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IDENTIFICATION AND PREVALENCE OF CD8$^+$ T-CELL RESPONSES DIRECTED AGAINST EPSTEIN-BARR VIRUS-ENCODED LATENT MEMBRANE PROTEIN 1 AND LATENT MEMBRANE PROTEIN 2

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Epstein-Barr virus (EBV) is associated with several human malignancies that each show different viral gene expression profiles. In malignancies such as Hodgkin's disease and nasopharyngeal carcinoma only Epstein-Barr nuclear antigen 1 (EBNA1) and varying levels of latent membrane proteins 1 and 2 (LMP1 and -2) are expressed. Since endogenously expressed EBNA1 is protected from CTL recognition, LMP1 and LMP2 are the most likely target antigens for anti-tumor immunotherapy. Therefore, we sought to identify in a systematic way CD8$^+$ T-cell responses directed against eptitopes derived from LMP1 and LMP2. Using IFNγ-ELISPOT assays of interferon-γ release, peripheral blood mononuclear cells (PBMC) of healthy donors were screened with peptide panels (15 mer overlapping by 10) spanning the LMP1 and LMP2 sequences of the prototype EBV strain B95.8. When positive responses were found, CD4$^+$ or CD8$^+$ T cells were depleted from donor PBMC to determine the origin of the responder population. We detected CD8$^+$ T-cell responses to LMP1 in 9/50(18%) donors and to LMP2 in 15/28 (54%) donors. In addition to the already described epitopes, 3 new LMP1- and 5 new LMP2-derived CD8$^+$ epitopes were identified. In most donors LMP1- and LMP2-specific CD8$^+$ precursor frequencies were low compared with precursors against immunodominant EBV epitopes from latent (EBNA3A, -3B and -3C) and lytic cycle antigens. These results demonstrate that CD8$^+$ memory T cell responses to LMP1 and especially to LMP2 do exist in Caucasians, albeit at low levels and could potentially be exploited for therapeutic use.

Key words: *CTL; LMP1; LMP2; Epstein-Barr virus*

Epstein-Barr virus (EBV) can induce fatal B lymphoproliferative lesions in immunocompromised patients and is etiologically linked to several malignancies arising in the immunocompetent host. Examples of the latter include all cases of endemic Burkitt's lymphoma (BL), all undifferentiated nasopharyngeal carcinomas (NPC), 10% of gastric carcinomas, certain T-/NK-cell lymphomas and 40–90% of Hodgkin's Disease (HD) cases.[1] EBV is transcriptionally active in the tumor cells but expresses only a restricted set of EBV-encoded proteins; expression patterns range from the Epstein-Barr nuclear antigen 1 (EBNA1) only in BL through to EBNA1 plus high levels of both latent membrane proteins 1 and 2 (LMP1, -2) in HD.[2–4] EBV-associated carcinomas are intermediate in that EBNA1 and LMP2 appear to be expressed, with or without LMP1.[5] Interestingly many EBV-positive carcinomas also show detectable transcription of a gene, BARF1, which is otherwise thought to be associated only with the virus lytic cycle.[6,7] Given the presence of at least some viral antigens in tumor cells, EBV-positive malignancies are potential candidates for immunotherapy. Because endogenously expressed EBNA1 is protected from proteasomal processing and therefore escapes cytotoxic T lymphocyte (CTL) recognition,[8] the prime targets for therapeutic responses in the context of HD and NPC are LMP1 and LMP2. Both are multiple membrane-spanning proteins with cytoplasmic N-terminal and C-terminal domains separated by 6 or 12 transmembrane runs, respectively.[9,10]

The LMP1- and LMP2-derived CD8$^+$ T cell epitopes described to date have been identified by *in vitro* stimulation of peripheral mononuclear blood monocytes (PBMC) either i) with the autologous lymphoblastoid cell line (LCL) followed by isolation of rare LMP-specific clones from the polyclonal effector population, or ii) with antigen presenting cells preloaded with LMP peptides selected by computer prediction for potential high affinity binding to particular HLA class I alleles.[11,12] The 2 known LMP1 epitopes were identified by the latter approach; both are HLA-A2 restricted and located in the membrane-associated domain of LMP1.[11] Such LMP1 epitope-specific CTL are potential applicable in many patients because the A2 family of alleles is common in human populations and these epitopes appear to be presented in the context of many different HLA-A2 subtypes. However, in limiting dilution assays *in vitro* the relevant CTL precursor frequencies in healthy HLA-A2 positive donors appeared to be low. Other *in vitro* studies have also detected the presence at low levels of anti-LMP1 CTL responses, without addressing the epitopes involved.[13–15] By these criteria, therefore, LMP1 appears to be only weakly immunogenic to CD8$^+$ T cells. LMP2 is also considered, at least from the small numbers of LMP2-specific clones that have been detected in LCL-stimulated effector populations to be a subdominant antigen for CD8$^+$ T cell responses. However, several LMP2 epitopes have been identified, restricted through HLA alleles such as HLA-A2.1, A11, A23, A24 and B60, all located in the membrane-associated domain of the molecule [reviewed in 12,14,16]. Interestingly all of these epitopes are located in the membrane-associated domain of the molecule and some are even presented via the HLA class I pathway in TAP-negative cell lines, indicating TAP-independent processing of the LMP2 protein.[17–19]

It is now clear that the conventional method of probing EBV latent antigen-specific CTL memory by autologous LCL stimulation *in vitro* does not give a complete picture of all reactivities; certain reactivities, drawn from the immunodominant EBNA3A, -3B and -3C family of latent proteins, tend to dominate the polyclonal T cell lines and derived T cell clones. In order to overcome the selection inherent in assays that require T cell expansion *in vitro*, here we analyzed CD8$^+$ T-cell responses to LMP1 and LMP2 in a different way by screening T-cell reactivity with peptide pools spanning the LMP1 and LMP2 B95.8 strain protein sequences and using immediate IFNγ release as the read out.[20] Such an approach has the potential to reveal even minor reactivities and has been used to confirm the specificity of responses to previously identified EBV epitopes in a variety of experimental settings.[20,21] Furthermore, screening in the IFNγ ELISPOT assay using peptide pools spanning the EBNA1 protein

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sequence has revealed unexpectedly strong $CD8^+$ T cell responses to epitopes from EBNA1,[22] which had not been identified by LCL stimulation. We therefore have used the same approach to conduct a systematic survey of $CD8^+$ T cell memory to LMP1 and LMP2.

MATERIAL AND METHODS

Donors

Whole blood was obtained form healthy laboratory donors with known HLA haplotypes ($n=11$) and buffy coats from anonymous blood bank donors ($n=53$) from the Birmingham Blood Transfusion Service. PBMC were isolated by Lymphoprep (Nycomed Pharma, Oslo, Norway) density grade centrifugation and were either used fresh or cryo-preserved until required. The EBV status of the donors was determined by serologic methods as previously described.[23]

Synthetic peptides

Panels of 15 mer peptides (overlapping by 10 mer) were synthesized covering the entire amino acid sequences of LMP1 and LMP2 from the Caucasian prototype 1 EBV strain B95.8. Peptides were synthesized by standard fluorenyl-methoxycarbonyl chemistry (Alta Bioscience, University of Birmingham, UK) and dissolved in DMSO. Their concentration was determined by biuret assay. Some peptides were synthesized by Pepscan Systems (Lelystad, The Netherlands).

ELISPOT assay for detection of IFNγ release

Ninety-six well polyvinylidene difluoride-backed plates (Millipore, Bedford, MA) were coated with 15 μg/ml of anti-IFNγ monoclonal antibody (MAb) 1-DIK (MABTECH, Stockholm, Sweden). PBMC (whole or CD4- or CD8-depleted) were added to duplicate wells at 5.10^5 cells in the presence of single or pooled 15 mer peptides or 8 mer peptides at a final concentration of 2 μM for each peptide. The plates were incubated overnight at 37°C in 5% CO_2. The cells were discarded the following day and a biotinylated anti-IFNγ MAb, 7-B6-1 (MABTECH), was added at 1 μg/ml and left for 2–4 hr at room temperature, followed by streptavidin-conjugated alkaline phosphatase (MABTECH) for an additional 2 hr. Individual cytokine producing cells were detected as dark spots after a 30 min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium using an alkaline phosphatase-conjugated substrate kit (Bio-Rad, Richmond, CA). The spots were counted using a dissection microscope. In all experiments, results from IFNγ-ELISPOT assays are expressed as spot forming cells (SFC) per 10^5 PBMC. Phytohaemagglutinin (PHA) was added to some wells at a final concentration of 10 μg/ml to provide a positive control; background levels of response were determined for wells given culture medium without peptide.

Depletion of $CD4^+$ and $CD8^+$ cells

Depletion of $CD4^+$ cells or $CD8^+$ cells prior to IFNγ-ELISPOT was carried out using DYNABEADS M450-CD4 and M450-CD8 (DYNAL, Oslo, Norway). Efficient depletion was confirmed by staining the depleted PBMC using a dual staining with FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 antibody (Serotec, UK) and flow-cytometry on a Coulter EPICS XL cytometer.

Analysis of HLA-binding

Since the HLA-haplotype of several donors with responses against the different LMP1 and LMP2 epitopes was unknown and could, even in retrospect, not be established, we have used the prediction of peptide binding to HLA motifs, described by Parker *et al.*,[24] available at http://bimas.dcrt.nih.gov/molbio/hla_bind, to determine possible restriction elements.

RESULTS

A large number of healthy EBV carriers (total $n=64$; $n=50$ for LMP1 and $n=28$ for LMP2) were assayed for responsiveness to the peptide panels covering the complete B95.8 strain sequences of LMP1 and LMP2. To minimize the initial screening assays, the peptides (15 mers overlapping by 10) were screened in pools of 3, each pool containing 3 adjacent peptides. When responses were observed in these initial screens, the assays were repeated of individually testing each of the 3 peptides within any positive pool. In all assays PHA-stimulated PBMC provided a positive control of IFNγ release, giving reproducible values of >300 SFC/10^5 PBMC and cells incubated without peptide provided a negative control.

$CD8^+$ T-cell responses to LMP1 in healthy donors

In the ELISPOT assays using LMP1 peptides 5/50 donors tested (10%) had a detectable $CD8^+$ T-cell response to 15 mer peptides from pool 7 and/or pool 9 and subsequently to the individual 15 mers 7.1 and/or 9.1 and 9.2, respectively. We noted that these 15 mers contained the previously described HLA-A2 supertype restricted epitopes YLLEMLWRL (YLL) and YLQQNWWTL (YLQ).[11] When the above 5 donors were subsequently tested with the relevant (9 mer) peptide sequences, the responses seen in the original screening assay were confirmed as being specific for the YLL and YLQ epitopes, as shown in Table I (upper panel). Indeed, the only donor of these 5 with known HLA-haplotype was A2. Since ~45% of the Caucasian population is HLA-A2 positive, this strongly suggests that $CD8^+$ T-cells specific for these 2 epitopes are only present at detectable levels in a minority of HLA-2-positive donors. The magnitude of these responses was within the range of 4–34 SFC/10^5 PBMC.

In addition, screening with the LMP1 peptide panel revealed novel responses in another 5/50 donors, leading to the identification of 3 new $CD8^+$ epitopes in the protein. One such result, for donor D22, is shown in Figure 1*a*. This donor exhibited significant reactivity above background to LMP1 peptide pool 27. The response was consistently observed on re-screening and mapped to a single 15 mer within the pool, peptide 27.2, DDDDPHGPVQLSYYD (aa 372–386), a sequence from the extreme C-terminus of LMP1. As illustrated in Figure 1*b*,*c*, depletion of the $CD4^+$ or $CD8^+$ T-cells from donor D22 PBMCs prior to the assay showed that the response could still be detected in the CD4-depleted but not in the CD8-depleted population, indicating that the response was CD8-mediated. The same results were observed for the other

TABLE I – CD8 RESPONSES TO LMP1 DETECTED BY IFNΓ-ELISPOT AND A 15 MER (10 MER OVERLAPPING) PEPTIDE LIBRARY (SFC/10^5 CELLS)[1]

Epitope	aa number	HLA allele[reference]	Donor								
			D22	D23	D28	D29	D37[2]	D53	D55	D56	D62
YLLEMLWRL	125–133	A2[11]					34	29	12	7	
YLQQNWWTL	159–167	A2[11]			20		33	34	7	4	
FWLYIVMSD	38–46	?[23]									30
FRRDLLCPLGA	72–82	B40[24] B61[24] B27.2[24]			8						
DPHGPVQLSYYD	375–386	B51.1[24]	12	12		13					

[1]Of the 50 donors screened, only the donors with a positive response are shown. In all experiments the positive control PHA yielded > 300 SFC/10^5.–[2]Donor with a known HLA-haplotype (see text).

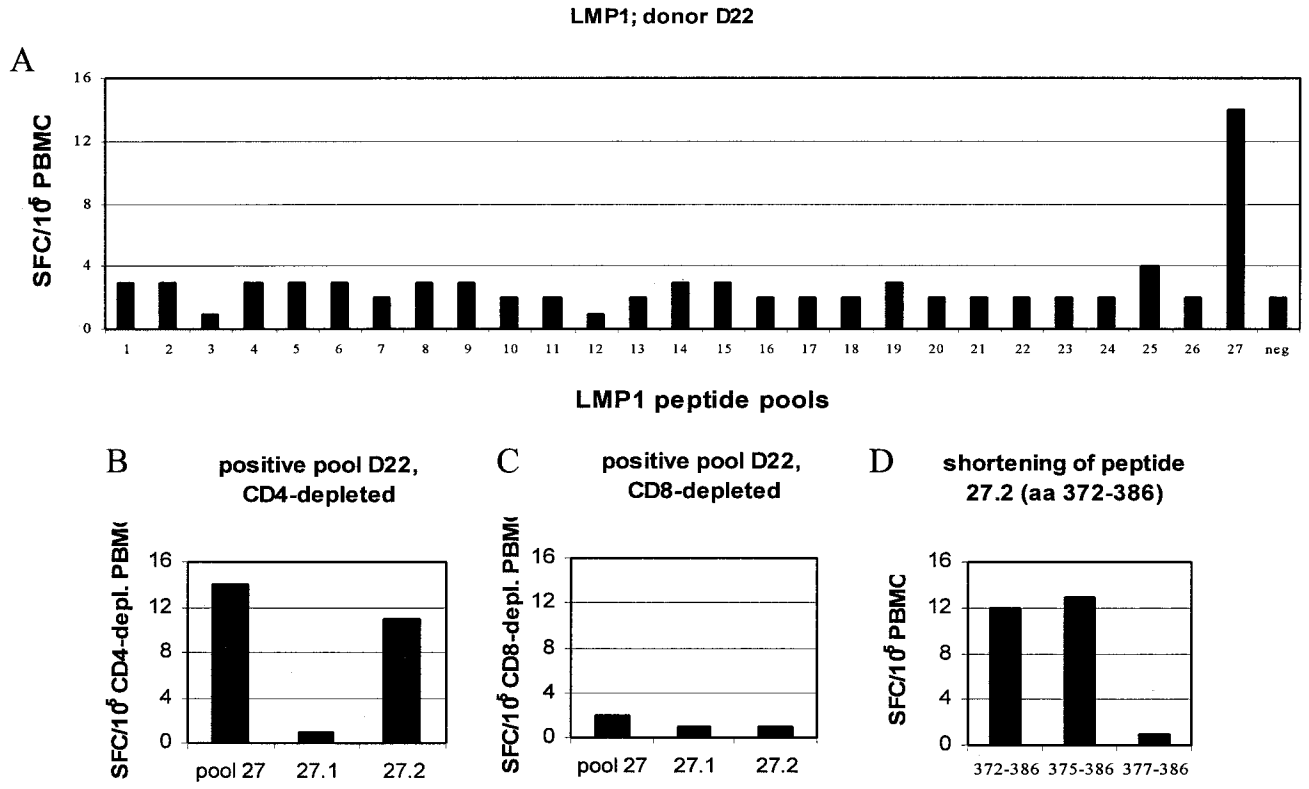

FIGURE 1 – Identification of CD8+ T-cell epitopes within LMP1 using IFNγ-ELISPOT and a 15 mer (10 mer overlapping) peptide library. PBMC were used at 5×10^5 cells per well and results are expressed as spot forming cells (SFC) per 10^5 cells. (*a*) PBMC of donor D22 are screened against peptides tested in pools of 3, (*b*) CD4-depleted PBMC of donor D22 are screened against the separate peptides of pool 27, (*c*) CD8-depleted PBMC of donor D22 are screened against the separate peptides of pool 27 and (*d*) PBMC of donor D29 are screened against peptide 372–386 and shortened sequences, being 375–386 and 377–386.

donor (D29) tested (data not shown). We detected responses to the LMP1 C-terminal epitope in 3 different donors, in each case at a relatively low magnitude of 12–13 SFC/10^5 PBMC. We attempted to identify the minimal epitope using shortened sequences within the 372–386 region. As shown in Figure 1*d*, now using PBMCs from another responsive donor D29, the 12-mer peptide LMP1 375–386 was recognized as well as the original 15 mer, whereas the 10 mer 377–386 was not. Although the HLA-haplotypes of these 3 donors were unknown, analysis of binding of the peptide suggested that B51.1 could be the restriction element.[24] This was the only HLA motif available in the data base in which the 15-mer and 12-mer peptide bound, whereas the 10-mer did not (Table I).

Table I also identifies responses, which mapped to other regions of the LMP1 molecule; each was observed in a single donor only. Donor D62 recognized 2 overlapping peptides LLLLALLFWLYIVMS (aa 31–45) and WLYIVMSDWTGGALL (aa 39–53) and donor D28 recognized peptide IIFIFRRDLLCPLGA (aa 68–82). These responses could be minimized to a 9 mer epitope, FWLYIVMSD (aa 38–46) and to an 11 mer epitope, FRRDLLCPLGA (aa 72–82), respectively, shown in Table II*a*,*b*. Again minimization yielded almost identical numbers of IFNγ-producing cells. The magnitude of these responses (8–30 SFC/10^5 PBMC) was within the same range as found for the YLL and YLQ epitopes. Prediction of binding to HLA motifs revealed no HLA-haplotype which bound the 9-mer peptide FWLYIVMSD. Possible restriction elements for FRRDLLCPLGA were B40, B60 and B27.[24]

CD8+ T-cell responses to LMP2 in healthy donors

A panel of 28 healthy positive donors was screened in a similar way using overlapping 15 mer peptides from the LMP2 sequence.

TABLE II – MINIMIZATION OF LMP1- AND LMP2-SPECIFIC CD8+ T-CELL EPITOPES DETECTED USING IFNγ-ELISPOT AND A 15 MER PEPTIDE LIBRARY

LMP1 peptide			SFC/10^5 cells
Number mer	Sequence	aa	
A: Minimization of LMP1 epitope aa 31–53, donor D62			
15 mer	LLLLALLFWLYIVMS	31–45	7
15 mer	WLYIVMSDWTGGALL	39–53	10
9 mer	FWLYIVMSD	38–46	10
B: Minimization of LMP1 epitope aa 68–82, donor D28			
15 mer	IIFIFRRDLLCPLGA	68–82	5
10 mer	IIFIFRRDLL	68–77	0
10 mer	FIFRRDLLCP	70–79	0
11 mer	FRRDLLCPLGA	72–82	9
C: Minimization of LMP2 epitope 289–306, donor D33			
14-mer	SSPGGLGTLGAALL	289–302	8
14-mer	GLGTLGAALLTLAA	293–306	5
9-mer	GLGTLGAAL	293–301	9

There was a high proportion (12/28) of responders to peptide pools 30 and 36. These responses were subsequently mapped to the individual 15 mer 30.2 and to the overlapping peptides 36.1 and 36.2, sequences that contained the previously identified HLA-A2.1-restricted LMP2 epitopes, FLYALALLL (FLY) and CLGGLLTMV (CLG), respectively[25] (Lautscham et al., article in preparation). These donors were then tested on the relevant 9 mer peptides and the responses were confirmed as being specific for the

FLY and CLG epitopes. As can be seen from the summary of data from positive responders shown in Table III, CLG responses were found in 10/28 (36%) healthy donors and FLY responses in 8/28 (29%) donors, with 6 individuals recognizing both epitopes. The magnitude of the responses was within the range of 4–76 SFC/105 PBMC. Surprisingly only 1 of the FLY and/or CLG responders exhibited any reactivity to a previously identified third A2.1 epitope from LMP2, namely, LLWTLVVLL. From 5 of the donors, responsive to the known A2-restricted epitopes of LMP2, the HLA-haplotype was known and was indeed A2. In addition to these responses to known A2.1 epitopes, responses were also detected to 4 other previously identified epitopes from LMP2, namely, SSCSSCPLSKI (A11-restricted), PYLFWLAAI (A23-restricted), TYGPVFMCL (A24-restricted) and IEDPPFNSL (B60-restricted). In each case, only a single donor in the panel exhibited these responses; this is not unexpected given the relatively low frequency of these alleles in Caucasian populations. From these donors, only the haplotype of D52 was known and was indeed A11.

Screening with the 15 mer peptides also revealed new LMP2 epitopes. As an example, Figure 2*a* shows the initial screening data from donor D50. In addition to the FLY and CLG reactivities in pools 30 and 36, respectively, significant reactivity was found to pool 11 and to pool 25. The response in pool 25 was mapped to 2 overlapping 15 mer peptides 25.1 (SSPGGLGTLGAALL, aa 289–302) and 25.2 (GLGTLGAALLTLAA, aa 293–306). As with the FLY and CLG reactivities, these new responses were clearly CD8-mediated since they were detectable in the CD4-depleted but not in the CD8-depleted PBMCs from donor D50 (Fig. 2*b*,*c*). We were particularly interested in the response to the 15 mer peptides 25.1 and 25.2, since this was seen in 5/28 individuals, 3 of whom were known HLA-A2 positive while the other 2 were probably also A2, since they both responded to the FLY and CLG epitopes (Table III). This raised the possibility that an A2.1-restricted epitope lay within the 10 mer overlap sequence GLGTLGAALL shared by peptides 25.1 and 25.2 and we noted that the 9 mer GLGTLGAAL (aa 293–301) conformed well to an A2.1 binding motif.[24] As exemplified in Table II*c* with donor D33, this 9 mer peptide (GLG) was indeed well recognized by all donors identified as responsive to pool 25 in the original LMP2 peptide screening assays. In addition to the GLG-specific response, peptide screening identified responses to 4 further novel LMP2 epitopes, each recognized by a single donor only (Table III). As is shown in Figure 2*b*, the response of donor D50 in pool 11 could be mapped to peptide 11.1, sequence NPVCLPVIVAPYLF (aa 121–134). Although D50 recognized 3 other A2-restricted LMP2 epitopes, analysis of HLA-binding of peptides[24] revealed that A2 was highly unlikely as the restriction element of NPVCLPVIVAPYLF, since this peptide did not contain a known A2 binding motif.

Overall the magnitude of the 5 newly identified LMP2 epitopes lay between 5 and 23 SFC/10^5 PBMC, about 2-fold lower than responses to previously described LMP2 epitopes. Combining all the positive results, LMP2-specific $CD8^+$ T-cell responses could be detected in 15/28 (54%) healthy donors and the magnitude of these responses was in the range of 4–76 SFC per 10^5 PBMC.

TABLE III–CD8 RESPONSES TO LMP2 DETECTED BY IFNγ-ELISPOT AND A 15 MER (10 MER OVERLAPPING) PEPTIDE LIBRARY (SFC/10^5 CELLS)[1]

Epitope	aa number	HLA allele[reference]	Donor												
			D33[2]	D37[2]	D41	D42	D44[2]	D45	D46	D48	D50	D51[2]	D52[2]	D53	D55	D56	D58
LLWTLVVLL	329–337	A2[12]		76													
FLYALALLL	356–364	A2[12]	18						39		10	20	6		9	18	8
CLGGLLTMV	426–434	A2[12]	47	9			16	9	40		8	16		35		19	8
SSCSSCPLSKI	340–350	A11[12]											4				
PYLFWLAAI	131–139	A23[12]													32		
TYGPVFMCL	419–427	A24[12]				7											
IEDPPFNSL	200–208	B60[12]												17			
EDPYWGNGDRHSDYQ	61–75									23							
NPVCLPVIVAPYLF	121–134										10						
ASCFTASVSTVVTA	141–154				7												
MFLACVLVLIVDAV	249–262									7							
GLGTLGAAL	293–301	A2[24]	5				9		10		9	21					

[1]Of the 28 donors screened, only the donors with a positive response are shown. In all experiments the positive control yielded > 300 SFC/10^5 PBMC.–[2]Donors with known HLA-haplotype (see text).

DISCUSSION

In contrast to the fatal EBV-positive lymphoproliferative diseases that arise in immunocompromised individuals and are sensitive to a restitution of T cell surveillance,[26] other EBV-associated tumors such as HD and NPC develop in patients whose EBV-specific immunity does not appear to be grossly impaired.[27,28] However, these tumors show patterns of viral antigen expression, which appear to be limited to EBNA1, LMP1 and LMP2. Since endogenously expressed EBNA1 is protected from presentation via the HLA class I pathway, attempts to target these tumors with EBV-specific CTLs depend upon the ability to boost responses to LMP1 and LMP2. Fundamental to this strategy is an overall understanding of the range of $CD8^+$ T cell epitopes present within

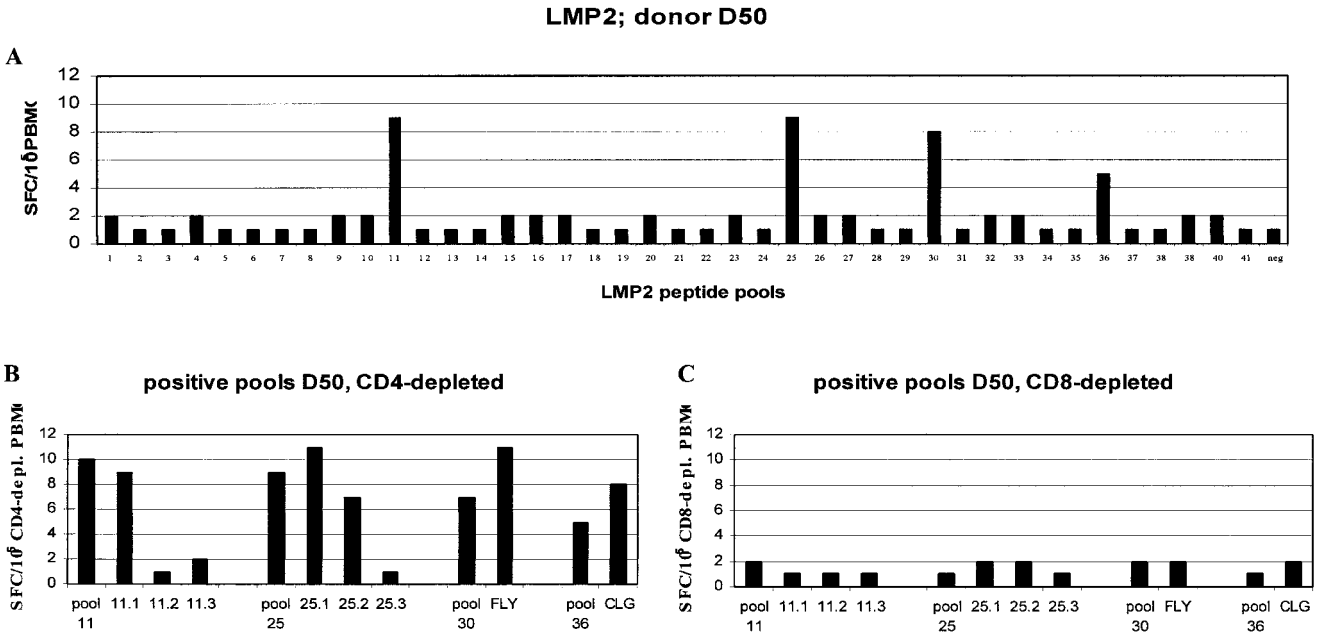

FIGURE 2 – Identification of CD8+ T-cell epitopes within LMP2 using IFNγ-ELISPOT and a 15 mer (10 mer overlapping) peptide library recognized by donor D50. PBMC were used at 5×10^5 cells per well and results are expressed as spot forming cells (SFC) per 10^5 cells. (*a*) PBMC of donor D50 are screened against peptides tested in pools of 3, (*b*) CD4-depleted PBMC are screened against the separate peptides of pool 11, 25, 30 and 36 and (*c*) CD8-depleted PBMC are screened against the separate peptides of pool 11, 25, 30 and 36.

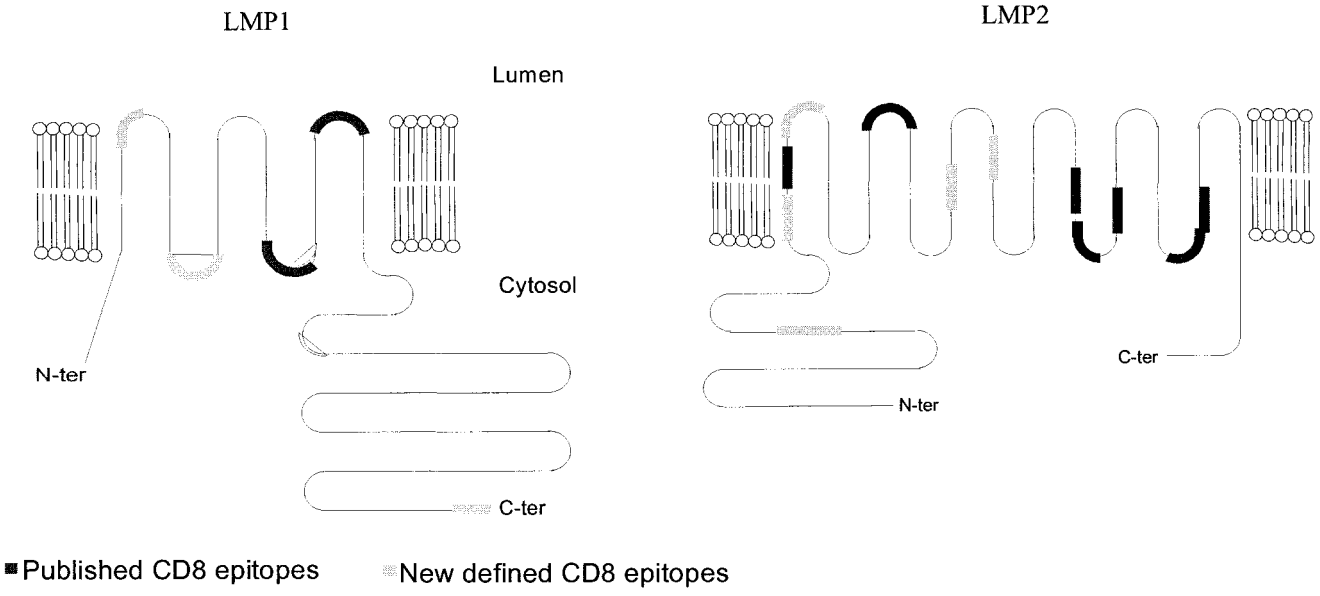

FIGURE 3 – Localization of new (gray) and previously (black) described CD8 epitopes on (*a*) LMP1 and (*b*) LMP2.

these proteins. We reasoned that studies to date based on the *in vitro* outgrowth of CTL clones from LCL-stimulated or peptide-stimulated PBMCs are likely to have given an incomplete picture of LMP-specific CD8+ T cell reactivities in virus-immune donors. The present study provides the first systematic analysis of CD8+ T cell memory by screening with overlapping 15 mer peptides covering the entire sequences of LMP1 and LMP2 and using rapid IFNγ release in ELISPOT assays as a means of quantitating the response.

Overall these assays revealed CD8+ T cell responses to LMP1 peptides in 9/50 (18%) and to LMP2 peptides in 15/28 (54%) of healthy Caucasian donors analyzed. These responses led to the identification of 3 new LMP1 epitopes in addition to the 2 previously recognized and of 5 new LMP2 epitopes in addition to the 7 previously recognized. The positions of all the new and previously known epitope sequences are shown in Figure 3 on diagrams of the native LMP1 and LMP2 molecules drawn in their presumed orientation in the cell membrane. In each case, all but 1 of the defined epitopes are derived from the membrane-associated domains of these molecules rather than from intracytosolic domains.

An important point to come from this work is that, although LMP-specific CD8+ T cells are detectable in a significant propor-

tion of Caucasians, the precursor frequencies of cells reactive to LMP1 (4–34 SFC/10^5 PBMCs) and to LMP2 (4–76 SFC/10^5 PBMCs) are low. Using the same kind of ELISPOT assay, the precursor frequencies of $CD8^+$ T cells to immunodominant epitopes from the EBNA-3A, -3B and -3C family of proteins are usually 10-fold higher.[20,29] Interestingly the newly identified epitopes, particularly in LMP2, tend to have lower precursor frequencies than seen for the known epitopes. We suggest that this is the reason these epitopes have remained unidentified in studies to date since representation of the relevant reactivities in LCL-stimulated polyclonal and clonal populations will be very low.

A second important point to note is the major contribution which HLA-A2-restricted T cells make to the overall response to these molecules. Thus in 5/9 donors recognizing LMP1 peptides and in 12/15 donors recognizing LMP2 peptides, much if not all of their response mapped to A2-restricted epitopes. Interestingly, all donors with a response against both A2-restricted LMP1 epitopes also displayed responses against at least 1 A2-restricted LMP2 epitope, whereas the reverse was not true. The relatively high frequency with which LMP1- or LMP2-specific responses were observed in this random screen of the Caucasian donors is therefore almost certainly a consequence of the high prevalence of the A2.1 allele in the Caucasian population. In turn, the dominance of A2.1 as a restricting allele for LMP1- and LMP2-specific responses reflects the preference of this HLA allele for hydrophobic peptide sequences and the fact that the LMP1 and LMP2 proteins contain 6 and 12 hydrophobic transmembrane spanning stretches, respectively. As one might have predicted, both A2-restricted LMP1 epitopes and all 4 A2-restricted LMP2 epitopes lie within the membrane-associated domains of the LMP1 and LMP2 molecules. In addition, however, the present study has identified the first epitopes to be mapped within intracytosolic domains of LMP1 and LMP2. This shows that these non-membrane-associated regions of the proteins are indeed available for processing and presentation to the HLA class I pathway. Although we can only speculate about the HLA restriction elements of these epitopes, analysis of HLA binding[24] was compatible with lack of A2 binding motifs. For LMP1 epitopes, restriction elements were most probably HLA-B alleles.

The overall impression from our study is that both LMPs and especially LMP1, are only weakly immunogenic to the $CD8^+$ T-cell response in Caucasians. All of the recorded responses are of relatively low magnitude and an unusually high proportion involve HLA-A2-restricted hydrophobic epitopes. It is formally possible that we have underestimated the level of $CD8^+$ T cell responses to these proteins because our work used only B95.8 strain peptides and there is some polymorphism in LMP gene sequences among Caucasian EBV strains.[30] However, the levels of sequence divergence are low and where amino acid changes relative to B95.8 have been observed to lie within epitopes, this has not affected the antigenicity of these epitopes.[25] In our view, therefore, sequence divergence is unlikely to have led to a significant underestimate of LMP-specific immunity. The present data indicating that the LMPs are only weakly immunogenic accord with the finding that the LMPs only induce detectable humoral responses in rare circumstances; thus anti-LMP1 antibodies have been detected in a subset of patients with HD or NPC but not in most healthy carriers,[23,31,32] while anti-LMP2 antibodies have only been described in NPC patients.[23,33,34] Furthermore, our recent studies have shown that LMP1 and LMP2 are also a poor source of epitopes for the $CD4^+$ T cell response.[35]

Why these molecules should be relatively poorly immunogenic is not understood. In the case of LMP1, it may be that the protein itself has immunosuppressive properties that favor the induction of T cell anergy.[36] When considering how these membrane proteins are viewed by $CD8^+$ T cells, it is clear that (unlike EBNA1) endogenously expressed LMP1 and LMP2 can access the HLA class I pathway and interestingly at least some of their derived epitopes even appear to be loaded on HLA class I molecules in a TAP-independent fashion.[17,18,19] It is therefore likely that the latently-infected growth-transformed B cells that appear transiently during primary EBV infection *in vivo*[37] can directly present LMP1- and LMP2-derived epitopes to the naïve CD8 repertoire. However, recent work has shown that EBNA1 also elicits a $CD8^+$ response in the host even though the endogenously-expressed protein is protected from presentation in naturally-infected cells;[22] this indicates that EBNA1 and perhaps other latent proteins induce $CD8^+$ T cell responses through cross-priming ,*i.e*., through proteins released from infected cells being taken up as exogenous antigens by dendritic cells, then processed and the antigen-derived epitopes presented at the dendritic cell surface via the class I as well as class II pathway.[38]In the case of LMP1 and LMP2, these proteins are shed from latently-infected cells in the form of exosomes[36] (Verkoeijen *et al*., unpublished). If hydrophobic membrane-associated proteins are for some reason difficult to process within dendritic cells, this could explain why the LMPs are generally poor inducers of both $CD8^+$ and $CD4^+$ T cell responses.

It can be concluded that $CD8^+$ immunity to LMP1 and LMP2 in healthy seropositive individuals constitutes a minor component of the virus specific T-cell response. Nevertheless, even though such responses are rare, the fact that a number of LMP1 and LMP2 epitopes do exist holds promise for future immunotherapy. Targeting EBV-positive tumors such as HD and NPC will require these minor components of the T cell response to be selectively amplified, either by *in vivo* vaccination with appropriate constructs[39] or by *in vitro* expansion[13,40,41] and re-infusion. Our data further contribute to the realization of this approach by identifying a more complete range of LMP-derived epitopes and determining the frequency of the responses they induce in the $CD8^+$ memory of healthy carriers.

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REFERENCES

1. Rickinson AB, Kieff E. Epstein-Barr virus. In: Fields BN, Knipe DM, Howley P, et al., eds. Fields virology. Philadelphia: Lippincott-Raven, 1996. 2397–446.
2. Pallesen G, Hamilton-Dutoit SJ, Rowe M, et al. Expression of Epstein-Barr virus latent gene products in tumour cells of Hodgkin's disease. Lancet 1991;337:320–22.
3. Herbst H. Dallenbach F, Hummel M, et al. Epstein-Barr latent membrane protein expression in Hodgkin and Reed-Sternberg cells. Proc Natl Acad Sci USA 1991;88:4766–70.
4. Niedobitek G, Kremmer E, Herbst H, et al. Immunohistochemical detection of the Epstein-Barr virus-encoded latent membrane protein 2A in Hodgkin's disease and infectious mononucleosis. Blood 1997; 90:1664–72.
5. Brooks L, Yao QY, Rickinson AB, et al. Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1 and LMP2 transcripts. J Virol 1992;66:2689–97.
6. Zur Hausen A, Brink AATP, Craanen ME, et al. Unique transcription pattern of Epstein-Barr virus (EBV) in EBV-carrying gastric adenocarcinomas: expression of the transforming BARF1 gene. Cancer Res 2000;60:2745–8.
7. Decaussin G, Sbih-Lammali F, de Turenne-Tessier M, et al. Expression of BAFR1 gene encoded by Epstein-Barr virus in nasopharyngeal carcinoma biopsies. Cancer Res 2000;60:5584–8.
8. Levitskaya J, Coram M, Levitsky V, et al. Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen 1. Nature 1995;375:685–8.
9. Liebowitz D, Mannick J, Takada K, et al. Phenotypes of Epstein-Barr virus LMP1 deletion mutants indicate transmembrane and amino-terminal cytoplasmic domains necessary for effects in B-lymphoma cells. J Virol 1992;66:4612–6.
10. Longnecker R, Druker B, Roberts TM, et al. An Epstein-Barr virus

protein associated with cell growth transformation interacts with a tyrosine kinase. J Virol 1991;65:3681–92.
11. Khanna R, Burrows SR, Nicholls J, et al. Identification of cytotoxic T cell epitopes within Epstein-Barr virus (EBV) oncogene latent membrane protein 1 (LMP1): evidence for HLA A2 supertype restricted immune recognition of EBV-infected cells by LMP1-specific cytotoxic T lymphocytes. Eur J Immunol 1998;28:451–8.
12. Rickinson AB, Moss DJ. Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection. Annu Rev Immunol 1997;15:405–31.
13. Sing AP, Ambinder RF, Hong DJ, et al. Isolation of Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes that lyse Reed-Sternberg cells: implications for immune-mediated therapy of EBV+ Hodgkin's disease. Blood 1997;89:1978–86.
14. Khanna R, Burrows SR. Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases. Annu Rev Microbiol 2000;54:19–48.
15. Subklewe M, Chahroudi A, Bickham K, et al. Presentation of Epstein-Barr virus latency antigens to CD8$^+$, interferon-γ-secreting, T lymphocytes. Eur J Immunol 1999;29:3995–4001.
16. Khanna R, Moss DJ, Burrows SR. Vaccine strategies against Epstein-Barr virus-associated diseases: lessons from studies on cytotoxic T-cell mediated immune regulation. Immunol Rev 1999;170:49–64.
17. Lee S P, Thomas WA, Blake NW, et al. Transporter (TAP)-independent processing of a multiple membrane-spanning protein, the Epstein-Barr virus latent membrane protein 2. Eur J Immunol 1996;26: 1875–83.
18. Khanna R, Burrows SR, Moss DJ, et al. Peptide transporter (TAP-1 and TAP-2)-independent endogenous processing of Epstein-Barr virus (EBV) latent membrane protein 2A: implications for cytotoxic T-lymphocyte control of EBV-associated malignancies. J Virol 1996; 70:5357–62.
19. Lautscham G, Mayrhofer S, Taylor G, et al. Processing of a multiple membrane spanning Epstein-Barr virus protein for CD8(+) T cell independent pathway. J Exp Med 2001;194:1053–68.
20. Yang J, Lemas VM, Flinn IW, et al. Application of the ELISPOT assay to the characterization of CD8$^+$ responses to Epstein-Barr virus antigens. Blood 2000;95:241–8.
21. Herr W, Ranieri E, Gambotto A, et al. Identification of naturally processed and HLA-presented Epstein-Barr virus peptides recognized by CD4$^+$ and CD8$^+$ T lymphocytes from human blood. Proc Natl Acad Sci USA 1999;96:12033–8.
22. Blake N, Haigh T, Shaka'a G, et al. The importance of exogenous antigen in priming the human CD8$^+$ T cell response: lessons from the EBV nuclear antigen EBNA1. J Immunol 2000;165:7078–87.
23. Meij P, Vervoort MBHJ, Aarbiou J, et al. Restricted low-level human antibody responses against Epstein-Barr virus (EBV)-encoded latent membrane protein 1 in a subgroup of patients with EBV-associated diseases. J Infect Dis 1999;179:1108–15.
24. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 peptides based on independent binding of individual peptide side-chains. J Immunol 1994;152:163–75.
25. Lee SP, Tierney RJ, Thomas WA, et al. Conserved (CTL) epitopes within EBV latent membrane protein 2, a potential target for CTL-based tumour therapy. J Immunol 1997;158:3325–34.
26. Haque T, Crawford DH. Role of adoptive immunotherapy in the prevention and treatment of lymphoproliferative diseases following transplantation. Br J Haematol 1999;106:309–16.
27. Lee SP, Chan AT, Cheung ST, et al. CTL control of EBV in nasopharyngeal carcinoma (NPC): EBV-specific CTL responses in the blood and tumors of NPC patients and the antigen-processing function of the tumor cells. J Immunol 2000;165:573–82.
28. Chapman ALN, Rickinson AB, Thomas WA, et al. Epstein-Barr virus-specific cytotoxic T lymphocyte responses in the blood and tumor site of Hodgkin's disease patients: implications for T-cell-based therapy. Cancer Res 2001;61:6219–26.
29. Tan LC, Gudgeon N, Annels NE, et al. A re-evaluation of the frequency of CD8$^+$ T-cells specific for EBV in healthy carriers. J Immunol 1999;162:1827–1835.
30. Sanvej K, Gratama JW, Munch M, et al. Sequence analysis of the Epstein-Barr virus latent membrane protein 1 gene and promoter region: identification of four variants among wild type EBV isolates. Blood 1997;90:323–30.
31. Chen HF, Kevan-Jah S, Suentzenich KO, et al. Expression of the Epstein-Barr virus latent membrane protein (LMP) in insect cells and detection of antibodies in human sera against this protein. Virology 1992;190:106–15.
32. Xu J, Ahmad A, D'Addario M, et al. Analysis and significance of anti-latent membrane protein-1 antibodies in the sera of patients with EBV-associated diseases. J Immunol 2000;164:2815–22.
33. Lennette ET, Winberg G, Yadav M, et al. Antibodies to LMP2A/2B in EBV-carrying malignancies. Eur J Cancer 1995;31A:1875–8.
34. Frech B, Zimber-Strobl U, Yip TTC, et al. Characterization of the antibody response to the latent infection terminal protein of Epstein-Barr virus in patients with nasopharyngeal carcinoma. J Gen Virol 1993;74:811–8.
35. Leen A, Meij P, Redchenko I, et al. Differential immunogenicity of Epstein-Barr virus latent-cycle proteins for human CD4(+) T-helper 1 responses. J Virol 2001;75:8649–59.
36. Dukers DF, Meij P, Vervoort MBHJ, et al. Direct immunosuppressive effects of EBV-encoded latent membrane protein 1. J Immunol 2000; 165:663–70.
37. Tierney RJ, Steven N, Young LS, et al. Epstein-Barr virus latency in blood mononuclear cells: analysis of viral gene transcription during primary infection and in the carrier state. J Virol 1994;68:7374–85.
38. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245–52.
39. Moss DJ, Schmidt C, Elliott S, et al. Strategies involved in developing an effective vaccine to EBV-associated diseases. Adv Cancer Res 1996;69:213–45.
40. Roskrow MA, Suzuki N, Gan YJ, et al. Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of patients with EBV-positive relapsed Hodgkin's disease. Blood 1998;91:2925–34.
41. Redchenko I, Rickinson AB. Accessing Epstein-Barr virus-specific T cell memory with peptide-loaded dendritic cells. J Virol 1999;73: 334–42.