# B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor–transduced T cells

James N. Kochenderfer,[1] Mark E. Dudley,[2] Steven A. Feldman,[2] Wyndham H. Wilson,[3] David E. Spaner,[4] Irina Maric,[5] Maryalice Stetler-Stevenson,[6] Giao Q. Phan,[2] Marybeth S. Hughes,[2] Richard M. Sherry,[2] James C. Yang,[2] Udai S. Kammula,[2] Laura Devillier,[2] Robert Carpenter,[1] Debbie-Ann N. Nathan,[2] Richard A. Morgan,[2] Carolyn Laurencot,[2] and Steven A. Rosenberg[2]

[1]Experimental Transplantation and Immunology Branch, [2]Surgery Branch, and [3]Metabolism Branch, National Cancer Institute (NCI), Bethesda, MD; [4]Sunnybrook Health Sciences Center, Toronto, ON; [5]Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, MD; and [6]Laboratory of Pathology, NCI, Bethesda, MD

**We conducted a clinical trial to assess adoptive transfer of T cells genetically modified to express an anti-CD19 chimeric Ag receptor (CAR). Our clinical protocol consisted of chemotherapy followed by an infusion of anti–CD19-CAR–transduced T cells and a course of IL-2. Six of the 8 patients treated on our protocol obtained remissions of their advanced, progressive B-cell malignancies. Four of the 8 patients treated on the protocol had long-term depletion of normal polyclonal CD19$^+$ B-lineage cells. Cells containing the anti-CD19 CAR gene were detected in the blood of all patients. Four of the 8 treated patients had prominent elevations in serum levels of the inflammatory cytokines IFNγ and TNF. The severity of acute toxicities experienced by the patients correlated with serum IFNγ and TNF levels. The infused anti–CD19-CAR–transduced T cells were a possible source of these inflammatory cytokines because we demonstrated peripheral blood T cells that produced TNF and IFNγ ex vivo in a CD19-specific manner after anti–CD19-CAR–transduced T-cell infusions. Anti–CD19-CAR–transduced T cells have great promise to improve the treatment of B-cell malignancies because of a potent ability to eradicate CD19$^+$ cells in vivo; however, reversible cytokine-associated toxicities occurred after CAR–transduced T-cell infusions. This trial was registered with ClinicalTrials.gov as NCT00924326. (*Blood*. 2012;119(12):2709-2720)**

## Introduction

Chimeric Ag receptors (CARs) are fusion proteins that incorporate Ag recognition moieties and T-cell activation domains.[1-3] The Ag recognition moieties of CARs are usually variable regions of mAbs.[1-3] T cells genetically modified to express CARs acquire the ability to specifically recognize targeted Ags.[2-8] CD19 is a protein that is expressed on almost all B-lineage cells.[9] Because expression of CD19 is limited to normal and malignant B-lineage cells, CD19 is an attractive target for immunotherapies aimed at B-cell malignancies.[9] Many groups have conducted preclinical experiments with T cells expressing anti-CD19 CARs, and these experiments have shown that anti–CD19-CAR–expressing T cells can recognize and destroy target cells in a CD19-specific manner.[10-18] The CARs used in these experiments have contained T-cell activation domains from molecules such as CD3ζ and a variety of costimulatory domains such as those from CD28 and 4-1BB.[12-17] Murine studies have shown that syngeneic T cells genetically modified to express anti-CD19 CARs can cure lymphoma and cause long-term eradication of normal B cells.[19,20] Based on these preclinical experiments, clinical trials of anti-CD19 CARs have been initiated, and some early results from these trials have been reported.[21-27] Similar to the murine studies, these early clinical reports have suggested an anti-malignancy effect of T cells expressing anti-CD19 CARs, and Ag-specific eradication of normal B cells has been demonstrated.[21,23,24,27]

Significant toxicities including hypotension, fevers, and renal insufficiency have occurred after infusions of anti–CD19-CAR–expressing T cells.[22-24,27] Three patients with elevations in serum levels of inflammatory cytokines such as IFNγ after anti–CD19-CAR–transduced T-cell infusions have been reported[22-24]; however, in one of these cases, the elevation in serum inflammatory cytokines was present before CAR-transduced T cells were infused.[22] Determining the causes of elevated cytokine levels after anti–CD19-CAR–transduced T-cell infusions is not straightforward because only a small number of patients with elevated serum cytokine levels have been reported, and there are other possible causes of elevated serum cytokines such as sepsis.[28] Inflammatory cytokines such as IFNγ and TNF (formerly known as TNFα) are produced by anti–CD19-CAR–transduced T cells in vitro.[10,12,15] IFNγ and TNF can cause significant toxicity in humans[29-32]; however, an association between inflammatory cytokine production by anti–CD19-CAR–transduced T cells and clinical toxicity has not been demonstrated. A better understanding of the relationship between cytokine production by CAR-transduced T cells and clinical toxicity is necessary to rationally plan future research aimed at increasing the safety of anti–CD19-CAR–transduced T cells.

We are conducting a clinical trial to assess the anti-malignancy efficacy, toxicity, and in vivo persistence of T cells transduced with

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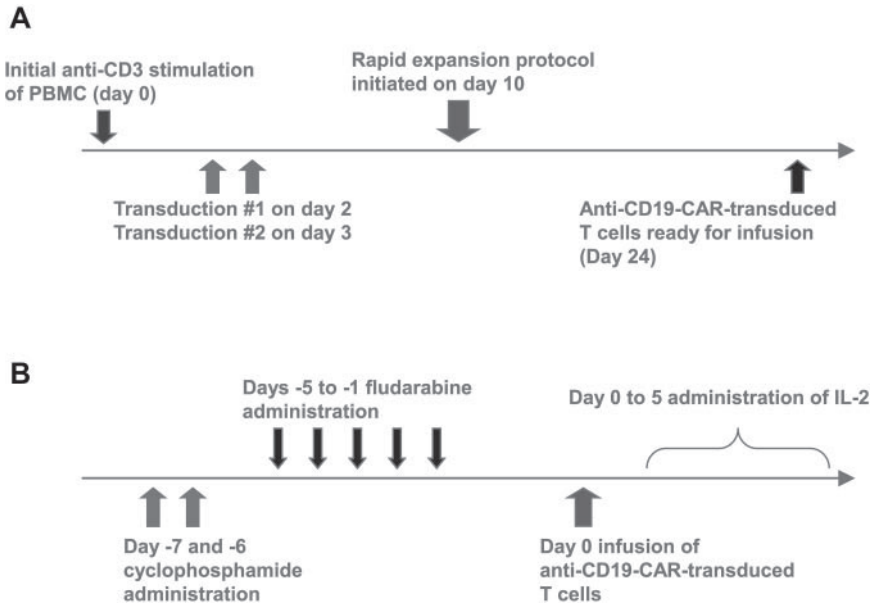


**Figure 1. Anti–CD19-CAR–transduced T-cell production and clinical treatment protocols.** (A) PBMCs were stimulated with the anti-CD3 mAb OKT3 on day 0. The cells were transduced with gammaretroviruses encoding the anti-CD19 CAR on days 2 and 3. On day 10, a rapid expansion protocol was started, and the cells were ready for infusion on day 24. (B) Patients received 60 mg/kg cyclophosphamide chemotherapy daily for 2 days. Next, patients received 25 mg/m$^2$ fludarabine chemotherapy daily for 5 days. One day later, the patients received a single infusion of anti–CD19-CAR–transduced T cells. Starting on the same day as the T-cell infusion, the patients received IV IL-2 every 8 hours.

an anti-CD19 CAR. All of the patients on our clinical trial had advanced, progressive B-cell malignancies that were incurable by any standard treatment except allogeneic stem cell transplantation. Six of the 8 patients treated on our trial obtained objective remissions of their malignancies, and 4 of 8 patients had long-term elimination of CD19$^+$ B-lineage cells. Significant toxicities that correlated with elevations in serum IFNγ and TNF occurred after infusions of anti–CD19-CAR–transduced T cells. In addition, we demonstrated CD19-specific IFNγ and TNF production by T cells from the blood of patients who had received infusions of anti–CD19-CAR–transduced T cells.

## Methods

### Clinical trial design

The trial was reviewed by the US Food and Drug Administration and the Institutional Review Board of the National Cancer Institute and allowed to proceed. Patients provided written informed consent before participation in this study in accordance with the Declaration of Helsinki. Patients underwent an apheresis to obtain PBMCs for producing anti–CD19-CAR–transduced T cells. Cyclophosphamide was administered daily on days −7 and −6 at a dose of 60 mg/kg. On days −5 through −1, patients received 25 mg/m$^2$ fludarabine daily. On day 0, patients received a single infusion of CAR-transduced T cells. Three hours after CAR-transduced T cells were administered, IL-2 was initiated. IL-2 was administered intravenously at a dose of 720 000 international units/kg every 8 hours until toxicity precluded additional doses. Remissions of chronic lymphocytic leukemia (CLL) or lymphoma were defined according to standard international criteria.[33,34]

### Anti-CD19 CAR retroviral vector design

We previously reported the design and construction of the murine stem cell virus–based splice-gag vector (MSGV)–FMC63-28Z that encoded the anti-CD19 CAR used in our clinical trial.[12]

### Anti–CD19-CAR–transduced T cell preparation

Anti–CD19-CAR–transduced T cells were prepared as described in supple-

### In vitro and ex vivo assays

Anti-Fab Ab staining and staining with labeled CD19 protein were used in flow cytometry to detect surface expression of the anti-CD19 CAR as described in the supplemental Methods. ELISAs, intracellular cytokine staining assays (ICCS), and CD107a degranulation assays were performed as described in supplemental Methods and as previously described.[12,21] Serum ELISAs were carried out as described in supplemental Methods. Immunohistochemistry and flow cytometry were carried out as detailed in the supplemental Methods and as previously described.[21]

### Real-time qPCR

Real-time quantitative PCR (qPCR) was carried out to determine the percentage of peripheral blood mononuclear cells containing the CAR gene as described in supplemental Methods.

### Calculation of SOFA scores

The sequential organ failure assessment (SOFA) score is an established method of quantifying the overall severity of illness.[36] The SOFA score includes an assessment of hypotension, the platelet count, and measurements of respiratory, liver, renal, and central nervous system function.[36,37] We calculated daily SOFA scores for each patient by using clinical records from the day of CAR-tranduced T-cell infusion and each of the first 10 days after CAR-transduced T-cell infusion.[36] For each patient, the sum of the SOFA scores from each day was calculated to give the total SOFA score.[36]

## Results

### Production of anti–CD19-CAR–transduced T cells

Autologous PBMCs were stimulated with an anti-CD3 mAb and transduced with gammaretroviruses encoding an anti-CD19 CAR (Figure 1A). The anti-CD19 CAR used in our clinical trial contained the variable regions of a murine anti–human-CD19 Ab, a portion of the CD28 molecule, and the signaling domain of the CD3ζ molecule.[12] The anti-CD19 CAR could be detected on

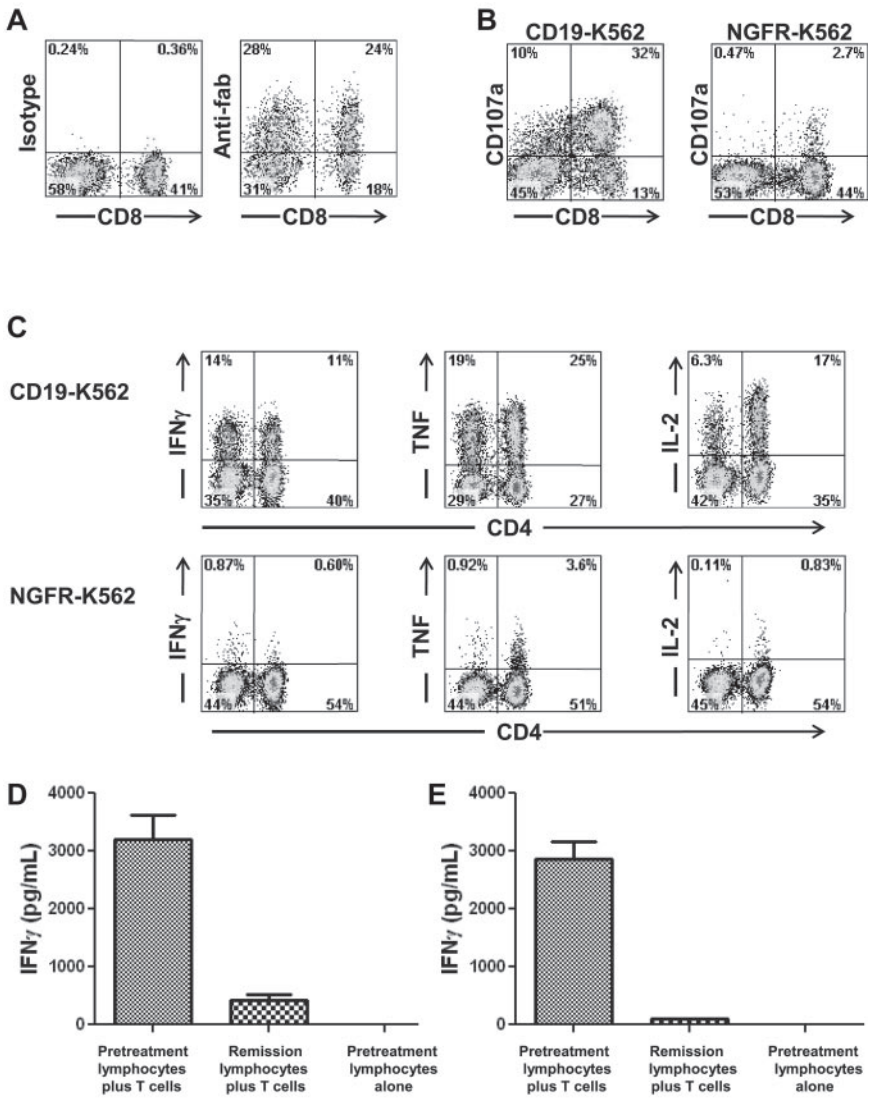


**Figure 2. Anti–CD19-CAR–transduced T cells produced cytokines in a CD19-specific manner and recognized autologous leukemia cells.** (A) Staining with an anti-Fab Ab revealed expression of the anti-CD19 CAR on the surface of T cells that were administered to patient 7. Staining with an isotype control Ab is also shown. Both plots were gated on $CD3^+$ lymphocytes, which made up 99% of the cells in the culture. (B) On the day of infusion, T cells of patient 7 up-regulated CD107a expression after a 4-hour culture with the $CD19^+$ target cell CD19-K562 but not the negative control cell NGFR-K562 that does not express CD19. (C) On the day of infusion, anti–CD19-CAR–transduced T cells of patient 7 produced IFNγ, TNF, and IL-2 when cultured for 6 hours with the $CD19^+$ target cell CD19-K562 but not the negative control cell NGFR-K562 that does not express CD19. The results shown in panels A through C are representative of the results obtained for all of the patients on the protocol. (D) Anti–CD19-CAR–transduced T cells of patient 3 were cultured with either autologous pretreatment lymphocytes or autologous remission lymphocytes overnight, and an IFNγ ELISA was performed on the supernatant. Anti–CD19-CAR–transduced T cells of patient 3 specifically recognized pretreatment lymphocytes but not remission lymphocytes obtained 7 weeks after CAR-transduced T-cell infusion. Sixty-four percent of the pretreatment lymphocytes were $CD19^+$ leukemia cells. The remission lymphocytes contained only 0.1% $CD19^+$ cells. (E) Anti–CD19-CAR–transduced T cells of patient 6 were cultured with either pretreatment autologous lymphocytes or autologous remission lymphocytes overnight and an IFNγ ELISA was performed on the supernatant. Anti–CD19-CAR–transduced T cells of patient 6 specifically recognized pretreatment lymphocytes but not remission lymphocytes obtained 2 weeks after CAR–transduced T-cell infusion. Seventy-six percent of the pretreatment lymphocytes were $CD19^+$ leukemia cells. The remission lymphocytes contained only 0.1% $CD19^+$ cells. In both panels D and E, pretreatment lymphocytes cultured alone did not produce detectable quantities of IFNγ.

expressing the anti-CD19 CAR at the time of infusion was 55% (Table 1). Most of the infused CAR-transduced cells were CCR7-negative, CD45RA-negative effector memory cells, but variable numbers of $CCR7^+$, CD45RA-negative central memory cells were also present (Table 2). The CAR-expressing T cells specifically up-regulated CD107a when cultured with CD19-expressing target cells but not when cultured with negative control target cells that lacked CD19 expression (Figure 2B).

**Table 1. Patient data**

| Patient | Age, y | Malignancy | No. of prior therapies | Total no. of infused cells/kg, $\times 10^7$ | Percentage of infused cells $CAR^+$ | No. of infused $CAR^+$ cells/kg $\times 10^7$ | Infused cells CD4/CD8 ratio | Doses of IL-2 administered | Response and time since treatment, mo* |
|---|---|---|---|---|---|---|---|---|---|
| 1a† | 47 | Follicular lymphoma | 4 | 0.5 | 64 | 0.3 | 29/63 | 8 | PR (7) |
| 1b† | 48 | Follicular lymphoma | 5 | 2.1 | 63 | 1.3 | 19/71 | 10 | PR (18+) |
| 2 | 48 | Follicular lymphoma | 5 | 0.5 | 65 | 0.3 | 23/73 | 9 | NE (died with influenza) |
| 3 | 61 | CLL | 3 | 2.5 | 45 | 1.1 | 35/53 | 2 | CR (15+) |
| 4 | 55 | Splenic marginal zone lymphoma | 3 | 2.0 | 53 | 1.1 | 72/24 | 4 | PR (12) |
| 5 | 54 | CLL | 4 | 0.6 | 50 | 0.3 | 87/12 | 2 | SD (6) |
| 6 | 57 | CLL | 7 | 5.5 | 30 | 1.7 | 37/57 | 1 | PR (7) |
| 7 | 61 | CLL | 4 | 5.4 | 51 | 2.8 | 58/41 | 2 | PR (7+) |
| 8 | 63 | Follicular lymphoma | 7 | 4.2 | 71 | 3.0 | 54/43 | 5 | PR (8+) |

PR indicates partial remission; NE, not evaluable; CR, complete remission; SD, stable disease; and CLL, chronic lymphocytic leukemia.

**Table 2. Memory phenotype of infused anti-CD19 CAR-expressing T cells**

| Patient | CD62L | CCR7$^+$CD45RA$^+$ | CCR7$^+$CD45RA$^-$ | CCR7$^-$CD45RA$^-$ | CCR7$^-$CD45RA$^+$ |
|---|---|---|---|---|---|
| 1a* | 21 | 7 | 30 | 53 | 10 |
| 1b* | 20 | 8 | 20 | 55 | 18 |
| 2 | 25 | 5 | 21 | 61 | 13 |
| 3 | 27 | 1 | 8 | 87 | 5 |
| 4 | 35 | 0 | 5 | 90 | 5 |
| 5 | 26 | 3 | 13 | 71 | 13 |
| 6 | 10 | 6 | 27 | 57 | 10 |
| 7 | 4 | 4 | 27 | 60 | 9 |
| 8 | 8 | 1 | 9 | 81 | 8 |

The phenotype of the infused CD19 CAR-expressing T cells was determined by flow cytometry. Values are the percentage of the CAR$^+$ CD3$^+$ cells that expressed the indicated markers.

*Patient 1 was treated twice.

Up-regulation of CD107a indicated degranulation, which is a prerequisite for perforin-mediated cytotoxicity.[38] The CAR-transduced T cells produced IFNγ, TNF, and IL-2 in a CD19-specific manner (Figure 2C, Table 3).

### Anti–CD19-CAR–transduced T cells can specifically recognize autologous leukemia cells

The blood of some of the patients on our trial contained large numbers of CD19$^+$ CLL cells. Access to pretreatment blood samples from these patients allowed us to determine whether anti–CD19-CAR–transduced T cells could specifically recognize unmanipulated autologous CLL cells. The blood lymphocytes of patient 3 contained 64% CD19$^+$ CLL cells before treatment on our protocol, and the blood lymphocytes of patient 6 contained 76% CD19$^+$ CLL cells before treatment on our protocol. After treatment when the patients were in remission, the blood lymphocytes of both patient 3 and patient 6 contained only 0.1% CD19$^+$ cells. As shown in Figure 2D and E, the anti–CD19-CAR–transduced T cells from each of these patients produced large amounts of IFNγ when cultured with the pretreatment lymphocytes that were mostly leukemia cells, but the CAR-transduced T cells produced only background levels of IFNγ when cultured with the lymphocytes that were obtained after treatment when the patients were in remission. IFNγ was not produced by the leukemia cells of either patient (data not shown).

### Six of the 8 treated patients obtained objective remissions

The 8 patients treated on our protocol had either B-cell lymphoma or CLL (Table 1). The patients all had progressive malignancy at the time of enrollment on our protocol despite a median of 4 prior therapies. All patients treated on our protocol received cyclophosphamide daily for 2 days followed by fludarabine daily for 5 days. One day after the last dose of fludarabine, the patients received a single IV infusion of anti–CD19-CAR–transduced T cells (Figure 1B). Three hours after the CAR-transduced T-cell infusion, a course of IV IL-2 was initiated (Figure 1B). The patients received doses of CAR-expressing T cells that ranged from $0.3 \times 10^7$ to $3.0 \times 10^7$ CAR$^+$ T cells/kg bodyweight. Patient 1 was treated twice. His first treatment course was previously reported.[21] Patient 1 developed progressive CD19$^+$ lymphoma 7 months after his first infusion of anti–CD19-CAR–transduced T cells. After his lymphoma progressed, patient 1 was treated a second time with the same regimen. He remains in a partial remission (PR) 18 months after the second treatment. Patient 2 died 18 days after CAR-transduced T-cell infusion with culture-proven influenza A pneumonia, nonbacterial thrombotic endocarditis, and cerebral infarction, so he is not evaluable for lymphoma response. Overall, 6 of the 7 evaluable patients treated on our trial obtained objective remissions (Table 1). Because the patients received chemotherapy with activity against B-cell malignancies immediately before the anti–CD19-CAR–transduced T-cell infusion, the contribution that the CAR-transduced T cells made to the remissions is unclear.

### Patient 3 had a prolonged complete remission and depletion of normal B cells

At the time of enrollment on our protocol, patient 3 had progressive CLL after receiving 3 prior therapies. Just before treatment on our protocol, he had a blood B-cell count of 1544 B cells/μL (Figure 3A). Ninety-six percent of the blood B cells were CLL cells. In addition, 50%-60% of his cellular BM was CLL (Figure 3B-C). A prominent clonal leukemia population that expressed the characteristic CD19$^+$ and CD5$^+$ phenotype of CLL was detected by flow cytometry of BM cells before treatment on our protocol (Figure

**Table 3. Cytokine production by infused cells**

| | IFNγ, pg/mL | | TNF, pg/mL | | IL-2, pg/mL | |
|---|---|---|---|---|---|---|
| Patient | CD19-positive target | CD19-negative target | CD19-positive target | CD19-negative target | CD19-positive target | CD19-negative target |
| 1* | 8190 | 411 | 448 | < 31 | 1156 | 48 |
| 2 | 9850 | 506 | 6250 | < 31 | 2002 | 139 |
| 3 | 19 000 | 916 | 9312 | < 31 | 1683 | 51 |
| 4 | 27 900 | 944 | 21 895 | < 31 | 2768 | 40 |
| 5 | 36 700 | 734 | 21 515 | < 31 | 2421 | 32 |
| 6 | 14 800 | 130 | 8288 | < 31 | 798 | 44 |
| 7 | 29 300 | 341 | 21 980 | < 31 | 1661 | 36 |
| 8 | 9960 | 240 | 9830 | 73 | 1697 | 46 |

Levels of the indicated cytokines were determined by standard ELISAs after an overnight culture of T cells from the time of infusion with either CD19-positive target cells or CD19-negative target cells. NGFR-K562 cells were used as CD19-negative target cells for all cultures. CD19-K562 cells were used as CD19-positive targets for the cultures

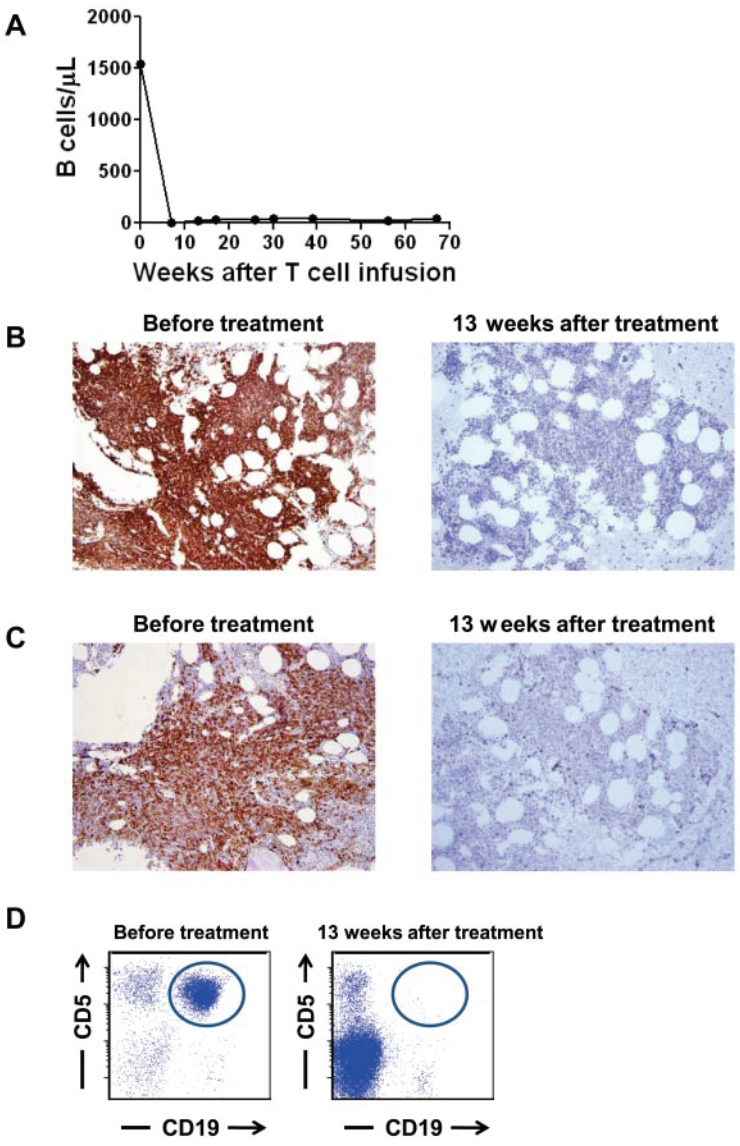


**Figure 3. Normal and malignant B-lineage cells were eliminated from the blood and BM of patient 3.** (A) Before treatment, the blood of patient 3 contained an elevated number of B cells, 96% of which were leukemia cells. After treatment, the blood B-cell count has remained below normal and patient 3 has been in complete remission for 67 weeks. B cells were quantitated by flow cytometry staining of $CD19^+$ cells. (B) CD19 IHC staining of the BM of patient 3 is shown before treatment and 13 weeks after treatment. The BM contained large numbers of $CD19^+$ cells before treatment. Thirteen weeks after treatment, $CD19^+$ cells were nearly absent. (C) CD20 IHC staining of the BM of patient 3 is shown before treatment and 13 weeks after treatment. The BM contained large numbers of $CD20^+$ cells before treatment. Thirteen weeks after treatment, $CD20^+$ cells were nearly absent. (D) Flow cytometric results of a BM aspirate from patient 3 are shown. Plots are gated on lymphoid cells by forward and side scatter. A monoclonal population of B cells expressing the characteristic $CD19^+$ and $CD5^+$ phenotype of CLL (circled) was present before treatment but not 13 weeks after treatment.

3D). After treatment, CLL was completely eradicated from the blood of patient 3, and the number of polyclonal blood B cells has stayed at below-normal levels of 17 to 40 B cells/μL for $>$ 15 months (lower limit of the normal 61 B cells/μL; Figure 3A). Patient 3's blood B cells that have returned after treatment were determined to be polyclonal by flow cytometry staining for Ig κ and λ proteins (data not shown). CLL was eliminated from the BM of patient 3 after treatment as shown by BM IHC staining (Figure 3B-C) and by multicolor flow cytometry analysis of the BM cells (Figure 3D). B-lineage cells were nearly absent from the BM, but other hematopoietic cells had recovered (Figure 3B-C). Overall, the cellularity of the BM varied between 20% and 70%. A normal BM cellularity for a 61-year-old patient is ~ 40%. Patient 3 continues in complete remission $>$ 15 months after treatment on our protocol.

### Patient 7 had a substantial reduction in adenopathy after CAR-transduced T-cell infusion

adenopathy occurred during the time between a pretreatment computed tomography (CT) scan and a second CT scan that was performed 32 days after treatment (Figure 4A). Regression of the adenopathy continued between day 32 and day 132 after the CAR-transduced T-cell infusion. This continued regression, which occurred $>$ 33 days after the last dose of chemotherapy suggested that the CAR-transduced T cells contributed to the regression of the adenopathy. In accordance with this possibility, anti–CD19-CAR–transduced T cells persisted in the blood of patient 7 until at least day 132 after infusion (Figure 5B). Because the patient received chemotherapy before the CAR-transduced T cells, an alternative explanation for the decreasing adenopathy between day 32 and 132 after CAR-transduced T-cell infusion is continued resolution of adenopathy because of clearance of leukemia cells that were killed by the chemotherapy.

### Patient 8 had a prolonged specific eradication of normal B cells

Patient 8 had a normal level of polyclonal blood B cells before

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