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*J Immunol* 2008; 180:1643-1654; doi: 10.4049/jimmunol.180.3.1643

http://www.jimmunol.org/content/180/3/1643
EBV Latent Membrane Proteins (LMPs) 1 and 2 as Immunotherapeutic Targets: LMP-Specific CD4+ Cytotoxic T Cell Recognition of EBV-Transformed B Cell Lines

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The EBV-latent membrane proteins (LMPs) 1 and 2 are among only three viral proteins expressed in EBV-associated Hodgkin’s lymphoma and nasopharyngeal carcinoma. Since these tumors are HLA class I and class II-positive, the LMPs could serve as both CD8+ and CD4+ T cell targets. In contrast to CD8 responses, very little is known about CD4 responses to LMPs. In this study, we describe CD4+ T cell clones defining four LMP1- and three LMP2-derived peptide epitopes and their restricting alleles. All clones produced Th1-like cytokines in response to peptide and most killed peptide-loaded target cells by perforin-mediated lysis. Although clones to different epitopes showed different functional avidities in peptide titration assays, avidity per se was a poor predictor of the ability to recognize naturally infected B lymphoblastoid cell lines (LCLs) expressing LMPs at physiologic levels. Some epitopes, particularly within LMP1, consistently mediated strong LCL recognition detectable in cytokine release, cytotoxicity, and outgrowth inhibition assays. Using cyclosporin A to selectively block cytokine release, we found that CD4+ T cell cytotoxicity is the key effector of LCL outgrowth control. We therefore infer that cytotoxic CD4+ T cells to a subset of LMP epitopes could have therapeutic potential against LMP-expressing tumors. The Journal of Immunology, 2008, 180: 1643–1654.

Virus-associated malignancies provide an important opportunity to develop immune T cell-based therapies targeting viral Ags expressed in tumor cells. The therapeutic potential of such an approach is best exemplified by an EBV-associated malignancy, posttransplant lymphoproliferative disease (PTLD),3 to which T cell-immunocompromised allograft recipients are particularly prone. PTLD lesions, particularly those arising early posttransplant, express the full spectrum of EBV-latent cycle proteins, the nuclear Ags EBNA1, 2, 3A, 3B, 3C, and –LP and the membrane proteins LMPs 1 and 2. In this respect, they closely resemble the lymphoblastoid cell lines (LCLs) generated when EBV transforms B cells into permanent growth in vitro. PTLD can be cured by adoptively transferring autologous or HLA-matched EBV-specific T cell preparations generated from PBMC by LCL stimulation in vitro (1–3). Such preparations tend to be limited to EBNA1, LMP1, and LMP2 (6). Of these, EBNA1 is now known to be an accessible CD8+ T cell target but EBNA1-specific CD8+ T cell memory is only detected in a small proportion of individuals with particular HLA class I alleles (7, 8). LMP1-specific responses are extremely rare, while LMP2 is more frequently immunogenic but almost always induces low-frequency subdominant responses (9, 10). Nevertheless, current trials of adoptive T cell therapy using LCL-stimulated preparations containing LMP2-specific CD8+ T cell reactivities have indicated possible clinical benefit against HL (11) and NPC (12). Further improvements to these protocols are clearly necessary and one attractive possibility would be to harness the power of the Ag-specific CD4+ T cell component.

CD4+ T cells play an important role in generating and maintaining effective CD8+ T cell immunity (13). More recently, however, attention has also focused on their potential role as direct effectors, capable of recognizing and killing target cells in situations where those targets express both MHC class II molecules and the relevant cognate Ag. This reflects the increasing number of examples in which endogenously expressed Ags are able to access the MHC class II presentation pathway within cells (14–20), an access once thought to be exclusive to exogenously acquired proteins. The fact that EBV-associated malignancies such as HL and NPC are HLA class II positive (6) has therefore prompted interest in the EBNA1, LMP1, and LMP2 proteins as targets of CD4+ T cells. Of these, EBNA1 appears to be a relatively strong immunogen for the CD4+ T cell response in EBV-infected individuals...
(21). However, although some in vitro-reactivated EBNA1-specific CD4+ T cell clones have been described which appear to recognize EBNA1-positive target B cells (17), the majority of such clones fail to recognize LCL target cells despite their endogenous expression of the EBNA1 protein (22–24). By contrast, very little is known about the LMPs as CD4+ T cell immunogens. This in fact reflects a general lack of information on multiple membrane-spanning proteins, of which LMPs are prime examples as CD4+ T cell targets. In this study, we describe the isolation of CD4+ T cell clones specific for a range of LMP1- and LMP2-derived epitopes, map their restricting alleles, and provide examples where such clones efficiently recognize latently infected targets and prevent their outgrowth through perforin-dependent cell killing.

Materials and Methods

**Donors, ELISPOT assays**

PBMCs from healthy EBV-seropositive adult donors of Chinese origin were depleted of CD8+ T cells using CD8 Dynabeads (Dynal) and tested in ELISPOT assays of IFN-γ release (Mabtech), as previously described (21), using individual 20-mer peptides (overlapping by 15 aa) spanning the entire LMP1 and LMP2 sequences as seen in typical Chinese EBV strains (25, 26). Some donors were HLA class II typed by PCR using sequence specific primers (performed by the National Blood Service, Birmingham, U.K.).

**In vitro reactivation and cloning protocols**

CD8-depleted PBMCs were pulsed for 1 h with 5 μM of the appropriate peptide(s), then washed and cultured in RPMI 1640 medium supplemented with 5% human AB serum (Sigma-Aldrich). Cells were harvested on day 7 of culture and cloned by limiting dilution in IL-2-supplemented medium as described elsewhere (9). Microcultures showing peptide reactivity by IFN-γ ELISA were expanded as previously described (23), now using FCS-supplemented medium. Microculture-derived T cell lines were analyzed for their CD4/CD8 status by dual-staining cells with FITC-Ab-ZB4 (2 anti-CD4 and PE-conjugated anti-CD8 (SeroSite) mAbs using standard protocols. T cell lines found to be exclusively CD4+ were assessed for clonality by PCR analysis of TCR Vβ chain usage using primers specific for each of the TCR Vβ chains.

**Target cells**

B lymphoblasts were generated by CD40L/IL-4 stimulation as previously described (27). EBV-transformed LCLs were generated by infection with the prototype CKL (Chinese) or B95.8 (Caucasian) EBV strains. Unless otherwise stated, all target cell recognition assays used CKL-transformed LCLs.

**ELISA of IFN-γ release and mAb blocking**

A range of numbers of CD4+ T cells was incubated in U-bottom microtest plate wells with a standard number of autologous, HLA-matched or HLA-mismatched LCL cells that had been either prepulsed for 1 h with 5 μM peptide or exposed to an equivalent concentration of DMSO solvent as a control, and then washed. Supernatant medium was harvested after 18 h and assayed for IFN-γ by ELISA (Endogen) following the manufacturer’s recommended protocol. In mAb-blocking assays, LCLs (either loaded with epitope peptide or exposed to DMSO alone as a control) were preincubated with mAbs specific for HLA-class I (W6/32 ATCC clone HB-95), HLA-DR (L243, ATCC clone HB-55), HLA-DQ (SPV-L3; SeroSite) and HLA-DP (B7.21, Cancer Research U.K. Research Services) at 10 μg/ml for 1 h before T cell addition, and the assay was conducted as above with mAb in the assay medium.

**Chromium release assays**

Effector CD4+ T cells were added to target LCL cells (peptide-loaded or DMSO-exposed as above) at known E:T ratios in 5- or 18-h chromium release experiments and results are expressed as percentage of specific lysis of the target line. In some experiments, T cells were pretreated for 2 h with 10 or 100 nM concanamycin A (Sigma-Aldrich) before addition to LCL targets in the presence of the same concentration of inhibitor. In other experiments, LCLs were pretreated for 2 h with the Fas-specific mAb ZB4 (1 μg/ml; Immunotech) before adding T cells in an equal volume of medium and conducting the assay as above. As a control for the ability of ZB4 to inhibit Fas-mediated lysis, in some cases ZB4-pretreated cells were then exposed to the agonistic anti-Fas mAb CH11 (0.5 μg/ml; Immunotech) or to an irrelevant control Ab instead of cells, and the assay was conducted as above.

**Perforin/granzyme staining of T cells**

T cells were first stained with anti-CD4 mAbs conjugated to appropriate fluorophores, then fixed in 1% paraformaldehyde for 10 min, permeabilized by incubation in 0.1% saponin in PBS for 30 min, and stained with perforin-PE or granzyme A-FITC Abs (BD Pharmingen). Cell staining was analyzed on a Coulter Epics Excel Flow Cytometer.

**Outgrowth assays**

Target cell lines (peptide loaded or DMSO exposed as above) were seeded into round-bottom microtest plate wells at doubling dilutions from 10,000 to 78 cells/well, with 6 replicate wells per dilution, with or without the addition of T cells (10,000 cells/well). All cultures were set up in standard LCL medium without cytokine supplements and were refed weekly by replacing half the medium in each well. Outgrowth was scored visually at 4 wk, with large pellets of actively growing CD19-positive cells indicating successful outgrowth of the seeded LCL cells. In contrast, only small quantities of cellular debris were present in wells where outgrowth had been inhibited by T cells. In some cases, the outgrowth assays were performed...
in parallel with or without the addition of cyclosporin A (CSA; Sandimmune; Novartis Pharmaceuticals) to standard culture medium at a final concentration of 0.25 μg/ml.

The range of cytokines produced by such cultures with and without CSA addition was determined using a multiplex bead assay detecting IFN-γ, TNF-α, IL-2, IL-4, and IL-10 (Beadlyte-Upstate Biotechnology).

**Results**

**CD4⁺ T cell clones against defined LMP1 peptide epitopes**

Based on screening CD8-depleted PBMCs from EBV-immune donors against a full panel of 74 LMP1 peptides in IFN-γ ELISPOT assays, we identified a number of relatively frequently recognized epitope regions and therefore selected for detailed study four individuals with clear evidence of responses to these regions. The ELISPOT results from these four donors are shown in Fig. 1 (left-hand column) as numbers of spot-forming cells per 10⁶ CD8-depleted PBMCs; reactivities were observed against LMP1 peptides 43/44 (recognized by donors 1 and 4), peptides 42/43 (recognized by donor 2), peptides 36/37 (recognized by donor 3), and peptides 3/4 (recognized by donors 2 and 4). ELISPOT responses to these four epitope regions were relatively common in the Chinese donors tested; 21% of healthy donors and 37% of NPC patients had reactivity to one or more of these epitopes (28). To characterize these responses in greater detail, we generated CD4⁺ T cell clones from each of the responses illustrated above by exposing CD8-depleted PBMCs for 1 h to the relevant peptide pair, then washing the cells and culturing for 7 days before limiting dilution cloning. For each epitope region, we selected two to three microcultures showing peptide reactivity by IFN-γ release in a screening assay and evidence of monoclonality based on being exclusively CD4⁺ and monotypic by PCR analysis of TCR Vβ chain usage (data not shown).

These selected clones were then assayed against target cells pre-exposed to titrated concentrations of the two epitope region peptides and their neighboring 20-mer in the LMP1 sequence. Representative results for one clone per epitope region are shown in Fig. 1 (right-hand column). Clones reactive to epitope region 43/44 (c1.63 from donor 1 and, data not shown, c4.2 and 4.4 from donor 4) all recognized peptide 43 slightly better than peptide 44; we therefore identified peptide 43 (LMP1 aa 211–230) as the optimal epitope and named this epitope SSH, based on the first three amino acids of the epitope sequence. Likewise, we identified the optimal epitope for the peptide 42/43 region as peptide 42 (LMP1 206–225, designated QAT), for the peptide 36/37 region as peptide 37 (LMP1 181–200, designated LIW) and for the peptide 3/4 region as peptide 3 (LMP1 11–30, designated GPP). These titration assays also allowed us to ascertain the functional avidity of each clone, defined as that concentration of epitope peptide giving half maximal IFN-γ release. Clones reactive to the same epitope, whether from the same or different donors, gave similar functional avidities.

**FIGURE 2.** HLA restriction of LMP1-specific CD4⁺ T cell clones. Representative clones specific for the SSH, QAT, LIW, and GPP epitopes within LMP1 were assayed for recognition of peptide-loaded LCL targets and the results are expressed as levels of IFN-γ release. **Upper panels,** Assays using the autologous LCL, a partially HLA-matched LCL, and two HLA-mismatched LCLs; the HLA-DR and HLA-DQ types of these targets are shown; and the relevant restricting allele is shown in bold. Note that these assays used a wider panel of HLA-typed allogeneic targets than shown, but all results were consistent with the identification of the bolded restricting allele. **Lower panels,** Assays using the peptide-loaded autologous LCL target conducted either in standard medium (no Ab) or in the presence of blocking mAbs to HLA class I, HLA-DP, HLA-DQ, or HLA-DR molecules.
avidities, but there were significant interepitope differences in avidity values, ranging from 1000 nM for QAT-specific clones to 1 nM for LIW-specific clones.

All clones were then analyzed for their HLA class II-restricting alleles: first, by testing a panel of partially HLA-matched cells as peptide presenters and, second, by assaying mAbs against HLA-DP, DQ, or DR Ags (and against HLA class I as a negative control) for their ability to block the recognition of peptide-loaded autologous cells. Representative data from such assays are shown in Fig. 2 for each of the epitope-specific responses. This showed that SSH-specific clones (in this case c4.2) were specifically blocked by the anti-HLA DQ mAb and that epitope presentation required a HLA-DQB1*0601-matched presenting cell; both donors who responded to the SSH epitope (donors 1 and 4) were indeed DQB1*0601 positive. The corresponding experiments with other epitope-specific clones identified the QAT epitope as DQB1*02 restricted and the LIW epitope as DRB1*16 restricted. Interestingly, the GPP epitope was found to be presented by two different DR alleles, DRB1*07 in the case of donor 2 and DRB1*09 in the case of donor 4 (Fig. 2 and data not shown).

**CD4+ T cell clones against defined LMP2 epitope peptides**

We then used the same approach to establish and characterize a panel of CD4+ T cell clones against LMP2 epitopes. Fig. 3 (left) shows the original ELISPOT screen data from three donors who responded to distinct epitope regions in LMP2, namely, peptides 86/87 (donor 5), peptides 40/41 (donor 6), and peptides 39/40 (donor 7). Again, 10% of healthy Chinese donors and 17% of NPC patients had ELISPOT reactivity to one or more of these epitopes (28). For each epitope region, two epitope-specific CD4+ T cell clones were selected as above for detailed study. Fig. 3 (right) shows representative results from peptide titration assays using the two epitope region peptides identified in the ELISPOT assay and neighboring peptides in the LMP2 sequence. Fig. 4 shows the corresponding restriction data from assays conducted with partially HLA-matched peptide-presenting cells and with blocking mAbs.

Thus, the peptide 86/87 response mapped to epitope peptide 86 (LMP2 419–438, designated TYG) in titration assays and to the HLA-DQB1*0601 allele in restriction assays. The peptide 40/41 response mapped to peptide 41 (LMP2 194–213, designated ICL).
and was HLA DRB1*1001 restricted, while the 39–40 response mapped to peptide 40 (LMP2 189–208, designated VTF) and was HLA DRB1*0901 restricted. In contrast to the earlier study of LMP1-specific clones, in this study we found greater differences in HLA DRB1*0901 restricted. In contrast to the earlier study of LMP1 and LMP2 sequences of a typical Chinese EBV strain, CKL, since all of the donors were of Chinese origin. There is slight sequence divergence at the latent gene loci between Chinese EBV strains and the prototype Caucasian strain B95.8 and this leads to between 1 and 3 aa changes in the 20-mer peptide sequences being studied here. For reference, we also conducted peptide titration assays on the corresponding B95.8 sequence peptides and the results are shown in Table II. For two of the LMP1 epitopes (SSH and QAT) and for all three LMP2 epitopes (TYG, ICL, and VTF), there was only a single amino acid change and in each case this had little effect on CD4+ T cell recognition. By contrast, the other two LMP1 epitopes (LIW and GPP) had two or three residues altered and this was reflected in much poorer recognition of the B95.8 sequence peptide. Note that subsequent LCL recognition experiments were conducted on CKL-transformed LCLs except for some assays using effectors against the LMP2 ICL and VTF epitopes where there was equivalent recognition of the CKL and B95.8 epitope peptides.

Table I. Summary of LMP1 and LMP2 epitope-specific CD4+ T cell clones

<table>
<thead>
<tr>
<th>Ag</th>
<th>Epitope Coordinates</th>
<th>Clone</th>
<th>Restriction Allele</th>
<th>Functional Aviditya (nM)</th>
<th>IFN-γ Recognition Efficiencyb (%)</th>
<th>Cytotoxicityc LCL</th>
<th>LCL + Peptide</th>
<th>Outgrowth Inhibitiond LCL</th>
<th>LCL + Peptide</th>
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<td>+ + + + + +</td>
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<td>+ + + + + +</td>
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<td></td>
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<tr>
<td>LMP1</td>
<td>206–225</td>
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<td>Caucasian</td>
<td>1000</td>
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<td>+ + + + + +</td>
<td>+ + + + + +</td>
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<td>Caucasian</td>
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</tr>
<tr>
<td>LMP2</td>
<td>419–438</td>
<td>c5.162</td>
<td>Caucasian</td>
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<tr>
<td>LMP2</td>
<td>419–438</td>
<td>c5.162</td>
<td>Caucasian</td>
<td>80</td>
<td>3</td>
<td>+ + + + + +</td>
<td>+ + + + + +</td>
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<td>300</td>
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<tr>
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<td>+ + + + + +</td>
<td>+ + + + + +</td>
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</table>

a Functional avidity is defined as the concentration of epitope peptide (CKL sequence) mediating 50% maximal IFN-γ release in peptide titration assays.

b Recognition of the unmanipulated LCL by IFN-γ release is expressed as a percentage of that seen in parallel against the same LCL optimally loaded with peptide epitope.

c Killing of LCL targets (unmanipulated or optimally loaded with epitope peptide) in 4-wk cocultivation assays; data summarized for three repeat assays per clone.

d Growth inhibition of LCL targets (unmanipulated or optimally loaded with epitope peptide) in 4-wk cocultivation assays; data summarized for three repeat assays per clone.

Results indicate the fold increase in mean LCL cell input required to obtain LCL outgrowth in the presence of CD4+ T cells compared to their absence. –, No increase; +, 1- to 2-fold increase; + +, 2- to 4-fold increase; + + +, 4- to 8-fold increase; + + + +, >8-fold increase.

Table II. Effect of EBV sequence polymorphism upon CD4+ T cell recognition

<table>
<thead>
<tr>
<th>Epitope Coordinates</th>
<th>Clone</th>
<th>Epitope</th>
<th>Functional Avidityb (nM)</th>
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<tbody>
<tr>
<td>LMP1 211–230</td>
<td>c2</td>
<td>Chinese</td>
<td>SSHESDSNSSNRHHHLLLVS</td>
</tr>
<tr>
<td>LMP1 206–225</td>
<td>c9</td>
<td>Chinese</td>
<td>QATDDSSSHESDSNSSNRHH</td>
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<td>LMP1 181–200</td>
<td>c3</td>
<td>Chinese</td>
<td>LIWMMYHGKRTHDEHHDDS</td>
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<td>c4</td>
<td>Chinese</td>
<td>GPRPRPLGPGLSSSGGLALL</td>
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<td>Chinese</td>
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<td>LMP2 194–213</td>
<td>c142</td>
<td>Chinese</td>
<td>ICCLTWRIEDPPFNSIFLALL</td>
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<tr>
<td>LMP2 189–208</td>
<td>c95</td>
<td>Chinese</td>
<td>VTFFAICLTWRIEDPPFNSI</td>
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</table>

a Each CD4+ T cell clone was tested in peptide titration assays with its cognate 20-mer peptide (based on the Chinese CKL strain of EBV) or the equivalent peptide synthesized according to the epitope sequence from the Caucasian B95.8 strain of EBV. One representative clone is shown for each epitope.

b Functional avidity is defined as the concentration of epitope peptide mediating 50% of each peptide’s maximal IFN-γ release in titration assays.
LCL recognition by CD4+ T cell clones

We next used assays first developed in work with EBNA-specific clones (23) to ask whether the above LMP1- and LMP2-specific CD4+ T cell clones were capable of recognizing unmanipulated (i.e., nonpeptide loaded) autologous LCL cells. For each clone, graded numbers of CD4+ T cells were added to a standard number of LCL cells from the autologous donor, from an appropriately HLA-matched donor, and from two HLA-mismatched donors. Each LCL was either left untreated or pre-exposed to the epitope peptide (at a dose known to produce an optimal response in the autologous presenting cell setting), and washed well before use; CD4+ T cell recognition was assayed by IFN-γ release after overnight coculture. Representative data are shown in Fig. 5 for all of the epitope reactivities analyzed. Recognition of peptide-loaded targets is denoted by gray bars, recognition of unmanipulated targets by black bars.

Among the LMP1-specific clones (Fig. 5A), although all four reactivities responded well to HLA-matched LCL targets pre-loaded with peptide, clones specific for the SSH and QAT epitopes also showed strong recognition of unmanipulated target cells. This recognition appeared to be specific since it was restricted to HLA-matched targets, increased proportionately with greater T cell input, and could be blocked by the anti-HLA DQ mAb, just as would be predicted for these DQB1*0601-restricted and DQB1*02-restricted epitopes. Note that these same CD4+ T cells showed no detectable recognition of autologous or HLA-matched B lymphoblasts activated by CD40L/IL-4 stimulation rather than by EBV (data not shown), again consistent with their virus specificity. As illustrated in Fig. 5A, levels of unmanipulated LCL recognition could be as high as 60% of that seen in the same assay against peptide-loaded targets. We found that, for all three SSH- and for both QAT-specific clones, autologous LCL recognition varied between experiments but was always in the range of 15–60% of the maximal response against the peptide-loaded LCL. By contrast, clones specific for the other two LMP1-derived epitopes, LIW and GPP, showed much lower recognition of unmanipulated LCL targets, <1% of the value seen with the corresponding peptide-loaded LCL. At these levels, significant IFN-γ release could only be detected in cocultures with the highest CD4+ T cell input. However, this IFN-γ response was reproducible between experiments and was never observed in cocultures with HLA-mismatched LCLs.

Data from individual LMP2-specific clones are illustrated in Fig. 5B. A clone specific for the DQB1*0601-restricted TYG epitope (c5.162) showed clear recognition of the unmanipulated HLA-matched LCL which increased with input T cell number and was specifically blocked by the anti-DQ mAb. However, the levels of recognition were relatively low, representing ~3% of the peptide-loaded LCL value. The other TYG specific but less avid clone, c5.48, failed to recognize unmanipulated LCL targets (data not shown). Both clones specific for the DRB1*1001-restricted ICL epitope recognized the unmanipulated HLA-matched LCL at levels varying from 9% of the peptide-loaded value for one of the clones (c6.142; Fig. 5B) to 2% for the other clone (c6.2; data not shown). Of the two clones specific for the DRB1*0901-restricted...
VTF epitope, c7.46 showed unmanipulated LCL recognition at ~5% of the peptide-loaded control value, whereas the less avid clone 7.95 was only capable of recognizing the peptide-loaded LCL. Data for all LMP1- and LMP2-specific clones analyzed are again summarized in Table I.

Cytotoxic capacity of CD4\(^{+}\) T cell clones

To examine their possible cytotoxic function, all clones were tested in 18-h cytotoxicity assays against unmanipulated and epitope peptide-loaded target LCLs, again both HLA matched and HLA mismatched with the relevant CD4 effectors (Fig. 6). Interestingly, all of the LMP1-specific clones mediated significant lysis of HLA-matched peptide-loaded LCL targets. Furthermore, the SSH- and QAT-specific CD4\(^{+}\) clones (i.e., those cells giving the highest recognition in IFN-\(\gamma\) release assay) also showed significant killing of unmanipulated LCL targets at E:T ratios as low as 2.5:1 (Fig. 6A and data not shown). By contrast, none of the LMP2-specific clones gave detectable lysis of the unmanipulated LCL, although five of the six such clones clearly killed peptide-loaded HLA-matched targets effectively. The sixth clone, VTF-specific c7.95, failed to kill the relevant peptide-loaded LCL even though these cells clearly recognized the same targets by IFN-\(\gamma\) release (cf VTF c7.95 data, Figs. 5B and 6B). This clone never gave detectable killing even when the E:T ratios were increased to 30:1.

Because target cell lysis by the above CD4\(^{+}\) T cells was less obvious in 5-h assays (the usual time scale for CD8\(^{+}\) T cell cytotoxicity assays), we sought to determine whether target cells were being killed by perforin/granzyme release (as used by CD8\(^{+}\) T cells) or by the slower pathway involving Fas ligation sometimes observed for CD4\(^{+}\) T cells (17, 29). Intracellular staining (Fig. 7A) showed that all of the cytotoxic clones were
FIGURE 7. Mechanism of cytotoxicity. A, Flow cytometric analyses of permeabilized CD4+ T cells stained with mAbs specific for perforin and for granzyme A. Data are shown from clones specific for three LMP1 epitopes (SSH, QAT, and LIW) and for two LMP2 epitopes (ICL and VTF). Note that the nontoxic clone VTF c7.95 is unique in lacking perforin expression. B, Cytotoxicity assays using the above CD4+ T cell clones and matched LCL targets (pretreated with epitope peptide) where the assays were conducted over 18 h in standard medium (control) in the presence of 10–100 nM concanamycin A (ConA) or in the presence of the anti-Fas-blocking mAb ZB4 (+ZB4). Results are expressed as in Fig. 6 at E:T ratios of 5:1 (■) and 2.5:1 (□) unless otherwise indicated on the figure. In the same assays, the LCL targets were exposed to the agonistic anti-Fas mAb CH11 in standard medium (control) or in the presence of the anti-Fas-blocking mAb ZB4 as above (+ZB4) and specific lysis is shown as hatched bars. Representative results from three independent experiments are shown.
Control of LCL outgrowth by CD4

inhibit lysis of the same target cells induced by the agonistic anti-Fas mAb ZB4, whereas in a parallel assay this anti-Fas mAb did not get cell lysis is significantly inhibited by concanamycin A but not killing (Fig. 7). The results clearly show that peptide-loaded targets in the presence of either concanamycin A for perforin expression. We then performed cytotoxicity assays using clear perforin and granzyme A positive, whereas the one non-cytotoxic clone, VTF c7.95, was distinct in lacking detectable perforin expression. We then performed cytotoxicity assays using peptide-loaded targets in the presence of either concanamycin A (30), to selectively block perforin/granzyme-dependent killing or the anti-Fas mAb ZB4 (31), to selectively block Fas-dependent killing (Fig. 7B). The results clearly show that peptide-loaded target cell lysis is significantly inhibited by concanamycin A but not by the mAb ZB4, whereas in a parallel assay this anti-Fas mAb did inhibit lysis of the same target cells induced by the agonistic anti-Fas mAb CH11 (31).

Control of LCL outgrowth by CD4+ T cell clones

All clones were then examined for their ability to prevent target cell outgrowth in 4-wk cocultures. In each case, two HLA-matched and two HLA-mismatched LCLs, with and without epitope peptide loading, were seeded into microtest plate wells across a range of cell inputs (10^2–78 cells/well). Outgrowth efficiency, defined as the minimum LCL input required to achieve successful outgrowth in 50% replicate wells, was then determined in the presence and absence of 10^4 added CD4+ T cells.

Representative results from such experiments are shown in Fig. 8. Outgrowth efficiency for LCLs cultured in the absence of T cells is shown by dotted lines and for LCLs in the presence of T cells by symbols (Fig. 8; ● and ■, unmanipulated LCL; ○ and □, peptide-loaded LCL). Almost all clones tested inhibited the outgrowth of peptide-loaded LCLs of the correct HLA type without affecting the corresponding HLA-mismatched targets. Importantly, clones specific for the LMP1 epitopes SSH and QAT, that had shown efficient recognition of the unmanipulated LCL in IFN-γ release and cytotoxicity assays, also significantly inhibited their outgrowth. Clones with lower levels of LCL recognition in those earlier assays, for example, clones to the LMP2 epitopes TYG and ICL, were correspondingly less active in outgrowth inhibition, but nevertheless still mediated a small effect apparent in HLA-matched but not HLA-mismatched LCL targets. By contrast, clones with only trace levels of unmanipulated LCL recognition in the earlier IFN-γ assays, for example, clones against the LMP1 epitopes LIW and GPP, had no effect on outgrowth inhibition (Fig. 8 and data not shown). Particularly interesting was the result from the clone VTF c7.95, which produced IFN-γ in response to the peptide-loaded HLA-matched LCL but failed to kill these cells; this was completely devoid of inhibitory activity in outgrowth assays.

This last observation prompted a final series of experiments which sought to determine the relative importance of cytokine release and cytotoxicity as effector mechanisms contributing to LCL growth control. In this study, we took advantage of the fact that following TCR ligation, perforin/granzyme release from CTLs can be distinguished from cytokine secretion (32) in that only the latter requires NFAT-dependent gene transcription (33). We therefore conducted preliminary assays in which the CD4+ T cell clones were exposed to peptide-loaded HLA-matched LCL targets in the presence or absence of CSA, a potent inhibitor of TCR signaling via the NFAT pathway (33). As shown by representative results from one such clone (Fig. 9A), target cell lysis was virtually unaffected by the presence of CSA, whereas IFN-γ production was almost completely blocked. This result was reproducible with many different clones. Furthermore, as shown in Fig. 9B, CSA also inhibited the production of other cytokines such as TNF-α and IL-2, which the LMP-specific clones were found to make in response to Ag stimulation; all cytokines were reduced to ~10% of control levels or less. The LCL outgrowth assays were therefore repeated, now seeding matched and mismatched LCLs at a range of dilutions with or without the addition of CD4+ T cells and in the presence or absence of CSA. Typical results for six different CD4+ T cell clones are shown in Fig. 9C, in this case using peptide-pulsed LCLs as targets to maximize the T cell-mediated effect. As before, all of these CD4+ T cell clones specifically inhibited outgrowth of the relevant HLA-matched peptide-loaded LCL but not of its HLA-mismatched counterpart. In each case, there was marked growth inhibition despite the presence of CSA in the culture medium. Measuring cytokines in supernatant medium harvested from the two sets of cultures at 24 h confirmed that CSA had reduced cytokine production by ≥90%; indeed, outgrowth inhibition frequently occurred in wells in which cytokine production had been abolished below detectable levels (data not shown).

Discussion

The LMPs are part of a very limited range of EBV-latent cycle proteins expressed in such malignancies as HL and NPC. Although at least one of these proteins, LMP2, can serve as a CD8+ T cell
target (9, 27, 34) it is debatable whether restoring LMP-specific CD8+ T cell responses in HL and NPC patients will on its own be sufficient to achieve a long-lasting therapeutic response. Including relevant CD4+ T cell reactivities could offer additional therapeutic benefit, either through their helper function or possibly as direct effectors. Before the present study, little was known about the LMPs as CD4+ T cell targets apart from some preliminary data from ELISPOT screening assays (21) and the presence of a LMP2-specific CD4+ T cell component within polyclonal effector populations stimulated in vitro either with the autologous LCLs (35) or with LMP2-transfected autologous dendritic cells (36). As summarized in Table I, in this study, we describe CD4+ T cell clones reactive to seven previously unidentified epitopes, four within LMP1 and three within LMP2, each restricted through a defined HLA class II allele. All of the clones displayed Th1-like patterns of cytokine production in response to Ag stimulation, secreting IFN-γ, TNF-α, and IL-2 in the absence of typical Th2-like cytokines such as IL-4 and IL-10. From peptide titration assays, the majority of clones analyzed showed functional avidities within the 1–100 nM range previously observed in our laboratory for CD4+ T cell clones against defined epitopes within the LMP1 sequence, SSH and QAT, elicited CD4+ T cell clones with markedly different avidities of 40 and 1000 nM.

With this as background, we went on to look in detail at the capacity of all of the clones to recognize unmanipulated LCL cells (i.e., HLA class II-positive targets expressing LMP1 and LMP2 at physiologic levels) as an indicator of their likely therapeutic value in targeting LMP-positive tumors. These assays showed that, for all seven epitopes studied, one or more of the epitope-specific clones was capable of LCL recognition but at levels which again varied substantially between epitopes. For example, recognition of the unmanipulated LCL by LMP1-specific clones ranged from 1% of that seen against peptide-loaded targets for clones against the LIW and GPP epitopes to between 15 and 60% for clones against the SSH and QAT epitopes. Note that these latter values exceeded the strongest LCL reactivity reported for any EBV-latent Ag-specific CD4+ T cell response, namely, against the well-characterized PRS epitope in EBNA2 (23, 37, 38).

An important conclusion from these experiments is that the ability to recognize unmanipulated LCL targets does not correlate with functional avidity of the response. Thus, clones specific for the LIW epitope in LMP1 were 1000-fold more avid in peptide titration assays than clones specific for the QAT epitope, also in LMP1, yet the latter showed much stronger LCL recognition. We infer that, just as for CD8+ T cell recognition (39), the cell surface level of CD4+ epitope display is likely to be a key factor influencing the potential of any one particular epitope as a therapeutic target. Thus, in LCLs with the relevant HLA type, there must be substantially greater levels of the QAT-DQ2 complex than the LIW-DR16 complex on the cell surface. Such differences in epitope display can clearly override the effects of response avidity and, as a result, one cannot assume that epitopes inducing the highest avidity responses will necessarily be the most effective therapeutic targets. Within the
context of any one epitope, however, clones with the highest avidity in peptide titration assays are likely to be the most useful therapeutically. For example, the more avid clones against LMP2 epitopes TYG and VTF gave detectable LCL recognition, whereas the less avid clones did not.

A second important conclusion from these experiments is that, although the different epitope responses vary greatly in their capacity for LCL recognition, this is not obviously reflected in the size of these responses in vivo as detected by ELISPOT screening. For example, responses to two DQ6-restricted epitopes, SSH and TYG, differ radically in LCL recognition, yet these responses are often of equivalent size (see Figs. 1 and 3). Larger scale ELISPOT screening of additional donors has confirmed that no one LMP1 or LMP2 epitope stands out as inducing either consistently high or consistently low responses (28). In light of these findings, it is clear that a numerically dominant response is not necessarily going to be the most valuable therapeutically. We infer that this lack of relationship between an LMP epitope’s immunogenicity and its level of display on naturally infected target cells reflects the fact that LMP-specific CD4+ T cell responses are unlikely to be primed by direct contact with EBV-infected B cells in vivo. More likely, these responses will be cross-primed by dendritic cells that have acquired the LMP proteins as exogenous Ags shed from infected cells. The implication, yet to be explored, is that these multiple membrane-spanning proteins are handled differently by the MHC class II processing pathway when exogenously acquired by APCs than when endogenously expressed in B cells.

Moving on to the functional analysis of the LMP-specific CD4+ T cell clones, we found that most were capable not only of secreting cytokines in response to peptide-loaded targets but also of killing these cells in cytotoxicity assays. As seen with cytotoxic CD4+ T cells to EBNA-derived epitopes (17, 23), killing was often better detected in overnight (18 h) than in shorter-term chromium release assays. The mechanism of killing by EBV-specific CD4+ clones remains unresolved in the literature with one report suggesting a Fas-dependent pathway (17), another a perforin-dependent pathway (36), and another a pathway that was perforin-independent but involved exocytosis of granulin (40). Our study of LMP1- and LMP2-specific CD4 responses strongly suggests target cell lysis by a perforin- and gran Lyzme-dependent mechanism. Thus, we noted that all 14 cytotoxic clones expressed both perforin and granzyme A, whereas the one noncytotoxic clone expressed granzyme A but was perforin negative; it is known that perforin is required for granyme effector proteins to mediate target cell killing (41). Furthermore, concanamycin A, an inhibitor of the vacuole acidification required for perforin-dependent killing (30), blocked target cell lysis by these CD4 clones, whereas the antagonistic anti-Fas mAb ZB4 had no blocking effect. CD4+ T cell killing via the perforin pathway has now been reported in a number of human cell studies in vitro (42–46). Given the limited sensitivity of direct ex vivo cytotoxicity assays, we have been unable to determine the cytotoxic function of LMP-specific CD4+ T cells before in vitro expansion. However, it is interesting to note that perforin-positive CD4+ T cells have also been detected in vivo both in healthy individuals (47) and in disease states (48), strongly suggesting that the cytotoxic capacity of the presently described clones is not purely an in vitro artifact.

Arguably, the best in vitro correlate of a T cell clone’s therapeutic potential is the ability to prevent the outgrowth of target cells in long-term coculture assays. In this study, we show that those clones with the strongest reactivity against unmanipulated LCL targets in IFN-γ and cytotoxicity assays are indeed by far the most efficient inhibitors of LCL outgrowth. This raised the question whether cytokine release or cell killing was the more important effector mechanism of growth control. In this regard, the only clone incapable of blocking the outgrowth of peptide-loaded LCL targets was the one clone that lacked cytotoxic ability. Furthermore, CSA was able to block cytokine release by our CD4+ T cell clones without affecting target cell lysis, and the presence of CSA in cocultures had little or no impact on outgrowth inhibition. Although we could not rule out an as-yet-unidentified cytokine whose production by CD4+ T cells is CSA independent, all our data point to cytotoxicity as the key property required by CD4+ T cells to act as direct effectors of growth control.

The present work, which represents the first description of CD4+ T cell clones against defined epitopes in LMP1 and LMP2, is important in two respects. First, it provides tools with which to examine the route(s) whereby such highly hydrophobic membrane proteins access the HLA class II pathway for presentation to CD4+ T cells. Second, it proves that there are components of CD4 memory that are specific for the LMPs, proteins expressed in a wide range of EBV-associated malignancies, and have the ability to directly recognize LMP-expressing target cells. Since LMP-specific CD4+ T cells are present in the blood of a sizeable proportion of patients with NPC, exploiting the therapeutic potential of these reactivities is an important priority for future work.

Acknowledgments

We thank A. Milner for advice on Fas blocking Abs, J. Curnow for help with the multiplex cytokine assay, and N. Gudgeon and D. Sauce for advice and assistance with flow cytometry (all from the University of Birmingham, Birmingham, U.K.). We are also grateful to E. Houssaint and E. Landais (University of Nantes, Nantes, France) for advice on intracellular staining for perforin and granzyme.

Disclosures

The authors have no financial conflict of interest.

References


