CD8+ T Cell Responses to Lytic EBV Infection: Late Antigen Specificities as Subdominant Components of the Total Response

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CD8⁺ T Cell Responses to Lytic EBV Infection: Late Antigen Specificities as Subdominant Components of the Total Response

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EBV elicits primary CD8⁺ T cell responses that, by T cell cloning from infectious mononucleosis (IM) patients, appear skewed toward immediate early (IE) and some early (E) lytic cycle proteins, with late (L) proteins rarely targeted. However, L Ag-specific responses have been detected regularly in polyclonal T cell cultures from long-term virus carriers. To resolve this apparent difference between responses to primary and persistent infection, 13 long-term carriers were screened in ex vivo IFN-γ ELISPOT assays using peptides spanning the two IE, six representative E, and seven representative L proteins. This revealed memory CD8⁺ T cell responses to 44 new lytic cycle epitopes that straddle all three protein classes but, in terms of both frequency and size, maintain the IE > E > L hierarchy of immunodominance. Having identified the HLA restriction of 10 (including 7 L) new epitopes using memory CD8⁺ T cell clones, we looked in HLA-matched IM patients and found such reactivities but typically at low levels, explaining why they had gone undetected in the original IM clonal screens. Wherever tested, all CD8⁺ T cell clones against these novel lytic cycle epitopes recognized lytically infected cells naturally expressing their target Ag. Surprisingly, however, clones against the most frequently recognized L Ag, the BNRF1 tegument protein, also recognized latently infected, growth-transformed cells. We infer that BNRF1 is also a latent Ag that could be targeted in T cell therapy of EBV-driven B-lymphoproliferative disease. The Journal of Immunology, 2013, 191: 000–000.
which inhibits the TAP-mediated peptide transport onto nascent HLA class I molecules (11, 12); BILF1, which specifically modulates HLA class I trafficking (13, 14); and BGLF5 and the viral IL-10 homolog, which have more general effects (15–17).

Given the apparent rarity of L Ag–specific responses in IM patients, it had generally been assumed that they would be at least as rare in memory, particularly given the huge contraction observed in CD8 responses to other lytic Ags following convalescence. Indeed, most surveys of CD8 memory in long-term virus carriers concentrated primarily on IE/E Ag targets recognized by both CD8 T cell lines to screen adult EBV carriers for ex vivo reactivity to peptide panels in IFN-γ ELISPOT assays. Our objectives were to determine the relative restriction of selected responses, particularly L Ag–specific responses, by CD8+ T cell cloning; and to re-examine IM PBMC preparations ex vivo for these newly identified reactivities.

In the present study, we re-examine the issue of immunodominance among EBV lytic proteins in CD8+ T cell memory. As representative target Ags, we selected the two IE proteins and a panel of six E and seven L proteins; these included some already studied by both ourselves (6) and by Orlova et al. (25), as well as others not hitherto investigated as CD8 targets. To avoid potential artifacts introduced by repeated LCL stimulation and polyclonal T cell expansion in vitro, we began by screening PBMCs from healthy virus carriers for ex vivo reactivity to peptide panels in ELISPOT assays. Our objectives were to determine the relative frequency of IE, E, and L peptide–specific responses in CD8+ T cell memory; to establish the lytic Ag specificity and HLA restriction of selected responses, particularly L Ag–specific responses, by CD8+ T cell cloning; and to re-examine IM PBMC preparations ex vivo for these newly identified reactivities.

Materials and Methods

EBV target Ags

The following 15 EBV lytic cycle proteins were selected for the study: the two IE transcription activator proteins, BZLF1 and BRLF1, six representative E proteins, BMLF1 and BMRFI (both transcriptional transactivators), BHRF1 (bc2 homolog), BaRF1 (small ribonucleotide reductase), BFRF1 (nuclear envelope protein), and BLF3 (dUTPase); and seven representative L proteins, gp350/BLF1, gp85/BXLF2, gp25/BKRF2 and gp42/BZLF2 (all viral glycoproteins), BFRF3 (small capsid protein), BVRF2 (capsid maturational protein), and BNRFI (major tegument protein). Synthetic peptides based on the B95.8 strain sequence were purchased from either AltaBioscience (University of Birmingham, Birmingham, U.K.) or Mimmotopes (Clayton, Australia) and dissolved in DMSO, and their concentrations were determined by biuret assay. Peptide panels were 15-mer or 20-mer overlapping by 10 or 11 aa for most Ags, as well as 20-mer overlapping by 15 aa for gp350, gp85, and gp42; both 15-mer and 20-mer panels were prepared for gp25 for comparison.

Donors

Written, informed consent was given by all donors for the collection and analysis of blood samples, and all experiments were approved by the West Midlands (Black Country) Research Ethics Committee (07/Q2702/24). Screening for memory CD8+ T cell responses was carried out on 13 EBV-seropositive donors with no history of IM and 1 EBV-seronegative control donor. Their HLA class I types are shown in Supplemental Table I and included many alleles commonly found in white populations. In addition, PBMCs from eight patients with acute IM of known HLA type were used in intracellular cytokine-staining experiments.

PBMC preparations

Sixty-milliliter blood samples were collected from all donors, and PBMCs were separated by Ficoll-Hypaque centrifugation into RPMI 1640 medium + 1% glutamine (Invitrogen) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 5% human serum or 10% FCS. PBMCs used in ELISPOT screening assays were depleted of CD4+ T cells using CD4 Dynabeads (Invitrogen), in accordance with the manufacturer’s protocol. Efficient depletion was confirmed by flow cytometry and >98% reduction in CD4+ cells was consistently achieved.

Screening for memory CD8+ T cell responses by IFN-α ELISPOT assay

CD4+–depleted PBMCs were tested in IFN-γ ELISPOT assays using cytokine capture and detection reagents, as previously described (26). Briefly, 96-well nitrocellulose plates (Millipore) were coated with anti–IFN-γ Abs, and CD4+–depleted PBMCs were added to replicate wells in the presence of single or pooled overlapping peptides (8–12 peptide/plate) at a final concentration of 5 µg/ml for each peptide. A total of 10 µg/ml PHA and an equivalent volume of DMSO were used as positive and negative controls, respectively. Generally, 150,000 cells were used in each replicate. After overnight incubation at 37°C in 5% CO2, the cells were discarded, and captured IFN-γ was detected with a biotinylated anti–IFN-γ Ab, followed by a streptavidin-conjugated alkaline phosphatase and an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad). The spots were counted using an automated plate counter (AID). In all experiments, results from ELISPOT assays are expressed as spot-forming cells (SFC)/million CD4+ cells. Positive responses were defined as those in which the mean number of spots in replicate wells exceeded the mean number of spots (+2 SD) in the replicate DMSO control wells.

Isolation and culture of T cell clones

Short-term polyclonal cultures of peptide-specific cells were generated by incubating whole PBMCs in RPMI 1640 medium + 1-glutamine (Invitrogen) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 5% human serum, and 20 ng/ml IL-7 after pre-exposing one fifth of the cells to 5 µg/ml the appropriate peptide(s) for 1–2 h and washing. IL-2 was added to the cultures on day 2 at a final concentration of 50 U/ml. On day 7, cultures were depleted of CD4+ T cells using anti-CD4 Dynabeads (Invitrogen), and the peptide-specific cells were enriched using a MACS IFN-γ Secretion Assay–Cell Enrichment and Detection Kit (Miltenyi Biotec), in accordance with the manufacturer’s protocols. T cell clones were isolated from these preparations by limiting dilution seeding in standard culture medium supplemented by IL-2, as previously described (27); irradiated preactivated allogeneic PBMCs (106/ml) were always included as feeders, with the addition of an anti-CD3 mAb (OKT3; Unipath, Basingstoke, U.K.) to a final concentration of 30 ng/ml. Growing microcultures reactive against the desired peptide(s) in IFN-γ ELISAs were further expanded and cultured by transfer into 2-ml wells using the same stimulation protocol as before.

Characterization of T cell clones by IFN-γ ELISA

The CD8+ T cell clones (5,000 or 10,000 cells/well) were incubated in V-bottom microtest plate wells with 10 times the number of target cells. The supernatant medium was harvested after 18 h and assayed for IFN-γ by ELISA (Endogen), in accordance with the manufacturer’s protocol. For HLA-restriction assays, the target cells were autologous, partially HLA-matched LCLs (chiefly from patients with lymphoma and included many alleles commonly found in white populations). In addition, clones were tested for recognition of lytically infected target cells using target LCLs of the relevant HLA type transformed with either wild-type B95.8 virus or, as a negative control, the replication-deficient BZLF1-knockout (BZ/KO) derivative strain of B95.8. For some specificities, the minimal epitope recognized by the CD8+ T cells was defined experimentally by testing on HLA-matched LCL cells pre-exposed to a panel of shorter peptides within the 15 mer/20 mer of interest, each at a range of peptide concentrations.

Identification of primary CD8+ T cell responses to EBV lytic epitopes by flow cytometry

Cryopreserved PBMCs from patients with acute IM were thawed, washed, and resuspended in RPMI 1640 medium + 1-glutamine (Invitrogen)
supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% FCS, and 50 μU/ml IL-2. The cells were transferred to FACS tubes (0.5–1 × 10^6 cells/tube) in 500 μl medium and incubated at 37°C in 5% CO2, with 5 μg/ml epitope peptide, with the equivalent volume of DMSO as a negative control or with 0.2 μg/ml staphylococcal enterotoxin B (Sigma) as a positive control. Brefeldin A (Sigma) was added to each tube after 1 h to a final concentration of 10 μg/ml. After an additional 5-h incubation period, the cells were washed in PBS and stained with LIVE/DEAD Fixable Far Red Dead Cell Stain (Invitrogen) for 15 min at room temperature. Following a wash with PBS, the cells were washed with staining buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA) before they were stained on ice for 15 min with saturating concentrations of the following anti-human Abs: PerCP-Cy5.5-conjugated CD8 mAb (clone RPA-T8; eBioscience), PE-conjugated CD4 mAb (clone RPA-T4; BD Pharmingen), and allophycocyanin-conjugated CD19 mAb (clone HIB19; eBioscience). Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% saponin for 5 min, and stained with FITC-conjugated IFN-γ mAb (clone 25723.11; BD FastImmune) for 30 min, all at room temperature. After a final wash, the cells were resuspended in 200 μl staining buffer and analyzed on a BD Accuri C6 flow cytometer (BD Biosciences). All data were processed using FlowJo software (TreeStar).

Results

Memory CD8+ T cell responses to EBV lytic cycle Ags

A first set of experiments used IFN-γ ELISPOT assays to screen CD4+ depleted PBMCs from healthy adult donors for ex vivo reactivity to peptide panels representing 15 EBV lytic cycle Ags. These were the two IE proteins (BZLF1 and BRLF1), six E proteins (BMLF1, BMRF1, BHRF1, BaRF1, BFRF1, and BLLF3), and seven L proteins (gp350, gp85, gp25, gp42, BFRF3, BVRF2, and BNRF1). For each protein, peptides were arranged into pools of 8–12 consecutive peptides for the initial screening and, where responses were detected, assays were repeated on the individual peptides within the pool to identify the relevant epitope region. Fig. 1 shows examples of results from two EBV-seropositive individuals. Responses in donor 5 could be detected to peptide pool 5 from the IE protein BZLF1, to pool 4 from the E protein BaRF1, to pool 4 from the L protein BFRF2, and to pools 15 and 20 from a second L protein, BNRF1 (Fig. 1A, left panels). Subsequent testing mapped these responses to individual peptides BZLF1 5.2 (aa 209–217), BaRF1 4.1 and 4.2 (sharing aa 149–159), BFRF2 4.2 and 4.3 (sharing aa 153–163), and BNRF1 15.9 and 15.10 (sharing aa 709–719) and 20.5 (aa 929–943) (Fig. 1A, right panels). Corresponding data from donor 9, again illustrating responses to peptides within IE (BZLF1), E (BLLF3), and L (BNRF1) Ags, are shown in Fig. 1B. All of the above responses represented novel reactivities. Indeed, of the five target Ags identified in Fig. 1, only BZLF1 had been reported as a CD8+ T cell target. Such results clearly showed that the peptide-screening approach was capable of detecting memory CD8 responses to hitherto unknown epitopes in IE, E, and L proteins of the lytic cycle. Note also that, in the examples shown in Fig. 1, response size (expressed as the number of SFC/10^6 CD4+-depleted PBMCs) decreased as one moved from IE through E to L Ag responses.

In total, 13 EBV-seropositive donors were screened in this way, alongside one EBV-seronegative donor as a control. Throughout this work, all assays on peptide pools and/or individual peptides were repeated using further bleeds of the donors in question to confirm that the reported responses were reproducible. Table I shows the combination of individual Ag responses seen in each donor. All 13 seropositive donors showed reactivity to at least 1 of the 15 lytic cycle Ags screened, with responses to a median of 6 Ags/donor, whereas the seronegative donor gave uniformly negative results. In line with their previously reported immunodominance, the IE proteins BZLF1 and BRLF1 were the most frequently recognized, with reactivities detectable in 12/13 and 10/13 seropositive subjects, respectively. Among the E proteins, the incidence of recognition among seropositive donors ranged from frequent (10/13) for BMLF1 to rare (1/12) for BHRF1, again reminiscent of the breadth of results obtained in earlier work examining E proteins as CD8 targets. Most interesting, however, were the data obtained with the L Ag peptide panel. Although
peptides from some Ags, such as the viral envelope glycoproteins, were rarely recognized, we found examples of four donors responding to peptides within the virus assembly protein BVRF2, five donors responding to peptides within the capsid protein BFRF3, and a remarkable total of nine donors responding to peptides within the tegument protein BNRF1. Overall, positive responses were obtained from 85% of individual donor/Ag combinations tested involving IE proteins, 38% of those involving E proteins, and 25% of those involving L proteins.

Most of the above responses were mapped to single-epitope regions in assays on individual peptides within the original positive pool, and the results are summarized in Fig. 2. Each protein is drawn to show its relative size, and the locations of CD8 epitopes within its sequence are identified as vertical bars. The two IE proteins and, in particular, BZLF1 (the smaller of the two) contained multiple epitopes, whereas epitope numbers in the E proteins ranged between 1 and 5. Patterns of epitope density were even more varied among the L proteins. Of the glycoproteins, gp350 and gp42 were completely devoid of detectable epitopes, whereas the occasional responses to gp85 and gp25 in each case mapped to a single peptide in the sequence. By contrast, the more frequently recognized BVRF2 and BFRF3 proteins each contained 3 epitopes, whereas the many responses detected against the large BNRF1 protein mapped to a total of 12 separate epitope regions. Overall, these screening assays detected memory CD8 responses to a total of 53 epitopes in the lytic cycle Ags chosen for study.

The detailed locations and sequences of these epitopes, as well as the individual donors that responded to them, are shown in Supplemental Table II. Remarkably, only nine of these responses targeted previously identified epitopes, all within the IE (BZLF1, BRLF1) or dominant E (BMLF1, BMRF1) proteins (2, 28). The remaining 44, marked with asterisks in Fig. 2 and in Supplemental Table II, represent novel reactivities. Clearly, the CD8 T cell pool of EBV carriers contained memory to multiple lytic cycle epitopes, many of which had not been identified before.

For each epitope region, the size of the IFN-γ ELISPOT response to the relevant screening peptide (15- or 20-mer) was expressed as mean SFC/10^6 CD4+-depleted PBMCs (minus the average DMSO control +2 SD). Combining results from all positive responses observed in the above assays, Supplemental Table II shows the range and median size of responses against each epitope. The data are summarized in Fig. 3 in terms of response size to Ags either grouped by kinetic class (Fig. 3A) or individually (Fig. 3B). Overall, IE-specific responses were slightly larger in size than responses to E proteins, and both were significantly larger than L-specific responses. Interestingly, when examined at the level of individual Ags, both IE proteins and the three most frequently recognized E proteins (BMLF1, BMRF1, BovRF1) could elicit numerically strong responses (>1000 SFC/10^6 CD4+-depleted PBMCs). However, responses to the L proteins, including

### Table 1. Summary of all memory CD8+ T cell responses to EBV lytic Ags identified by ex vivo IFN-γ ELISPOT assay.

<table>
<thead>
<tr>
<th>IE</th>
<th>Number of positive responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>BZLF1</td>
<td>12/13</td>
</tr>
<tr>
<td>BRLF1</td>
<td>10/13</td>
</tr>
<tr>
<td>BMLF1</td>
<td>9/13</td>
</tr>
<tr>
<td>BHRF1</td>
<td>7/13</td>
</tr>
<tr>
<td>BMRF1</td>
<td>7/13</td>
</tr>
<tr>
<td>BarF1</td>
<td>6/13</td>
</tr>
<tr>
<td>BFRF1</td>
<td>6/13</td>
</tr>
<tr>
<td>BLLF3</td>
<td>6/13</td>
</tr>
</tbody>
</table>

For each epitope region, the size of the IFN-γ ELISPOT response to the relevant screening peptide (15- or 20-mer) was expressed as mean SFC/10^6 CD4+-depleted PBMCs (minus the average DMSO control +2 SD). Combining results from all positive responses observed in the above assays, Supplemental Table II shows the range and median size of responses against each epitope. The data are summarized in Fig. 3 in terms of response size to Ags either grouped by kinetic class (Fig. 3A) or individually (Fig. 3B). Overall, IE-specific responses were slightly larger in size than responses to E proteins, and both were significantly larger than L-specific responses. Interestingly, when examined at the level of individual Ags, both IE proteins and the three most frequently recognized E proteins (BMLF1, BMRF1, BovRF1) could elicit numerically strong responses (>1000 SFC/10^6 CD4+-depleted PBMCs). However, responses to the L proteins, including

![Figure 2](http://www.jimmunol.org/)
the frequently recognized BNRF1, are typically very low, with the one exception being responses to the single epitope in gp25.

**CD8\(^{+}\) T cell clones against lytic Ag-derived epitopes**

To further characterize some of these responses, we selected 10 of the 44 novel reactivities identified in the initial screening assays for detailed analysis by CD8\(^{+}\) T cell cloning. These 10 epitopes are marked by double asterisks in Fig. 2 and are named in subsequent experiments using the first 3 aa of the sequence; two are IE epitopes (one in BZLF1 and one in BRLF1), one is an E epitope (in BaRF1), and seven are L epitopes (one each in gp25 and BFRF3, two in BVRF2, and three in BNRF1). In each case, CD8\(^{+}\) T cells reactive to peptide stimulation in short-term PBMC cultures were selected using IFN-\(\gamma\)-capture assay, seeded by limiting

FIGURE 3. Magnitude of memory CD8\(^{+}\) T cell responses to EBV lytic Ags identified in the 13 healthy EBV-seropositive donors screened by ex vivo IFN-\(\gamma\) ELISPOT assay. (A) Scatter dot plots (with line at median) show the size of responses to individual epitopes from Ags expressed during IE, E, and L phases of the lytic cycle, as determined in assays using the original screening peptide (15- or 20-mer). Each symbol represents the mean number of SFC/ million CD4\(^{+}\)-depleted PBMCs from replicate wells after subtraction of baseline reactivity to DMSO (mean of replicate DMSO wells +2 SD). When analyzed statistically by the Kruskal–Wallis test with post hoc Dunn test, both the IE and E Ag–specific responses were significantly larger than the L Ag–specific responses (*\(p < 0.05\)). Where a response to two adjacent screening peptides was identified, the larger response is shown. Reactivities that have only been identified against pool(s) of overlapping peptides and have not yet been mapped to individual peptides within the pool(s) are not included. (B) The same results in (A) are subdivided to show responses to the individual Ags expressed within each phase.
dilution into cloning medium; thereafter, growing cultures were screened for peptide specificity by IFN-γ ELISA, and selected clones were expanded further, as previously described (7). All selected clones were confirmed as uniformly CD8+ by mAb staining and in many, but not all, cases, the optimal epitope was identified in titration assays using shorter peptides within the originally recognized sequence (data not shown). For all 10 cloned responses, Table II gives the currently defined epitope sequence and its coordinates within the source Ag. Access to cloned populations made two objectives possible: first to identify the epitope’s HLA-restricting allele and second, using an appropriate panel of target LCLs, to check whether these lytic epitope-specific T cells could indeed recognize lytically infected cells within LCL populations.

Representative results from the analyses of HLA restriction are shown in Fig. 4A, with data from clones against four of the selected epitopes. Using IFN-γ ELISA as the readout, clones were tested for recognition of epitope peptide-loaded target cells either from the autologous donor or from allogeneic donors partially matched through specific HLA-A, HLA-B, or HLA-C alleles. For example, the epitope recognized by the BZLF1 (VST)-specific clone (Fig. 4A, top panel) was only presented by target cells sharing HLA-B58 with the T cell donor, showing this to be the restricting allele. Likewise, the results shown for other clones identified HLA-A*0201 as the restricting allele for the BaRF1 (LLI) epitope, HLA-B*0702 for the BFRF3 (TPS) epitope, and HLA-B60 for the gp25 (VED) epitope. All 10 cloned reactivities were studied in this way, and their restricting alleles are shown alongside the epitopes in Table II. Overall, two of these novel epitopes were restricted through HLA-A*0201, two through HLA-B*0702, and two through HLA-B*5801, whereas the other four were restricted through individual HLA-A, HLA-B, or HLA-C alleles. Knowing the restriction, it was now possible to go back to the original panel of 13 seropositive donors and ask how many of those with the relevant HLA-restricting allele actually made an epitope response. As shown in Table II, several of these newly defined epitopes were inducing detectable responses in only a subset of individuals with the relevant HLA allele, a pattern often associated with subdominant epitopes (10). For example, typically weak responses to the TPS epitope in L Ag BFRF3 were detected in just 2/4 B*0702-positive donors. Even the intermediate-size response to the LLI epitope in the E protein BaRF1 was seen in only 4/8 A*0201-positive donors, clearly showing that this epitope is subordinate to the well-known YVL (BRLF1) and GLC (BMLF1) epitopes against which most A*0201-positive donors respond (Supplemental Table II). However, the gp25 epitope VED (B60-restricted) was again an interesting exception; all three B60-positive donors tested had a detectable memory response to this L epitope.

All clones were then tested for their ability to recognize lytically infected cells. Assays were conducted on LCL target lines, with and without the relevant HLA-restricting allele, and transformed with the wild-type B95.8 EBV strain; note that such lines are semipermissive for virus replication, with typically 1–5% of cells in the lytic cycle. As a control, the assays included matched LCLs established using BZ-K/O-deleted B95.8 virus (27) and, therefore, devoid of lytically infected cells. Examples of results from such assays are presented in Fig. 4B, using clones directed against the same lytic epitopes as in Fig. 4A. In each case, there was clear recognition of wild-type B95.8 LCLs with the relevant restricting allele but not of matching BZ-K/O LCLs. This pattern of results was seen in all assays using clones against the IE proteins BZLF1 and BRLF1, the E protein BaRF1, and the L proteins gp25, BFRF3, and BVRF2. In each case, such results were consistent with the clone’s presumed lytic Ag specificity and with its ability to recognize that Ag when expressed in lytically infected cells.

However, a pattern of results different from that described above was observed using clones specific for epitopes in BFRF1, the L Ag against which memory CD8+ T cell responses had been detected most frequently. Fig. 5A shows representative data from multiple experiments with clones specific for the HLA-A*0201–restricted WQW epitope in BFRF1. These again show the expected recognition of wild-type B95.8 LCLs from HLA-A*0201–positive (but not negative) donors. However, this is always accompanied by significant, albeit slightly lower, recognition of the corresponding A*0201-matched BZ-K/O LCL. Likewise in Fig. 5B, the same result was reproducibly obtained using clones to the HLA-B*0702–restricted YPR epitope in BFRF1, with recognition of B*0702–matched targets whether transformed with wild-type or BZ-K/O virus strains. Both sets of assays included, as an additional control, CD8 clones with the same restricting allele as the BFRF1 effectors but specific for other lytic cycle Ags. Fig. 5C and 5D show the control data from an A*0201-restricted clone against a previously identified epitope FLD in the late lytic glycoprotein gp110 (6) and from a B*0702-restricted clone against a recently identified epitope RPF (RPGRPLAGFYA) in the early lytic protein BNLF2b (L. Quinn, unpublished observations), respectively. Just as noted with the other lytic cycle effectors tested in Fig. 4, CD8+ T cells specific for gp110 and BNLF2b showed no recognition of BZ-K/O LCLs, confirming that these target lines are indeed devoid of lytically infected cells. As a further check, we searched among a panel of A*0201- and/or B*0702-positive wild-

### Table II. Summary of epitope-specific CD8+ T cell clones

<table>
<thead>
<tr>
<th>Phase</th>
<th>Protein</th>
<th>Function</th>
<th>Epitope Coordinates</th>
<th>Sequence</th>
<th>HLA Restriction</th>
<th>No. of Responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE</td>
<td>BZLF1</td>
<td>Transcription activator</td>
<td>66–75a</td>
<td>VSTAPTGSWFa</td>
<td>B58.01</td>
<td>1/2</td>
</tr>
<tr>
<td></td>
<td>BRLF1</td>
<td>Transactivator</td>
<td>101–115</td>
<td>LACPIVMRYVLDHLI</td>
<td>B58.01</td>
<td>1/2</td>
</tr>
<tr>
<td>E</td>
<td>BaRF1</td>
<td>Ribonucleotide reductase</td>
<td>151–159</td>
<td>LLIEGIFF</td>
<td>A2.01</td>
<td>4/10</td>
</tr>
<tr>
<td>L</td>
<td>gp25</td>
<td>Membrane fusion (gL)</td>
<td>120–129</td>
<td>VEDLGFLGAL</td>
<td>B60</td>
<td>3/3</td>
</tr>
<tr>
<td>BFRF1</td>
<td>Trigerm</td>
<td>700–719a</td>
<td>GGYKYGKSLDLa</td>
<td>B41</td>
<td>1/1</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>1247–1257</td>
<td>YPRNPTEQGNI</td>
<td>B7.02</td>
<td>3/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1281–1289</td>
<td>WQWEHIPPAA</td>
<td>A2.01</td>
<td>2/10</td>
<td></td>
</tr>
<tr>
<td>BFRF3</td>
<td>Capsid</td>
<td>127–137</td>
<td>TPSVSSISSL</td>
<td>B7.02</td>
<td>2/4</td>
<td></td>
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<tr>
<td>BVRF2</td>
<td>Assembly</td>
<td>153–163a</td>
<td>AVYGTDLAWVLa</td>
<td>C3</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>341–351a</td>
<td>QTFTSPGGYYAa</td>
<td>A29</td>
<td>1/1</td>
<td></td>
</tr>
</tbody>
</table>

Epitopes are referred to by the first three amino acids of their sequence, as underlined. The No. of Responders column gives the number of healthy donors that were shown to have a CD8+ cell response to the specified peptide by IFN-γ ELISPOT/number of healthy donors screened with the correct HLA class I restriction. Note that the subtype of the HLA-C3-positive donors has not been determined.

*Minimal epitopes have not been defined experimentally; coordinates and sequences given are the overlapping amino acids from the peptides to which the clones respond.
We have used B95.8 LCLs to select lines lacking spontaneous lytic cycle entry and found that these were also recognized by the BNRF1 effectors but not by effectors against other lytic cycle Ags (data not shown). This showed that the unexpected detection of BNRF1 in a latent growth-transforming infection was not simply an artifact of the recombinant BZ-K/O virus construct.

Primary CD8* T cell responses to EBV lytic cycle Ags in IM patients

The work to this point had revealed a surprisingly large number of CD8 responses to IE, E, and also L Ags in healthy virus carriers, many more than had been identified in the original T cell cloning work with IM patients. Therefore, we turned our attention back to IM, asking whether responses to these novel epitopes were indeed detectable during primary infection or had arisen as accompaniments of long-term virus carriage. We again used peptide-induced IFN-γ production as the marker of a response; however, in this case, we stained for intracellular IFN-γ after a 6-h peptide exposure because activated, apoptosis-prone, CD8* T cells in IM blood are inefficiently detected in an 18-h ELISPOT assay. First, we focused on HLA-A*0201–positive IM patients, for which earlier ex vivo cloning assays already identified abundant responses to two A*0201-restricted epitopes: YVL from the IE protein BRLF1 and TLD from the E protein BMRF1 (9). These peptides were used to stimulate IM PBMC preparations in parallel with two A*0201-restricted epitopes identified in the present work, LLI in the E protein BaRF1 and WQW in the L protein BNRF1, as well as with a previously identified weak A*0201 epitope from another L protein, gp110 (6).

**FIGURE 4.** Characterization of representative IE, E, and L Ag–specific CD8* T cell clones. Recognition of target LCLs was measured by IFN-γ ELISA, and results are mean ± 1 SD of duplicate wells. (A) HLA restriction of CD8* T cell clones. T cells were incubated overnight with peptide-loaded cells of the autologous B95.8 LCL and of partially HLA class I–matched allogeneic LCLs (for which the matching alleles are given). (B) CD8* T cell recognition of unmanipulated LCL targets. T cells were incubated overnight with LCL cells from HLA-matched (or autologous) and HLA-mismatched donors. Where possible, a pair of B95.8 and BZ-K/O LCLs from an HLA-matched donor was included. All HLA-matched B95.8 and BZ-K/O LCLs were strongly recognized by the relevant T cells if they were pre-exposed to target peptide, with ≥8 ng/ml IFN-γ released in all cases (data not shown). Note that levels of B95.8 LCL recognition by IE versus E versus L Ag–specific clones cannot be compared directly because the percentage of lytically infected cells in these LCLs differs between cell lines.
patients screened for B*0702-restricted reactivity to the known RPQ epitope in E protein BMRF1 and to the new L protein epitopes YPR and TPS. Again, a reasonably strong RPQ response was seen in most patients, but smaller responses to the L epitopes were also detectable. These novel A*0201- and B*0702-restricted responses had been identified as typically subdominant components of long-term memory; therefore, their detection in IM, even at low levels, is significant. These studies were extended to include the one L Ag–specific response, against the B60-restricted VED epitope in gp25, which we had found was reasonably abundant in long-term memory. We sought to detect this response in acute IM, comparing it with that seen against another B60-restricted IE epitope, SEN in BZLF1 (29); note that SEN is a weak epitope against which responses are detectable in some, but not all, B60-positive carriers (Supplemental Table II). Fig. 6D shows the data examining these two epitope responses in three B60-positive IM patients. The VED response is detectable in all three cases, albeit at quite low levels but always larger than that seen against SEN. We conclude that the novel L Ag responses, which the present work identified by memory screening, are not products of long-term virus carriage but are already present as components of the primary response.

Discussion

This article addresses the issue of immunodominance among EBV lytic cycle proteins as targets for CD8+ T cells. Our earlier studies, cloning T cells from IM blood and screening on a limited range of IE, E, and L Ags, suggested that the primary CD8 response was markedly skewed toward the two IE proteins and a subset of E proteins, whereas L proteins constituted only rare subdominant targets (6, 7, 9). However, studying memory responses in the rhesus model of LCV infection and subsequently in healthy EBV carriers, Orlova et al. (25) found that polyclonal T cell lines made by repeatedly stimulating PBMCs with the autologous semipermmissive LCL regularly contained CD8+ T cell reactivities against one or more L proteins. Interestingly, in the rhesus LCV model, such reactivities were detected more frequently in older animals than in younger animals, prompting the investigators to suggest that L Ag–specific responses may develop slowly over time, perhaps as a result of on-going virus replication during the virus carrier state (25). This certainly seemed plausible in the context of EBV infection, because prospective studies on IM patients had provided examples in which the balance between CD8 responses to individual Ags or epitopes appeared to change over time (9, 30); furthermore, with another human herpesvirus, CMV, some, but not all, components of the CD8 response expand considerably with life-long viral carriage (31, 32). Therefore, the present work sought to re-examine the CD8+ T cell memory of healthy EBV carriers to selected IE, E, and L Ags; to identify a representative range of new IE, E, and L target epitopes and their restricting alleles; and then to compare the relative abundance of these reactivities in primary versus persistent infection. The results provide a much more detailed picture of CD8 responses to lytic cycle Ags, with multiple subdominant IE, E, and L epitope reactivities present alongside those against dominant IE/E epitopes.
(IE > E > L) is apparent in both the primary and memory phases of the response.

To avoid the possibility that in vitro expansion might distort the composition of T cell memory, we screened CD4-depleted PBMCs from long-term EBV carriers ex vivo using IFN-ELISPOT assays and peptides covering the primary sequences of 15 selected lytic cycle Ags. In addition to EBV’s two IE trans-activator proteins, the Ag panel included six E proteins with a range of different lytic cycle functions and seven L proteins ranging from virion capsid (BFRF3), tegument (BNRF1), and envelope (gp25, gp42, gp85, gp350) components to an assembly protein (BVRF2) that is not detected in mature virus particles (33). Although there was some overlap with previous panels used by us (6) and other investigators (25), three E (BaRF1, BFRF1, BLLF3) and three L proteins (gp25, gp42, BFRF3) were examined as CD8 T cell targets for the first time, to our knowledge. The ELISPOT assays quickly showed that EBV carriers had detectable CD8 memory cells against a broad array of lytic cycle Ags; overall, individuals recognized a mean of six Ags on the panel, and every individual tested had at least one response. The relative importance of IE, E, and L target Ags can be judged in terms of the frequency of positive responses among donors tested (Table I), the number of epitopes identified in these proteins (Fig. 2), and the size of response to those epitopes (Fig. 3, Supplemental Table II). The IE Ags, BZLF1 and BRLF1, remain the strongest targets by all three criteria, with responses detectable to one or both proteins.
in all 13 individuals, with eight new epitopes added to the six already known, and with the largest median size of response; interestingly, BZLF1 was stronger than BRLF1 in all three respects. As observed in the original IM clonal analysis, E Ags were more variable as CD8 targets. Thus, the two transactivator proteins, BMLF1 and BMRF1, and a newly studied protein, BaRF1, were recognized more frequently than the other three E Ags tested, contained more epitopes than they did (including 11 of the 16 new E epitopes identified), and elicited numerically stronger responses. Leaving aside the special case of BNRF1 (discussed below), the other five L proteins tested were also variable as CD8 targets. Overall, however, they showed lower frequencies of recognition than the six E proteins, contained just eight epitopes (all newly identified), and, for seven of these eight epitopes, elicited weak responses.

Therefore, the above findings are broadly consistent with an IE > E > L hierarchy of immunodominance among lytic cycle Ags as targets for the memory CD8 response, in line with the hierarchy first suggested from studies of the primary response in acute IM (6, 7). That said, there are important caveats to the conclusions drawn from a study of this kind. First, we detected memory responses using IFN-γ secretion; although it is the most sensitive cytokine in this viral system, it nevertheless underestimates the true number of epitope-specific cells (26). Second, given the limited size of the donor panel (13 individuals), our study covers most, but certainly not all, HLA types that are relatively common in white populations and so the analysis is incomplete. Indeed, just as seen with responses to latent cycle Ags (2, 34), individual HLA alleles can have idiosyncratic preferences among lytic cycle targets that appear to contradict the general trend; for example, the three L Ag–specific memory responses that are clearly larger than the rest all came from B60-positive donors responding to a single B60-restricted epitope in gp25. Third, our target Ag panel represents just a subset (20–25%) of all E and L proteins, and there is a clear need to extend the panel before drawing too firm conclusions. In that regard, among the L proteins tested, the apparent difference in antigenicity between the four viral envelope glycoproteins (gp25, gp42, gp85, gp350) and the nonenvelope BFRF3 (capsid) and BVRF2 (assembly) proteins raises the possibility that a glycoprotein bias has lowered the overall frequency of L Ag responses. It should be noted that three of these glycoproteins were represented by 20-mer, rather than 15-mer, peptide libraries; using the longer peptides was reported to slightly reduce the efficiency of detection in CD8 memory screens in some studies (35), whereas no such difference was found in other studies (36). We conducted several preliminary experiments (including screening gp25 with both 15- and 20-mers) to show that, under our assay conditions, 20-mer screening detected the same epitope responses as did 15-mers but tended to give slightly lower SFC counts. On these grounds, we are confident that the infrequency of detectable ELISPOT responses seen against these glycoproteins genuinely reflects their poor immunogenicity for CD8+ T cells. In that regard, other investigators reported the existence of subdominant A2-restricted CD8 epitopes in gp350 and gp85 (23), but even rescreening the A2-positive donors in our study with the published minimal epitope peptides did not detect such gp350 and gp85 responses (data not shown).

We then went on to establish CD8+ T cell clones against 10 of the 44 new epitopes identified by ELISPOT screening, thereby opening the way to functional studies. One particular issue, bearing upon the biological relevance of these newly identified responses, was to determine whether they were capable of recognizing lytically infected cells. The results (Fig. 4B) show that this was indeed the case, not just for IE and E epitope–specific effectors but even for clones directed against L epitopes. Semi-permissive B95.8 LCL targets with the correct HLA-restricting allele were recognized, whereas (for all but the BNRF1 effectors) the matching BZ/KO LCLs were not. That recognition of semipermissive lines must have been directed toward the lytically infected cells themselves, because within LCL cultures we have never observed any cross-presentation of released lytic cycle Ags to CD8+ T cells [(37) and data not shown]. Therefore, despite the virus’ immune-evasion strategies becoming ever more effective with progress through the lytic cycle (38), there is still enough epitope display, even of L Ag–derived epitopes, to allow some level of T cell recognition. This no doubt attests to the potent Ag-presenting function of LCL cells and the difficulty of completely eliminating epitope display. We conclude that even L Ag–specific responses, although a minor component of the total lytic Ag response, have the capacity to recognize lytically infected cells of the kind found within EBV-driven lymphoproliferative lesions in vivo (39–41); this could be important in preventing virus release, secondary infection, and the recruitment of new B cell transformati ons into these lesions.

More interesting in that regard, however, were the unexpected findings with clones specific for BNRF1, a virion tegument protein (42, 43) hitherto thought to be expressed exclusively in late lytic cycle but encoded by a gene lying immediately downstream of the latent membrane protein LMP2 gene in the EBV genome. Clones against two independent BNRF1 epitopes recognized not just semipermissive B95.8 LCL targets of the appropriate HLA type but also the matched BZ/K-O LCLs included as lytic cycle–deficient controls (Fig. 5), as well as matched LCLs carrying wild-type virus but identified as tightly latent through their absence of BZLF1 expression (data not shown). We infer that, in addition to its established status as a late lytic cycle protein, BNRF1 is expressed as a latent protein in growth-transforming infection. Indeed, recognition of the BZ/K-O LCL in the above experiments was in many cases only slightly lower than that of the semipermissive line, indicating that most recognition reflects epitope display on latent lytically infected cells, with only a small contribution from cells in the lytic cycle. Note that these findings are not indicative of a general promiscuity of lytic gene expression in BZ/K-O LCLs or indeed in the BZLF1-negative B95.8 LCLs tested because, both in this study (Figs. 4B, 5) and elsewhere (11), T cell assays with other lytic Ag–specific effectors make it clear that genuine lytic cycle proteins are not detectably expressed in such lines. Fortuitously, therefore, the present work identified BNRF1 as a protein expressed in both latent and lytic infections that contains multiple CD8 epitopes and elicits detectable, although usually weak, responses in the majority of EBV carriers. Such BNRF1 epitope responses can be amplified in vitro and, thereby, add to the arsenal of latent Ag–specific T cells with therapeutic potential against EBV-driven B lymphoproliferative disease (4).
reactivities are all present in the primary response in proportions that broadly reflect their order of abundance in memory. These findings suggest that L Ag–specific responses are not selectively expanded with long-term virus carriage (25) but, like most of the better-studied IE and E Ag responses (9), undergo a phase of primary expansion following by contraction into a smaller memory population that is then stably maintained. Observations of rare responses to A2-restricted gp350 and gp85 epitopes (22, 23) also support this conclusion. However, the door is now open to address the issue more systematically using tetramers to track L epitope–specific responses in individuals following IM.

In summary, our work makes it clear that CD8+ T cell responses to lytic EBV infection contain considerably more components than were apparent from earlier studies based on T cell cloning. Relative paucity of L Ag–specific CD8+ T cell responses is persistent infection. Thus, the numerically dominant responses directed toward subdominant epitopes, a subset of E proteins. However, these are accompanied by many low-abundance responses directed toward subdominant epitopes, some drawn from IE, some from E, and some from L proteins. The persistently infected IM carriers.

References


