Treatment of nasopharyngeal carcinoma with Epstein-Barr virus–specific T lymphocytes

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Conventional treatment for nasopharyngeal carcinoma (NPC) frequently fails and is accompanied by severe long-term side effects. Since virtually all undifferentiated NPCs are associated with Epstein-Barr virus (EBV), this tumor is an attractive candidate for cellular immunotherapy targeted against tumor-associated viral antigens. We now demonstrate that EBV-specific cytotoxic T-cell (CTL) lines can readily be generated from individuals with NPC, notwithstanding the patients' prior exposure to chemotherapy/radiation. A total of 10 patients diagnosed with advanced NPC were treated with autologous CTLs. All patients tolerated the CTLs, although one developed increased swelling at the site of pre-existing disease. At 19 to 27 months after infusion, 4 patients treated in remission from locally advanced disease remain disease free. Of 6 patients with refractory disease prior to treatment, 2 had complete responses, and remain in remission over 11 to 23 months after treatment; 1 had a partial remission that persisted for 12 months; 1 has had stable disease for more than 14 months; and 2 had no response. These results demonstrate that administration of EBV-specific CTLs to patients with advanced NPC is feasible, appears to be safe, and can be associated with significant antitumor activity. (Blood. 2005;105:1898-1904)

Introduction

Nasopharyngeal carcinoma (NPC) occurs worldwide and is the third most common malignancy in Southern China, where the incidence is as high as 50 per 100 000.[1] NPC is a radiosensitive tumor and local control rates of more than 80% can be obtained. However, a significant number of patients relapse, particularly when disease is advanced at diagnosis—the most common presentation due to a lack of early symptoms.[2] Moreover, radiation and chemotherapy are accompanied by severe short- and long-term side effects including secondary malignancies.[3] Hence, there is a need for therapies that will improve disease-free survival and that may be associated with reduced toxicity.

Epstein-Barr virus (EBV) is present in virtually all poorly and undifferentiated nonkeratinizing NPCs regardless of geographic origin,[4] and the viral antigens expressed by the tumor provide potential target antigens for immunotherapy. Adoptive transfer of cytotoxic T cells (CTLs) specific for EBV antigens has proved safe and effective as prophylaxis and treatment for EBV-associated lymphoproliferative disease in bone marrow and solid organ transplant recipients.[5-11] These highly immunogenic lymphomas express all latent EBV antigens, including the immunodominant EBV nuclear antigens (EBNA) 3A, 3B, and 3C, and are therefore ideal targets for immunotherapy. By contrast, NPC expresses a restricted set of less immunogenic viral antigens, namely EBNA1, and latent proteins (LMPs) 1 and 2. EBNA1 is expressed in all NPCs, and although its processing through the HLA class I pathway is inhibited by a glycine-alanine repeat, peptides derived from incompletely translated proteins may be presented to $CD8^+$ T cells.[12-15] Expression of LMP1 and/or LMP2 is detectable in at least 50% of NPC tumors.[16,17] Since NPCs also express major histocompatibility complex (MHC) class I molecules as well as the peptide transporters TAP1 and TAP2, they are capable of processing and presenting these antigens in the context of HLA class I molecules for recognition by CTLs.[18] LMP1- and LMP2-specific T cells are indeed present in the peripheral blood of NPC patients, albeit at lower frequency than in healthy donors,[19,20] and could potentially be activated and expanded for immunotherapeutic strategies.

We hypothesized that ex vivo expansion of EBV-specific CTLs in the absence of tumor inhibitory factors[21,22] and the subsequent adoptive transfer of these cells may be of benefit to patients with EBV-positive NPC. Here we confirm the feasibility of this approach, and in 10 patients show evidence for safety and activity.

Patients, materials, and methods

Study entry criteria and patient details

This protocol was approved by the institutional review board (IRB) at Baylor College of Medicine and the Food and Drug Administration (FDA). Patients were eligible for study if they had stage III or IV nasopharyngeal carcinoma at diagnosis (according to American Joint Committee for Cancer

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Staging and End-Results Reporting staging system 1997[23]) and were either in remission or had refractory or relapsed disease, and if their tumor was EBV-positive as determined by in situ hybridization or polymerase chain reaction (PCR) amplification for Epstein-Barr virus–encoded RNA (EBER). Patients were treated on 3 escalating dose levels and received either 2 doses of 2×10^7 CTLs/m^2 (dose level 1) or one dose of 2×10^7 CTLs/m^2 and 1 dose of 1×10^8 CTLs/m^2 (dose level 2) or 1 dose of 1×10^8 CTLs/m^2 and 1 dose of 2×10^8 CTLs/m^2 (dose level 3). CTLs/m^2 were given intravenously with a 2-week interval between each dose. Peripheral blood was obtained before and at multiple time points after CTL infusion for evaluation of toxicity and EBV immunity.

Generation of EBV-transformed B-cell lines and EBV-specific CTLs

After informed consent, peripheral blood (40-60 mL) from patients with EBV-positive NPC was used to generate both EBV-transformed lymphoblastoid B-cell lines (LCLs) and EBV-specific CTL lines.[24] Briefly, for LCL generation, 5×10^6 peripheral blood mononuclear cells (PBMCs) were incubated with concentrated supernatant of B95-8 cultures, in the presence of 1 μg/mL cyclosporin A (Sandoz, Vienna, Austria) to establish an LCL. Subsequently, PBMCs (2×10^6 per well of a 24-well plate) were stimulated with LCLs irradiated at 4000 rads at an effector-stimulator (E/S) ratio of 40:1. After 9 to 12 days, viable cells were restimulated with irradiated LCLs (at 4:1 E/S ratio). Subsequently, CTLs were expanded by weekly stimulations with LCLs (at 4:1 E/S ratio) in the presence of recombinant human interleukin-2 (rhIL-2, Proleukin; Chiron Emeryville, CA) (40-100 U/mL). After expansion, CTLs were tested for sterility, HLA identity, immunophenotype, and EBV specificity and cryopreserved. Specificity was tested in a 4-hour Cr^{51} release assay. In 8 lines, the CTLs showed a significantly higher killing of the autologous LCLs (mean, 56.6%; range, 38%-92%) compared with HLA antigen–mismatched LCLs (mean, 6.1%; range, 0%-27%; $P < .0001$) or to HSB-2 (mean, 21.5%; range, 6%-55%; $P < .005$) at an effector-target ratio of 20:1. In 2 CTL lines, lysis of the HLA-mismatched LCLs was observed, which was significantly reduced by depletion of T-cell receptor γδ (TCRγδ)–positive cells. Autoreactivity was excluded by the absence of lysis of autologous phytohemagglutinin (PHA)–stimulated lymphoblasts in all 10 CTL lines.

Peptides

The following peptides were used for analysis of EBV-specific T-cell populations according to the patients' HLA specificity: LMP1, HLA-A2: YLQQNWWTL, YLLEMLWRL; LMP2, HLA-A2: LLWTLVVLL, CLGGLLTMV, FLYALALLI, GLGTLGAAI, TVCGGIMFL, LTAGFLIFL, LIVDAVLQL; HLA-A11: SSCSSCPLSKI; HLA-A24: TYGPVFMCL; HLA-A23/24: PYLFWLAAI; HLA-A68: FTASVSTVV, ASCFTASVSTVVTAT (15-mer); HLA-B27: RRRWRRLTV, RRWRRLTVCGGIMFL (15-mer), RRLTVCGGIMFL; HLA-B60: IEDPPFNSL; EBNA1, HLA-B35: HPVGEADYFEY; EBNA2, HLA-A2: DTPLIPLTIF; EBNA3, HLA-A2: LLDFVRFMGV; HLA-A3: RLRAEAQVK; HLA-A11: AVFDRKSDAK, IVTDFSVIK, LPGPQVTAVLLHHEES, DEPASTEPVHDQLL, NPTQAPVIQLVHAVY; HLA-A24: RYSIFFDY, TYSAGIVQI; HLA-B7: RPPIFIRLL, QPRAPIRPI; HLA-B27: RRIYDLIEL; HLA-B35: YPLHEQHGM, AVLLHEESM; HLA-B44: VEITPYKPTW, EGGVGWRHW, EENLLDFVRF, KEHVIQNAF; BZLF1, HLA-B35: EPLPQGQLTAY; BRLF1, HLA-A2: YVLDHLIVV; HLA-A11: ATIGTAMYK; HLA-A24: DYCNVLNKEF; BMLF1, HLA-A2: GLCTLVAML; and BMRF1, HLA-A2: TLDYKPLSV (listed in Khanna and Burrows[25]; Houssaint et al[26]; and K.C.M.S., Ann Leen, M.H.H., H.E.H., C.M.R., and C.M.B., manuscript in preparation). HLA-A2–restricted cytomegalovirus pp65–derived peptide NLVPMVATV was used as a control. Peptides were synthesized by either Martin Campbell, Synthetic Antigen Laboratory, The University of Texas M. D. Anderson Cancer Center, Houston, TX, or Genemed Synthesis (South San Francisco, CA). In this paper, the peptides are referred to by the first 3 amino acids as underlined.

Tetramer staining

To identify LMP1- and LMP2-specific T cells, a selection from the following tetramers was used, as determined by the HLA type of the patient: LMP1: HLA-A*0201-YLQQNWWTL; and LMP2: HLA-A*0201-CLGGLLTMV, HLA-A*0201-FLYALALLI, HLA-A*0201-LLWTLVVLL, HLA-A*0201-TVCGGIMFL, HLA-A*1101-SSCSSCPLSKI, HLA-A*2301-PYLFWLAAI, HLA-A24-TYGPVFMCL, HLA-A68-FTASVSTVV, HLA-B*2705-RRRWRRLTV, and HLA-B*2705-RRLTVCGGIMF. Tetramers were prepared by the National Institute of Allergy and Infectious Diseases (NIAID) tetramer core facility (Atlanta, GA) or by the Baylor College of Medicine Tetramer Core Facility (Houston, TX). CTLs or PBMCs ($5\text{-}10 \times 10^5$) were incubated at room temperature for 30 minutes in phosphate-buffered saline (PBS)/1% fetal calf serum (FCS) containing the phycoerythrin (PE)–labeled tetrameric complex. Samples were costained with anti-CD8 fluorescein isothiocyanate (FITC) and anti-CD3 peridinin chlorophyll-alpha protein (PerCP). Appropriate isotype controls were included. Stained cells were fixed in PBS containing 0.5% paraformaldehyde. For each sample, a minimum of 100 000 cells was analyzed using a FACS Calibur with the Cell Quest Software (Becton Dickinson, San Jose, CA).

Enzyme-linked immunospot (ELISPOT) assay

The frequency of EBV- and LMP2-specific T cells in the infusion product as well as in the peripheral blood before and at multiple time points after CTL infusion was measured using an interferon-γ (IFN-γ) ELISPOT assay. The 96-well filtration plates (MultiScreen, no. MAHAS4510; Millipore, Bedford, MA) were coated overnight with 10 μg/mL anti–IFN-γ antibody (Catcher-mAB91-DIK; Mabtech, Cincinnati, OH). PBMCs were thawed 24 hours before the assay in complete media supplemented with 50 U/mL Benzonase (Novagen, Madison, WI), rested overnight in complete media, and plated at 1 to 2×10^5 cells/well and 2 to 3 serial dilutions for LCL targets and 3 to 4×10^5/well for peptide targets. CTLs were rested overnight in complete media and plated at 1×10^5 cells/well and 2 serial dilutions. Cells were stimulated with either irradiated (40 Gy) autologous LCLs (1×10^5/well) or 5 μg/mL peptide. In HLA-A2–positive patients, the cytomegalovirus (CMV) pp65–encoded HLA-A2–restricted peptide NLVPMVATV was used as control. After 18 to 24 hours, the plates were washed and incubated with the secondary biotin conjugated anti–IFN-γ monoclonal antibody (Detector-mAB [7-B6-1-Biotin]; Mabtech). After incubation with avidin–biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit [Standard], no. PK6100; Vector Laboratories, Burlingame, CA), plates were developed with 3-amino-9-ethylcarbazole (AEC) substrate (Sigma, St Louis, MO). Plates were sent for evaluation to Zellnet Consulting (New York, NY). Spot-forming units (SFCs) per 1×10^5 CTLs or per 1×10^6 PBMCs were calculated by linear regression analysis when serial dilutions were performed and subsequent subtraction of background of nonstimulated T cells. If an epitope-specific T-cell population had been identified in the infusion product, EBV- and LMP2-specific immunity was monitored in patient peripheral blood using this IFN-γ ELISPOT assay and, when enough PBMCs were available and HLA type was informative, by tetramer staining.

PCR for EBV load in PBMCs

PBMCs were isolated from peripheral blood on a Ficoll (Lymphoprep; Axis-Shield, Oslo, Norway) gradient and washed with PBS. DNA was isolated from 3 to 5×10^6 PBMCs using an anion exchange column (Qiagen, Valencia, CA). DNA (500 ng) was then used for real-time polymerase chain reaction (PCR) to quantitate EBV genome copy number and was reported as copies (cp)/μg DNA.[27]

Results

Patient characteristics

A total of 10 patients were enrolled in the study, and all had poorly differentiated or undifferentiated nasopharyngeal carcinoma (WHO II/III) at diagnosis. At the time of CTL infusion, 4 patients at high risk for relapse were in remission and 6 patients had failed multiple rounds of radiotherapy and chemotherapy

Table 1. Characteristics of patients on study

Patient no.	Dose	Sex/age	HLA	Ethnicity	Stage	Previous treatment
Treated in remission						
729	$2 \times 10^7/m^2 \times 2$	M/50	A2/11 B56/61	Asian	IV	RT, cisplatin, 5-FU
606	$2 \times 10^7/m^2 \times 2$	F/59	A2/2 B60/61	White	IV	RT, cisplatin, 5-FU
697	$2 \times 10^7/m^2 \times 2$	F/11	A1/2 B37/44	African American	III	RT, cisplatin, MTX, 5-FU
815	$1 \times 10^8/m^2 \times 1$, $2 \times 10^8/m^2 \times 1$	M/19	A33/36 B53/72	African American	IV	RT, cisplatin, MTX, 5-FU
Treated with relapsed or refractory disease						
845	$2 \times 10^7/m^2 \times 1$	M/11	A3/68 B7/35	White	IV	RT, cisplatin, MTX, 5-FU, paclitaxel, carboplatin, VP16, vinorelbine, gemcitabine
894	$2 \times 10^7/m^2 \times 1$, $1 \times 10^8/m^2 \times 1$	M/36	A1/32 B27/35	White	III	RT, cisplatin, 5-FU, carboplatin, ifosphamide, paclitaxel, radioactive seed implants, gemcitabine
389	$2 \times 10^7/m^2 \times 1$, $1 \times 10^8/m^2 \times 1$*	F/17	A2/3 B44	White	IV	RT, cisplatin, MTX, 5-FU, carboplatin, paclitaxel
918	$2 \times 10^7/m^2 \times 1$, $1 \times 10^8/m^2 \times 1$	M/16	A11/68 B49/52	Hispanic	IV	RT, cisplatin, MTX, 5-FU
1042	$1 \times 10^8/m^2 \times 1$, $2 \times 10^8/m^2 \times 1$	F/46	A2/24 B51/61	Asian	IV	RT, cisplatin, 5-FU, docetaxel, CPT-11
1046	$1 \times 10^8/m^2 \times 1$, $2 \times 10^8/m^2 \times 1$	M/16	A30/68 B18/42	African American	IV	RT, cisplatin, MTX, 5-FU, docetaxel, oxaliplatin, epirubicin, gemcitabine, etoposide

M indicates male; RT, radiotherapy; 5-FU, 5-fluoruracil; F, female; and MTX, methotrexate.
Stage according to American Joint Committee for Cancer Staging and End-Results Reporting staging system 1997.[23]
VP16 indicates etoposide; CTP-11 is irinotecan.
*This patients received additional doses of 1×10^8 CTLs/m^2 at 6 months, 9 months, and 12 months after the initial CTL infusions.

and had relapsed/refractory disease. Patient characteristics and previous treatment are summarized in Table 1.

CTL lines contain LMP2-specific T-cell populations

Autologous LCLs and EBV-specific CTLs were successfully generated from 10 of 10 NPC patients. The phenotype of these CTL lines is shown in Table 2. The presence of LMP1- and LMP2-specific T cells within these CTL lines was evaluated by IFN-γ ELISPOT after stimulation with LMP1/2 peptides. In 8 of 9 CTL lines for which informative peptides were available based on HLA type, T cells specific for at least 1 LMP2 epitope were detected (Table 3). In addition, in 1 of 5 CTL lines evaluable for LMP1 specificity an LMP1-YLL–specific T-cell population was identified. As measured by tetramer staining, up to 5.5% of the total $CD8^+$ population was specific for a single LMP2 epitope (data not shown). In 4 lines, T cells specific for multiple (up to 5) different LMP2 epitopes were present; in 2 cases these were restricted through different HLA alleles. Such T-cell responses targeted toward multiple tumor antigen-derived epitopes are important to reduce the risk of tumor escape through antigen deletion. Overall the T-cell responses against these subdominant LMP antigens were weaker than those against epitopes derived from the immunodominant lytic and EBNA3 latent antigens (Table 3), but in the same range as detected in LCL-reactivated CTL lines from healthy donors.[28] Moreover, the identified T-cell populations specific for individual peptides reflect the minimum LMP2 specificity present and likely underestimate the total number of LMP2-specific T cells.

Safety of EBV-specific CTLs

Upon administration of EBV-specific CTLs, no immediate or long-term toxicity was observed in the 4 patients without detectable disease and in 5 of 6 patients with refractory/relapsed disease (Table 4). However, in one patient (P845) with bulky disease, pre-existing facial swelling increased markedly 2 days after infusion of the first dose of CTLs ($2 \times 10^7/m^2$) requiring a tracheostomy. A needle biopsy of this mass showed tumor cells and no inflammatory cells suggesting tumor progression as the causative factor, but a contributory effect from CTL cannot be excluded.

Changes in EBV immunity after CTL administration

Viral load and the frequency of EBV-specific T cells were monitored in the peripheral blood at multiple time points after

Table 2. Phenotype of patient CTL lines for infusion

Patient no.	$CD3^+/TCR\alpha\beta^+$, %	$CD3^+/TCR\gamma\delta^+$, %	$CD3^+/CD4^+$, %	$CD3^+/CD8^+$, %	$CD3^+/CD56^+$, %	$CD3^-/CD56^+$, %	$CD3^-/CD16^+$, %
729	92.3	5.6	5.4	87.8	46.9	3.1	3.4
606	96.4	0.9	1.0	95.1	5.7	2.6	2.7
697	85.9	8.6	17.1	73.2	31.7	5.1	5.2
815	86.1	12.0	3.7	78.7	10.1	1.9	1.6
845	71.7	30.2	4.6	67.2	25.4	0.2	0.0
894	83.8	2.4	11.1	72.9	12.8	13.8	10.4
389	98.0	0.1	0.2	97.0	24.9	0.1	0.1
918	95.7	1.4	4.8	91.7	9.7	0.7	0.6
1042	84.2	15.6	1.4	83.5	22.4	2.8	2.7
1046	92.9	1.4	0.3	94.9	31.7	0.9	0.0

Table 3. T-cell populations specific for EBV antigens (SFCs/1 × 10^5 CTLs) in infusion product

Patient no.	LMP1	LMP2	EBNA1	EBNA2/3	Lytic cycle
729	YLQ: 0	CLG: 0	ND	DTP: 0	TDL: 3.5
	YLL: 0	GLG: 0		LLD: 0	YVL: 31
		FLY: 1988		AVF: 0	GLC:1236
		LLW: 0		IVT: 0	ATI: 0
		LTA: 0		NPT: 0	
		TVC: 0		LPG: 0	
		LIV: 0		DEP: 0	
		SSC: 27			
606	YLQ: 0	CLG: 45	ND	DTP: 24	TDL: 50
	YLL: 30	GLG: 0		LLD: 0	YVL: 45
		FLY: 6			GLC: 1824
		LLW: 0			
		LTA: 0			
		TVC: 82			
		LIV: 750			
		IED: 830			
697	YLQ: 0	CLG: 33	ND	DTP: 0	TDL: 12
	YLL: 0	GLG: 0		LLD: 256	YVL: 60
		FLY: 156		VEI: 0	GLC: 480
		LLW: 4		EGG: 96	
		LTA: 0		KEH: 0	
		TVC: 0		EEN: 3	
		LIV: 0			
845	ND	FTA: 0	HPV: 0	RPP: 125	EPL: 0
				QPR: 0	
				RLR: 0	
				YPL: 0	
				AVL: 0	
894	ND	RRR: 3	HPV: 1588	RRI: 160	EPL: 1124
		RRL: 52		YPL: 26	
				AVL: 72	
389	YLQ: 0	CLG: 26	ND	DTP: 0	TDL: 4
	YLL: 0	GLG: 0		LLD: 0	YVL: 7
		FLY: 0		VEI: 0	GLC: 934
		LLW: 0		EGG: 398	
		LTA: 0		KEH: 0	
		TVC: 0		EEN: 506	
		LIV: 0		RLR: 0	
918	ND	SSC: 8	ND	AVF: 1214	ATI: 0
		FTA: 0		IVT: 1420	
				NPT: 0	
				LPG: 0	
				DEP: 0	
1042	YLQ: 0	CLG: 0	ND	DTP: 0	DYC: 0
	YLL: 0	GLG: 0		LLD: 0	TDL: 0
		FLY: 0		RYS: 0	YVL: 0
		LLW: 0		TYS: 0	GLC: 0
		LTA: 0			
		TVC: 317			
		LIV: 0			
		PYL: 0			
		TYG: 0			
1046	ND	FTA: 29	ND	ND	ND

CTL lines were screened for the presence of T-cell populations specific for the indicated antigens by IFN-γ ELISPOT. The panel of peptides used for stimulation was based on the HLA type of the patient (Table 1). The sequence of the peptides referred to by the first 3 amino acids is listed in "Patients, materials, and methods."

ND indicates not done, as no informative peptides were available.

For patient 815, there were no informative peptides available.

CTL infusion to evaluate in vivo persistence and activity of the infused CTLs. Of 9 patients with a detectable amount of EBV-DNA in PBMCs prior to CTL infusion, EBV load fell within 6 weeks after infusion in 6 patients (Table 5). A decrease in EBV viral load in the peripheral blood likely reflects the lysis of EBV-infected B cells, and therefore demonstrates in vivo activity of the infused EBV-specific CTLs.

In 9 of 10 patients, the low normal frequency of EBV-specific T cells in the peripheral blood (mean: 274, range: 197-384 SFCs/1 × 10^5 PBMCs), as measured by IFN-γ secretion of PBMCs upon stimulation with autologous LCLs, remained unchanged after CTL infusion (data not shown). In one patient (P845) with a low number of circulating EBV-specific CTLs prior to CTL infusion (24 SFCs/1 × 10^5 PBMCs), a transient 3-fold increase in the number of EBV-specific CTLs was measured. In addition, the LMP2-specific T-cell populations identified in the infusion product were monitored in the peripheral blood after CTL infusion. In 5 HLA-A2+ patients, using IFN-γ ELISPOT analysis, the number of T cells specific for a cytomegalovirus pp65–derived epitope was determined at the same time points to control for natural variations in viral immunity. In 4 of 8 evaluated patients, the number of T cells specific for LMP2 epitopes increased more than 2-fold, whereas the pp65-specific immunity remained stable over this time period (Table 5). However, this increase in LMP2 immunity was transient as the number of LMP2-specific T cells was similar to baseline 6 weeks after CTL infusion in 3 of these 4 patients. Additional tetramer analysis of the frequency of LMP2-specific T cells in the peripheral blood after CTL infusion in 3 patients failed to detect a persistent increase in LMP2 immunity (data not shown).

Clinical responses after CTL therapy indicate antitumor activity

Clinical responses were evaluated from computed tomography and magnetic resonance imaging scans before and after CTL therapy, using the international criteria proposed by the Response Evaluation Criteria in Solid Tumors Committee.[29] All 4 patients who were in remission at the time of enrollment on the study remained in complete remission 19 to 27 months after CTL therapy (Table 4). Of the 6 patients with refractory/relapsed disease, 2 patients had no response, 1 patient has had stable disease for more than 14 months without additional therapy, 1 patient had a partial response sustained for 12 months, and 2 patients attained complete remission (CR). One of the patients who attained CR (P389) with refractory relapsed disease had a 24% reduction in tumor size after the initial 2 CTL infusions on dose level 2. Because of this partial response, this patient received 3 additional doses of 1 × 10^8 CTLs/m^2 at 6 months, 9 months, and 12 months after the initial CTL infusions with IRB and FDA approval. During this period, the patient did not receive other treatment and showed continuing response. Positron emission tomography imaging at 15 months after the first CTL infusion showed normal isotope uptake consistent with a complete response and residual fibrosis. In the second patient who had a CR (P894), a biopsy of the nasopharynx prior to CTL infusion showed poorly differentiated EBER-positive NPC. Multiple biopsies taken 6 months after CTL therapy were all negative for tumor indicating a complete remission (Figure 1). Of the 2 patients who had no direct response to CTL infusion, 1 (P845) came off study at 2 weeks because of progressive disease, but subsequently developed a partial response to palliative chemotherapy (gemcitabine and carboplatin), to which the disease had been previously unresponsive. The condition of this patient remained stable for 4 months until the tumor again progressed.

Table 4. Toxicity and clinical response after CTL therapy

Patient no.	Toxicity	Clinical response	Outcome
Treated in remission			
729	None	N/A	Remains in remission > 27 mo
606	None	N/A	Remains in remission > 26 mo
697	None	N/A	Remains in remission > 25 mo
815	None	N/A	Remains in remission > 19 mo
Treated with relapsed or refractory disease			
845	Swelling at tumor site	No response then PR after chemotherapy	PR for 4 months then progressed and died at 12 mo
894	None	CR	Remains in remission > 23 mo after CTLs
389	None	CR	Remains in remission > 11 mo after CTLs
918	None	PR	PR for 12 mo after CTLs then relapsed
1042	None	Stable disease	Stable disease for > 14 mo
1046	None	No response	Died of disease at 3 mo

N/A indicates not applicable; CR, complete remission; and PR, partial response according to the international criteria proposed by the Response Evaluation Criteria in Solid Tumors Committee.[29]

Discussion

Although patients with advanced, relapsed NPC have been exposed to intensive radiation and chemotherapy, EBV-specific CTLs can readily be reactivated from their PBMCs. Adoptive transfer of these CTL lines appears safe in this patient group, although caution may be required in patients with bulky disease. The infused lines contained cytotoxic T cells specific for LMP2 (an EBV antigen usually expressed by NPC tumor cells), and were biologically active in vivo, reducing levels of EBV DNA in peripheral blood mononuclear cells. Although there was no persistent rise in the frequency of circulating T cells specific for LMP2 after infusion, the CTLs appeared to have significant antitumor activity. Of 6 patients with disease that was resistant to, or had relapsed after, intensive chemotherapy and radiation, 2 have had complete and sustained remissions. A third patient had a partial response and a fourth has stable disease. All 4 patients who were in remission at the time of CTL infusion remained disease free after 19 to 27 months.

The EBV-specific CTLs used in this study were reactivated using LCLs that express all EBV latent antigens. LCLs are excellent antigen-presenting cells that are readily available for all patients, as only a limited amount of blood is required to establish an LCL line. As expected using this method, only a minority of the expanded T cells were specific for the subdominant antigen LMP2. However, upon encounter with NPC cells in vivo these LMP2-specific T cells may expand in number. Although such an increase in the frequency of LMP2-specific T cells was not detectable in the peripheral blood in the majority of patients using ELISPOT assays or tetramers, only a small number of T cells were infused ($4\text{-}30 \times 10^7$ CTL/m^2) and less than 10% were LMP2 specific. An expansion of several logs would be required to detect a significant increase in the peripheral blood, and it may be that the infused T cells instead accumulate and expand at local sites of tumor antigen presentation rather than circulate in the periphery. In addition to LMP2-directed immune responses, immunity to other EBV antigens may have contributed to these tumor responses. Recent insights in the processing and presentation of EBNA1 suggest that although a glycine-alanine repeat prevents the processing of the full-length protein, peptides derived from incompletely translated proteins may be available for T-cell recognition.[12-15] Of note, the CTL line from P894, who attained a complete response, contains a relatively large T-cell population specific for an EBNA1-derived, HLA class I–restricted epitope (Table 3). In addition, clinically relevant doses of chemotherapy can induce the expression of EBV lytic cycle antigens in NPC tumors in vivo.[30] Similarly, gamma-irradiation at clinically relevant doses can induce lytic EBV infection in EBV-positive B-cell tumors in vivo.[31] Patient 845, who progressed 2 days after CTL therapy, received chemotherapy

Table 5. Virologic and immunologic responses to CTL infusion

Patient no.	EBV load (cp/μg DNA) in PBMCs			LMP2-specific T cells (SFCs/1 × 10^6 PBMCs)				pp65-specific (SFCs/1 × 10^6 PBMCs)			
	Before	2 wk after	6 wk after	Epitope tested	Before	2 wk after	6 wk after	Epitope tested	Before	2 wk after	6 wk after
729	10	46	0	FLY	8	26	15*	NLV	2623	2521	1896*
606	295	114	324	IED	9	5	50	NLV	995	958	1181
				LIV	4	ND	26				
697	31	193	519	FLY	14	0	9	NLV	144	143	96
815	367	174	147	ND				ND			
845	797	286	103	ND				ND			
894	0	0	0	RRL‡	11	44	3†	ND			
389	347	120	156	CLG	16	10	18	NLV	114	100	95
918	87	27	0	SSC	15	63	20	ND			
1042	664	120	367	TVC	116	171	84	NLV	2510	2960	2596
1046	67	56	54	FTA‡	0	0	0	ND			

cp indicates copies; wk, weeks; ND, not done as not enough PBMCs or no informative peptide was available.
*At 3 months after CTLs, as a sufficient number of PBMCs was not available at 6 weeks after CTLs time point.
†At 8 weeks after CTLs, as a sufficient number of PBMCs was not available at 6 weeks after CTLs time point.
‡Pentadecamers containing minimum epitope were used for stimulation.

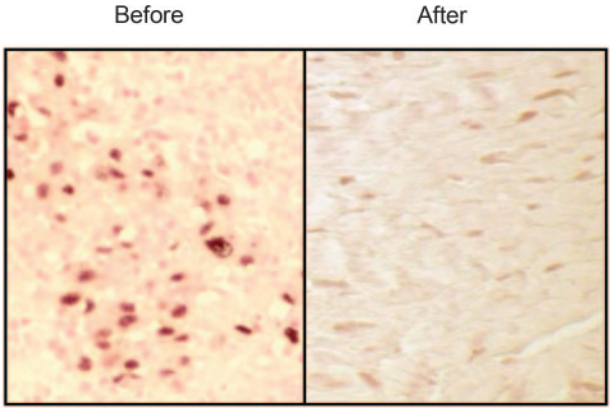

Figure 1. Absence of NPC tumor cells in nasopharynx after treatment. Biopsies taken before (left) and after (right, representative of 7 biopsies) the administration of EBV-specific CTLs as adjuvant treatment in a patient with refractory NPC (P894) were analyzed for the presence of EBV-positive tumor cells by in situ hybridization for EBER using a digoxigenin/alkaline phosphatase-labeled probe (Sigma-Genosys, The Woodlands, Texas). Stained slides were examined with a Zeiss Microscope (Axioscope, Carl Zeiss, Thornwood, NY) at 200 × magnification using a Pan NeoFluar lens (20 ×/0.50). Images were captured using a Spot Insight camera and software (3.4 P.C.) (Diagnostic Instruments, Sterling, MI).

shortly after CTLs. These chemotherapeutic agents had no antitumor effect at an earlier stage, whereas when combined with CTLs a partial tumor response was induced. This might be the result of chemotherapy-induced expression of lytic EBV antigens and thus sensitization of the tumor for lytic antigen-specific T cells present in the CTL lines and would provide a rationale for combination of CTL therapy with chemotherapy and/or radiation to enhance CTL efficacy.

Previous efforts have been made to recruit the immune system to destroy EBV-positive NPC cells in vivo. Adoptive transfer of similar quantities of autologous EBV-specific CTLs as used in this study induced antiviral responses but no clinical responses in 4 NPC patients treated on a pilot study in China.[32] This lack of tumor response may be explained by the fact that these patients all had end-stage disease with a large tumor burden. Adoptive transfer of an allogeneic EBV-specific CTL line in one patient with relapsed NPC resulted in a temporary stabilization of disease.[33] Vaccination with dendritic cells loaded with LMP2 peptides induced or boosted LMP2-specific CD8+ T-cell responses in 75% of the patients with advanced stage NPC.[34] In 2 of these patients in whom the LMP2-directed immune response was sustained for 3 months, a partial tumor response was induced. How may the success rate of immunotherapy for NPC be increased? The CTLs we transfer may undergo only limited in vivo expansion, so that strategies aimed at increasing the number of LMP1- and LMP2-specific T cells in the infusion product may be of value. We are currently using dendritic cells and/or LCLs that overexpress these subdominant antigens to produce order of magnitude increments in the proportion of cells in CTL lines specific for the EBV latency antigens that are expressed by the tumor.[35,36] In addition, antitumor activity after CTL infusion may be augmented by vaccinating patients with an LMP1 polyepitope adenovirus vaccine,[37] LMP2 peptide-loaded dendritic cells,[34] or EBNA1-LMP2–transduced dendritic cells.[38] Finally, depletion of the patients' endogenous T cells may promote the in vivo expansion of the subsequently infused CTLs, a strategy that has been successfully explored by Dudley et al,[39] and which may underlie the greatly increased expansion of infused T cells after hematopoietic stem cell transplantation.[7,40] Given the feasibility and apparent safety of preparing and administering EBV-specific CTLs to patients with advanced NPC, it will be of interest to discover if these and other manipulations further increase the tumor response rate.

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