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Cytotoxic T Lymphocyte Therapy for Epstein-Barr Virus^{+} Hodgkin's Disease

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Abstract

Epstein Barr virus (EBV)$^{+}$ Hodgkin's disease (HD) expresses clearly identified tumor antigens derived from the virus and could, in principle, be a target for adoptive immunotherapy with viral antigen–specific T cells. However, like most tumor-associated antigens in immunocompetent hosts, these potential targets are only weakly immunogenic, consisting primarily of the latent membrane protein (LMP)1 and LMP2 antigens. Moreover, Hodgkin tumors possess a range of tumor evasion strategies. Therefore, the likely value of immunotherapy with EBV-specific cytotoxic effector cells has been questioned. We have now used a combination of gene marking, tetramer, and functional analyses to track the fate and assess the activity of EBV cytotoxic T lymphocyte (CTL) lines administered to 14 patients treated for relapsed EBV^{+} HD. Gene marking studies showed that infused effector cells could further expand by several logs in vivo, contribute to the memory pool (persisting up to 12 mo), and traffic to tumor sites. Tetramer and functional analyses showed that T cells reactive with the tumor-associated antigen LMP2 were present in the infused lines, expanded in peripheral blood after infusion, and also entered tumor. Viral load decreased, demonstrating the biologic activity of the infused CTLs. Clinically, EBV CTLs were well tolerated, could control type B symptoms (fever, night sweats, and weight loss), and had antitumor activity. After CTL infusion, five patients were in complete remission at up to 40 mo, two of whom had clearly measurable tumor at the time of treatment. One additional patient had a partial response, and five had stable disease. The performance and fate of these human tumor antigen–specific T cells in vivo suggests that they might be of value for the treatment of EBV^{+} Hodgkin lymphoma.

Key words: immunotherapy • lymphoma • Epstein-Barr virus • LMP2 • gene marking

Introduction

Immunotherapy with CTLs has successfully prevented and treated EBV-associated lymphoproliferative diseases occurring after hematopoietic stem cell and solid organ transplants (1–8). EBV lymphoproliferative diseases are generally highly immunogenic, express all of the EBV latent antigens (type 3 latency), and arise only in patients with severe T cell dysfunction. By contrast, EBV^{+} Hodgkin's disease (HD) arises in the immunocompetent host and must evade preexisting immune responses to EBV, which control virus-infected normal B cells and epithelial cells. Hence, the malignant Reed-Sternberg cells of HD have developed a multiplicity of immune evasion mechanisms (9), including the down-regulation of the immunodominant EBV nuclear antigen (EBNA)3A, EBNA3B, and EBNA3C antigens to which the bulk of the CTL response to latent antigens is directed. Nevertheless, the tumor cells continue to express the im-

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Abbreviations used in this paper: EBER, EBV-encoded small RNA; EBNA, EBV nuclear antigen; HD, Hodgkin's disease; LCL, lymphoblastoid cell line; LMP, latent membrane protein; Q-PCR, quantitative real-time PCR; SCT, stem cell transplant; SFC, spot-forming cell.

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munologically subdominant viral latency proteins latent membrane protein (LMP)1 and LMP2, and are therefore potential targets for immunotherapy with EBV-specific CTLs. Because patients with HD have a high rate of morbidity after conventional treatment and a poor prognosis after relapse (10, 11), we decided to explore the value of EBV CTL therapy for this disease.

EBV CTLs were generated using patient-derived EBV-transformed B lymphocytes (B lymphoblastoid cell lines [LCLs]) as APCs. These LCLs predominantly induce proliferation of T cells specific for the early lytic cycle transactivators BZLF1 and BMLF1 and for the latency-associated EBNAs (3A, 3B, and 3C), which are antigens that are not expressed by the EBV^{+} tumor cells of HD (12). However, LCLs also reactivate T cells specific for LMP2, an EBV latency antigen regularly expressed by the tumor (13, 14). We hypothesized that any clones recognizing tumor-associated antigens would expand in vivo (15). To track the in vivo persistence and fate of the infused EBV CTLs, we genetically marked the CTLs of some patients by transduction with a retroviral vector expressing the neomycin resistance (*neo*) gene (2, 16) and analyzed the numbers of LMP-2–reactive cells using tetramers and functional assays. In patients with measurable disease, we also assessed the impact of the CTLs on tumor burden. Our results showed both in vivo expansion and persistence of tumor-reactive T lymphocytes and evidence that these cells can have substantial antitumor activity.

Materials and Methods

Patients and EBV Status of the Tumors. The investigation was approved by the Food and Drug Administration, the Recombinant DNA Advisory Committee, and the hospitals' Institutional Review Boards. Of our referrals, 26% had tumors that were positive for EBV. 13 patients were treated on these protocols in the USA, and 1 was treated in Germany after regulatory approval in that country (Table I). Patients were eligible if they had received treatment for relapsed HD and had been off other systemic lymphoma therapy for 1 mo before CTL infusion and had either measurable disease or were considered at high risk of relapse. In

Table I. *Patient Characteristics*

Patient ID	Age	Sex	Disease stage at diagnosis	Most recent chemo (time before CTL)	Gene marked	Dose level	Toxicity attributed to CTL	Response to CTL	Outcome
1	21	M	IA	SCT→relapse 5 mo later→CTL (8 mo)	Yes	1	None	Stable disease	DOD 13 mo after CTLs
2	18	M	IVB	3 wk hemipelvis RT (stopped 1 d before CTL no. 2)	Yes	1	None	Stable disease (hilar/mediastinal/ pulmonary)	DOD 10 mo after CTLs
3	24	M	IIIA	Vinblastine weekly (1 mo)	Yes	1	Transient malaise	PR	DOD 12 mo after CTLs
4	36	F	$II_{E}A$	Paraspinal RT and αIFN (1 mo)	Yes	1	None	NR	DOD 2 mo after CTLs
5	19	F	IIIA	Mantle/lung RT (1 mo)	Yes	1	None	Stable disease	DOD 10 mo after CTLs
6	36	M	IIIA	MOPP (2 mo)	Yes	1	None	Stable disease then allo BMT	In remission 56 mo after CTLs
7	40	F	IIIB	ABVD (2 mo)	No	2	None	Stable disease	DOD 20 mo after CTLs
8	24	M	IIA	MTX and 6TG (2 mo)	Yes	2	None	NR	DOD 7 mo after CTLs
9	20	M	IVB	SCT (10 mo)	No	1	None	Remains in remission	In remission 24 mo after CTLs then lost to follow-up
10	27	F	IIB	SCT (4 mo) RT (2 mo)	No	1	None	CRU[a]	In remission 24 mo after CTLs
11	16	M	IIB	SCT (2 mo) αIFN (1 wk)	No	1	None	CRU[a]	In remission 38 mo after CTLs
12	18	M	IIA	SCT (3 mo)	No	2	None	CR	In remission 27 mo after CTLs
13	29	F	IIB	SCT (3 mo)	No	2	None	NR	DOD 4 mo after CTLs
14	8	F	IA	RT (9 mo)	No	2	Transient swelling and pain in cervical lymph node	CR	In remission 9 mo after CTLs

DOD, died of disease; PR, partial response; NR, no response; CR, complete response; CRU, complete remission undetermined; RT, radiotherapy; MOPP, mechlorethamine, oncovin (vincristine), procarbazine, and prednisone; ABVD, adriamycin, bleomycin, vinblastine, and dacarbazine; MTX, methotrexate; 6TG, 6-thioguanine.

[a]These two patients had residual mediastinal masses after autograft at the time they received CTLs, which eventually resolved but could not be classified as having definite disease, as gallium scans were negative.

all patients, tumor samples had been established as EBV^{+} using immunohistochemistry for LMP-1 and/or in situ hybridization for the small nonpolyadenylated viral RNA (EBV-encoded small RNA [EBER]1; reference 14).

Generation of EBV-transformed B Cell Lines. After consent, the patients donated 40–70 cc peripheral blood. $5–10 \times 10^6$ PBMCs were used for the establishment of an EBV-transformed LCL (EBV-LCL) by infection of PBMCs with concentrated supernatants from the B95-8 working cell banks (17). EBV-LCLs were grown for at least 2 wk in acyclovir before being used as APCs to prevent the release of infectious virus. This treatment targets the virus thymidine kinase and inhibits lytic virus production, but does not affect the expression of the early lytic cycle transactivators.

Generation of EBV-specific CTL Cultures. To generate EBV-specific CTLs, PBMCs were cocultured with γ-irradiated (40 Gy) autologous LCLs at a responder/stimulator ratio of 40:1 in a 24-well plate. Starting on day 10, the responder cells were restimulated weekly with irradiated LCLs at a responder/stimulator ratio of 4:1. Two weekly doses of rhIL-2 (R&D Systems) were added from day 14. CTL lines were cultured in complete medium (RPMI 1640 medium; GIBCO BRL or Hyclone) containing 10% FCS (Hyclone), and 2 mM L-glutamine ($n = 11$; Biowhittaker), or complete medium supplemented with 45% EHAA ($n = 3$; Irvine Scientific). To increase the rate of expansion when the cell line was growing poorly, 12 CTL lines were treated with a "superexpansion cocktail" consisting of irradiated allogeneic PBMCs from blood bank–approved donors, irradiated autologous EBV-LCL, 50 ng/mL anti-CD3 (Orthoclone OKT3; Ortho Biotech), and 100 U/mL IL-2 (14). To avoid stimulating allospecific clones, this cocktail was used only from the third stimulation.

Gene Marking of EBV-specific CTLs. Seven patients received CTLs genetically marked by transduction with the G1Na (five patients) or LNSJ1 (two patients) retroviral vectors, which contain the *Escherichia coli*–derived neomycin resistance gene (*neo*). Clinical grade G1Na or LNS-containing supernatant from a PA317 amphotropic packaging cell line (provided by Genetic Therapy Inc.) was incubated for 6 h with the CTLs at a multiplicity of infection of 10:1 in the presence of 50–100 U/mL IL-2 and 4 μg/mL protamine sulfate in a 75-cm^2 flask (14). After transduction, the CTLs were returned to their regular growth schedule. To determine the efficiency of transduction, DNA was extracted from an aliquot of the transduced cells and analyzed by real-time PCR as described below. Transduction efficiencies ranged from 1.01 to 15.6%.

Cytotoxicity Assays. The cytotoxic specificity of each CTL line was analyzed in a standard 4-h chromium-51 release assay using effector/target ratios of 40:1, 20:1, 10:1, and 5:1 (3). The following target cells were tested: autologous LCL; HLA class I– and II–mismatched LCL; HSB-2, which is sensitive to killing by lymphokine-activated killer cells; and autologous lymphoblasts that had been stimulated with PHA blasts (17). To determine whether the EBV-specific CTL lines recognized the EBV antigens expressed by Hodgkin's cells, the killing of target cells expressing LMP2 or LMP1 alone was tested. Autologous or HLA-matched dermal fibroblast lines were treated for 24 h with IFN-γ to increase the expression of MHC molecules and infected for 48 h with recombinant adenovirus containing either LMP2a or GFP genes (provided by A. Davis, Baylor College of Medicine, Houston, TX; reference 18). The chromium release assay was harvested after 6 h.

Immunophenotyping. CTL lines were stained with CD3, CD4, CD8, CD16, CD56, TCR αβ, TCR γδ, CD19, CD62L, CD45RA, and CD45RO (Becton Dickinson). For Vβ usage, cells were stained with perCP anti-CD3 (Becton Dickinson) and PE- or FITC-conjugated anti-Vβ mAbs (Beckman Coulter). For each sample, 10,000 cells were analyzed by FACSCalibur using CELLQuest software (Becton Dickinson).

Tetramer Staining. To detect LMP2 peptide–specific T cells in the CTL lines and PBMCs, soluble HLA-A02*01-CLG-GLLTMV, HLA-A02*01-FLYALALLL, HLA-A11*01-SSCSSCP-LSKI, HLA-A24*01-PYLFWLAAI, HLA-A24-TYGPVFMSL, and HLA-B35*01-MGSLEMVPM PE-conjugated tetramers were prepared by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility and by the Baylor College of Medicine Tetramer Core Facility. The peptides were synthesized either by M. Campbell (University of Texas Anderson Cancer Center, Houston, TX) or by Genemed Synthesis Inc. 5×10^5 CTLs or 10^6 PBMCs were incubated at room temperature for 30 min in PBS with 1% FCS containing the PE-labeled tetrameric complex, anti-CD8 FITC, and anti-CD3 PerCP. IgG-PE was used for isotype control. Stained cells were fixed in PBS containing 0.5% paraformaldehyde. For each sample, 100,000 cells were analyzed as described above.

Monitoring of Gene-marked T Cell Lines In Vivo. The forward and reverse primers and probe sequences specific for the G1Na vector were CAGCCCTCACTCCTTCTCTAGG, CGAAAC-GATCCTCATCCTGTCTCTTG (GIBCO BRL), and CCG-CTACAATTCC, respectively (Applied Biosystems). The forward and reverse primers and probe sequences specific for LNSJ1 vector were CCCTTTATCCAGCCCTCACTCC, CTCATC-CTGTCTCTTGATCAGATCGG, and TCTAGGCGCCGG-AAT, respectively. The G1Na and LNSJ1 probes were 5′ VIC and 3′ MGBHQ conjugated (Applied Biosystems). The forward and reverse primers and probe sequences specific for C-reactive protein gene (used as the internal control) were CTTGAC-CAGCCTCTCTCATGC, TGCAGTCTTAGACCCCACCC, and TTTGGCCAGACAGGTAAGGGCCACC, respectively. C-reactive protein probes were 3′ FAM and 5′ MGBHQ conjugated (Applied Biosystems). A standard was generated from K562 cells transduced with each vector with a single retroviral integrant diluted with nontransduced K562 cells to the following clinically relevant dilution range: 0.01, 0.069, 0.29, 1.27, 5.4, 33, and 100%. 250 ng genomic DNA was used as the template with TaqMan PCR mastermix (Applied Biosystems) diluted to 1× with nuclease-free water. Thermocycling and fluorescent analysis was performed with ABI PRISM 7700 (Applied Biosystems; reference 19). FAM and VIC signals were measured simultaneously. Real-time fluorescence measurements were taken, the threshold cycle value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit, and the percentage of transduced cells was calculated from the standard curve.

Detection of EBV DNA in PBMCs by Quantitative Real-Time PCR (Q-PCR). DNA was isolated from $3–5 \times 10^6$ PBMCs using an anion exchange column (QIAGEN). 500 ng DNA was analyzed by Q-PCR as described previously (20) to quantitate EBV genome copy number per microgram.

In Situ Hybridization for neo. To document the presence of gene-marked, EBV-specific T cells in tumor tissue, we examined paraffin sections of tumor for *neo*-bearing cells using in situ PCR as described previously (3). Paraffin-embedded Burkitt's lymphoma cell line (BL2) and G1Na-transduced K562 served as negative and positive controls, respectively.

ELISPOT Assay. ELISPOT analysis was used to quantitate the frequency of LMP2 peptide–specific CTLs or to compare the peripheral blood T cells responding to LCLs or peptide-pulsed

PHA blasts by IFN-γ secretion before and after CTL infusion. All studies for each patient were performed in one single assay using batched-frozen PBMCs to avoid interassay variability. LCLs or autologous PHA blasts alone or pulsed with the LMP2 peptides MGSLEMVPM (MGS) or SSCSSCPLSKI (SSC) were used as stimulators. As controls, PBMCs were stimulated with 1 μg/ml staphylococcal enterotoxin B (Sigma-Aldrich) and 1 μg/ml CMV lysate (Advanced Biotechnologies), or PHA blasts were pulsed with CMV peptides NLVPMVATV (A02*01 restricted) or TPRVTGGGAM (B07*01 restricted) in patients with the appropriate HLA types.

Stimulators were irradiated (40 Gy), washed, and then resuspended at 2×10^5/ml. Responder PBMCs were thawed 24 h before assay set-up, cultured in CTL media (without IL-2), and then harvested and resuspended at 2×10^6/ml. 96-well filtration plates (MultiScreen, no. MAHAS4510; Millipore) were coated with 10 μg/mL anti–IFN-γ antibody (Catcher-mAB91-DIK; Mabtech) overnight at 4°C and then washed and blocked with ELISPOT medium for 1 h at 37°C. Responder and stimulator cells were incubated on the plates for 20 h. The plates were then washed and incubated with the secondary biotin-conjugated anti–IFN-γ mAb (Detector-mAB [7-B6-1-Biotin]; Mabtech) followed by incubation with avidin–biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit [Standard], no. PK6100; Vector Laboratories), and then developed with AEC substrate (Sigma-Aldrich; reference 21). Plates were sent for evaluation to Zellnet Consulting. Spot-forming cells (SFCs) and input cell numbers were plotted.

Patient Characteristics. 14 patients received CTLs (Table I). Of these patients, 12 had nodular sclerosing HD and 2 had mixed cellularity. Their ages ranged from 8 to 40 yr, and their initial disease presentation ranged from stage IA to IVB (22). Samples for CTL generation were collected from patients after a first or subsequent relapse. Nine patients received CTL doses of 4×10^7 cells/m^2, and five received 1.2×10^8 cells/m^2 (Table I). As shown in Table I, all except patient 2 received CTL therapy at least 4 wk from other chemotherapy/radiotherapy or at least 2 mo from stem cell transplant (SCT). Patient 2 had extensive disease with bone, hilar, mediastinal, and pulmonary involvement and was receiving localized radiotherapy to the right hemipelvis for pain control during his course of CTL therapy. The hemipelvis was not included in our evaluation of disease response after CTL therapy. Analysis of disease response to CTL therapy was performed using standard Eastern Cooperative Oncology Group criteria (23).

Results

CTL Line Characteristics

EBV-specific CTL Lines Contained LMP2-specific T Cell Populations on Tetramer Analysis. CTL lines from patients grew more slowly than lines from healthy controls and required frequent mitogenic stimulation. However, they were ultimately indistinguishable by immunophenotype and cytolytic activity from lines generated from normal individuals based on the phenotypic markers CD3, CD4, CD8, CD56, TCR αβ, and TCR γδ (Table II and reference 14). The patient EBV CTL also had an effector–memory phenotype ($CD62L^-$ $CD45RA^-$ $CD45R0^+$; references 14 and 24–27). Analysis of vβ expression by flow cytometry (Fig. 1) did not reveal any stereotyped pattern of vβ usage in CTL lines from normal individuals or Hodgkin patients (six of each illustrated). Nor was there any difference in the frequency of T cells specific for well-characterized LMP2 epitopes in CTL lines from patients or healthy controls. Table II shows flow cytometric analysis performed on patients with informative HLA types. HLA-A02*01, -A11, -B35, and -A24 LMP2 tetramers bound to <1–6% (median 0.75%) of the CD8 T cells in CTLs from six normal EBV-seropositive donors and to <1–6.8% (me-

Table II. *CTL Line Characteristics*

Patient ID	$CD3^+/CD4^+$ (%)	$CD3^+/CD8^+$ (%)	$TCR\alpha\beta^+/CD3^+$ (%)	$TCR\gamma\delta^+/CD3^+$ (%)	$CD56^+/CD3^+$ (%)	LMP2 tetramer positivity	HLA type
1	10	91	99	1.3	5.5	not tested	A3;11/B27;51
2	3	94	85	0.3	4	not tested	A2;3/B44;62
3	15	56	72	1.9	25	yes	**A2**;3/B18;35
4	1.5	85	96	0.9	9	not tested	A3/B7;40(61)
5	17	84	61	34	15	not tested	A3;24/B7
6	1.2	98	98	1.2	0.7	yes	**A2**;3/B7;51
7	15	58	48	45	16	yes	**A2**;32/B18;71
8	0.1	99	99	0.1	29	yes	**A2**;3/B7;18
9	3	78	51	57	5	no	A31;68/B35;39
10	38	63	90	7	34	yes	A1;**24**/B18;53
11	0.6	95	95	12	1.8	yes	A1;68/**B35**;81
12	1	98	96	2.5	0.7	yes	**A11**;29/B7;40(61)
13	49	39	86	15	0.1	yes	A1;**24/B35**;61
14	24	54	83	15	0.8	no	A2;26/B44;50

HLA specificity of the tetramer is highlighted in bold.

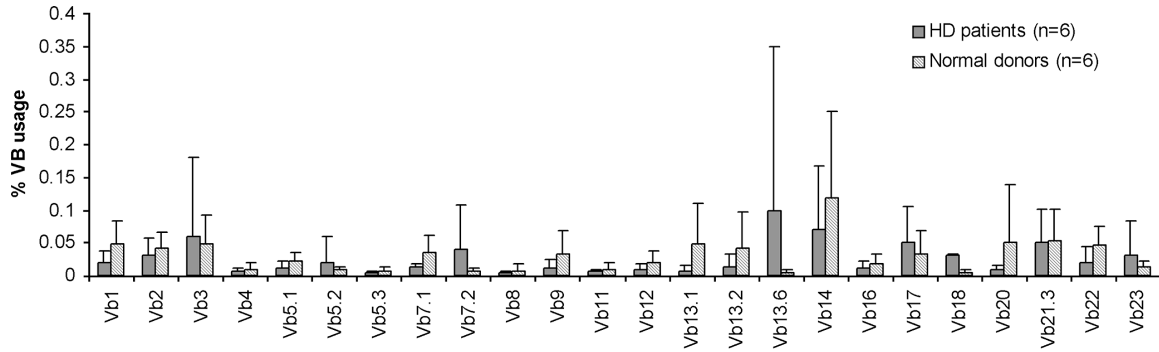

Figure 1. All CTL lines generated were polyclonal with unique Vβ repertoires. To analyze the Vβ repertoires of the CTL lines generated from six patients (gray) and six normal donors (shaded), the CTLs were stained with anti-CD3 and 24 Vβ antibodies grouped into 8 vials. Surface immunofluorescence was analyzed by flow cytometry.

dian 1.03%) of the CTLs from Hodgkin patients (Fig. 2). A broad range of epitopes were recognized by patient lines, although there was a particularly high representation of the B35-restricted LMP2 epitope MGS in the CTL line generated from patient 11. Flow cytometric analysis was also performed on CTL lines from HLA-A02*01 patients using available HLA-A02*01–restricted LMP1 and HLA B07*01–restricted EBNA1 tetramers, but no detectable LMP1 or EBNA1-specific populations were identified. Therefore, the frequency of LMP2-specific effectors in the CTL lines generated from HD patients was not significantly different from the frequency seen in the CTL lines generated from normal donors ($P = 0.16$). This result is unexpected. LMP2-specific T cells are readily detectable by tetramer and ELISPOT analysis in the peripheral blood of healthy donors but appear to be absent in HD patient blood. Therefore, we assume that growth and survival of LMP2-specific T cells is compromised in patients in vivo but might be restored during ex vivo culture (9).

In Vivo Behavior of Adoptively Transferred EBV-specific CTL Lines

Infused CTLs Expand In Vivo, Persist in the Circulation, and Traffic to Sites of Tumor. Seven patients received CTLs that had been genetically marked with a retrovirus containing the neomycin resistance gene (*neo*; reference 3). *Neo*$^+$ cells were then tracked in the peripheral blood using Q-PCR. Gene-marked CTLs could be detected in PBMCs for 3–12 mo after infusion (Fig. 3). From our knowledge of the transduction efficiency and the number of the CTLs infused, we calculated that the levels of the *neo* gene in peripheral blood implied that marked T cells had expanded up to at least 100-fold in vivo. For example, patient 1 received a total CTL dose of 8×10^7 CTLs, of which 1.6% were gene marked (1.28×10^6). His lymphocyte count was 6% of the total white cell count (6,700/mm^3). If we assume that the CTLs remained in the peripheral blood, which has a volume of approximately 5,000 ml, this would give a total of 8×10^9 total mononuclear cells, of which 1.63% (1.28×10^8) were gene marked at 9 wk after the CTLs. This equates to a 100-fold expansion (Fig. 3).

The above assessment likely profoundly underestimates the true level of expansion because it assumes that all infused CTLs remain in the circulation and do not enter lymphoid organs or tumor sites. In fact, marked T cells readily leave the circulation, as shown in Fig. 4, in which an analysis of postmortem tissues from patient 4 using in situ PCR showed gene-marked cells infiltrating a site of disease. Further evidence that the CTL lines traffic and accumulate at tumor sites came from patient 3. Q-PCR amplification

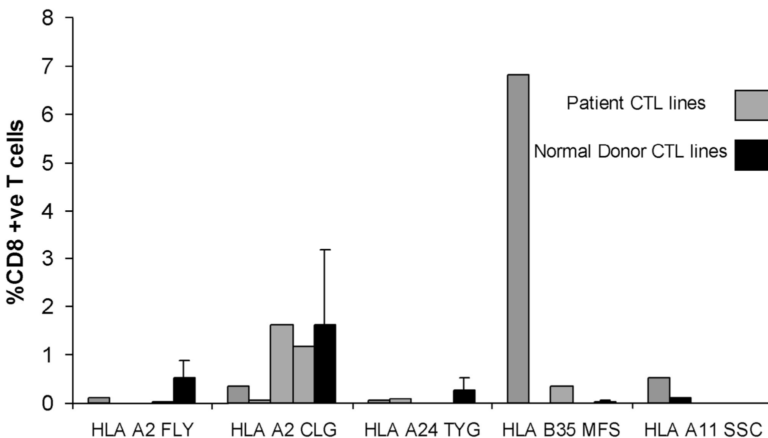

Figure 2. EBV-specific CTL lines derived from Hodgkin patients and normal donors contained LMP2-specific T cell populations on tetramer analysis. The frequency of LMP2-specific T cells in CTL lines generated from 11 patients with relapsed HD were compared with CTL lines from 7 normal donors with relevant HLA alleles. CTLs were costained with PE-conjugated tetramers and FITC-conjugated CD8 and PerCP-conjugated CD3. Tetramers used to test the lines were as follows: HLA-A02*01-CLGGLLTMV, HLA-A02*01-FLYALALLL, HLA-A11*01-SSCSSCPLSKI, HLA-A23*01-PYLFWLAAI, HLA-A24-TYGPVFMSL, and HLA-B35*01-MGSLEMVPM. The average (± SD) of the results obtained from the normal donor lines are shown in black compared to the results obtained from each Hodgkin line tested, which are shown in gray.

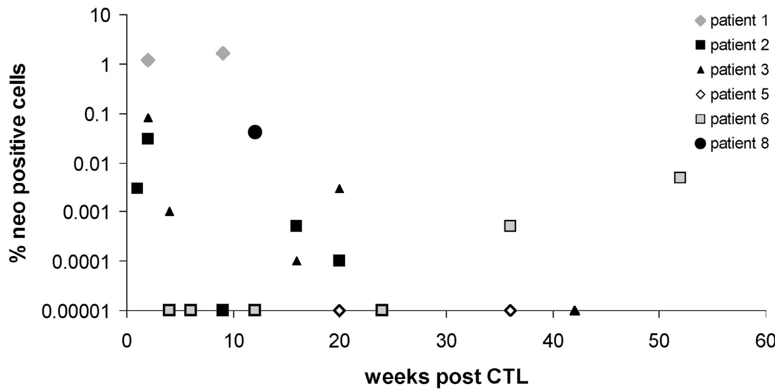

Figure 3. Infused CTLs gene marked with the neomycin resistance gene can persist in vivo for up to 12 mo. We used Q-PCR to quantify the presence of the marker gene DNA in PBMCs after CTL infusion in the patients who received gene-marked cells. Control DNA was prepared by diluting G1Na-transduced K-562 cells (one integrant per cell) with nontransduced K-562 to give mixtures containing 0.01–10% neo^+ cells. Therefore, results are reported as a percent of neo^+ cells in PBMCs after infusion.

analysis of his malignant pleural effusion 3 wk after CTL infusion revealed that 0.65% of the cells were marked versus just 0.001% in the peripheral blood. Hence, infused effector–memory T cells can expand and persist in vivo and will traffic to sites of HD.

The Frequencies of EBV- and LMP2-specific T Cells Increase after Infusion of Polyclonal EBV-specific CTLs

Having shown an increase in the overall number of infused gene-marked CTLs, we next determined whether peripheral blood and tumor sites also showed the expected corresponding increase in the frequency of EBV- and LMP2-specific cells. LMP2 tetramer analysis of $CD8^+$ T cells in the peripheral blood of the four HLA-A2 patients showed a 2–20-fold increase for up to 9 mo after infusion (Fig. 5 A). As argued for the measurement of gene-marked CTLs in the circulation, this increase indicates a several log expansion of the infused LMP2-specific cells in vivo because such small numbers of LMP2-specific T cells were infused (Fig. 2).

In six patients for whom LMP2 peptide tetramer reagents were lacking, we used ELISPOT assays to measure IFN-γ release by $CD8^+$ CTLs after stimulation with autologous LCLs. This measure of EBV-specific CTL precursor frequency showed a 1.3–4-fold increase in LCL-reactive cells by 4 wk after infusion (Fig. 5 B). Because these experiments gave no information about the LMP2-specific component, we further studied two of these patients by measuring IFN-γ secretion by $CD8^+$ T cells after stimulation with LMP2 peptide–pulsed PHA blasts in ELISPOT assays. As shown in Fig. 5 C, there was a 2.5-fold increase in IFN-γ SFCs in response to the B35-restricted LMP2 peptide MGS 8 wk after CTL infusion in patient 11, whereas patient 12 had a fourfold rise in the response to the HLA-A11–restricted LMP2 peptide SSC (Fig. 5 D). These data show that there is an increase in LMP2-specific CTLs in vivo after infusion of small numbers of ex vivo–cultured cells, and the infused cells are functional.

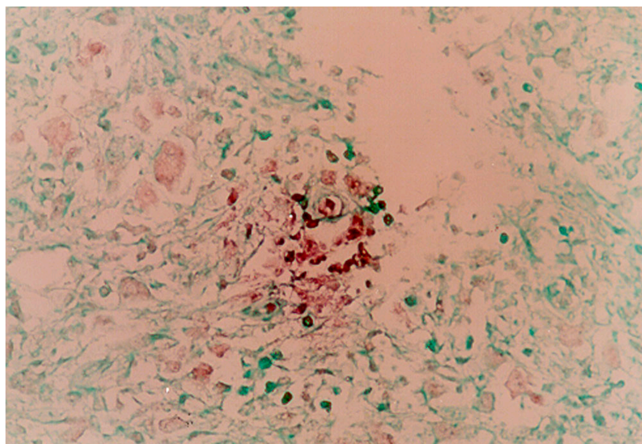

Figure 4. Infused CTLs can home to tumor sites. In situ PCR analysis of a mediastinal Hodgkin tumor obtained at autopsy 2 mo after receiving gene-marked EBV-specific CTLs demonstrates gene-marked, tumor-infiltrating lymphocytes.

To demonstrate that this rise in LMP2-specific cells did not simply parallel the general tempo of immune recovery after SCT, we analyzed the response to irrelevant antigens. As shown in Fig. 5 (C and D), the frequency of CMV and superantigen-specific T cells failed to show the same increase as the LMP2-specific T cells, suggesting that the increase was specific to the infused T cells. Moreover, the Vβ phenotype of the LMP2 $tetramer^+$ T cells in the peripheral blood detected after infusion in patient 11 matches the LMP2 $tetramer^+$ cells in the infused CTL line, further supporting the assertion that the rise in LMP2-specific T cells is attributable to the infused CTLs rather than to a generalized tide of immune recovery (Fig. 5 E). Finally, expansion of LMP2-specific T cells in the peripheral blood can be accompanied by selective expansion or accumulation at tumor sites because analysis of the malignant pleural effusion from patient 3 showed a higher proportion of LMP2-specific T cells in effusion than in peripheral blood (Fig. 5 F).

EBV-specific CTLs Have Antiviral Activity in Patients with Relapsed HD

To determine if the infused CTLs had biological activity after transfer, EBV DNA levels in patient PBMCs were measured before and after CTL infusion using Q-PCR. 9 of 10 patients with measurable EBV DNA in PBMCs had a fall in viral DNA within 8 wk of CTL infusion, and in 6 of these, EBV DNA became undetectable. Amongst these six were the

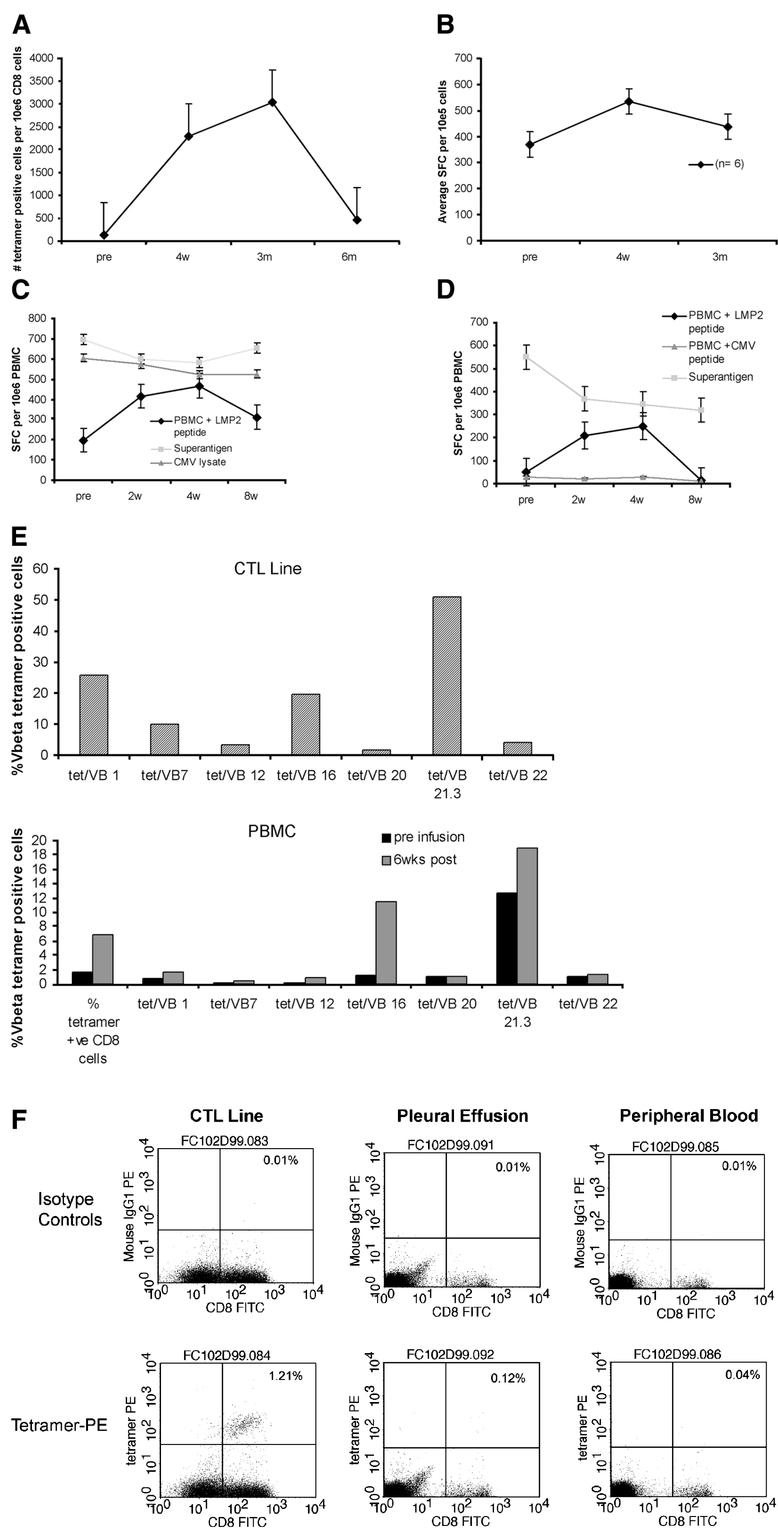

Figure 5. The frequency of EBV- and LMP2-specific T cells increase after infusion of polyclonal EBV-specific CTLs. (A) Tetramer analysis was used to compare the frequency of LMP2-specific CTLs before and after infusion in the four patients who were HLA-A02*01. The mean number ± SD of A02*01 tetramer$^+$ cells per 10^6 CD8$^+$ T cells are shown before and after infusion. (B) In six patients where tetramer reagents were not available or where sample volumes were small, ELISPOT analysis was used to compare the mean frequency of peripheral blood T cells (± standard error) responding to LCLs by IFN-γ secretion before and after CTL infusion. (C) In patients 11 and (D) 12 where the HLA-restricted LMP2 peptide was known, peripheral blood T cells were incubated with LMP2 peptide–pulsed PHA blasts (black diamond) or CMV lysate/PHA blasts pulsed with CMV peptide (gray triangle) or superantigen (gray square). The number of IFN-γ SFCs per 10^6 mononuclear cells was measured. The average IFN-γ SFCs ± standard error in response to the B35-restricted LMP2 peptide MGS before and after CTL infusion in patient 11 are shown in C, whereas the LMP2 peptide$^+$ populations before and after CTL measured with the HLA-A11–restricted LMP2 peptide SSC in patient 12 are shown in D. (E) The Vβ phenotype of the PE-labeled LMP2 tetramer$^+$ T cells from patient 11 were analyzed by flow cytometry using available FITC-labeled Vβ antibodies in the infused CTL line (black stripes) and were compared to the phenotype of peripheral blood LMP2 tetramer$^+$ cells obtained before (black bars) and 6 wk (gray bars) after CTL infusion. (F) In the patient who had a partial response (patient 3), gene-marked cells were detected in the pleural effusion at the level of 0.65% using Q-PCR. A sample of this patient's pleural fluid was also tested for the presence of LMP2-specific CTLs using the HLA-A02*01 tetramer CLG. As shown in F, 1.21% of the patient's CTL line was positive for this tetramer, and these LMP2 tetramer–specific CTLs were detected in the pleural fluid at a level three times higher than that in the peripheral blood. (G) The EBV DNA levels as determined by Q-PCR for patients 11 (black) and 12 (gray).

patients in whom a rise in LMP2-specific T cells could be directly established using peptides. Levels of EBV DNA fell as the numbers of LMP2-specific cells increased and rose again as LMP2-specific cells declined (Fig. 5, C, D, and G).

Although the decline in EBV DNA after CTL infusion likely reflects the killing of virus-infected normal B cells rather than of tumor cells, it may nonetheless be a surrogate marker of LMP2 effector function. The pattern of latency generally found in the peripheral blood of immunocompetent patients is "type 0," in which there is expression of LMP2, EBERs, and low levels of EBNA1 (15). Because EBNA1 is poorly presented by infected cells and EBERs

have no protein product, the presumptive target antigen for the CTL is LMP2. A decrease in the peripheral blood EBV DNA levels may thus indicate LMP2-specific cytolytic activity in the infused CTL.

Toxicity and Clinical Outcomes

No toxicities were observed immediately after CTL infusion, although two patients with measurable disease developed transient flu-like symptoms. One patient with bulky mediastinal disease died from an erosion of the tumor through the left upper lobe bronchus 3 mo after receiving CTLs. We do not believe this was a toxicity due to CTL infusion because in situ PCR for the marker gene showed no gene-marked CTLs at the site of erosion, although foci of gene-marked T cells were detected in other areas of disease (Fig. 4).

Clinical responses became evident within weeks of CTL infusion and were observed in the patient group who had clinical and radiological evidence of progressive/relapsed (as opposed to stable) disease before CTL infusion (Table I). Three of four patients had resolution of their B symptoms (fever, night sweats, and weight loss; reference 22). Of the 11 patients with measurable tumor, 3 were evaluated as no response to CTLs, 5 as stable disease, and 1 as a partial response. Two patients, however, had complete responses to therapy. The first of these (patient 14) was initially diagnosed with chronic active EBV infection. 2 yr later, she developed an enlargement of the mediastinum with suspected hyperplasia of the thymus. Secondary EBV$^+$ Hodgkin lymphoma (mixed cellularity type) was diagnosed (stage IIA). Over a 3-mo period, she received chemotherapy according to the German HD 95 protocol (28) followed by fractionated radiotherapy (19.8 Gy) for 6 wk. After this therapy, residual cervical lymphadenopathy persisted. She then developed rising EBV DNA levels. Her EBV IgM was negative and she did not have clinical or laboratory evidence of infectious mononucleosis. Biopsy results remained consistent with EBV$^+$ HD. She received no other therapy until receiving two doses of EBV CTLs (total cell dose of $1.2 \times 10^8/m^2$), which were given 9 mo after completing the course of radiotherapy. 4 wk after the first CTL infusion, the cervical nodes became swollen and painful. A biopsy performed at this time showed a prominent infiltrate of CD8$^+$ T cells and a marked decrease in LMP1$^+$ (and EBER$^+$) tumor cells as

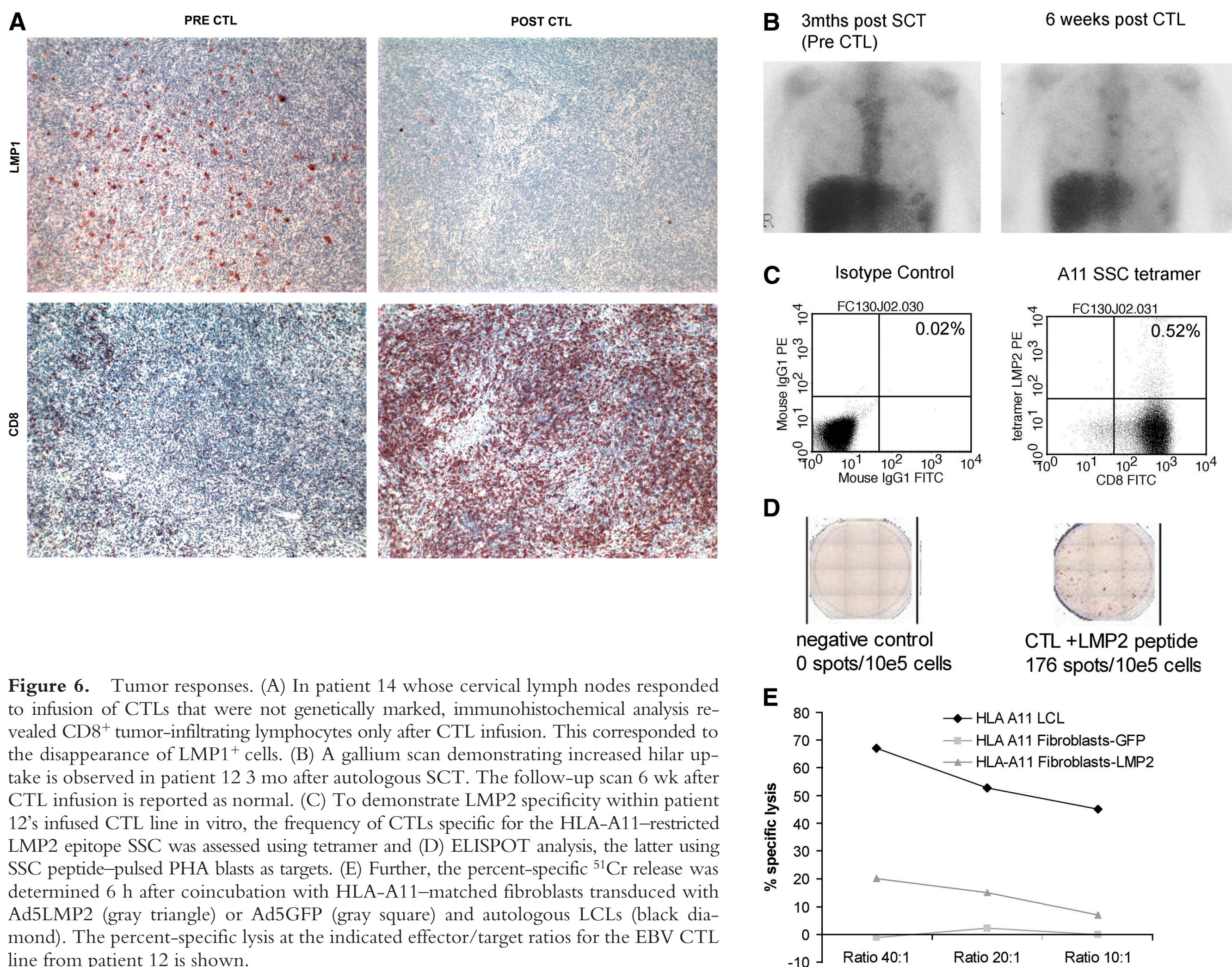

Figure 6. Tumor responses. (A) In patient 14 whose cervical lymph nodes responded to infusion of CTLs that were not genetically marked, immunohistochemical analysis revealed CD8$^+$ tumor-infiltrating lymphocytes only after CTL infusion. This corresponded to the disappearance of LMP1$^+$ cells. (B) A gallium scan demonstrating increased hilar uptake is observed in patient 12 3 mo after autologous SCT. The follow-up scan 6 wk after CTL infusion is reported as normal. (C) To demonstrate LMP2 specificity within patient 12's infused CTL line in vitro, the frequency of CTLs specific for the HLA-A11–restricted LMP2 epitope SSC was assessed using tetramer and (D) ELISPOT analysis, the latter using SSC peptide–pulsed PHA blasts as targets. (E) Further, the percent-specific ^{51}Cr release was determined 6 h after coincubation with HLA-A11–matched fibroblasts transduced with Ad5LMP2 (gray triangle) or Ad5GFP (gray square) and autologous LCLs (black diamond). The percent-specific lysis at the indicated effector/target ratios for the EBV CTL line from patient 12 is shown.

compared to the pre-CTL biopsy sample (Fig. 6 A). The nodes subsequently reverted to normal, and the patient is currently disease free for >9 mo. In the second complete response (patient 12), a persistently abnormal gallium scan after autologous SCT resolved after CTL infusion, and the patient remains in remission after >27 mo (Fig. 6 B). The specificity of this patient's CTL line could be mapped to the known HLA-A11 LMP2 peptide SSC (Fig. 6, C and D), and the line produced significant LMP2-specific killing (20% Cr^{51} release at an E/T ratio of 40:1) of HLA-A11–matched fibroblast targets expressing LMP2 (Fig. 6 E). Using this information, it was possible to track the SSC-specific population in the peripheral blood before and after CTL infusion. As shown in Fig. 5 D, the clinical response correlated with a rise in the LMP2-specific precursor frequency in the peripheral blood.

An additional three patients who had no clearly measurable disease at the time of CTL infusion also remain disease free (requiring no additional therapy after CTL) 10–40 mo later (Table I and footnote).

Discussion

We have used LCL-activated, EBV-specific cytotoxic T lymphocytes (EBV CTLs) to treat patients with multiply relapsed HD. We have shown that it is possible to generate from these patients polyclonal EBV-specific CTL lines with an effector–memory phenotype containing clones specific for the subdominant tumor antigen LMP2 expressed by the malignant Reed-Sternberg cells. Gene marking showed that the infused cells further expanded by two logs or more in vivo and persisted for up to 1 yr after infusion, trafficking to tumor sites. It is likely that actual expansion is greater than this estimate because the infused T cells do not remain exclusively in the blood, circulating as well throughout the lymphoid tissues and entering tumor sites (as shown in Figs. 4 and 5 F). Therefore, estimates based on measurement in the peripheral blood alone may underestimate the total expansion of infused cells in vivo. These CTLs appeared functional, reducing EBV DNA levels in PBMCs and producing IFN-γ ex vivo in response to LMP2 peptides. EBV-specific CTLs were safe at the dose levels used, and administration was followed by measurable tumor responses, including complete remissions.

Successful prevention and eradication of EBV lymphomas after transplant indicates that EBV CTLs can correct an underlying EBV-specific immunodeficiency state and eradicate even bulky EBV antigen–expressing tumor cells (3). Because in situ hybridization studies have localized EBV RNA and antigens to the neoplastic Reed-Sternberg cells in about 40% of cases of HD (29–31), it has been postulated that EBV CTL therapy might also be of value in this group of patients. However, most posttransplant lymphomas express the full panoply of EBV-associated latency antigens, whereas EBV^+ Hodgkin tumors express a much more restricted array, including LMP1, LMP2, EBNA1, and perhaps proteins derived from the BamHI A region of the viral genome (type 2 latency; references 30 and 32). Of these antigens, LMP1 and LMP2 are weakly immunogenic, whereas EBNA1 might be presented poorly to $CD8^+$ cytotoxic T cells (33–35). LMP1-specific T cells are rarely detected in EBV CTLs (36) and indeed we did not detect LMP1-specific T cells in any of our lines using known tetramers. Although LMP2 is also considered weakly immunogenic, the majority of the lines we tested using tetramer or ELISPOT analysis contained cells with specificity for this antigen. We were able to demonstrate that infusion of CTLs was followed by in vivo expansion of both the total numbers of EBV CTLs (measured by levels of gene marking) and the specific fraction that were LMP2 specific (measured by tetramer or ELISPOT analysis). The LMP2–specific T cells were functional in vitro, responding to LMP2-expressing target cells and LMP2 peptides. They also appeared active in vivo because their administration was associated with a decrease in circulating EBV-infected B cells, whose only CTL target antigen appears to be LMP2 (15, 37). Although $LMP1^+$ tumor cells disappeared after CTL infusion (Fig. 6 A), we believe this event to be secondary to the activity of LMP2-specific effector cells, rather than to cryptic LMP1 activity in the infused line.

The gene-marked CTLs were able to traffic to sites of tumor, as were LMP2-specific T cells. Although we cannot directly demonstrate that the infused LMP2-specific CTLs were the cause of the tumor responses observed, the evidence supports such a linkage. For example, patient 12 had normalization of a gallium-67 scintigraphy scan within 8 wk of receiving CTL therapy (Fig. 6 B), and this patient's CTL line showed LMP2 specificity (Fig. 6, C–E). Normalization of the scan occurred as he developed a rise in LMP2 peptide–specific T cells (measured by an IFN-γ ELISPOT; Fig. 5 D) and a fall in EBV DNA levels (Fig. 5 G). Similarly, in patient 3 who had a partial response to CTL therapy, in vivo expansion followed by selective accumulation of gene-marked cells and LMP2 $tetramer^+$ cells at a site of disease was followed by a partial disease response (Fig. 5 F).

Patients with EBV^+ HD are able to control their EBV-infected normal B cells and epithelial cells without difficulty. Therefore, it is paradoxical that their apparently immunogenic tumors coexist with circulating T cells with specificity for the tumor antigens. It is not obvious why T cells that were previously ineffectual in vivo should become more active when reinfused as small numbers of effector–memory cells after ex vivo activation. We suggest that endogenous LMP2-specific T cells are prevented from exerting their potential effector function by the multiple immunoregulatory strategies adopted by Hodgkin Reed-Sternberg cells (9, 38). Hodgkin Reed-Sternberg cells secrete immunosuppressive cytokines such as IL-10 and TGF-β, as well as the chemokine TARC, which selectively recruits IL-4–secreting Th2 cells, thereby inhibiting the Th1 cell CTL response (9, 39). Hodgkin Reed-Sternberg cells also recruit $CD4^+25^+$ negative regulatory T cells into the tumor environment (40). Removal of LMP2-specific T cells from this immunoregulatory environment may

allow the activation and expansion of this previously inhibited population as well as completion of ex vivo–initiated expansion and effector programs, even after the lymphocytes have been returned to an inhibitory environment. However, it is likely that even infused CTLs may become subject to tumor inhibition, reducing their effectiveness.

Although the results we report show that our adoptively transferred T cells had biologic activity and could induce even complete tumor responses, they also reveal limitations with the current approach. Three patients with bulky disease showed no response at all, and in several of the remaining patients with measurable disease, response was limited and transient. Nonetheless, given the difficulty of treating relapsed EBV$^+$ HD and the toxicities associated with conventional therapy, our observations support further development of CTL therapy for this lymphoma and perhaps for other weakly immunogenic tumors as well.

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