Adoptive T cell therapy as treatment for Epstein Barr Virus-associated malignancies : strategies to enhance potential and broaden application

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Chapter 3

Treatment of Nasopharyngeal Carcinoma with Epstein-Barr Virus-Specific T-Lymphocytes

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Abstract

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 lines (CTL) can readily be generated from individuals with NPC, notwithstanding the patients' prior exposure to chemotherapy/radiation. Ten patients diagnosed with advanced NPC were treated with autologous CTL. All patients tolerated the CTL, although one developed increased swelling at the site of pre-existing disease. Four patients treated in remission from locally advanced disease remain disease free 19 to 27 months after infusion. Of 6 patients with refractory disease prior to treatment, 2 had complete responses, and remain in remission > 11-23 months after treatment, 1 had a partial remission that persisted for 12 months, 1 has had stable disease for > 14 months and 2 had no response. These results demonstrate that administration of EBV-specific CTL to patients with advanced NPC is feasible, appears to be safe and can be associated with significant anti-tumor activity.

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 greater than 80% can be obtained. However, a significant number of patients relapse, particularly when disease is advanced at diagnosis - the commonest 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 non-keratininzing NPCs regardless of geographical origin[4] and the viral antigens expressed by the tumor provide potential target antigens for immunotherapy. Adoptive transfer of cytotoxic T-cells (CTL) specific for EBV antigens has proven 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 EBNA3 antigens, and are therefore ideal targets for immunotherapy. By contrast, NPC expresses a restricted set of less immunogenic viral antigens, namely EBNA1, LMP1 and LMP2. 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 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 CTL.[18] LMP1 and LMP2 specific T-cells are indeed present in the peripheral blood of NPC patients, albeit at lower frequency than in normal donors,[19,20] and could potentially be activated and expanded for immunotherapeutic strategies.
We hypothesized that ex vivo expansion of EBV-specific CTL 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, material and methods

Study entry criteria and patient details

This protocol was approved by the institutional review board at Baylor College of Medicine and the Food and Drug Administration. Patients were eligible for study if they had stage III or IV nasopharyngeal carcinoma at diagnosis (according to American Joint Committee for Cancer 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 PCR-amplification for Epstein Barr Virus-Encoded RNA (EBER). Patients were treated on 3 escalating dose levels and received either 2 doses of $2x10^7$ CTL/m^2 (dose level 1), or one dose of $2x10^7$ CTL/m^2 and 1 dose of $1x10^8$ CTL/m^2 (dose level 2) or 1 dose of $1x10^8$CTL/m^2 and 1 dose of $2x10^8$/m^2 (dose level 3). CTL were given intravenously with a 2-week interval between each dose. Peripheral blood was obtained pre and at multiple time points post CTL infusion for evaluation of toxicity and EBV-immunity.

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

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 (LCL) and EBV-specific CTL lines.[24] Briefly, for LCL generation, $5x10^6$ peripheral blood mononuclear cells (PBMC) 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, PBMC (2×10^6 per well of a 24 well plate) were stimulated with LCL irradiated at 4000 rads at an effector: stimulator ratio of 40:1. After 9-12 days, viable cells were restimulated with irradiated LCL (at 4:1 E:S ratio). Subsequently, CTL were expanded by weekly stimulations with LCL (at 4:1 E:S ratio) in the presence of recombinant human interleukin-2 (rhIL-2, Proleukin, Chiron Corporation, 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 CTL showed a significantly higher killing of the autologous LCLs (mean 56.6%: range 38-92%) as compared to HLA antigen mismatched LCLs (mean 6.1%, range 0-27%, $p<0.0001$) or to HSB-2 (mean 21.5%, range 6-55% $p<0.005$) at an E/T ratio of 20:1. In two CTL lines, lysis of the HLA-mismatched LCL was observed, which was significantly reduced by depletion of TCRγδ-positive cells. Auto-reactivity 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, HLAB44: VEITPYKPTW, EGGVGWRHW, EENLLDFVRF, KEHVIQNAF, **BZLF1**: HLA-B35:

EPLPQGQLTAY, **BRLF1**: HLA-A2: YVLDHLIVV, HLA-A11: ATIGTAMYK, HLA-A24: DYCNVLNKEF, **BMLF1**: HLA-A2: GLCTLVAML, **BMRF1**: HLA-A2: TLDYKPLSV (listed in Khanna *et al*[25] and Houssaint *et al*[26], and Straathof *et al*, manuscript in preparation). HLA-A2-restricted Cytomegalovirus pp65-derived peptide NLVPMVATV was used as a control. Peptides were either synthesized by Martin Campbell, Synthetic Antigen Laboratory, The University of Texas MD Anderson Cancer Center, Houston, TX, or Genemed Synthesis Inc. (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 (5-10x10^5) were incubated at RT for 30 minutes in PBS/1% FCS containing the PE-labeled tetrameric complex. Samples were costained with anti-CD8 FITC and anti-CD3 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).

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 pre and at multiple time points post CTL infusion was measured using an IFN-γ ELISPOT assay. 96-well filtration plates (MultiScreen, #MAHAS4510, Millipore, Bedford, MA) were coated overnight with 10 μg/mL anti-IFN-γ antibody (Catcher-mAB91-DIK, Mabtech, Cincinnati, OH). PBMC 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 1-2x10^5 cells/well and 2-3 serial dilutions for LCL targets and 3-4x10^5/well for peptide targets. CTL were rested overnight in complete media and plated at 1x10^5 cells/well and 2 serial dilutions. Cells were stimulated with either irradiated (40 Gy) autologous LCL (1x10^5/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 18-24 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), #PK6100, Vector Laboratories, Burlingame, CA) plates were developed with AEC substrate (Sigma, St. Louis, MO). Plates were sent for evaluation to Zellnet Consulting, New York, NY. Spot-forming units (SFC) per 1x10^5 CTL or per 1x10^6 PBMC were calculated by linear regression analysis when serial dilutions were performed and subsequent subtraction of background of non-stimulated T-cells. If an epitope-specific T-cell population had been identified in the infusion product, EBVand LMP2-specific immunity was monitored in patient peripheral blood using this IFN-γ ELISPOT assay and, when enough PBMC were available and HLA type was informative, by tetramer staining.

PCR for EBV-load in PBMC

PBMC were isolated from peripheral blood on a Ficoll (Lymphoprep, Axis-Shield, Oslo, Norway) gradient and washed with PBS. DNA was isolated from 3-5x10^6 PBMC using an anion exchange column (Qiagen, Valencia, CA). Five hundred nanograms of DNA 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

Ten patients were enrolled on the study and all had poorly differentiated or undifferentiated nasopharyngeal carcinoma (WHO II/III) at diagnosis. Four patients at high risk for relapse were in remission at the time of CTL infusion and six patients had failed multiple rounds of radiotherapy and chemotherapy and had relapsed/refractory disease. Patient characteristics and previous treatment are summarized in Table 1.

Table 1. Characteristics of patients on study

Patient no.	Dose	Sex/age	HLA	Ethnicity	Stage	Previous treatment
Treated in remission						
729	2 x 10^7/m^2 x 2	M/50	A2/11 B56/61	Asian	IV	RT, cisplantin, 5-FU
606	2 x 10^7/m^2 x 2	F/29	A2/2 B60/61	White	IV	RT, cisplantin, 5-FU
697	2 x 10^7/m^2 x 2	F/11	A1/2 B37/44	African American	III	RT, cisplantin, MTX, 5-FU
815	1 x 10^8/m^2 x 1, 2 x 10^8/m^2 x 1	M/19	A33/36 B53/72	African American	IV	RT, cisplantin, MTX, 5-FU
Treated with relapsed or refactory disease						
845	2 x 10^7/m^2 x 1	M/11	A3/68 B7/35	White	IV	RT, cisplantin, MTX, 5-FU, paclitaxel, carboplantin, VP16, vinorelbine, gemcitabine
894	2 x 10^7/m^2 x 1, 1 x 10^8/m^2 x 1	M/36	A1/32 B27/35	White	III	RT, cisplantin, 5-FU, carboplantin, ifospamide, paclitaxel, radioactive seed implants, gemcitabine
389	2 x 10^7/m^2 x 1, 1 x 10^8/m^2 x **1***	F/17	A2/3 B44	White	IV	RT, cisplantin, MTX, 5-FU, carboplantin, paclitaxel
918	2 x 10^7/m^2 x 1, 1 x 10^8/m^2 x 1	M/16	A11/68 B49/52	Hispanic	IV	RT, cisplantin, MTX, 5-FU
1042	1 x 10^8/m^2 x 1, 2 x 10^8/m^2 x 1	F/46	A2/24 B51/61	Asian	IV	RT, cisplantin, 5-FU, docetaxel, CPT-11
1046	1 x 10^8/m^2 x 1, 2 x 10^8/m^2 x 1	M/16	A30/68 B18/42	African American	IV	RT, cisplantin, MTX, 5-FU, docetaxel, oxaliplantin, epirubicin, gemcitabine, etoposide

*M = male, F = Female, RT = radiotherapy, MTX = Methotrexate, 5-FU = 5-Fluoruracil. Stage according to American Joint Committee for Cancer Staging and End-Results Reporting staging system 1997.[23] * = this patients received additional doses of 1x10^8 CTL/m^2 at 6 months, 9 months and 12 months after the initial CTL infusions*

CTL lines contain LMP2-specific T-cell populations

Autologous LCL 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 out 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 towards 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.

Table 2. Phenotype of patient CTL lines for infusion

Patient no.	$CD3^{+/}$ $TCR\alpha\beta^{+}$,%	$CD3^{+/}$ $TCR\gamma\delta^{+}$,%	$CD3^{+/}$ $CD4^{+}$,%	$CD3^{+/}$ $CD4^{+}$,%	$CD3^{+/}$ $CD56^{+}$,%	$CD3^{-/}$ $CD56^{+}$,%	$CD3^{-/}$ $CD16^{+}$,%
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	5.5
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

Safety of EBV-specific CTL

Upon administration of EBV-specific CTL no immediate or long-term toxicity was observed in the 4 patients without detectable disease and in 5 out of 6 patients with refractory/relapsed disease (Table 4). However, in one patient (P845) with bulky disease, pre-existing facial swelling increased markedly two days after infusion of the first dose of CTL ($2x10^7/m^2$) 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.

Table 3. T-cell populations specific for EBV antigens (SFC/1x10^5 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		LGP: 0	
		LIV: 0		DEP: 0	
		IED: 830			
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	HVP: 0	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 (see: Table 1). The sequence of the peptides referred to by the first 3 amino acids is listed in the Methods section. ND = not done as no informative peptides available.

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 post CTL infusion to evaluate persistence and activity of the infused CTL. Of 9 patients with a detectable amount of EBV-DNA in PBMC prior to CTL infusion, EBV load fell within 6 weeks post 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 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 SFC/1x10^5 PBMC), as measured by IFN-γ secretion of PBMC upon stimulation with autologous LCL, remained unchanged post CTL infusion (data not shown). In one patient (P845) with a low number of circulating EBV-specific CTL prior to CTL infusion (24 SFC/1x10^5 PBMC) a transient 3-fold increase in the number of EBV-specific CTL was measured. In addition, the LMP2-specific T-cell populations identified in the infusion product were monitored in the peripheral blood post 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 > 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 post CTL therapy indicate anti-tumor activity

Clinical responses were evaluated from CT and MRI scans pre and post 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 remain in complete remission 19-27 months post CTL therapy (Table 4). Of the 6 patients with refractory/relapsed disease, two patients had no response, 1 patient has stable disease for > 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 1x10^8 CTL/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

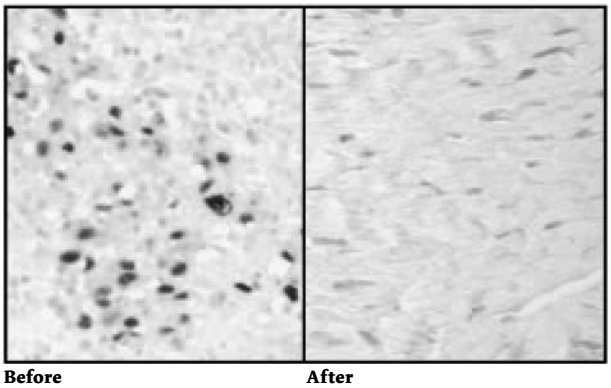

Figure 1. Absence of NPC tumor cells in nasopharynx post treatment
Biopsies taken pre (left) and post (right, representative of 7 biopsies) the administration of EBV-specific CTL as adjuvant treatment in a patient with refractory NPC (P894) were analyzed for the presence of EBV-positive tumor cells by in situ hybridization for EBER 1 (EBV-encoded small nuclear RNA). EBER-positive cells stain red-brown. The absence of EBER-positive cells post treatment demonstrates a complete response.
Full colour image available at: http://www.bloodjournal.org/cgi/content/full/105/5/1898

Table 4. Toxicity and clinical responses 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 refactory 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 afther CTLs
389	None	CR	Remains in remission > 11 mo afther 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 = not applicable, CR = complete remission, PR = partial response according to the international criteria proposed by the Response Evaluation Criteria in Solid Tumors Committee.[29]

Table 5. Virological and immunological response to CTL infusion

	EBV load (cp/µg DNA) in PBMCs			LMP2-specific T cells (SFCs/1x10^6 PBMCs)				pp65-specific (SFCs/1x10^6 PBMCs)			
Patient no.	**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[a]	NLV	2623	2521	1896[a]
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[c]	11	44	3[b]	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[c]	0	0	0	ND			

wk = weeks, cp = copies, [a] = 3 months post CTL, [b] = 8 weeks post CTL, as not sufficient number of PBMC available at 6 weeks post CTL time point, [c] = pentadecamers containing minimum epitope were used for stimulation. ND = not done as not enough PBMC or no informative peptide available.

treatment and showed continuing response. PET 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 post CTL therapy were all negative for tumor indicating a complete remission (Figure 1). Of the two patients who had no direct response to CTL infusion, one (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.

Discussion

Although patients with advanced, relapsed NPC have been exposed to intensive radiation and chemotherapy, EBV-specific CTL can readily be reactivated from their PBMC. 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 , 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 CTL appeared to have significant anti-tumor activity. Two of six patients with disease that was resistant to, or had relapsed after, intensive chemotherapy and radiation, 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 19-27 months.

The EBV-specific CTL used in this study were reactivated using LCL that express all EBV latent antigens. LCL 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 ($4\text{-}30x10^7$ 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 .[30] Similarly, gamma-irradiation at clinically relevant doses can induce lytic EBV infection in EBV-positive B-cell tumors .[31] Patient 845, who progressed 2 days after CTL therapy, received chemotherapy shortly after CTLs. These chemotherapeutic agents had no anti-tumor effect at an earlier stage, whereas when combined with CTL 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 CTL as used in this study induced anti-viral 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 CTL we transfer may undergo only limited *in vivo* expansion, so that strategies aimed at increasing the number of LMP1 and LMP2 specific T-cell in the infusion product may be of value. We are currently using dendritic cells and/or LCL that over express 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, anti-tumor 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 expansion of the subsequently infused CTL, 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 hemopoietic stem cell transplantation.[7,40] Given the feasibility and apparent safety of preparing and administering EBV-specific CTL 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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