Cytotoxic CD4⁺ T Cell Responses to EBV Contrast with CD8 Responses in Breadth of Lytic Cycle Antigen Choice and in Lytic Cycle Recognition

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J Immunol 2011; 187:92-101; Prepublished online 27 May 2011;
doi: 10.4049/jimmunol.1100590
http://www.jimmunol.org/content/187/1/92

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Cytotoxic CD4+ T Cell Responses to EBV Contrast with CD8 Responses in Breadth of Lytic Cycle Antigen Choice and in Lytic Cycle Recognition

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EBV, a B lymphotropic herpesvirus, encodes two immediate early (IE)-, >30 early (E)-, and >30 late (L)-phase proteins during its replication (lytic) cycle. Despite this, lytic Ag-induced CD8 responses are strongly skewed toward IE and a few E proteins only, all expressed before HLA I presentation is blocked in lytically infected cells. For comparison, we examined CD4+ T cell responses to eight IE, E, or L proteins, screening 14 virus-immune donors to overlapping peptide pools in IFN-γ ELISPOT assays, and established CD4+ T cell clones against 12 defined epitopes for target-recognition assays. We found that the lytic Ag-specific CD4+ T cell response differs radically from its CD8 counterpart in that it is widely distributed across IE, E, and L Ag targets, often with multiple reactivities detectable per donor and with IE, E, or L epitope responses being numerically dominant, and that all CD4+ T cell clones, whether IE, E, or L epitope-specific, show strong recognition of EBV-transformed B cell lines, despite the lines containing only a small fraction of lytically infected cells. Efficient recognition occurs because lytic Ags are released into the culture and are acquired and processed by neighboring latently infected cells. These findings suggested that lytic Ag-specific CD4+ responses are driven by a different route of Ag display than drives CD8 responses and that such CD4 effectors could be therapeutically useful against EBV-driven lymphoproliferative disease lesions, which contain similarly small fractions of EBV-transformed cells entering the lytic cycle. The Journal of Immunology, 2011, 187: 92–101.

Epstein-Barr virus, a B lymphotropic γ-herpesvirus widespread in human populations, normally persists as a lifelong asymptomatically infected under the control of host T cell surveillance (1). Yet this same virus has B cell growth-transforming ability and, in acutely T cell-compromised individuals, can drive the outgrowth of fatal posttransplant lymphoproliferative disease (PTLD) lesions (2). These are typically composed of growth-transformed, latently infected B cells expressing the full spectrum of eight latent-cycle Ags plus a subpopulation of cells that recently switched into the lytic (virus-replicative) cycle with sequential expression of two immediate early (IE), some 30 early (E), and 30 late (L) lytic Ags culminating in infectious virus production (3, 4). Likewise, B cell transformation by the virus in vitro yields semipermissive lymphoblastoid cell lines (LCLs), which similarly contain latently infected and smaller lytically infected subpopulations (1). Therefore, virus-infected B cells provide a potentially rich array of immunogens for host T cell responses and, given the virus’ causal role in PTLD and suspected role in certain other malignancies, mapping the full breadth of those virus-specific responses is an important goal.

Most attention to date has focused on the latent-cycle Ags, namely the nuclear Ags EBNA1, 2, 3A, 3B, 3C, and -LP and latent membrane proteins LMP1 and 2. Both CD8+ and CD4+ T cell responses are broadly targeted across all eight proteins. There are trends in target Ag choice, such that several (but not all) epitopes eliciting the strongest CD8 responses derive from the EBNA3 Ags, whereas up to half of the currently defined CD4 epitopes (but not necessarily those eliciting the strongest responses) derive from EBNA1 (1). However, a more detailed study showed that no latent Ags are completely ignored by CD8+ or by CD4+ T cell responses; furthermore, any apparent trends in immunodominance bear no relation to the order in which these Ags first appear during the initiation of B cell-transforming infections (5). When analyzed functionally by T cell cloning, interesting differences between CD8 and CD4 responses become apparent. Thus, essentially all latent epitope-specific CD8+ T cell clones recognize HLA I-matched target LCLs both in IFN-γ release and in appropriately designed cytotoxicity assays and prevent LCL outgrowth in long-term cocultures (6). By contrast, CD4+ T cell clones against many latent Ag epitopes, although capable of recognizing target cells loaded with epitope peptide or expressing cognate Ag in an HLA II-pathway-directed form, nevertheless recognize native LCLs poorly, if at all (7–10), indicating that latent proteins endogenously expressed from the resident EBV genome in LCL cells have limited access to the HLA II-presentation pathway (11).

Turning to the lytic-cycle Ags as T cell targets, current understanding is largely confined to CD8+ T cell responses. These responses are markedly skewed toward the two IE Ags (BZLF1 and BRLF1) and just a subset of E Ags, notably those such as BMLF1 that are expressed soon after the IE to E transition (12). Some responses have been detected against epitopes in other E
Ags, such as BMRF1, which are expressed at slightly later times; however, interestingly, the first of these responses to be studied in detail was found to disappear over time following primary infection (13). Responses to L Ags are only rarely detectable, even in primary infection (12). Importantly, this marked hierarchy of immunodominance among lytic-cycle proteins matches the efficiency with which these proteins are presented for CD8+ T cell recognition on the surface of lytically infected cells (14). Thus IE-specific CD8+ T cell clones recognize lytically infected cells within LCL target lines quite well, generally better than do E-specific clones and much better than do L-specific clones (15), despite the fact that the latter clones tend to have higher functional avidity for their cognate epitope in peptide-titration assays (12). This progressive impairment of lytic Ag presentation with progress through the lytic cycle led to the identification of three virus-coded immune-evasion proteins, BNLF2a, BGLF5, and BILF1, which first appear during the E phase and act by different mechanisms to progressively inhibit the HLA I-presentation pathway (15–17). Such concordance between CD8 immunodominance among lytic-cycle Ags and their kinetics of expression in lytically infected cells is a specific feature of the EBV system and is not seen, for example, in another herpesvirus encoding multiple inhibitors of the HLA I pathway, human CMV (18).

With this as background, we wondered how the CD4+ T cell response is targeted across the IE, E, and L proteins and, if one can generate CD4+ T cell clones against these different Ag classes, whether one could use the CD4+ T cell response to drug-resistant virus and CD8-depleted preparations to detect or purify virus from infected cells. We were unable to detect a CD4+ T cell response to virus-infected cells, however, interestingly, the first of these responses to be studied in detail was found to disappear over time following primary infection (13). Responses to L Ags are only rarely detectable, even in primary infection (12). Importantly, this marked hierarchy of immunodominance among lytic-cycle proteins matches the efficiency with which these proteins are presented for CD8+ T cell recognition on the surface of lytically infected cells (14). Thus IE-specific CD8+ T cell clones recognize lytically infected cells within LCL target lines quite well, generally better than do E-specific clones and much better than do L-specific clones (15), despite the fact that the latter clones tend to have higher functional avidity for their cognate epitope in peptide-titration assays (12). This progressive impairment of lytic Ag presentation with progress through the lytic cycle led to the identification of three virus-coded immune-evasion proteins, BNLF2a, BGLF5, and BILF1, which first appear during the E phase and act by different mechanisms to progressively inhibit the HLA I-presentation pathway (15–17). Such concordance between CD8 immunodominance among lytic-cycle Ags and their kinetics of expression in lytically infected cells is a specific feature of the EBV system and is not seen, for example, in another herpesvirus encoding multiple inhibitors of the HLA I pathway, human CMV (18).

Materials and Methods
Ethics statement and donors

All experiments were approved by the South Birmingham Local Research Ethics Committee (07Q2702/24), and all donors provided written informed consent for the collection of blood samples and their subsequent analysis. The donor cohort included one EBV-seronegative donor and 14 EBV-seropositive donors with no history of infectious mononucleosis (IM), who represented a heterogeneous sampling of HLA class II alleles, including most of those commonly found in Caucasian populations.

Cell preparations and cell lines

PBMCs were separated from healthy EBV-immune and -naive donors by Ficoll-Hypaque centrifugation into RPMI 1640 medium + l-glutamine (Invitrogen) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (standard medium) plus 5% human serum (HD Supplies). PBMCs were depleted of CD8+ T cells using CD8 Dynabeads (Dynal) in accordance with the manufacturer’s protocol. LCLs were generated from donor B cells by in vitro infection with B95.8 strain EBV (B95.8 LCLs) or a replication-defective B95.8 recombinant virus lacking the IE gene BZLF1, BZLF1 knockout (k/o) LCLs (BZ k/o LCLs). All LCLs were cultured in standard medium plus 10% FCS, supplemented with 200 μM acyclovir (ACV) where described.

Synthetic peptides

Epitope peptides and 15mer peptides overlapping by 10 aa and covering the primary sequences of BZLF1, BRLF1, BMLF1, and BMRF1 were purchased from Alte BioScience, University of Birmingham, and 20mer peptides overlapping by 15 aa and covering the primary sequences of BHRF1, BKF2, BZLF2, and BXLF2 were purchased from Mimotopes (Clayton, Australia); all were based on the B95.8 strain sequence. Peptides were dissolved in DMSO, and their concentrations determined by biuret assay.

ELISPOT assays

CD8-depleted preparations were tested in ELISPOT assays of IFN-γ release, as previously described (23), against either pools of overlapping peptides from the Ags of interest (6–10 peptides/pool) or individual peptides, all at a final concentration of 5 μg/ml for each peptide. An equivalent volume of DMSO and 10 μg/ml PHA were added to separate wells as negative and positive controls, respectively.

Isolation and culture of T cell clones

CD8-depleted PBMCs from healthy donors or whole PBMCs from acute IM donor IM225 were exposed for 1–3 h to 5 μM the appropriate peptide(s) and then washed. Responding cells were isolated either immediately by IFN-γ-secreting cell enrichment (Miltenyi) or after 1–2 wk of culture in RPMI 1640 supplemented with 5% HuS or 10% FCS for IM225. T cell clones were isolated from the above preparations by limiting dilution seeding in IL-2-supplemented RPMI 1640 medium supplemented with 5% HuS or 10% FCS, as described (7). Growing microcultures reactive against peptide-loaded autologous LCLs in IFN-γ ELISAs were further expanded and cultured as described (7).

Virus purification

Cell-free virus preparations were overlaid on a 50% solution of Optiprep (Axis Shield) buffered with 0.85% (w/v) NaCl, 60 mM HEPES-NaOH (pH 7.4), and centrifuged in a SW-40 rotor at 160,000 × g for 2 h to band the virus sharply at the Optiprep interface. All of the supernatant was removed except for a volume equal to the volume of Optiprep, and the contents were mixed and transferred to 4.9-ml vertical rotor Beckman tube and centrifuged at 350,000 × g for 2.4 h in a V60Ti vertical rotor to band the virus in a self-generated gradient of Optiprep, with controlled deceleration. The visible virus band was harvested with a syringe and metal cannula, and the recovered virus was quantitated by the quantitative PCR assay amplifying the BALF5 gene (24, 25).

ELISAs of IFN-γ release

A range of numbers of CD4+ T cell clones (100–500/triplicate test well) was incubated in V-bottom microtest plate wells with standard numbers (5 × 10^3/well) of autologous, HLA-matched or HLA-mismatched LCL cells that had been pre-exposed for 1 h either to 5 μM epitope peptide or to an equivalent volume of DMSO solvent as a control and then washed. The supernatant medium was harvested after 18 h and assayed for IFN-γ by ELISA (Endogen), in accordance with the manufacturer’s recommended protocol. In HLA-restriction assays, LCL targets were preincubated with mAbs specific for HLA-DR (L243, ATCC clone HB-55) or HLA-DQ (SPV-L, Serotec) at 10 μg/ml for 1 h before addition of T cells to the assay. In virus-recognition assays, LCLs were pre-exposed to purified EBV at a multiplicity of infection of 100 for 2 h and then washed before addition to the assay.

Cytotoxicity assays

Target LCLs were pre-exposed for 1 h either to 5 μM epitope peptide or to an equivalent volume of DMSO solvent as a control and then washed. Effector CD4+ T cells were added at known E:T ratios in 5- or 12-h chromium-release assays (7). Results are expressed as the percentage of specific lysis of the target line.

Flow cytometry

T cell clones were stained for surface CD4 by incubating the cells on ice for 20 min with saturating concentrations of the following anti-human Abs: PE-conjugated 5-saturating CD4 mAb (clone SK3), BD PharMingen), or PE-conjugated CD4 mAb (BD PharMingen). T cells stained with CD4 mAbs conjugated to appropriate fluorophores, either immediately or after a 12-h stimulation with unmanipulated or peptide-loaded LCL, were fixed in 1% paraformaldehyde for 10 min, permeabilized with 0.1% saponin for 30 min, and stained with PE-conjugated perforin mAb (BD PharMingen) or FITC-conjugated granzyme B mAb (BD PharMingen). Cells were analyzed on either an LSRII flow cytometer (Beckman Coulter) or an Epics flow cytometer (Beckman Coulter), and all data were processed using FlowJo software (TreeStar).
**Results**

**EBV lytic-cycle Ags as targets of the CD4⁺ T cell response**

We selected eight lytic-cycle Ags for detailed study: the two IE proteins, BZLF1 and BRLF1, known to be strong CD8 targets; three E proteins, BMLF1, which is a strong CD8 target, BMRF1, which also elicits CD8 responses that in some cases do not persist, and BHRF1, which is an infrequent CD8 target; and three L proteins, BKRF2, BXLF2, and BZLF2, against which CD8 responses are rarely, if ever, detected. Based on the B95.8 EBV strain sequence, we synthesized 20mer or 15mer peptides (overlapping by 15 or 10 aa respectively) covering the entire primary sequence of these proteins. Peptides were arranged into pools of 8–10 consecutive peptides and used in IFN-γ ELISPOT assays to screen for CD4⁺ T cell memory in fresh CD8-depleted PBMCs from healthy adult donors. Routine CD8 depletion consistently achieved >98% reduction in CD8⁺ T cells, as confirmed by flow cytometry. Fig. 1 shows results from two EBV-seropositive donors. In Fig. 1A, Donor 2 was tested against multiple peptide pools from IE (BZLF1), E (BMLF1), and L (BKRF2) proteins. This donor has detectable reactivities to peptide pools 1, 2, and 3 from BZLF1; pool 1 from BMLF1; and pools 2 and 3 from BKRF2. The same donor was retested against the individual peptides within each of the relevant pools to identify the epitope being recognized. Thus, the three BZLF1 reactivities in this donor each mapped to single BZLF1 peptides, 1.3, 2.3, and 3.4 (aa 11–25, 61–75, and 116–130, respectively). Likewise, the BMLF1 response mapped to BMLF1 peptide 1.9 (aa 41–55), and the BKRF2 responses mapped to BKRF2 peptides 2.7 and 2.8 (sharing aa 81–95) and peptide 3.8 (aa 118–137). Interestingly, in further screening experiments, Donor 2 responded to all eight Ags in the panel (Fig. 2A). Similarly, Fig. 1B shows sample results from Donor 6, who also responded to multiple Ags (Fig. 2A). For this donor, sample ELISPOT-screening assays with BRLF1 (IE), BMRF1 (E), and BXL2 (L) are presented, showing detectable responses that could be mapped to individual epitopes: BRLF1 pep 3.2 (aa 106–120), BMRF1 pep 5.8 (aa 61–76), and BXL2 pep 3.8 (aa 136–155). As illustrated here and seen consistently in such ex vivo IFN-γ ELISPOT screens, the numbers of CD4⁺ T cells in peripheral blood that were specific for any one lytic epitope always fall within a range (15–100 spot forming cells per 5 × 10⁵ CD8-depleted PBMCs), similar to that seen for latent epitope-specific CD4 responses (7) and up to 10-fold lower than that seen for individual lytic epitope-specific CD8 responses (26, 27). The size of the CD4 response to any particular Ag varied between individual donors, and there was no apparent overall difference in magnitude of responses to proteins from any particular phase of the lytic cycle.

Overall, we screened CD8-depleted PBMCs from 14 EBV-seropositive and 1 EBV-seronegative donor; HLA typing confirmed that many of the HLA II alleles most commonly found in Caucasian populations were represented in the donor panel. The assay results are summarized in Fig. 2A. Remarkably, although we...
were screening on peptide pools representing just eight lytic-cycle Ags, all 14 seropositive donors showed reactivity to at least one, and usually to more (median, 3), of those eight proteins; as a control, the seronegative donor did not give a detectable response to peptide pools from any of the Ags tested. As shown in Fig. 2B, there was no obvious correlation between the number of seropositive donors reactive to a particular protein and either the temporal class of that protein (IE, E, L) or its size. Thus, the two most frequently recognized proteins were the IE protein BZLF1 (245 aa) and the L protein BXLFL2 (706 aa), whereas the two least frequently recognized were the IE protein BRLF1 (605 aa) and the late L protein BKRF2 (137 aa). Fig. 2C summarizes the results from the mapping of Ag-specific responses to single-epitope regions, showing the primary sequence of each protein according to its size and identifying the position of CD4 epitopes within each sequence. Perhaps not surprisingly, the least frequently recognized proteins BRLF1 and BKRF2 contain the smallest number of identified epitopes (4 and 2, respectively), whereas the most frequently recognized proteins BZLF1 and BXLFL2 contain larger numbers, 7 and 17 epitopes, respectively. However, epitope content is not the only factor influencing the frequency of donors responding to a particular Ag; another is the identity of the HLA II alleles that present those epitopes and the representation of those alleles in the cohort of donors being tested.

**Characterization of CD4+ T cell clones against lytic Ag-derived epitopes**

To determine the HLA class II restriction and effector function of lytic Ag-specific CD4+ T cell responses in greater detail, we isolated CD4+ T cell clones specific for four IE epitopes (three in BZLF1, one in BRLF1), three E epitopes (one each in BHRF1, BMLF1, and BHRF1), and three L epitopes (one each in BKRF2, BZLF2, and BXLFL2) from individual donors in our panel (Fig. 2C, *). Epitope-specific T cells were selected using IFN-γ capture assay after a 3-h peptide stimulation, either ex vivo or after 7–14 d of culture postinital peptide stimulation, after which selected cells were cloned by limiting dilution, and peptide-responsive T cell clones were expanded as described (7). In addition, we included clones similarly raised against two previously defined epitopes within other L proteins: BLLFL1 (gp350) (20) and BXLFL4 (gB/gp110) (19). All clones exhibited an effector memory phenotype, with detectable surface expression of CD4, CD45RO, and CD28 and no detectable expression of CD27, CCR7, or CD62L by flow cytometric analysis (data not shown). Details of all 12 epitopes studied are given in Table I.

**Using approaches established in earlier work, we initially identified the relevant HLA class II-restricting allele of the clones to each of our 12 selected epitopes, by assaying first against the autologous peptide-loaded LCL in the presence of HLA-DR–, -DQ–, and -DP–blocking mAbs to narrow the restriction down to HLA-DR, -DQ, or (by inference) -DP, and second against peptide-loaded LCLs from allogeneic donors partially matched through specific HLA-DR, -DQ, or -DP alleles, as appropriate. Representative results in Fig. 3 show, for example, that epitope recognition by the BZLF1 (VKF)-specific clone was specifically blocked by the anti–HLA-DR mAb and that the epitope was only presented by an allogeneic LCL sharing the HLA-DRB3*01 allele with the T cell donor. Corresponding experiments shown for the other clones identified the HLA class II-restricting allele for the BMLF1 (DED) epitope as HLA-DQB1*07 and for the BXLFL2 (LEK) epitope as HLA-DRB5*01. Table I summarizes the overall results of these assays showing the epitope sequence and restriction element for each clonal reactivity.

**CD4+ T cell recognition of EBV-transformed LCLs**

Our next experiments examined to what extent these IE, E, and L Ag-specific CD4+ T cell clones were able to recognize semipermissive LCLs (i.e., cells transformed with wild-type [wt] EBV, in which typically 2–5% of cells spontaneously enter the lytic cycle). As described elsewhere (28), the control targets in these...
assays were paired nonpermissive LCLs (i.e., cells from the same donors but transformed with a replication-defective BZLF1-knockout virus BZ k/o, therefore devoid of lytically infected cells) (29). Fig. 4 presents results of experiments in which increasing numbers of IE, E, and L Ag-specific CD4+ T cells were simultaneously tested against a particular wt/BZ k/o LCL pair matched for the relevant restricting HLA II alleles; as an internal control, the clones were simultaneously tested against the same LCLs pre-loaded with an optimal concentration of the epitope peptide. In the first such experiment (top panel), the IE (BZLF1), E (BMLF1) and L (BLLF1) Ag-specific clones all recognized these peptide-loaded targets well. Interestingly, all three clones also showed significant recognition of the unmanipulated wt-LCL, at levels that increased with T cell number, whereas the lytic Ag-negative BZ k/o LCL was never recognized. Fig. 4 (middle and bottom panels) illustrates results from similar experiments in which CD4+ T cell clones specific for different IE, E, and L targets were tested on different wt/BZ k/o LCL pairs with similar conclusions. Throughout all such experiments we found that, irrespective of the lytic Ag class against which they were directed, lytic epitope-specific CD4+ T cell clones showed significant recognition of appropriately HLA II-matched semipermissive LCLs. Levels of IFN-γ release, expressed relative to that seen in the same assay against the peptide-loaded control target, differed between clones specific for different epitopes. However, there was no suggestion in any of these experiments that any one class of lytic Ag was preferentially presented by semipermissive LCLs. Thus, in the three experiments in Fig. 4, the strongest relative recognition of the wt-LCL was by CD4+ T cells against the L (BLLF1) Ag in the first case, the E (BMLF1) Ag in the second, and the IE (BRLF1) Ag in the third.

Because in earlier studies many EBV-latent Ag-specific CD4+ T cell clones, identified initially by IFN-γ production in response to Ag stimulation, had also shown cytotoxic activity (7), we tested our lytic Ag-specific CD4+ T cells against HLA-matched wt and BZ k/o LCLs in 5- and 12-h chromium-release assays, again with and without epitope peptide loading of the targets. Fig. 5A illustrates the results obtained with clones specific for the IE (BZLF1), E (BMLF1), and L (BLLF1) Ags. In each case, the peptide-loaded wt and BZ k/o LCLs were equally well killed, with lysis being evident within 5 h and elevated by 12 h (there was no lysis of peptide-loaded but HLA-mismatched LCLs included in the same assay; data not shown). Moreover, in 12-h assays, these same effectors also caused significant lysis of the unmanipulated wt-LCL but not of the BZ k/o LCL control. We observed this result with all the lytic Ag-specific clones tested, and, in all cases, killing was associated with cell surface mobilization of CD107a, an indicator of in vitro degranulation associated with cytolytic function (30) (data not shown). Note that the L Ag-specific clone illustrated in Fig. 5A was unique in mediating detectable killing of the wt-LCL target within 5 h. Interestingly this clone, which was the only clone to be established from an acute IM patient rather than a healthy EBV carrier, was also the only one to contain preformed perforin (Fig. 5B).

\section*{Intracellular Ag transfer in LCL cultures can sensitize cells to CD4+ T cell recognition}

We then studied in more detail the pathway whereby lytic cycle Ags, being expressed by the small fraction of lytically infected cells in semipermissive LCLs, were being presented for CD4+ T cell recognition. Previous work showed that the EBV-latent Ags that are constitutively expressed in LCLs gain access to the HLA

\begin{table}[h]
\centering
\caption{Summary of epitope-specific CD4+ T cell clones}
\begin{tabular}{llllll}
\hline
Phase & Protein & Epitope Coordinates & Sequence & HLA Restriction & Responders \\
\hline
IE & BZLF1 & 11–25 & VKFTDPYQVFPVQA & DRB3*01 & 2/5 \\
BZLF1 & 61–75 & LTAVHYSTAPGTSWF & DRB3*02 & 2/4 \\
BZLF1 & 116–130 & PDNSTVQTAANAVF & DRB1*13 & 1/1 \\
BRLF1 & 407–421 & PPYRKKQLRDSREG & DRB1*08 & 1/1 \\
E & BMLF1 & 41–55 & DEDPTPAHAPARPS & DQB1*07 & 2/4 \\
BHRF1 & 122–133 & PYYVVDLSVRRGM & DRB1*04 & 1/4 \\
BMRF1 & 136–150 & VKEMGYDVKVKS & DRB1*0301 & 2/4 \\
L & BKR2F & 116–135 & GFSSVERFGLANLNYAHR & DPB1*04 & 4/6 \\
BZLF2 & 186–205 & QFGSHCITYSKFSTVPVSH & DRB1*16 & 2/2 \\
BLLF1 & 61–81 & LDLQGQLPHTHKAYQPR & DRB1*15 & 3/3 \\
BALF4 & 575–589 & DNEHFLKTMKTEVCQ & DRB1*08 & 1/1 \\
BXL2F & 126–140 & LEQQLFYGGIMLPNTRPHS & DRB5*01 & 3/5 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a}Previously published epitopes (19, 20, 22). Epitopes are hereby referred to by the first three amino acids of their sequence, as underlined.

\textbf{FIGURE 3.} Characterization of IE, E, and L Ag-specific CD4+ T cell clones. \textit{Top row:} Autologous and HLA-mismatched allogeneic B95.8 LCLs (5 \times 10^4/well) were incubated for 1 h in the presence of 5 \mu M epitope peptide and then washed. Targets were incubated for an additional 2 h in the presence of mAbs to HLA DR (αDR) or HLA DQ (αDQ), or media alone, before the addition of T cells (1000well). \textit{Bottom row:} T cells were incubated overnight with cells of the autologous B95.8 LCL and of partially HLA class II-matched allogeneic LCLs, for which the matching alleles are shown; all were pre-exposed to epitope peptide. Results are mean ± 1 SD of triplicate wells and are expressed as IFN-γ release (ng/ml), as determined by ELISA.
II-presentation pathway either within the Ag-expressing cell itself, by processes such as macroautophagy (EBNA1) (11, 31), or by release into culture supernatant, followed by uptake and processing as exogenous Ag by neighboring cells (EBNA2, 3B, 3C, BHRF1) (28, 32, 33). Therefore, we set up 7-d cocultures between an HLA-mismatched, semipermissive wt-LCL (i.e., Ag+ donor cells) and an HLA-matched, BZ k/o LCL (Ag donor cells), after which the coculture was tested for recognition by lytic Ag-specific CD4+ T cell clones. Such assays also included the donor and recipient lines cultured alone, as negative controls, and the appropriate HLA-matched, wt-LCL as a positive control; all targets were additionally tested following pre-exposure to 5 \( \mu M \) epitope peptide. Fig. 6 shows typical results obtained with IE, E, and L specific CD4+ T cells, all tested in parallel on the same LCL targets. Although there was no background recognition of the donor cells or recipient cells alone, all three CD4 clones showed significant recognition of the donor–recipient coculture, at levels that in two cases were equal to that seen against the HLA-matched, wt-LCL control. This strongly suggested that, in semipermissive LCLs, IE, E, and L Ags can access the HLA II-presentation pathway via Ag release and intercellular transfer.

Included as an important control was an IE (BZLF1) Ag-specific CD8+ T cell clone, again HLA matched with the same recipient LCL; in accord with earlier findings (12), there was no recognition of the same coculture by these CD8 effectors, indicating that Ag exogenously acquired by recipient B cells preferentially accesses the HLA II pathway rather than the HLA I pathway.

In semipermissive LCLs, those cells in the lytic cycle are destined to die and release into supernatant medium virus particles that, in reinfected neighboring cells, may act as vehicles of Ag transfer. Therefore, we sought to determine to what extent the presentation of lytic-cycle Ags to CD4+ T cells was dependent on full virus replication, taking advantage of the fact that ACV, a nucleoside analog, inhibits viral DNA polymerase (34) and blocks entry into the late lytic cycle. Therefore, semipermissive wt-LCLs of appropriate HLA type were cultured for 14 d in ACV. In each case, immunofluorescence staining for IE (BZLF1) and L (VCA) expression confirmed that ACV did not alter the number of IE and E Ag-expressing cells in the culture, but it reduce L Ag-expressing cell numbers (data not shown). These ACV-treated LCL cells were then used as targets in T cell assays, alongside the corresponding untreated control wt-LCL, the matching BZ k/o LCL, and, as a control for virion-mediated Ag-transfer, the BZ k/o LCL that had been pre-exposed to a purified virion preparation. Again, all targets were also tested in peptide-loaded form. Fig. 7 shows the data from such experiments involving three different pairs of IE and L Ag-specific clones and three different sets of paired LCLs. In each case, ACV treatment strongly reduced recognition of the wt-LCL target by the L-specific clones (consistent with the drug’s marked inhibition of L Ag expression) but had no effect on recognition by the IE and (data not shown) E Ag-specific clones. Thus, the intercellular transfer of IE and E Ags for HLA II-restricted presentation seems to be independent of full virus replication and virion release. Conversely, when BZ k/o LCLs were deliberately exposed to a virion preparation as a source of Ag, they were sensitized to recognition by L-specific clones, as might be expected because the three L Ags in question (BLLF1, BXLF2, BALF4) are structural proteins of the virion (19) but were not sensitized to IE-specific clones.

Discussion

The present work was prompted by our earlier findings that CD8+ T cell responses to lytic Ags were markedly skewed toward IE and certain E proteins (12). Ags expressed before viral inhibitors of HLA I Ag presentation come into play (14); indeed the few L Ag-specific responses detected, despite being of high avidity, showed very poor recognition of semipermissive LCLs (12). We set out to determine the range and frequency of CD4+ T cell responses to
representative IE, E, and L Ags and compare their ability to recognize these same LCLs. In contrast to the pattern seen with CD8+ T cells, we found that CD4 responses are broadly distributed across the three lytic Ag classes and yield CD4+ T cell clones that, irrespective of the kinetics of their target Ag expression, recognize and kill semipermissive LCLs with unexpected efficiency. These results point to a potentially important role for lytic Ag-specific CD4+ T cells in the control of semipermissive PTLD lesions in vivo.

Earlier studies on CD4+ T cell responses to EBV lytic-cycle Ags largely focused either on detecting reactivity to one or two particular proteins in ex vivo assays (19, 20, 35, 36) or on analyzing specificities detectable in T cell preparations generated by LCL stimulation and expansion in vitro (21, 37, 38). We chose to address the question of in vivo immunodominance among the different lytic Ag classes more directly by selecting representative Ags from each class and screening PBMCs for reactivity against Ag-spanning peptide pools in ex vivo ELISPOT assays. Although using IFN-γ as the readout limits our detection to Th1-like responses, there is already much evidence from our laboratory and others that EBV-specific CD4 responses are strongly skewed in this direction (7, 10, 19, 39). Our observations on the range of lytic Ag-specific responses are immediately apparent from Figs. 1 and 2. Remarkably, although our analysis was limited to just 8 selected proteins (out of >60 encoded by the virus in the lytic cycle), all 14 virus-carrying donors gave evidence of CD4 reactivity to one or more of these proteins and often to more than one epitope/protein.

We draw attention to the fact that the peptide panels available for this work were 15mers overlapping by 10 aa for the two IE proteins (BZLF1 and BRLF1) and two E proteins (BMRF1 and BMLF1) and 20mers overlapping by 15 aa for the remaining E protein (BHRF1) and the L proteins (BKRF2, BZLF2, and BXLF2). Because naturally processed MHC class II epitopes are usually 12–26 aa in length (40), it is possible that the breadths of responses to some proteins in our panel were underestimated by screening on the shorter peptides. However, we consider it unlikely that this seriously affected the overall pattern of results. Multiple sequence analyses of peptides eluted from MHC class II alleles revealed that presented epitopes are made up of families of heterogeneous peptides sharing a common nine amino acid-binding core with varying-length flanking regions (reviewed in Ref. 40). Thus, all possible 9mer peptides are covered by both 15mer and 20mer peptides overlapping by 10 and 15 residues, respectively. Indeed, a study designed to determine the optimal overlapping peptide set to stimulate IFN-γ production from human virus-specific T cells concluded that although there was a trend toward detecting more CD4+ T cell responses with longer peptides, there was no statistically significant difference between peptides ranging from 15–20 aa (41).

Certainly, our results make clear that, in contrast to the corresponding CD8 response, the CD4+ T cell response to EBV lytic-cycle Ags is widely spread across IE, E, and L protein targets, as are the 54 individual peptide epitopes that were mapped as a result. Perhaps the only hint of unequal epitope distribution that we
detected involved the two IE proteins (both screened using peptides of 15 aa); in relation to their size, BZLF1 was relatively epitope rich, and BRLF1 was relatively epitope sparse, a trend that needs to be investigated further using larger donor panels and longer peptides. But, viewing the results overall, we detected no skewing of CD4+ T cell responses toward Ags appearing in a particular phase of the lytic cycle.

The ELISPOT data also showed that CD4+ T cell responses to lytic Ags resemble the previously analyzed latent Ag-specific CD4 memory responses (7, 23) in two important respects: all individual epitope-specific populations fall within a similar fairly narrow size range, with lytic Ag responses typically outnumbering those against latent Ags, and there are much more consistent trends in Ag/epitope dominance (1). These differences between EBV-specific CD4 and CD8 memory in long-term virus carriers may be reflections of events occurring in primary infection where, albeit not defined at the epitope level (21, 37). Hence, the total pool of lytic Ag-specific CD4+ T cells in virus carriers could be much larger than the coresident latent Ag-specific pool.

To study the function of these responses in more detail, we generated CD4+ T cell clones against four IE, three E, and three L Ag-derived epitopes, wherever possible establishing multiple clones/epitope. To these we added clones raised in a similar way on the potential importance of such cells as effectors against foci of lytic infection in vivo. We addressed this using pairs of

**FIGURE 6.** CD4+ T cell recognition of target cell mixtures. CD4+ T cells specific for VKF (BZLF1 11–25), VKL (BMRF1 136–150), and LDL (BLLF1 61–81) and CD8+ T cells specific for RAK (BZLF1 190–197) were simultaneously tested against a pair of B95.8 and BZ k/o LCLs expressing the relevant HLA-restricting allele of each clone (HLA-DRB3*0101, -DRB1*0301, and -DRB1*1501 and -B*08) and a mismatched B95.8 LCL negative for the restricting allele of each clone. In the same assay, all T cells were tested for recognition of a 1:1 mixture of the above HLA-matched BZ k/o LCL and HLA-mismatched B95.8 LCL that had been cocultured for 7 d before the assay (coculture). Each clone was assayed at 2000 cells/well against 1 × 105 target cells/well, either unmanipulated or preloaded with 5 μM epitope peptide, as in Fig. 5. Results are mean ± 1 SD of triplicate wells and are expressed as IFN-γ release (ng/ml), as determined by ELISA.

**FIGURE 7.** Presentation of L Ags, but not IE or E Ags, is blocked by ACV. CD4+ T cell clones (2000 cells/well) were tested for recognition of the pairs of B95.8 and BZ k/o LCLs used in Fig. 5, and in the same assay for recognition of the B95.8 LCL grown for 2 wk in ACV and the BZ k/o LCL pulsed with purified virus particles at a multiplicity of infection of 100. All targets were either unmanipulated or preloaded with 5 μM epitope peptide and plated at 5 × 104 cells/well. Results are the mean + 1 SD of triplicate wells and are expressed as IFN-γ release (ng/ml), derived epitopes (42). As described above, lytic and latent Ag-induced CD4 responses are similar in certain respects; however, our results also suggested one important difference. Thus, although responses to individual lytic epitopes may be small, the fact that every donor reacted to one or more of just eight lytic Ags tested suggested that the overall range of such responses will be extremely large, with any one donor reactive to many of the >60 lytic Ags that EBV encodes. This notion is supported by two recent studies in which LCL-stimulated polyclonal CD4+ T cell cultures contained reactivities against multiple lytic proteins, albeit not defined at the epitope level (21, 37). Hence, the total pool of lytic Ag-specific CD4+ T cells in virus carriers could be much larger than the coresident latent Ag-specific pool.

We then sought to determine how well such CD4+ T cell clones could recognize lytically infected target cells, because this reflects on the potential importance of such cells as effectors against foci of lytic infection in vivo. We addressed this using pairs of
semipermissive (wt virus-transformed) and tightly latent (BZ k/o virus-transformed) LCLs as Ag+ and Ag− targets, respectively. By choosing LCLs of appropriate HLA type, one could test IE, E, and L Ag-specific clones simultaneously on the same target lines. Such experiments (e.g., Fig. 4) consistently revealed specific recognition of the wt-LCL by all of the CD4+ T cell clones tested, irrespective of the phase of lytic cycle in which their target Ag was expressed. Indeed, the levels of recognition, although variable between different epitopes, were regularly >10% of that seen when the same LCLs were optimally loaded with peptide epitope, and, in several cases, they were >50% of that optimum value. Perhaps surprisingly, these levels of wt-LCL recognition far exceeded those seen in earlier work using latent Ag-specific CD4+ T cell clones with a similar range of functional avidities, in which almost all clones recognized the LCL at <5% of optimum values (7). Recognition was also apparent in cytotoxicity assays, in which the lytic epitope-specific clones were clearly cytotoxic for wt-LCL target, albeit in 12-h, rather than 5-h, assays. This was associated with these CD4+ T cells being positive for granzyme B and undergoing degranulation upon stimulation, as measured by cell surface mobilization of CD107a (30).

These findings contrast slightly with the only other study directly comparing different classes of lytic epitope-specific CD4+ T cell clones in LCL-recognition assays (19). In that report, clones against L Ag-derived epitopes (in BLLF1 and BALF4) recognized semipermissive LCLs, whereas clones against two IE epitopes (in BZF1L1) did not. Because our work involved CD4+ T cells against three other BZF1L1 epitopes, the discrepancy in results may reflect interepitope differences in presentation or the isolation of clones with different functional avidities. A more recent report, in which BZF1L1 specificities were detected in T cell lines expanded in vitro by LCL stimulation (37), supports the view that at least some BZF1L1-derived epitopes are presented by semipermissive LCLs.

The earlier studies of Mautner and colleagues (19, 44) clearly showed that late lytic-cycle proteins, such as BLLF1 and BALF4, which are structural components of virus particles, can access the HLA II-presentation pathway in LCLs through virus release from latently infected cells, followed by receptor-mediated entry into neighboring latently infected cells and processing as exogenous Ag. Accordingly, L structural Ag-specific CD4+ T cell efficiently recognize Ag+ LCLs exposed to virus-like particles [virions devoid of viral DNA (45)] as a source of Ag (46). Using appropriate mixtures of Ag-donor and Ag-recipient LCLs, we found that nonstructural IE (BZF1L1, BRLF1) and E (BMLF1, BMRF1) proteins were also presented via a pathway involving intercellular transfer. However, that presentation was independent of virion production and release, because ACV treatment of semipermissive LCLs to inhibit late cycle entry (34) did not reduce IE or E Ag presentation. Note that a similar process of Ag transfer was shown to underlie the HLA II-restricted presentation of the EBNA2 and EBNA3 latency proteins by LCLs, which, because it also occurs in BZ k/o lines, is clearly independent of any lytic cycle entry (28).

Similarly, HLA class II presentation of intercellularly transferred Ag may explain the expansion of nonstructural Ag-specific CD4+ T cells from PBMCs by LCL stimulation (21, 37, 44).

Importantly, Ag transfer and processing by coresident, latently infected cells in the culture also explain why semipermissive LCLs are recognized so efficiently by lytic Ag-specific CD4+ T cell clones in both IFN-γ and cytotoxicity assays (Figs. 4, 5), when only 2–5% of cells in these target lines are actually in the lytic cycle. Indeed, if, as reported, the HLA II-presentation pathway is blocked in cells after entry into the lytic cycle (47, 48), then lytic epitope-specific CD4+ T cell recognition of semipermissive LCLs may be entirely dependent upon such Ag transfer, whether occurring by noninvariant (IE, E)- or virion (L)-mediated routes.

In summary, we described some essential features of the CD4+ T cell response to EBV lytic-cycle Ags, as seen in the blood of long-term virus carriers, which have interesting implications in two contexts. First, we showed that the CD4 response differs markedly from the corresponding CD8 response in its range; thus, it is not skewed toward just a few immunodominant proteins but is spread widely against IE−, E−, and L-phase targets and is likely composed of many coresident epitope-specific populations of comparatively similar size and functional avidity. We suggested that such differences reflect an important role for direct contact with lytically infected cells in driving the CD8+ T cell response in vivo compared with a more important role for cross-presented Ag in driving CD4 responses. Additionally, we showed that semipermissive LCL targets are efficiently recognized by L Ag-specific clones, via virion-mediated Ag transfer, as well as by clones specific for IE and E Ags presented via nonvirion-mediated transfer. In that regard, some evidence suggested that the lytically infected cells typically present within PTLD lesions express IE and E Ags but do not always progress to being L Ag− (4, 49). Thus, we inferred that IE and E Ag transfer within these lesions will render them sensitive to IE and E Ag-specific CD4+ T cell. Indeed, the likely presence of such cells within the LCL-reactivated preparations used as adoptive T cell therapy for PTLD could help to explain the greater clinical effectiveness noted using preparations with a significant CD4+ T cell content (50).

Disclosures
The authors have no financial conflicts of interest.

References


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