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Functional Reversion of Antigen-Specific CD8⁺ T Cells from Patients with Hodgkin Lymphoma following In Vitro Stimulation with Recombinant Polyepitope

Corey Smith,* Leanne Cooper,* Melinda Burgess,* Michael Rist,* Natasha Webb,* Eleanore Lambley,* Judy Tellam,* Paula Marlton,† John F. Seymour,‡ Maher Gandhi,*† and Rajiv Khanna*‡

Recent studies on Hodgkin’s lymphoma (HL) have indicated that patients with active disease display functional impairment of Ag-specific CD8⁺ T cells due to expansion of regulatory T cells at sites of disease and in the peripheral blood. Adoptive cellular immunotherapy based on EBV-specific CD8⁺ T cells has been explored with limited success to date. It has been proposed that improved targeting of these CD8⁺ T cells toward viral Ags that are expressed in HL may enhance future therapeutic vaccine strategies. In this study, we have developed a novel replication-deficient adenoviral Ag presentation system that is designed to encode glycine alanine repeat-deleted EBV nuclear Ag 1 covalently linked to multiple CD8⁺ T cells from healthy virus carriers, and patients with HL with this adenoviral construct in combination with IL-2, was sufficient to reverse the functional T cell impairment and restored both IFN-γ production and cytolytic function. More importantly, these activated CD8⁺ T cells responded to tumor cells expressing membrane proteins and recognized novel EBNA1 epitopes. Flow cytometric analysis revealed that a large proportion of T cells expanded from patients with HL were CD62L⁺ and CD27⁺, and CCR7⁻, consistent with early to mid effector T cells. These findings provide an important platform for translation of Ag-specific adoptive immunotherapy for the treatment of EBV-associated malignancies such as HL and nasopharyngeal carcinoma. The Journal of Immunology, 2006, 177: 4897–4906.

Immunosurveillance by CTLs plays a critical role in the detection and killing of a wide range of malignant cells (1, 2). Conversely, the ability to evade recognition by CTL is thought to promote the survival and spread of malignant cells (3, 4). Unlike a number of other malignancies, Ag processing and presentation functions remain intact in the EBV-associated malignancies, Hodgkin’s lymphoma (HL), and nasopharyngeal carcinoma (NPC) (5, 6). Recent studies have provided evidence that the onset of HL is coincident with an expansion in regulatory T cells and the presence of an indolent phenotype in Ag-specific cells, which have impaired production of IFN-γ ex vivo in response to CD8⁺ T cell epitopes from the HL-associated EBV Ags, including latent membrane protein (LMP)-1 and -2 (7, 8). This suggests that the immune response may be subverted by regulatory T cell-mediated suppression of the LMP-specific T cells, leading to failure to clear malignant cells. Restoration of normal immunological function in these T cells occurs following remission from active HL (8).

Although successful in the majority of cases, current therapies used to treat HL and NPC are less effective in patients who relapse or have advanced stage disease (9). Therefore, augmentation of T cell responses against the HL and NPC-associated Ags, LMP1, LMP2A, and EBV nuclear Ag 1 (EBNA1), either via the adoptive transfer of CTL or therapeutic vaccination, offers an attractive alternative to current treatment strategies (1, 10–13). CTL immunotherapy has been effectively used in the past to treat EBV-associated posttransplant lymphoproliferative disorder (14–17). The lymphoblastoid cell line (LCL)-mediated expansion of CTL used to treat posttransplant lymphoproliferative disorder typically yields T cells reactive against the immunodominant EBNA3 Ags, which are not expressed in HL and NPC (14, 15). Although LCL-mediated stimulation has been shown to induce the expansion of LMP-specific T cells, these cells typically constitute only a small fraction of the resulting CTL population, and immunotherapy with LCL-expanded CTL has usually had little therapeutic effect in advanced HL or NPC (10, 11). Strategies aimed at generating T cells specific for the HL and NPC-associated Ags are therefore required to optimize development of Ag-specific CTL for immunotherapy.

One strategy under current investigation within our laboratory involves activation of virus-specific CD8⁺ T cells using polyepitope technology (12, 13). The polyepitope technology allows expression of weak CD8⁺ T cell epitopes as a string-of-beads without flanking sequences, which limits any potential oncogenic effects of LMP sequences. In a recent study, we demonstrated that immunization of HLA class I transgenic mice with this polyepitope-based vaccine was effective in inducing Ag-specific CD8⁺
T cell responses (12, 13). Translation of these findings to patients with HL is constrained by the impairment of Ag-specific T cell function in vivo and immune evasive mechanisms used by malignant cells (1, 18). To overcome these potential limitations, we have developed a novel T cell activation strategy based on polyepitope technology, which completely reverses the impaired T cell function seen in patients with HL, and rapidly expands CD8/HLA class I-restricted T cells specific for LMP1, LMP2A, and EBNA1. More importantly, these expanded T cells efficiently recognize tumor cells expressing a limited array of EBV Ags.

Materials and Methods

Construction and expression of EBNA1-LMP polyepitope

EBNA1-coding DNA without the glycine alanine (GA) repeat (EBNA1GA) (19) was amplified by PCR and cloned into the BamH1/EcoRI sites of pBluescript KS (Stratagene). Previous studies have shown that GA repeat within EBNA1 inhibits its self synthesis and blocks proteasomal degradation (20). Therefore, to optimize EBNA1 expression and endogenous processing, the GA repeat was removed. A synthetic polyepitope nucleotide sequence, including 16 HLA class I-restricted CD8+ T cell epitopes, was constructed using epitope sequence-specific primers and a technique based on mutual priming and overlap extension as described in Material and Methods. This E1-LMPpoly insert was initially cloned into a pShuttle vector and then subcloned into Ad5F35 expression vector. The recombinant Ad5F35 vector was packaged into infectious adenovirus by transfecting HEK 293 cells, and recombinant adenovirus (referred to as AdE1-LMPpoly) was harvested from transfected cells by repeated freeze-thawing cycles.

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Table I. List of donors used in this study

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<th>Gender</th>
<th>Histology</th>
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*All volunteers were serologically EBV positive. Advanced stage classified as stages IIb/III/IV, staged as per Ann Arbor Criteria (with Cotswold modification). NS, Nodular sclerosing; MC, mixed cellularity; LR, lymphocyte rich; NL, nodular lymphoma; NA, not applicable; PD, Postdiagnosis; M, male; F, female.
were selected using G418. Expression of LMP1 or GFP was confirmed by immunoblotting. These cell lines were maintained in growth medium, whereas the HEK 293 cell line was maintained in DMEM containing 10% FCS.

### Expansion of LMP/EBNA1-specific T cells from healthy donors and patients with HL

A panel of 14 human volunteers (4 healthy EBV carriers and 10 patients with HL) were recruited for this study (for clinical details, see Table I). Each volunteer was asked to sign the consent form as outlined in the institutional ethics guidelines. For the expansion of specific T cells, PBMC were cocultured in multiwell tissue culture plates in growth medium with either autologous-irradiated LCLs (8000 rad) or PBMC (2000 rad) infected with AdE1-LMPpoly (multiplicity of infection (MOI) of 10:1) at a responder to stimulator ratio of 2:1. On day 3, and every 3–4 days thereafter, the cultures were supplemented with growth medium containing rIL-2 (donated by National Institutes of Health AIDS Research and Reference Reagent Program), IL-7, and/or IL-15 (donated by Amgen). These T cell cultures were assessed for EBV epitope-specific reactivity on days 10–17. In some experiments, a polyepitope construct based on HLA class I-restricted human CMV epitopes (23) was also used in this study (referred to as AdCMVpoly).

### IFN-γ ELISPOT assay

The ELISPOT assay used has been described previously (24). Briefly, 96-well multiscreen plates were coated overnight at 4°C with 1 μg/ml anti-human IFN-γ mAb 1-D1K (Mabtech). The plates were washed with PBS and blocked with culture medium. Between 2.5 × 10⁴ and 2 × 10⁵ cells/well were incubated for 24 h at 37°C with 1 μg/ml relevant peptide (Mimotopes). The plates were washed to remove cells, and cytokine bound to the nitrocellulose was detected using biotinylated anti-human IFN-γ, 7-B6-1 (Mabtech), followed by streptavidin alkaline phosphatase (Sigma-Aldrich). ELISPOTs were developed using the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich) and counted automatically using image analysis software.

### MHC-peptide pentamer staining and T cell phenotyping

MHC-peptide pentamers were supplied by Prolimmune. MHC-peptide pentamers for the HLA A2-restricted epitopes CLGGLLLTMV (LMP2A), YL LEMLWRL (LMP1), YLQQNWWTTL (LMP1), and FLYALALL (LMP2A), the HLA A1-restricted epitope SSCSSCPLSKI (LMP2A), and the HLA B35-restricted epitope HPVGEADYFEY (EBNA1) were used to detect epitope-specific CD8⁺ T cells. PBMC or cultured T cells were incubated with PE-labeled pentamers for 30 min at 4°C, washed, and incubated for a further 30 min at 4°C with PE-Cy5-conjugated anti-CD8 and with FITC-conjugated anti-CD27, anti-CD38, anti-CD62L, or an appropriate isotype control. For CD127 and CCR7 staining, following pentamer staining, cells were incubated with anti-CD127 or anti-CCR7 for 30 min at 4°C. Cells were then washed and incubated with FITC-conjugated anti-mouse Ig for 30 min at 4°C, washed, and incubated with 10% mouse serum, washed again and incubated with PE-Cy5 conjugated anti-CD8. Cells were then analyzed on a FACSCanto (BD Biosciences). Abs were supplied by BD Pharmingen and Caltag Laboratories.

### Cytotoxicity assay

Target cells were infected with recombinant vaccinia virus (MOI 10:1). Following incubation for 5–6 h, target cells were washed in growth medium, labeled with ⁵¹Cr, and used as targets in standard 5-h ⁵¹Cr-release assays (25). In some assays, the HRS cell line L1236 expressing LMP1 or GFP were also used as target cells.

### Intracellular cytokine staining

T cells stimulated with AdE1-LMPpoly were incubated for 5 h at 37°C with LMP or EBNA1 peptide epitopes (1 μg/ml) in growth medium supplemented with brefeldin A (BD Pharmingen), or with FITC-conjugated anti-CD107a and monensin (BD Pharmingen) for analysis of CD107a and IFN-γ expression. These cells were then washed and incubated with PerCP-conjugated anti-CD8, FITC-conjugated anti-CD4, andallophycocyanin-conjugated anti-CD3 at 4°C for 30 min. Cells were washed, then fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) at 4°C for 20 min. Cells were washed in perm/wash (BD Pharmingen), incubated with PE-conjugated anti-IFN-γ (BD Pharmingen) at 4°C for 30 min, washed again with perm/wash, resuspended in PBS, and analyzed on a FACSCanto (BD Biosciences).

### Results

#### Comparison of AdE1-LMPpoly and LCL-mediated expansion of LMP1 and LMP2A-specific T cells

Despite repeated attempts to exploit adoptive immunotherapy as a therapeutic tool for advanced HL and NPC, polyepitopic LCL-stimulated virus-specific T cells have achieved only limited success in clinical trials, with eventual disease progression or relapse (1, 26, 27). Although a number of possible tumor-associated mechanisms may be responsible for the poor efficacy, the most evident limitation of the current strategy of generating Ag-specific T cells is that the LCL-based stimulation limits the expansion of T cells that are relevant for controlling the expansion of HRS cells (18). Over the last few years, a number of attempts have been made to modify T cell activation strategies to preferentially expand T cells specific

FIGURE 2. Expansion of LMP1 and LMP2A-specific T cells following AdE1-LMPpoly stimulation. PBMC from three different healthy EBV carriers (D1, D2, and D3) were incubated with irradiated AdE1-LMPpoly-infected (MOI 10:1) autologous PBMC or LCLs at a ratio of 2:1. These cultures were supplemented with rIL-2 (10 U/ml) on day 3 and every 3–4 days thereafter. A, Assessment of IFN-γ production by AdE1-LMPpoly, LCL-stimulated or ex vivo T cells using ELISPOT assays as described in Material and Methods. The epitopes tested for T cell reactivity were CLG, FLY, TYG from LMP2A and YLL, YLQ and IAL from LMP1. The results are expressed as spot forming cells (SFC)/10⁶ cells. B, MHC-peptide pentamer staining of LMP1 or LMP2A-specific T cells. AdE1-LMPpoly-stimulated cells were incubated with PE-labeled MHC-peptide pentamers specific for CLG (LMP2A), FLY (LMP2A), or YLL (LMP1) and tricolor-labeled CD8 Ab. Data presented in upper right quadrant is the percentage of MHC-peptide pentamer-positive cells within the total lymphocytes. C, CTL lysis of target cells expressing LMP2A or LMP1 Ags by AdE1-LMPpoly-stimulated T cells. Target cells used in these assays were MHC-matched human fibroblast cell line infected with Vacc.LMP2A or a HRS cell line, L1236 stably expressing LMP1 protein. Target cells infected with Vacc.TK- or expressing GFP alone were used as control. These CTL effectors were tested at the E:T ratios of 20:1 or 10:1.
for LMP1 or LMP2A Ag (28, 29). An ideal strategy should result in the expansion of effector T cells that recognize LMP1, LMP2A, and EBNA1. Earlier studies from our laboratory have reported one such strategy based on linking minimal CD8\(^+\) T cell epitopes from LMP1 and LMP2A as a polypeptide, which allows rapid activation of Ag-specific T cells from healthy EBV carriers (12). Recent studies (30, 31) have also indicated that EBNA1 should also be considered as a potential immunological target for EBV-associated malignancies. Considering these findings, we decided to modify the polypeptide technology in such a way that it allowed the activation of T cells specific for not only LMP1 and LMP2A but also EBNA1.

In the first set of experiments, we compared the stimulation of LMP-specific CTLs using autologous LCLs and an adenoviral construct, which encodes EBNA1\_AGA covalently linked to multiple minimal LMP1 and LMP2A CD8\(^+\) T cell epitopes (AdE1-LMP\_poly; Fig. 1). Representative data from three healthy virus carriers are presented in Fig. 2. Two different techniques based on ELISPOT and MHC-peptide pentamers were used to assess the expansion of virus-specific T cells. Although both AdE1-LMP1 and autologous LCLs induced rapid expansion of LMP1 and LMP2A-specific T cells as determined by ELISPOT responses (Fig. 2A), the proportion of LMP-specific T cells in AdE1-LMP\_poly-stimulated cultures were 2- to 15-fold higher when compared with LCL-stimulated cultures. These observations were also confirmed by MHC-peptide pentamer staining, which showed that stimulation of T cells with AdE1-LMP\_poly induced 30- to 150-fold expansion of LMP epitope-specific T cells within 10 days of stimulation (Fig. 2B). More importantly, these T cells also efficiently recognized HLA-matched target cells endogenously expressing either LMP2A or LMP1 proteins (Fig. 2C). Of particular interest was the recognition of the HRS cell line L1236 expressing LMP1, which showed that endogenously processed LMP1 epitopes presented by HRS cells can be efficiently recognized by AdE1-LMP\_poly-stimulated T cells.

The influence of \(\gamma\)C-cytokines on the expansion and phenotype of LMP-specific T cells

There is increasing evidence from humans and murine models that different \(\gamma\)C-cytokines (e.g., IL-2, IL-7, and IL-15) can influence the proliferation and the phenotype of in vitro-expanded tumor Ag-specific T cells (32–34). To address the effect of \(\gamma\)C-cytokines on the AdE1-LMP\_poly-stimulated T cells and to optimize conditions for the expansion of LMP-specific T cells, PBMC were stimulated with AdE1-LMP\_poly in the presence of different \(\gamma\)C-cytokines combinations. At 10 and 17 days poststimulation, these cultures were analyzed for absolute cell number and for the percentage of CD8\(^+\) and CLG-pentamer\(^+\) cells. It was evident at day 10 that culture medium supplemented with IL-15 or IL-7 alone failed to support optimal expansion of T cells, whereas IL-2 alone was sufficient to rapidly induce proliferation of activated cells (Fig. 3A). These differences were further exaggerated by day 17, whereby there were 6-fold and 30-fold greater number of cells in cultures supplemented with IL-2 when compared with IL-15 and

**FIGURE 3.** Influence of different \(\gamma\)C-cytokines on the expansion of LMP1 and LMP2A-specific T cells following stimulation with AdE1-LMP\_poly. PBMC from a healthy virus carrier (D1) were incubated with irradiated autologous PBMC infected with AdE1-LMP\_poly (MOI 10:1) at a ratio of 2:1. These cultures were supplemented with different \(\gamma\)C-cytokines (10 U/ml IL-2, 100 U/ml IL-7, and/or 10 ng/ml IL-15) on day 3 and every 3–4 days thereafter. These cells were analyzed for Ag specificity and for the expression of various phenotypic markers on day 0, 10, and 17. A, Longitudinal analysis of the expansion of cells following stimulation with AdE1-LMP\_poly in the presence of different \(\gamma\)C-cytokines. The absolute numbers of cells were calculated by trypsin blue exclusion. B, Longitudinal analysis of the expansion of CD8\(^+\) and LMP2 epitope (CLG)-specific T cells in cultures stimulated with AdE1-LMP\_poly and supplemented with different \(\gamma\)C-cytokines. Cells were either stained with anti-CD8 alone or in combination with the CLG MHC-peptide pentamer. C, Analysis of T cell markers on AdE1-LMP\_poly-stimulated cells. These cells were costained with CD8-specific Ab, MHC-peptide pentamer, and CD27, CD38, CD62L CD127, or CCR7-specific Abs. Representative data based on the CLG MHC-peptide pentamer is presented. D, Longitudinal phenotypic analysis of AdE1-LMP\_poly-stimulated T cells cultured in the presence of different \(\gamma\)C-cytokines. Ag-specific T cells were stained with CD27, CD38, CD62L CD127, or CCR7-specific Abs on days 0, 10, and 17. Representative data based on the CLG MHC-peptide pentamer is presented.
PGPQ (LSR) is presented. Data presented in the ELISPOT assays were tested with individual peptides using intracellular cytokine assay. Representative data based on the peptide LSRLPFGMAPG were used in these assays. The results are expressed as SFC/10^6 cells.

To address the influence of γC-cytokines and AdE1-LMPpoly on the phenotype of responding T cells, ex vivo PBMC and AdE1-LMPpoly-stimulated cells were analyzed for the expression of CD27, CD38, CD62L, CD127, and CCR7. Before AdE1-LMPpoly stimulation, CD8^+ LMP-specific T cells (data presented for CLG epitope-specific T cells) were typically positive for CD27, CD127, and CCR7, showed an intermediate phenotype for CD62L, and were negative for CD38 (Fig. 3C). Stimulation with AdE1-LMPpoly in the presence of IL-2 resulted in a down-regulation of CD27, CD127, and CCR7, an up-regulation in the expression of CD38, and a similar level of expression of CD62L. This phenotype was maintained by day 17 in culture, although there was a decrease in the percentage of CD62L-positive cells, and was not significantly influenced by the addition of other cytokines (Fig. 3D).

Incubation with IL-15 alone, or with IL-7 and IL-15 in combination with IL-2, did not influence the phenotype of the responding Ag-specific T cells. Incubation with IL-7 alone led to greater expression of CD27. However, this increase was not evident following incubation with IL-7 in combination with IL-2 or IL-15.

Characterization of EBNA-1-specific T cell response following stimulation with AdE1-LMPpoly

In addition to LMP1 and LMP2A, both HL and NPC tumor cells express EBNA1. Although initial evidence suggested that EBNA1 was not endogenously processed through the MHC class I or class II pathway, recent investigations have shown that EBNA1 is a target for both CD8^+ and CD4^+ CTL (30, 31, 35, 36). We compared the stimulation of EBNA1-specific CTLs using autologous LCLs and AdE1-LMPpoly. T cell cultures were assessed for reactivity toward previously defined EBNA1 CD8^+ T cell epitopes HPV, RPQ, and IPQ, respectively. As shown in Fig. 4A, a strong HPV- and IPQ-specific IFN-γ response could be expanded following AdE1-LMPpoly stimulation. This response was of a

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**FIGURE 4.** Expansion of EBNA1-specific T cells following stimulation with AdE1-LMPpoly. A, AdE1-LMPpoly- and LCL-stimulated T cells from healthy virus carriers (D2 and D3) were assayed for IFN-γ production using ELISPOT assays. T cell epitopes restricted through HLA B35 (HPV) and HLA B7 (RPQ and IPQ) were used in these assays. The results are expressed as SFC/10^6 cells. B, Comparison of EBNA1-specific T cell numbers in unstimulated PBMC or AdE1-LMPpoly-stimulated cultures. T cells were costained with anti-CD8 and the HPV MHC-peptide pentamer. Data presented in the upper right quadrant are the percentage of MHC-peptide pentamer-positive cells within the total lymphocyte population. C, Recognition of an endogenously processed EBNA1 epitope by T cells stimulated with AdE1-LMPpoly. Data presented in the upper right corner represent the percentage of CD8^+ HPV-pentamer-lymphocytes expressing IFN-γ following stimulation with autologous and allogeneic LCLs. D, Mapping of novel EBNA1-specific reactivity from T cell cultures stimulated with AdE1-LMPpoly. AdE1-LMPpoly-stimulated cultures were incubated with overlapping EBNA1 peptide pools and assayed for IFN-γ production by ELISPOT. The results are expressed as SFC/10^6 cells. E, Fine mapping of EBNA1-specific T cell reactivity. T cells showing reactivity in ELISPOT assays were tested with individual peptides using intracellular cytokine assay. Representative data based on the peptide LSRLPFMAGP (LSR) is presented. Data presented in the upper right corner represent the percentage of CD8^+ CD3^+ lymphocytes expressing IFN-γ following stimulation with LSR. F, Mapping of HLA-restriction for the novel LSR epitope. CTLs specific for LSR were exposed to either autologous or MHC-matched PHA blasts (HLA A2, HLA B57 or HLA B62). An E:T ratio of 20:1 was used in this assay. G, Minimalization of the LSR epitope. PHA blasts presensitized with overlapping 9-aa long peptides were exposed to LSR-specific T cells at an E:T ratio of 20:1. H, Titration of the most active minimal EBNA1 peptides. PHA blasts were sensitized with varying concentrations of PLSRLPFGM and LSRLPFMA and then exposed to LSR-specific CTLs. I, Expression of IFN-γ by AdE1-LMPpoly-stimulated T cells following incubation with EBNA1 peptides. This figure shows representative data based on stimulation of T cells from donor 4 with the peptide GGSKTSYLNLRRGTA. Numbers indicated in the box represents the percentage of IFN-γ producing CD8^+ CD3^+ lymphocytes.
similar magnitude to the LMP-specific responses detected (see Fig. 2A). In addition, MHC-peptide pentamer staining of T cells confirmed that stimulation lead to a significant expansion of EBNA1-specific cells, whereby a 380-fold increase in the percentage of HPV-pentamer-positive cells was detected 10 days poststimulation (Fig. 4B). These EBNA1-specific T cells efficiently recognized processed HPV epitope on autologous LCLs (Fig. 4C).

In addition to the detection of known EBNA1 CD8⁺ T cell responses, we also analyzed these expanded cultures for the presence of novel EBNA1 reactivity. T cell cultures stimulated with AdE1-LMPpoly were assessed for reactivity toward EBNA1 using an ELISpot assay based on a matrix of overlapping 15-mer peptides. IFN-γ-producing cells could be detected in pools 4, 5, 6, 14, and 15 (Fig. 4D) following stimulation of donor 1 T cells, suggesting possible reactivity to peptides 42, 43, 44, and 51, 52, and 53 in the EBNA1-overlapping peptide set. Subsequent analysis of IFN-γ production by intracellular cytokine staining revealed that this epitope was restricted through HLA B57 (Fig. 4E). The minimal sequence of the novel epitope was identified by using overlapping 9-mer peptides based on the sequence CRLTPLSRLPFGMAPGPGPQ. This assay indicated strong reactivity toward two overlapping 9-mer epitopes, HLSRLPFGGM and HLSRLPFGMAP.
successful in activating LMP and EBNA1-specific T cells in healthy virus carriers, it was important to demonstrate that this strategy can be transferred into a clinically relevant setting, such as newly diagnosed patients with HL. A total of 10 patients with HL (Table I) were recruited and assessed for LMP and/or EBNA1-specific T cell responses following stimulation with AdE1-LMPPoly. At day 10 poststimulation, cultures were assessed for reactivity toward LMP epitopes by IFN-γ ELISPOT, and where possible, responses were compared with those detected ex vivo. Following stimulation with AdE1-LMPPoly, a lack of IFN-γ responsiveness could be completely reversed. Representative data from a patient with newly diagnosed HL (D11) is presented in Fig. 5A. Although LMP-specific IFN-γ production was not detected against the majority of epitopes at the time of diagnosis, LMP-specific T cells were rapidly expanded following stimulation with AdE1-LMPPoly from PBMCs at both time points (Fig. 5A). A comparison of the response generated following AdE1-LMPPoly and LCL-stimulation was also assessed from two of the HL-patients, confirming the previous observation in healthy donors that AdE1-LMPPoly was much more efficient in expanding LMP-specific T cells (Fig. 5B). A complete analysis of the AdE1-LMPPoly-expanded T cell responses revealed that a broad range of LMP-specific T responses could be detected in all donors, including those who had been undergoing treatment for >6 mo or are long-term survivors (Fig. 5C), and newly diagnosed (Fig. 5D).

In addition to LMP-specific T cell responses, we also assessed EBNA1-specific reactivity in these expanded cultures from patients with HL. Data presented in Fig. 5D shows that AdE1-LMPPoly stimulation of T cells from D13 induced a strong ELISPOT response toward the HLA B35-restricted HPV epitope. To determine whether any novel reactivity against EBNA1 epitopes could

(Fig. 4G). However, peptide titration of these two 9-mer epitopes revealed LSRLPGFMA as the minimal epitope sequence (Fig. 4H). A similar analysis of T cells expanded from another donor (D4) revealed reactivity toward the peptide, GGSKLSSLNR RTA (Fig. 4I), which likely corresponds to the previously described HLA-B8-restricted CD8 epitope, YNLRRGTAL (31).

Restoration of functional responses in T cells from patients with HL following stimulation with AdE1-LMPPoly

The results obtained from healthy virus carriers indicated that AdE1-LMPPoly is highly efficient in rapidly expanding LMP1, LMP2A, and EBNA1-specific CTLs that are capable of recognizing endogenously presented Ags from HRS cells. Recent studies from our laboratory have shown that during active HL, there is a selective loss of function in LMP1 and LMP2A-specific T cells, coincident with the marked increase in CD4+ regulatory T cells at disease sites and in the peripheral blood (8). Although we were presented in the upper right hand corner of the box represent the number of CD8+ lymphocytes expressing IFN-γ alone.

Table II. Novel EBNA1 responses mapped from HL patients

<table>
<thead>
<tr>
<th>Donor</th>
<th>Peptide</th>
<th>% IFN-γ of CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>RPSCIGCKTGGTGTG</td>
<td>0.55</td>
</tr>
<tr>
<td>8</td>
<td>GVVYGSGGKSLSLYNL</td>
<td>6.56</td>
</tr>
<tr>
<td>11</td>
<td>GAVKTSGLYRLRGTAA</td>
<td>1.44</td>
</tr>
<tr>
<td>12</td>
<td>PPGPGGNGLQEGGD</td>
<td>7.36</td>
</tr>
<tr>
<td>13</td>
<td>YFMVLIFTHFAEVL</td>
<td>3.56</td>
</tr>
<tr>
<td></td>
<td>CPPVQGQAAEDEGDDG</td>
<td>1.78</td>
</tr>
<tr>
<td></td>
<td>GVFYGGKSSLSLYNL</td>
<td>0.14</td>
</tr>
</tbody>
</table>

a Cells from patient cultures stimulated with AdE1-LMPPoly were assessed for responsiveness to the EBNA1-overlapping peptide matrix (1 μg/ml of each peptide), the phenotype of responding cells was then determined with individual peptide by flow cytometry.
be detected, AdE1-LMPpoly-stimulated T cells from patients with HL were assessed using IFN-γ ELISPOT and intracellular cytokine assays. A summary of the data based on intracellular cytokine assays is presented in Table II. Of particular interest was the strong reactivity detected against the peptide GYFVYGGSKTSLYNL in the T cell cultures from patient D8, and against three epitopes (PGTGPNGGLGKEKGD, YFMVFLQTHIFAEVL, and PPMVEGAAEGDDGDG) from patient D12, indicating that AdE1-LMPpoly is highly efficient in activation T cells specific for not only LMP1 and LMP2A but also EBNA1. A fine mapping and HLA restriction of these novel epitopes is currently in progress.

To determine whether AdE1-LMPpoly stimulation of T cells from different stages of treatment in patients with HL had any impact upon the expansion and phenotype of responding cells, cell numbers were assessed at 10 and 17 days poststimulation and analyzed for the expression of CD27, CD38, CD62L, CD127, and CCR7 on CD8+ T cells. The overall level of expansion in cell numbers for patients with newly diagnosed HL was comparable to the patients in remission (6 mo postdiagnosis) and healthy virus carriers (Fig. 5E). Similar to healthy virus carriers, the optimal expansion of T cells from HL patients was observed in the presence of IL-2 when compared with IL-7 and IL-15 (Fig. 5F). Furthermore, MHC-pentamer analysis also revealed that E1-LMPpoly stimulation in presence of IL-2 was more efficient in expanding EBV epitope (HPV)-specific T cells (Fig. 5G). Finally, AdE1-LMPpoly-stimulated CD8+ T cells from patients with newly diagnosed HL displayed a phenotype which was very similar to that observed for T cells from healthy virus carriers (Fig. 5H). Both total CD8+ and MHC-peptide pentamer-positive T cells were typically CCR7 and CD127 negative, CD38 positive, and intermediately for the expression of CD62L and CD27.

An important issue to be addressed was whether T cells expanded from patients with HL are capable of recognizing endogenously processed epitopes from LMP and EBNA1 Ags. To address this issue, we used AdE1-LMPpoly-stimulated T cells from five separate patients with HL and exposed these to LMP1, LMP2A, or EBNA1-expressing HRS cells, normal fibroblasts, and LCLs, respectively. Data presented in Fig. 6 clearly shows that these in vitro-expanded T cells efficiently recognized HLA-matched normal human fibroblasts expressing LMP2A Ag, HRS cells (L1236-LMP1GFP) expressing LMP1, and EBNA1-expressing LCLs. In addition, to demonstrate that the expanded LMP and EBNA1-specific T cells generated from HL patients were functionally similar to other viral Ag-specific CTLs, the expression of CD107α and IFN-γ was assessed following stimulation with AdE1-LMPpoly or AdCMVpoly. Data presented in Fig. 6C clearly demonstrates that T cells stimulated with either AdE1-LMPpoly or AdCMVpoly showed comparable levels of CD107α and IFN-γ. Taken together, these experiments clearly demonstrate that AdE1-LMPpoly is highly efficient in restoring functional capability in virus-specific T cells from patients with HL.

**Discussion**

Recent studies (7, 8) have suggested that expansion of regulatory T cells contributes toward the loss of function in Ag-specific T cells from patients with HL. Reversion of this phenotype is coincident with recovery from active HL. The reconstitution of cellular immunity through augmentation of antiviral T cell responses, either via vaccination or T cell therapy, may therefore be used as an adjunct to chemotherapy and for treatment of patients who fail to respond to conventional treatment (1, 9). Previous studies have successfully used autologous EBV-transformed B cells to expand virus-specific T cells from patients with HL; however, their therapeutic efficacy is rather limited due to the predominance of T cells that are not relevant for controlling the expansion of HRS cells (10, 37). In this study, we have developed a novel Ag presentation system referred to as AdE1-LMPpoly, which is highly effective in expanding T cells specific for the HL-associated EBV Ags, LMP1, LMP2A, and EBNA1, across a range of HLA types. More importantly, AdE1-LMPpoly-stimulated a higher frequency of T cells specific for HL-associated Ags when compared with LCL-mediated expansion.

Over recent years, γC-cytokines (e.g., IL-2, IL-7, and IL-15) have been recognized for their critical role in T cell homeostasis and in the maintenance and proliferation of memory T cells (32, 33). Furthermore, there is growing evidence from murine models which suggests that exposure of tumor Ag-specific T cells to γC cytokines, such as IL-7 and IL-15, can alter their phenotype and thus improves their therapeutic efficacy (34, 38, 39). However, the role of these cytokines in a clinical setting is less clearly defined.

We used the AdE1-LMPpoly expression system in combination with γC-cytokines to determine their effect on T cell expansion and function. In contrast to murine models, we found that IL-7 or IL-15, alone or in combination, failed to support the expansion of Ag-specific T cells. Our data clearly showed that IL-2 alone was sufficient to drive expansion of T cells following stimulation with AdE1-LMPpoly. Studies on murine models have also shown that expansion of Ag-specific T cells in the presence of IL-15 can influence T cell phenotype, resulting in the generation of a predominantly central memory population. Central memory T cells express CD27, CD62L, CCR7, and CD127, which are typically down-regulated in effector memory cells (40). Central memory T cells have a greater proliferative capacity in vivo, but typically have reduced effector function in comparison to effector memory cells (41–43). It was therefore hypothesized that expansion of T cells in the presence of IL-15 may influence the resulting phenotype of effector cells stimulated with AdE1-LMPpoly, which may be of benefit in altering trafficking to the tumor site in lymph nodes, and enhancing proliferation upon Ag exposure following adoptive transfer (34, 43). However, we found no evidence which indicated that IL-15 influenced the phenotype of EBV-specific T cells, alone or in combination with IL-2. It is possible that the adenoviral vector, which has previously been shown to induce the up-regulation of immunostimulatory cytokines, may itself influence the resulting T cell phenotype (44). In addition, most of the murine studies that have demonstrated differences between IL-2 and IL-15 have used naive T cell models, whereas application of adoptive immunotherapy in clinical settings like HL is primarily based on the expansion of memory T cells, which may have a preprogrammed potential to expand into a particular phenotype.

Our studies on the phenotypic profiling of EBV-specific T cells following stimulation with AdE1-LMPpoly revealed that most of these effector cells were typical of an activated effector memory population, whereby CD27, CCR7, and CD127 were down-regulated, and CD38 was up-regulated. However, a significant proportion of specific CD8+ T cells expressed CD62L, poststimulation. There is evidence that the down-regulation of CD62L expression occurs following multiple exposures to Ag, which may indicate the terminal differentiation of effector memory T cells (34, 43, 45). It has also been demonstrated that CD62L+CD8+ T cells display very poor proliferative potential and are less effective in controlling tumor growth (34, 43). The expression of CD62L in a significant population of AdE1-LMPpoly-stimulated T cells suggests that these cells may retain the capacity to proliferate upon additional antigenic exposure, such as upon encountering HRS cells in vivo. Furthermore, CD62L is required for entry into lymph nodes, which may assist T cells to access HL tumor sites.
To effectively use AdE1-LMPpoly as a tool for the expansion of T cells for immunotherapeutic treatment of HL and other EBV-associated malignancies, or as a therapeutic vaccine, it was necessary to determine whether stimulation with AdE1-LMPpoly could overcome the indifferent nature of the LMP-specific T cells in patients with HL. Indeed, our studies showed that a single stimulation with AdE1-LMPpoly was sufficient to restore T cell function from patients with HL, leading to the generation of IFN-γ-producing CTL capable of lysing targets cells presenting endogenous Ags. Importantly, these cells were also capable of inducing lysis of the HRS cell line, L1236. The ability of AdE1-LMPpoly stimulation to overcome the unresponsive nature of the LMP-specific T cells suggests that in addition to generating peptide for presentation via MHC class I molecules, AdE1-LMPpoly may also provide the immunostimulatory signals required to overcome the suppression of LMP-specific T cells from patients with HL, allowing their activation and expansion. In addition to the results obtained during these studies, other studies have previously shown that LMP-specific responses can be expanded from patients with HL, indicating that although LMP-specific responses from patients with newly diagnosed HL are unresponsive ex vivo; provision of the correct immunostimulatory signals can overcome this unresponsiveness (10, 11). Recent evidence has suggested that, in addition to LMP1 and LMP2A, EBNA1 may also offer a viable antigenic target in the treatment of EBV-associated malignancies (30, 31, 35, 36). Evidence obtained here revealed that AdE1-LMPpoly is an effective tool for the expansion of EBNA1-specific T cells. In addition to the detection of EBNA1-specific T cells following AdE1-LMPpoly stimulation in the majority of healthy individuals and patients with HL, we were successful in mapping novel EBNA1 epitopes.

The results obtained in this study provide clear evidence that a single stimulation with AdE1-LMPpoly can be used effectively to rapidly expand T cells directed against the HL and NPC-associated Ags, LMP1, LMP2A, and EBNA1. In the treatment of EBV-associated malignancies by adoptive immunotherapy, the ability to rapidly generate a broad specificity of autologous T cells, as occurs following AdE1-LMPpoly, may offer advantages over other strategies that use single specificity autologous or allogeneic T cells. It is plausible that T cells with different specificities may be required for optimal killing of tumor cell lines in different individuals, either due to antigenic variation or immunological differences between individuals. Therefore, strategies aimed at providing a range of epitopes may be required to generate optimal CTL populations for immunotherapy. Although overcoming the poor immunogenicity of the full-length HL-associated Ags, AdE1-LMPpoly encodes a multitude of epitopes across a broad range of HLA-types, providing broad specificity in the expansion of CTL for the treatment of EBV-associated malignancies.

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Disclosures

The authors have no financial conflict of interest.

References


