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Phenotypic and Functional Heterogeneity of EBV Epitope-Specific CD8+ T Cells

Michelle D. Catalina, John L. Sullivan, Robin M. Brody, and Katherine Luzuriaga

High frequencies of EBV-specific CD8+ T cells have been detected during acute EBV infection, yet persistent infection inevitably results. To address this issue, we characterized the phenotype and function of epitope-specific CD8+ T cell populations from presentation with acute through latent infection. Considerable phenotypic and functional heterogeneity within, as well as between, two different epitope-specific populations was observed over time following acute infection. B7 EBV-encoded nuclear Ag (EBNA)-3A-specific CD8+ T cells expressed only CD45RO from acute through latent EBV infection. A2 BMLF-1-specific CD8+ T cells expressed CD45RO during acute infection and either CD45RA or CD45RO during latent EBV infection. This difference in CD45 isoform expression between the two epitope-specific populations did not translate into differences in perforin content, the ability to produce IFN-γ, or the ability to proliferate in response to Ag in vitro. In individuals with latent EBV infection, the frequencies of A2 BMLF-1- or B7 EBNA-3A-specific CD8+ T cells that expressed CD45RA, CD45RO, CD62 ligand, CCR7, and perforin were stable over time. However, the expression of CD62 ligand and CCR7 was significantly higher among EBNA-3A-specific CD8+ T cells than among BMLF-1-specific CD8+ T cells. Further work is necessary to understand how phenotypic and functional differences between EBV epitope-specific CD8+ T cells are related to the biology of the virus and to the equilibrium between the virus and the host during persistent infection. The Journal of Immunology, 2002, 168: 4184–4191.

Epstein-Barr virus infects >90% of the world’s population. During acute EBV infection, the virus replicates in the tonsillar epithelium and B cells, expressing an array of >100 lytic and latent proteins (1). The infection of B cells results in a lifelong EBV carrier state in which EBV gene expression is thought to be limited (reviewed in Ref. 2). Viral and host factors that contribute to the establishment of this carrier state are not completely understood.

Several lines of evidence suggest that memory EBV-specific CD8+ T cells play an important role in controlling viral replication or preventing disease in established (or latent) infection, and EBV is an important source of morbidity and mortality in individuals with compromised cell-mediated immunity. For this reason several groups, including our own, have thought it important to characterize the development and maintenance of these CD8+ T cell responses over the course of infection.

Adolescents and adults with acute EBV infection frequently present with acute infectious mononucleosis (AIM) syndrome and may be easily distinguished by the detection of heterophile Abs in peripheral blood. It has long been recognized that CD8+ T cells are markedly expanded and highly activated during primary infection. Callan et al. (3) were among the first to report that the majority of these CD8+ T cells are EBV-specific. They and others (4, 5) have documented preferential recognition of lytic as opposed to latent epitopes early in acute EBV infection. EBV lytic and latent epitope-specific memory CD8+ T cell responses have also been persistently detected in established (or latent) infection (4, 6, 7).

Characterization of the evolution of the CD8+ T cell repertoire and effector functions should help us to better understand virus-host interactions that contribute to persistent EBV infection. Murine models of lymphocytic choriomeningitis virus (LCMV) infection (a nonpersistent viral infection) suggest that the memory CD8+ T cell repertoire largely reflects the repertoire generated during acute infection, but less is known regarding the relationship between acute and memory epitope-specific CD8+ T cell responses in persistent human viral infections. We have recently reported that the epitope specificity of EBV-specific memory CD8+ T cells varies over the course of infection, and is not simply reflective of CD8+ T cell responses detected during primary infection (5). This suggests that the generation of memory EBV epitope-specific CD8+ T cell responses does not fit the stochastic model proposed on the basis of murine models of viral infection.

The use of HLA class I peptide tetramers together with mAb staining to cell surface Ags and functional (cytokine secretion or cytolyis) assays has allowed examination of the phenotype and function of epitope-specific CD8+ T cells at the single cell level. Using this approach, several investigators have proposed that differential cell surface expression of the CD45 isoforms (RA vs RO), CD27, CD28, and CCR7 delineates a maturational pathway for CD8+ T cell responses (8, 9). Although these studies provide provocative evidence for the heterogeneity of memory CD8+ T cell surface phenotype, strict correlation of these phenotypes with function has been called into question. Recently, Hislop and colleagues (10) have reported a relationship between memory CD8+ T cell phenotype and EBV epitope specificity (lytic vs latent) in individuals with latent infection. EBV latent epitope-specific CD8+ T cells were polarized toward a CD45ROhigh
CD45RA−CD28+ phenotype, whereas EBV lytic epitope-specific CD8+ T cell responses were CD45RA− or CD45RO+ and were more commonly CD28+. These phenotypic differences did not correlate with cytokine secretion or cytolytic activity. Lytic and latent epitope-specific CD8+ T cells were heterogeneous for CCR7 expression, but there was a trend toward greater CCR7 expression in the latent epitope-specific CD8+ T cells. Interestingly, effector cell function appeared to be limited to the CCR7− subset of cells.

In our prior studies evaluating EBV epitope-specific CD8+ T cell responses, most of the changes in epitope specificity and epitope-specific CD8+ T cell activation occurred over the first year following presentation with symptoms of AIM. Therefore, we felt it important to begin to evaluate epitope-specific CD8+ T cell phenotype and function at presentation with AIM through at least 1 year following infection. Using an HLA-A2 tetramer made with an epitope derived from BMLF-1 (an early lytic gene; Refs. 1, 11, and 12) and an HLA-B7 tetramer made with an epitope derived from BMLF-1 (an early lytic gene; Refs. 1, 11, and 13, 14), we sequentially characterized EBV epitope-specific CD8+ T cell responses in HLA-A2 and HLA-B7 individuals presenting with AIM and compared them with memory CD8+ T cell responses detected in HLA-matched individuals with established infection. Phenotypic and functional differences were observed over time within and between these two epitope-specific populations. The potential implications for the establishment of persistent EBV infection are discussed.

Materials and Methods

Patients

These studies were conducted in adolescents (aged 17−24 years old) presenting to the clinic at the University of Massachusetts/Amherst Student Health Service (Amherst, MA) with clinical symptoms consistent with AIM (fever, rash, fatigue, and hepatosplenomegaly). Following informed consent, students gave five blood samples (50 ml each) at the following time points: at presentation with symptoms (V-1), and 1 wk (V-2), 2 wk (V-3), 6 mo (V-4), and 1 year (V-5) following presentation. Entry to the study was based on a positive Monospot test and presence of atypical lymphocytes. Acute EBV infection was confirmed by the detection of IgM for the EBV viral capsid Ag.

All participants were analyzed using HLA class I tetramers upon blood receipt (within 4 h of blood draw) and participants with tetramer-binding CD8+ T cells were further analyzed using a panel of activation and adhesion markers (see below). The HLA type of all study participants was compared with each other, non-T cell populations, thus cells were first gated through both CD3 and CD8 to insure that the tetramer-positive cells were all CD8+ T cells. Cells were analyzed with a tetramer conjugated to allophycocyanin, CD8 FITC (Sigma Aldrich, St. Louis, MO), CD3 PerCP (BD Biosciences), and one of the following Abs: HLA-DRPE, CD45ROPE, CD45RAPE, CD69PE, CD62l (CD62L)PE, CD28PE, and CD38PE (BD Pharmingen). Isotype controls IgG1 FITC, IgG2b PerCP, and one of each IgG1PE, IgG2aPE, and IgG2bPE were done on each patient at each time point. Permeabilization for staining with perforin (BD Pharmingen) was done after tetramer staining and lysis of the RBC (FACSlyse, BD Biosciences). Permeabilized isolate control Abs were run alongside each analysis for perforin; 100,000 lymphocyte events were collected per sample.

Detection of cytokine-secreting CD8+ T cells following in vitro stimulation with EBV peptides

Fresh whole blood (0.3 ml/test, heparin) was incubated for 1 h at 37°C with 0.5–10 μM EBV or control HIV-1 peptides. Because staphylococcal enterotoxin B (Toxin Technology, Sarasota, FL) is able to nonspecifically stimulate T cells to secrete IFN-γ, it was used as a positive control. None of the HLA-A2 latent donors used in this study was HIV-1 infected, so the HIV-1 gag (SLYNTVATL) peptide was used as a negative control. After a 1-h incubation with peptide, Golgiplug (BD Pharmingen) was added to the cells and they were incubated an additional 5 h. After incubation the cells were incubated with 2 μM EBV for 15 min with vigorous vortexing every few minutes. Cells to be analyzed by tetramers were then stained with the allophycocyanin-A2 BMLF1, allophycocyanin-B7 EBNA-3A, allophycocyanin-A2 latent membrane protein-2, or an allophycocyanin-HIV-1 gag tetramer for 20 min. After tetramer staining, the cells were lysed with BD lyse (BD Biosciences), fixed, and permeabilized using Cytofix/Cytoperm solution (BD Biosciences). The cells were stained with combinations of the following Abs for tetramer staining: CD8 CyChrome, IFN-γ FITC, CD69PE, CD69 FITC, CD28PE, CD82PE, and CD45RO PE (BD Pharmingen). For cells not stained with tetramers, after the permeabilization step the cells were stained with IFN-γ allophycocyanin, CD3 PerCP, CD69PE, and CD8 FITC. The cells were incubated at room temperature for 30 min, and then washed with 1% FBS in PBS. Samples were analyzed immediately by four-color flow cytometry. Appropriately isolated negative- and positive-control effects were used to define positive and negative cell populations. Background IFN-γ production in this assay was 0.02 ± 0.01% for stimulation of individuals with either a peptide derived from HIV-1 or EBV-derived non-HLA binding peptides. Significant IFN-γ production was considered to be greater than the mean of the background plus three SD, or 0.05% of CD8+ T cells.

In vitro culture and generation of cell lines

Cell lines were generated from Ficoll separated, and in some cases sorted PBMC and cultured in RPMI with 10% FCS (R-10) and 20 U/ml of IL-2 by adding 5 μM EBV-specific peptide or control HIV-1 gag peptide. These cell lines were fed twice weekly and maintained for 1 mo. Alternatively, Ficoll-separated and in some cases sorted PBMC were incubated with EBV peptide or control peptide-pulsed, psoralen-fixed autologous B lymphoblastoid cell lines (BLCL) at a ratio of 1 BLCL:5 PBMC. These cell lines were also fed twice weekly with 20 U/ml of IL-2 in R-10 and restimulated with peptide-pulsed BLCL every 2–3 wk. These cell lines were maintained for up to 3 mo.

Statistical analysis

Means and SDs were calculated and compared with each other using the Student t test to determine p values. The Wilcoxon signed rank test, a nonparametric paired t test, was used to determine p values for the differences in the range of tetramer-staining CD8+ T cells between study time
Results

We have previously used two EBV epitope-specific HLA class I tetramers to characterize the generation and maintenance of epitope-specific memory CD8\(^+\) T cells in acute and latent EBV infection. Responses to these two epitopes are detected in the majority of EBV-infected individuals (5). Four-color flow cytometry was done to determine the percentage of tetramer-binding CD8\(^+\) T cells coexpressing phenotypic markers (Fig. 1). A total of 18 (10 HLA-A2, 4 HLA-B7, and 4 HLA-A2 and HLA-B7) individuals with AIM were studied. For comparison, 16 (9 HLA-A2, 2 HLA-B7, and 5 HLA-A2 and HLA-B7) healthy EBV seropositive individuals were studied.

Differential expression of the CD45 isoforms by A2 BMLF-1 and B7 EBNA-3A tetramer-binding effector and memory T cells

Analyses of individuals with acute EBV infection showed that all HLA-A2-restricted, BMLF-1-specific CD8\(^+\) T cells coexpressed CD45RO at presentation with AIM. By 2 wk postpresentation, 3–15% of the A2 BMLF-1 tetramer-binding CD8\(^+\) T cells expressed CD45RA and were CD45RO negative. There were significant differences in the expression of CD45RO and CD45RA by postpresentation expressed only CD45RO. Additionally, all B7 EBNA-3A tetramer-binding CD8\(^+\) T cells coexpressed CD62L and CD45RA (Fig. 2a). Significantly more of the B7 EBNA-3A tetramer-binding CD8\(^+\) T cells were CD45RO negative. There were significant differences in the expression of CD45RO and CD45RA by B7 EBNA-3A tetramer-binding CD8\(^+\) T cells at presentation, and at 6 mo or 1 year following acute infection (RO, \(p = 0.001\); RA, \(p = 0.001\); Fig. 2, a and b). By 6 mo and 1 year, there was no statistically significant difference between the acute patients and healthy EBV seropositive donors in the percentages of A2 BMLF-1 tetramer-binding CD8\(^+\) T cells that expressed CD45RA vs CD45RO. Indeed, varying percentages of CD45RA/A2 BMLF-1 tetramer-binding cells were detected in all of the EBV seropositive donors (range 14.8–76.8%; Fig. 2b). None of the B7-1 tetramer-binding CD8\(^+\) T cells in the long-term EBV seropositive donors coexpressed both CD45RA and CD45RO (data not shown). The percentages of CD45RO\(^+\)/CD45RA\(^-\) A2 BMLF-1-specific CD8\(^+\) T cells remained stable in individuals with latent infection over four measurements repeated during an 18-mo time period.

At presentation, all B7 EBNA-3A tetramer-binding CD8\(^+\) T cells expressed CD45RO (\(n = 3\)). However, in contrast to the A2 BMLF-1 tetramer-binding CD8\(^+\) T cells, all of the B7 EBNA-3A CD3\(^+\)/CD8\(^+\) tetramer-binding cells detected one and two weeks postpresentation expressed only CD45RO. Additionally, all B7 EBNA-3A tetramer-binding CD8\(^+\) T cells detected in individuals at 6 mo postpresentation (8/8; 99 ± 0.5%), 1 year postpresentation (5/5; 99 ± 0.5%), and in EBV, seropositive individuals (7/7; 99 ± 0.3%) coexpressed CD45RO.

Because the peptides used were not only derived from different proteins, but also restricted by different HLA molecules, we wanted to determine whether CD8\(^+\) T cells that bound to HLA-A2 peptide tetramers made with latent protein epitopes also expressed only the CD45RO isoform. Analyses of HLA-A2 latent donors with an A2 LMP-2 tetramer revealed that 100% of the CD8\(^+\) cell populations binding to these tetramers also expressed only CD45RO (\(n = 8\); data not shown). These data demonstrate that for two latent epitopes restricted by distinct HLA alleles, the coexpression of the CD45RO isoform predominated.

Differential expression of cell surface homing molecules on A2 BMLF-1 and B7 EBNA-3A tetramer-binding CD8\(^+\) T cells

The lymphocyte cell surface molecule CD62L has been shown to play a role in the specific homing of T cells to peripheral lymph nodes (16). CD62L expression on A2 BMLF-1 tetramer-binding CD8\(^+\) T cells. For presentation through 1 year, each symbol represents the same donor. Latent donors were a minimum of 10 years post acute EBV infection.

![Figure 1](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)  
**FIGURE 1.** a, Percentage of CD8\(^+\) T cells binding to the A2BMLF-1 tetramer in an HLA-A2 individual. b, Percentage of CD8\(^+\) T cells binding to the B7EBNA-3A tetramer in an HLA-B7 individual. Small lymphocytes were gated through both CD3\(^+\) and CD8\(^+\) (c) for analyses of activation marker expression on tetramer binding CD3\(^+\) and CD8\(^+\) T cells (d).
the CD45RO isofrom, and also expressed very high levels of CD26L, we wanted to determine whether there were differences in the coexpression of CCR7 on A2 BMLF-1 compared with B7 EBNA-3A tetramer-binding CD8\(^+\) T cells. Fig. 3d demonstrates that B7 EBNA-3A tetramer-binding CD8\(^+\) T cells in latent individuals more commonly expressed CCR7 (54 ± 24% CCR7\(^+\)) than A2 BMLF-1 tetramer-binding CD8\(^+\) T cells (18 ± 10% CCR7\(^+\); \(p < 0.001\)). For each individual, the percentage of CCR7\(^+\) tetramer-binding CD8\(^+\) T cells was always less than the percentage of CD62L\(^+\)-positive tetramer-binding CD8\(^+\) T cells. For HLA-A2 individuals, the small percentages of CCR7\(^+\) tetramer-binding CD8\(^+\) T cells were distributed between both the CD45RA and CD45RO populations.

**No differences in IFN-γ production by A2 BMLF-1 or B7 EBNA-3A tetramer-binding CD8\(^+\) T cells**

Because of the observed differences of A2 BMLF-1 and B7 EBNA-3A with respect to the expression of the CD45RO isofrom over time, we wanted to determine whether these phenotypic differences corresponded with the ability to produce IFN-γ during AIM and latent EBV infection. AIM study participants and long-term EBV seropositive individuals were studied by tetramer staining combined with the in vitro stimulation assay for cytokine production to determine the percentage of tetramer-binding cells expressing CD69 and IFN-γ. IFN-γ production by A2 BMLF-1 tetramer-binding CD8\(^+\) T cells was lowest at presentation with symptoms (V-1; 33 ± 12%), and was significantly different (\(p < 0.05\)) from 6 mo postpresentation (V-4, 48 ± 11%) and from the long-term latent individuals (65 ± 17%; \(p < 0.001\); Fig. 4a). There was also a significant difference (\(p < 0.05\)) in IFN-γ production between the 6-mo time point and the long-term latent individuals. Although all IFN-γ producing A2 BMLF-1 CD8\(^+\) T cells coexpressed CD69, many CD69-positive T cells did not produce IFN-γ (Table I).

Similar to the data for the A2 BMLF-1 tetramer-binding CD8\(^+\) T cells, IFN-γ production by B7 EBNA-3A tetramer-binding CD8\(^+\) T cells was lowest at presentation with symptoms (V-1; 41 ± 12%), increased by 6 mo postpresentation (V-4, 66 ± 17%; not significant) and was significantly different from the long-term latent individuals (82 ± 17%; \(p < 0.01\); Fig. 4b). There was no significant difference between the 6-mo time point and the long-term latent individuals for B7 EBNA-3A tetramer-binding CD8\(^+\) T cells. There were also no significant differences in the percentage of IFN-γ producing B7 EBNA-3A and A2 BMLF-1 CD8\(^+\) T cells when compared at similar time points.

As shown in Fig. 2, we detected populations of A2 BMLF-1-specific CD8\(^+\) T cells that coexpressed either CD45RA or CD45RO, but only populations of CD45RO\(^+\)B7 EBNA-3A-specific CD8\(^+\) T cells. Because we determined that not all tetramer-binding CD8\(^+\) T cells produced IFN-γ, we wanted to determine which subpopulations of epitope-specific CD8\(^+\) T cells did not produce IFN-γ, or express CD69. Fig. 5 shows a representative analysis (\(n = 8\)) to evaluate the ability of A2 BMLF-1 tetramer-staining cell subsets to produce IFN-γ. This figure clearly demonstrates that both CD45RO\(^+\) (Fig. 5a) and CD45RA\(^+\) (Fig. 5b) BMLF-1 tetramer-binding CD8\(^+\) T cells were able to produce cytokines in the short-term assay at the 2 μM concentration of peptide.

Cell surface expression of CD28 by A2 BMLF-1 and B7 EBNA-3A-specific CD8\(^+\) T cells was variable. CD28\(^+\) and CD28\(^-\) CD8\(^+\) T cell populations of both specificities were able to produce cytokines in the short-term assay at concentrations of peptide ranging from 0.5–10 μM. Because CD26L is rapidly lost upon in vitro Ag stimulation, evaluation of IFN-γ production by CD26L\(^+\) cells could not be determined by this method.
The perforin content of A2 BMLF-1 and B7 EBNA-3A tetramer-binding CD8\(^+\) T cells does not correlate with cell surface expression of the CD45 isoform.

We were not able to determine significant differences in the abilities of the A2 BMLF-1 and B7 EBNA-3A tetramer-binding CD8\(^+\) T cells to produce IFN-\(\gamma\), nor were we able to detect differences in IFN-\(\gamma\) production by the subpopulations of CD45RA\(^+\), CD45RO\(^+\), or CD28\(^+\) positive tetramer-binding CD8\(^+\) T cells. Thus, we wanted to determine whether differences in perforin content existed between A2 BMLF-1 and B7 EBNA-3A tetramer-binding CD8\(^+\) T cells. Fig. 6a demonstrates that A2 BMLF-1 tetramer-binding CD8\(^+\) T cells had variable levels of perforin at presentation (V-1) and 6 mo post EBV infection. Perforin was detected in up to 50% of tetramer-binding cells through 1-year post EBV infection and in latent individuals (Fig. 6a). No statistically significant differences between the perforin content of A2 BMLF-1 and B7 EBNA-3A tetramer-binding CD8\(^+\) T cells were detected during presentation (V-1) through 6 mo (V-4). However, HLA-B7 EBNA-3A tetramer-binding cells containing perforin were uncommonly detected after 6 mo and the overall percentage of perforin containing B7 EBNA-3A tetramer-binding CD8\(^+\) T cells was significantly lower (1.9 \pm 3.2%; \(p < 0.05\); Fig. 6b) than the percentage of perforin containing A2 BMLF-1 tetramer-binding CD8\(^+\) T cells (19.1 \pm 17.8%; Fig. 6a) at 1 year. Additionally, the percentage of perforin containing B7 EBNA-3A CD8\(^+\) T cells in long-term EBV seropositive individuals (5.8 \pm 8.7%; Fig. 6b) was significantly lower (\(p < 0.05\)) than the percentage of perforin containing A2 BMLF-1 tetramer-binding CD8\(^+\) T cells in long-term EBV seropositive individuals (17.7 \pm 12.9%; Fig. 6a). Interestingly, the percentage of perforin containing A2 BMLF-1 or B7 EBNA-3A CD8\(^+\) T cells remained stable over three repeated measurements over a 1-year time period for all of our long-term EBV seropositive donors.

Given the difference in CD45 isoform expression by the A2 BMLF-1 and B7 EBNA-3A tetramer-staining cells, we went on to examine whether CD45 isoform expression correlated with perforin content. Fig. 7 shows an example of FACS analysis of two long-term EBV seropositive individuals (both HLA-A2, B7) and the perforin content of A2 BMLF-1 (donor 001) or B7 EBNA-3A (donor 006) tetramer-binding CD8\(^+\) T cells within the CD45RA\(^+\) and CD45RO\(^+\) subsets. Table II demonstrates the variability in the percentages of perforin containing A2 BMLF-1 and B7 EBNA-3A T cells for 14 HLA-A2 long-term EBV seropositive individuals tested, and like Fig. 7, shows that for this epitope the majority of perforin-containing cells were within the CD45RA\(^+\) and CD45RO\(^+\) populations of CD8\(^+\) T cells. Thirteen of 14 had detectable perforin in their BMLF-1 tetramer-binding CD8\(^+\) T cells (range 0–34%). Only donor 007 had more CD45RO\(^+\) than CD45RA\(^+\) BMLF-1 tetramer-binding CD8\(^+\) T cells containing perforin. Table III demonstrates low percentages of perforin-containing cells in HLA-B7 EBNA-3A tetramer-staining cells; four of seven HLA-B7 long-term EBV seropositive donors had no detectable perforin.
Both CD45RA⁺ and CD45RO⁺ CD8⁺ T cells are capable of in vitro proliferation

Because we could not distinguish functional differences between A2 BMLF-1 and B7 EBNA-3A-specific CD8⁺ T cells directly ex vivo, we derived cell lines from individuals who were both HLA-A2⁺ and HLA-B7⁺ to determine whether differences existed between the abilities of CD45RA⁺ and CD45RO⁺ T cells to proliferate in vitro. CD3⁺ T cells from three HLA-A2⁻B7⁻ individuals were sorted into CD45RA⁺ or CD45RO⁺ populations (Fig. 8, a, e, and i; representative donor 003). The sorted cells were then stimulated with either peptide-pulsed autologous BLCL or peptide only and propagated in vitro. Fig. 8, b–d, shows that after only 4 days in culture the small population of CD45RA⁺ BMLF-1-tetramer-binding CD8⁺ T cells present after sorting (Fig. 8a) had expanded and switched their CD45 isoform expression to CD45RO (Fig. 8, b and c). The expansion with peptide only was smaller, but still resulted in the expression of CD45RO by BMLF-1-tetramer-binding CD8⁺ T cells (Fig. 8d). Because BLCL express 10 latent proteins from EBV, we detected a switch in isoform expression for many non-tetramer-binding CD8⁺ T cells which were cultured with BLCL (Fig. 8, b and c), but not for many non-tetramer-binding CD8⁺ T cells when the cultures were just stimulated with peptide (Fig. 8d).

The CD45RO⁺ BMLF-1-tetramer-binding CD8⁺ T cells also expanded after culturing, although the expansion was much less pronounced than for the CD45RA⁺ BMLF-1-tetramer-binding CD8⁺ T cells. Fig. 8e shows the percentage of tetramer-binding CD8⁺ T cells after sorting, and Fig. 8, f and g, shows after culture with A2 BMLF-1 peptide-pulsed BLCL. Of note, none of these cells switched their isoform to CD45RA in culture.

Culture of the sorted CD45RO⁺ (Fig. 8i) or CD45RA⁺ T cells with B7 EBNA-3A-pulsed BLCL (Fig. 8, j and k) or with B7 EBNA-3A peptide only (Fig. 8i) showed expansion only in cultures derived from the CD45RO⁺ T cells, as expected. Similarly, the CD45RO⁺ B7 EBNA-3A-specific CD8⁺ T cells expanded quite readily in vitro and all retained their CD45RO⁺ phenotype (Fig. 8, j and k).

Discussion

The availability of HLA class I peptide tetramers has provided the tools to distinguish populations of effector and memory CD8⁺ T cells on the basis of their epitope specificity (18). To our knowledge, this is the first study to follow a large cohort of individuals from acute through established EBV infection to characterize the phenotype of effector and memory CD8⁺ T cells. Considerable phenotypic and functional heterogeneity within, as well as between two different EBV epitope-specific CD8⁺ T cell populations was observed over time following acute infection.

Many groups have begun to evaluate the cell surface phenotype of Ag-specific CD8⁺ T cells. Champagne and colleagues (9) have reported a correlation between cell surface phenotype and function in HIV-1-specific CD8⁺ T cells, and from this have proposed a differentiation model for CD8⁺ T cells in which fully differentiated effector cells are of the CD45RA⁺, CD27⁻, CD28⁻, and CCR7⁻ phenotype. The present study does not support this model for EBV infection. At presentation with AIM, 100% of lytic and latent epitope-specific CD8⁺ T cells expressed only the CD45RO isoform (Fig. 2); as acute infection resolved, the BMLF-1-specific CD8⁺ T cells coexpressed either the RA or the RO isoform, while the EBNA-3A- and LMP2-specific CD8⁺ T cells continued to express only the RO isoform. Others have shown that lytic protein-specific CD8⁺ T cells (HLA A2-restricted, BMLF-1-specific CD8⁺ T cells and HLA B8-restricted, BZLF-1-specific CD8⁺ T cells from individuals with established infection can express either the CD45RA or CD45RO isoforms; Refs. 3 and 6). The CD45RO⁺ B7 EBNA-3A-specific CD8⁺ T cells were capable of IFN-γ production and contained perforin. The CD45RA⁺ BMLF-1-specific CD8⁺ T cells were clearly not end stage differentiated effector cells, as has been suggested, because they were easily expanded in culture (8, 9).

Lytic epitope-specific CD8⁺ T cell responses are detected earlier in AIM than latent epitope-specific CD8⁺ T cell responses and are generally higher in magnitude (5). The differences in CD45 isoform expression on memory CD8⁺ T cells might simply reflect differences in the magnitude of the epitope-specific CD8⁺ T cell response generated during acute infection. However, the frequencies of BMLF-1-specific CD8⁺ T cell responses measured 1–2 wk following presentation with AIM varied 100-fold among individuals (5), and there was no discernible relationship between the magnitude of the epitope-specific response measured during acute infection and expression of CD45RA during convalescence.

Coexpression of either the CD45RA or RO isoforms by epitope-specific CD8⁺ T cells has also been reported in individuals with established cytomegalovirus infection (19). High early viral replication concurrent with the generation of the epitope-specific response might contribute to the observed differences in CD45 isoform expression. In acute EBV infection, up to 25% of circulating B cells may be infected with EBV (2). In this regard, it is interesting to note that the CD45RA⁺ A2 BMLF-1-specific CD8⁺ T cells reverted to CD45RO (Fig. 8, a–d) following reexposure to Ag in vitro.
The production of IFN-γ by BMLF-1 and EBNA-3A-specific CD8⁺ T cells was low in early infection but increased with time. Variability was noted in IFN-γ production by epitope-specific CD8⁺ T cells in latently infected individuals. In mice infected with low doses of LCMV, IFN-γ production appears to correlate well with the number of tetramer-binding CD8⁺ T cells detected during acute infection (20–22). However, in mice infected with high doses of LCMV, with resultant persistent infection, this concordance is lost (23). This same discordance between tetramer-staining cells and the ability to produce IFN-γ has been shown in acute SIV infection (24) and acute hepatitis C virus infection (25), and has been proposed as a mechanism for the establishment of persistent infection.

It is possible that the in vitro assay might not accurately reflect the in vivo functional capabilities of the EBV epitope-specific CD8⁺ T cells. During acute EBV infection, Ag-specific CD8⁺ T cells are already highly activated (5) and either may not be capable of producing IFN-γ or may enter apoptotic pathways following reexposure to Ag in vitro. As the viral load diminishes, the cells detected during latent infection are not highly activated and may be more capable of producing IFN-γ in the in vitro assay.

Cell surface CD62L and CCR7 expression was significantly higher on EBNA-3A-specific CD8⁺ T cells, than on BMLF-1-specific CD8⁺ T cells from convalescent or latently infected individuals. These data are comparable with data from Hislop et al. (10), who also reported differences in CCR7 and CD62L expression between lytic and latent epitopic-specific CD8⁺ T cells. Murine models have shown that there are phenotypic differences between memory CD8⁺ T cells in the lamina propria and other tissues (26), and this suggests that subpopulations of memory cells will express receptors which allow them to interact with APCs and/or a specific microenvironment. The ligands for CD62L (L-selectin), peripheral lymph node addressin, are expressed at very high levels in tonsillar tissue (27). It could be that the B7 EBNA-3A-specific CD8⁺ T cell populations retain CD62L and CCR7 expression to gain re-entry into this site. Other recently published studies also suggest that CCR7 expression on T cells (and its ligand, EBV-induced molecule 1 ligand chemokine, expression on lymph node and tonsillar tissue) are important for the trafficking of lymphocytes to these lymphoid sites (28, 29).

While both of the epitope-specific CD8⁺ T cell populations we studied demonstrated in vitro function, EBV is never eliminated. One important caveat of persistent infections in humans is that the virus may persist for years with limited gene expression. Thus, even though the host has functional virus-specific CD8⁺ T cells, virus-infected cells might be unapparent to immune surveillance.

In individuals with latent EBV infection, the percentages of A2 BMLF-1 or B7 EBNA-3A-specific CD8⁺ T cells remain remarkably stable over time (5). Interestingly, analysis of the perforin content of A2 BMLF-1 and B7 EBNA-3A-specific CD8⁺ T cells in long-term EBV seropositive donors showed that similar percentages of perforin-containing epitope-specific CD8⁺ T cells were always found within the same individual over a period of 1 year. This is similar to the consistency in the overall percentages of tetramer-binding CD8⁺ T cells coexpressing CD45 RA or RO in these individuals. Work by Khan et al. (30), has elegantly shown that the peripheral blood EBV load remains constant in individuals over time periods of several years. Thus, for the two populations of EBV-epitope-specific CD8⁺ T cells studied, the expression of CD45RA, CD45RO, CD62L, CCR7, and perforin appears to be stable over time, reflecting equilibrium between the virus and the host immune system. Further work is necessary to understand how perturbations in the EBV-specific CD8⁺ T cell repertoire relate to disease manifestations in individuals with compromised cell-mediated immunity.

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