Complete Responses of Relapsed Lymphoma Following Genetic Modification of Tumor-Antigen Presenting Cells and T-lymphocyte transfer.

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Short Title for running head: Cytotoxic T cells for EBV-positive Lymphoma
ABSTRACT

Epstein Barr Virus (EBV) associated tumors developing in immunocompetent individuals present a challenge to immunotherapy, since they lack expression of immunodominant viral antigens. However, the tumors consistently express viral proteins including LMP2, which are immunologically “weak” but may nonetheless be targets for immune T cells. We previously showed that a majority of cytotoxic T lymphocytes (CTL) reactivated using EBV-transformed B lymphoblastoid cells lines (LCL) contained minor populations of LMP2-specific T cells and homed to tumor sites. However, they did not produce remissions in patients with bulky disease. We have now used gene transfer into antigen presenting cells (APC) to augment the expression and immunogenicity of LMP2. These modified APC increased the frequency of LMP2-specific CTLs by up to 100 fold compared to unmodified LCL-APCs. The LMP2 specific population expanded and persisted in vivo without adverse effects. Nine of ten patients treated in remission of high-risk disease remain in remission, and five of six patients with active relapsed disease had a tumor response, which was complete in 4 and sustained >9 months. It is therefore possible to generate immune responses to weak tumor antigens by ex vivo genetic modification of APC and the CTL so produced can have substantial anti-tumor activity. This study is registered at http://www.cancer.gov/clinicaltrials (Protocol IDs: BCM-H-9936, NCT00062868, NCT00070226).
INTRODUCTION

Infused monoclonal antibodies enjoy significant success as cancer immunotherapies, but it has proved harder to exploit the equivalent promise of infused T lymphocytes.1-3 Many tumor-associated target antigens are functionally weak stimulators of the immune response.4 Consequently, preparation of tumor antigen-specific T cells for infusion requires specialized APC. Since these APC are required during both the induction and the amplification phase of the anti-tumor T cell response, large numbers of APC are necessary. This is problematic in many cancer patients whose APC may be numerically and functionally defective, due to the disease or its treatment.5

Even when tumor-specific cytotoxic (CD8+) T cells are successfully prepared 6, lack of T helper (CD4+) cell function in vivo may lead to lack of in vivo expansion, a problem that is not overcome by infusion of large numbers of cells. 6-8 Finally, although successful responses to adoptively transferred T cells have been intermittently observed in patients with relapsed or resistant cancer, the overall response rate has been low.6;7;9

For example, our previous experience using Epstein Barr Virus (EBV) specific cytotoxic T lymphocytes (CTL) for relapsed EBV-associated Hodgkin Disease (HD) generated CTL lines with low frequencies of cells specific for the weak EBV-derived antigens expressed by the tumor cells (e.g. LMP2) and produced responses only in a small proportion of patients, whose disease was limited.10;11

Of the potential CTL target antigens expressed in EBV positive Hodgkin’s Disease (HD) and non-Hodgkin’s Lymphoma, EBNA-1- is not processed for presentation on class I antigens by tumor cells 12 while wild type LMP1 is toxic when expressed in antigen presenting cells.13 LMP2A, however, is consistently expressed on the malignant
populations in Lymphoma tumors (e.g. Hodgkin Reed-Sternberg cells) and its epitopes are conserved between viral strains and amongst lymphoma biopsy samples. 14;15

To bias the EBV-specific CTL response to LMP2, we have replaced LCLs as APCs for the first stimulation, with dendritic cells (DCs) engineered to express LMP2 from an adenovirus (Ad) vector and used LCLs overexpressing LMP2 from the same Ad vector for subsequent stimulations.16;17 This approach expands polyclonal populations of both helper (CD4+) and cytotoxic (CD8+) T lymphocytes specific for this weak tumor antigen. After infusion into patients diagnosed with LMP2-positive lymphomas, LMP2-reactive T cells increased in number in the circulation and at tumor sites and produced sustained tumor responses.

MATERIALS AND METHODS

Patients and LMP status of the tumors

The protocol for the use of LMP2-specific CTL as therapy for Lymphoma was approved by the Food and Drug Administration, the Recombinant DNA Advisory Committee and the Baylor College of Medicine Institutional Review Board and Institutional Biosafety Committees. Informed consent was provided according to the Declaration of Helsinki. Patients were eligible for this study if they had Epstein Barr Virus (EBV)-associated Type II latency Hodgkin’s disease or Non-Hodgkin’s lymphoma (NHL) detected by immunohistochemistry for LMP1 and/or in situ hybridization for EBER.11

Patients had either relapsed after receiving standard therapy (Table 1) or were considered high risk for relapse (Table 2) and received two infusions of T cells two weeks apart in the General Clinical Research Center (GCRC) at Texas Children’s Hospital or The Methodist Hospital, where their vital signs were monitored before and
immediately after each infusion. On level 1 patients received 2 doses of cells at 2x10^7/m^2. At level 2 they received one dose of 2x10^7/m^2 and a second dose of 1x10^8/m^2 and at level 3 patients received one dose of 1x10^8/m^2 and a second dose of 2x10^8/m^2. If patients had a partial response or stable disease eight weeks after CTL they were eligible to receive additional CTLs, consisting of the same number of cells as their second injection. Total doses of CTLs received are shown in Tables 1 and 2. Complete blood counts were obtained, and electrolytes, renal and liver function were evaluated at 2, 4 and 6 weeks post-infusion and then at 3, 6, 9, and 12 months. Blood from patients was also monitored at regular intervals for EBV-DNA levels and immunologic assessment. Analysis of disease response to CTL therapy was performed using RECIST criteria.\textsuperscript{18}

**Generation of LMP-2 specific CTLs and EBV-specific CTLs**

5 x 10^6 PBMC from each patient were used for the establishment of an EBV transformed B lymphoblastoid cell line (LCL) for use as APCs.\textsuperscript{16} For DC production, PBMCs were plated overnight, then non-adherent cells were cryopreserved and adherent cells were cultured with 800 U/ml GM-CSF (Sargramostim Leukine; Immunex, Seattle, WA) and 500 U/ml IL-4 (R&D Systems, Minneapolis, MN, USA) for 7 days. IL-4 and GM-CSF were again added on day 3. On day 5, immature DCs were harvested by vigorous pipetting, transduced with Ad5f35LMP2 vector, and treated with TNF\textsubscript{α} (R&D systems) and PGE1 (Merck) for maturation. Before co-culture with non-adherent PBMCs the DCs were gamma-irradiated (30Gy). From day 10, responder T cells were restimulated weekly with irradiated LCL transduced with the same LMP2 vector. At the time of final cryopreservation (30-50 days of culture), the patient-derived CTL had an effector-memory phenotype (CD62L\textsuperscript{−}, CCR7\textsuperscript{−}, CD28\textsuperscript{−}, CD45RA\textsuperscript{−}, CD45R0\textsuperscript{+}) and comprised a mean of 24.2% (range 0.4%- 94.2%) CD4\textsuperscript{+} and a mean of 71.5% (range 6.62%-99.4%)
CD8+ T cells. Less than 1% of cells expressed monocytoid or B cell markers\textsuperscript{10,19-22}. The LMP2 specificity of the CTL lines was determined using HLA-peptide multimers, cytotoxicity assays and IFN-\textgreek{g} ELISPOTs. In ten out of 16 CTL lines, LMP2-specific activity was confirmed using cytotoxicity\textsuperscript{16} and/or \textgreek{g}-IFN Elispot assays with overlapping LMP2 peptides\textsuperscript{23}. In the remaining 6 CTL lines, LMP2-specific activity could not be confirmed. In the case of two predominantly CD4+ HLA-Class II restricted CTL lines, this may have been because the 15mer overlapping peptide library did not detect class II epitopes. EBV-specific CTL lines were produced as previously described\textsuperscript{24}. Briefly, PBMCs were cocultured with autologous irradiated (40Gy) LCLs at a responder : stimulator ratio of 40:1 for 9 days and then restimulated weekly with the autologous LCL at a 4:1 ratio. The cultures were supplemented with 40 units per mL of IL-2 from day 13.

**Cytotoxicity assays**

The cytotoxic specificity of each CTL line was analyzed in a standard 4-hour chromium\textsuperscript{51} release assay using effector:target ratios of 40:1, 20:1, 10:1, and 5:1.\textsuperscript{25} The target cells tested were LMP2-positive: autologous LCL; HLA class I and II mismatched LCL; HLA-matched fibroblasts transduced with Ad5f35LMP2; or PHA blasts pulsed with the LMP2 pepmix (Jerini, Berlin, Germany). As additional controls, we used LMP2-negative target cells; autologous phytohemagglutinin–stimulated PBMC (PHA blasts) alone or pulsed with irrelevant peptides from a CMV pp65 pepmix,(Jerini), and HLA matched fibroblasts transduced with Ad5f35GFP.

**Immunophenotyping**
CTL lines were stained with CD3, CD4, CD8, CD16, CD56, TCRαβ, TCRγδ, CD19, CD28, CD62L, CCR7, CD45RA and CD45RO (Becton Dickinson, San Jose, CA). For each sample, 10,000 cells were analyzed by FACS Calibur using Cell Quest software (Becton Dickinson).

Detection of EBV-DNA in PBMCs by Quantitative Real time PCR

DNA was isolated from 3 to 5×10⁶ PBMCs using an anion exchange column (Qiagen, Valencia, CA). Five hundred nanograms of DNA was analyzed by EBV EBER-specific Q-PCR as previously described to quantitate EBV genome copy number per µg.²⁶

LMP2-multimers and peptides

To detect LMP2-specific T cells in the CTL lines and PBMC, we used the soluble HLA-peptide tetramers prepared by the Baylor College of Medicine Tetramer Core Facility or pentamers Proimmune Inc (Springfield, Virginia) (together termed multimers). Multimers were HLA-A*0201-CLGGLLTMV, HLA-A*0201-FLYALALLL, HLA-A*0201-LLWTLVLL, HLA-A*1101-SSCSSCPLSKI, HLA-A*2301-PYLFWLAAI, HLA-A24-PYLFWLAAI, HLA-A24-TYGPVFMSL and HLA-A*0201-CLGGLLTMV, HLA-A*0201-FLYALALLL, HLA-A*1101-SSCSSCPLSKI, HLA-A24-PYLFWLAAI, HLA-A24-TYGPVFMSL, HLA B*2705-RPPIFIRRL, B*2705-RRIYDLIEL and HLA-B*3501-MGSLEMVPM. Peptides were synthesized by the Baylor College of Medicine Protein Core Facility or by Genemed Synthesis Inc. (South San Francisco, California). Tetramer staining of CTLs (5 x 10⁶) or PBMC (1 x 10⁶) is previously described.¹⁰ For pentamer staining, CTLs or PBMC were incubated with unlabeled pentamer followed by Pro5™ Flurotag (PE) (Proimmune Inc, Springfield, Virginia) according to the manufacturer’s instructions. For each sample, 100,000 cells were analyzed as described above.
Panels of 15mer peptides (overlapping by 11 amino acids) covering the entire amino acid sequence of LMP2 from the Caucasian prototype EBV strain B95-8 were synthesized as previously described.\textsuperscript{16,27} Twenty-three peptide pools comprising 2 to twelve 15mer peptides were prepared, so that each 15mer peptide was represented in two pools.\textsuperscript{16,28} This LMP2 peptide library was designed to identify all possible HLA class I restricted epitopes, which have a length of 9-11 amino acids, however, it is not ideal for the identification of HLA class II restricted epitopes, which have lengths of 13 to 17 amino acids.\textsuperscript{29}

**Enzyme-Linked Immunospot (ELISPOT) assay**

ELISPOT analysis was used to determine the frequency and function of T cells secreting IFN-$\gamma$ in response to LMP2 pepmix\textsuperscript{TM} (Jerini, Berlin, Germany), which contains all 15mer peptides of LMP2 in one pool, or to LCL.\textsuperscript{10} ELISPOT assays were performed on the CTL lines and on PBMC pre- and post-CTL infusion. In addition, to define the CD4 and CD8 restricted LMP2-specific activity we pulsed OKT3 blasts with either LMP2 pepmix or CMV pepmix\textsuperscript{17} and used them as antigen presenting cells to stimulate either CD8$^+$ or CD4$^+$ T cells sorted from CTL lines using flow cytometry. To reduce inter-assay variability, patient PBMC samples were cryopreserved and batched for ELISPOT analysis after >6 weeks of follow-up. PBMC stimulated with CMV pepmix and Staphylococcal Enterotoxin B (1$\mu$g/ml) (Sigma-Aldrich Corporation, St Louis, MO) served as controls. Spot-forming cells (SFC) and input cell numbers were plotted after plates were evaluated by Zellnet Consulting (New York, New York).

**Statistical Analysis**

Descriptive statistics were calculated to summarize SFC for LMP2-or EBV-specific T cells at pre- and post-infusion time points as well as the changes in SFC from pre-
infusion. Assessments of data normality on these outcomes were performed and data were log-transformed if indicated. Comparisons between pre- and post-infusion LMP2-specific and EBV (LCL)-specific T cells SFCs were performed using paired t-tests on log-transformed data. Comparison of repeated measurements of LMP2 and EBV SFC levels over time between patients with vs. without LMP2 sensitivity in their CTL lines were performed using random coefficient models. P values < 0.05 were considered statistically significantly different.

RESULTS

Patient Characteristics

All patients had EBV positive lymphoma. 8 had Hodgkin Disease (4 Nodular Sclerosing Hodgkin Disease and 4 Mixed Cellularity) and 8 had non-Hodgkin Lymphoma (2 diffuse large B cell, 1 anaplastic large cell, 1 T cell severe chronic active EBV infection (SCAEBV), and 4 nasal NK/Tcell NHL). Their ages ranged from 7 to 66 years (median 30 years) and their initial disease presentation ranged from stage IA to IVB. Samples for CTL generation were collected from patients either at diagnosis (n=2) or after a first or subsequent relapse (n=14). Pre CTL infusion, the 16 patients had lymphocyte counts ranging from 333.9 to 3494.4/mm$^3$ (median 850cells/mm$^3$). Lymphopenia as defined as a peripheral blood lymphocyte count of $<1,000$/mm$^3$ was observed in nine out of 16 patients.

CTLs are LMP2-specific and polyclonal

The specificity of the LMP2-CTL lines was determined using HLA-peptide multimers when available. Specific function was determined, using cytotoxicity assays and IFN$\gamma$ ELISPOT assays. The cytolytic activity of the responder cells was tested against a panel of $^{51}$Cr-labeled autologous and allogeneic target cells. Ten of the 16 lines infused had
demonstrable LMP2-specific activity. LMP2-specific CTLs generated from a patient representative of these twelve patients killed autologous LCL and HLA-matched fibroblasts only if transduced with Ad5f35LMP2A (30% at an E: T ratio of 20:1 (Figure 1a)) whereas the LCL-activated EBV-specific CTL line demonstrated no LMP2 specific activity (data not shown). Killing was not due to adenovirus-directed CTLs, since fibroblasts infected with recombinant adenovirus encoding GFP were not recognized. There was no cytotoxic activity against HLA-mismatched LCLs, (Figure 1a) or untransduced fibroblasts (data not shown).

This same patient’s HLA type was A2;29/B13;27. Using HLA A2, A29 and B27 multimers we demonstrated that CTL generated using Ad5f35LMP2-transduced APCs (LMP2-CTL) were markedly enriched for T cells specific for LMP2 epitopes compared to the EBV-CTL line generated from the same patient using non-transduced LCL alone. Further, the frequency of T cells specific for the HLA B27-restricted non-tumor-associated immunodominant EBV epitope RRIYDLIEL (RRI) in EBNA 3C was markedly decreased in the LMP2-CTL line (0.11%) compared to the population detectable in the EBV-CTL line (12%). (Figure 1b)

Figure 1c illustrates that LMP2-specific lines can secrete IFNγ in response to several different LMP2 peptides, representing discrete epitopes on the LMP2 antigen. Furthermore, using OKT3 blasts expressing LMP2 peptides as APCs we were able to demonstrate that both the CD4+ and CD8+ T cells present in the polyclonal CTL lines have LMP2 specificity (Figure 1d). Hence the lines infused, though reactive to LMP2 are multispecific and are not restricted to predetermined peptide epitopes of LMP2, thereby reducing the probability of tumor immune-escape due to outgrowth of a single-epitope loss variant.
Adoptively-transferred LMP2-specific CTL lines expand in vivo

Sixteen patients received total numbers of CTL ranging from $4 \times 10^7$ to $3.2 \times 10^8$ cells/m². Multimer and ELISPOT assays were performed to measure the frequency of LMP2-specific CTL in peripheral blood before and after infusion. During the follow-up period, LMP2-specific T cells increased up to 5-fold following infusion of the first dose of T cells as measured by multimer assay (Figure 2a). Antigen responsiveness based on ELISPOT analysis also showed a significant increase in cells that secreted IFNγ in response to LMP2 pepmix. In the ten patients with a measurable LMP2-specific response in their CTL line, there was a significant increase in LMP2-specific T cells in the peripheral blood 1 week after infusion (mean difference from pre-infusion = 1.4 log units, p=0.018) and 2 weeks post-infusion (mean difference from pre-infusion = 1.0 log units, p=0.037) and a similar outcome was also seen at 4 weeks (mean difference from pre-infusion = 1.3 log units, p=0.051) (Figure 2b). In the remaining six patients for whom LMP2 specificity could not be quantified, we observed a similar pattern of increase in peripheral blood T cells responding to autologous EBV-LCLs, which present all 8 latency-associated EBV proteins as well as early lytic cycle proteins. This trend did not, however, reach statistical significance in this smaller group (mean difference from pre = 0.57, 0.69, 1.05 log units at 1, 2, and 3 weeks, respectively) (Figure 2c). There was a significant difference in LMP2 CTL expansion over time between those patients with versus without LMP2 specificity in their CTL lines (p = 0.006). The elevation in LMP2 and LCL-specific T cells in the peripheral blood persisted for > 3 months following infusion. There was no corresponding rise in T cells with specificity to CMV antigens (p > 0.05) (data not shown). No relationship between the number of CTLs infused and the degree of in vivo expansion or final frequency of LMP2 reactive cells in peripheral blood was detected.
Outcome

No short or long-term toxicities were observed after CTL infusion. Of the ten patients treated in remission of high-risk disease, 9 have remained in complete remission for up to 37 months post CTL (Table 2). The tenth patient progressed within 8 weeks of receiving CTL.

Of the six patients with relapsed disease that was refractory to standard treatment (Table 1), four had complete clinical responses and one had a very good partial response, all as defined by RECIST criteria. Responses were seen in both Hodgkin’s and non-Hodgkin’s lymphomas, and in patients receiving multiple doses of CTL at either 2x10^7/m^2/dose or 1x10^8/m^2/dose or 2x10^8/m^2/dose (total dose range 8x10^7/m^2 to \( \geq 3x10^8/m^2 \) CTL). Of the four complete responders, the first patient (pt 3) had Hodgkin’s disease involving the bone marrow that had proved resistant to multiple courses of chemotherapy and now remains in complete remission 34 months post CTL. The second patient (pt 6) had residual Hodgkin’s disease evident on imaging post-chemotherapy that resolved eight weeks after receiving CTL. She remains in CR at 15 months post CTL. Figure 3a shows imaging data from a third responding patient (pt 2) who had refractory NHL with multiple disease sites that cleared after CTL infusion. This patient also had a follow up needle biopsy of a previously involved cervical node (Figure 3b), which showed no residual LMP2+ tumor cells, but a heavy infiltrate of CD8+ T cells. Tetramer staining (Figure 3c) showed many of these cells were specific for an LMP2 epitope strongly represented in the line. The fourth patient (pt 4) had a relapsed NK/T non-Hodgkin lymphoma whose resolution is shown in Figure 4a together with an associated increase in LMP2-specific CTL activity and corresponding fall in the level of his tumor marker EBV-DNA (Figure 4b). A follow-up nasopharyngeal tissue biopsy (Figure 4c)
revealed no residual LMP2+ cells and a marked increase in infiltrating CD4+ T cells, consistent with the predominant phenotype of this patient’s LMP2-specific T cell line.

DISCUSSION

Efforts to exploit the promise of T lymphocytes for the targeted therapy of cancer have been hindered by the limited immunogenicity of most tumor-associated antigens. They have also been impaired by lack of subsequent in vivo expansion and homing of tumor specific T cells, since substantial numbers of effector cells are required to control established human tumors, which are often bulky and widely dispersed.

We have used cytotoxic T lymphocytes (CTL) targeting the subdominant tumor-associated viral antigen LMP2 to treat patients with EBV-associated Hodgkin and non-Hodgkin Lymphoma. Our results show how the genetic modification of APCs so that they over-express LMP2, allows them to amplify an in vitro T cell response against this weak lymphoma-associated antigen, expanding both CD4+ (helper) (Figure 1d) and CD8+ (cytotoxic) T cells directed to an array of LMP2 epitopes (Figure 1c). Infusion of these T cells was followed by a substantial in vivo increase in the frequency of tumor reactive T cells, so that as few as 80 million infused T cells were able to produce tumor responses in 5/6 patients with measurable lymphoma.

In our previous clinical studies, we treated patients with relapsed EBV positive Hodgkin Disease (HD) using EBV-CTL in which only a small component of the line was specific for the weak EBV-derived antigens expressed by the tumor cells: only 2/11 patients had a complete clinical response, and both had only limited disease.10 By genetically modifying the antigen presenting cells used to stimulate the cytotoxic T cells (CTL), we have been able to direct the response away from the immunodominant EBV antigens
(EBNA 3A, 3B, 3C) that are absent on the malignant cells, and towards the weak tumor associated antigen LMP2.

LMP2 is an EBV encoded latency protein expressed in up to 40% of all lymphomas. It is apparent that EBV-associated lymphomas arising in the immunocompetent host lack expression of viral proteins that dominate the human T cell response following exposure to the virus. Most EBV positive lymphomas arising in the immunocompetent host are characterized as viral latency pattern II, and express the EBV antigens EBNA-1, LMP1 and LMP2. We chose LMP2 as the target antigen for the current trial since EBNA1 is processed and presented poorly to classical CD8+ T cells and LMP1 shows significant sequence variability between viral strains. By contrast, LMP2 is consistently expressed, and its epitopes are conserved.

To express whole LMP2 protein in APCs, we used an adenovirus vector, Ad5f35, encoding LMP2 as a transgene. This choice allows APCs and T cells to “self-select” the processed peptides they present and react with, so that generation of LMP2-specific T cells is not restricted to patients whose HLA-polymorphisms are appropriately matched to the individual peptides used. The use of full-length antigen may have two additional benefits. First, T cells may recognize multiple epitopes within the protein as observed in multimer and ELISPOT assays. This decreases the chances of tumor escape due to emergence of epitope loss variants within the malignant population. While such events cannot be entirely avoided, they are likely to be reduced in frequency if the immune response is directed to multiple distinct regions of a target molecule rather than to a single peptide epitope. Second, combinations of both CD4 and CD8 epitopes that recruit both CD4+ and CD8+ T lymphocytes can be presented, increasing the likelihood that both types of effector cells will be generated, favoring the subsequent sustained
expansion of transferred cells and eradication of established lymphoma. Although we do not know if the \textit{in vivo} expansion we observed was directly attributable to the mix of CD4$^+$ and CD8$^+$ LMP2 specific T cells we infused, clinical effects were observed with as few as 80 million infused cells, and the increase in the frequency of T cells in peripheral blood implied significant \textit{in vivo} expansion had occurred. Although these increases in LMP2-specific T cells were observed without prior lymphodepletion, the majority of the patients were lymphopenic at the time of infusion and this may have contributed to subsequent in vivo proliferation.

How essential is our technique of LMP2-CTL enrichment for the anti-tumor activity we observe? Although we could not confirm that amplification of LMP2 specificity had occurred in six lines (two that were exclusively CD4$^+$) there is consistent corroborative evidence from the remaining 10/16 patients to support the value of enrichment. In these individuals, infusion of LMP2 enriched lines produced an increase in the number of LMP2-antigen binding and tumor-antigen-reactive cells not only in the circulation, but also at sites of tumor. Moreover, this accumulation occurred reciprocally to the decline in LMP2+ tumor cells.

Although our study is not randomized, these lymphomas do not undergo spontaneous complete remission, so that the clinical responses we observed are likely causally related to the anti-tumor activity of the CTLs we infused. Of note, benefits were obtained in the absence of the severe morbidity and mortality that are an inevitable accompaniment to conventional salvage chemo-radiation.

Our technique using genetically modified APC that overexpress weak antigens may have utility for preparing effector cells that target other "weak" tumor associated...
antigens, since it favors expansion of T cells present at low frequencies, and with low antigen affinity (Figure 1a). This current study, however, modulates the response to a weak viral-derived tumor antigen and it is possible that it will be less effective for tumor-antigens that are of host origin. Even if such a limitation applies, this approach should nonetheless prove valuable for the targeted treatment of other viral-associated malignancies of the head and neck, the cervix, and the hemopoietic system. Furthermore, the clinical outcome we report, taken in conjunction with successful studies in which the T lymphocytes themselves are genetically modified to express tumor specific receptors, demonstrates that the convergence of cellular immunotherapy and gene transfer can be of clinical value.

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AUTHOR CONTRIBUTIONS

**CMB**: participated in the development of the clinical study, was a co-Principal investigator on the clinical trial and cared for the patients enrolled on this study, manufactured CTL lines and performed the characterization of these CTL lines, performed many of the immune reconstitution studies and contributed to the writing of the paper. **SMG** participated in the development of the clinical study and cared for some of the patients enrolled on this study. **AML**: participated in the generation and administration of the CTL for this study. **HW and MW**: provided statistical support for this study. **KCS**: participated in the development of the clinical study and performed characterization of CTL lines. **GC**: participated in the clinical study by caring for some of the lymphoma patients enrolled. **MK**: performed the characterization of CTL lines and performed the immune reconstitution studies. **MHH**: supervised CTL preparation and quality assurance. **CCC and MVG**: reviewed tumor samples for EBV positivity. **APG**: performed quality assurance on all CTL lines prior to clinical use. **MKB**: participated in the development of the clinical study and contributed to the writing of the paper. **CMR**: developed the clinical study and was a co-Principal Investigator and contributed to the writing of the paper. **HEH**: developed the clinical study and was a co-Principal investigator on the clinical trial, cared for some of the patients enrolled on this study and contributed to the writing of the paper.
FIGURE LEGENDS:

**Figure 1. LMP2-specific CTL lines derived from Lymphoma patients contained functional LMP2-specific T cell populations**

This figure shows the LMP2 specific activity in a CTL line generated from a patient with relapsed Lymphoma. The patient is representative of the 10 patients whose CTL lines recognized LMP2. To demonstrate cytolytic-specificity of patient CTL lines *in vitro*, percent specific $^{51}$Cr release was determined 6-hours after coincubation with HLA matched fibroblasts transduced with Ad5LMP2 (black square), or Ad5GFP (open square), autologous LCLs (black triangle) and allogeneic LCL (open triangle). The LMP2-specific CTL line from a representative patient shown in Figure 1b showed killing of autologous LCL and HLA-matched fibroblasts only if they were transduced with Ad5LMP2 (30% at an E: T ratio of 20:1). Killing was not due to adenovirus-directed CTLs, since fibroblasts infected with recombinant adenovirus encoding GFP were not recognized. There was no killing of HLA-mismatched LCLs, (Figure 1a) or non-transduced fibroblasts (data not shown). Figure 1b shows the frequency of LMP2-specific T cells in the CTL line generated from the same patient (HLA A2;29/B13;27). CTL were co-stained with PE-conjugated multimers CD8-FITC and CD3-PerCP. Multimers were: LMP2-HLA-A$^{*}$0201-LLW, HLA-A$^{*}$0201-FLYALALLL, HLA A29-ILL, and EBNA 3C- HLA-B27-RRI. When compared to the EBV-LCL-activated CTL line, the LMP2-activated CTL line showed an increased in the frequency of T cells reactive with all three LMP2 tetramers, but decreased frequency in the EBNA3C tetramer reactivity (Figure 1b). To assess the breadth and function of the LMP2-specific response the CTL were co-incubated with overlapping LMP2 peptides and IFN-$\gamma$ release in response to specific 15mer and 8-mer peptides was measured in an ELISpot assay. (Figure 1c) This patient's polyclonal CTL line was then sorted for CD4$^+$ and CD8$^+$ T cells. Subsequently recognition of LMP2 by these separated CD4$^+$ and CD8$^+$ T cells was
determined in an IFN-γ ELISPOT assay using OKT3 blasts pulsed with either LMP2pepmix or CMVpepmix as the APCs (Figure 1d).

**Figure 2. The frequency of LMP2-specific T cells increase after infusion of polyclonal LMP2-specific CTLs**

LMP2 multimer analysis was also used to compare the frequency of LMP2-specific CTL pre- and post-infusion. The average number of LMP2 and CMV multimer-reactive cells per $10^6$ CD8+ T cells are shown for one patient pre- and post-infusion (Figure 2a). This patient was representative of the 10 patients who received CTL where specific LMP2 epitopes had been characterized. In these 10 patients in whom the HLA-restricted LMP2 peptide(s) were available, peripheral blood T cells were incubated with LMP2pepmix and the number of IFN-γ spot forming cells per $2\times10^5$ mononuclear cells was measured (Figure 2b). In the six patients in whom LMP2 peptides were not detected, peripheral blood T cells were incubated with autologous LCL and the number of IFN-γ spot forming cells per $2\times10^5$ mononuclear cells was measured (Figure 2c).

**Figure 3. Induction of complete clinical response and LMP2-specific T cell accumulation at tumor site after CTL infusion**

A positron emission tomography (PET) scan demonstrating abnormal fluorodeoxyglucose (FDG) uptake in supraclavicular and para-aortic lymph nodes was observed pre CTL infusion in a patient with NHL (pt 2). The follow-up scan 8 weeks post-CTL infusion is reported as normal (Figure 3a). Using immunohistochemistry CD8+ infiltrating T cells were seen in lymph node biopsy post-CTL infusion which corresponded to a clearance of LMP2+ tumor cells (Figure 3b). In addition, the percent
CD8+/LMP2 tetramer+ T cells in the lymph node and peripheral blood were compared after CTL infusion by flow cytometry (Figure 3c).

**Figure 4. Induction of complete clinical response and T cell accumulation at tumor site after CTL infusion**

A PET scan demonstrating abnormal FDG uptake in the nasopharyngeal region was observed pre CTL infusion in another patient (pt 4) with NK/T cell lymphoma. The follow-up scan 8 weeks post-CTL infusion was reported as normal (Figure 4a). EBV DNA levels in this patient (pt 4) PBMC measured by quantitative real time PCR pre- and post-CTL were plotted against the EBV(LCL)-specific T cell response post infusion as measured by IFN-γ secretion in ELISPOT assay (Figure 4b). Using immunohistochemistry, CD4+ infiltrating T cells were seen in a lymph node biopsy post CTL infusion, and corresponded to a clearance of LMP2 positive tumor cells in patient 4 (Figure 4c).
REFERENCES


<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age/Sex</th>
<th>Disease (stage at diagnosis)</th>
<th>Number of relapses</th>
<th>Most recent treatment (time pre CTL)</th>
<th>Total CTL Dose</th>
<th>Toxicity Attributed to CTL</th>
<th>Response to CTL (survival months)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>8yrs/M</td>
<td>T cell SCAEBV</td>
<td>2</td>
<td>syngeneic SCT - Bu/CY/Campath (5mths)</td>
<td>$4 \times 10^7/m^2$</td>
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<td>PR (&gt;36mths)</td>
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<td>2*</td>
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<td>anti-CD30 (11mths)</td>
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<td>CR (relapsed 10mths, died 33mths)</td>
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<td>3*</td>
<td>30yrs/F</td>
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<td>2</td>
<td>ABVD (12mths) rituximab (1mth)</td>
<td>$8 \times 10^7/m^2$</td>
<td>None</td>
<td>CR (&gt;32mths)</td>
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<td>R-CHOP (1mth)</td>
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<td>None</td>
<td>CR (relapsed 9mths, died 18mths)</td>
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<td>3</td>
<td>autologous SCT- BEAM (3mths)</td>
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<td>VP16/Dox/Rituximab (1mth)</td>
<td>$3 \times 10^6/m^2$</td>
<td>None</td>
<td>CR (&gt;13mths)</td>
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</table>

SCAEBV = severe chronic active EBV infection, NHL = non-Hodgkin Lymphoma, HD = Hodgkin disease, SCT = stem cell transplant, BU = busulfan, CY = cyclophosphamide, ABVD = doxorubicin, bleomycin, vinblastine and dacarbazine, R-CHOP = Rituximab plus Cyclophosphamide, Doxorubicin, Vincristine and Prednisone, BEAM = BCNU, Etoposide, Cytarabine and Melphalan. VP-16 = etoposide, Dox = doxorubicin. PR = partial response, CR = complete response, NR = no response.

*Patients who received 4 doses of CTL.
<table>
<thead>
<tr>
<th>UPN</th>
<th>Age/Sex</th>
<th>Disease (stage at diagnosis)</th>
<th>Number of relapses</th>
<th>Most recent treatment (time pre CTL)</th>
<th>Total CTL Dose</th>
<th>Toxicity Attributed to CTL</th>
<th>Response to CTL (survival months)</th>
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<tbody>
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<td>7</td>
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<td>3</td>
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<td>remains in remission (&gt;37mths)</td>
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<td>NR (died 22mths)</td>
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<td>COP (1mth)</td>
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<td>remains in remission (3mths)</td>
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<td>NK/T cell NHL</td>
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<td>CHOP and RT (3mths)</td>
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<td>Remains in remission (8weeks)</td>
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<td>mixed cellularity HD stage Ia (post lung transplant)</td>
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<td>Rituximab (8mths)</td>
<td>3x10⁹/m²</td>
<td>None</td>
<td>remains in remission (&gt;18mths)</td>
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</tbody>
</table>

NHL = non-Hodgkin Lymphoma, HD = Hodgkin disease, SCT = stem cell transplant, BU = busulfan, MEL = melphalan, ABVD = doxorubicin, bleomycin, vinblastine and dacarbazine, RT = radiotherapy, BEAM/BEAM-R = BCNU, Etoposide, Cytarabine, Melphalan +/- rituximab.
Figure 1
Figure 2

A

Pre CTL | 1 week | 2 weeks

Pentamer | LMP2(CLG) | 0.09% | 0.16% | 0.46%

Pentamer | CMVpp65(NLV) | 4.56% | 4.12% | 4.83%

B

SFC per 2x10^6 cells

CD3

time post infusion

C

SFC per 2x10^6 cells

time post infusion
Figure 3

A  
Pre CTL infusion  2 months post CTL infusion

B  
Pre CTL infusion  Post CTL infusion

C  
LMP2 tetramer (TYG)  
Lymph node  Peripheral blood

CD8
Figure 4

A

Pre CTL

Post CTL

B

Pre CTL

Post CTL

C

Pre CTL infusion

Post CTL infusion

LMP2

CD4
Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer

Catherine M. Bollard, Stephen Gottschalk, Ann M Leen, Heidi Weiss, Karin C Straathof, George Carrum, Mariam Khalil, Meng-fen Wu, M Helen Huls, Chung-Che Chang, M Victoria Gresik, Adrian P Gee, Malcolm K Brenner, Cliona M Rooney and Helen E Heslop