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Differential Immunogenicity of Epstein-Barr Virus Latent-Cycle Proteins for Human CD4$^+$ T-Helper 1 Responses

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Human CD4$^+$ T-helper 1 cell responses to Epstein-Barr virus (EBV) infection are likely to be important in the maintenance of virus-specific CD8$^+$ memory and/or as antiviral effectors in their own right. The present work has used overlapping peptides as stimulators of gamma interferon release (i) to identify CD4$^+$ epitopes within four EBV latent-cycle proteins, i.e., the nuclear antigens EBNA1 and EBNA3C and the latent membrane proteins LMP1 and LMP2, and (ii) to determine the frequency and magnitude of memory responses to these proteins in healthy virus carriers. Responses to EBNA1 and EBNA3C epitopes were detected in the majority of donors, and in the case of EBNA1, their antigen specificity was confirmed by in vitro reactivation and cloning of CD4$^+$ T cells using protein-loaded dendritic cell stimulators. By contrast, responses to LMP1 and LMP2 epitopes were seen much less frequently. EBV latent-cycle proteins therefore display a marked hierarchy of immunodominance for CD4$^+$ T-helper 1 cells (EBNA1, EBNA3C $\gg$ LMP1, LMP2) which is different from that identified for the same proteins with respect to CD8$^+$-T-cell responses (EBNA3C > EBNA1 > LMP2 $\gg$ LMP1). Furthermore, the range of CD4$^+$ memory T-cell frequencies in peripheral blood of healthy virus carriers was noticeably lower and narrower than the corresponding range of latent antigen-specific CD8$^+$-T-cell frequencies.

Epstein-Barr virus (EBV), a B-lymphotropic gammaherpesvirus widespread in human populations and linked to a range of malignancies, has provided important insights into the human CD8$^+$-T-cell response to viral infection. In particular, CD8$^+$ T cells recognizing EBV latent proteins have attracted interest because of their potential use as effectors targeting virus-positive malignancies (18, 38, 40). The virus encodes eight antigenically distinct latent-cycle proteins, the nuclear antigens EBNA1, -2, -3A, -3B, -3C, and -LP and latent membrane proteins LMP1 and -2, all of which are expressed in EBV-transformed B-lymphoblastoid cell lines (LCLs). These proteins display a marked hierarchy of immunodominance for the CD8$^+$-T-cell response (38). Epitopes derived from the EBNA3A, -3B, and -3C family of proteins tend to induce the strongest responses across a range of different HLA class I alleles. This is apparent both from functional studies on LCL-reactivated T-cell lines in vitro and from ELISPOT assays of peptide-induced gamma interferon (IFN-γ) release on fresh peripheral blood mononuclear cells (PBMCs) (21, 33, 46). Responses to EBNA1-derived epitopes are seen in fewer donors, but, in the context of particular HLA class I alleles, EBNA1-specific CD8$^+$-T-cell numbers in memory can reach the same high levels as for EBNA3-derived epitopes (5). Of the other latent proteins, LMP2 has been identified as a source of epitopes for several HLA class I alleles, but the numbers of reactive T cells are always low, whereas responses to EBNA2, EBNA-LP, and LMP1 are rare (21, 22, 23, 26, 33). The basis of these differences in immunogenicity is still not understood. However, they may reflect the differential access of individual latent-cycle proteins to the HLA class I presentation pathway either in virus-infected B cells themselves or in the dendritic cells (DCs), which are thought to be involved in priming T-cell responses in vivo through their capacity to acquire viral proteins exogenously and present them to the CD8$^+$ repertoire (2). This latter cross-priming pathway appears to be important at least in the case of the EBNA1-specific CD8$^+$ response (5), since in infected cells the endogenously expressed EBNA1 protein is protected from HLA class I presentation by virtue of its internal glycine-alanine repeat (GAr) domain (28).

Much less is known about CD4$^+$-T-cell responses to EBV latent antigens (24, 25), although by analogy with murine models such cells may well be needed to maintain the functional competence of CD8$^+$ T cells specific for the virus (8), as well as possibly serving as antiviral effectors in their own right (34). In this context, the polyclonal LCL-stimulated T-cell preparations used to treat EBV-positive lymphoproliferative lesions of immunosuppressed patients contain CD4$^+$ as well as CD8$^+$ components, and both components may be necessary for the clinical effectiveness of this adoptive T-cell therapy (18, 40). It is therefore important to determine which latent antigens elicit CD4$^+$ responses and what is the frequency of such T cells in the memory pool. Recently Munz and colleagues (32) have approached this question by stimulating the CD4$^+$ T cells of virus-immune donors with autologous DCs expressing individual EBV latent proteins from recombinant vaccinia virus vectors and then assessing the resultant T-cell population for evidence of antigen specificity using either blastogenesis or

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IFN-γ production as a readout. It appeared that particular proteins, especially EBNA1 and also LMP1, were frequently capable of inducing specific responses; this result was of particular interest not just because of the contrast with the $CD8^+$ response but also because EBNA1 and LMP1, along with LMP2, constitute a subset of latent proteins that are selectively expressed in EBV-positive tumors such as Hodgkin's disease and nasopharyngeal carcinoma (NPC) (37). However, the work of Munz et al. (32) was based on a limited number of individuals, and none of the apparently antigen-specific reactivities were confirmed at the peptide epitope level. Here we have addressed the question in a different way by screening the $CD4^+$ T cells of immune donors with peptide pools from latent-cycle proteins and using rapid IFN-γ release as the readout. In this initial study we have concentrated on four of the eight latent-cycle proteins, namely, EBNA1, the two latent membrane proteins LMP1 and LMP2, and one of the immunodominant $CD8^+$ target antigens, EBNA3C.

MATERIALS AND METHODS

Donors and cells. Whole blood was obtained from healthy laboratory personnel, and buffy coats were obtained from blood donations to the Birmingham Blood Transfusion Service. EBV status was determined by serological staining for immunoglobulin G antibodies for viral capsid antigen, and all donors were HLA class II typed at the HLA-DR and -DQ loci using PCR-based DNA typing. PBMCs were isolated by Lymphoprep (Nycomed Pharma, Oslo, Norway) density-grade centrifugation and were either used fresh or cryopreserved until required. PBMCs to be used as responder populations in ELISPOT assays were routinely depleted of $CD8^+$ T cells using Dynabeads M450-CD8 (DYNAL United Kingdom Ltd.) according to the manufacturer's instructions. Efficient depletion was confirmed by staining CD8-depleted PBMCs using a dual-staining fluorescein isothiocyanate-conjugated anti-CD8 and phycoerythrin-conjugated anti-CD4 antibody (Serotec, Oxford, United Kingdom) followed by flow cytometric analysis on a Coulter EPICS XL cytometer; depletion of >95% of the $CD8^+$ cells was consistently achieved. In some cases aliquots of PBMCs were used to establish EBV-transformed LCLs using the B95.8 virus strain, and LCLs were maintained in RPMI 1640 containing 2 mM glutamine, 10% (vol/vol) fetal calf serum, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml (growth medium).

Synthetic peptides and baculovirus-expressed protein. Peptides were synthesized by standard fluorenyl-methoxycarbonyl chemistry (Alta Bioscience, University of Birmingham, Birmingham, United Kingdom) and dissolved in dimethyl sulfoxide, and their concentration was determined by biuret assay. Full-length EBNA1 protein (bEBNA1) was prepared using the baculovirus expression system as previously described (14) and was a kind gift from Lori Frappier (University of Toronto, Toronto, Canada). Human papillomavirus protein E4 (bE4), prepared using the baculovirus expression system (39) and used as a control, was a kind gift from Sally Roberts (University of Birmingham).

ELISPOT assay for detection of IFN-γ release. Ninety-six-well polyvinylidene difluoride-backed plates (Millipore, Bedford, Mass.) were precoated with a 15-μg/ml concentration of an anti-IFN-γ monoclonal antibody (MAb), 1-DIK (MABTECH, Stockholm, Sweden). CD8-depleted PBMCs were added to duplicate wells at known cell numbers in the presence of single or pooled 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-IFN-γ MAb, 7-B6-1 (MABTECH), was added at 1 μg/ml and left for 2 to 4 h at room temperature, followed by streptavidin-conjugated alkaline phosphatase (MABTECH) for an additional 2 h. Individual cytokine-producing cells were detected as dark spots after a 30-min reaction with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium using an alkaline phosphatase-conjugated substrate kit (Bio-Rad, Richmond, Calif.). The spots were counted under a dissection microscope. In all experiments, results from ELISPOT assays are expressed as spot-forming cells (SFC) per 10^6 CD8-depleted PBMCs.

Reactivations of $CD4^+$ T cells. To provide an antigen-presenting cell population, DCs were first prepared by seeding PBMCs onto six-well plates (Costar, Cambridge, Mass) at 10^7 cells/well. After 2 h at 37°C, nonadherent cells were removed and the adherent population was cultured in growth medium supplemented with 50 ng of granulocyte-macrophage colony-stimulating factor (GM-CSF) per ml and 1,000 U of interleukin-4 (IL-4) per ml. The cultures were refed on days 2 and 4 by replacing half of the medium with fresh medium as described above. On day 6 or 7 the cells were harvested by gentle pipetting action, resuspended in 500 μl of AIM-V serum-free medium (Life Technologies, Paisley, United Kingdom) containing full-length EBNA1 protein (bEBNA1) at 5 μg/ml, and incubated for 14 h at 37°C. Cells were then washed twice with RPMI 1640 (with no supplements) and resuspended in growth medium containing GM-CSF and IL-4 as described above but also supplemented with tumor necrosis factor alpha (50 ng/ml) as a maturation stimulus. Cells were cultured for a further 24 h before being seeded as stimulators at 10^5 cells/2-ml well in growth medium supplemented with IL-7 at 5 ng/ml. Responder PBMCs were added at 2×10^6 cells/well to give a responder/stimulator ratio of 20:1. Cultures were fed twice weekly as described above, restimulated on days 7 and 21 with DCs pulsed with EBNA1 protein as described above, and from day 7 fed with growth medium supplemented with IL-7 (5 ng/ml) and IL-2 (20 U/ml). Aliquots of the bulk responder population were screened for EBNA1 specificity in proliferation assays carried out from day 12 onwards. On day 24, the bulk responder population was seeded at limiting dilutions of 3 and 0.3 cells/round-bottomed microtest plate well using irradiated (4,000 rads) autologous LCL stimulators prepulsed for 1 h with peptide at 20 μg/ml, and the cultures were maintained in IL-2-conditioned medium as described previously (45)

Proliferation assay. LCL stimulator cells were either pulsed with peptide as described above or pulsed overnight with full-length EBNA1 protein (bEBNA1) at 5 μg/ml in AIM-V serum-free medium. The LCL was then washed once in RPMI plus L-glutamine, resuspended in 5 ml of RPMI plus 8% fetal calf serum, and gamma irradiated as described above. The peptide- or protein-pulsed LCLs were washed and added to the responder T cells at a ratio of 1:1 in 96-well round-bottomed plates and then incubated at 37°C in 5% CO_2 for 96 h; all cocultures were set up in triplicate. Wells were pulsed with 1 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech) for the last 12 to 16 h of incubation and then harvested using a micro cell harvester (Skatron, Lier, Norway) and counted in a Betaplate 1205. MAb blocking assays were conducted using the HLA-DR-specific MAb L243 (19) at a final concentration of 5 μg/ml and an isotype-matched control MAb at the same concentration.

Identification of resident EBV strains. Some donors were analyzed to determine the identities of the EBNA1 and LMP1 alleles in their resident EBV strains by direct PCR amplification and sequencing of viral DNA from PBMCs (6). EBNA1 sequences were determined over the polymorphic region from codon 460 to 510 using published primers (16), and LMP1 sequences were determined over the polymorphic region from codon 318 to 386, again using published primers (20).

RESULTS

Mapping of $CD4^+$-T-cell responses to EBNA1 peptides. Using the ELISPOT assay of rapid peptide-induced IFN-γ release, we first undertook a series of experiments to map $CD4^+$-T-cell epitopes within the EBNA1 protein. CD8-depleted PBMC preparations were screened using a panel of peptides (20-mers overlapping by 15 amino acids [aa]) covering the 409-aa unique sequence of the EBNA1 protein (B95.8 strain) plus three overlapping 20-mers representing repeat sequences within the internal 232-residue GAr domain. To minimize the size of the initial screening assays, we used 27 pools each containing three adjacent peptides from the EBNA1 sequence, an approach already established as a means of identifying $CD8^+$-T-cell epitopes within the protein (5). Detailed results of the initial assays from three representative donors are shown in Fig. 1. Donor 1 (Fig. 1, top panel) showed significant reactivity above background to four EBNA1 peptide pools (no. 5, 16, 17, and 19), whereas donors 2 (middle panel) and 3 (bottom panel) each responded to three pools (no. 16, 17, and 19 and 16, 17, and 22, respectively). All of these responses were consistently observed on rescreening, whereas the weak additional response to pool 18 originally seen in donor 3 did not prove to be reproducible (data not shown).

To check that reactivities to peptide pools did indeed reflect

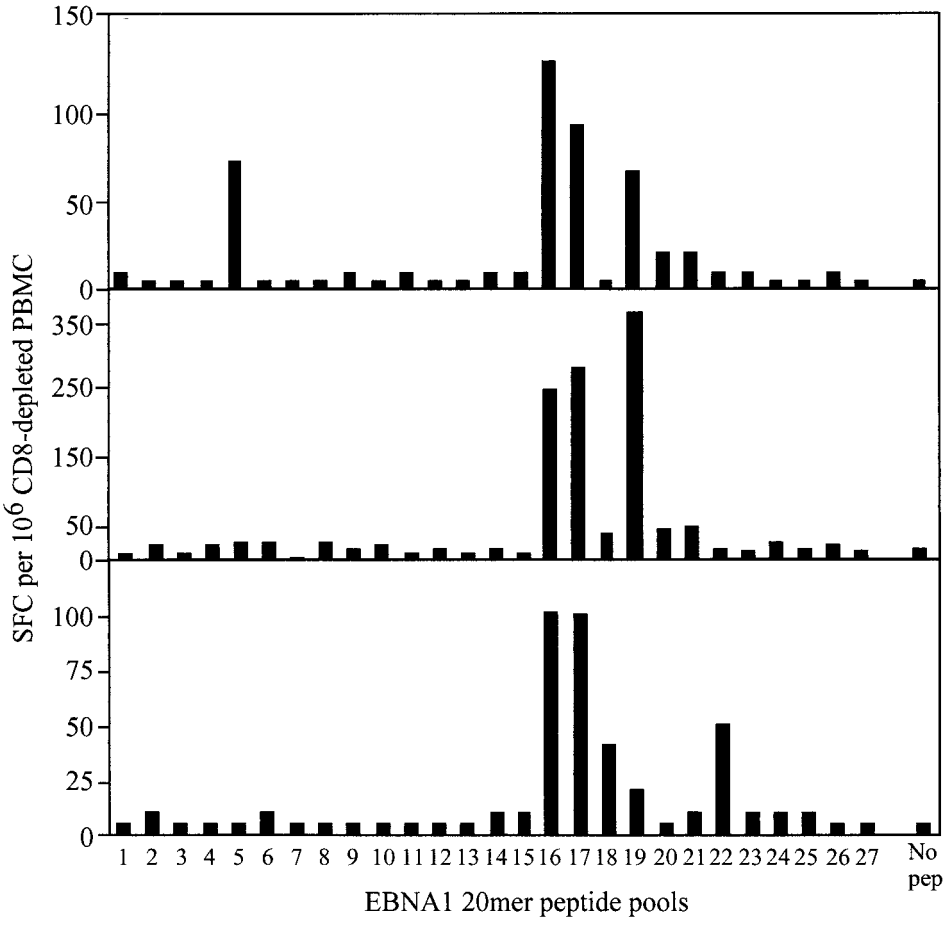

FIG. 1. Identification of CD4+-T-cell epitopes within EBNA1 using the ELISPOT assay of peptide-induced IFN-γ release. CD8-depleted PBMCs from donors 1 (top panel), 2 (middle panel), and 3 (bottom panel) were screened using a panel of peptides (20-mers overlapping by 15 aa) spanning the unique sequence of the B95.8 strain EBNA1 protein. The 20-mer peptides were tested in pools of three, generating 26 pools; three additional peptides were used to represent the internal GAr domain of EBNA1 (pool 27). PBMCs were used at 2×10^5 cells per well, and results are expressed as spot-forming cells (SFC) per 10^6 CD8-depleted PBMCs. The background observed when no peptide was added is shown (No pep).

responses to unique epitopes, each of these donors was then assayed against the individual 20-mers from the relevant pools and against shorter peptides from these same regions. Examples of such mapping are shown in Fig. 2. The response of donor 1 to pool 5 (Fig. 2A) mapped to two overlapping peptides (aa 66 to 85 and aa 71 to 90) and could subsequently be minimized to a 15-mer epitope, EBNA1 aa 71 to 85, RRPQKRPSCIGCKGT (designated RRP). Likewise, as shown in Fig. 2B, this same donor's response to two of the original pools, 16 and 17, reflected recognition of one peptide within pool 16 (aa 469 to 488) and of an overlapping peptide within pool 17 (aa 474 to 493) and could subsequently be minimized to a 15-mer epitope, EBNA1 aa 475 to 489, NPKFENIAEGLRALL (designated NPK). Similarly, the responses seen both in donor 2 and in donor 3 to pools 16 and 17 also mapped to the NPK epitope (aa 475 to 489) (data not shown). In addition, the response of donor 2 to pool 19 (Fig. 2C) minimized to a 14-mer peptide from aa 515 to 528, TSLYNLRRGTALAI (designated TSL), which has already been identified as a DR1-restricted EBNA1 epitope (24), whereas the response of donor 3 to pool 22 (Fig. 2D) mapped to the 15-mer epitope from aa 563 to 577, MVFLQTHIFA EVLKD (designated MVF). It is worth noting that the magnitude of the responses to CD4 epitopes within EBNA1 ranges from a minimum of 60 IFN-γ-producing cells (donor 3, MVF epitope) to a maximum of 350 IFN-γ-producing cells (donor 2, TSL epitope) per 10^6 CD8-depleted PBMCs. All subsequent responses to EBNA1 epitopes seen in other donors (see below) fell within this range.

EBNA1 antigen specificity of mapped responses. To determine whether these ELISPOT responses to synthetic peptides represented authentic components of EBNA1-specific memory, we attempted to reactivate the relevant responses by stimulating PBMCs with autologous DCs preloaded with purified EBNA1 protein. Immature DC preparations, produced by culturing adherent PBMCs in the presence of GM-CSF and IL-4 for 6 days, were incubated overnight with baculovirus-expressed EBNA1 protein and subsequently matured by treatment with tumor necrosis factor alpha for 24 h. Fresh PBMCs were then cocultured with these stimulator cells and restimulated on two further occasions before limiting-dilution cloning. For each of the three donors tested, screening of the derived

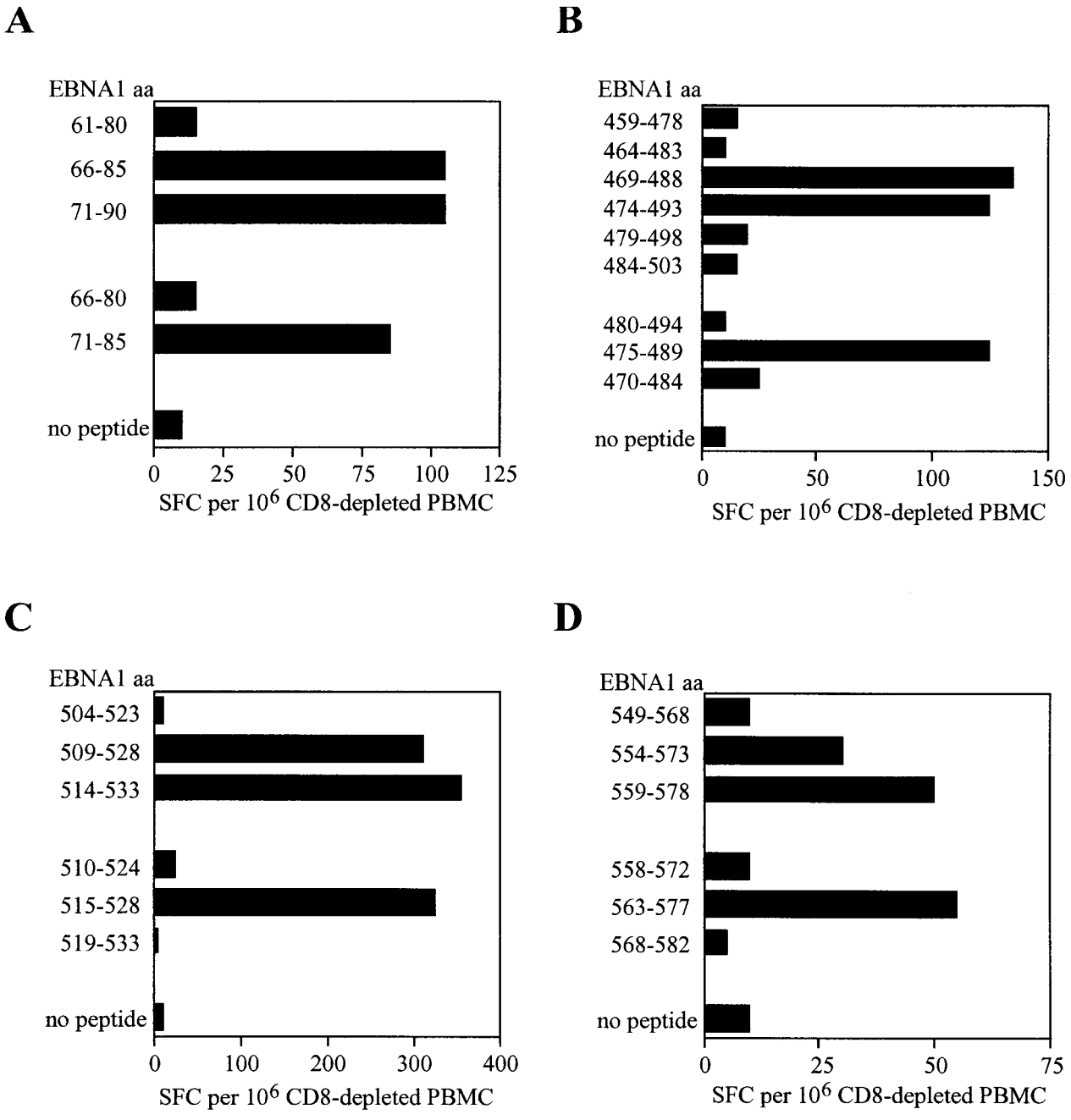

FIG. 2. ELISPOT mapping of the minimal EBNA1 $CD4^+$-T-cell epitopes from donors 1, 2, and 3, using individual 20-mer peptides from the original pool(s) plus 15-mers for the relevant epitope region. (A) CD8-depleted PBMCs from donor 1 tested with pool 5 peptides. (B) CD8-depleted PBMCs from donor 1 tested with pool 16 and pool 17 peptides. (C) CD8-depleted PBMCs from donor 2 tested with pool 19 peptides. (D) CD8-depleted PBMCs from donor 3 tested with pool 22 peptides. All results are expressed as in Fig. 1.

T-cell clones in proliferation assays identified a number which were specific for one of the predicted epitope peptides (NPK, TSL, or MVF) and which were subsequently shown to recognize stimulator cells preexposed to the EBNA1 protein. Staining with CD4 and CD8 MAbs confirmed that each of these cloned populations was >90% CD4 positive and was uniformly CD8 negative.

Figure 3 shows examples of proliferation assays confirming the specificity and HLA class II restriction of such clones. Clone 63 from donor 3 (Fig. 3A, upper panel) specifically responded to autologous LCL cells preloaded either with EBNA1 protein or with the EBNA1 peptide MVF (aa 563 to 577) but did not respond to the same LCL cells used either alone or preloaded with an irrelevant baculovirus expressed protein, human papillomavirus E4. Epitope-specific proliferation was inhibited in the presence of an HLA-DR-specific MAb, L243. To identify the restriction element for donor 3 clone 63, proliferation assays were carried out using the autol-

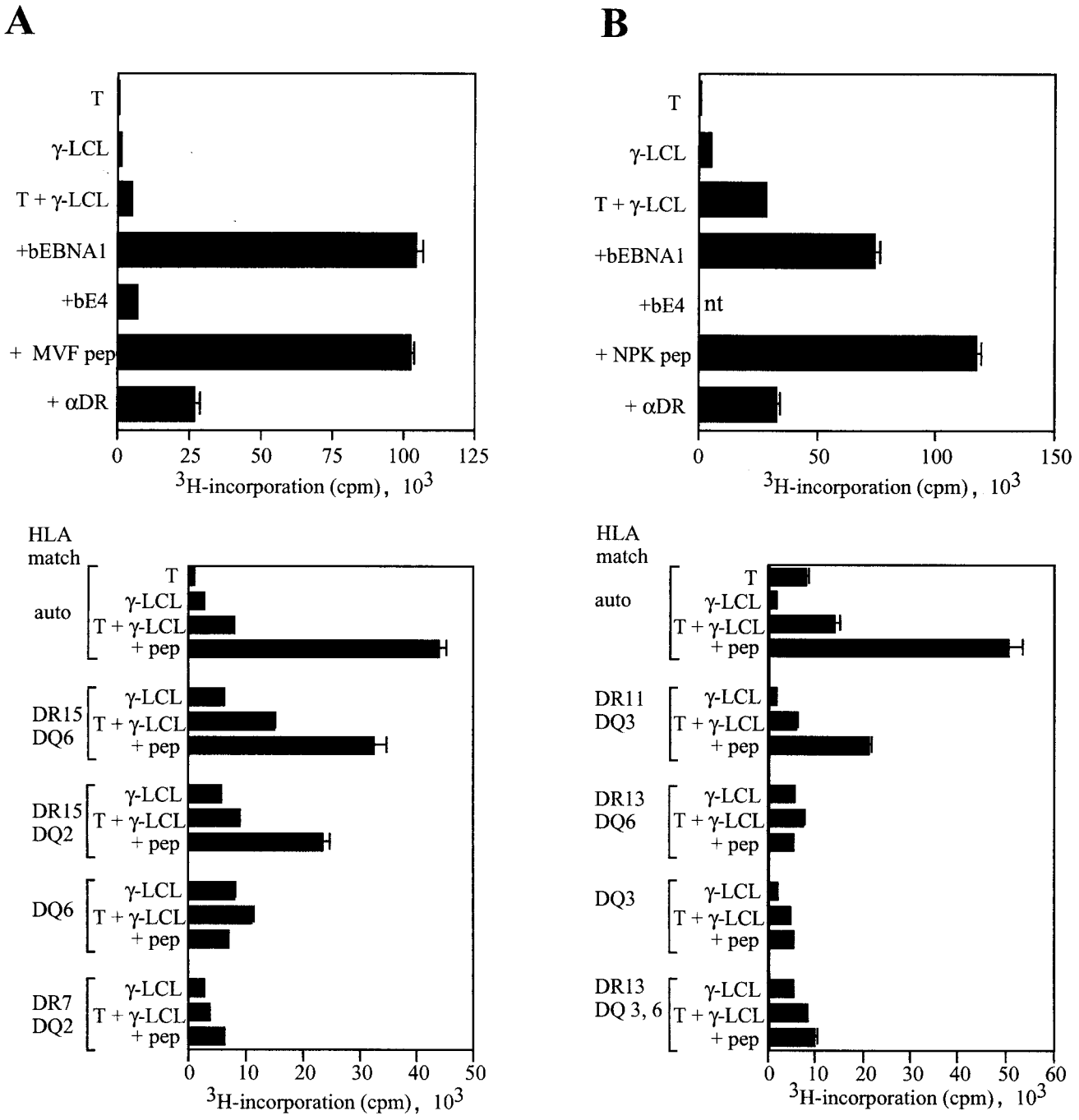

FIG. 3. In vitro reactivation and HLA restriction of EBNA1-specific CD4+-T-cell clones from donor 3 (A) and donor 1 (B) generated by PBMC stimulation in vitro with DCs loaded with EBNA1 protein. The upper panels represent proliferation assays using donor 3 clone 63 specific for EBNA1 epitope MVF (aa 563 to 577) (A) and donor 1 clone 14 specific for EBNA1 epitope NPK (aa 475 to 489) (B). T-cell clones were cultured either alone (T), with the gamma-irradiated autologous LCL (T + γ-LCL), or with the γ-LCL loaded with either EBNA1 protein (bEBNA1), control human papillomavirus protein (bE4), or specific epitope peptide (pep). In both cases peptide-induced proliferation was blocked by addition of an anti-DR MAb, L243 (αDR). nt, not tested. In the lower panels, the T-cell clones were tested in proliferation assays against gamma-irradiated autologous (auto) or HLA-matched LCL targets (T + γ-LCL) or with the LCL targets pulsed with specific epitope peptide (+ pep). All results are expressed as the incorporation of [3H]thymidine and are the means and standard deviations of triplicate values.

ogous or HLA-matched LCL stimulators either alone or pulsed with the MVF epitope peptide. As shown in Fig. 3A (lower panel), epitope-specific proliferation was observed only using HLA DR15-matched LCL stimulators, indicating restriction through this allele. Figure 3B shows parallel data from another representative clone, donor 1 clone14, in this case specific for the NPK epitope (EBNA1 aa 475 to 489). Again the clone was capable of recognizing the autologous LCL ei-

TABLE 1. $CD4^+$-T-cell epitopes identified in EBNA1

EBNA1 aa[a]	Epitope sequence	No. of responders	Potential restriction[b]		Known restriction[c]
			DR allele(s)	DQ allele(s)	
B95.8 sequence					
71–85	RRPQKRPSCIGCKGT	1	11, 13	3, 6	
403–417	RPFFHPVGEADYFEY	2		2	
429–448	VPPGAIEQGPADDPGEGPST	1	1, 3	2, 5	
455–469	DGGRRKKGGWFGRHR	1	7, 15	1, 2	
475–489	NPKFENIAEGLRALL	6			DR11
485–499	LRALLARSHVERTTD	7	[11, 13, 15]	[1, 3, 6]	
509–528	VYGGSKTSLYNLRRGTALAI	1	11, 13	3, 6	
515–528	TSLYNLRRGTALAI	5			DR1
519–533	NLRRGTALAIPQCRL	1	4	3	
529–543	PQCRLTPLSRLPFGM	5	[13]	[3, 5, 6]	
544–563	APGPGPQPGPLRESIVCYFM	1	1, 3	2, 5	
554–573	LRESIVCYFMVFLQTHIFAE	1	1, 3	2, 5	
563–577	MVFLQTHIFAEVLKD	2			DR15
574–593	VLKDAIKDLVMTKPAPTCNI	1	1, 3	2, 5	
594–613	RVTVCSFDDGVDLPPWFPPM	2	None	None	
Q/T variant					
424–443	DGEPDMPPGAIEQGPADDPG	1	1, 4	1, 8	
514–533	KTSLYNLRRGIALAIPQCRL	3	1		
589–608	PTCNIKATVCSFDDGVDLPP	3	1		

[a] Data are from screening on the entire B95.8 strain sequence of EBNA1 and on 22 variant peptides incorporating all sequence changes in the Q/T strain EBNA1.
[b] Potential restriction elements based on HLA-DR and -DQ typing of donors. Where the response to a peptide was limited to one donor only, the DR and DQ types of that donor are shown. Where more than one donor responded to a peptide, DR and DQ alleles shared by the responsive donors are shown; if there were no common alleles, then DR and DQ alleles shared by the majority of donors are shown in brackets.
[c] Known restriction elements from the functional analysis of derived clones; note that not all responders to the TSL or NPK epitope carried the relevant DR1 or DR11 allele, so these epitopes may have at least one other restriction element.

ther preloaded with EBNA1 protein or pulsed with the NPK epitope peptide. Recognition was again blocked by the anti-DR MAb, and assays on peptide-loaded HLA-matched LCL stimulators identified HLA DR11 as the restriction element. In the same way, HLA-DR1-restricted $CD4^+$-T-cell clones were isolated from donor 2 and shown to be specific for the previously identified TSL epitope; interestingly, some of the TSL-specific clones showed cytolytic as well as proliferative responses to epitope-loaded target cells, whereas clones reactive to the MVF and NPK epitopes displayed proliferative but not cytolytic function (data not shown).

Having thus validated the ELISPOT screening as a means of identifying authentic $CD4^+$-T-cell memory, we extended the screening to a wider panel of donors. Of 26 EBV-seropositive donors tested in this way, positive responses to one or more peptide pools were observed in 19 individuals. In each of these cases, the responses could be mapped to an individual 20-mer or 15-mer epitope. Overall, this allowed 15 individual $CD4^+$ epitopes to be identified within the EBNA1 protein sequence. The epitopes are listed in Table 1 along with either their known HLA class II restricting alleles (from functional analysis of clones) or potential restricting alleles from HLA-DR and -DQ typing data (see Table 1, footnote *b*). Note that we never observed responses to peptide epitopes from the GAr domain of EBNA1. In fact, all but one of the identified epitopes lay within what appears to be an immunodominant 210-aa C-terminal region of the protein (aa 403 to 613); four of the epitopes within this region (aa 485 to 499, 475 to 489, 515 to 528, and 529 to 543) reflected common responses recognized by five or more donors. As a control in these screening assays, we also studied five EBV-seronegative individuals and found no significant response to any of the EBNA1 peptide pools.

Responses to variant EBNA1 epitope peptides. It is known that a significant fraction of healthy Caucasian donors carry an EBV strain with an EBNA1 allele distinct from that present in the prototype B95.8 strain. This allele carries 13 amino acid changes vis-à-vis B95.8 and is referred to as the Q/T variant on the basis of signature changes at positions 16 and 487 (16). We therefore extended the peptide screen to include 22 variant peptides incorporating all of the potentially new epitope sequences within Q/T EBNA1. All seven EBV-seropositive donors who had given no response to B95.8 peptide pools were tested in this way, as were four seronegative donors as controls. The latter again gave uniformly negative results, while responses to two or more Q/T sequence peptides were detected from three of the seropositive donors, identifying a total of three variant sequence-specific epitopes (Table 1). Sequencing of the resident EBV strains from PBMCs of these donors confirmed the presence of a Q/T EBNA1 allele (data not shown). The results from one such responder, donor 4, are shown in Fig. 4. This donor reproducibly gave no significant responses in a screen using B95.8 peptide pools (Fig. 4, upper panel) but showed a clear response to two of the variant peptides, aa 514 to 533 and 589 to 608 (Fig. 4, lower panel). The peptide from aa 514 to 533 was in fact recognized by all three responders identified in the screening assays with Q/T sequence peptides. Interestingly, this peptide contains the variant version of the DR1-restricted TSL epitope, and all three responders proved to be DR1 positive and to recognize the minimized variant version of the TSL epitope sequence from aa 515 to 527. By contrast the sequence from aa 589 to 608

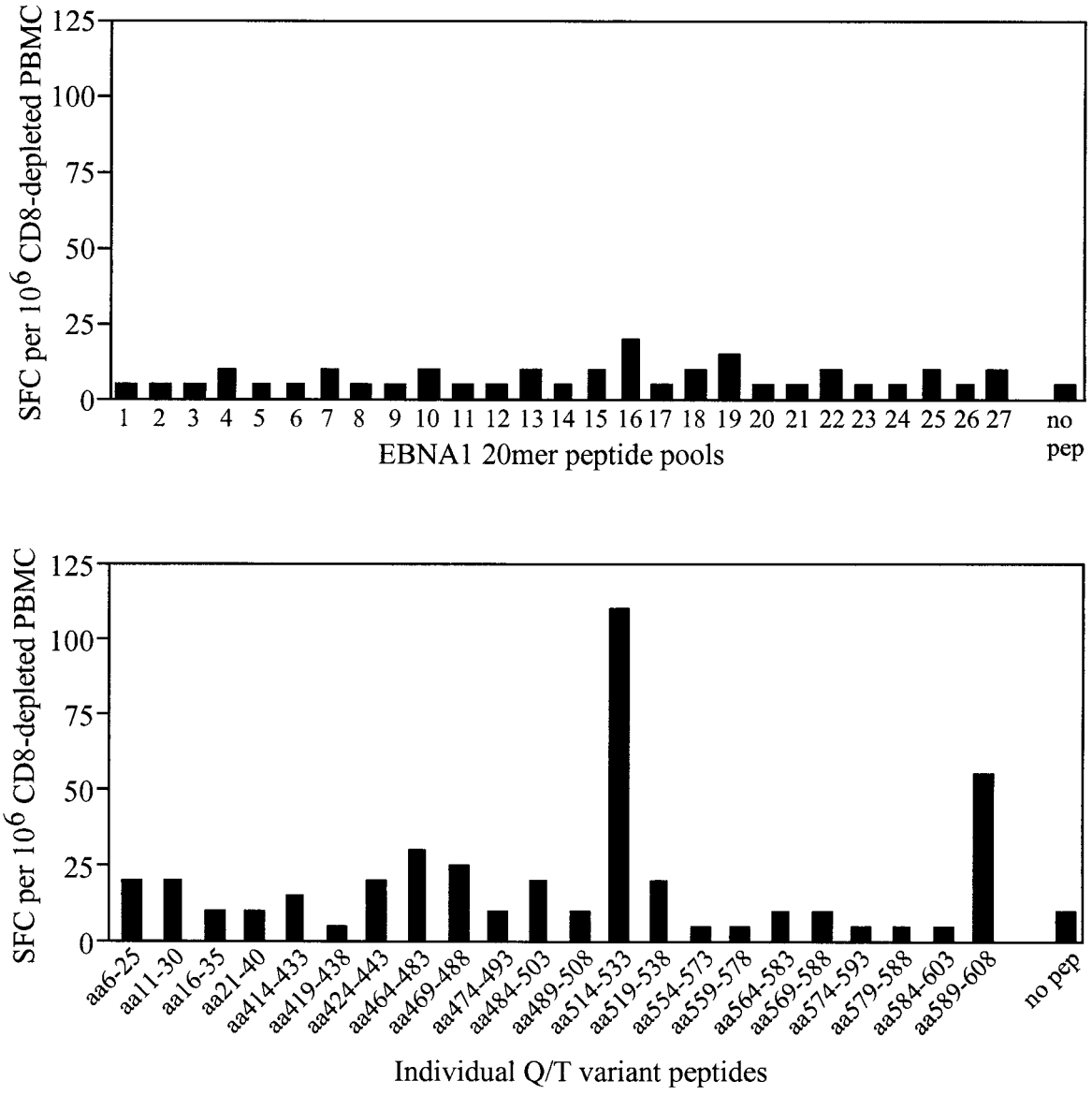

FIG. 4. Identification of CD4+-T-cell epitopes in the Q/T EBNA1 variant by peptide-induced IFN-γ release. CD8-depleted PBMCs from donor 4 were screened in an ELISPOT assay against B95.8 EBNA1 peptides, using 20-mers overlapping by 15 aa, as pools of three as for Fig. 1 (upper panel) or against 22 individual peptides incorporating all of the potential new epitopes within the Q/T variant EBNA1 protein (lower panel). Results are expressed as in Fig. 1.

(Fig. 4) and the other variant peptide sequence identified (aa 424 to 443) appear to represent Q/T EBNA1-specific epitopes, since responses to the equivalent sequences had not been observed in the original B95.8 peptide assays.

We noted that 10 of the 15 EBNA1 epitopes already identified using B95.8 sequence peptides (epitopes RPF, VPP, NPK, LRA, VYG, TSL, NLR, LRE, MVF, and VLK in Table 1) were altered at one or two amino acid residues in the Q/T EBNA1 sequence. All EBV-seropositive donors who had responded to B95.8 peptides in the original assays were therefore retested against the entire panel of Q/T variant peptides, including the variant versions of the original B95.8 epitopes to which they had responded initially. Interestingly, we noted cross-reactive responses in every case, indicating that despite small sequence variations many CD4+-T-cell epitopes in EBNA1 were antigenically conserved between the two EBNA1 alleles. This second screen did not reveal any responses to additional Q/T-specific epitopes (data not shown).

Identification of EBNA3C-derived CD4+-T-cell epitopes. Using the same ELISPOT screening approach as for EBNA1, we proceeded to analyze the CD4+-T-cell response to the B95.8 strain EBNA3C protein, which is known to be a dominant target of the CD8+ response. Synthetic peptides representing the entire 992 aa of the EBNA3C protein were available as 15-mer peptides overlapping by 10 aa. From the 18 virus-positive healthy donors screened, we observed IFN-γ secretion induced by specific EBNA3C peptide pools in 13 donors. Each of these responses again mapped to an individual

TABLE 2. $CD4^+$-T-cell epitopes identified in EBNA3C[a]

EBNA3C aa	Epitope sequence	No. of responders	Potential restriction[b]	
			DR	DQ
66–80	NRGWMQRIRRRRRRR	2	1, 13	5, 6
141–155	ILCFVMAARQRLQDI	1	1, 13	5, 6
386–400	SDDELPYIDPNMEPV	6	1	
401–415	QQRPVMFVSRVPAKK	1	11, 14	3, 5
546–560	QKRAAPPTVSPSDTG	1	1, 13	5, 6
586–600	PPAAGPPAAGPRILA	1	1, 13	5, 6
626–640	PPVVRMFMRERQLPQ	1	11, 14	3, 5
649–660	PQCFWEMRAGREITQ	2	None	None
916–930	PSMPFASDYSQGAFT	3	4	
961–986	AQEILSDNSEISVFPK	4	[4]	[2, 3, 5]

[a] The sequence of EBNA3C is derived from the B95.8 prototype strain of EBV.
[b] Potential restriction elements were identified as described for Table 1. None, responders had no shared DR or DQ allele.

15-mer within the pool, and in that way a total of 10 epitopes were identified. These data are summarized in Table 2, showing a list of epitopes along with their potential restriction elements (from HLA-DR and -DQ typing results). Again, some of the EBNA3C epitopes were recognized by several donors. In particular, six donors, all sharing the DR1 allele, responded to the sequence SDDELPYIDPNMEPV (designated SDD; aa 386 to 400), and three donors, all sharing the DR4 allele, responded to the sequence PSMPFASDYSQGAFT (designated PSM; aa 916 to 930). Again, some peptides (for example, aa 961 to 986) were recognized by multiple donors without a common DR or DQ restriction element. The magnitude of these EBNA3C epitope-specific responses (70 to 200 SFC per 10^6 CD8-depleted PBMCs) was within the range seen in the EBNA1 epitope screen.

Identification of LMP1- and LMP2-derived $CD4^+$-T-cell epitopes. We then extended the screening using both 20-mer (overlapping by 15 aa) and 15-mer (overlapping by 10 aa) peptide pools, covering both the LMP1 and LMP2 sequences. A much lower frequency of positive responses was observed in such assays. In total, only 5 of 53 seropositive donors were found to respond to LMP1 peptides, identifying three epitopes in all. Likewise, only 7 of 45 seropositive donors were found to respond to LMP2 peptides, and only four epitopes were identified. Table 3 presents these epitope sequences as either 20-mers or 15-mers. Any one individual only ever responded to a single epitope in either protein, but where positive responses were observed, their magnitude (40 to 204 and 58 to 238 SFC per 10^6 CD8-depleted PBMCs, respectively) generally lay within the broad range seen in the EBNA1 or EBNA3C peptide screening assays. In many cases where donors lacked detectable LMP1- or LMP2-specific reactivity, assays conducted at the same time with EBNA1 peptides as a positive control confirmed the presence of EBNA1-specific responses in the same PBMC preparation.

DISCUSSION

The present study of $CD4^+$-T-cell responses to EBV latent-cycle antigens was motivated by the presumed role of $CD4^+$ T-helper 1 cells (conventionally assessed by IFN-γ production) in the maintenance of $CD8^+$-T-cell immunity in vivo and possibly also as direct antiviral effectors in their own right (8, 34, 40, 49). We were particularly interested in the relative immunogenicities of the different latent-cycle proteins for the $CD4^+$ response, since these proteins show a marked hierarchy of immunodominance for $CD8^+$ T cells, which can be summarized as EBNA3A, -3B, -3C > EBNA1 > LMP2 ≫ EBNA2, EBNA-LP, LMP1 (38). An earlier study, using $CD4^+$ blastogenesis and IFN-γ production in response to antigen-expressing DCs as an assay of responsiveness, had suggested that both EBNA1 and LMP1 were strong inducers of $CD4^+$-T-cell immunity, with generally weak responses to the EBNA3 proteins and to LMP2 (32). The present work approached the same question using synthetic overlapping peptides spanning the sequences of EBNA1, EBNA3C, LMP1, and LMP2 as the stimulus and IFN-γ production as the readout. The overall results, summarized in Table 4, clearly show that EBNA1- and EBNA3C-specific responses are detectable in the majority of healthy EBV-seropositive individuals, whereas responses to LMP1 and LMP2 are much rarer.

The first set of experiments using peptide pools of the B95.8 strain EBNA1 sequence (Fig. 1 and 2) detected responses in 19 of 26 individuals and led to the identification of 15 $CD4^+$-T-cell epitopes (Table 1). We believe that these responses from ELISPOT assays do indeed represent authentic EBV-specific immunity, since (i) EBV-seronegative donors did not show any significant responses in the screening assays, and (ii) in three immune donors studied in detail, HLA class II-restricted

TABLE 3. $CD4^+$-T-cell epitopes identified in LMP1 and LMP2[a]

Antigen and aa	Epitope sequence	No. of responders	Potential restriction[b]	
			DR	DQ
LMP1				
130–144	LWRLGATIWQLLAFF	1	1, 4	1, 3
212–226	SGHESDSNSNEGRHH	3		2
340–354	TDGGGGHSHDSGHGG	1	ND[c]	ND
LMP2				
149–163	STVVTATGLALSLLL	2	1, 13	5
169–182	SSYAAAQRKLLTPV	2	1, 13	5, 6
224–243	VLVMLVLLILAYRRRWRRLT	2	None	None
385–398	STEFIPNLFCMLLL	1	ND	ND

[a] The sequences of LMP1 and LMP2 are derived from the B95.8 prototype strain of EBV.
[b] Potential restriction elements were identified as described for Table 1.
[c] ND, not determined.

TABLE 4. Summary of EBV-specific CD4+-T-cell responses in EBV-seropositive donors[a]

Antigen	No. of donors	No. of responders	No. of epitopes identified
EBNA1	26	19 + 3 var	15 + 2 var
EBNA3C	18	13	10
LMP1	53	5	3
LMP2	45	7	4

[a] Responses were mapped using the B95.8 strain sequences, with the exception of EBNA1, where addition analysis included the Q/T variant strain (var).

CD4+-T-cell clones with the predicted epitope specificity could be generated by stimulating their PBMCs in vitro with purified EBNA1 protein, and the derived clones also recognized autologous antigen-presenting cells preexposed to EBNA1 protein (Fig. 3). Interestingly all three epitopes identified by clonal analysis proved to be HLA-DR restricted, as have a number of other reported CD4+-T-cell clones against EBV antigens (24, 32, 35, 47). However, not all donors in our panel who responded to the DR11-restricted NPK epitope or to the previously published DR1-restricted TSL epitope carried the expected DR11 or DR1 allele, implying that these EBNA1 peptides can also be presented by other restriction elements; such promiscuity is well documented for CD4+ epitopes in various systems (10, 17). We also stress that in Tables 1 to 3, in cases where there are no functional studies on epitope-specific clones, potential DR or DQ restriction elements are listed for reference purposes only. The absence of a shared DR or DQ allele among all of the responders to a particular epitope might reflect promiscuity of the epitope or, possibly, restriction through a shared HLA-DP allele (29).

Because there is some sequence variation at the EBNA1 locus among Caucasian EBV strains, we extended the analysis to include variant peptide sequences covering each of the 13 amino acid changes between B95.8 and the other common EBNA1 allelic sequence, Q/T (16). Interestingly, this revealed EBNA1-specific responses in three of seven EBV-seropositive donors who had not given responses to the B95.8 peptide pools (Fig. 4; Table 1) and identified three additional epitopes, one of which represented the variant version of the B95.8 TSL epitope and the others of which were Q/T specific (i.e., there were no examples of responses to the equivalent B95.8 peptide sequences). Of 15 epitopes recognized in the B95.8 screening assays, 5 were completely conserved in the Q/T allelic sequence and 10 were altered in one or two residues. Even in the latter cases, however, further experiments showed that both the B95.8 and Q/T peptides were recognized in ELISPOT assays by PBMCs from donors identified as B95.8 responders in the original screening assays. We infer that many, though not all, CD4+ epitopes in EBNA1 are antigenically conserved between the common EBNA1 alleles in Caucasian populations. It is striking that all but 1 of the 17 CD4+ epitopes identified overall in EBNA1 map within a fragment of the protein (aa 403 to 613) against which much of the humoral response appears to be directed (9, 31); whether this is coincidental or an example of linked T- and B-cell epitopes (15) remains to be determined. Interestingly, however, we did not observe any CD4+-T-cell responses to peptides from the other region of the protein recognized by the antibody response, that is, the GAr domain (11).

A subsequent series of experiments with EBNA3C peptides also showed evidence of responsiveness in the majority of donors tested. Arguably, these assays underestimated the frequency of EBNA3C-specific responses, since the screening was carried out only with 15-mer, and not 20-mer, peptides. Our experience with EBNA1 screening indicates that at least six of the EBNA1 epitopes were identified only by 20-mer peptide screening and could not be mapped to component 15-mers. For these reasons we suggest that EBNA3C may be as immunogenic as EBNA1 for CD4+ T-helper 1 responses. Again we identified some EBNA3C epitopes, for example, SDD, PSM, and AQE, which were recognized by multiple donors with, in two of the three cases, a common DR allele (Table 2). Interestingly, many of the EBNA3C epitopes identified here lay within a fragment of the 992-aa protein (aa 376 to 668) which another recent study found to be the optimal fusion protein fragment for eliciting CD4+-T-cell proliferation in vitro (44). However, neither that study nor the present work detected significant CD4+-T-cell responses to the 13-aa repeat region of EBNA3C (aa 741 to 779) which has been reported by others to contain a number of promiscuous overlapping CD4+ epitopes as well as being an immunodominant target for the antibody response (35).

The frequency of CD4+ epitope detection in EBNA1 and EBNA3C is in sharp contrast to the results from LMP1 and LMP2 peptide screens (Table 3). In the latter cases, even though the donor groups were significantly larger than for EBNA epitope screening, only 9 and 16% of donors tested gave responses, and then only to a single LMP1 or LMP2 epitope in any one individual. These assays were conducted using both 20-mer and 15-mer peptide pools for each antigen and so are unlikely to have underestimated responsiveness. Furthermore, in independent assays, the same 15-mer peptide pools were capable of detecting CD8+-T-cell memory to LMP1 and, in particular, to LMP2 epitopes, indicating that the hydrophobic nature of many of these sequences was not a bar to their operation in ELISPOT assays (P. Meij et al., unpublished data). Note that there is some sequence polymorphism in LMP1 (42) and, although it has been less well studied, in LMP2 (3) among Caucasian strains. It is possible, therefore, that screening on B95.8 peptides could have underestimated the incidence of CD4+-T-cell responses to LMP1 and/or LMP2 in our donors. However, we analyzed the EBV strain carried by a subset of donors who did not respond in the LMP1 assays, amplifying the resident LMP1 sequence across a known polymorphic region of the gene. This showed that most such donors carried an LMP1 sequence that was close to the B95.8 prototype (data not shown), as would be expected from the distribution of B95.8-like LMP1 alleles in earlier work on Caucasian EBV isolates (groups A and B in reference 42). Based on these observations and on the fact that many EBNA1 CD4+ epitopes are conserved antigenically between different viral strains, sequence variation is unlikely to explain the low level of positive results in the LMP peptide screens. We conclude that, in contrast to EBNA1 and EBNA3C, LMP1 and LMP2 are poorly immunogenic for CD4+ T-helper 1 responses. These findings contrast somewhat with those of Munz et al. (32), who, using in vitro stimulation with B95.8 strain EBV antigen-ex-

pressing DCs, reported proliferative and IFN-γ responses to EBNA1 in 10 of 10, to LMP1 in 6 of 10, and to EBNA3C in 1 of 10 donors tested. It is possible that the differences between the two studies reflect differences in experimental approach. However, another possible factor is the relatively low number of donors assayed in the earlier work (32).

We conclude from the present work that EBV latent proteins do indeed show a marked hierarchy of immunodominance for $CD4^+$-T-cell responses (namely, EBNA1, EBNA3C $\gg$ LMP1, LMP2) and that this is different from the hierarchy already identified for $CD8^+$ responses (EBNA3C > EBNA1 > LMP2 $\gg$ LMP1). It is also interesting that the absolute frequency of $CD4^+$-T-cell memory to the EBV latent-cycle epitopes defined here seems to lie within a range of 50 to 280 IFN-γ-producing cells per 10^6 PBMCs (values calculated from the ELISPOT data to allow for the effect of CD8 depletion). This is noticeably lower and narrower than the corresponding frequency range for latent epitope-specific $CD8^+$ T cells (50 to 2,500 IFN-γ-producing cells per 10^6 PBMCs), again measured by ELISPOT assay (5, 46). A similar trend is also apparent during the primary response to EBV infection in infectious mononucleosis, where there is a preferential amplification of $CD8^+$ T cells (36), the majority of which are virus specific (7), leading to an inversion of CD4/CD8 ratios in peripheral blood. Such observations may reflect the fact that $CD4^+$ T cells are predominantly regulators of immune responses and may therefore be subject to less expansion in vivo than are $CD8^+$ effector populations.

We also point out that the present assays of $CD4^+$-T-cell responsiveness are restricted to IFN-γ release and are presumably detecting T-helper 1-like activity of the kind thought to favor $CD8^+$-T-cell response induction (1). Assays of T-helper 2-associated cytokines such as IL-4, IL-5, and IL-13, which are thought to reflect help for humoral responses, may provide a different picture, but reports to date leave this issue unresolved (4, 44). Nevertheless, it is striking that the relative immunogenicity of EBV latent proteins for $CD4^+$ T cells seen in the present work is not too dissimilar from immunogenicity as defined serologically. Thus, EBNA1 is consistently recognized by sera from healthy virus carriers and EBNA3C is less so (38, 43), whereas antibodies to the LMPs are very rare and, even where detectable, present at very low titers (27, 30, 41, 48), except in the special case of antibodies to LMP2 in NPC patients (13, 27). It may be that the hydrophobic nature of the LMPs renders these proteins less susceptible to uptake and processing by DCs in vivo (2) and hence less well presented to the $CD4^+$-T-cell repertoire. Additionally, there is evidence that LMP1 itself contains a motif which can impair T-cell responses (12). Whatever the basis of these differences in immunogenicity for $CD4^+$ T cells among the latent-cycle proteins, the fact that such differences exist has important implications for immunotherapeutic strategies. Thus, vaccines aiming to boost T-cell responses to antigens expressed in EBV-positive malignancies may well need to include appropriate $CD4^+$ as well as $CD8^+$ epitopes. In the context of EBV-positive malignancies such as Hodgkin's disease or NPC, LMP2 would be the preferred $CD8^+$ target antigen (27, 41). However, on the basis of the present results, LMP2 seems unlikely to serve as a reliable source of T-helper epitopes. Vaccine constructs which include relevant EBNA1 sequences could overcome that difficulty.

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