Published OnlineFirst January 26, 2012; DOI: 10.1158/0008-5472.CAN-11-3399

Cancer Research

Effective Treatment of Metastatic Forms of Epstein-Barr Virus–Associated Nasopharyngeal Carcinoma with a Novel Adenovirus-Based Adoptive Immunotherapy

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Abstract

Nasopharyngeal carcinoma (NPC) is endemic in China and Southeast Asia where it is tightly associated with infections by Epstein-Barr virus (EBV). The role of tumor-associated viral antigens in NPC renders it an appealing candidate for cellular immunotherapy. In earlier preclinical studies, a novel adenoviral vector–based vaccine termed AdE1-LMPpoly has been generated that encodes EBV nuclear antigen-1 (EBNA1) fused to multiple $CD8^+$ T-cell epitopes from the EBV latent membrane proteins, LMP1 and LMP2. Here, we report the findings of a formal clinical assessment of AdE1-LMPpoly as an immunotherapeutic tool for EBV-associated recurrent and metastatic NPC. From a total of 24 patients with NPC, EBV-specific T cells were successfully expanded from 16 patients with NPC (72.7%), whereas six patients with NPC (27.3%) showed minimal or no expansion of virus-specific T cells. Transient increase in the frequencies of LMP1&2- and EBNA1-specific T-cell responses was observed after adoptive transfer to be associated with grade I flu-like symptoms and malaise. The time to progression in these patients ranged from 38 to 420 days with a mean time to progression of 136 days. Compared with patients who did not receive T cells, the median overall survival increased from 220 to 523 days. Taken together, our findings show that adoptive immunotherapy with AdE1-LMPpoly vaccine is safe and well tolerated and may offer clinical benefit to patients with NPC. *Cancer Res; 72(5); 1116–25. ©2012 AACR.*

Introduction

Nasopharyngeal carcinoma (NPC) is endemic in southern China, including Hong Kong (1). After primary treatment with radiotherapy or chemoradiotherapy, more than one third of patients will relapse with locoregional recurrence and distant metastases (2). Most patients who recur or develop distant metastases are only amenable to palliative chemotherapy or radiotherapy and overall survival after recurrence is poor with reported median survival ranging from 7.2 to 22 months (3–5). Epstein-Barr virus (EBV) is present in virtually all poorly differentiated and undifferentiated nonkeratinizing NPC (WHO type II and III). Consequently, the viral antigens expressed by the tumor cells are attractive targets for immunotherapy and may be a potential avenue for the development of new therapies for the treatment of NPC (6, 7).

The broad application of adoptive immunotherapeutic approaches using CTLs to treat EBV-associated posttransplant lymphoproliferative disorders (PTLD; refs. 8–10) and the known safety profile suggest that CTL-based therapeutic approaches can be extended to other EBV-associated malignancies, including NPC. Several phase I and II studies have been completed using approaches based on those used to generate CTLs for PTLD and have shown some potential efficacy against NPC (11–15). However, unlike PTLD, NPC cells express a limited array of EBV antigens, including latent membrane proteins (LMP)1 and 2, and EBV nuclear antigen 1 (EBNA1; refs. 16, 17). Poor immunogenicity of these antigens and the subsequent induction of subdominant T-cell responses are likely to play a key role in promoting immunoevasion by EBV-positive malignant cells (18–21).

Current approaches using lymphoblastoid cell lines (LCL) predominantly generate CTLs which target the immunodominant EBNA3–6 antigens. In the context of NPC, immunotherapeutic approaches which target only LMP1&2 and

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

C. Smith and J. Tsang have contributed to this study equally and their order should be considered arbitrary.

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doi: 10.1158/0008-5472.CAN-11-3399

Published OnlineFirst January 26, 2012; DOI: 10.1158/0008-5472.CAN-11-3399

EBNA1 should not only improve the specificity of CTL lines but also avoid the requirement to generate LCLs. We have previously reported on the use of an adenovirus-based vector, referred to as AdE1-LMPpoly, which encodes multiple CTL epitopes from LMP1&2 fused to a truncated EBNA1 without an internal glycine–alanine repeat sequence. We have shown in preclinical studies that AdE1-LMPpoly can be used to rapidly expand LMP1&2- and EBNA1-specific T cells from patients with cancer (22, 23). Here, we report the outcome of a phase I clinical study, investigating for the first time, the use of a polyepitope-based vector as a therapeutic tool for recurrent or metastatic NPC. Data presented here show that infusion of AdE1-LMPpoly–generated T cells was well tolerated and was associated with stabilization of disease and possible delayed tumor progression with prolonged survival.

Materials and Methods

Patients

This study was approved by the Queensland Institute of Medical Research (Brisbane, Qld, Australia) and University of Hong Kong (Hong Kong) Ethics Committees and is registered under the Australia New Zealand Clinical Trial Registry (ACTRN12609000675224). During the period from January 2008 to April 2010, patients with NPC who had locoregional recurrence or distant metastatic disease, and progressed after standard palliative radiotherapy, chemotherapy, and/or surgery in the Department of Clinical Oncology, Queen Mary Hospital, The University of Hong Kong, were eligible for this study. Informed consent was obtained from all patients. A total of 24 patients (22 males, 2 females), mean age being 48.3 years, with recurrent or metastatic EBV (EBER)-positive NPC were recruited for this study. Two of these patients were excluded from the study, including one self-withdrawn patient shortly after informed consent was obtained and another due to the detection of active hepatitis B infection. Twenty-two patients were included in this analysis. The detailed clinical characteristics and treatment history of patients prior to T-cell expansion in this study are shown in Table 1. Seventeen patients had distant metastases and 5 patients had only locoregional recurrence at the time of accrual. All patients received concurrent chemoirradiation for locoregional control for their primary nasopharyngeal tumor site, and following this, they received a range of further lines of palliative chemotherapy before the T-cell expansion (Table 1).

AdE1-LMPpoly vector

The AdE1-LMPpoly vector has been described previously (22, 23) and encodes a polyepitope of defined $CD8^+$ T-cell epitopes from LMP1&2 (see Supplementary Table) fused to a Gly-Ala repeat–deleted EBNA1 sequence. Clinical grade material was generated at the Cell and Gene Therapy Centre Vector Development Laboratory, Baylor College of Medicine, Houston, TX. The use of this vector for this study was approved by the Gene and Related Therapies Research Advisory Panel (GTRAP), Australia.

Generation and characterization of LMP/EBNA1-specific T cells

LMP/EBNA1-specific T cells were generated using the AdE1-LMPpoly adenoviral vector in accordance with Good Manufacturing Practice in the Q-Gen facility at the Queensland Institute of Medical Research. Peripheral blood mononuclear cells (PBMC) were isolated from 100 to 200 mL of peripheral blood using Ficoll-Paque gradient. After extensive wash with PBS, PBMCs were resuspended in RPMI-1640 supplemented with 10% FBS (growth medium) and then cocultured in T75 tissue culture flasks with irradiated autologous PBMCs infected with AdE1-LMPpoly (multiplicity of infection of 10:1) at a responder to stimulator ratio of 7:3. On day 3, and every 3 to 4 days thereafter, the cultures were supplemented with growth medium containing recombinant interleukin-2 (IL-2; Komtur Pharmaceuticals). On day 14, cells were harvested and cryopreserved. Before cryopreservation, cells were tested for sterility and for LMP- and EBNA1-specific T cells using an IFN-γ intracellular cytokine assay.

Intracellular cytokine assay

PBMCs or cultured T cells were stimulated with a pool of defined $CD8^+$ T-cell epitopes from LMP1&2 or EBNA1 (Supplementary Table) or with an overlapping set of peptides encompassing the whole EBNA1 protein (peptides were supplied by Mimotopes and GenScript). Cells were incubated in the presence of GolgiPlug (BD Biosciences) for 4 hours and then washed and incubated with perCP or perCPCy5.5-conjugated anti-CD8, fluorescein isothiocyanate (FITC)-conjugated anti-CD4, and allophycocyanin (APC)-conjugated anti-CD3. Cells were washed, then fixed and permeabilized with cytofix/cytoperm, washed and incubated with phycoerythrin (PE)-conjugated anti-IFN-γ (BD Pharmingen). Cells were then washed, resuspended in PBS, and acquired using a FACSCanto II with FACSDiva software (BD Biosciences). Postacquisition analysis was conducted using FlowJo software (TreeStar). T-cell responses were deemed significant if more than 0.1% of the total lymphocyte population showed reactivity (IFN-γ production) to the LMP or EBNA1 epitope pool.

Adoptive cell transfer

Table 2 shows the T-cell expansion history of all the patients. Patients who had successful T-cell expansion received 3 to 8 infusions of 2×10^7 to 3×10^7 (median, 2.3×10^7) AdE1-LMPpoly–expanded T cells per infusion. Infusions were administered at fortnightly intervals. Peripheral blood samples were harvested prior to the first infusion and for up to 6 months postinfusion to assess the impact of T-cell infusion on the frequency of LMP/EBNA1-specific T cells and to monitor plasma EBV load. Patients were monitored for tolerability and safety following infusion. MRI or computed tomographic scanning was conducted on the subjects to assess baseline tumor load prior to infusion and serially at 1, 2, 3, 4, and 6 months after first infusion. Best treatment responses were determined on the basis of WHO criteria and/or Response Evaluation Criteria in Solid Tumors (RECIST).

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Table 1. Characteristics of patients with NPC and detailed treatment history prior to T-cell expansion

Subject code	Age at infusion, y	Site of recurrence or metastasis	Pre-CTL courses of RT to NP	RT other than RT to NP	No. of lines of pre-CTL chemo for recurrence/ metastases	Pre-CTL surgery detail
01	42	Liver, lung	1	Lung met	6	N/A
02	44	Locoregional	3	N/A	3	N/A
03	42	Locoregional, liver, lung	2	N/A	2	N/A
04	42	Neck nodes, lung	1	Neck nodes	2	N/A
05	50	Bone, liver	1	Bone, liver	2	N/A
06	48	Neck nodes, bone, liver, lung	1	Neck nodes	1	Neck dissection
07	55	Locoregional	2	N/A	5	N/A
08	60	Bone, lung	1	Bone	3	N/A
09	50	Neck nodes, distant nodes	1	N/A	3	Craniofacial Excision Neck dissection
10	34	Neck nodes, bone	1	Bone	2	N/A
11	50	Locoregional, distant nodes	2	Neck nodes	3	N/A
12	50	Neck nodes, lung	1	Neck nodes	3	N/A
13	46	Locoregional, lung	1	N/A	1	N/A
14	53	Bone, liver, lung, distant nodes	1	Bone (T12-L3, then L4-S1)	4	N/A
16	52	Locoregional, bone, distant nodes	1	Neck nodes	2	N/A
17	50	Locoregional	2	N/A	4	N/A
19	50	Locoregional, brain	2	Brain	8	Craniotomy
20	42	Bone, liver, distant nodes	1	Distant lymph nodes × 2, Liver	3	Radiofrequency ablation to liver metastases
21	58	Locoregional, bone, liver, lung	1	Bone (T9-L1, then T4-T7)	1	N/A
22	51	Bone, liver, distant nodes	1	Neck nodes	2	N/A
23	44	Locoregional	2	N/A	1	nasopharyngectomy
24	37	Locoregional	2	N/A	2	nasopharyngectomy

NOTE: The shaded boxes refer to those patients who did not receive any CTL infusion: 6 failures of T-cell expansion with 2 patients died before the arrival of T cells.

Abbreviations: N/A, not available; NP, nasopharyngeal.

IFN-γ ELISPOT assay

The ELISPOT assay was conducted using the Mabtech Human IFN-γ ELISpotPRO Kit (Mabtech AB). Precoated 96-well plates were washed with sterile PBS and blocked for 1 hour with culture medium containing 10% fetal calf serum. Blocking medium was removed and PBMCs (2×10^5 cells per well) were incubated for 24 hours at 37°C with a pool of LMP1&2- or EBNA1-encoded $CD8^+$ T-cell peptide epitopes (Supplementary Table). The plates were washed to remove cells and incubated with the one-step detection reagent for 2 hours. ELISPOTS were developed using BCIP/NBT-plus and counted automatically using AID image analysis software. ELISPOT results are expressed as spot-forming cells per 10^6 $CD3^+CD8^+$ cells calculated by the percentage of $CD3^+CD8^+$ in the PBMC sample as determined by flow cytometry and the number of peptide-specific spots per well.

Quantitative real-time PCR determination of EBV DNA load

Quantitative real-time PCR was carried out using StepOne Real-Time PCR System (Applied Biosystems). Each reaction contained 25 μL of reaction mixture, including 10 μL purified DNA template, 12.5 μL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μL of forward primer, 1 μL of reverse primer, 0.25 μL of TaqMan probes, and 0.25 μL of nuclease-free water. The primers for this assay were designed

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Table 2. Patients with NPC and their T-cell treatment history

Subject code	Time for T-cell infusion from the time of blood taking, d	Total cells expanded	Cells per infusion	Number of infusions	Best response to T-cell therapy	Time to the diagnosis of progressive disease after first CTL infusion, d	Post-CTL/further chemo no.
01	N/A	Insufficient cells	N/A	0	N/A	N/A	0
02	81	1.23×10^8	2.44×10^7	5	SD	420	0
03	51	6.12×10^7	2.04×10^7	3	SD	182	0
04	N/A	Insufficient cells	N/A	0	N/A	N/A	2
05	77	9.18×10^7	1.84×10^7	4	SD	63	2
06	N/A	Insufficient cells	N/A	0	N/A	N/A	0
07	N/A	Insufficient cells	N/A	0	N/A	N/A	0
08	87	1.14×10^8	2.28×10^7	5	SD	123	1
09	64	7.22×10^7	2.41×10^7	3	PD	38	0
10	70	1.08×10^8	2.16×10^7	5	SD	230	0
11	51	1.09×10^8	2.18×10^7	4	SD	68	4
12	N/A	Insufficient cells	N/A	0	N/A	N/A	N/A
13	85	1.15×10^8	2.3×10^7	4	SD	310	2
14	74	8.8×10^7	2.2×10^7	3	PD	53	0
16	42	9.22×10^7	2.31×10^7	4	SD	63	1
17	44	6.09×10^7	2.03×10^7	3	SD	65	0
19	47	1.15×10^8	Died before therapy	0	N/A	N/A	0
20	107	1.55×10^8	2.59×10^7	6	PD	64	1
21	N/A	Insufficient cells	N/A	0	N/A	N/A	1
22	79	9.36×10^7	Died before therapy	0	N/A	N/A	0
23	70	2.53×10^8	3.17×10^7	8	SD	174	1
24	51	2.23×10^8	2.79×10^7	3	PD	49	1

NOTE: The shaded boxes refer to those patients who did not receive any CTL infusion.
Abbreviations: N/A, not available; PD, progressive disease; SD, stable disease.

to target the *Bam*HI-W region. The forward primer (5′-GGTCGCCCAGTCCTACCA-3′, EBV coordinate: 14732–14749), the reverse primer (5′-GCTTACCACCTCCTCTTCTTGCT-3′, EBV coordinate: 14817–14795), and fluorogenic probe (5′-6-FAM-CCAAGAACCCAGACGAGTCCGTAGAAGG-TAMRA-3′, EBV coordinate: 14757–14784) were custom-synthesized by Applied Biosystems. These primers were validated using OptiQuant EBV DNA Quantification Panel. They were also optimized to detect at least one copy of EBV in 100 μL of the extracted DNA template. Each 48-well plate included patient samples run in parallel with positive and negative controls. The positive control plasmid standards were prepared using pCRII-TOPO vector (Invitrogen) which contains the target viral sequences. Cycling conditions consisted of a 10-minute hold at 95°C; 50 cycles of 95°C for 15 seconds; and 60°C for 1 minute. The positive controls were prepared as a 10-fold dilution series (1 to 1×10^8 copies/μL) to create a standard curve to quantify the viral loads. All specimens, including the controls, were analyzed in duplicate and the mean of the 2 runs are reported. The EBV DNA load was expressed as number of copies per mL of plasma.

Statistical analysis

The survival analysis was conducted using the Kaplan–Meier method. The progression-free survival was calculated from the time of first T-cell infusion, whereas overall survival was calculated from the time the first blood sample was drawn for T-cell expansion.

Results

In vitro expansion and characterization of AdE1-LMPpoly–expanded T cells

LMP1/EBNA1-specific T cell lines were successfully generated from 16 of 22 patients with NPC recruited for the study. The average time for T-cell infusion from the time of blood taking from the patients was 67.5 day. Among the patients for whom T-cell therapy was not generated, failed expansion of T cells correlated with a significantly lower number of white blood cells, whereas no significant difference was observed for age, lymphocyte count, or previous treatment with chemo/radiotherapy (Table 3). The T cell lines generated for therapy contained a high frequency of $CD3^+$ lymphocytes, ranging from

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Table 3. Impact of clinical profile on T-cell expansions from patients with NPC

	T-cell expansion success, $n = 16$ (72.7%)	T-cell expansion failure, $n = 6$ (27.3%)	*P*
Age at consent, y	48.60	47.60	0.79
Lymphocyte count	0.67	0.93	0.07
WBC	10.23	5.20	0.01[a]
Pre–T-cell chemo	3.75	2.67	0.59
Pre–T-cell RT	1.50	1.75	0.56
Gender (M/F)	14/2[a]	6/0	NA
EBV DNA load	2.05×10^5 copies/mL	4.83×10^5 copies/mL	0.60

Abbreviations: NA, not available; WBC, white blood cell.

[a]These include the 2 patients who have had successful T-cell expansion but died before the arrival of T-cell infusion.

49% to 100% (median, 84.1%). These $CD3^+$ populations contained a heterogeneous number of $CD8^+$ T cells, ranging from 17% to 74% (median, 38.9%). Analysis of the T-cell specificity revealed a substantial increase in the proportion of both LMP1&2- and EBNA1-specific T cells following AdE1-LMPpoly stimulation (Fig. 1A). While the majority of donors showed no detectable *ex vivo* reactivity against either LMP1&2- or EBNA1-encoded $CD8^+$ T-cell epitopes, 11 of 16 T cell lines tested contained $CD8^+$ T cells reactive to both LMP- and EBNA1-encoded $CD8^+$ T-cell epitopes (Fig. 1B). The remaining T cell

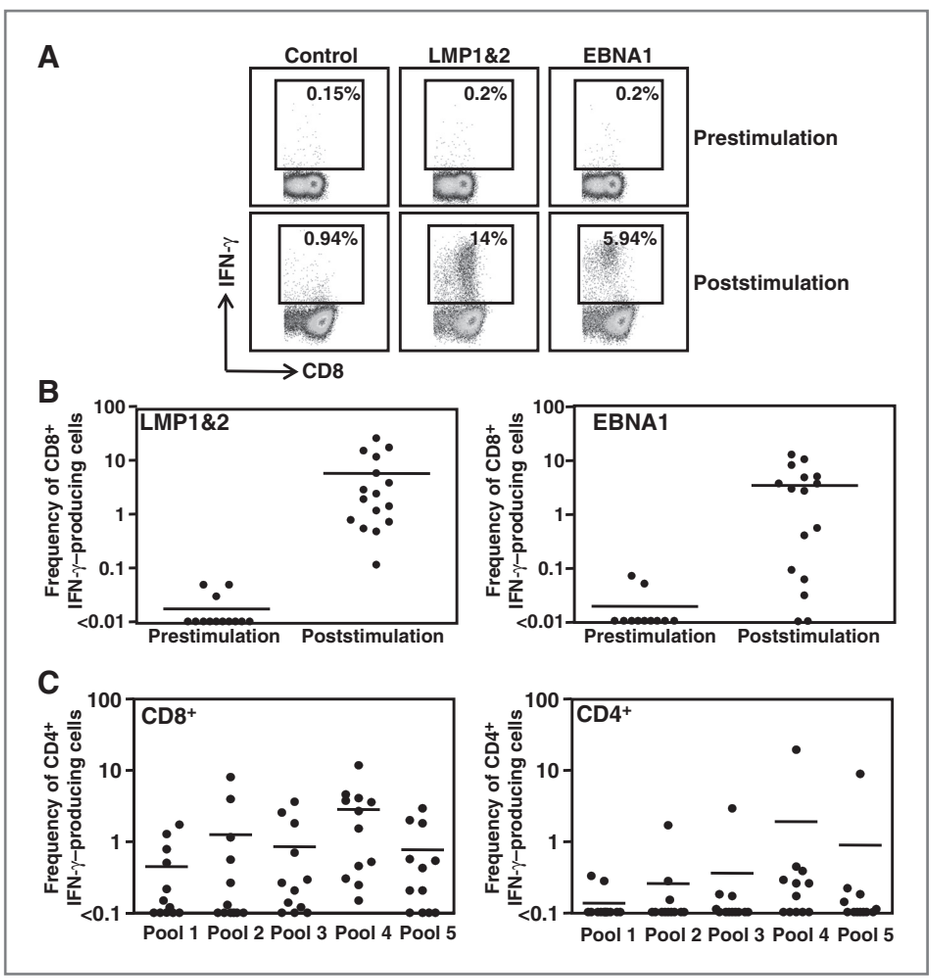

Figure 1. Expansion of LMP1&2- and EBNA1-specific T cells from patients with NPC following AdE1-LMPpoly stimulation. PBMCs or cultured T cells were incubated with the relevant peptide pools in the presence of GolgiPlug for 4 hours. Cells were then incubated with antibodies specific for CD3, CD4, and CD8 and then assessed for intracellular IFN-γ production. A, representative data from a single donor following stimulation with and without a pool of LMP1&2- or EBNA1-encoded $CD8^+$ T-cell epitopes are shown. B, data represent the percentage of $CD3^+CD8^+$ lymphocytes from individual donors expressing IFN-γ following stimulation with either a pool of LMP1&2- or EBNA1-encoded $CD8^+$ T-cell epitopes. C, data represent the percentage of $CD3^+CD8^+$ or $CD3^+CD4^+$ lymphocytes expressing IFN-γ following stimulation with pools of overlapping peptides encoded by EBNA1.

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Table 4. Toxicity grading of T-cell therapy in patients with NPC ($n = 14$)

Symptoms	G0	G1	G2	G3	G4
Flu-like symptoms	13/14	1/14	—	—	—
Malaise	12/14	3/14	—	—	—
Dry cough	13/14	2/14	—	—	—
Low blood pressure	13/14	1/14	—	—	—

lines showed reactivity against LMP1&2-encoded $CD8^+$ T-cell epitopes alone. Analysis of the reactivity of both $CD4^+$ and $CD8^+$ T cells to overlapping peptides from the whole of the EBNA1 antigen revealed a bias toward the generation of EBNA1-specific $CD8^+$ T cells that recognized peptides across the whole antigen (Fig. 1C).

Safety analysis of AdE1-LMPpoly T-cell–based immunotherapy

Of the 16 patients for whom LMP/EBNA1-specific T cells were generated, 14 patients received 3 to 8 T-cell infusions (18.4×10^6 to 31.7×10^6 cells per infusion). The remaining 2 patients died prior to the availability of T cells for infusion. These 2 patients were included in the analysis for T-cell expansion but excluded from toxicity and survival analysis. Infusion of EBV-specific T cells was safe and only grade I and/or II toxicities including flu-like symptoms, malaise, dry cough, and low blood pressure were observed (Table 4). These toxicity grading were assigned according to the National Cancer Institute Common Toxicity Criteria Scale 2.0. A single case of a serious adverse event (SAE) was recorded for one patient (patient code 17). One month after the completion of EBV-specific T-cell therapy, this patient with progressive disease involving the nasopharynx was admitted to hospital because of severe epistaxis from both nostrils and treated with transamine and packing by ENT surgeons but later died after discharge. Computed tomographic imaging of the patient after admission was reviewed and both the clinical and radiological features pointed to progressive disease. The investigators discussed this patient with both the radiologists and the ENT surgeons at that time and deemed that the SAE was not associated with T-cell therapy but was probably due to progressive disease.

Clinical evaluation following infusion of AdE1-LMPpoly–expanded T cells

Clinical response was assessed by comparing imaging studies before and after T-cell therapy. A summary of this analysis is presented in Table 2. Of the 14 patients treated with T-cell therapy, 10 patients showed stable disease following T-cell therapy. The time to the diagnosis of progressive disease following T-cell therapy ranged from 38 to 420 days with a mean time to progression of 136 days (Fig. 2A). NPC patient 2 showed the longest stabilization of disease with well more than 400 days of progression-free survival. Patient 2 had no other treatment subsequent to CTL infusion. The median overall survival of patients with and without CTL treatment were 523 and 220 days, respectively ($n = 14$ for CTL-infused patients vs. $n = 6$ for CTL-naive patients). There was no correlation between the number of LMP/EBNA1-specific T cells transferred and the time to progression (Fig. 2B). These analyses show that adoptive transfer of AdE1-LMPpoly–stimulated T cells is safe and may stabilize disease in patients with recurrent or metastatic NPC. Among the 14 patients who had received T-cell infusion therapy, one patient (code 11) had an unexpected good response to chemotherapy after T-cell infusion. He had 3 lines of palliative chemotherapy before T-cell infusion and the disease was chemoresistant. When disease progressed after T-cell infusion, he was started on palliative chemotherapy with paclitaxel. There was rapid regression of tumor after one cycle of paclitaxel (Fig. 2C). However, the response was transient and he subsequently succumbed to disease.

Immunologic and virological responses following infusion of AdE1-LMPpoly–expanded T cells

In the final set of experiments, we conducted comprehensive longitudinal *ex vivo* profiling of LMP/EBNA1-specific T cells and EBV load in the peripheral blood of patients with NPC who had been treated with autologous AdE1-LMPpoly–stimulated T cells. To assess the EBV-specific T-cell responses, we conducted IFN-γ ELISPOT assays at different time points and compared these responses with those seen prior to the first infusion. Representative data from 6 patients with NPC are presented in Fig. 3A–F. These analyses revealed that in many patients there was an increase in the precursor frequency of the LMP1&2- and/or EBNA1-specific T cells either during or after the completion of adoptive immunotherapy. However, in almost all patients, this increase was only transient and the number of antigen-specific T cells returned to baseline after the completion of adoptive immunotherapy. Both LMP1&2- and EBNA1-specific T cells displayed contemporaneous fluctuations during the follow-up period. To evaluate the impact of T-cell infusion on plasma EBV load, which has previously been correlated to disease status in patients with NPC, quantitative real-time PCR on plasma samples was carried out prior to the first T-cell infusion and at different time point after infusion. These analyses showed minimal changes in EBV load during T-cell infusion, although there was a trend toward lower DNA load at the late time point (Fig. 3G).

Discussion

Anti-viral T cells have emerged as powerful therapeutic tools for the treatment of EBV-associated malignancies. Early studies based on adoptive transfer of virus-specific T cells

Published OnlineFirst January 26, 2012; DOI: 10.1158/0008-5472.CAN-11-3399

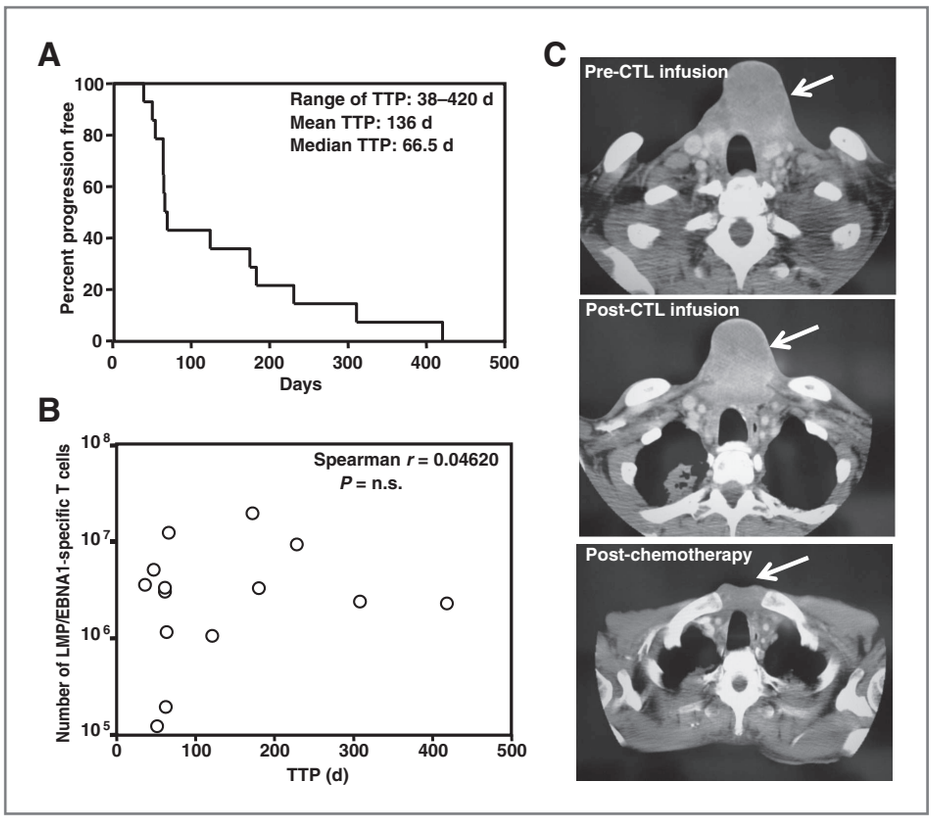

Figure 2. A, time to progression following AdE1-LMPpoly T-cell infusion. Progression-free survival was determined following the first T-cell infusion. B, correlation of the LMP/EBNA1-specific T-cell dose and time to progression following infusion. Data represent the correlation between the number of adoptively transferred LMP/EBNA1-specific T cells received by each patient, as determined using the intracellular IFN-γ analysis, and the time to progression (TTP) following the first T-cell infusion. C, clinical response after AdE1-LMPpoly infusion and postinfusion chemotherapy in one of the T-cell–infused patients with an initial bulky suprasternal mass. n.s., nonsignificant.

stimulated with EBV-transformed B cells reported successful treatment of posttransplant lymphomas in stem cell transplant and solid organ transplant patients (8, 9). Extension of this adoptive immunotherapy to other EBV-associated malignancies such as NPC and Hodgkin lymphoma has provided mixed success (9, 11–13). One limitation to this approach is that T cells used in these studies were expanded using EBV-transformed LCLs which preferentially expand T cells specific for the immunodominant EBNA3 proteins which are not expressed in NPC and Hodgkin lymphoma cells. To overcome this limitation, we designed a novel adenoviral expression system, AdE1-LMPpoly, which encodes multiple $CD8^+$ T-cell

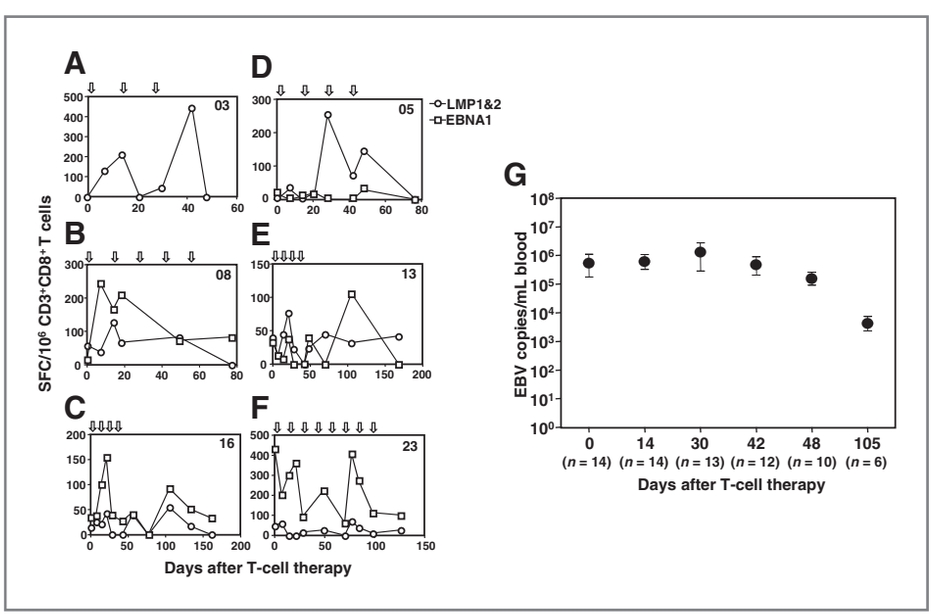

Figure 3. A–F, LMP1&2- and EBNA1-specific T-cell responses in the peripheral blood following AdE1-LMPpoly T-cell infusion. PBMC and plasma samples from patients prior to the first infusion and at the indicated time points postinfusion were assessed for LMP-specific T-cell responses by ELISPOT. Data represent the number of spot-forming cells (SFC) per 10^6 $CD3^+CD8^+$ cells. G, impact of AdE1-LMPpoly T-cell infusion on EBV load. Plasma samples taken prior to the first infusion and at the indicated intervals after T-cell infusion were assessed for EBV load by quantitative PCR. Data represent the EBV copy number per mL of blood.

Published OnlineFirst January 26, 2012; DOI: 10.1158/0008-5472.CAN-11-3399

epitopes from LMP1&2 and the EBNA1 protein. Our preclinical studies have shown that this adenoviral vector is highly efficient in activating T cells specific for LMP1&2 and EBNA1 proteins both *in vitro* and *in vivo* (22, 23). Data presented in this study provide the first clinical evaluation of AdE1-LMPpoly in recurrent or metastatic patients with NPC. A number of important conclusions can be drawn from this study.

First, we found that the AdE1-LMPpoly vector is highly efficient in expanding antigen-specific T cells from patients with advanced recurrent or metastatic NPC disease and these expanded T cells display high levels of functional capacity as assessed by IFN-γ expression. One of the major advantages of the AdE1-LMPpoly expression vector is that a large number of antigen-specific T cells can be expanded within 2 weeks, which is much quicker than the previously published protocol where EBV-transformed LCLs were used to expand T cells. More importantly, the AdE1-LMPpoly expression system selectively expands T cells specific for LMP1&2 and EBNA1 with minimal expansion of T cells directed toward viral proteins (e.g., EBNA3–6) which are not expressed in NPC tumor cells. Of the 22 patients with NPC, we were able to expand virus-specific T cells from 16 patients (72.7%), whereas there was minimal or no expansion of LMP1&2- and/or EBNA1-specific T cells from 6 patients (27.3%). These *in vitro* expanded polyclonal T cells included both $CD8^+$ and $CD4^+$ T cells directed toward multiple epitopes from LMP1&2 and EBNA1. The failure of T-cell expansions from 6 patients with NPC was not due to the lack of appropriate human leukocyte antigen (HLA)-matched T-cell epitopes within the AdE1-LMPpoly vector. These patients showed a significantly lower white blood cell count which may reflect the reduced number of professional antigen-presenting cells in PBMCs and thus limit the ability of AdE1-LMPpoly to stimulate antigen-specific T cells. Another potential indicator, although not statistically significant, was the high viral load in patients who failed to expand a sufficient number of antigen-specific T cells. Previous studies based on murine models have shown that high viral load can induce T-cell exhaustion and/or anergy. Indeed previous studies by Li and colleagues have shown that functional inactivation of EBV-specific T cells is frequently seen in patients with NPC (24).

Second, adoptive transfer of AdE1-LMPpoly–expanded T cells was completely safe with minimal toxicities. One of the patients with NPC reported an SAE after the completion of adoptive immunotherapy; however, a careful clinical assessment revealed that this event was not associated with the T-cell therapy but was due to progressive disease. Although concomitant chemoradiotherapy is highly effective against recurrent, locoregional NPC and provides greater long-term survival, these therapies induce considerable acute mucosal and hematologic toxicity and thus profoundly reduce quality of life (4). Data presented in this study and previously published work clearly show that adoptive T-cell therapy displays limited toxicities and future studies should aim to develop more efficient strategies to provide "of-the-shelf" access for such therapies for patients with NPC. Indeed recent studies by Haque and colleagues have shown that HLA-matched allogeneic EBV-specific T cells can be used for the treatment of EBV-associated lymphomas (10, 25). It will be important to explore the safety and therapeutic profile of allogeneic EBV-specific T cells for patients with NPC as well.

Third, as part of the secondary objectives, clinical follow-up analyses of the patients showed that adoptive transfer of LMP/EBNA1-specific T cells was coincident with disease stabilization with prolonged progression-free survival in some patients (median, 66.5 days). While we were unable to see partial or complete regression of tumor in any of our patient cohort, such responses have been primarily observed in locoregional disease but rarely in recurrent or metastatic disease (13). The median overall survival in patients with NPC who received T-cell therapy was 523 days whereas that of the patients with NPC who have not received the T-cell therapy in our study was 220 days. Earlier studies have reported that median survival after relapse for metastatic NPC range from 7.2 to 22 months (3–5). When we further reviewed our own institutional data with regard to those recurrent or metastatic patients with NPC who were treated during the time of the study from January 2008 to April 2010 but who were not recruited to this study, the medial overall survival for this corresponding cohort was 10.3 months. Thus, the median overall survival of patients receiving T-cell therapy was longer than our institutional average during the same period and is at the higher end of reported survival in literature. However, these preliminary observations will require confirmation in a formal phase II randomized clinical trial.

One of the unexpected outcomes of this analysis was that there was no correlation between the number of LMP/EBNA1-specfic T cells adoptively transferred and duration of progression-free survival after T-cell infusion. This was also reflected in our follow-up analysis of LMP- and EBNA1-specific T cells following adoptive immunotherapy. Although some donors showed an increase in virus-specific T cells in the peripheral blood following the first few infusions, this effect was transient and the number of antigen-specific T cells returned to baseline after the completion of adoptive immunotherapy. Moreover, we did not observe any correlation between the number of antigen-specific T cells in peripheral blood and clinical outcome (i.e., progression-free survival or overall survival). Although the precise reason for this lack of correlation is not known, it is probable that the tumor microenvironment and disease burden influence the clinical response to adoptive immunotherapy (26, 27). It will be important to explore complementary strategies to enhance the therapeutic outcome of adoptive immunotherapy. A combination of adoptive immunotherapy and chemo/radiotherapy has emerged as a potential alternative to standard treatment options especially for bulky, recurrent or metastatic disease (28). Indeed, preliminary data from a single patient (patient code 11) showed that adoptive immunotherapy followed by a single cycle of paclitaxel was effective in tumor regression. This patient already had 3 lines of palliative chemotherapy before T-cell therapy including platinum-based chemotherapy which is considered as the most effective agent for NPC. The patient was

Published OnlineFirst January 26, 2012; DOI: 10.1158/0008-5472.CAN-11-3399

nonresponsive and the disease was considered chemoresistant. Previous studies by Au and colleagues reported that paclitaxel treatment of chemonaive metastatic NPC generated partial response in 5 of 24 (21.7%) patients with no complete response (29). Thus, in patient 11 with chemoresistant disease, the dramatic shrinkage of tumor with single-agent paclitaxel was unexpected. We are uncertain whether the prior immunotherapy enhances the response to chemotherapy and this strategy needs to be formally tested.

Taken together, adoptive immunotherapy with AdE1-LMPpoly–stimulated T cells is safe and may provide long-term clinical benefits. These studies provide an important platform for a formal assessment of AdE1-LMPpoly–based immunotherapy in both therapeutic and prophylactic settings. If successful, we would like to propose that the AdE1-LMPpoly vector should be considered for potential use as a prophylactic vaccine for the prevention of recurrent or metastatic disease in high-risk individuals.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

Acknowledgments

The authors thank Natasha Stevens for her role as clinical trial coordinator and Drs. Sandro Porceddu and Ben Panizza for their intellectual input on the analysis of the data and also for reviewing the manuscript. This study is part of the Center for NPC Research (CNPCR) under the aegis of the Area of Excellence scheme (Hong Kong).

Grant Support

This study was supported by funding from Ester Lee and Chew Pik Foundation, Croucher Foundation, and many other generous donors. R. Khanna is supported by a National Health and Medical Research Council (Australia) Senior Principal Research Fellowship.

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Received October 14, 2011; revised November 28, 2011; accepted December 14, 2011; published OnlineFirst January 26, 2012.

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Published OnlineFirst January 26, 2012; DOI: 10.1158/0008-5472.CAN-11-3399

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Published OnlineFirst January 26, 2012; DOI: 10.1158/0008-5472.CAN-11-3399

Cancer Research
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Cancer Res 2012;72:1116-1125. Published OnlineFirst January 26, 2012.