7 and IL-15 replacing IL-2 took place during the trial (43). The characteristics of the T cell products are summarized in Figure 2 and Supplemental Table 1. Some patients had T cell lines made during the change in manufacturing and had both products available. Nontransduced, control T cells were grown in parallel from each patient in our manufacturing facility, starting from the same

### Figure 6. Correlative biological studies after CD30.CAR-Ts.

(A) There was no significant generation of human anti-mouse antibody (HAMA) after CD30.CAR-T infusions. (B) Shown are percentages of CD4+CD25+ (top graph) and CD4+FoxP3+ (bottom graph) T cells for the 9 infused patients. Each symbol denotes a different patient (UPINs), and the dashed line indicates the mean values. (C) Pearson correlation between peak of CD30.CAR-T molecular signals (log transformed) and sCD30 cell preinfusion for the 9 infused patients. (D) CD30.CAR-T molecular signals and sCD30 levels in the peripheral blood for patient 6. (E) The graph shows the percentage of PD1+ CD30.CAR-Ts in the infused products (shown are means ± SD; n = 7). On the right, dot plots from 2 representative patients are shown.
PBMC product. Generation of CAR-Ts was successfully completed for all PBMCs in which the process was initiated. Products were generated from stored PBMCs, after collection from consenting patients.

**Immunophenotyping.** We used phycoerythrin-, fluorescein isothiocyanate–, peridinin chlorophyll protein–, allophycocyanin, Alexa Fluor 750–, Alexa Fluor 700–, phycoerythrin cyanin 7–, Pacific Blue–, or Krome–Orange–conjugated CD3 (clone SK7), CD4 (SK3), CD8 (SK1), CD56 (B159), CD19 (SJ25C1), TCR-αβ (T10B9), TCR-γδ (B1), CD62L (DREG-56), CD27 (M-T271), CD28 (CD28.2), CD45RA (HI100 or 2H4), CD45RO (UCHLI), CCR2 (clone 48607), CCR4 (G11), CCR5 (2D7), CCR7 (3D12), CXCR3 (IC6), CXCR4 (12G5), and PD1 (MH4) (Becton Dickinson/Pharmingen or Beckman Coulter) antibodies to stain the T cell products. We included control samples labeled with appropriate isotype antibodies and used a “fluorescence minus one” strategy for multicolor staining. We detected the CD30.CAR-Ts with an Fc-specific cyanine-Cy5–conjugated (Fc-Cy5) monoclonal antibody (catalog 109-606-088; Jackson ImmunoResearch), which recognizes the IgG1.CH2–CH3 component of the CAR. We analyzed the cells by FACScan equipped with a filter set for 4 fluorescence signals, using CellQuest software, or a FACS-Canto II, using DIVA software (Becton Dickinson). In some cases cells were collected using the Gallios Flow Cytometer (Beckman Coulter) and analyzed using FlowJo software version 9.3 (Tree Star). Data analysis was performed using FlowJo Software (Tree Star) or Kaluza software (Beckman Coulter).

**Cytotoxicity assays.** The cytotoxic specificity of each CD30.CAR-T cell line was measured in a standard 4-hour 51Cr release assay, using effector-to-target ratios of 40:1, 20:1, 10:1, and 5:1. As target cells, we used HDLM-2 (CD30+ tumor cells) and Raji (CD30+ tumor cells). The target cells were labeled simultaneously for 1 hour with 51Cr. We calculated the percentage of specific lysis as [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100.

**Real-time qPCR of CD30.CAR transgene.** We quantified the integrated genome of the retrovirus encoding the CD30.CAR by real-time qPCR. After extracting DNA from peripheral blood samples with the QIAamp DNA Blood Mini Kit (Qiagen) following the manufacturer’s instructions, we amplified the DNA with primers and probes (Applied Biosystems), and HAMA (Alpha Diagnostic) resources of the Dan L. Duncan Cancer Center support grant 5P30CA125123 (NCI), and the National Gene Vector Biorepository. We thank the following for financial support: the NIH National Cancer Institute (grants 3P50CA126752 and R01CA131027), the National Heart, Lung, and Blood Institute (R01HL114564), and the Leukemia and Lymphoma Society (LLSTR 6227-08). The clinical trial also received support from the Institute for Clinical and Translational Research at Baylor College of Medicine and shared resources of the Dan L. Duncan Cancer Center support grant 5P30CA125123 (NCI), and the National Gene Vector Biorepository. We also thank Andreas A. Hombach and Hinrich Abken (University of Cologne, Cologne, Germany) for providing the original scFv.

**Statistics.** We used descriptive statistics (means, median, ranges, and standard deviations or standard errors) to summarize the data. We used Student’s t test (2-tailed) or the nonparametric Wilcoxon rank-sum test for between-group comparisons, while the nonparametric Wilcoxon signed-rank test was applied when observations were made within subject. Pearson correlation was used to test the statistical relationship between 2 variables. A P value less than 0.05 was considered statistically significant.

**Study approval.** This study was approved by the US FDA, the Recombinant DNA Advisory Committee, and the IRB of Baylor College of Medicine and was conducted in accordance with Declaration of Helsinki principles. All participants provided written informed consent upon enrollment.

**Author contributions**

CAR, GD, BG, CMR, MKB, HEH, and BS conceptualized the overall strategy and developed its clinical translation and implementation. The clinical protocol was written by CAR and MKB. CAR was the principal investigator of the protocol, and BG, MKB, and HEH were the investigational new drug (IND) sponsors. Manufacturing of T cells, flow cytometry, and qPCR acquisition of clinical samples were performed by BB, HZ, and OD, supervised by BS and CMR, and directed by APG and ZM. The manuscript was written by CAR, BS, and GD, and all authors discussed and interpreted the results. MFW and HL performed statistical analyses. CAR, CMB, BHC, and HEH enrolled patients in the protocol and/or managed the patients, and MB was the study research nurse, assisting with enrollment, sample acquisition, and data safety monitoring of patients.

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