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CD4⁺ T-Cell Responses to Epstein-Barr Virus (EBV) Latent-Cycle Antigens and the Recognition of EBV-Transformed Lymphoblastoid Cell Lines

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There is considerable interest in the potential of Epstein-Barr virus (EBV) latent antigen-specific CD4⁺ T cells to act as direct effectors controlling EBV-induced B lymphoproliferations. Such activity would require direct CD4⁺ T-cell recognition of latently infected cells through epitopes derived from endogenously expressed viral proteins and presented on the target cell surface in association with HLA class II molecules. It is therefore important to know how often these conditions are met. Here we provide CD4⁺ epitope maps for four EBV nuclear antigens, EBNA1, -2, -3A, and -3C, and establish CD4⁺ T-cell clones against 12 representative epitopes. For each epitope we identify the relevant HLA class II restricting allele and determine the efficiency with which epitope-specific effectors recognize the autologous EBV-transformed B-lymphoblastoid cell line (LCL). The level of recognition measured by gamma interferon release was consistent among clones to the same epitope but varied between epitopes, with values ranging from 0 to 35% of the maximum seen against the epitope peptide-loaded LCL. These epitope-specific differences, also apparent in short-term cytotoxicity and longer-term outgrowth assays on LCL targets, did not relate to the identity of the source antigen and could not be explained by the different functional avidities of the CD4⁺ clones; rather, they appeared to reflect different levels of epitope display at the LCL surface. Thus, while CD4⁺ T-cell responses are detectable against many epitopes in EBV latent proteins, only a minority of these responses are likely to have therapeutic potential as effectors directly recognizing latently infected target cells.

Epstein-Barr virus (EBV), a herpesvirus with B-cell growth transforming ability and lymphomagenic potential, provides one of the most instructive systems in which to study T-cell responses to viral infection in humans (11, 25). Primary infection is usually asymptomatic but in some individuals can present as infectious mononucleosis, a self-limiting lymphoproliferative disease where the symptoms are coincident with the appearance of a large reactive T-cell response. Following primary infection, the virus is carried for life as a latent infection of the circulating memory B-cell pool (1), with low-level reactivation from latency into virus productive (lytic) infection at oropharyngeal sites. Immune T-cell responses clearly play some role in the maintenance of this virus-host balance since T-cell-immunocompromised individuals show increased virus replication in the oropharynx (4) and an increased risk of EBV-driven B-lymphoproliferative disease (22). One of the outstanding issues to resolve in this respect is the relative contribution made by CD4⁺ and CD8⁺ T-cell responses to this host control.

The HLA class I-restricted CD8 response has attracted the most attention, for two reasons. Firstly, primary T-cell responses seen in the blood of infectious mononucleosis patients are largely of CD8⁺ T-cell origin; indeed, many of these in vivo-primed reactivities have been mapped to epitopes drawn

from EBV lytic- and latent-cycle antigens (28, 29). Secondly, memory T-cell responses reactivated from immune donors by in vitro stimulation with the virus-transformed B-lymphoblastoid cell line (LCL) are likewise dominated by CD8⁺ effectors. Many such effectors recognize epitopes drawn from the latent-cycle proteins that are expressed in all LCLs, namely, nuclear antigens EBNA1, -2, -3A, -3B, -3C, and -LP and latent membrane proteins LMP1 and -2, with responses to EBNA3A-, -3B-, and -3C-derived epitopes frequently in the majority (8, 18). Such CD8 responses to EBV latent antigens are of particular interest because of their ability to recognize and kill virus-transformed B cells in vitro and their therapeutic potential against EBV-driven B-lymphoproliferative disease in vivo (24).

The growing interest in HLA class II-restricted CD4⁺ T-cell responses to the virus reflects not only an appreciation of the general role that CD4⁺ T cells are thought to play in the maintenance of effective CD8 immunity (7, 26, 30) but also the fact that EBV infects target cells in which the HLA class II pathway of antigen presentation is active (33). This raises the possibility that virus-specific CD4⁺ T cells are able to recognize infected cells directly and, if they are, could act (like CD8⁺ T cells) as effectors in their own right. Certainly there are examples where indicator antigens have been expressed endogenously within LCLs and appear to have gained direct intracellular entry into the HLA class II processing pathway (2, 20, 23, 34), in some way bypassing the usual means of HLA class II presentation involving uptake as exogenously acquired antigen (33). The first CD4⁺ T-cell clones to EBV latent pro-

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teins, specific for an EBNA1-derived and an EBNA2-derived epitope, respectively, were identified as rare components of LCL-reactivated memory T-cell preparations (9, 10). Of these, only the EBNA2-specific clone appeared to be capable of recognizing LCLs directly in cytotoxicity assays (10). Since that time, CD4⁺ recall responses to more latent-cycle epitopes have been generated by a variety of protocols, many involving *in vitro* stimulation with dendritic cells either overexpressing antigen from vaccinia virus vectors or preloaded with exogenous antigen or with epitope peptides (13, 17, 21, 27, 32). Although these clones display specificity for antigen in protein loading assays, there are differing reports of their ability to recognize LCLs naturally expressing cognate antigen from the resident EBV genome. It is not clear whether these divergent results reflect technical differences in the way in which the clones are generated *in vitro* or genuine differences in the way in which either individual antigens or individual epitopes are processed and presented. The present study (i) provides detailed CD4 epitope maps for four EBV latent-cycle antigens, EBNA1, -2, -3A, and -3C; (ii) establishes CD4⁺ T-cell clones to 12 selected epitopes from these antigens; and (iii) characterizes these clones in terms of their HLA class II restriction, their functional avidity in peptide titration assays, and their ability to recognize autologous LCL targets in gamma interferon (IFN- γ) release, cytotoxicity, and outgrowth assays.

MATERIALS AND METHODS

Cell preparations and cell lines. Peripheral blood mononuclear cells (PBMCs) were separated from healthy, EBV-immune donors by Ficoll-Hypaque centrifugation into RPMI 1640 medium (Invitrogen) supplemented with 2 mM glutamine, 100 IU of penicillin per ml, 100 μ g of streptomycin per ml, and 5% autologous serum. Where specified, PBMCs were depleted of CD8⁺ T cells with CD8 Dynabeads (Dyna) in accordance with the manufacturer's recommended protocol. Dendritic cells were prepared as previously described (13), by 6-day culture of adherent PBMCs in the above medium supplemented with granulocyte-macrophage colony-stimulating factor and interleukin-4, and then matured for 24 h in 50 ng of tumor necrosis factor alpha per ml. EBV-transformed LCLs were prepared with prototype 1 strain B95.8 or prototype 2 strain Ag876. All LCLs were cultured in medium (as described above) containing 10% fetal calf serum, and all assays involved B95.8 virus-transformed LCLs unless otherwise stated.

Synthetic peptides and protein preparations. Epitope peptides were synthesized by 9-fluorenylmethoxycarbonyl chemistry (Alta Bioscience, University of Birmingham) and dissolved in dimethyl sulfoxide (DMSO), and their concentrations were determined by biuret assay. The preparation and purification of baculovirus-expressed EBNA1 protein have been described elsewhere (13). EBNA2 protein and appropriate control preparations made in baculovirus expression systems were kindly provided by Friedrich Grässer, Homburg-Saar, Germany (6). EBNA3A and EBNA3C protein preparations and controls were likewise made from appropriate baculovirus vectors by infection of insect cells and purification of nuclear proteins.

ELISPOT assays. CD8-depleted PBMC preparations were tested in ELISPOT assays of IFN- γ release as previously described (13), with pools of overlapping peptides (three or four peptides per pool) from the antigen sequence of interest, followed by assays on individual peptides within positive pools. The EBNA1 and EBNA2 panels were 20-mer peptides (overlapping by 15 residues), and the EBNA3A and -3C panels were 15-mer peptides (overlapping by 10 residues), all based on the B95.8 EBV strain sequence.

In vitro reactivation protocols. CD4⁺ T-cell clones were generated from cultures of CD8-depleted PBMCs 7 days after *in vitro* stimulation either with 5 μ M epitope peptide directly loaded onto the cells for 1 h or with autologous dendritic cells loaded either with peptide or with protein (13) or (as for CD8⁺ T-cell activation) with gamma-irradiated autologous LCLs (18). On day 7, cells were cloned by limiting dilution at 3 cells per well on autologous gamma-irradiated LCLs (10⁴/well) loaded with the relevant peptide at 5 μ M and allogeneic gamma-irradiated, phytohemagglutinin-treated PBMCs (10⁵/well) in *in*

terleukin-2-supplemented medium with 5% autologous serum as previously described (12). Growing microcultures were screened for peptide reactivity by IFN- γ enzyme-linked immunosorbent assay (ELISA), and selected clones were expanded as described above with fetal calf serum-supplemented medium.

ELISAs of IFN- γ release and MAbs blocking. Cloned T cells were incubated in U-bottom or V-bottom microtest plate wells with standard numbers of autologous, HLA-matched, or HLA-mismatched LCLs that were either unmanipulated or prepulsed for 1 h with 5 μ M peptide (or an equivalent concentration of DMSO solvent as a control) or preexposed in serum-free medium for 2 h to specific EBV antigen preparations (or to control antigen preparations) and then washed. The supernatant medium harvested after 18 h was assayed for IFN- γ by ELISA (Endogen) in accordance with the manufacturer's recommended protocol. In blocking assays, LCLs were preincubated with monoclonal antibodies (MAbs) specific for HLA-DR (L243; ATCC clone HB-55), HLA-DQ (SPV-L3; Serotec), and HLA-DP (B7.21; kindly provided by A. M. de Jong, Leiden University, Leiden, The Netherlands) for 1 h before addition of T-cells to the assay.

Chromium release assays. CD4⁺ T-cell clones were tested for killing of target cells at known effector/target ratios in 5- and 18-h chromium release assays, and results were expressed as percent specific lysis of the target line. Targets were HLA-matched or HLA-mismatched LCLs preexposed for 1 h to 5 μ M epitope peptide or to an equivalent concentration of DMSO solvent as a control.

Outgrowth assays. Target LCLs (HLA matched and HLA mismatched, either unmanipulated or prepulsed with 5 μ M epitope peptide and then washed) were serially diluted into replicate U-bottom microtest plate wells at 10⁴ to 300 per well, and T cells were added to half of the replicates at 10⁴/well. Plates were incubated at 37°C in 5% CO₂ with weekly refeeding, and LCL outgrowth was scored after 4 weeks. Results are expressed as the minimum seeding of LCLs required for successful outgrowth.

RESULTS

CD4 epitope mapping of EBV latent-cycle antigens. In initial epitope mapping experiments, we increased the number of EBV-seropositive donors screened for CD4⁺ T-cell reactivity to EBNA1 and EBNA3C peptide panels (13) and extended the analysis to new peptide panels covering the primary sequences of EBNA2 and EBNA3A. The results of these assays are summarized in Fig. 1, showing all of the individual peptides against which CD4⁺ T-cell memory was detected; numbers refer to the coordinate of the first amino acid in the peptide sequence. For each peptide, the histogram indicates the overall percentage of seropositive donors who made a detectable response. These results, based on the screening of 23 to 32 donors per peptide panel, confirm that a 200-amino-acid stretch in the C-terminal half of EBNA1 is a rich source of CD4 epitopes, with 75% of the donor cells reactive to 1 or more of the 17 epitopes in this area. In addition 70% of the donor cells also responded to 1 or more of 11 epitopes in the much larger EBNA3C protein. Of note are individual epitopes, for example, EBNA1 515 (TSL) and EBNA3C 386 (SDD), that were recognized by about 30% of the donors tested. Although almost as large as EBNA3C, the 944-amino-acid EBNA3A protein proved to be much less immunogenic to the CD4⁺ T-cell response, with only three epitope peptides detected and <25% of the donors reactive to any one of these epitopes. Screening of EBNA2, a protein with a unique sequence similar in size to that of EBNA1, revealed only six epitopes; however, one of these (EBNA2 276, PRS) was recognized by 40% of the donors tested, and this was largely responsible for the overall frequency of EBNA2-reactive donors reaching 65%.

Throughout these ELISPOT assays, we consistently found that the size of CD4 epitope-specific memory populations in peripheral blood lay between the detection threshold of 30 spot-forming cells (SFC) and a maximum value of 350 SFC per 10⁶ CD8-depleted PBMCs. This range is 10-fold smaller than

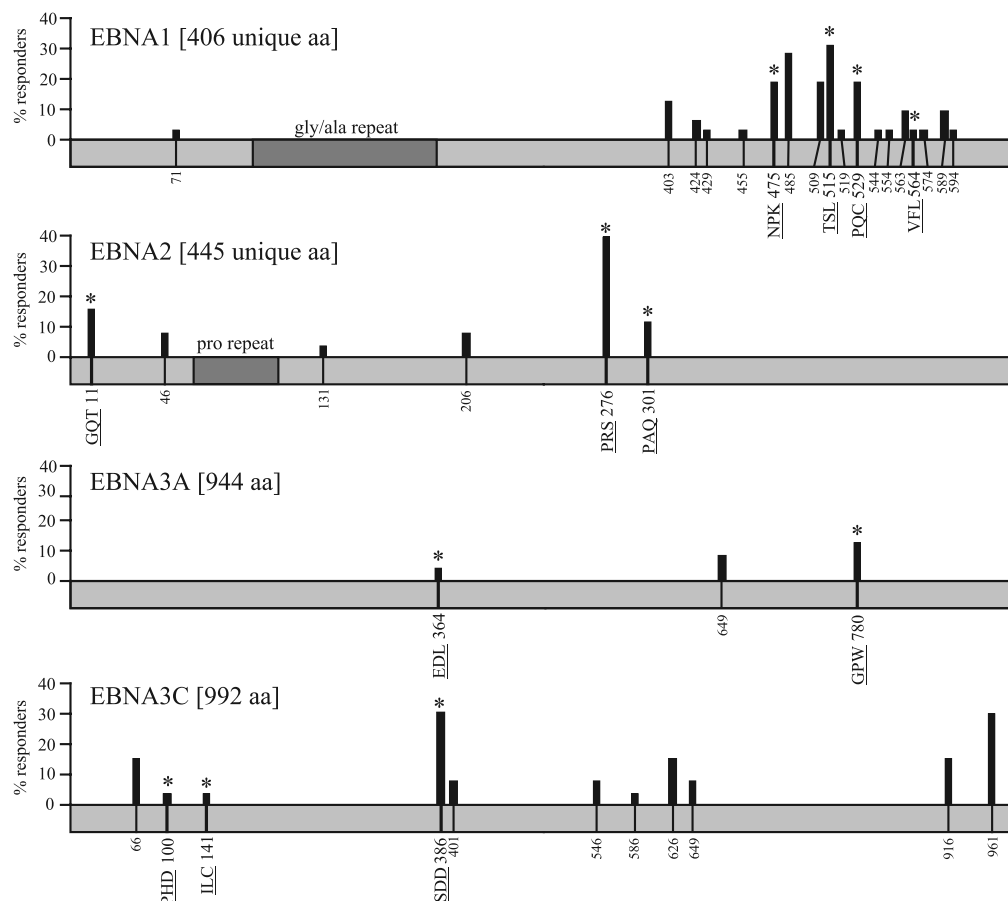


FIG. 1. Mapping of CD4⁺ epitopes within the primary sequences of EBNA1, -2, -3A, and -3C. The position of each epitope is identified by a number that refers to the coordinate of the first amino acid in the protein as encoded by EBV strain B95.8. The bar above each epitope position indicates the percentage of all EBV-seropositive donors tested who responded to the relevant epitope peptide in ELISPOT assays. Epitopes against which CD4⁺ T-cell clones were later derived are also identified by a three-letter code corresponding to the first three amino acids of the epitope sequence and by an asterisk above the bar. For each protein, the number of unique amino acids (aa) is shown; this excludes the 233-amino-acid glycine-alanine repeat domain (residues 93 to 325) for EBNA1 and the 42-amino-acid polyproline repeat domain (residues 59 to 100) for EBNA2.

the equivalent range of EBV latent epitope-specific CD8⁺ T-cell memory, which, for immunodominant CD8 epitopes, can reach up to 3,000 SFC/10⁶ PBMCs (5, 31). Furthermore, the size of the CD4 response to any particular epitope peptide varied between individual donors across the full range, again in contrast to certain immunodominant CD8 epitopes, which in donors with the appropriate HLA type consistently produce large responses in the ELISPOT assay (5, 31; data not shown).

Antigen specificity of CD4⁺ T-cell clones generated by epitope-peptide stimulation. On the basis of the above assays, we selected 12 epitopes (4 from EBNA1, 3 from EBNA2, 2 from EBNA3A, and 3 from EBNA3C) against which to generate CD4⁺ T-cell clones. These epitopes included some that were recognized by a large proportion of donors, for example, EBNA1 515 (TSL), EBNA2 276 (PRS), and EBNA3C 386 (SDD), and others that represented relatively rare responses.

Using donors with detectable CD4⁺ T-cell memory to the individual peptides in ELISPOT assays, we generated CD4⁺ T-cell clones by limiting-dilution cloning of PBMCs 7 days after peptide stimulation *in vitro*. All proliferating cultures were first screened for peptide reactivity in ELISAs of IFN- γ

release, and peptide-specific clones were checked for antigen specificity by testing on autologous antigen-presenting cells that had been loaded with an exogenous supply of the relevant EBV protein preparation. Figure 2 shows representative results from these antigen specificity assays, with clones raised against the EBNA1 515 (TSL), EBNA2 276 (PRS), EBNA3A 780 (GPW), and EBNA3C 386 (SDD) epitopes. In each case, we were able to confirm that the clones recognized not only the epitope peptide but also processed antigen, whereas there was no significant response to control epitope or antigen preparations. Specific recognition of antigen-loaded cells clearly required processing and could not be ascribed to contamination of the protein preparation with preformed peptide, since prefixing the presenting cells eliminated the response to antigen but not that to exogenously loaded peptide (data not shown). Figure 2 also shows representative MAb blocking assays in which peptide-loaded autologous cells were used as targets in the presence of high concentrations of HLA-DP-, -DQ-, and -DR-specific MAbs. The results show that the TSL-, PRS- and GPW-specific clones were all restricted through an HLA-DR allele and the SDD-specific clones were all restricted through

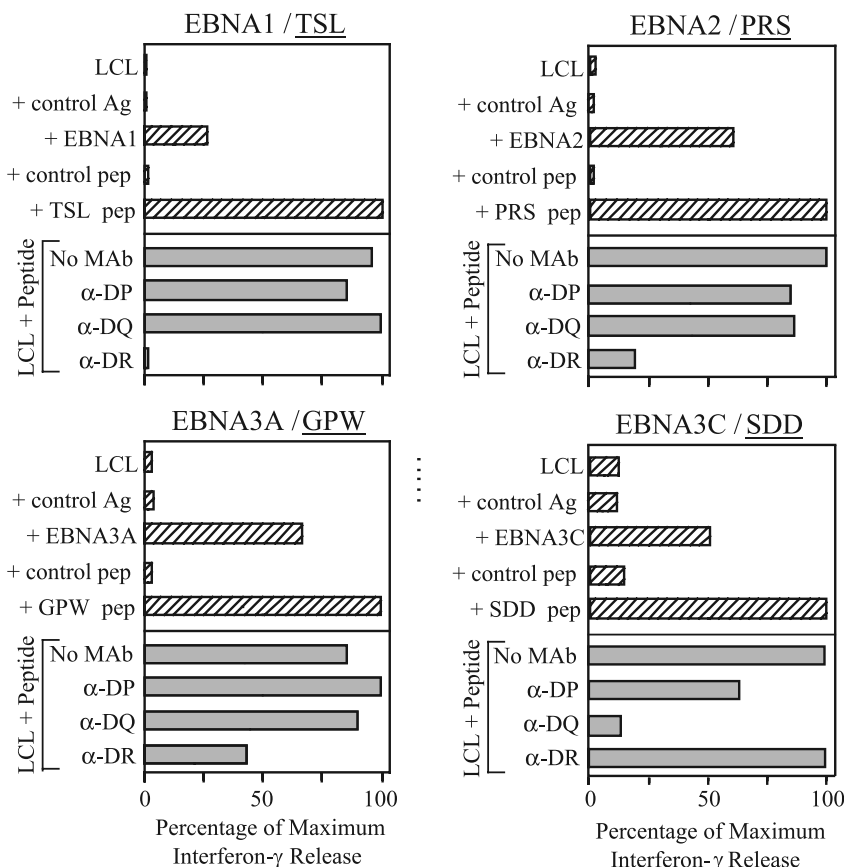


FIG. 2. Antigen (Ag) specificity of CD4⁺ T-cell clones raised against the TSL (EBNA1 515), PRS (EBNA2 276), GPW (EBNA3A 780), and SDD (EBNA3C 386) epitopes. (Top panels) Clones (500 T cells per well) were stimulated overnight with autologous LCLs that were either unmanipulated, prepulsed for 1 h with 5 μM epitope peptide (pep; or an equivalent concentration of control peptide), or preexposed in serum-free medium for 2 h to specific EBV antigen preparations (or to a control antigen) and then washed before the assay. Responses are expressed as percentages of the maximum IFN-γ induced by peptide-loaded target cells in each case. (Bottom panels) The results shown in the bottom panels are from a separate experiment in which autologous LCLs were preexposed for 1 h to epitope peptide at a concentration mediating half-maximal responses, washed, and then incubated for a further hour in the presence of MAb to HLA-DP, HLA-DQ, or HLA-DR or in medium as a control (No MAb) before the addition of 500 T cells to the cell suspension. Results are expressed as described above. Note that assays conducted with the PRS-specific clones were carried out with LCLs transformed with the type 2 Ag876 EBV strain, in which the PRS epitope sequence is mutated (10), thereby reducing background LCL recognition to zero.

an HLA-DQ allele. In each case, the relevant allele could be determined by screening partially HLA-matched target cells for the ability to present the peptide. This identified the restricting alleles as HLA-DR103 for TSL, HLA-DR52 for PRS, HLA-DR1 for GPW, and HLA-DQ5 for SDD (data not shown). In this way, restriction was mapped to defined HLA-DR or HLA-DQ alleles for 10 of the 12 epitopes being studied and to HLA-DP alleles for the other 2 epitopes (see Table 1).

LCL recognition by CD4⁺ T-cell clones: IFN-γ release assays. For the 12 sets of clones reactive to individual epitopes, we then conducted a series of IFN-γ release assays (i) to determine each clone's functional avidity, defined in peptide titration assays as that peptide concentration mediating 50% maximal recognition, and (ii) to determine the efficiency with which each clone recognized the unmanipulated LCL, expressing this as a percentage of the maximal response seen on the same targets loaded with an optimal concentration of epitope peptide. All of the clones generated against the same epitope-HLA allele combination were functionally similar to one an-

other, and so the data for individual epitopes are illustrated with a representative clone in each case.

Figure 3 presents the results for three CD4 epitopes in EBNA1, PQC (529) restricted through HLA-DR14, VFL (564) restricted through an HLA-DP allele, and TSL (515) restricted through HLA-DR103. Peptide titrations (top panels) determined the functional avidities as 15 nM for PQC-specific clones, 30 nM for VFL-specific clones, and 90 nM for TSL-specific clones. The same clones were then assayed at a range of T-cell inputs (500 to 5,000 per well) against the autologous LCL and an HLA-mismatched LCL (seeded at 5 × 10⁴ per well) where the LCL targets had either been preexposed to the cognate peptide at a 5 μM concentration and then washed (middle panels) or left untreated (bottom panels). The PQC-specific clone showed strong recognition of the peptide-loaded autologous LCL, with levels of IFN-γ release increasing with increased T-cell input, and not of peptide-loaded control targets. However, this clone, and several other PQC-specific clones, showed no detectable recognition of the autologous LCL without added peptide. This was also true of clones di-

TABLE 1. Summary of epitope-specific CD4⁺ T-cell clones

Protein and epitope	Epitope coordinates	HLA restriction	Functional avidity ^a (concn [nM])	% Recognition of LCL ^b
EBNA1				
PQC	529–543	DR14	15	0
NPK	475–494	DP	50	0
VFL	564–583	DP	30	0.2
TSL	515–529	DR103	90	3
EBNA2				
PAQ	301–320	DR17	100	3
GOT	11–30	DR4	80	15
PRS	276–295	DR52a	6	1
PRS	276–295	DR52c	7	1
PRS	276–295	DR7	30	15
PRS	276–295	DR52b	3	35
EBNA3A				
GPW	780–799	DR1	30	1
EDL	364–383	DR15	10	4
EBNA3C				
ILC	141–155	DR13	50	0
PHD	100–119	DR16	100	3
SDD	386–400	DQ5	30	7

^a Functional avidity is defined as the concentration of epitope peptide mediating 50% maximal IFN- γ release in peptide titration assays. Values shown are the means of results of assays on several epitope-specific clones (except for PHD and DR52c PRS, where only one clone was available).

^b Recognition of the unmanipulated autologous LCL, as measured by IFN- γ release, is expressed as a percentage of the IFN- γ release seen in the same assay against the same LCL optimally loaded with epitope peptide. The value for each epitope is the mean from assays on several epitope-specific clones (except for PHD and DR52c PRS, where only one clone was available).

rected against a different EBNA1 epitope, NPK (475) (data not shown). By contrast, clones specific for the VFL epitope again responded well to peptide-loaded autologous targets but in this case also showed low-level IFN- γ release in response to the autologous LCL itself. This recognition was reproducible, titrated against the input T-cell number, and also was blocked specifically by the same anti-HLA-DP MAb as had blocked the recognition of exogenously loaded peptide in the earlier assays. However, comparing the observed levels of release of IFN- γ against peptide-loaded and unloaded targets in the same assay gave an efficiency of recognition of the unmanipulated LCL of only 0.2%. Other VFL-specific clones gave similarly low values. Interestingly, CD4 clones to the third EBNA1 epitope, TSL, were also capable of recognizing the untreated autologous LCL, but in this case at higher levels representing around 3% of that seen on peptide-loaded targets. Recognition again titrated against the input T-cell number and was blocked with the appropriate antibody, in this case the anti-DR MAb.

The latter result was of particular interest since different groups have reported either no LCL recognition by TSL-specific CD4⁺ T-cell clones (9) or recognition that appeared to be strong, albeit never directly compared with that seen on peptide-loaded cells (17). To determine whether such differences might have arisen wholly or in part through the use of different in vitro reactivation protocols, we established TSL-specific CD4⁺ T-cell clones by standard peptide stimulation and compared these to clones generated from the same donor by stimulation with EBNA1 protein-loaded dendritic cells. We found

that the two sets of clones were very similar in the above types of assay (data not shown), and indeed their functional avidities were similar to those reported by others for TSL-specific clones reactivated by autologous LCL stimulation (9).

Figure 4 shows corresponding data for CD4⁺ T-cell clones raised against EBNA2 epitope PAQ (301), EBNA3A epitope GPW (780), and EBNA3C epitope SDD (386). These showed functional avidities of 100, 30, and 30 nM, respectively, a range not dissimilar from that seen with the EBNA1 epitope-specific clones. Again, all three epitope-specific reactivities in Fig. 4 showed strong recognition of peptide-loaded autologous (but not HLA-mismatched) LCL targets and also recognized the unmanipulated autologous LCL at levels representing 3% (for PAQ), 1% (for GPW), and 7% (for SDD) of the optimal levels seen on peptide-loaded targets. These values were reproducibly observed both on repeat testing of the particular clones illustrated and on testing of other clones generated against the same epitopes. In this case, we also compared SDD-specific clones generated by standard peptide stimulation against clones produced by autologous LCL stimulation and found that the two sets were again functionally very similar (data not shown).

Similar experiments were conducted on a further five epitopes from the EBNA2, EBNA3A, and EBNA3C proteins, and we again observed that each individual epitope was associated with its own characteristic level of LCL recognition (for a summary, see Table 1). Of particular interest was EBNA2 epitope PRS (276) because it had been recognized by 40% of the seropositive donors tested in ELISPOT assays (Fig. 1) and in an earlier study (21) appeared to be presented in several different HLA class II contexts. On the basis of our ELISPOT screening, we selected four PRS-responsive donors with disparate HLA class II types and from each generated PRS-specific clones that, when tested in MAb blocking assays, were all found to be restricted through an HLA-DR allele. Since all individuals possess two DRB1 alleles and, in some cases, also one or two additional DR alleles (designated DR51, -52a, -52b, -52c, and -53), we screened each set of clones on a large panel of fully DR-typed target cells loaded with the PRS peptide. Figure 5 shows data from a representative range of targets sufficient to map the HLA restricting allele in each case. We found that PRS-specific clones from the four different donors used four different alleles, DR52a, DR52b, DR52c, and DR7.

The four different sets of clones were then tested for functional avidity and for autologous LCL recognition. As shown in Fig. 6, all clones restricted through DR52 alleles had unusually high avidities in peptide titration assays, with 50% end points at 6 nM (DR52a), 3 nM (DR52b), and 7 nM (DR52c), whereas the value of 30 nM for DR7-restricted clones was in the range seen earlier for clones restricted through other DRB1 alleles. Interestingly, all four sets of clones showed detectable recognition of the unmanipulated autologous LCL but at widely different efficiencies. These ranged from 1% of that seen on peptide-loaded targets for the DR52a- and DR52c-restricted clones to 15% for the DR7-restricted clones and to as high as 35% for DR52b-restricted clones. To check the reproducibility of the latter result, we generated PRS-specific clones from a second DR52b-positive donor by standard peptide stimulation and from the same donor by two other in vitro reactivation protocols, namely, stimulation with peptide-loaded dendritic

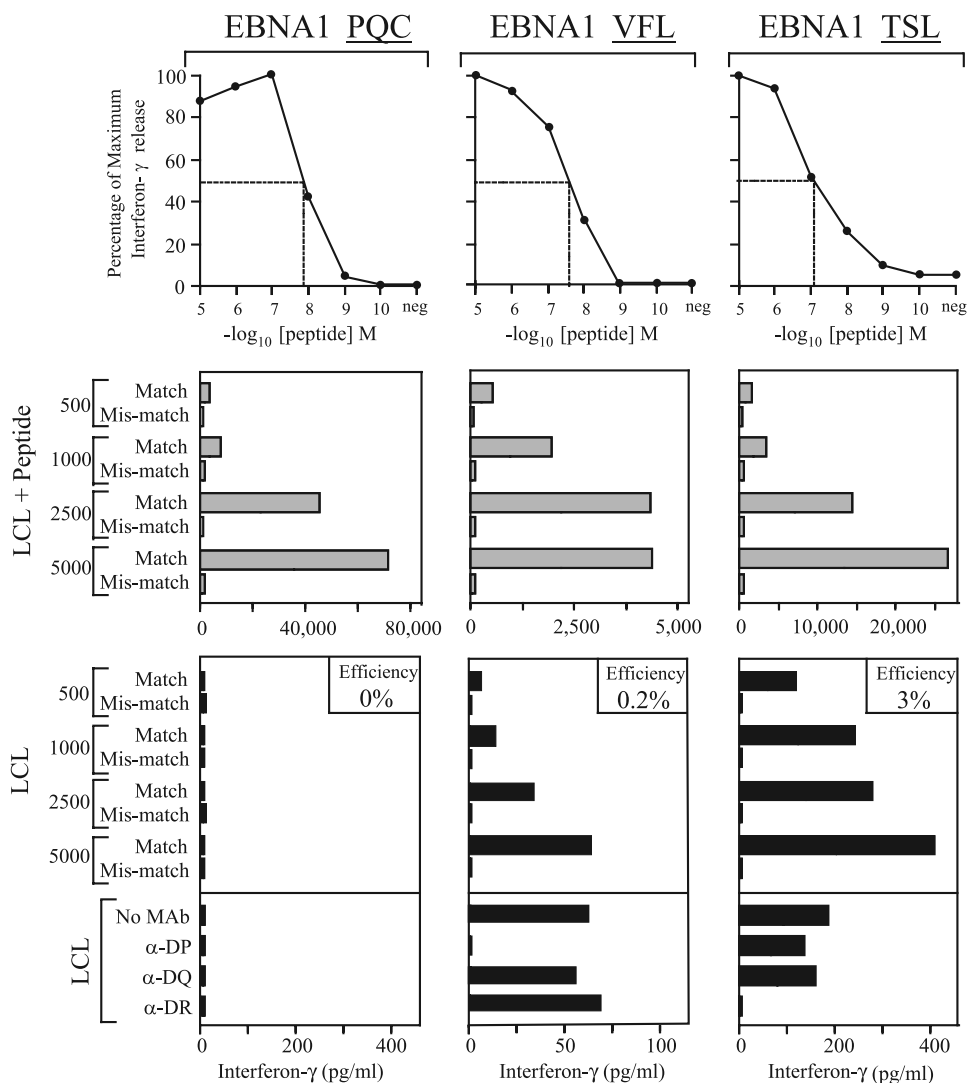


FIG. 3. Functional analysis of CD4⁺ T-cell clones against the PQC (EBNA1 529), VFL (EBNA1 564), and TSL (EBNA1 515) epitopes. (Top panels) Clones (100 T cells per well) were stimulated overnight with autologous LCLs (5×10^4 per well) either unmanipulated (neg) or loaded with epitope peptide at 10^{-5} to 10^{-10} M concentrations. Responses were assayed by IFN- γ release and expressed as a percentage of the maximum peptide-induced response. (Middle panels) Clones (500 to 5,000 T cells per well) were stimulated as described above with the autologous LCLs (match) or with HLA class II-mismatched LCLs (5×10^4 per well), both previously exposed to 5 μ M epitope peptide and then washed before the assay. Responses are expressed as IFN- γ release in picograms per milliliter. (Bottom panels, upper section) Clones were tested at the same time as above, on the same autologous and HLA class II-mismatched LCLs but with no exogenous peptide treatment. Responses are expressed as IFN- γ release in picograms per milliliter. The efficiency with which each clone is able to recognize unmanipulated autologous LCL targets is expressed as a percentage of the maximal response seen on the same targets loaded with peptide (box at upper right of each graph). (Bottom panels, lower section) The results shown in the lower section of the bottom panels are from a separate experiment in which the responses of 500 T cells to the autologous LCL (non-peptide loaded) were assayed either alone (no MAb) or in the presence of MAbs to HLA-DP, HLA-DQ, or HLA-DR as described above.

cells and stimulation with the LCL alone. All epitope-specific DR52b-restricted clones, regardless of the stimulation protocol or individual donor, gave a similar pattern of results.

Table 1 summarizes the data obtained with CD4⁺ T-cell clones to all 12 of the epitopes studied, including the PRS epitope in its four different HLA contexts. Epitopes are grouped in accordance with their source antigen and, within each group, ordered by the efficiency of LCL recognition shown by epitope-specific clones; the functional avidity of these clones is shown alongside.

LCL recognition by CD4⁺ T-cell clones: cytotoxicity and LCL outgrowth assays. The ability of EBV latent-epitope-specific CD4⁺ T cells to recognize naturally infected B-cell targets implies that such T cells might have a direct effector role in the control of EBV infection. For that reason, we examined whether the CD4⁺ T-cell recognition of LCLs observed by IFN- γ release was also detectable with other functional readouts, namely, short-term cytotoxicity and longer-term LCL outgrowth assays. This work was conducted with CD4⁺ T-cell clones to five selected epitopes; these were (in

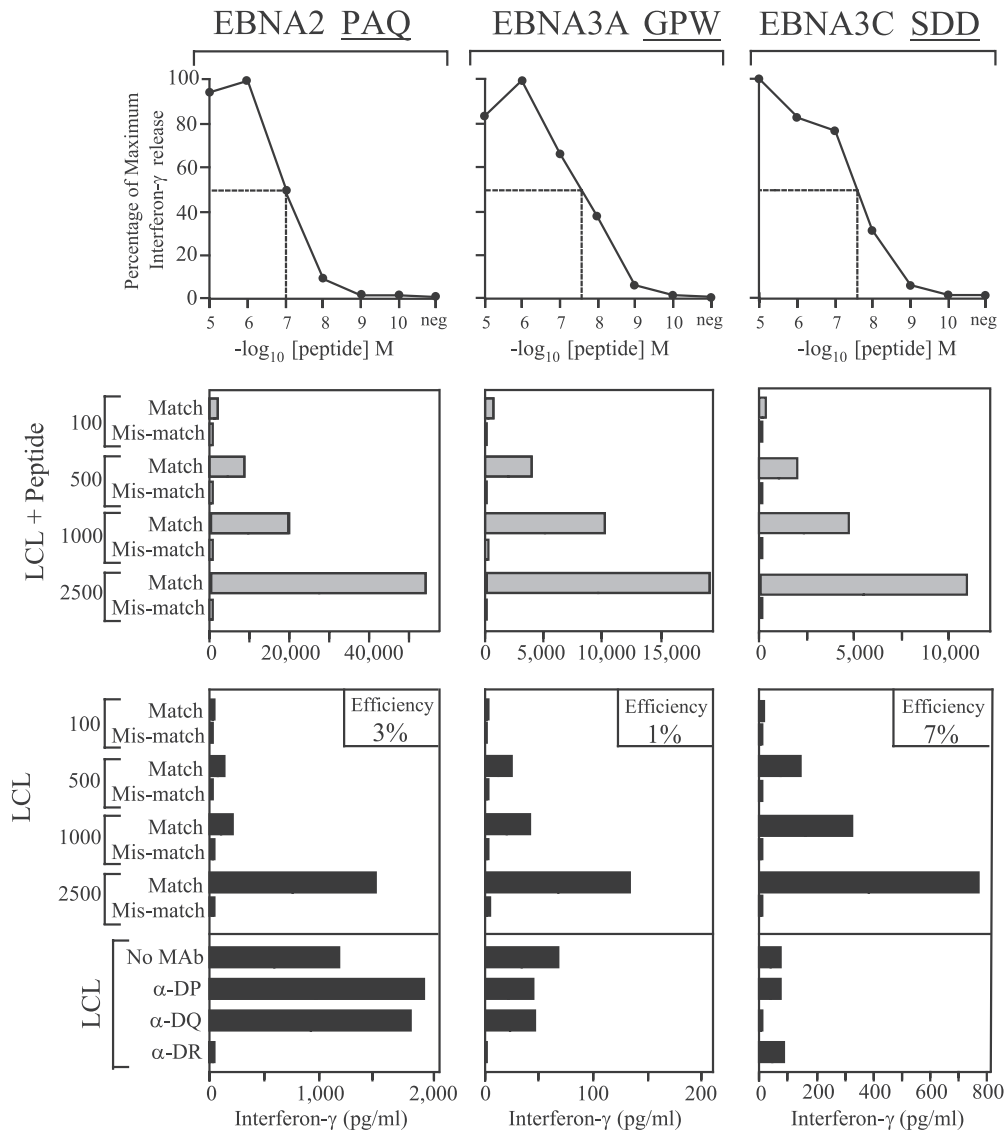


FIG. 4. Functional analysis of CD4⁺ T-cell clones against the PAQ (EBNA2, 301), GPW (EBNA3A, 780), and SDD (EBNA3C, 386) epitopes. The experimental design and expression of results are the same as in Fig. 3, except that the range of T-cell numbers used in LCL stimulation experiments extended from 100 to 2,500 per well.

order of increasing efficiency of LCL recognition by IFN- γ release) clones specific for EBNA1 epitopes PQC and TSL, EBNA2 epitope PAQ, EBNA3C epitope SDD, and DR52b-restricted EBNA2 epitope PRS.

Figure 7 presents chromium release assay data showing the levels of killing observed against HLA class II-matched and mismatched target LCLs, each tested with and without preloading with the relevant epitope peptide. Because pilot experiments had shown that killing was not always apparent within the conventional 5-h assay period, we measured specific lysis after both 5 and 18 h. All of the clones showed killing of the HLA-matched peptide-loaded LCL, apparent within 5 h and stronger by 18 h, and no killing of the mismatched peptide-loaded target. However, the clones differed in the ability to kill the unmanipulated HLA-matched LCL. The PQC (data not shown)- and TSL-specific CD4⁺ effectors did not lyse these

targets significantly, even within 18 h. The PAQ-specific clone gave marginal killing, even after 18 h only, while the SDD-specific and PRS-specific clones both gave a hint of killing within 5 h and clearly detectable lysis at the later time.

We then set up outgrowth assays in which replicate cultures of HLA-matched and mismatched LCLs, either untreated or preloaded with the relevant epitope peptide, were seeded into U-bottom microtest plate wells at doubling dilutions of 10⁴ to 300 cells per well; to some wells at each seeding, a standard number of CD4⁺ T cells (10⁴ cells per well) were then added from the same clones as tested above. The cultures were maintained in standard cell culture medium for 3 to 4 weeks and examined for successful LCL outgrowth. Although these experiments could be accurately scored by microscopic inspection of the cultures, in several cases we confirmed by CD19 staining that successful outgrowth involved the LCL and not

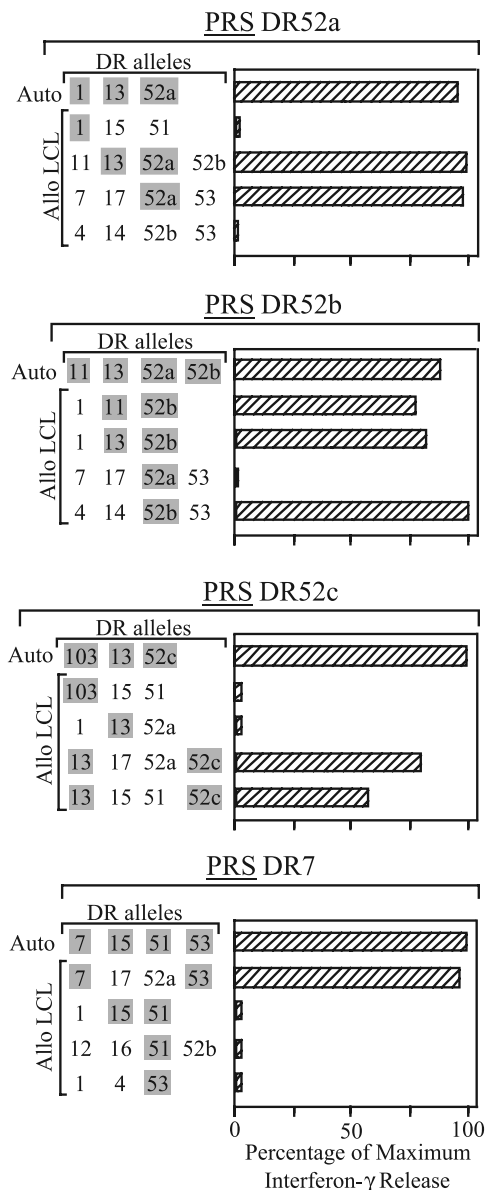


FIG. 5. Analysis of HLA class II allele restriction of CD4⁺ T-cell clones specific for the PRS (EBNA2, 276) epitope. Clones were established from four donors whose HLA-DR types are in each case identified (Auto). These clones were then tested as described in the legend to Fig. 3 (middle panels) against peptide-loaded cells of the autologous LCL and of allogeneic LCLs of known HLA-DR type (Allo LCL). Alleles matched with the autologous cells are identified by shading. Results are expressed as a percentage of the maximum IFN-γ release observed in the assay.

surviving T cells. Figure 8 expresses the results of these assays as the minimum number of each LCL required for successful outgrowth under the various conditions. The results are consistent with the earlier experiments in that clear evidence of LCL growth inhibition was limited to T-cell-LCL combinations with the higher levels of LCL recognition as determined by IFN-γ release. Thus, PQC (data not shown)-, TSL-, and PAQ-specific CD4⁺ T-cell clones, which had shown levels of unmanipulated LCL recognition of 0 to 3% of that seen

against peptide-loaded targets, markedly inhibited outgrowth of HLA-matched LCLs if they had been peptide loaded but had no effect on the corresponding nonloaded cells; as a specificity control, these same clones had little if any effect on HLA-mismatched LCLs with or without peptide loading. By contrast, clones specific for the SDD and PRS epitopes, which had shown stronger recognition of autologous LCL in IFN-γ assays, clearly were able to inhibit the outgrowth of HLA-matched (but not mismatched) LCLs even without peptide loading.

DISCUSSION

The extent to which EBV latent-specific CD4⁺ T cells are able to recognize naturally infected LCL targets is an important in vitro indicator of their likely potential as direct effectors controlling EBV-driven lymphoproliferations in vivo. Most in vitro studies to date have focused on CD4⁺ T-cell responses to just one of the available latent proteins, EBNA1 (9, 13, 17, 27, 32) and, even when studying responses to the same epitope (9, 17), have reported discordant results with respect to LCL recognition. To address this issue in a more systematic way, the present work set out to identify CD4 epitopes in a broader range of latent-cycle antigens, EBNA1, -2, -3A, and -3C, and then to generate CD4⁺ T-cell clones to a representative panel of epitopes drawn from these four proteins.

Screening with peptide panels showed that three antigens, EBNA1, -2, and -3C, are each recognized by a majority (65 to 75%) of EBV-seropositive donors, whereas only a small number (<25%) respond to EBNA3A. Interestingly certain epitopes, for example, TSL in EBNA1, PRS in EBNA2, and SDD in EBNA3C, were recognized by 30 to 40% of the donors tested. Such a high frequency is explained in the case of the PRS epitope by its capacity to elicit responses in the context of several different HLA alleles (Fig. 5) (21). The same also appears to be true of the TSL epitope because, although we established epitope-specific clones that were DR103-restricted, not all responders to TSL in ELISPOT screening assays express this allele (13; data not shown). However, this was not the case for the SDD epitope; this was restricted to HLA-DQ5, a high-incidence allele in Caucasian populations (16), and all responders to this epitope indeed proved to be HLA-DQ5 positive. It is important to note that, while a high percentage of immune donors might respond to certain epitopes, these are not necessarily immunodominant responses in terms of absolute size. Thus, both here and in an earlier study (13), we found that responses to all CD4 epitopes fell within a rather narrow size range and the strongest responses did not consistently map to a particular set of epitopes or to epitopes from a particular antigen. This contrasts with the CD8 response to EBV latent-cycle antigens, which is not only much larger than the CD4 response but also tends to focus preferentially on immunodominant epitopes from the EBNA3A, -3B, and -3C proteins (11, 25).

Having established CD4⁺ T-cell clones to 12 representative epitopes and confirmed their specificity for the relevant EBV target antigen in protein loading assays, we determined their functional avidity by peptide titration. There were two important findings in this regard. Firstly, all clones produced by peptide stimulation of PBMCs and specific for the same

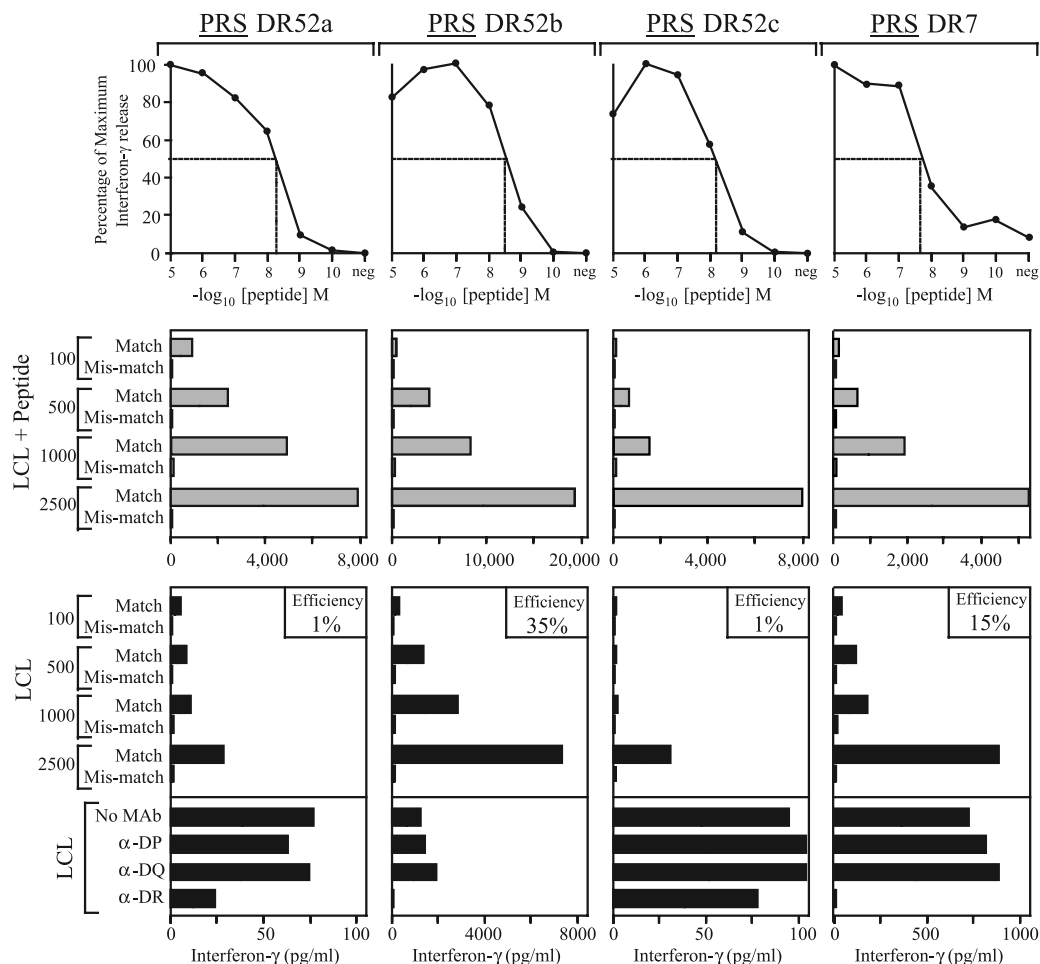


FIG. 6. Functional analysis of CD4⁺ T-cell clones specific for the PRS (EBNA2, 276) epitope derived from different EBV-seropositive donors and restricted through HLA-DR52a, -DR52b, -DR52c, and -DR7, respectively. The experimental design and expression of results are the same as in Fig. 4. Note that peptide titration assays involving the DR52b-restricted PRS clones were conducted with the Ag876 virus-transformed LCL as in Fig. 2.

epitope-HLA allele combination, whether from the same donor or different donors, gave similar peptide titration curves. Secondly, in several cases we compared epitope-specific CD4⁺ T-cell clones that had been generated from individual donors either by conventional peptide stimulation, by peptide- or antigen-loaded dendritic cells, or by LCL stimulation and, whenever we tested them, found no significant differences in functional avidity. We infer that stimulation protocols are not major sources of artifacts in these experiments and that the CD4⁺ T-cell clones being used are genuinely representative of the epitope-specific memory populations present in our EBV-immune donors.

We then turned to the question of LCL recognition. To allow comparisons to be made between individual clones to a single epitope and between clones specific for different epitopes, in each case we expressed the level of recognition of the unmanipulated LCL as a percentage of that seen in parallel against the same LCL preloaded with an optimal concentration of epitope peptide. The overall findings, summarized in Table 1, allow a number of conclusions to be drawn. Firstly, the level of LCL recognition is consistent among clones with the

same epitope specificity but differs markedly, from 0 to 35% of optimal peptide-loaded values, between clones with different specificities. Secondly, these interepitope differences are not obviously related to the antigenic source of the epitope. Thus, levels of LCL recognition ranged from 0 to 3% of peptide-loaded values for EBNA1 epitopes, from 1 to 35% for EBNA2 epitopes, from 1 to 4% for EBNA3A epitopes, and from 0 to 7% for EBNA3C epitopes. Thirdly, the differences in LCL recognition cannot solely be explained by differences in the functional avidities of the CD4⁺ T-cell clones. For example, clones to the PQC epitope in EBNA1 had an avidity of 15 nM and showed no LCL recognition whereas clones to the TSL epitope also in EBNA1 recognized the LCL at an efficiency of 3% yet required a sixfold higher peptide concentration (90 nM) for half-maximal IFN- γ release in peptide titration assays. Likewise, among EBNA2-specific clones, those against the GQT epitope showed a 15% efficiency of LCL recognition yet were 10-fold less avid in peptide titration assays than DR52a-restricted, PRS-specific clones, which show much less efficient (1%) LCL recognition. We conclude that, as for CD8⁺ T-cell clones in this viral system (3, 14, 15), the observed level of LCL

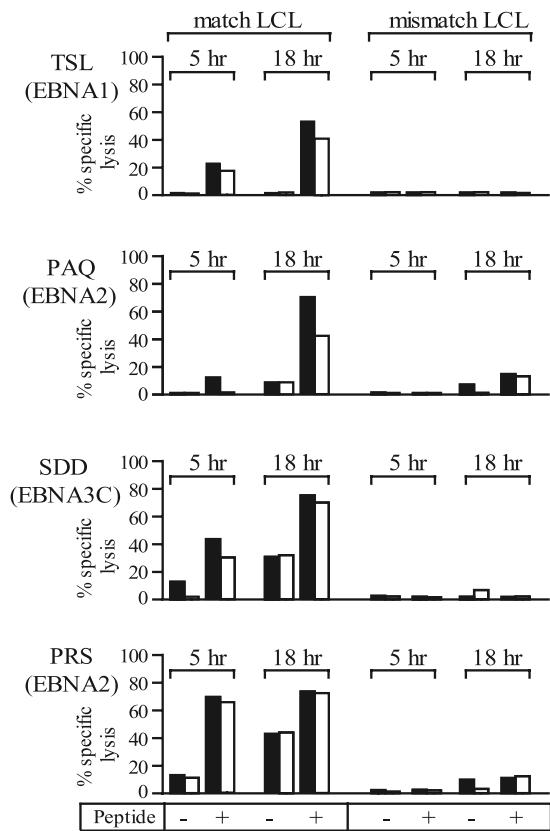


FIG. 7. Killing of LCL targets by CD4⁺ T-cell clones against the TSL (EBNA1 515), PAQ (EBNA2 301), SDD (EBNA3C 386), and PRS (EBNA2 276) epitopes. Five- and 18-h chromium release assays were conducted with HLA class II-matched and mismatched LCL targets either unmanipulated or previously exposed to 5 μ M epitope peptide and then washed before the assay. Results are expressed as percent specific chromium release from target cells at effector/target ratios of 5:1 (■) and 2.5:1 (□).

recognition will be a function both of the inherent avidity of the CD4⁺ T-cell clones and of the degree of representation of the epitope-HLA complex on the LCL surface.

The example of the PRS epitope is particularly interesting in this regard. It was already clear from the literature that this epitope can elicit responses in the context of several different HLA class II alleles. Thus, Khanna et al. reported strong killing of the autologous LCL by a PRS-specific clone reactivated by LCL stimulation and restricted through an HLA-DQ allele (10). Subsequently, Omiya and colleagues generated PRS-specific clones by peptide stimulation from several different donors and mapped their restriction to five different HLA alleles. Interestingly, all clones recognized the autologous LCL by IFN- γ release, although at levels that were never compared to the maximal peptide-induced response, whereas only clones restricted through an unidentified DR52 allele killed LCL targets in cytotoxicity assays (21). Our work extends this analysis by accurately quantitating LCL recognition by PRS-specific clones restricted through four different HLA class II alleles. Recognition ranged from 1% efficiency for DR52a- and DR52c-restricted clones through 15% efficiency for DR7-restricted clones to 35% efficiency for DR52b-restricted clones.

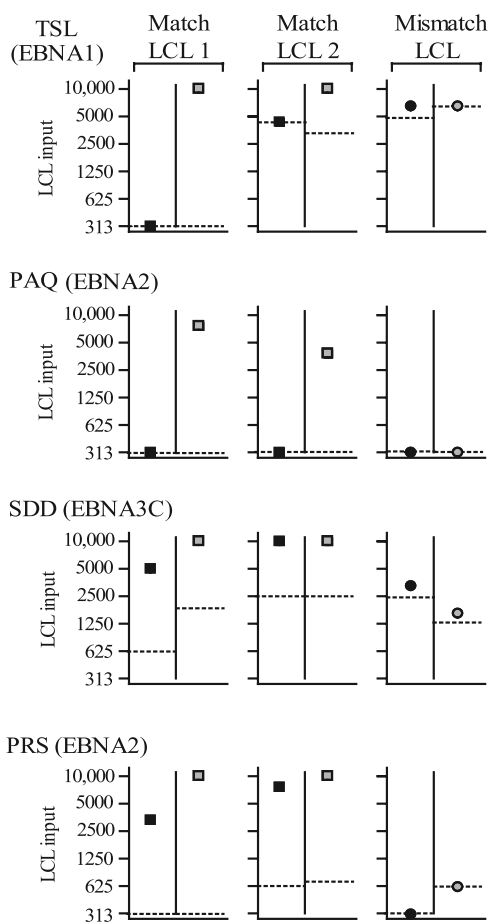


FIG. 8. Inhibition of LCL outgrowth by the epitope-specific CD4⁺ T-cell clones used in Fig. 7. For each clone, two HLA class II-matched LCLs and one mismatched LCL were seeded at doubling dilutions of 10⁴ to 300 cells per well either alone or with the addition of 10⁴ CD4⁺ T cells. The LCLs were either unmanipulated or previously exposed to 5 μ M epitope peptide and then washed before seeding. Results are expressed as the minimum LCL seeding required for successful outgrowth in each case. For each clone, the results from LCL-T-cell cocultures are shown for the unmanipulated LCL (■) and in the adjacent column for the peptide-loaded LCL (◼). These values are in each case compared with the corresponding results for outgrowth of the unmanipulated LCL or of the peptide-loaded LCL cultured in the absence of T cells (dotted lines).

Again, these differences cannot be explained by differences in functional avidity but instead reflect how the level of representation of the PRS epitope on the LCL surface is critically influenced by the identity of the restricting allele.

A similar phenomenon may underlie the apparent discrepancy in the literature with respect to CD4⁺ T-cell clones specific for the TSL epitope in EBNA1. Khanna et al. reported no LCL recognition in cytotoxicity assays by a DR1-restricted TSL clone (9). This is reminiscent of the DR103-restricted clones described in the present work, which again did not kill the LCL but showed low-level (3%) efficiency of recognition by IFN- γ release. In contrast, the TSL-specific clones with strong LCL killing described by Munz and colleagues (17) may be restricted through a different HLA class II allele that mediates more efficient epitope presentation at the LCL surface. With

promiscuous epitopes such as PRS and TSL, one can begin to look for correlations between the level of epitope presentation on infected cells and immunogenicity of the epitope *in vivo*. Clearly in the case of PRS, even low-level presentation in the context of the HLA-DR52a or -52c allele can elicit a response; however, we found that only a small number of individuals with the DR52a or DR52c allele screened actually made a detectable response to the peptide in ELISPOT assays. By contrast, most individuals positive for HLA-DR7 or -DR52b, an allele that mediates more efficient presentation of the epitope, did have detectable PRS-specific memory (data not shown). These questions will be better addressed once the restricting alleles for other apparently promiscuous EBV epitopes have been identified.

One of our main motivations for this work was to address the potential importance of EBV latent epitope-specific CD4⁺ T cells as direct effectors capable of recognizing and eliminating EBV-driven lymphoproliferations *in vivo*. Our final series of experiments, measuring both short-term cytotoxicity and long-term inhibition of target cell outgrowth *in vitro*, show firstly that all of the epitope-specific CD4⁺ T-cell clones tested were cytotoxic on epitope-loaded LCL targets and could inhibit their outgrowth in cocultivation assays. However, parallel assays on unmanipulated LCL targets split the clones into two groups. Clones with an efficiency of LCL recognition at or below 3% in IFN- γ assays showed no detectable killing in 18-h assays and no detectable inhibition of outgrowth against the unmanipulated LCL, whereas clones with efficiencies of 7% or greater were active in both situations. These results, and those of other studies with CD4⁺ T-cell clones against as yet undefined targets on the LCL surface (5), are all consistent with the view that inhibition of LCL outgrowth correlates strongly with cytotoxic activity. The present work confirms that CD4⁺ T cells specific for some EBV latent-cycle epitopes can prevent LCL outgrowth *in vitro* (17, 19, 21) and therefore, like their CD8⁺ T-cell counterparts, could act directly against EBV-driven lymphoproliferative lesions *in vivo* (24). However, our data suggest that this is only true of CD4⁺ T cells against a minority of epitopes, namely, those epitopes represented on the surface of latently infected cells above a critical threshold. This is not to imply that T cells specific for other latent-cycle epitopes do not play an important role *in vivo*; for example, they may act to help CD8⁺ responses but such helper activity is more likely to be induced by specific recognition of antigen exogenously acquired and presented by dendritic cells rather than of antigen endogenously expressed by infected cells themselves. From a therapeutic standpoint, identifying the subset of epitope-HLA combinations that can mediate direct T-cell recognition and determining the route whereby these epitopes access the HLA class II pathway in infected cells represent important priorities for future work.

ACKNOWLEDGMENT

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