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CD8$^+$ immunodominance among Epstein-Barr virus lytic cycle antigens directly reflects the efficiency of antigen presentation in lytically infected cells

Victoria A. Pudney, Alison M. Leese, Alan B. Rickinson, and Andrew D. Hislop

Institute for Cancer Studies, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

Antigen immunodominance is an unexplained feature of CD8$^+$ T cell responses to herpesviruses, which are agents whose lytic replication involves the sequential expression of immediate early (IE), early (E), and late (L) proteins. Here, we analyze the primary CD8 response to Epstein-Barr virus (EBV) infection for reactivity to 2 IE proteins, 11 representative E proteins, and 10 representative L proteins, across a range of HLA backgrounds. Responses were consistently skewed toward epitopes in IE and a subset of E proteins, with only occasional responses to novel epitopes in L proteins. CD8$^+$ T cell clones to representative IE, E, and L epitopes were assayed against EBV-transformed lymphoblastoid cell lines (LCLs) containing lytically infected cells. This showed direct recognition of lytically infected cells by all three sets of effectors but at markedly different levels, in the order IE $>$ E $\gg$ L, indicating that the efficiency of epitope presentation falls dramatically with progress of the lytic cycle. Thus, EBV lytic cycle antigens display a hierarchy of immunodominance that directly reflects the efficiency of their presentation in lytically infected cells; the CD8$^+$ T cell response thereby focuses on targets whose recognition leads to maximal biologic effect.

CORRESPONDENCE
A.B. Rickinson:
A.B.Rickinson@bham.ac.uk

Abbreviations used: ACV, acyclovir; E, early; IE, immediate early; IM, infectious mononucleosis; L, late; LCL, lymphoblastoid cell line.

CD8$^+$ T cells control virus infection by recognizing short peptides derived from the intracellular breakdown of viral proteins and presented at the infected cell surface by MHC class I molecules. There is often a marked focusing of CD8$^+$ T cell responses against a small number of immunodominant viral epitopes (1–3), and many aspects of peptide generation, transport, and MHC class I loading have been considered as potential determinants for epitope immunodominance (4, 5). However, in more complex systems where not all viral proteins may access the MHC class I pathway equally, the antigen source itself may also be an important factor. This is particularly the case for herpesviruses, where immediate early (IE), early (E), and late (L) viral proteins are expressed at different phases of the replication cycle and where the antigen-presenting capacity of the infected cell may itself change as the cycle progresses (6). Indeed, determining whether there is a marked hierarchy of immunodominance among IE, E, and L proteins and whether this is related to the antigen-processing function of the lytically infected cells could have implications for the pathway whereby the virus-specific CD8$^+$ T cell response is primed.

By definition, hierarchies of immunodominance should be consistently observed across a wide range of MHC backgrounds. As a result, their existence in herpesvirus systems is likely to emerge more clearly from studies in man, rather than in mouse models where work tends to focus on a small number of inbred strains (6–8). Studies to date on human CD8+ T cell responses to herpes simplex virus, an α-herpesvirus, suggest that they are preferentially directed against late structural proteins of the virus (9, 10). Likewise with cytomegalovirus, a β-herpesvirus, the pp65 viral tegument protein expressed late in lytic cycle is well established as an immunodominant antigen (11, 12)—although not to the exclusion of some more recently discovered IE and E target proteins (13, 14). However, for both agents the issue of immunodominance among viral antigens, and the effect that viral immune evasion proteins might have on immunogenicity in vivo (6), remains to be fully resolved.

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Here, we turn our attention to EBV, a γ1-herpesvirus which preferentially infects B lymphocytes. During primary infection as seen in infectious mononucleosis (IM) patients, the virus replicates at permissive sites in the oropharynx and induces a strong CD8$^+$ T cell response to lytic cycle antigens that dominate the circulating T lymphocyte pool (15, 16). Although viral epitopes of varying strengths have been identified within IE and some E proteins examined to date (17–20), these studies were based on a small number of lytic cycle proteins or selected epitopes, with virtually no representation of L antigens as potential targets. This paper describes two interdependent sets of experiments. In the first, we screen primary CD8$^+$ T cell responses for reactivities to the 2 IE proteins, 11 representative E, and 10 representative L proteins and demonstrate the existence of a marked hierarchy of immunodominance between these different groups of antigens. In the second, we use CD8$^+$ T cell clones specific for representative IE, E, and L epitopes as probes to ask whether the above hierarchy correlates in any way with the efficiency of epitope presentation in lytically infected cells.

RESULTS

Antigen mapping experiments

CD8$^+$ T cell clones were established from in vivo–activated cells in the blood of acute IM patients by limiting dilution cloning and their specificity determined in cytotoxicity assays against autologous target lymphoblastoid cell lines (LCLs) infected with recombinant vaccinia viruses expressing one of the panel of selected EBV lytic cycle proteins. The 23 targets selected included the 2 IE, 11 representative E, and 10 representative L proteins (see Materials and methods). Examples of the range of specificities identified, and the subsequent mapping of those specificities to individual peptide epitopes are given for two patients (see Figs. 1 and 2).

For patient IM73, responses were directed against just 3 of the 23 target antigens (BRLF1, BMLF1, and BALF2) and the screening results from clones representing these three reactivities are shown in Fig. 1 A. Overall, there were 15 clones specific for the IE protein BRLF1, 4 for the E protein BMLF1, and 7 for the E protein BALF2. Throughout these experiments, once the relevant HLA-restricting allele had been identified from a second round of vaccinia assays on HLA-matched LCL targets, the peptide epitope specificity of each clone was determined wherever possible. Clones were tested either against known epitopes derived from the protein of interest (where such epitopes existed), or against an overlapping peptide library for the protein (where the library was available), or against potential epitope predictions based on the peptide binding motif of the restricting HLA allele. Fig. 1 B shows the relevant results from all of the antigen-specific clones derived from IM73. By HLA restriction, the 15 BRLF1-specific clones contained three different reactivities. Four clones restricted through HLA-A*0201 were found to recognize a known BRLF1 epitope YVLDHLIVV (hereafter, all epitopes are subsequently identified by three or four letter codes as initially underlined); interestingly two

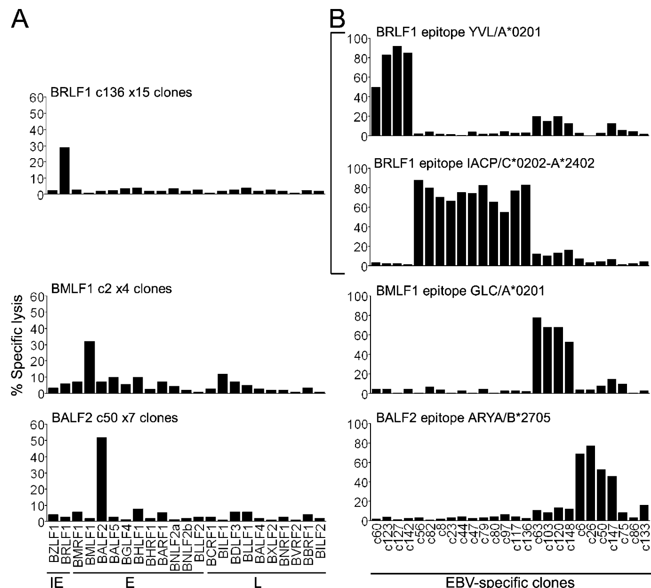

Figure 1. Assays on EBV-specific CD8$^+$ T cell clones from IM 73. (A) Representative antigen mapping results from 1 of 15 BRLF1-specific clones, 1 of 4 BMLF1-specific clones, and 1 of 7 BALF2-specific clones tested on autologous LCL targets preinfected with the panel of 23 recombinant vaccinias expressing individual IE, E, and L proteins of the EBV lytic cycle. (B) Epitope mapping results from the 26 EBV lytic antigen-specific clones tested on autologous LCL targets preexposed to the BRLF1-derived YVL and IACP peptides, to the BMLF1-derived GLC peptide, and to the BALF2-derived ARYA peptide (restriction elements as shown). All results are expressed as a percentage of specific lysis in 5-h chromium release assays.

further clones restricted through HLA-A*2402 and nine further clones restricted through HLA-C*0202 all mapped to epitopes encoded within a 15mer BRLF1 peptide IACPIVMRYVLDHLI that overlapped, but was distinct from, the YVL sequence. As for the other reactivities in IM73, all four BMLF1-specific clones recognized the known HLA-A*0201–restricted epitope GLCTLVAML, whereas the seven BALF2-specific clones included four HLA-B*2705–restricted clones that mapped to a novel epitope ARYAYYLQF found by screening predicted B27-binding peptides, and three HLA-B*0702–restricted clones for which no epitope was identified among predicted B7-binding peptides from the BALF2 sequence.

Fig. 2 shows the corresponding data for a second patient, IM179, with a broader range of responses. In this case, clonal reactivities were identified against six different EBV lytic proteins; these included the IE protein BRLF1 (12 clones), the E proteins BMRF1 (4 clones), BMLF1 (1 clone) and BALF2 (4 clones), and the two L proteins BALF4 (4 clones) and BILF2 (1 clone). HLA restriction and epitope mapping of the IM179 responses (Fig. 2 B) identified two types of BRLF1 (IE) reactivity; four HLA-A*0201–restricted clones were specific for the YVL epitope, and eight HLA-C*0202–restricted clones recognized the same IACP peptide as seen by the C*0202-restricted clones from IM73. Of the E antigen–specific responses in IM179, the BMRF1- and BMLF1-specific clones mapped to known HLA-A*0201–restricted

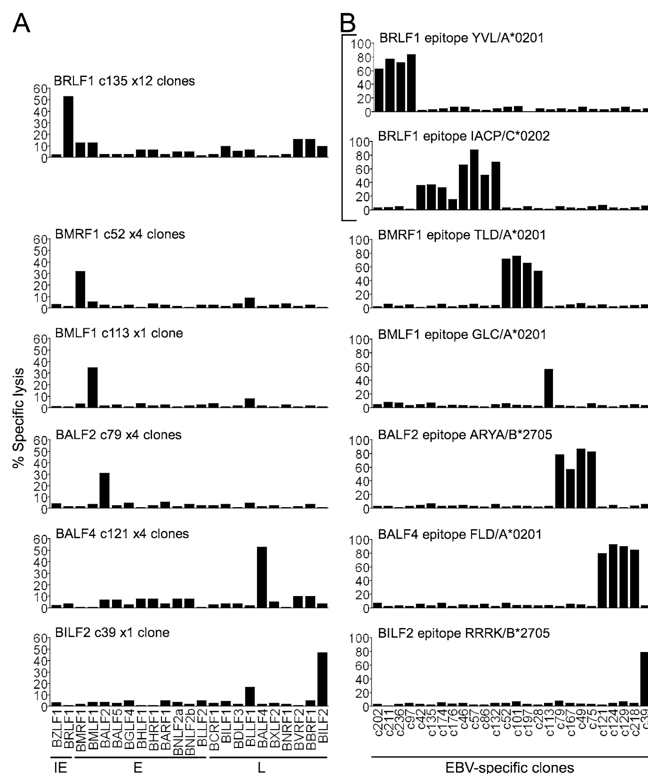

Figure 2. Assays on EBV-specific CD8$^+$ T cell clones from IM 179. (A) Representative antigen mapping results from 1 of 12 BRLF1-specific clones, 1 of 4 BMRF1-specific clones, 1 BMLF1-specific clone, 1 of 4 BALF2-specific clones, 1 of 4 BALF4-specific clones, and 1 BILF2-specific clone. (B) Epitope mapping results from the 26 EBV lytic antigen–specific clones tested on autologous LCL targets preexposed to the BRLF1-derived YVL and IACP peptides, to the BMRF1-derived TLD peptide, to the BMLF1-derived GLC peptide, to the BALF2-derived ARYA peptide, to the BALF4-derived FLD peptide, and to the BILF2-derived RRRK peptide (restriction elements as shown). Results expressed as in Fig. 1.

epitopes in these proteins (TLDYKPLSV and GLC, respectively), whereas the BALF2-specific clones were HLA-B*2705–restricted and recognized the ARYA 9mer epitope. The L antigen specificities in IM179 were of particular interest because examples of CD8$^+$ T cell responses to late proteins are extremely rare in the literature (21). The BALF4-specific clones proved to be HLA-A*0201–restricted and were mapped by testing predicted peptides to a novel epitope FLDKGTYTL; the BILF2 reactive clone was restricted by HLA-B*2705 and again was mapped by peptide prediction to a novel epitope, RRRKGWIPL.

For all patients, clones were established by stimulating IM PBMCs with a mixture of the γ-irradiated autologous LCL and pooled allogenic γ-irradiated PBMCs as feeder cells. Our earlier work had found that, providing IL2 was available, specific antigen stimulation in vitro was not necessary in order to expand in vivo–activated CD8$^+$ T cells from the blood of IM patients (18). However, we were concerned to check whether inclusion of the autologous LCL, which often contains a small percentage of lytically infected cells, in the cloning mixture was in some way biasing the range of specificities obtained. Therefore, in the case of IM179 and of another patient IM146, parallel clonings were performed using two substitutes for the autologous LCL; namely, an irradiated partially HLA-matched LCL that could not enter lytic cycle and therefore could not provide any lytic antigen-specific stimulus (22) or exposure to an anti-CD3 Mab as a pan-T cell stimulus. In both cases, the range of lytic antigen-specific clones recovered using these alternative protocols was similar to that seen using the autologous LCL in the cloning mix (see Fig. 3's legend).

A total of 11 acute IM patients, yielding a mean of 20 EBV lytic antigen-specific clones per patient, were studied in the antigen mapping experiments and Fig. 3 summarizes the pattern of results obtained. In each case, positive responses are indicated by shaded boxes and the depth of shading indicates the proportion of clones that mapped to the antigen in question. Some 9/11 patients responded to one or both EBV-coded IE proteins; and in five cases, an IE protein

Table I. EBV lytic cycle antigens and epitopes identified by CD8$^+$ T cell clones

Temporal expression	EBV antigen	Epitope coordinates	Epitope sequence	HLA allele	Reference
IE	BZLF1	190–197	RAKFKQLL	B*0801	(54)
		101–115	LQHYREVAA	C*0802	New
	BRLF1	109–117	YVLDHLIVV	A*0201	(55)
		148–156	RVRAYTYSK	A*0301	(56)
		91–99	AENAGNDAC	B*4501	New
		101–115	IACPIVMRYVLDHLI	A*2402	New[a]
		101–115	IACPIVMRYVLDHLI	C*0202	New[a]
E	BMLF1	280–288	GLCTLVAML	A*0201	(17, 18)
	BMRF1	268–276	YRSGIIAVV	B*3906	New
		86–100	FRNLAYGRTCVLGK	C*0304	(18)[a]
		208–216	TLDYKPLSV	A*0201	(16)
		116–128	RPQGGSRPEFVKL	B*0702	New
		20–28	CYDHAQTHL	A*2402	New
	BALF2	418–426	ARYAYYLQF	B*2705	New
		ND	ND	B*0702	New
		ND	ND	C*0702	New
		ND	ND	B*1401	New
		ND	ND	B*4501	New
	BALF5	ND	ND	A*0201	New
		ND	ND	A*0301	New
		ND	ND	B*4501	New
	BHLF1	ND	ND	A*0201	New
		ND	ND	B*1501	New
	BGLF4	ND	ND	C*0101	New
	BNLF2b	ND	ND	B*1401	New
L	BALF4	276–284	FLDKGTYTL	A*0201	New
	BILF2	240–248	RRRKGWIPL	B*2705	New
	BBRF1	ND	ND	A*0201	New

[a]Minimal epitope to be determined.

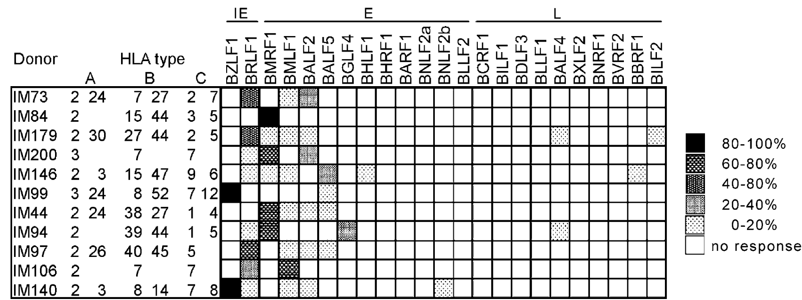

Figure 3. Summary of antigen mapping data from 11 acute IM patients (HLA class I types shown). A mean of 20 EBV-specific clones were analyzed per patient. For each patient, the EBV lytic antigens recognized by CD8$^+$ T cell clones are identified as shaded boxes, and the percentage of CD8$^+$ T cell clones from each donor recognizing each target antigen is reflected by the intensity of the shading. Note that in two cases, IM 179 and IM 140, clonings were also performed without γ-irradiated autologous LCL feeders, either using a γ-irradiated allogeneic BZLF1-LCL feeder that could not provide any lytic antigen–specific stimulus or using an anti-CD3 mAb as a pan-T cell stimulus. Both protocols yielded the same spectrum of antigen specificities in proportions that were not significantly different from those illustrated above. Combining the results from these alternative protocols, for IM 179 (of 20 clones) 50% clones were IE antigen specific, 25% clones E antigen specific, and 25% clones L antigen specific; for IM 140.1, (of 30 clones) 90% clones were IE antigen specific and 10% E antigen specific.

was the immunodominant target. In addition, all 11 patients responded to one or more of the E proteins and, in six cases, these were the dominant responses. Interestingly, the great majority of E protein–specific clones that were detected mapped to a particular subset of target proteins, namely BMRF1, BMLF1, BALF2, and BALF5. In contrast, L protein–specific responses were detected in only 3/11 patients and, in every case, these responses (to BALF4 in two patients, and to BILF2 and BBRF1 each in one patient) were represented by only a small number of clones. Table I gives the full range of EBV antigen/HLA class I combinations that were recognized by T cell clones in these experiments. The majority of these responses are novel and, in 10 cases, led to the identification of new CD8 epitopes. Of the immunodominant subset of IE and E antigens (BZLF1, BRLF1, BMLF1, BMRF1, BALF2, BALF5), all but one (BMLF1) contained between two and six epitopes, whereas responses to other E antigens or to any of the L antigens were almost all detected as isolated reactivities to a single epitope.

Antigen presentation experiments

A system to examine CD8$^+$ T cell recognition of lytically infected cells. We next sought to establish a means of assaying IE, E, and L antigen–specific CD8$^+$ T cell responses for their ability to recognize lytically infected target cells. The lack of a permissive in vitro system for EBV replication has meant that this could never be addressed in conventional cytotoxicity assays. However, the use of IFN γ release as an alternative readout of CD8$^+$ T cell function allowed the development of an ELISPOT assay where effector clones were challenged with the appropriately HLA-matched LCL targets in which the percentage of cells spontaneously entering lytic cycle (usually 2–5%) could be determined by lytic antigen–specific mAb staining. These lines were established with a recombinant wild-type EBV and are referred to as BZLF1+ LCLs. Essential control targets in all of these experiments were BZLF1− LCLs established from the same HLA-matched donors using a recombinant EBV deleted for the BZLF1 IE gene and, therefore, incapable of entering lytic cycle.

Fig. 4 illustrates two types of experiments that have been conducted with all the lytic antigen–specific clones used in this work. These particular experiments used HLA-B*0801–derived clones specific for the IE epitope RAKFKQLL derived from BZLF1. In the first experiment, inspection of ELISPOT plates showed clear recognition of an HLA-B*0801–positive BZLF1+ LCL but not of the matching BZLF1− target (Fig. 4 A). Additional targets were reconstructed mixtures of BZLF1− LCL cells in which 1, 2, or 5% of the cells had been previously loaded with an optimal concentration of epitope peptide, washed well, and added back. Clearly, these mixtures also induced IFN γ release from the CD8$^+$ T cell clones, thereby confirming the sensitivity of the assay. The counts of spot-forming cells per well are shown as histograms in Fig. 4 B. A second type of experiment sought to determine whether positive responses to the BZLF1+ LCL actually reflected direct recognition of the lytically infected subpopulation or recognition of the antigen that had been released from the lytically infected cells, taken up, processed, and re-presented via the class I pathway by neighboring latently infected cells in the culture. To discriminate between the two possibilities, a BZLF1+ but HLA-B*0801–negative "donor" LCL was cocultured for 7 d with the BZLF1−, HLA-B*0801–positive "recipient" LCL, and then the mixture was used as a target in the ELISPOT assay. As shown by the results (Fig. 4, C and D), there was no recognition of the mixed LCL targets even though recipient cells were clearly still present in the coculture and were able to present RAK peptide exogenously loaded on the mixed LCL cell population immediately before the ELISPOT assay. This indicates that recognition of the BZLF1+, HLA-B*0801–positive LCL target line by RAK-specific T cells reflects the presentation of endogenously expressed antigen by lytically infected cells themselves and does not result from antigen transfer and cross-presentation within the culture.

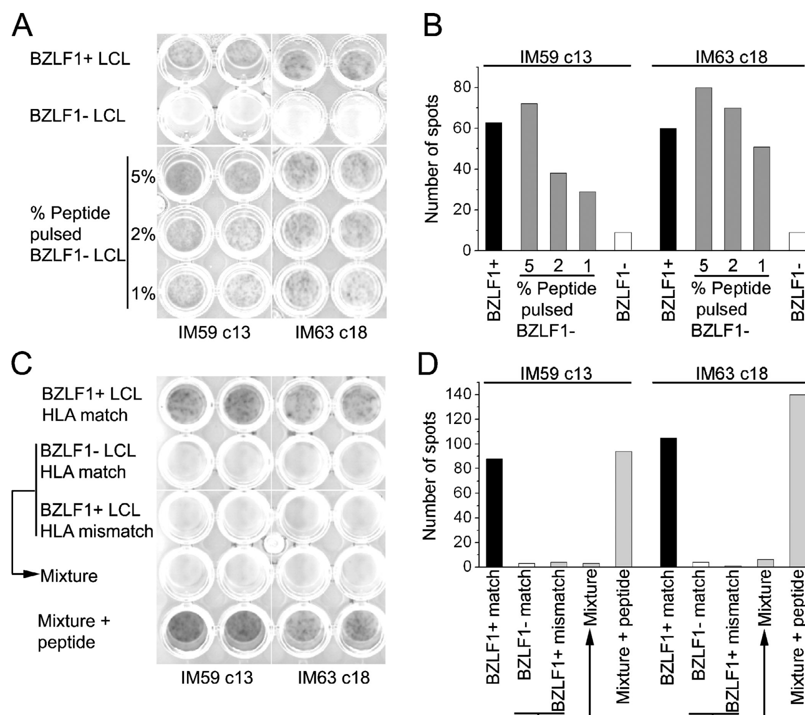

Figure 4. Antigen presentation assays using two CD8$^+$ T cell clones specific for the BZLF1-derived RAK peptide (HLA-B*0801 restricted). A and B show data from an ELISPOT assay on the BZLF1+ LCL in which 4% cells expressed lytic cycle antigens, on a paired BZLF1− LCL from the same HLA-B*0801–positive donor, and on the BZLF1− LCL in which 1, 2, or 5% of cells had been preexposed to the RAK peptide. C and D show data from an ELISPOT assay on the above BZLF1+ and BZLF1− LCLs, on a BZLF1+ LCL from a HLA-B*0801–negative donor (HLA mismatch) and on a mixture of the BZLF1− HLA-B*0801–positive LCL and the BZLF1+ HLA-B*0801–negative LCL that had been cocultured for 7 d before the assay and then used as targets either without further manipulation or exposed to the RAK peptide immediately before the assay. (A and C) The original ELISPOT plate is shown alongside the histogram recording the number of spot-forming cells observed (B and D).

LCL recognition by IE, E, and L antigen–specific CD8$^+$ T cells. Using this approach, we set out to examine the efficiency with which epitopes from different temporal phases of the EBV replication cycle were presented. Multiple CD8$^+$ T cell clones to each of three IE epitopes, the HLA-B*0801–restricted RAK and HLA-B*3501–restricted EPLPQGQLTAY epitopes both from BZLF1 and the HLA-A*0201–restricted YVL epitope from BRLF1, were tested against appropriately HLA-matched BZLF1+ and BZLF1− LCL pairs and representative results are shown in Fig. 5. For all three epitope specificities, there was consistently good recognition of the lytic antigen–expressing target cell line, usually at levels equivalent to those seen on reconstructed targets in which between 2 and 5% of the control BZLF1− LCL had been preloaded with epitope peptide. In each case, control experiments were performed using appropriate donor and recipient cell mixtures as described in Fig. 4 (C and D) and again gave no evidence of antigen transfer and cross-presentation (not depicted).

Parallel experiments looking at the presentation of E antigens were performed using CD8$^+$ clones specific for the BMLF1-derived GLC epitope and the BMRF1-derived TLD epitope, both restricted through HLA-A*0201, and the BALF2-derived ARYA epitope restricted through B*2705. As before, several independently derived clones for each epitope were incubated with appropriate BZLF1+ and BZLF1− LCL pairs and recognition assayed by ELISPOT. As illustrated in Fig. 6 using representative clones to each of the epitopes, significant recognition was observed in each case but only at levels equivalent to or less than those seen in reconstructed targets with 1% peptide-loaded cells. The apparently lower levels of E antigen compared with IE antigen recognition could not be explained by reduced numbers of lytically infected cells in the BZLF1+ LCL targets because the assays in Fig. 6 used the same target lines as used for the EPL- and YVL-specific effectors in Fig. 5. Again donor and recipient LCL mixing experiments gave no evidence of E antigen transfer within cocultures (not depicted).

Although CD8$^+$ T cell responses to viral proteins expressed at the late stage of EBV replication are rare, we were able to carry out similar experiments with three clones specific for the BALF4-derived FLD epitope restricted through HLA-A*0201 and two clones specific for the BILF2-derived RRRK epitope restricted through HLA-B*2705; for each epitope, the clones were derived from two different donors. Representative results in Fig. 7 show that the L antigen–specific T cell recognition is detectable only at very low levels—well below that seen against reconstructed targets in which 1% of the cells had been loaded with epitope peptide. Importantly, we observed these results despite the fact that

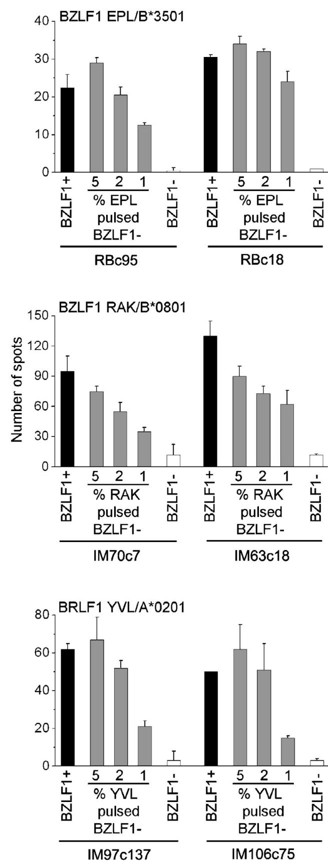

Figure 5. **Antigen presentation assays using IE antigen–specific CD8+ T cell clones.** Clones specific for the BZLF1-derived EPL (HLA-B*3501 restricted) and RAK (HLA-B*0801 restricted) epitopes and the BRLF1-derived YVL (HLA-A*0201 restricted) epitope were tested in ELISPOT assays against pairs of BZLF1+ and BZLF1− LCLs with the relevant HLA class I–restricting allele (where 5, 10, and 5% of the BZLF1+ LCL cells, respectively, were in lytic cycle) and on the BZLF1-LCL in which 1, 2, or 5% of cells had been preexposed to the epitope peptide. Results are shown as the number of spot-forming cells per well, and the error bars represent 1 SD. Data from the two EPL-specific clones are representative of results obtained on the only available HLA-B*3501–positive LCL pair. Data from the two RAK-specific clones and the two YVL-specific clones are in each case representative of results obtained from three clones of each specificity tested on three different LCL pairs.

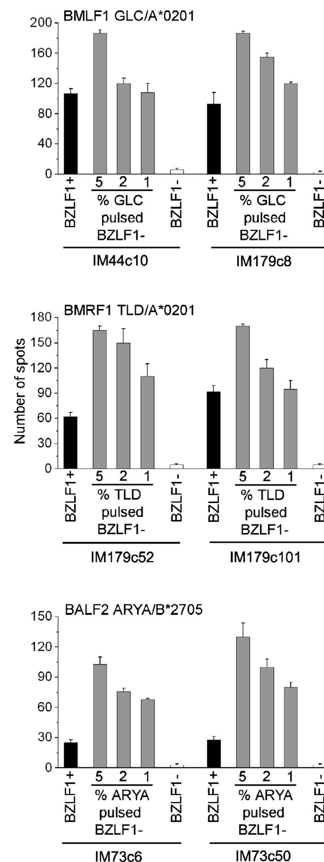

Figure 6. **Antigen presentation assays using E antigen–specific CD8+ T cell clones.** Clones specific for the BMLF1-derived GLC (HLA-A*0201 restricted), the BMRF1-derived TLD (HLA-A*0201 restricted) and the BALF2-derived ARYA (HLA-B*2705 restricted) epitopes were assayed as in Fig. 5; in each case, 5% of the BZLF1+ LCL cells were in lytic cycle. Data from the two GLC-specific and TLD-specific clones are each representative of results obtained for three epitope-specific clones tested on two different HLA-A*0201–positive LCL pairs, and data from the two ARYA-specific clones are representative of results obtained from four such clones on two different HLA-B*2705–positive LCL pairs.

mAb staining against one of the target antigens, BALF4, confirmed that some 4–5% of the cells in these particular BZLF1+ LCLs were indeed in late lytic cycle. To ensure that the result was not an artifact of working with B95.8 virus–transformed cell lines (23), we repeated the assays using HLA-A*0201– or HLA-B*2705–positive LCLs carrying other wild-type EBV strains and confirmed that these were also poorly recognized by the L epitope–specific effectors despite the presence of significant numbers of cells in lytic cycle (unpublished data). As a further control, BZLF1+ LCLs were cultured in acyclovir (ACV) to block late lytic protein production and recognition was indeed abolished (Fig. 7, ACV); this contrasted with the results observed using IE or E antigen–specific clones where ACV treatment had no effect on BZLF1+ LCL recognition (unpublished data).

The possibility of antigen cross-presentation within LCL cultures is particularly relevant for antigens such as BALF4 and BILF2, as these proteins are present within virus particles themselves; however, we again observed no cross-presentation in donor and recipient LCL mixtures, suggesting that the above experiments were indeed measuring L epitope presentation by lytically infected cells.

Comparing LCL recognition by IE, E, and L antigen–specific clones of known avidities

Throughout such experiments, we checked in parallel the functional avidity of the various effector T cell clones by titration in ELISPOT assays on appropriately matched BZLF1− LCLs preloaded with 10-fold dilutions of epitope peptide in the 10^{-5}–10^{-13} M range. We found that IE epitope–specific clones gave 50% optimal recognition at 10^{-9} to 5×10^{-10} M peptide concentrations, which was similar to the E epitope–specific clones with 50% endpoints $\sim 10^{-9}$ M.

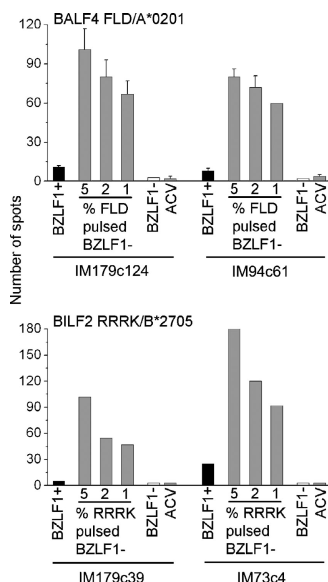

Figure 7. Antigen presentation assays using L antigen–specific CD8+ T cell clones. Clones specific for the BALF4-derived FLD (HLA-A*0201 restricted) and the BILF2-derived RRRK (HLA-B*2705 restricted) epitopes were assayed as in Fig. 5; for each set of effectors, 4% of the BZLF1+ LCL cells were in late lytic cycle. These assays also included the ACV-treated BZLF1+ LCL (with no cells in late lytic cycle) as an additional target (ACV). Data from the FLD-specific clones are representative of results from three such clones tested on two different HLA-A*0201–positive LCL pairs, and data from the two RRRK-specific clones are representative of results obtained on two different HLA-B*2705–positive LCL pairs.

Interestingly, all five clones specific for L epitope specificities FLD and RRRK were reproducibly more avid with 50% endpoints between 2×10^{-10} and 10^{-11} M peptide. Therefore, it seemed very unlikely that poor LCL recognition by the latter clones could be ascribed to low avidity. To check this further, different epitope-specific clones restricted through the same HLA allele were tested together on the same BZLF1+ and BZLF1− LCL target pairs, and their avidity was simultaneously determined on the peptide-loaded BZLF1− LCL. Fig. 8 presents the results of one such experiment involving HLA-A*0201–restricted clones to the YVL (IE), GLC (E), and FLD (L) epitopes and involving HLA-B*2705–restricted clones to the ARYA (E) and RRRK (L) epitopes, all of which were tested on the same pair of HLA-A*0201–, HLA-B*2705–positive LCLs; because the BZLF1+ LCL member of the pair showed that 5% of cells were in the lytic cycle, the reconstructed target used had 5% BZLF1− LCL cells preloaded with the relevant epitope peptide. The HLA-A*0201–restricted clones reproduced the familiar pattern of results in which LCL recognition through the IE epitope was more efficient than through the E epitopes, and both were more efficient than through the L epitope. Yet, the L epitope (FLD)–specific effector clone was clearly the most avid in the peptide titration assay, with 50% optimal recognition at a peptide concentration (10^{-11} M) that was 50–100-fold lower than that for the IE and E epitope–specific clones (Fig. 8 A). Likewise with the HLA-B*2705–restricted clones, the ARYA (E) epitope–specific clone showed much better LCL recognition than the RRRK (L) epitope–specific clones even though the latter had a significantly higher avidity by peptide titration (Fig. 8 B).

DISCUSSION

There is still relatively little known about hierarchies of immunodominance among antigens expressed during a viral infection. This reflects both the logistic problems of analyzing responses across a large range of MHC alleles and the fact that viruses with large coding capacities, which are most likely to display such hierarchies, are the most difficult to analyze comprehensively. Here, we address the question in the context of EBV, a large human γ1-herpesvirus which has the capacity to encode ∼70 lytic cycle proteins expressed in three temporal stages: 2 IE followed by >30 E followed by >30 L proteins. For this purpose, we expanded the small target antigen panel used in earlier work (18) to include the two IE proteins, 11 representative E and 10 representative L proteins, selected so that the sum of unique E and L sequences being screened was roughly equivalent and that the L proteins included virus capsid, tegument and envelope components. The results show a clear focusing of CD8+ T cell responses toward epitopes drawn from the two IE and just four of the E proteins. The trend is consistent with all 11 donors analyzed and is based on the identification of 28 different lytic antigen-specific responses restricted through a total of three HLA-A, seven HLA-B, and four HLA-C alleles.

Our work exploited the fact that primary infection in IM is associated with the activation of a large lytic antigen–specific response (15, 16, 18, 19) that can be directly cloned in vitro (16, 18). Good correlations have been found between the range of clones generated in this way and the epitope-specific response as monitored by HLA class I peptide tetramer staining of IM T cells ex vivo (16). However, it is possible that our screening assays, based on representative rather than the full panel of E and L proteins, may have missed responses to immunodominant antigens not included in the screen. Nevertheless, the consistent pattern of results obtained from different patients with different HLA backgrounds, added to more limited data from an earlier study (18), suggests that the hierarchy of immunodominance observed is indeed real. The relationship between immunodominance and time of antigen expression in the lytic cycle is further emphasized by the fact that, of the four most commonly recognized antigens within the E protein group, BMLF1 and BMRF1 are proteins whose synthesis is directly induced by the BZLF1 and BRLF1 IE transactivators (24–27), whereas BALF5, and potentially also BALF2, synthesis is induced by BRLF1 acting in concert with cellular transcription factors (28). Therefore, the expression of such proteins would be among the first markers of progression into early phase.

We then asked to what extent these lytic antigen–specific responses are capable of recognizing lytically infected cells, which is an issue that could never be addressed by conventional cytotoxicity assays as there is no fully permis-

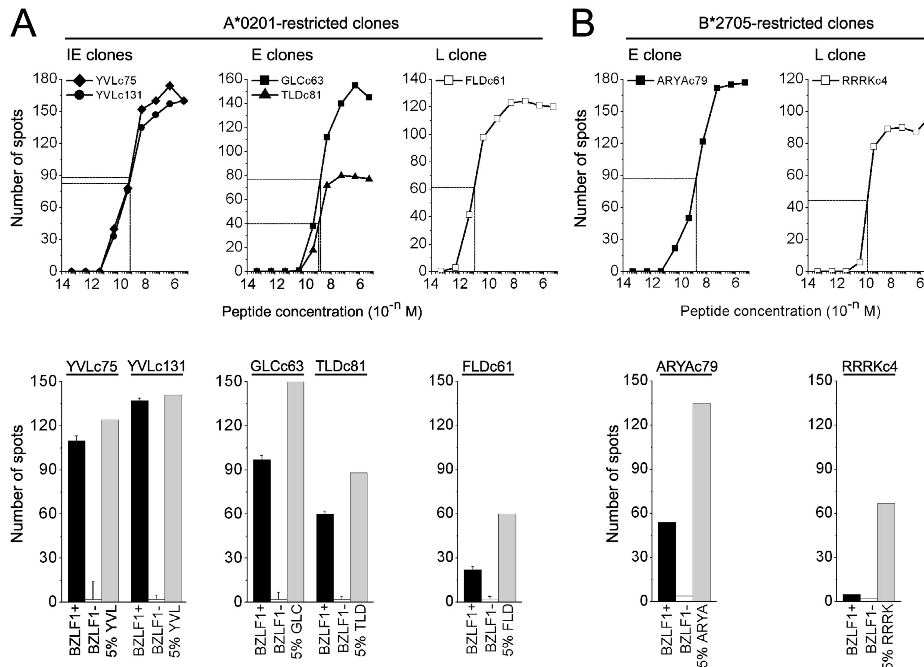

Figure 8. Comparative antigen presentation and functional avidity assays using representative CD8$^+$ T cell clones specific for IE, E, and L protein–derived epitopes. (A) HLA-A*0201–restricted epitopes, YVL from the IE protein BRLF1, GLC from the E protein BMLF1, TLD from the E protein BMRF1, and FLD from the L protein BALF4, and (B) HLA-B*2705–restricted epitopes, ARYA from the E protein BALF2 and RRRK from the L protein BILF2. In each case, the top half of the figure shows the results of peptide titration assays (performed on a peptide-loaded BZLF1− LCL) plotting number of spot-forming cells against peptide concentration; this allows functional avidity of the CD8$^+$ T cell clone to be determined as the peptide concentration mediating 50% optimal recognition. The bottom half of the figure in each case shows the results of antigen presentation assays on a pair of BZLF1+ and BZLF1− LCL target cells (where 5% of the BZLF1+ LCL cells were in lytic cycle) and on the BZLF1− LCL in which 5% cells had been loaded with epitope peptide. Note that these results are from a single experiment in which all 7 clones were tested simultaneously on the same BZLF1+ and BZLF1− LCL pair derived from a HLA-A*0201, B*2705-positive donor, and titrated on the same BZLF1− LCL loaded with cognate peptide. Data are expressed as in Fig. 5.

sive system supporting virus replication in vitro. The alternative approach, based on ELISPOT detection of IFNγ release, is well suited to situations where only a small fraction of the target cell population expresses cognate antigen. Using the paired BZLF1− LCLs as negative controls, our data reproducibly show recognition of BZLF1+ LCLs that is strong for IE effectors, slightly weaker for E effectors and poor for L effectors. This pattern of results cannot be explained by differences in the functional avidities of the clones; indeed, it was notable that all five L epitope–specific clones obtained had the highest avidities of all the lytic epitope-specific clones expanded from IM blood. Furthermore, these results must reflect direct recognition of lytically infected cells because experiments using appropriate mixtures of donor and recipient LCLs as targets failed to show any evidence of the intercellular transfer and cross-presentation of lytic cycle antigens in vitro. We believe that this is a significant negative result because essentially similar experiments have successfully detected intercellular transfer of EBV antigens into the HLA class II–processing pathway (unpublished data; Taylor, G.S., personal communication). Therefore, although LCLs can present exogenously acquired antigen to CD8$^+$ T cells if the antigen is supplied at sufficiently high concentrations (29–31), the process does not appear to make a significant contribution to lytic antigen presentation in LCL cultures.

The present data strongly suggest that the efficiency of antigen presentation in EBV-infected cells falls progressively during lytic cycle transit. This is interesting in view of the recent finding that HLA class I levels at the surface of LCL cells in lytic cycle are on average four- to fivefold lower than those on the coresident latently infected population (32). In that study, it was notable that a small fraction of BZLF1-stained cells retained good HLA class I expression, which is consistent with the view that antigen presentation is intact at the initiation of lytic cycle and begins to fall in the early phase. Importantly, even though BZLF1 and BRLF1 proteins are detectable by immunofluorescence staining throughout the IE, E, and most of the L phases in lytically infected cells (33), their mRNA transcripts are present only during the short IE phase (34) and so de novo synthesis of these antigens is limited to that period. Because defective products of de novo synthesis (rather than the products of mature protein turnover) are now thought to be the major source of antigens furnishing the MHC class I pathway (35, 36), this would explain why IE-derived epitopes appear to be well presented on cells throughout the lytic cycle. Likewise, those E proteins such as BMLF1 and BMRF1, whose

expression is directly activated by the IE proteins, are likely to be synthesized at a time when the HLA class I presentation pathway is still relatively intact. Thereafter, antigen presentation becomes less efficient, as the present experiments with L antigen–specific effectors show. It is not yet known whether this is a nonspecific consequence of lytic cycle transit or evidence of an active EBV immune evasion mechanism, which is analogous to those seen in some α-, β-, and γ2-herpesviruses (37–39). In opposition to the nonspecific cytopathic effect theory is the fact that cells remain viable even late in the EBV lytic cycle in vitro (40). Furthermore, HLA class I down-regulation on lytically infected LCL cells was not accompanied by a loss of other B cell surface molecules and was equally apparent on cells whose entry into the late phase was blocked by acyclovir (32), thus, raising the possibility that an early lytic cycle gene product is actively targeting the HLA class I antigen-processing pathway.

Our experiments revealed a striking correlation between the hierarchy of immunodominance among EBV lytic cycle antigens for $CD8^+$ T cell responses (Figs. 1–3) and the efficiency with which these antigens are processed and presented to $CD8^+$ T cells in lytically infected cells (Figs. 4–8). This focusing of the response on IE and a subset of E proteins expressed early in the lytic cycle, at a time when processing function remains relatively intact, is likely to maximize the effectiveness of $CD8^+$ T cell control recognizing and removing lytically infected cells before they progress to the point at which infectious virions are produced. It is interesting to contrast this situation with that seen for other human herpesviruses. The human $CD8^+$ T cell response to HSV, an α-herpesvirus predominantly replicating in skin and mucosal epithelium, is still quite poorly characterized; however, the evidence to date, at least for HSV type 2, suggests that virus structural proteins of the late lytic cycle constitute some of the major targets (9, 10). CMV, a β-herpesvirus with a wider cell tropism, is more closely studied and its immunodominant antigens include late structural proteins—in particular the pp65 tegument component—as well as some IE and E proteins (11–14). Given these differences, we infer that immunodominance hierarchies in antigenically complex viral systems may not follow a common pattern but may be unique to each agent, reflecting the particular biology of infection by that agent in vivo. Key determinants are likely to be the identity and location of the cells replicating virus during primary infection, the extent to which viral immune evasion mechanisms affect antigen presentation in these cells, and the relative importance of cross-priming versus direct priming (41, 42) in the induction of $CD8^+$ T cell responses.

In the specific context of EBV, it is still not clear whether the initial focus of virus replication in the oropharynx involves mucosal epithelium or B lymphocytes as the most important permissive cell type (43). Resolving this could have an impact on the question of priming pathways, because virus replication at localized epithelial sites would imply a necessity for cross-priming, whereas mucosa-associated B cell–replicating virus might be able to directly prime the response. The present results are interesting in this regard. Cross-priming, a process in which viral proteins shed from infected cells at the site of replication are acquired as exogenous antigen by local dendritic cells and then presented to the T cell repertoire (41, 42), might be expected to induce responses against a broad range of lytic cycle antigens, including the abundantly expressed late structural proteins of the virus. Superficially this seems at odds with the marked focusing of responses that is actually observed. However a broad range of responses might indeed be induced very early after EBV infection but, as the IE and E antigen–specific components restrict the movement of cells into late lytic cycle, the antigen supply would favor the selective expansion of those same components and produce the narrow spectrum of responses seen by the time the infection becomes clinically manifest as IM. Nevertheless, the concordance described here between antigen immunodominance and the efficiency of antigen presentation on lytically infected cells is particularly striking and this leaves open the possibility that the marked $CD8^+$ T cell response to this B-lymphotropic virus is indeed directly primed.

MATERIALS AND METHODS

Selection of EBV target proteins and generation of vaccinia recombinants. The following EBV lytic cycle proteins (number of amino acid residues identified in brackets) were selected for study: the 2 IE proteins, BZLF1 (245) and BRLF1 (605); 11 representative E proteins, BMLF1 (438), BMRF1 (404), BALF2 (1120), BALF5 (1015), BGLF4 (455), BHLF1 (660), BHRF1 (191), BARF1 (221), BNLF2a (60), BNLF2b (101), and BLLF2 (148); and 10 representative L proteins, BCRF1 (170), BILF1 (312), BNRF1 (1318), BVRF2 (605), BBRF1 (613), BLLF1 (907), BALF4 (857), BXLF2 (706), BDLF3 (234), BILF2 (248). The E and L proteins were chosen to be directly comparable in size range and to include a range of different functions (44). Thus, the E proteins included transcriptional transactivators (BMLF1 and BMRF1), known or presumed components of the viral DNA replication machinery (BALF2, BALF5, BGLF4, and BHLF1), a bcl2 homologue (BHRF1), a secreted protein (BARF1), and proteins of unknown function (BNLF2a, BNLF2b and BLLF2). The L proteins included virus capsid and tegument components (BNRF1, BVRF2, and BBRF1), known or presumed viral glycoproteins (BILF1, BILF2, BLLF1, BALF4, BXLF2, and BDLF3), and a secreted IL-10 homologue (BCRF1). The relevant EBV (B95.8 strain) genes were expressed from vaccinia virus vectors either as previously described (18, 45–48) or using the WR strain to express BARF1, BDLF3, and BXLF2, the RB12 strain (49) to express BNLF2a, BNLF2b, BILF1, BILF2, BLLF2, and BALF4, or the modified vaccinia Ankara (MVA) strain (50) to express BBRF1, BVRF2, and BNRF1. For RB12 and MVA recombinants the EBV genes were tagged at their 3′ end with sequences encoding an antibody-defined epitope from the influenza virus hemagglutinin protein and expression of the relevant protein confirmed by immunoblotting with the epitope-specific antibody.

IM patients. 30–60-ml blood samples were collected from serologically confirmed cases of acute IM under protocols approved by the South Birmingham Research Ethics Committee. Most PBMCs were cryopreserved until required, but some were used to generate an EBV-transformed B LCL by culturing cells in standard culture medium (RPMI-1640, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% [vol/vol] FCS) supplemented with 0.1 μg/ml cyclosporin A either without the addition of exogenous virus (spontaneous transformation by the patient's resident virus) or after exposure to B95.8 strain EBV. Patients' HLA types were identified by genotype assay of LCL-derived DNA.

T cell cloning, antigen mapping, and epitope identification. CD8$^+$ T cell clones were generated from the blood of IM patients by limiting dilution cloning of PBMCs at 0.3 and 3 cells/well in standard culture medium supplemented with IL-2 as described previously (18); pooled γ-irradiated allogeneic PBMCs (10^5 cells/well) were always included as feeders, usually with γ-irradiated cells (10^4 cells/well) of the autologous LCL. In some experiments, the autologous LCL was replaced (a) by γ-irradiated cells of a partially HLA-matched LCL transformed with an EBV strain incapable of lytic cycle entry (see next paragraph) and (b) by exposure to an anti-CD3 mAb OKT3 at 30 ng/ml. Clones with specific cytotoxicity against EBV antigens were identified in standard 5-h chromium release assays by testing on EBV-transformed B LCL targets (autologous and allogeneic HLA class I matched) preinfected with recombinant vaccinias expressing one of a series of individual EBV lytic cycle antigens as described previously (18). Where possible, the epitope specificity of the clones was identified first by testing on HLA-matched LCL cells preexposed to each of a panel of peptides (15-mer overlapping by 10, used at 5 μg/ml) representing the primary sequence of the relevant EBV-antigen (B95.8 strain), and then by testing shorter peptides within the 15-mer of interest at a range of peptide concentrations. When a library of overlapping peptides was not available, epitope prediction analysis was conducted on the relevant protein sequence using both the HLA Peptide Binding Predictions program from the Bioinformatics and Molecular Analysis Section (http://bimas.dcrt.nih.gov/molbio/hla_bind) (51), and the SYFPEITHI database (52). Predicted epitopes were synthesized and used to sensitize target cells in cytotoxicity assays as above. Peptides were purchased from Alta Biosciences and dissolved in DMSO (Fisher Chemicals).

Target cells for antigen presentation experiments. For these experiments, pairs of target LCLs were generated from individual HLA-typed laboratory donors by in vitro infection of peripheral blood B cells with recombinant strains of EBV generated by bacterial artificial chromosome cloning (53). One LCL in each pair (referred to as the BZLF1+ LCL) carried a wild-type B95.8 virus genome, the other (referred to as the BZLF1− LCL) carried a B95.8 genome that had been rendered incapable of lytic cycle entry by disruption of the BZLF1 gene (22). All LCLs were cultured in standard culture media, except in some experiments where late antigen expression in BZLF1+ LCLs was inhibited by growth for 7 d in 200 μM acyclovir. The lytic antigen-positive and lytic antigen-negative status of BZLF1+ and BZLF1− LCLs in each pair was confirmed both by immunoblotting using selected human sera with strong lytic antigen reactivities (33) and immunofluorescence staining with mAbs BZ1 to the IE antigen BZLF1, R3 to the E antigen BMRF1, and L2 to the L antigen BALF4 (32). Frequencies of antigen-positive cells were determined from counts on >2,000 cells per cell line. Frequencies of BZLF1- and BMRF1-expressing cells were very similar and only slightly higher than that of BALF4-expressing cells; typical percentages were between 2 and 5% for BZLF1 and BMRF1 staining, and between 1.5 and 4.5% for BALF4 staining. No staining was ever detected in BZLF1− LCLs.

ELISPOT assays for antigen presentation experiments. The lytic antigen-specific CD8+ T cell clones were generated either from the present panel of IM donors or from other IM patients as described previously (18); the only exceptions were the IE epitope-specific clones RBc 95 and RBc 18 that were generated by autologous LCL stimulation from a healthy EBV carrier and the L epitope–specific clone IM73c4 that was generated by peptide stimulation from a rare memory cell in post-IM PBMCs. The capacity of these CD8$^+$ T cell clones to recognize lytically infected cells within LCLs of the relevant HLA type was measured by IFNγ ELISPOT assays using cytokine capture and detection reagents according to the manufacturer's instructions (Mabtech). In brief, anti-IFN γ antibodies were coated on the wells of a 96-well nitrocellulose plate and replicate wells were seeded with 500 cloned CD8$^+$ T cells and 50,000 target cells of either the BZLF1+ LCL or, as a negative control, the paired BZLF1− LCL or, as a positive control, the BZLF1− LCL in which 1, 2, or 5% of the cells had been preincubated with an optimal concentration of the epitope peptide and washed well before adding back to the untreated BZLF1− LCL population. After incubation for 16 h, captured IFNγ was detected with a biotinylated anti-IFNγ antibody followed by development with streptavidin horseradish peroxidase complex and chromogenic substrate, and spots counted using an automated plate counter (AID). Additional ELISPOT assays were performed to determine whether, in BZLF1+ LCLs, antigens released from the subpopulation of cells in lytic cycle could be acquired by latently infected cells in the same culture and represented via the HLA class I pathway. In these experiments, BZLF1+ LCL cells lacking the relevant HLA restricting allele (donor cells) were mixed with an equal number of BZLF1− LCL cells with the relevant HLA allele (recipient cells) and the mixture cocultivated for 7 d, and then used as targets in ELISPOT assays either without further manipulation or preexposed to an optimal concentration of epitope peptide immediately before the assay. The donor and recipient LCLs alone served as negative control targets in such experiments, and a BZLF1+ LCL with the relevant HLA allele served as a positive control.

Assays of CD8$^+$ T cell functional avidity. CD8$^+$ T cell clones were assayed for functional avidity using the ELISPOT assay of IFNγ release to quantitate antigen recognition. Target cells were BZLF1− LCLs of the appropriate HLA type that had been preexposed to epitope peptide across a range of 10-fold dilutions from 10^{-5} to 10^{-13} M, washed well and used immediately as targets. Functional avidity was calculated from a graph of recognition versus peptide concentration as that concentration mediating 50% optimal recognition.

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REFERENCES

1. Doherty, P.C., and J.P. Christensen. 2000. Accessing complexity: the dynamics of virus-specific T cell responses. *Annu. Rev. Immunol.* 18: 561–592.
2. McMichael, A.J., and R.E. Phillips. 1997. Escape of human immunodeficiency virus from immune control. *Annu. Rev. Immunol.* 15:271–296.
3. Klenerman, P., V. Cerundolo, and P.R. Dunbar. 2002. Tracking T cells with tetramers: new tales from new tools. *Nat. Rev. Immunol.* 2:263–272.
4. Yewdell, J.W., and J.R. Bennink. 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu. Rev. Immunol.* 17:51–58.
5. Cresswell, P., N. Bangia, T. Dick, and G. Diedrich. 1999. The nature of the MHC class I peptide loading complex. *Immunol. Rev.* 172:21–28.
6. Reddehase, M.J. 2002. Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. *Nat. Rev. Immunol.* 2:831–844.
7. Stevenson, P.G., G.T. Belz, J.D. Altman, and P.C. Doherty. 1999. Changing patterns of dominance in the CD8+ T cell response during acute and persistent murine gamma-herpesvirus infection. *Eur. J. Immunol.* 29:1059–1067.
8. Woodland, D.L., E.J. Usherwood, L. Liu, E. Flano, I.J. Kim, and M.A. Blackman. 2001. Vaccination against murine γ-herpesvirus infection. *Viral Immunol.* 14:217–226.
9. Tigges, M.A., D. Koelle, K. Hartog, R.E. Sekulovich, L. Corey, and R.L. Burke. 1992. Human CD8+ herpes simplex virus-specific cytotoxic T-lymphocyte clones recognize diverse virion protein antigens. *J. Virol.* 66:1622–1634.
10. Koelle, D.M., Z. Liu, C.L. McClurkan, R.C. Cevallos, J. Vieira, N.A. Hosken, C.A. Meseda, D.C. Snow, A. Wald, and L. Corey. 2003. Immunodominance among herpes simplex virus-specific CD8 T cells expressing a tissue-specific homing receptor. *Proc. Natl. Acad. Sci. USA.* 100:12899–12904.
11. Riddell, S.R., M. Rabin, A.P. Geballe, W.J. Britt, and P.D. Greenberg. 1991. Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require en-

dogenous viral gene expression. *J. Immunol.* 146:2795–2804.
12. Wills, M.R., A.J. Carmichael, K. Mynard, X. Jin, M.P. Weekes, B. Plachter, and J.G. Sissons. 1996. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. *J. Virol.* 70:7569–7579.
13. Elkington, R., S. Walker, T. Crough, M. Menzies, J. Tellam, M. Bharadwaj, and R. Khanna. 2003. Ex vivo profiling of CD8+-T-cell responses to human cytomegalovirus reveals broad and multispecific reactivities in healthy virus carriers. *J. Virol.* 77:5226–5240.
14. Manley, T.J., L. Luy, T. Jones, M. Boeckh, H. Mutimer, and S.R. Riddell. 2004. Immune evasion proteins of human cytomegalovirus do not prevent a diverse CD8+ cytotoxic t cell response in natural infection. *Blood.* 104:1075–1082.
15. Callan, M.F., L. Tan, N. Annels, G.S. Ogg, J.D. Wilson, C.A. O'Callaghan, N. Steven, A.J. McMichael, and A.B. Rickinson. 1998. Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo. *J. Exp. Med.* 187:1395–1402.
16. Hislop, A.D., N.E. Annels, N.H. Gudgeon, A.M. Leese, and A.B. Rickinson. 2002. Epitope-specific evolution of human $CD8^+$ T cell responses from primary to persistent phases of Epstein-Barr virus infection. *J. Exp. Med.* 195:893–905.
17. Scotet, E., J. David-Ameline, M.A. Peyrat, A. Moreau-Aubry, D. Pinczon, A. Lim, J. Even, G. Semana, J.M. Berthelot, R. Breathnach, et al. 1996. T cell response to Epstein-Barr virus transactivators in chronic rheumatoid arthritis. *J. Exp. Med.* 184:1791–1800.
18. Steven, N.M., N.E. Annels, A. Kumar, A.M. Leese, M.G. Kurilla, and A.B. Rickinson. 1997. Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus-induced cytotoxic T cell response. *J. Exp. Med.* 185:1605–1617.
19. Catalina, M.D., J.L. Sullivan, K.R. Bak, and K. Luzuriaga. 2001. Differential evolution and stability of epitope-specific CD8(+) T cell responses in EBV infection. *J. Immunol.* 167:4450–4457.
20. Bharadwaj, M., S.R. Burrows, J.M. Burrows, D.J. Moss, M. Catalina, and R. Khanna. 2001. Longitudinal dynamics of antigen-specific CD8+ cytotoxic T lymphocytes following primary Epstein-Barr virus infection. *Blood.* 98:2588–2589.
21. Saulquin, X., M. Bodinier, M.A. Peyrat, A. Hislop, E. Scotet, F. Lang, M. Bonneville, and E. Houssaint. 2001. Frequent recognition of BCRF1, a late lytic cycle protein of Epstein-Barr virus, in the HLA-B*2705 context: evidence for a TAP-independent processing. *Eur. J. Immunol.* 31:708–715.
22. Feederle, R., M. Kost, M. Baumann, A. Janz, E. Drouet, W. Hammerschmidt, and H.J. Delecluse. 2000. The Epstein-Barr virus lytic program is controlled by the co-operative functions of two transactivators. *EMBO J.* 19:3080–3089.
23. Neuhierl, B., R. Feederle, W. Hammerschmidt, and H.J. Delecuse. 2002. Glycoprotein gp110 of Epstein-Barr virus determines viral tropism and efficiency of infection. *Proc. Natl. Acad. Sci. USA.* 99:15036–15041.
24. Rooney, C.M., D.T. Rowe, T. Ragot, and P.J. Farrell. 1989. The spliced BZLF1 gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. *J. Virol.* 63: 3109–3116.
25. Kenney, S., E. Holley-Guthrie, E.C. Mar, and M. Smith. 1989. The Epstein-Barr virus BMLF1 promoter contains an enhancer element that is responsive to the BZLF1 and BRLF1 transactivators. *J. Virol.* 63: 3878–3883.
26. Holley-Guthrie, E.A., E.B. Quinlivan, E.C. Mar, and S. Kenney. 1990. The Epstein-Barr virus (EBV) BMRF1 promoter for early antigen (EA-D) is regulated by the EBV transactivators, BRLF1 and BZLF1, in a cell-specific manner. *J. Virol.* 64:3753–3759.
27. Ragoczy, T., and G. Miller. 1999. Role of the epstein-barr virus RTA protein in activation of distinct classes of viral lytic cycle genes. *J. Virol.* 73:9858–9866.
28. Liu, C., N.D. Sista, and J.S. Pagano. 1996. Activation of the Epstein-Barr virus DNA polymerase promoter by the BRLF1 immediate-early protein is mediated through USF and E2F. *J. Virol.* 70:2545–2555.
29. Liu, T., X. Zhou, C. Orvell, E. Lederer, H.G. Ljunggren, and M. Jondal. 1995. Heat-inactivated Sendai virus can enter multiple MHC class I processing pathways and generate cytotoxic T lymphocyte responses in vivo. *J. Immunol.* 154:3147–3155.
30. Schirmbeck, R., K. Melber, and J. Reimann. 1995. Hepatitis B virus small surface antigen particles are processed in a novel endosomal pathway for major histocompatibility complex class I-restricted epitope presentation. *Eur. J. Immunol.* 25:1063–1070.
31. Blake, N., S. Lee, I. Redchenko, W. Thomas, N. Steven, A. Leese, P. Steigerwald-Mullen, M.G. Kurilla, L. Frappier, and A. Rickinson. 1997. Human CD8+ T cell responses to EBV EBNA1: HLA class I presentation of the (Gly-Ala)-containing protein requires exogenous processing. *Immunity.* 7:791–802.
32. Keating, S., S. Prince, M. Jones, and M. Rowe. 2002. The lytic cycle of Epstein-Barr virus is associated with decreased expression of cell surface major histocompatibility complex class I and class II molecules. *J. Virol.* 76:8179–8188.
33. Rowe, M., A.L. Lear, D. Croom-Carter, A.H. Davies, and A.B. Rickinson. 1992. Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. *J. Virol.* 66:122–131.
34. Takada, K., and Y. Ono. 1989. Synchronous and sequential activation of latently infected Epstein-Barr virus genomes. *J. Virol.* 63:445–449.
35. Schubert, U., L.C. Anton, J. Gibbs, C.C. Norbury, J.W. Yewdell, and J.R. Bennink. 2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature.* 404:770–774.
36. Reits, E.A., J.C. Vos, M. Gromme, and J. Neefjes. 2000. The major substrates for TAP in vivo are derived from newly synthesized proteins. *Nature.* 404:774–778.
37. Fruh, K., A. Gruhler, R.M. Krishna, and G.J. Schoenhals. 1999. A comparison of viral immune escape strategies targeting the MHC class I assembly pathway. *Immunol. Rev.* 168:157–166.
38. Hengel, H., U. Reusch, A. Gutermann, H. Ziegler, S. Jonjic, P. Lucin, and U.H. Koszinowski. 1999. Cytomegaloviral control of MHC class I function in the mouse. *Immunol. Rev.* 168:167–176.
39. Stevenson, P.G. 2004. Immune evasion by gamma-herpesviruses. *Curr. Opin. Immunol.* 16:456–462.
40. Inman, G.J., U.K. Binne, G.A. Parker, P.J. Farrell, and M.J. Allday. 2001. Activators of the Epstein-Barr virus lytic program concomitantly induce apoptosis, but lytic gene expression protects from cell death. *J. Virol.* 75:2400–2410.
41. Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature.* 392:245–252.
42. Norbury, C.C., and L.J. Sigal. 2003. Cross priming or direct priming: is that really the question? *Curr. Opin. Immunol.* 15:82–88.
43. Rickinson, A.B., and E. Kieff. 2001. Epstein-Barr virus. *In* Fields Virology. D. M. Knipe and P. M. Howley, editors. Lippincott Williams and Wilkins, Philadelphia, 2575-2627.
44. Kieff, E., and A.B. Rickinson. 2001. Epstein-Barr virus and its replication. *In* Fields Virology. D. M. Knipe and P. M. Howley, editors. Lippincott Williams & Wilkins, Philadelphia, 2511–2573.
45. Lear, A.L., M. Rowe, M.G. Kurilla, S. Lee, S. Henderson, E. Kieff, and A.B. Rickinson. 1992. The Epstein-Barr virus (EBV) nuclear antigen 1 BamHI F promoter is activated on entry of EBV-transformed B cells into the lytic cycle. *J. Virol.* 66:7461–7468.
46. Kurilla, M.G., S. Swaminathan, R.M. Welsh, E. Kieff, and R.R. Brutkiewicz. 1993. Effects of virally expressed interleukin-10 on vaccinia virus infection in mice. *J. Virol.* 67:7623–7628.
47. Lee, S.P., L.E. Wallace, M. Mackett, J.R. Arrand, P.F. Searle, M. Rowe, and A.B. Rickinson. 1993. MHC class II-restricted presentation of endogenously synthesized antigen: Epstein-Barr virus transformed B cell lines can present the viral glycoprotein gp340 by two distinct pathways. *Int. Immunol.* 5:451–460.
48. Kurilla, M.G., T. Heineman, L.C. Davenport, E. Kieff, and L.M. Hutt-Fletcher. 1995. A novel Epstein-Barr virus glycoprotein gp150 expressed from the BDLF3 open reading frame. *Virology.* 209:108–121.
49. Blasco, R., and B. Moss. 1995. Selection of recombinant vaccinia viruses on the basis of plaque formation. *Gene.* 158:157–162.
50. Chakrabarti, S., K. Brechling, and B. Moss. 1985. Vaccinia virus expression vector: coexpression of beta-galactosidase provides visual

screening of recombinant virus plaques. *Mol. Cell. Biol.* 5:3403–3409.

51. Parker, K.C., M.A. Bednarek, and J.E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J. Immunol.* 152:163–175.
52. Rammensee, H.G., J. Bachmann, N.N. Emmerich, O.A. Bachor, and S. Stevanovic. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics.* 50:213–219.
53. Delecluse, H.J., T. Hilsendegen, D. Pich, R. Zeidler, and W. Hammerschmidt. 1998. Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. *Proc. Natl. Acad. Sci. USA.* 95:8245–8250.
54. Bogedain, C., H. Wolf, S. Modrow, G. Stuber, and W. Jilg. 1995. Specific cytotoxic T lymphocytes recognize the immediate-early transactivator Zta of Epstein-Barr virus. *J. Virol.* 69:4872–4879.
55. Saulquin, X., C. Ibisch, M.A. Peyrat, E. Scotet, M. Hourmant, H. Vie, M. Bonneville, and E. Houssaint. 2000. A global appraisal of immunodominant CD8 T cell responses to Epstein-Barr virus and cytomegalovirus by bulk screening. *Eur. J. Immunol.* 30:2531–2539.
56. Pepperl, S., G. Benninger-Doring, S. Modrow, H. Wolf, and W. Jilg. 1998. Immediate-early transactivator Rta of Epstein-Barr virus (EBV) shows multiple epitopes recognized by EBV-specific cytotoxic T lymphocytes. *J. Virol.* 72:8644–8649.