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

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Chapter 4

The Generation and Characterization of LMP2-specific CTLs for Use As Adoptive Transfer From Patients with Relapsed EBV-Positive Hodgkin Disease

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Introduction

Adoptive immunotherapy with cytotoxic T-lymphocytes (CTL) has been used successfully to treat Epstein-Barr virus associated malignant disease. The results have been most striking for the EBV positive B-immunoblastic lymphomas occurring in immunocompromised hosts following stem cell transplant (SCT). In this disorder, the malignant cells express the full spectrum of nine EBV latent cycle antigens (latency Type III) and may also express lytic cycle EBV antigens. Hence, EBV specific CTL lines (EBV-CTL) can be generated simply by stimulating peripheral blood mononuclear cells (PBMC) with irradiated EBV-transformed B-cells (EBV-lymphoblastoid cell lines or LCL), which express an identical pattern of viral genes, and are excellent antigen presenting cells.

In the immunocompetent host, EBV is associated with malignancies that express a more limited array of viral genes. For example, in EBV-positive Hodgkin's Lymphoma, only subdominant EBV antigens such as latent membrane antigens (LMP) 1 and 2, and Epstein-Barr nuclear Ag (EBNA) 1 are expressed – termed Type II latency. Our clinical studies of CTL for the treatment of relapsed EBV positive Hodgkin Disease (HD) showed that *ex vivo* expanded EBV-CTL survived in the patients' circulation for up to 9 months, homed to sites of disease and reduced EB viral load, but produced limited tumor responses.[1] This limitation may be because the bulk of each EBV-CTL line was directed against the immunodominant EBV antigens (EBNA 3A, 3B, 3C) that are strongly expressed by the stimulator LCL or against early lytic cycle antigens, which are lacking from the tumor cells themselves.[2;3]

Of the potential CTL target antigens expressed in HD, EBNA-1- is not processed for presentation on class I antigens by tumor cells[4] while LMP1 displays heterogeneity between virus strains,[5] so that CTL raised against B-cell (B95-8 prototype) derived LMP1 may not recognize the LMP1 tumor variants.[6] LMP2A, however, is consistently expressed on Reed-Sternberg cells and its epitopes are conserved between viral strains and amongst Hodgkin's lymphoma biopsy samples.[7-9] The existence of LMP2-specific CTLs in patients with HD has previously been reported.[10] We have also reported the production of LMP2-specific CTL lines from healthy donors, using Ad5-LMP2-transduced DC as APC. However, this method was ineffective when applied to mononuclear cells from patients with relapsed HD. We were unable to produce sufficient DC for the expansion of adequate numbers of LMP2-specific CTL and CTL restimulated with transduced DC failed to expand.

We now describe how LMP2-specific CTLs, from patients with relapsed HD, can be reactivated from patient peripheral blood using LMP2-expressing DC and expanded using autologous LCLs overexpressing LMP2 from an adenovirus vector. We used a chimeric adenoviral vector Ad5F35 that has been optimized for the transduction of human hematopoietic cells, and effectively expresses transgene in both DC and LCL.[11,12] This modification increases the frequency of LMP2-specific T-cells produced and ensures that every patient line contains an LMP2-specific component. The CTL lines contained both CD4+ T-helper cells and CD8+ CTLs that and recognized multiple LMP-2 epitopes, favoring prolonged *in vivo* survival and reducing the risk of tumor-antigen loss variants respectively.[13]

Material and methods

Cell lines and blood donors

The LMP2-specific CTL lines were derived from 2 patients with relapsed EBV-positive Hodgkin disease. Both patients had received standard HD chemotherapy and radiotherapy and patient 1 had also received an autologous stem cell transplant. At relapse, peripheral blood was collected to generate the cell lines before instituting rescue chemotherapy. The HLA class I types of the patients were as follows: Patient 1 HLA-A2, -A3, -B7, -B51 and patient 2: HLA-A11, -29, -B7, -40(61). EBV-transformed Lymphoblastoid Cell Lines (LCL) were established, using a standard protocol.[14] As positive controls, LMP2-specific CTL lines were generated from normal EBV seropositive donors with HLA types A2, B27, B51 and A11, A34, B35, B40(61). Primary skin fibroblasts were isolated and cultured as previously described.[15]

Construction of the recombinant adenovirus AdLMP2A

A recombinant adenovirus Ad5f35LMP2A for the transduction of DC and LCL was generated by Dr Alan Davis in our Gene Vector Laboratory (Baylor College of Medicine, Houston, TX). The structure and synthesis of the Ad5f35 vector has been described previously.[11] The 1.8 kb fragment containing the LMP2A gene was removed via Hind III and Xba I sites from pBluescriptLMP2A[15] and cloned into the *Hind III* and *Xba* I sites of the pShuttle-X (Clontech) shuttle vector. Clontech's adeno-X expression system protocol was used and the I-Ceu/PI-SceI fragment of pShuttle-X was cloned into Adeno-X viral DNA backbone containing an Ad5 gene with a modified fiber containing the knob and shaft of the Ad35 and the tail of Ad5.[11;16] The titer of the vector was 1 x 10^{12} vp/ml. The recombinant vector was stored at -80°C until use. The recombinant adenoviruses Ad5GFP for the transduction of fibroblast targets in cytotoxicity assay were also supplied by Dr. Alan Davis (Baylor College of Medicine, Houston, TX).[15]

Generation of Dendritic Cells (DC)

DCs were generated as previously described by Gahn et al with some modifications.[15] 40-60 mls of peripheral blood was collected from the patients. The PBMCs were then purified by Ficoll (Lymphoprep; Nycomed, Oslo, Norway) gradient separation and frozen at 5x10^6/ml in Origen® DMSO freeze medium (Irvine Scientific, Santa Ana, CA). Once the LCL line was established 10-20x10^6 PBMC were thawed and washed twice in CellGenix media (CellGenix USA, Antioch, IL) and plated at 5x10^6 cells per well in DC medium (CellGenix media plus 2 mM L-glutamine) (GlutaMAX, Invitrogen, Carlsbad, CA) in a 6-well plate (Costar, Corning, NY) for 2 hr at 37°C in a humidified CO2 incubator. Nonadherent cells were removed by rinsing with 1X PBS (GibcoBRL, Gaithersburg, Maryland), and loosely adherenT-cells were cultured in DC media 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, cells were harvested by vigorous washing.

Adenoviral transduction of Dendritic Cells

Immature DCs were harvested on day 5, counted and resuspended at 2x10^6/ml in DC media. 500 µl of cells were then added to 24-well plates (Costar, Corning, NY) and transduced with Ad5f35LMP2A at a multiplicity of infection (MOI) of 30,000 vp/cell. Plates were incubated 37°C for 90 minutes. Afterward, DCs were cultured in DC media for 2 days supple-

mented with 1000 U/ml IL-4, 800 U/ml GM-CSF, 10 ng/ml TNF-α (R&D Systems) and 1 μg/ml PGE2 (Sigma, St Louis, MO, USA) and then irradiated and used to stimulate LMP2-specific CTLs.

Adenoviral transduction of LCLs

Two days before CTL stimulation, LCL were harvested, pelleted and incubated with Ad-5f35LMP2A at a multiplicity of infection (MOI) of 100,000 vp/cell for 90 minutes at 37°C. The cells were then resuspended at $5x10^5$ cells/ml of complete media and transferred to a 24 well plate at 2 mls per well.

Initiation and expansion of LMP2-specific CTL (LMP2-CTL)

Frozen PBMC were thawed and washed and resuspended in 45% RPMI (Hyclone) and 45% CLICKS (Irvine Scientific) with 10% FCS plus GlutaMAX™-I (CTL medium). Cells were resuspended at $2x10^6$/ml and co-cultured with autologous, transduced DCs at a ratio of 40 PBMC to 1 DC. Cultures were restimulated on day 10 with Ad5f35:LMP2-transduced LCL at a responder-to-stimulator ratio of 4:1 and after that weekly with irradiated autologous Ad5f35LMP2-transduced LCL at a responder-to-stimulator ratio of 4:1. IL-2 (50-100 U/ml, Proleukin; Chiron, Emeryville, CA) was added 3 days after the second stimulation and added twice weekly.

Generation of EBV-specific CTL cultures (EBV-CTL)

EBV-CTLs were prepared by stimulating PBMC with the autologous EBV-transformed LCL as previously published.[17;18] Briefly, PBMC ($2x10^6$) were co cultured with $5x10^4$ gamma-irradiated (40 Gy) autologous LCL per well in a 24-well plate. Starting on day 10, the responder cells were restimulated weekly with irradiated (40Gy) LCL at a responder: stimulator ratio of 4:1. Two weekly doses of rhIL-2 (50-100 IU/ml) were added from day 14.

Enzyme-Linked Immunospot (ELISPOT) assay

ELISPOT analysis was used to estimate the frequency of T-cells secreting IFNγ in response to LMP2 peptides. To analyze the HLA-restriction of the LMP2 CTL clone, we measured IFNγ release after culture with peptide pulsed allogeneic LCL lines, matched at only one class I antigen. Ninety six-well filtration plates (MultiScreen, #MAHAS4510, Millipore, Bedford, MA) were coated with 10 μg/mL anti-IFN-γ antibody (Catcher-mAB91-DIK, Mabtech, Cincinnati, OH) overnight at 40C and then washed and blocked with RPMI 1640 containing 5% Human Serum and L-glutamine (ELISPOT medium) as previously described.[19] To determine the LMP2 peptide specificity of the CTL lines, the responder LMP2-CTL were thawed and cultured in CTL media with 25 U/ml IL-2 for 24 hour. The CTL were harvested and resuspended at $1x10^6$/ml ELISPOT medium without IL-2. Peptides were initially diluted in 1ml 1XPBS and 1ml ELISPOT medium to 10 μg/ml. To identify the HLA A29 LMP2 peptide epitope, serial logarithmic dilutions of 8-mer, 9-mer and 10-mer peptides were made. 100 μl of CTL were then added to 100 μl of diluted peptide. LCL generated as previously described were used either alone, or pulsed with peptide. The LCL were washed, irradiated (40 Gy), washed twice and then transferred to sterile polypropylene tubes at (500 μl of $1x10^6$/ml) and 10 ug peptide (diluted to 20 μg/ml in PBS) was added. 1hr later, the cells were washed and resuspended in 2mls ELISPOT medium. Responder and stimulator cells were first prepared in a replica 96-well U-bottom plate and then transferred to coated 96-well plate. After 20hr incubation, the plates were washed, incubated with the secondary biotin conjugated anti-

IFN-γ monoclonal antibody (DetectormAB (7-B6-1-Biotin), Mabtech) followed by incubation with Avidin: biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit (Standard), #PK6100, Vector Laboratories, Burlingame, CA) and then developed with AEC substrate (Sigma, St. Louis,MO) as previously described.[19] Plates were sent for evaluation to Zellnet Consulting, New York, NY. The frequency of peptide-specific T-cells was expressed as specific Spotforming units (SFC).

LMP2-peptides and LMP-2 peptide Pools

Panels of 15-mer 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 described by Meij et al.[20] Twenty-three peptide pools comprising 2 to twelve 15-mer peptides were prepared, so that each 15-mer peptide was represented in two pools, as previously described.[21] To identify the minimal epitope from a reactive 15-mer HLA-A29 LMP2 epitope, 8-mer, 9-mer and 10-mer peptides were created using published predictions of peptide binding to HLA A29.[22] All peptides were synthesized by Genemed Synthesis Inc. (South San Francisco CA) and dissolved in DMSO under nitrogen.

T-cell Cloning and Identifying the HLA restriction of LMP2 epitope

$CD8^+$ T-cell clones were generated from the LMP2-CTL line of patient 2 by limiting dilution cloning of the CTL on allogeneic feeder cells in interleukin 2-conditioned medium with irradiated LCL transduced with the Ad5f35 LMP2A adenoviral vector and pulsed with the 10-mer peptide KILLARLFLY identified from the reactive 15-mer. The specificity and HLA restriction of the clones was then determined by ELISPOT assay.

Flow cytometry

Expression of the LMP2A protein was analyzed by FACS 2 days after transduction. The anti-LMP2A MAb, derived from clone 8c3, was kindly provided by E. Kremmer (Munich, Germany). Briefly, transduced and control DCs or LCL were fixed for 20 minutes at RT with 4% paraformaldehyde, permeabilized for 30 minutes with 1% saponin (Sigma) and stained with primary rat anti-LMP2A Mab as described previously.[15] Expression of the surface molecules on DC were measured on non-fixed, non-permeabilized DCs using PEconjugated MAbs: anti-CD3, -CD16, -CD19, -CD56, (Becton Dickinson, Mountain View, CA) -CD83, (Caltag, San Francisco, CA) and -DR PerCP (Becton Dickinson) and FITC-, PE- and PerCP-conjugated, isotype-matched mouse IgGs were used as controls (Becton Dickinson). CTL lines were analyzed with anti-CD8 FITC, -CD56 PE, -CD3 PerCP, and CD4 PE antibodies (Becton Dickinson). The TCR Vβ analysis was performed on the CTL lines using the IOTest® Beta Mark TCR Vβ Repertoire Kit according to the manufacturers instructions (Immunotech, a Beckman Coulter Company, Marseille, France). Samples were acquired on a FACScan flow cytometer (Becton Dickinson) and the data analyzed using *CellQuest* software (Becton Dickinson).

Tetramer staining

Soluble HLA-A02*01-CLGGLLTMV, A02*01-FLYALALLL, A29*01-ILLARLFLY, A11*01-IVTDFSVIK, A11*01AVFDRKSDAK and B07*01-RPPIFIRRL PE-conjugated tetramers were prepared by the National Institute of Allergy and Infectious Diseases (NIAID) tetramer core facility (Atlanta, GA) and the Baylor Tetramer Core Facility (Houston TX). The CLGGLLTMV peptide was also prepared by Synthesis Inc. (South San Francisco CA). CTLs (1×10^6) were in-

cubated at RT for 30 min in PBS with 1% FCS containing the PE-labeled tetrameric complex. Samples were additionally incubated with anti-CD8 FITC and anti-CD3 PerCP. IgG-PE was used for isotype control. Stained cells were fixed in PBS containing 0.5% paraformaldehyde. Samples were analyzed by FACS as described.

Cytotoxicity assay

CTLs were tested for specific cytotoxicity against fibroblasts, either uninfected or infected with Ad5LMP2A or Ad5GFP. Autologous LCLs, and HLA class I-mismatched LCLs were also tested.[51] Cr-labeled target cells were mixed with effector cells at doubling dilutions to produce the effector: target (E: T) ratios specified. TargeT-cells incubated in complete medium or 5% Triton X-100 (Sigma) were used to determine spontaneous and maximal ^{51} Cr release, respectively. After 4 hours (LCLs) or 6 hours (fibroblasts), supernatants were collected and radioactivity was measured on a gamma counter. The mean percentage of specific lysis of triplicate wells was calculated as 100 x (experimental release - spontaneous release)/ (maximal release - spontaneous release).

Statistical analysis

The Student *t* test was used to test for significance in each set of values, assuming equal variance. Mean values ± SE are given unless otherwise stated.

Results

Expansion of LMP2-CTL in patients with relapsed Hodgkin Disease

We previously generated LMP2-specific CTLs (LMP2-CTL) in normal donors by repeated stimulation of peripheral blood mononuclear cells (PBMCs) with DCs expressing LMP2a from a recombinant adenovirus (Ad5).[15] This method induced CTLs that lysed LMP2a-expressing target cells more effectively than CTLs stimulated with LCLs. However, this approach required large numbers of dendritic cells as antigen presenting cells to activate and expand LMP2-specific CTL and Ad5LMP2-transduced DC were unable to expand LMP2-specific T-cells from patients with relapsed HD (data not shown).

To reduce the requirement for large numbers of DC, we used LCL over expressing LMP2 to expand CTL. Thus HD patient PBMCs were stimulated with Ad5f35LMP2A-transduced autologous DCs on day 0 and then with Ad5f35LMP2A-transduced autologous LCL on days 10 and 17, 25 and 32. The transduction efficiencies of the DC and LCL ranged from 48.8% to 63% (average 55.9%) and 0.8% to 8.7% (average 4.2%) respectively (data not shown). The resultant CTL lines, termed LMP2-CTL, were compared phenotypically to EBV-CTL lines generated using unmodified LCL as stimulators from day 0. Figure 1 shows that on day 24, all lines contained both CD4 and C8 cells, and that apart from a small increase in CD4 positive cells in the LMP2-CTL lines, there were no striking differences between LMP2 and EBV CTL.
To assess the repertoire diversity of our CTL lines, we used antibodies against the T-cell receptor beta chain v region. Flow cytometric analysis (Figure 2) confirmed that both EBV and LMP2-specific lines were polyclonal, and that the majority of Vβ families were represented. However, all the lines were dominated by relatively few families, the most extreme example being the LMP2-CTL line of patient 2, which was dominated by Vβ 5.1 and Vβ 13.6. Vβ usage differed between the LMP2 and EBV-specific CTL lines consistent with a skewed antigen specificity in the LMP-2 stimulated lines. Interestingly in both patients 1 and 2, only

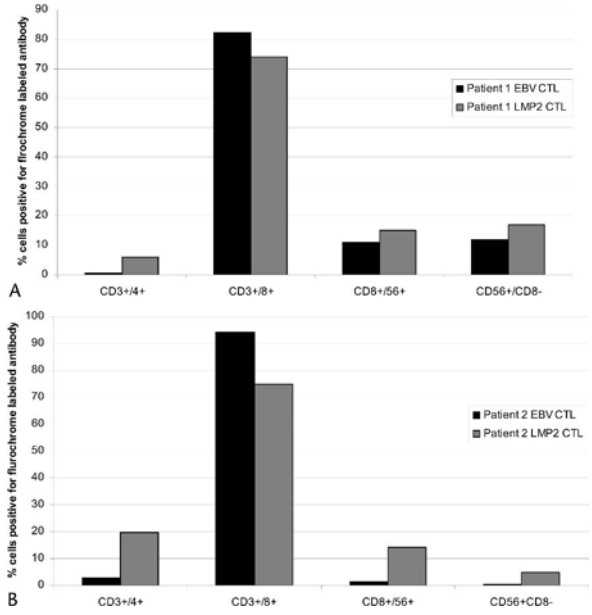


Figure 1. LMP2-CTL lines have more CD4 positive cells compared to EBV-CTL lines generated from the same patient. *LMP2 specific CTL (gray) and EBV specific CTL (black) lines generated from patients 1 (Figure 1a) and patient 2 (Figure 1b) were stained with antibodies against T-cell surface antigens CD3, CD4, CD8, and CD56. Surface immunofluorescence was analyzed by flow cytometry.*

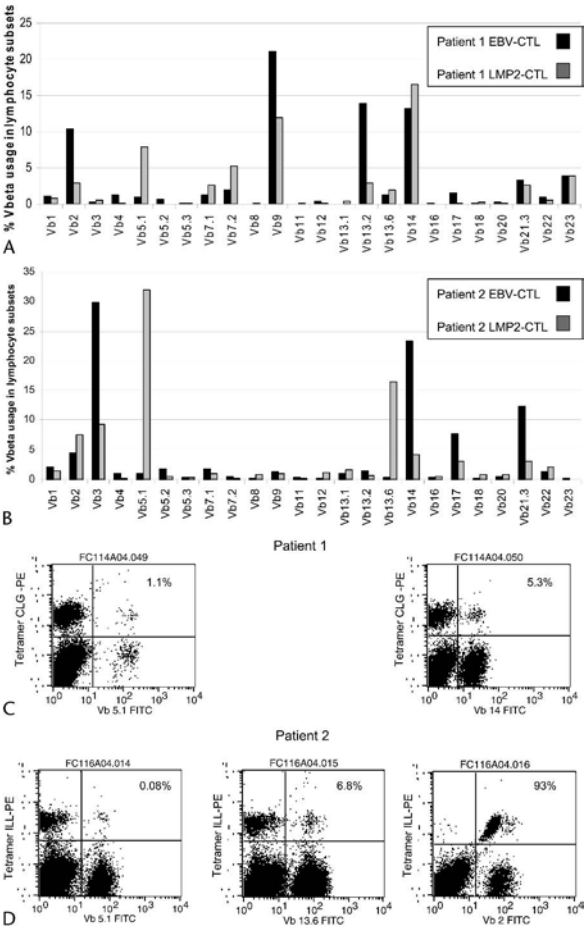


Figure 2. All CTL lines generated were polyclonal with unique Vβ repertoires. *Toanalyze the Vβ repertoires of the LMP2 specific (gray) and EBV specific (black) CTL lines, the CTL generated from patient 1 (Figure 2a) and patient 2 (Figure 2b) were stained with anti-CD3 and 24 Vβ antibodies grouped into 8 vials and surface immunofluorescence was analyzed by flow cytometry. To analyze the Vβ specificity of LMP2 tetramer positive cells the polyclonal LMP2 specific CTL lines from patient 1 (Figure 2c) and 2 (Figure 2d) were stained with anti-CD8 PerCP and either CLGtetramer- PE (patient 1) or ILL-tetramer-PE (patient 2) and single FITC-labeled Vβ antibodies.*

a relatively small proportion of the tetramer positive cells were positive for the dominating Vβ's tested on the polyclonal lines and the epitope specific T-cells demonstrated varying Vβ specificities (Figures 2c and 2d). In patient 1, Vβ 5.1, 9 and 14 predominated on the polyclonal LMP2-CTL line (Figure 2a). A FITC-labeled Vβ 9 antibody was not available. However, using the HLA A2 restricted LMP2 tetramers CLG and FLY we showed that no FLY positive cells were positive for these Vβ subtypes. (data not shown) In contrast, 1.1% of CLG tetramer positive cells were positive for Vβ5.1 and 5.3% were positive for Vβ14. (Figure 2c) In patient 2, the HLA A11 restricted LMP2-tetramer (SSC) positive population in the polyclonal line was negative for both Vβ5.1 and 13.6 (data not shown). Only 0.08% of the HLA A29 restricted LMP2-tetramer (ILL) positive population in the polyclonal line were positive for Vβ5.1, 7% were positive for Vβ13.6 and 93% positive for Vβ 2. (Figure 2d) However, as shown in Figure 2d there are substantial populations that are positive for Vβ 5.1, and 13.6 that are negative for this tetramer.

Figure 3a and 3b shows that the LMP2-CTL and EBV-CTL generated from patients 1 and 2 grew at similar rates. Hence, while the growth characteristics of the patient CTL lines were essentially the same, the Vβ usage differences were suggestive of an altered specificity.

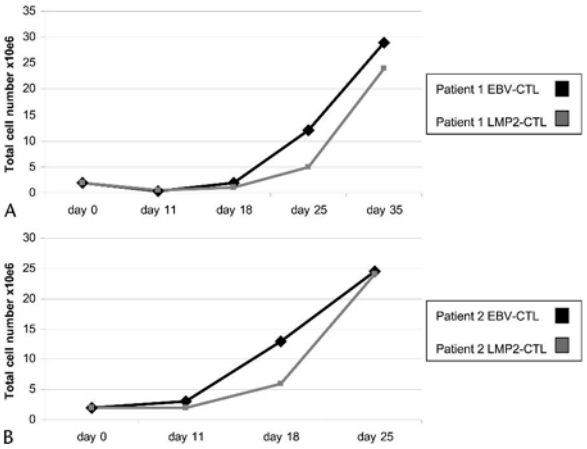
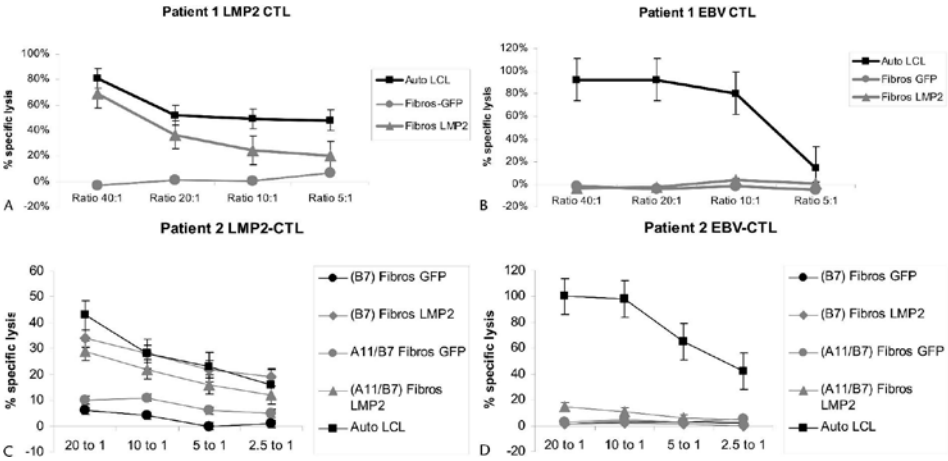


Figure 3a and 3b. The expansion rates of autologous EBV-specific versus LMP-2–specific CTL were similar.
For the generation of EBV-specific CTL (EBV-CTL), a total of 2x10⁶ peripheral blood mononuclear cells (PBMC) were activated with autologous lymphoblastoid cell lines (LCL) and were stimulated weekly with LCL and fed twice weekly with IL-2. LMP2-specific CTL (LMP2-CTL) were activated with Ad5f35LMP2- transduced dendritic cells (DC) followed by weekly stimulations with Ad5f35LMP2- transduced LCL (LMP2-CTL) and twice weekly IL-2 feeds. Figure 3a (patient 1) and Figure 3b (patient 2) represent CTL cell numbers (x10⁶) recorded from weekly cell counts comparing the expansion rates of EBV-CTL (black) versus LMP2-CTL (gray).

Patient-derived LMP2A-specific CTLs kill Ad5LMP2 Fibroblast targets.

The cytolytic activity of the responder cells was tested against a panel of ^{51}Cr-labeled autologous and allogeneic target cells. LMP2-specific CTLs generated from patient 1 killed autologous fibroblasts only if transduced with Ad5LMP2A (68% at an E: T ratio of 40:1 (Figure 4a)) whereas the EBV-specific CTL line demonstrated no LMP2 specific activity (Figure 4b). Both EBV and LMP2 CTL lines lysed autologous LCLs. Similarly, LMP2-CTLs generated from patient 2 lysed both autologous LCLs and Ad5LMP2Ainfected fibroblasts matched at HLA A11*01 and B07*01 (Figure 4c). By comparison, EBV-CTL generated from patient 2 showed strong cytolytic activity against autologous LCL (100% at E: T ratio of 20:1) but little lysis of HLA A11 fibroblasts expressing LMP2 (15% specific at an E: T ratio of 20:1) and no killing of

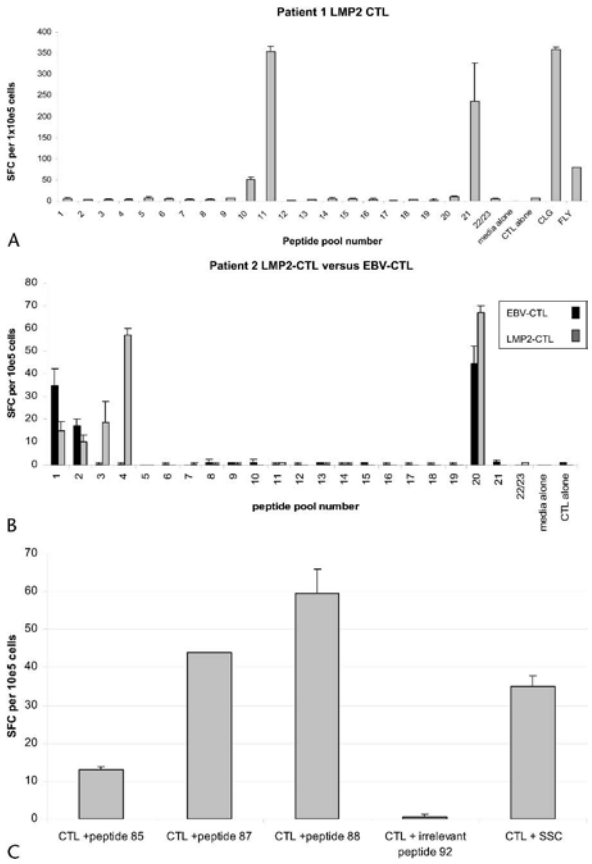


Figures 4a-4d LMP2-specific CTL demonstrate specific lysis of Ad5LMP2- transduced fibroblasts but not Ad5GFP-transduced fibroblasts.
Percent specific ^{51}Cr release was determined 6-hours after coincubation with fibroblasts transduced with Ad5LMP2 (gray triangle), or Ad5GFP (gray circle) and autologous LCLs (black square). The percent specific lysis at the indicated effector to target ratios for the LMP2-CTL line (Figure 4a) and EBV CTL line (Figure 4b) from patient 1 and the LMP2-CTL line (Figure 4c) and EBV CTL line (Figure 4d) from patient 2 are shown. The fibroblast targets used for patient 1 were autologous. In absence of autologous fibroblasts for patient 2, allogeneic fibroblasts class I HLA matched at A11 and B7 (gray triangle) or B7 only (gray diamond) were transduced with Ad5LMP2 and used as targets. As controls, HLA A11;B7 matched (gray circle) and HLA B7 matched (black circle) fibroblasts transduced with Ad5GFP were also tested in this assay.

HLA B07*01-matched fibroblasts expressing LMP2 (Figure 4d). 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 untransduced fibroblasts or HLA-mismatched LCLs (data not shown).

LMP2 epitope specificity was detected in patient CTL lines using LMP2 peptide pools

To characterize the epitope specificity of the CTL lines, they were incubated with LMP2 peptide pools and IFNγ release was measured by ELISPOT assays. Patient 1 had a detectable T-cell response to 15-mer peptides from pools 10, 11 and 21 (Figure 5a). The 15-mers common to these three pools were 106 (GPVFMCLGGLLTMVA) and/or 107 (MCLGGLLTMVAGAVW) spanning the region 421-439. Both peptides contain the known HLA A02*01 LMP2 9-mer epitope CLGGLLTMV (CLG). Subsequent ELISPOT analysis revealed that the LMP2-CTL line shows specificity for the CLG peptide (Figure 5a). There was additional specificity for another known HLA A02*01 LMP2 9mer epitope FLYALALLL (FLY) (Figure 5a), even though reactivity with peptide pools 4, 5, and 20, which also contain the FLY peptide, was not seen. By contrast, analysis of lines prepared with nontransduced LCL stimulators, showed no IFNγ release with any LMP2 peptide pool as targets (data not shown).



Figures 5a-5c. Identification of CD8+ T-cell epitopes within LMP2 using IFNγ- ELISPOT and a 15-mer (11-mer overlapping) peptide library.

*CTL were plated at 1x10^5 cells per well and results are expressed as spot forming cells (SFC) per 10^5 cells. Figure 5a shows the reactivity of the LMP2-CTL line from patient 1 with the peptide pools and two known HLA A02*01 restricted 9mer peptides CLGGLLTMV (CLG) and FLYALALLL (FLY). For patient 2 (Figure 5b) the EBV-CTL line (black) and the LMP2-CTL line (gray) are screened against the 15-mer LMP2 peptide library. LMP2-specific CTL from patient 2 were then tested against separate 15-mer peptides within pools 1, 3, 4 and 20 and the known HLA A11*01 restricted 11-mer peptide SSCSSCPLSKI (SSC)(Figure 5c). As a negative control the CTL are also incubated with an irrelevant 15-mer peptide not contained within pools 1, 3, 4, and 20.*

The LMP2 CTL line from patient 2 had a detectable T-cell response to pools 1,2,3,4 and 20 (Figure 5b). The most prominent responses were seen with peptide pools 3, 4 and 20, all of which contain peptides 87 (CPLSKILLARLFLYA) and/or 88 (KILLARLFLYALALL) (region 345-364 of LMP2). Peptide pools 1, 2 and 20 also contained the 15-mer peptides LICSSCSSCPLSKIL and SCSSCPLSKILLARL (#87 and 88) which incorporate the known HLA A11*01 restricted LMP2 epitope SSCSSCPLSKI (SSC) (region 337-352). There is a marked response to two 15-mer peptides (#87 and 88) tested separately and to another peptide (#85) that contained the SSC epitope. (Figure 5c) Testing of this patient's EBVspecific CTL line generated using non-transduced LCL as stimulators, showed a more limited ability to recognize LMP-2 epitopes, with responses only to LMP2 peptide pools 1,2 and 20 consistent with specificity for the SSC epitope only (Figure 5b).

Detection of a new HLA A29*01 LMP2-specific epitope using ELISPOT assay

To identify the minimal epitope contained in peptides 87 and 88 (CPLSKILLARLFLYA and KILLARLFLYALALL), we used shortened sequences within the 345-364 region of LMP2 as shown in Table 1 predicted based on published HLA A29 restricted T-cell epitopes.[22] Serial dilutions of the peptides were incubated with the LMP2 CTL from patient 2. CTL incubated with peptide 2 KILLARLFLY (KIL), secreted IFN-γ at all peptide concentrations in contrast to other peptides tested (Figure 6a). In limiting dilution, the 9 mer ILLARLFLY (ILL) (peptide 7) and 10-mer KIL induced similar levels of IFNγ release, (Figure 6a) therefore ILL was determined to be the minimal epitope.

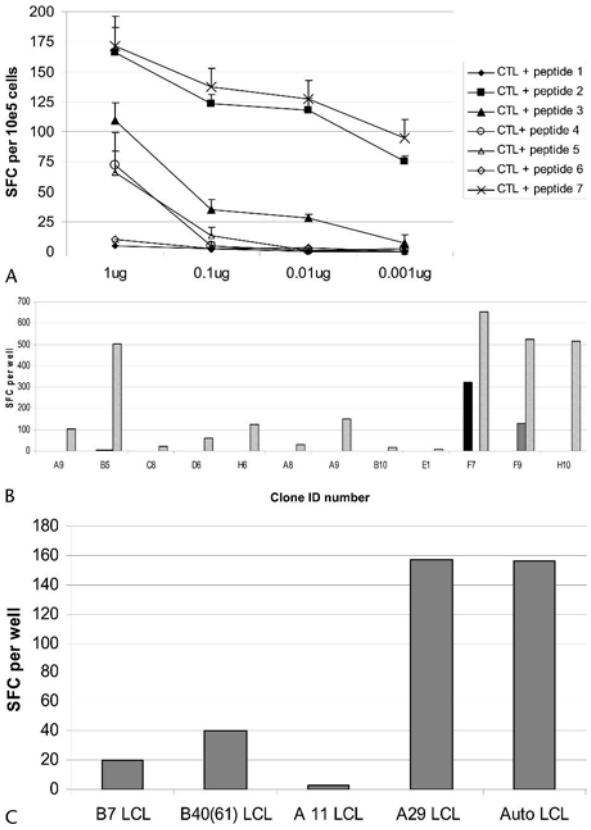


Figure 6a-6c. Identification of new HLA29*01 restricted LMP2 epitope.
Figure 6a shows the polyclonal LMP2-specific CTL line generated from patient 2 screened against shortened peptides listed in Table 1 comprising the region 345-360 of the LMP2 antigen using an IFNγ ELISPOT assay. Log dilutions of each peptide were performed before adding to the CTL. The CTL were plated at 1x10⁵ cells/well and results are expressed as spot forming cells (SFC) per 10⁵ cells. In Figure 6b LMP2-CTL clones generated from patient 2 were screened against autologous LCL (gray bars), KILLARLFLY (KIL) peptide (black bar) and PMA-ionomycin (gray stripes). As a negative control, clones were incubated in media only (black stripes). The CTL clones were plated at 10 μL/well and results are expressed as spot forming cells (SFC). Figure 6c shows the HLA restriction of clone F7. Allogeneic LCL matched at one class I locus with patient 2 whose HLA type was A11, A29, B7, B40(61) were irradiated, incubated with the KIL peptide for 1 hour and then washed and plated at 1x10⁵ cells per well. As a positive control, irradiated autologous LCL pulsed with KIL peptide were also plated as above. 10 μl of clone F7 was added to each LCL target and results are expressed as spot forming cells (SFC).

Table 1. Minimization of the LMP2-Specific CD8+ T-Cell Epitope KILLARLFLYA (Region 345–364) Detected Using the IFN-γ ELISPOT and the 15 Mer LMP2 Peptide Library

Peptide identification number	Sequence	Region
1	KILLARLF	349–356
2	KILLARLFLY	349–358
3	LLARLFLY	351–358
4	LLARLFLYA	351–359
5	LLARLFLYAL	351–360
6	KILLARLFL	349–357
7	ILLARLFLY	350–358

To determine the HLA restriction of this new LMP2 epitope, single cell clones were expanded from the LMP2-CTL line from patient 2 and tested against the KIL peptide or LCL (Figure 6b). One clone (F7) recognized KIL but not the autologous LCL. This clone was then incubated with 4 allogeneic LCL lines, matched at one class I locus each. These LCL were also pulsed with KIL. Clone F7 reacted most strongly to the HLA A29*01 LCLpulsed with KIL peptide cells, and to a minor extent with LCL targets expressing A11*01, B7*01 or B40 (61). These data indicate that this LMP2 epitope is A29*01 restricted (Figure 6c). Despite the ELISPOT results showing that ILL and KIL and induced equal responses at all concentrations, the ILL peptide was able to fold easily into the HLA A29 tetramer compared to the KIL peptide thus confirming that ILL was the minimal epitope of the KIL peptide.

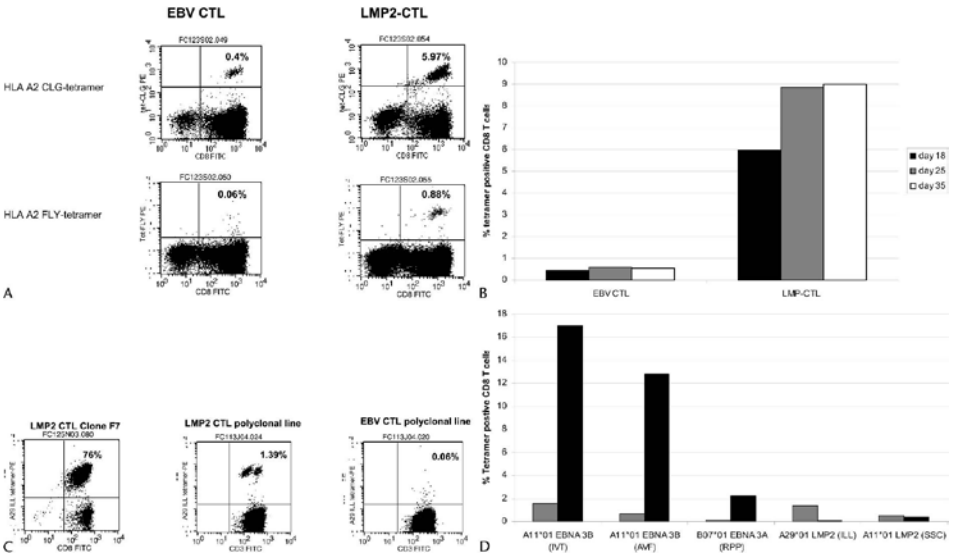


Figure 7a-7d LMP2-expressing dendritic cells effectively increase the LMP2 tetramer-specific CTL populations compared to unmodified LCL.

The specificity of EBV CTL or LMP2 CTL from patient 1 were compared by tetramer analysis using HLAA02 01-CLGGLLTMV (CLG) and HLA-A02*01-FLYALALLL (FLY) tetramers on days 18, 25 and 35 (Figures 7a and 7b). In Figure 7a, the percentage of FLY and CLG tetramer+CD8+ cells is indicated on histograms. Figure 7b shows the percentage CLG tetramer+CD8+ cells over time in the EBV-CTL line versus the LMP2-CTL line. In Figure 7c the EBV-CTL line, the polyclonal LMP-CTL line and the LMP2-CTL clone F7 generated from patient 2 were analyzed using the A29*01-ILLARLYFLY (ILL) tetramer. The percentage of ILL tetramer+CD8+ cells is indicated on histograms. Figure 7d compares the percentage of tetramer positive CD8+ cells specific for the HLA 11*01 restricted LMP2 peptide SSC and the HLA A29*01 restricted LMP2 peptide ILL with HLA A11*01 restricted EBNA 3B peptides IVTDFSVIK (IVT) and AVFDRKSDAK (AVF) and the B07*01 restricted EBNA 3A peptide RPPIFIRRL (RPP) in the EBV-CTL versus LMP2-CTL lines generated on patient 2.*

The frequency of LMP2 epitope-specific CTL is increased by stimulation with Ad5f35LMP2 transduced stimulator cells

To confirm the results of the ELISPOT assays we used tetramer analysis with the CLGA02*01 (A2-CLG) and FLY-A02*01 (A2-FLY) tetramers to estimate the frequency of LMP2A-specific CTLs before and after stimulation with LMP-2a transduced DCs and LCL. In the unstimulated peripheral blood lymphocytes of patient 1, 0.05% and 0% of CD8+ T-cells were positive for the A2-CLG tetramer and A2-FLY tetramers respectively (data not shown). After stimulation with Ad5f35LMP2A-transduced DC and LCL, 5.97% of $CD8^+$ cells reacted with the A2-CLG tetramer and 0.88% with the A2-FLY tetramer (Figure 7a,b). In comparison, unmodified autologous LCLs as stimulators induced only 0.4% of EBV-CTLs specific for the A2-CLG tetramer and 0.06% for the A2-FLY tetramer. For Patient 2 we used the 9-mer ILL-A29*01 tetramer (A29-ILL). A29-ILL tetramer reactive T-cells could not be detected in unstimulated PBMC nor in the EBV-specific CTL line generated using non-transduced LCL. By comparison, 1.39% of CD8+ cells were detected in the LMP2-CTL line (Figure 7c). Only 0.5% of the LMP2-CTL line from patient 2 reacted with the known HLA-A11*01 restricted SSC epitope using tetramer analysis. This was similar to the frequency seen in the EBV-CTL line (Figure 7d). A significant population of the EBV-CTL from Patient 2 were specific for the known immunodominant HLA A11*01 EBNA 3B epitopes IVTDFSVIK (IVT) and AVFDRKSDAK (AVF) as well as for the B07*01 EBNA 3A peptide RPPIFIRRL (RPP) (Figure 7d). In contrast, LMP2-CTLs generated from this same patient show a decreased frequency for these immunodominant EBNA epitopes (Figure 7d). These data indicate that stimulation of patient T-cells using DC and LCL expressing LMP-2a selectively expands clones capable of recognizing LMP2 specific tetramers/peptides, whilst reducing the proportion of CTL specific for conventionally immunodominant EBV antigens such as EBNA 3A, B or C. Finally, we also observed that the frequency of peptide positive cells is higher using tetramer assay compared to ELISPOT analysis. In the LMP2-CTL line from patient 1 at total of 5.97% of CD8+ve cells recognized the CLG peptide using tetramer analysis (Figure 7a) compared to 352 spots/ 100,000 cells (0.35%) observed using ELISPOT (Figure 5a). A difference was also seen in the LMP2-CTL line from patient 2 where 1.39% of CD8+ve cells were positive for the ILL peptide (Figure 7c) versus 0.08% cells producing IFNγ in response to this peptide (data not shown). Thus ELISPOT can be used to identify specificity, but does not reveal the frequency of CTL specific for the identified epitopes.

Discussion

One means of improving immunotherapeutic strategies against EBV+ tumors in immunocompetent patients is to specifically target subdominant antigens such as LMP2 that are expressed as part of the Type II latency pattern of virus gene expression. Efforts to expand LMP2-specific CTL by stimulating PBMC with Ad5LMP2 transduced DC and expanding with transduced DC or non-transduced LCL were successful for normal donors, who have measurable level of CTL reactive with LMP2 and whose DC and CTL are uncompromised and expand readily [15]. However this approach consistently failed when patient samples were used. We now describe an approach to generate LMP2 specific CTL that is effective in patient with relapsed HD and that avoids the requirement for multiple stimulation with DCs, a technical challenge in patients heavily treated with chemoradiotherapy. [23] Our method takes advantage of an Ad5F35 vector that readily transduces B-LCL, allowing them to express high levels of transgenic LMP2a. [12] A sequence of one stimulation with LMP2a expressing DCs, to specifically reactivate LMP2- specific T-cells, followed by expansion with multiple stimulations with LMP2a-LCL substantially enriches CTL specific for LMP2a protein and peptides, whilst also decreasing the proportion of cells specific for other, conventionally immunodominant, EBV antigens. Hence by transducing DCs and LCL with LMP2a and using them as stimulators, we can bias recognition towards the subdominant LMP2A antigen rather than the highly immunodominant EBNA epitopes. Our approach significantly reduced the volume of patient blood necessary to generate the LMP2-specific CTL (LMP2-CTL) when compared to generating the LMP2-CTL using DC as the primary APC. Further, the LMP2-CTLs were generated in large numbers with growth kinetics similar to the conventional EBV-CTL lines generated from the same patients. The use of whole protein antigen expressed as a transgene from recombinant adenovirus is advantageous over single peptide antigens, since host alleles are able to select multiple HLA-restricted epitopes for presentation. By contrast, the use of single peptides restricts patient eligibility based on their HLA type and increases the chance that the antigen chosen may not be expressed by endogenous virus.

Analysis of single HLA-restricted tetramers within a polyclonal CTL line may significantly under-estimate the frequency of LMP2-specific CTLs. This is highlighted by patient 2 for whom the only previously identified LMP2-restricted epitope was the HLA A11*01- restricted epitope SSC. Only 0.5% of LMP2-CTLs from patient 2 were specific for this SSC epitope using tetramer analysis (data not shown). To identify other LMP2 epitopes we screened the LMP2-CTL lines using LMP2 peptide pools spanning the entire LMP2A antigen. We identified a new LMP2 epitope which mapped to HLA A29*01, and revealed a frequency of CTL specific for the new LMP2 epitope (ILL) that was 2 fold greater than for the previously defined A11*01-LMP2 SSC epitope. Thus the cocktail library will aid in characterizing LMP2 epitopes in CTL lines for which LMP2 epitopes have not yet been identified, and will assist the tracking of infused cells in peripheral blood.

While LMP2 peptide pools may identify multiple epitopes recognized by CTL, even this method underestimates the true breadth of the response to the viral antigen. Class I presented peptides are usually 8-10 amino acids long, and degradation of the 15-mer peptides relies on the proteasome and surface proteases.[24] It is however not clear how effective this machinery is in T-cells versus professional antigen presenting cells. Hence, 15-mer peptides

added directly to T-cells may fail to elicit responses because of a lack of proteolysis of the 15-mer to an presentable 8 to 10-mer.[24 25] This problem was observed in patient 2, where the detection of HLA-B7 restricted LMP2 killing implies that epitopes not identified by the cocktail pools were also targeted. Similarly, in patient 1 only one epitope (the HLA A02*01 restricted CLG peptide) was identified using the peptide pool screening, but additional testing revealed reactivity with the known HLA A02*01 restricted FLYALALLL (FLY) 9-mer LMP2 peptide which is unusual in its dependence upon the immunoproteasome.[26;27] These problems may be specific to T-cells as APC. Of note, comparison of peptide-specific T-cell precursor frequencies by tetramer and ELISPOT analysis (Figures 5a, 5c, and 6a versus Figures 7a, 7c and 7d) shows that in concordance with previously published results performed on PBMC, the ELISPOT assay underestimates epitope-specific T-cell frequency in CTL lines by about tenfold, compared to the tetramer assay. [28;29] This may be because fewer activated T-cells secrete γ-IFN in response to specific antigen or because of activation-induced cell death in the responder population. Thus, while the ELISPO assay is useful in characterizing the epitope specificity of the CTL line, the frequencies obtained by ELISPOT do not reflect the true frequency of epitope-specific T-cells within each CTL line.

The HLA restriction of the ILL epitope was determined using T-cell clones derived from the polyclonal LMP2-CTL line. It is of note that the ILL-specific clone secreted IFNγ when incubated with the ILL peptide but not with the autologous LCL and that the EBV-CTL line generated using autologous LCL also lacked detectable ILL–specificity. This may be because a low affinity clone was selected by the cloning procedure, or that the unmodified LCL does not present the ILL peptide. Thus to generate ILL-specific CTL, over expression of LMP2a following gene transfer was required. Low-level expression of LMP2 epitopes by unmodified LCLs may be related to requirements such as immunoproteasome dependence for the generation of this peptide within LCL and/or tumor cells. [26] This is supported by the fact that the ILL peptide was calculated to be highly hydrophobic (8.73) which is predictive for proteasome dependence.[26;30] However the existence of precursor T-cells specific for this LMP2 epitope suggests that it may be relevant *in vivo*. Further work is therefore required to study the requirements for the generation of this and other "tumorassociated" epitopes within cells and determine whether these requirements are met in the Hodgkin Reed Sternberg cells themselves to allow killing of the tumor cells by the epitope-specific CTL.

In conclusion, the use of DCs and LCLs transduced with an LMP2A-encoding adenovirus vector efficiently stimulates and expands LMP2-specific CTLs derived from patients with relapsed Hodgkin Disease. Expression of the whole protein allows the activation of both CD4 and CD8 T-cells important for persistence and presentation of as yet undefined antigen epitopes, such as the new HLA A29*01 restricted epitope we describe. This approach is now being used in clinical adoptive immunotherapy protocols targeting EBV+ Hodgkin disease.

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