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Characterization of Latent Membrane Protein 2 Specificity in CTL Lines from Patients with EBV-Positive Nasopharyngeal Carcinoma and Lymphoma

Karin C. Straathof,* Ann M. Leen,* Elizabeth L. Buza,* Graham Taylor,‖ M. Helen Huls,* Helen E. Heslop,*‡†‡ Cliona M. Rooney,*‡§ and Catherine M. Bollard‡*‡†¶

Viruses expressed by EBV-associated tumors provide target Ags for immunotherapy. Adoptive T cell therapy has proven effective for posttransplant EBV-associated lymphomas in all EBV latent Ags are expressed (type III latency). Application of immunotherapeutic strategies to tumors such as nasopharyngeal carcinoma and Hodgkin’s lymphoma that have a restricted pattern of EBV Ag expression (type II latency) is under investigation. Potential EBV Ag targets for T cell therapy expressed by these tumors include latent membrane proteins (LMP) 1 and 2. A broad panel of epitopes must be identified from these target Ags to optimize vaccination strategies and facilitate monitoring of tumor-specific T cell populations after immunotherapeutic interventions. To date, LMP2 epitopes have been identified for only a limited number of HLA alleles. Using a peptide library spanning the entire LMP2 sequence, 25 CTL lines from patients with EBV-positive malignancies expressing type II latency were screened for the presence of LMP2-specific T cell populations. In 21 of 25 lines, T cell responses against one to five LMP2 epitopes were identified. These included responses to previously described epitopes as well as to newly identified HLA-A*0206-, A*0204/17-, A29-, A68-, B*1402-, B27-, B*3501-, B53, and HLA-DR-restricted epitopes. Seven of the nine newly identified epitopes were antigenically conserved among virus isolates from nasopharyngeal carcinoma tumors. These new LMP2 epitopes broaden the diversity of HLA alleles with available epitopes, and, in particular, those epitopes conserved between EBV strains provide valuable tools for immunotherapy and immune monitoring.


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3 Abbreviations used in this paper: NPC, nasopharyngeal carcinoma; EBNA, EBV-encoded nuclear Ag; HL, Hodgkin’s lymphoma; LCL, EBV-transformed B cell line; LMP, latent membrane protein; NHL, non-HL; SFC, spot-forming cell.

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plates were developed with AEC substrate (Sigma-Aldrich). Plates were sent for evaluation to Zellnet Consulting. Results are shown as spot-forming cells (SFC) per 1 × 10^6 CTL. For the screening with LMP2 peptide pools, all assays were performed once in duplicate. Before using this method as screening for patient CTL lines, the reproducibility of this method was first confirmed using CTL lines from two healthy donors (data not shown). Those responses that exceeded 5× background level of nonstimulated CTL and were at least 5 SFC/1 × 10^6 CTL were regarded as significant. For 2 of 25 CTL lines screened, this threshold level was lowered to 1× background level. The relevance of the identified LMP2-specific responses was subsequently confirmed in ELISPOT assays using single LMP2 peptides, again using a threshold level of 5× background and >5 SFC/1 × 10^5. Responses to the identified LMP2 epitopes were consistently detected in the CTL lines studied; however, the strength of these responses varied between assays. The average response to each epitope within the same CTL line is reported in Table I.

### Determining HLA restriction of identified epitopes

To determine the HLA restriction of the novel LMP2 class I peptides, CTL with specificity for the LMP2 peptide were plated at 1 × 10^6/well in an IFN-γ ELISPOT assay with partially HLA-matched PHA-activated lymphoblasts (40 Gy irradiated) used as APCs either alone or pulsed with peptide (10 μg/ml for 30 min at 37°C). All immunogenic peptides were analyzed for the presence of anchor sites for HLA alleles expressed by the patient using prediction databases from K. Parker (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) (http://bimas.dccn.nih.gov/molbio/hla_bind/index.html#112601) and H.-G. Rammensee (Heidelberg, Germany) (www.syfpeithi.de) and the HLA Factsbook (38). For seven of nine epitopes, the HLA restriction was confirmed by staining with the HLA tetramer derived from the newly identified epitope. To confirm HLA class II restriction of identified epitopes, T cells were stained with CD4 and CD8 mAbs (BD Biosciences), sorted on a MoFlow Cytometer, and subsequently used in an IFN-γ-ELISPOT assay. HLA-DR restriction was confirmed by incubating the T cells for 30 min at 37°C with HLA-DR-blocking Ab (1 μg/ml) before addition of the peptide.

### Tetramer staining

Tetramers were prepared by the National Institute of Allergy and Infectious Diseases tetramer core facility, or by the Baylor College of Medicine tetramer core facility. CTL or PBMC (5–10 × 10^5) were incubated at room temperature for 30 min in PBS/1% FCS containing the PE-labeled tetrameric complex. Samples were stained with anti-CD8 FITC and anti-CD3 PerCP. Appropriate isotype controls were included. Stained cells were fixed in PBS containing 0.5% paraformaldehyde. For each sample, a minimum of 100,000 cells was analyzed using a FACSCalibur with the CellQuest software (BD Biosciences).

### Results

#### LMP2-specific T cells within patient CTL lines

Using LCL as APC, EBV-specific T cells were reactivated and expanded from the peripheral blood of 25 patients with EBV type II latency tumors: 13 patients with NPC, 10 patients with HL, and 2 patients with NHL. The presence of LMP2-specific and thus tumor-specific T cells within these patient CTL lines was assessed using a peptide library representing the entire LMP2a sequence (B95-8 strain). In 21 of 25 patient CTL lines, LMP2-specific T cells were detectable in an IFN-γ ELISPOT assay after overnight incubation with each of the peptide pools. This result demonstrates that T cells specific for a dominant EBV-Ag can regularly be reactivated using LCL even in patients. An example of one CTL line is shown in Fig. 1A: T cells that produced above background levels of IFN-γ were detectable after stimulation with pools 1, 12, 17, and 18. In two patient CTL lines, the spontaneous IFN-γ secretion of nonstimulated T cells resulted in a signal to noise ratio that was too high to detect LMP2-specific T cell responses. In two other patient CTL lines, none of the peptide pools induced IFN-γ secretion of the T cells, although incubation with LCL resulted in a measurable response.
Determining minimal recognized LMP2 sequence

Following the initial screening with each of the LMP2 peptide pools, the minimal recognized T cell epitopes were identified. Based on the LMP2 peptide pools that induced IFN-γ secretion, individual pentadecamers that were present in two of the peptide pools that tested positive were selected, e.g., pentadecamers 49, 60, 61, and 72 for the patient CTL line shown as example (Fig. 1B). Stimulation of the T cells with these individual pentadecamers identified the sequence of peptides 60 and 61, most likely the overlapping 11 aa, as the CTL epitope. For example, within the RLTVCGGIMFL sequence, TVCGGIMFL was shown to represent the minimum recognized epitope, whereas RLTVCGGIM and LTVCGGIMF were not recognized by the CTL (Fig. 1D). Using this strategy, seven nonamers representing new LMP2 epitopes were identified (Table I, epitopes in bold). Two of the epitopes identified in this study (LPVIVAPYL and FTASVSTVV) represent the minimum epitope within regions of LMP2 earlier reported as CD8⁺ T cells recognition sites in PBMC of healthy donors (33).

FIGURE 1. Identification of LMP2 epitopes using an LMP2 peptide library. A, LCL-reactivated CTLs (1 × 10⁵/well) from a patient with NPC (HLA-A*0206, A24, B51, B61) were stimulated with an LMP2 peptide library pooled into 23 pools. Responses were measured in an 18-h IFN-γ ELISPOT assay. Shown are mean and SD of duplicate wells. The black horizontal line shows the threshold level used to determine significance (5 × number of SFC/10⁵ unstimulated CTL). B, All peptides were divided into 23 pools in such a way that each peptide is present in 2 pools. This method allows determining those single peptides that most likely induced responses to the peptide pools. Thus, responses to pools 1 and 17, 1 and 18, 12 and 17, and 12 and 18 may be induced by single peptides 49, 61, 60, and 72, respectively. C, Testing of these individual pentadecamers identifies the sequence of peptides 60 and 61, most likely the overlapping 11 aa, as the CTL epitope. D, Testing of the three potential nonamers within this sequence at indicated concentrations identifies the minimum recognized epitope.
HLA-A*0206-matched APC were available, A*0201-typed APC pulsed APC matched for the HLA-A2 allele, but not after stimulation and HLA-B53 restricted (Table I).

HLA-A29, HLA-A68, HLA-B*1402, HLA-B27, HLA-B*3501, HLA-B*4403, and HLA-B*5701 identified LMP2 epitopes were found to be HLA-A*0204 or A*0217, whereas no CD8 T cells secreted IFN-γ upon stimulation with autologous (A*0206) and A*0201-typed APC pulsed with different concentrations of the TVCGGIMFL peptide stimulating was further confirmed by the identification of a T cell population staining positive with HLA-A*0201-TVCGGIMFL tetramer (Fig. 2C). Using this same strategy, the other newly identified LMP2 epitopes were found to be HLA-A*0204 or A*0217, HLA-A29, HLA-A68, HLA-B*1402, HLA-B27, HLA-B*3501, and HLA-B53 restricted (Table I).

Identification of a CD4 epitope

Although the LMP2 peptide library used in this study was designed to identify HLA class I-restricted epitopes with a length of 9–11 aa, an HLA class II-restricted epitope was identified in one of the patient CTL lines. T cells present in this CTL line recognized LMP2 sequence DYQPLGTQDQSLYLG (aa 73–87), but none of the shorter (9–14 aa) peptides derived from this pentadecamer (data not shown). Therefore, the pentadecamer appeared to represent the minimum recognized epitope. As the binding groove of MHC class II molecules can accommodate peptides with a length of up to 20 aa, we reasoned that this LMP2 peptide may be recognized in the context of HLA class II. Indeed, separation of CD4+ and CD8+ T cells within this polyclonal CTL line demonstrated that this peptide induces a CD4-mediated T cell response, whereas no CD8+ T cells were activated (Fig. 3A). The recognized pentadecamer contains anchor residues that are predicted for binding to HLA-DR4 (DYQPLGTQDQSLYLG), one of the HLA class II alleles of this patient (HLA-DR4/16, DQ5/7, DP not done). Complete abrogation of peptide recognition in the presence of an HLA-DR-blocking Ab confirmed this predicted HLA-DR restriction (Fig. 3B). Other class II epitopes may have been missed in this study because of the design of the peptide library, which for detection of class II epitopes should optimally be composed of overlapping 20-mers (39).

LMP2 epitopes partially conserved in NPC tumors

The LMP2 peptide library used in this study is based on the prototype EBV type I strain B95-8. However, different EBV strains may be present in the tumor, depending on the geographical origin of the patient (40). For these newly identified LMP2 epitopes to be useful for immunotherapy, their sequence must be conserved between the B95-8 strain and the EBV strain present in the tumor.

For one epitope, RRLTVCGGIMFL (aa 240–250), the minimal recognized sequence consisted of 11 aa rather than 9 aa, analogous to a previously described LMP2 epitope SSCSSCPLSKI (aa 340–350). Interestingly, this epitope is located within a region that contains four overlapping CD8 epitopes, VLVMLILILAYRRRWRRLTVCGGIMFL, VLVMLILILAYRRRWRRLTVCGGIMFL, and VLVMLILILAYRRRWRRLTVCGGIMFL, and one CD4 epitope, VLVMLILILAYRRRWRRLTVCGGIMFL. Similarly, ILLARLFYL is located in an epitope hotspot: SSCSSCPLSKI, and ILLARLFYALALLL, SSCSSCPLSKILLARFLYALALLL, and SSCSSCPLSKILLARFLYALALLL. HLA restriction of identified CD8 epitopes

To determine the HLA restriction of the identified LMP2 epitopes, we took advantage of described peptide-binding motifs (see Materials and Methods). The HLA type of the patient used as an example is as follows: A*0206, A24, B51, B61 (Fig. 2A). The identified epitope TVCGGIMFL contains a valine at position 2 and a leucine at position 9, which are anchor residues predicted to bind to HLA-A*0206. We subsequently confirmed this HLA restriction by using partially HLA-matched PHA-activated lymphoblasts pulsed with this LMP2 peptide as APC in an ELISPOT assay (Fig. 2A). T cells secreted IFN-γ upon stimulation with all peptide-pulsed APC matched for the HLA-A2 allele, but not after stimulation with peptide-pulsed APC matched for HLA-A24. As no HLA-A*0206-matched APC were available, A*0201-typed APC were used in this experiment. Comparing IFN-γ secretion upon stimulation with autologous (A*0206) and A*0201-typed APC pulsed with different concentrations of the TVCGGIMFL peptide demonstrates that, although less efficient, this epitope can also be demonstrated in the context of HLA-A*0201 (Fig. 2B). HLA-A2 restriction was further confirmed by the identification of a T cell population staining positive with HLA-A*0201-TVCGGIMFL tetramer (Fig. 2C). Using this same strategy, the other newly identified LMP2 epitopes were found to be HLA-A*0204 or A*0217, HLA-A29, HLA-A68, HLA-B*1402, HLA-B27, HLA-B*3501, and HLA-B53 restricted (Table I).

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LMP2 epitopes partially conserved in NPC tumors

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### Table I. LMP2-specific T cell populations in patient CTL lines

<table>
<thead>
<tr>
<th>Minimum Epitope</th>
<th>Amino Acids</th>
<th>HLA Restriction</th>
<th>No. Responding/No. Tested</th>
<th>SFC/10^5 CTL (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLGGLLTMV</td>
<td>416–434</td>
<td>A*0201/06/07/09</td>
<td>4/12</td>
<td>84 (19–236)</td>
</tr>
<tr>
<td>GLGTTLGAAI</td>
<td>293–301</td>
<td>A2</td>
<td>1/12</td>
<td>459</td>
</tr>
<tr>
<td>LTAGFLFLIL</td>
<td>453–461</td>
<td>A2</td>
<td>0/12</td>
<td></td>
</tr>
<tr>
<td>FLYALALLL</td>
<td>356–364</td>
<td>A*0201</td>
<td>7/12</td>
<td>381 (7–1990)</td>
</tr>
<tr>
<td>LIVDAVLQPL</td>
<td>257–265</td>
<td>A<em>0204 or A</em>0217</td>
<td>1/12</td>
<td>651</td>
</tr>
<tr>
<td>TVCGGIMFL</td>
<td>243–251</td>
<td>A*0101/06</td>
<td>2/12</td>
<td>198 (175–222)</td>
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<tr>
<td>LLWTLVVL</td>
<td>329–337</td>
<td>A*0101</td>
<td>2/12</td>
<td>19 (14–24)</td>
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<tr>
<td>FTAASVSTYV</td>
<td>144–152</td>
<td>A68</td>
<td>2/6</td>
<td>38 (23–53)</td>
</tr>
<tr>
<td>SSCSSCPLSKI</td>
<td>340–350</td>
<td>A11</td>
<td>3/3</td>
<td>43 (8–90)</td>
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<tr>
<td>TYGPVFML</td>
<td>419–427</td>
<td>A24</td>
<td>2/5</td>
<td>58 (16–101)</td>
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<tr>
<td>PYLFWLAAI</td>
<td>131–139</td>
<td>A23/24</td>
<td>3/6</td>
<td>462 (12–1132)</td>
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<td>ILLARLFYLYL</td>
<td>349–358</td>
<td>A29</td>
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<td>6</td>
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<td>237–245</td>
<td>B*1402</td>
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<td>41</td>
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<td>B*27/04/05/09</td>
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<td>16</td>
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<td>B*3501</td>
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<td>DYOPLGTQDQSLYLG</td>
<td>73–87</td>
<td>DR4 or DR16</td>
<td>1/1</td>
<td>57</td>
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</table>

* Listed are the amino acid sequence of newly identified (in bold) as well as previously described LMP2 epitopes (27, 29–33); their location in the LMP2 molecule; HLA restriction; the number of CTL lines from NPC, HL, and NHL patients in which responses to these epitopes were identified; and the strength of these responses. When responses to the indicated epitope were found in more than one patient, CTL line average response and range are shown.
two mutations were present (Table II). Analysis of the immunogenicity of these variant epitopes with those derived from the B95-8 sequence shows that recognition of the CD8-restricted epitopes LPVIVAPYL and MGSLEMVPM is disrupted when indicated amino acids are altered (Fig. 4). The altered amino acids most likely compromise HLA binding (e.g., proline-leucine mutation at an anchor site for B53 binding of the LPVIVAPYL epitope) or TCR recognition of these epitopes. T cell responses to variants of the CD4-restricted epitope DYQPLGTQDQSLYLG, although reduced in number, appear to be preserved possibly because MHC class II-restricted epitopes are often promiscuous in their binding to HLA molecules (Fig. 4C). Using those LMP2 epitopes that are conserved between viral isolates for immunotherapeutic strategies is preferred so as to allow for their application in large patient groups worldwide.

Assessing the breadth of the LMP2 response

EBV-specific CTL reactivated and expanded using LCL as APC contain both CD4+ (mean 7.4%, range 0.1–50.0%) and CD8+ (mean 83.8%, range 39.4–98.8%) T cells that can potentially recognize multiple LMP2-derived epitopes. Screening the patient CTL lines with the LMP2 peptide pools allows for assessment of the breadth of the LMP2-directed specificity. In 12 CTL lines, detectable LMP2 reactivity was directed against a single epitope, whereas in 9 CTL lines T cell responses against 2–5 LMP2 epitopes were present (Table III). In two CTL lines that were known to contain a FLYALALLL-specific T cell population as determined by tetramer staining, no IFN-γ-secreting cells were detected upon stimulation with peptide pools that contained a pentadecamer representing the FLYALALLL sequence (data not shown). This observation suggests that screening with peptide pools may underestimate the true breadth of the LMP2 response in some cases. An example of a CTL line containing broad LMP2 specificity is shown in Fig. 5. Initial screening with the LMP2 peptide pools indicated recognition of multiple LMP2 sequences (Fig. 5A). Subsequently, these responses were mapped to 4 LMP2 epitopes: the earlier described HLA-A2-restricted FLYALALLL and LLWTLVVL, the HLA-B27-restricted RRRWRRLTV epitopes, and the newly identified HLA-A29-restricted ILLARFLYL epitope (Fig. 5B). The presence of T cells recognizing multiple epitopes is desirable, as this reduces the risk of immune escape by the tumor, and of strain sequence variations.

Monitoring of LMP2-specific T cell populations postadoptive T cell therapy

The ability to monitor the frequency of LMP2-specific T cell populations in the peripheral blood or infiltrating at the tumor site is crucial to determine the efficacy of immunotherapeutic interventions. In our ongoing Food and Drug Administration-approved clinical studies, patients with relapsed EBV-positive NPC, HD,
and NHL are being treated with autologous EBV-specific CTL. LMP2-specific T cell populations are identified in the infusion product by screening with the LMP2 peptide library and subsequent staining with tetramers derived from the identified epitopes. For example, 11.0% of the CD8\(^+\) T cells within the CTL line from a patient with EBV-positive Hodgkin’s disease were specific for one of the newly described LMP2 epitopes, MGSLEMVPM, which was found to be HLA-B*3501 restricted. Following infusion of this EBV-specific CTL line, the LMP2-specific T cell population was monitored in peripheral blood using tetramer analysis (Fig. 6). Preinfusion, 1.98% of CD8\(^+\) T cells in the peripheral blood were specific for this LMP2 epitope. The frequency of MGSLEMVPM-specific T cells increased to 5.37% 6 wk post-CTL infusion. These results indicate that the infused CTL (4 \times 10^7/m^3) proliferate in vivo and persist for at least 6 wk postinfusion and demonstrate the value of monitoring tools derived from LMP2 epitopes.

**Discussion**

Detailed characterization of LMP2-directed T cell specificity will greatly enhance the potential application of immunotherapeutic strategies and our ability to evaluate their effect as treatment of EBV latency type II malignancies. Stimulation of patient EBV-specific cytotoxic T cell lines with an LMP2 peptide library using IFN-\(\gamma\) secretion as readout proves to be a fast and sensitive method to evaluate the strength and breadth of the LMP2-specific immune response. As this technique is effective regardless of the patient’s HLA type, it can be applied to all patients. LMP2-specific T cell

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</table>

Amino acid sequences of the identified LMP2 epitopes as derived from the reference B95-8 strain were compared with the previously described sequences of: cell line section, one NPC tumor cell line (C15); or cell line/isolate section, three NPC tumor cell lines (C15, C18, and C19) as well as eight fresh EBV isolates from NPC tumors (41). Variations from the B95-8 sequence are indicated in bold.
responses were detectable in 84% of LCL-reactivated CTL lines. This result may be viewed as surprising, as these patients have developed EBV-positive malignancies in the presence of a competent immune system. However, EBV⁺ve tumors expressing type II latency use multiple strategies to evade the immune response. For example, HL cells secrete the immunosuppressive cytokine, TGβ, and recruit regulatory T cells, which together have devastating effects on CTL proliferation and function (42). The apparent lack of efficacy of endogenous tumor-specific CTL circulating in the patient peripheral blood provides a rationale for ex vivo expansion of the tumor-specific CTL in isolation from tumor-derived immune suppressive factors.

The LMP2-specific T cell responses were mapped either to previously described epitopes or to nine newly identified HLA class I- and class II-restricted epitopes. The latter were shown to be HLA-A*0204/17, A*0206, A29, A68, B*1402, B27, B*3501, B53, and DR (most likely DR4) restricted, mostly alleles for which no LMP2 epitopes have previously been identified. Interestingly, the new class II epitope identified, which partially overlaps with a previously reported CD8⁺ recognition site 33, and the class I epitope MGSLEMVPM identified in this study are the only LMP2 epitopes located in the cytoplasmic region of LMP2, whereas all other LMP2 epitopes are located in the transmembrane region (Fig. 7).

Although NPC, HL, and NHL occur worldwide, NPC is endemic in southern China (43), whereas EBV-positive HL is more common among Hispanics (44). As HLA-A*0206 is a common HLA allele in the Asian population and HLA-A29, A68, and B*3501 are common HLA alleles among the Hispanic populations (45), these are valuable additions to the panel of LMP2 epitopes particularly when conserved among geographically separated virus isolates.

Initial methods to identify LMP2 epitopes relied on the generation of CTL clones expanded from LCL-reactivated T cell lines from healthy donors, a relatively time-consuming process (27, 29, 30). More recently, an epitope-screening strategy based on a peptide library developed by Kern et al. (37) was used to analyze

![FIGURE 4. CTL recognition of LMP2 epitopes with altered amino acid sequences. CTL (1 × 10⁵/well) were stimulated with the B95-8-derived LMP2 epitopes LPVIVAPYL (A), MGSLEMVPM (B), and DYQPLGTQDQS LYLG (C), as well as altered versions as identified in non-B95-8 EBV strains (see Table II), and responses were measured in an IFN-γ ELISPOT assay. Average and SD of CTL stimulated with 500 ng/ml peptide are shown.](http://www.jimmunol.org/)
EBNA1-, LMP1-, and LMP2-specific immune responses in the peripheral blood of healthy donors (33, 39). This method with IFN-γ release as measured in an ELISPOT assay as readout significantly simplified the epitope identification, and was therefore our method of choice. LCL-reactivated T cell lines increased the frequency of the LMP2-specific T cells compared with that in PBMC. Nevertheless, LMP2 specificity could rarely be detected in cytotoxicity assays because of the low frequency of the LMP2-specific component. However, LMP2 tetramer-reactive cells that were isolated and expanded demonstrated LMP2-specific cytotoxic effector function (data not shown). In this study, we have shown that, using LCL as APC, LMP2-specific T cells can be reactivated and expanded for the vast majority of patients with type II latency malignancies irrespective of the patient’s age, sex, type of cancer, and disease stage. LMP2-specific T cell populations that represent <0.1% of CD3+ T cells could be detected using the ELISPOT assay, suggesting underdetection of LMP2 specificity in previous studies.

**FIGURE 5.** Breadth of the LMP2-specific T cell responses in patient CTL line. A, EBV-specific CTL from a patient with NHL (HLA type A2, A29, B13, B27) (1 × 10^5/well) were stimulated with the indicated LMP2 peptide pools. The black horizontal line shows the threshold level used to determine significance (5 × number of SFC/1 × 10^5 unstimulated CTL). B, Responses to these peptide pools could subsequently be mapped to four different HLA-A2 (FLYALALLL and LLWTLVVL)-, A29 (ILLARLFLY)-, and B27 (RRWRRLTV)-restricted LMP2 epitopes, demonstrating a broad LMP2 specificity in this CTL line.

**FIGURE 6.** Monitoring of an LMP2-specific T cell population in PBMC post-CTL infusion. A, Using an HLA-B35 LMP2aa 1–9:MGSLEMVPM tetramer, an LMP2-specific T cell population was detected in the CTL line from a patient with HL. B, Using the same HLA-B35 tetramer, the number of T cells specific for this epitope was determined in PBMC before and (C) 6 wk after the infusion of this CTL line to monitor the persistence and expansion of the infused CTL in vivo.
whether they can express the immunoproteasome after IFN-
be capable of peptide trimming to a certain extent, it is not clear
for its processing from whole Ag (29). Although T cells appear to
other epitopes described, in that it relies on the immunoproteasome
epitopes (46–48). The FLYALALLL epitope differs from the
samples are required for presentation of C terminus-extended
epitopes (46–48). The FLYALALLL epitope differs from the
other epitopes described, in that it relies on the immunoproteasome
its processing from whole Ag (29). Although T cells appear to
be capable of peptide trimming to a certain extent, it is not clear
whether they can express the immunoproteasome after IFN-γ
induction (49, 50), and thus may be unable to complete C-terminal
trimming of the FLYALALLL epitope. Therefore, the absence of
professional APC in our screening assay may explain why FLYA
LALLL-specific T cell responses were not detected in all cases. In
addition, when APC are exposed to peptide mixtures, competition
for binding to HLA molecules may lead to underdetection of
LMP2 specificity.

If T cells specific for LMP2 epitopes are to have antitumor ef-
fects, epitopes originating from the Caucasian-derived B95-8 vari-
ant of LMP2 (51) must be conserved in the tumor strain of EBV.
Comparison of the LMP2 epitope sequences in B95-8 with Asian
and Mediterranean EBV isolates from NPC tumors showed that
1 or 2 aa were altered in 3 of the newly identified epitopes. Simi-
larly, previous analysis showed amino acid alterations in 6 of
11 described/predicted LMP2 epitopes (including LPVIVAPYL
characterized in this work) in isolates from NPC and HL tumors
(30, 52). We and others (30) have shown that for 5 of these
epitopes, the alterations did not disrupt CTL recognition. Mutations
in 2 epitopes (LPVIVAPYL and MGSLEMVP) were shown to compromise their interaction with CTL, and for 1 epitope
(RRRWRLTLV) CTL recognition is predicted to be decreased
(52). Overall, the majority of, but not all, LMP2 epitopes appear to
be antigenically conserved among different isolates. These results
imply that one should ideally use the LMP2 protein as expressed
in the tumor as source of Ag for immunotherapeutic strategies.
However, as this is not feasible in the manufacturing of a clinical
grade therapeutic product, multiple LMP2 epitopes, including iso-
late-specific variant epitopes, should be used to activate tumor-
specific T cells.
The ability of tumor cells to delete certain Ags or epitopes to
escape from the immune response, as described both for the mell-
oma Ag MART1 and an immunodominant HLA-A11-restricted
EBV EBNA3 epitope, further stresses the importance of targeting
multiple tumor epitopes preferentially from multiple tumor Ags
(34, 53). Although broad LMP2 specificity was found in a number of
the LCL-reactivated CTL lines studied, in a significant number of
CTL lines the LMP2 response was targeted toward a single
epitope and 4 CTL lines lacked detectable numbers of LMP2-
specific T cells. LMP1-specific responses were only detected in 1
of 25 CTL lines (data not shown). This is most likely a result of the
preferential activation of immunodominant EBNA3 and lytic
EBV-Ag-specific T cells using LCL as APC to establish these CTL
cells. To improve tumor-Ag reactivity, reactivation and expansion
methods using APC overexpressing LMP1 and LMP2 have been
developed. Using this approach, the frequency of LMP-specific T
cells and the number of epitopes that these are targeted toward can
be increased (54, 55). Similarly, for vaccination approaches, vec-
tors encoding whole protein or, to avoid possible oncogenicity of
the Ag, multiple LMP1 and LMP2 epitopes (polytope approach)
are being developed instead of single peptides, to boost LMP-
specific T cells with a broad specificity (21). Incorporation of the
LMP2 epitopes identified in this study into current polytopes will
enhance the number of tumor Ag-derived epitopes targeted and
allow for application of this strategy to an even broader patient
group.

Valuable tools for immune monitoring following immunother-
APE interventions can be derived from LMP2 epitopes. These
include tetramers and peptides for stimulation of T cells in quanti-
tative and functional assays to detect cytokine secretion, and in
this study we have demonstrated how the in vivo expansion and
Persistence of LMP2-specific T cells can be monitored in the pe-
ipheral blood using LMP2 tetramers. Such immune studies that
provide insight into functional changes in tumor immunity are cru-
ical to evaluate efficacy and further optimize immunotherapeutic
strategies. These newly identified LMP2 epitopes will contribute to
a detailed characterization of the LMP2-directed T cell immunity
required to achieve this goal.

Disclosures
The authors have no financial conflict of interest.
References


