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EBV-Specific CD8+ T Cell Memory: Relationships Between Epitope Specificity, Cell Phenotype, and Immediate Effector Function

Andrew D. Hislop,‡ Nancy H. Gudgeon,‡ Margaret F. C. Callan,‡ Chrysoula Fazou,‡ Hitoshi Hasegawa,§ Michael Salmon,§ and Alan B. Rickinson§‡

EBV infection in humans induces CD8+ T cell memory to viral epitopes derived from both lytic and latent cycle Ags. We have analyzed the relationship between the phenotype and function of the memory pool of T cells specific for these Ags. Lytic epitope-specific populations were heterogeneous in terms of CD45RO/RA and CD28 expression, whereas latent epitope-specific populations were uniformly CD45RO+ and CD28+, consistent with the higher antigenic challenge from lytic epitopes driving some memory cells toward a CD45RA+, CD28- phenotype. However, both types of memory population showed immediate epitope-specific cytotoxicity and type 1 cytokine production in ex vivo assays. Cytotoxic function was not associated with preactivated T cells, as EBV-specific populations were negative for activation markers such as CD69 or CD38, nor could cytotoxic function be ascribed to CD27- or CD56- subsets, as such cells were not detected in EBV-specific memory. Furthermore, cytotoxicity was not limited to CD45RA+ and/or CD28- fractions, but also was observed in CD45RO+, CD28+ populations in lytic and latent epitope-specific memory. Cytokine (IFN-γ, TNF-α) responses, measured by intracytoplasmic staining after peptide stimulation, also were detectable in CD45RO+ and RA- subsets as well as CD28+ and CD28- subsets. Of other markers that were heterogeneous in both lytic and latent epitope populations, CCR7 gave the best discrimination of functionality; thus, CCR7+ cells consistently failed to give an IFN-γ or TNF-α response, whereas many CCR7- cells were responsive. Our data are consistent with effector functions having a broad distribution among phenotypically distinct subsets of “effector memory” cells that have lost the CCR7 marker. The Journal of Immunology, 2001, 167: 2019–2029.

Primary infection with a virus often leads to an acute T cell-mediated response characterized by the expansion of an effector population of virus-specific CD8+ T cells. Once the infection is brought under control, many of these effectors die by apoptosis, but a small proportion of the primed CD8+ T cell population survive to generate a long-lived memory cell pool (1, 2). These memory cells then are capable of mounting an enhanced response to any subsequent rechallenge by the virus. For nonpersistent viruses, this rechallenge will come from a second exogenously acquired strain and for persistent viruses from a recrudescence of the initial infection.

Although the primary effectors of a CD8+ T cell-mediated response in man are phenotypically identifiable as activated cells expressing the CD45RO isoform and activation markers such as CD38, CD69, and HLA-DR (3–5), the assignment of Ag-specific memory to a phenotypically distinct subset of circulating CD8+ T cells has proved controversial. Even before single-cell assays allowed Ag-specific memory cells to be individually identified in lymphocyte preparations ex vivo, it was suggested that the Ag-experienced human CD8+ T cell pool could be distinguished from the naive pool on the basis of increased CD11a/CD18 (LFA1) adhesion molecule expression (6). However, in terms of other putative markers of differentiation such as CD45RO, the other isoform CD45RA (once thought to be unique to naive cells), CD27, CD57, and CD62L, the LFA1+ population is quite heterogeneous (7–9). This led to numerous suggestions that these phenotypically distinct subsets were functionally distinct, and in particular that an immediate effector function such as cytotoxicity was confined to particular CD8+ subsets, especially those defined as CD45RA+ CD27- (7, 8, 10) and/or CD28+ (11–13), whereas the capacity for immediate cytokine (IFN-γ, TNF-α) release was more widespread. In this latter context, a more recent study based on the chemokine receptor CCR7 as a discriminatory marker has suggested that the capacity for immediate IFN-γ/TNF-α release is restricted to a CCR7- subset of Ag-experienced CD8+ T cells (14). However, these studies focused on all of the cells within a particular CD8+ subpopulation and not on cells responsive to a particular Ag. Accordingly, effector functions were assayed in a non-Ag-specific manner. Cytotoxicity was assessed by anti-CD3 mAb-stimulated (redirected) lysis of Fe receptor-positive target cells and immediate cytokine production by PMA/ionomycin stimulation, approaches which may well not be representative of Ag-specific responses.

The identification of viral peptide epitope against which the human CD8+ T cell response to common pathogens is directed has opened the way for new assays, based on staining with HLA class I/peptide tetramers (15) and on peptide-induced cytokine release (16, 17), which allow epitope-specific T cells in memory to be identified at the single-cell level. The present work focuses on one
such pathogen, the EBV, a persistent γ-herpesvirus that establishes both lytic and latent infections in vivo and where both types of infection elicit detectable CD8+ responses (18). Studies on patients with primary EBV infection, manifesting as infectious mononucleosis (IM),4 have shown that the primary EBV-specific response accounts for much if not all of the highly expanded pool of activated CD45RO+ CD8+ T cells seen in the blood during this disease, with the response to lytic cycle Ags being amplified to 10-fold higher levels than that to latent Ags (3). Those expansions, visualized by tetramer staining, also are reflected functionally when IM effectors are tested on peptide-loaded targets in ex vivo cytotoxicity assays (19, 20). In the subsequent life-long virus carrier state (both in post-IM patients and in individuals who had asymptomatic primary EBV infection), small numbers of both lytic and latent Ag-specific cells are maintained and together may account for 1–3% of the circulating CD8+ pool, with lytic epitope reactivities again usually in the majority (21). Interestingly, not all of these cells appear to be functionally equivalent because estimates of epitope-specific T cell numbers in the blood of healthy carriers from ELISPOT assays of peptide-induced IFN-γ release are generally 20–40% of the numbers assessed by tetramer staining (21). It is not known how differences in the capacity for IFN-γ production might relate to phenotypic heterogeneity seen within EBV-specific memory populations, nor whether any of these cells might also be capable of a second immediate effector function, such as epitope-specific target cell lysis. As with all persistent viruses, it might be argued that at any one time, chronic antigenic stimulation might be driving a small number of memory cells to an activated effector phase like that seen in the acute primary infection and that any detectable cytotoxicity would be restricted to cells with this phenotype.

In the present study, we have developed the work looking for relationships between EBV-specific memory CD8+ phenotype and effector capacity in three ways: 1) by comparing memory populations derived from the highly amplified primary response to EBV lytic cycle Ags with those derived from the smaller response to latent cycle Ags; 2) by using intracellular staining as a more rapid assay of the epitope-induced cytokine response (22), where the phenotype of responsive cells can be simultaneously identified; and 3) by asking whether any of these phenotypically distinct memory populations might mediate detectable epitope-specific cytolyis in ex vivo cytotoxicity assays.

Materials and Methods

Peptides and HLA class I tetramers

The following EBV-epitope peptides were used in this study: the A2-restricted epitope GLCTLVAML derived from the lytic cycle protein BMLF1 (23), YVDDHLIV derived from the lytic cycle protein BMRF1 (24), and CGLGGTLTMV derived from the latent cycle protein LMP2 (25). The B8-restricted epitope peptides used in this study were RAKFQQL derived from the lytic cycle protein BZLF1 (26), FLRGRAYGL derived from the latent cycle protein EBNA3A (27), and QAKWRLQTL derived from the latent cycle protein EBNA3A (28). All epitope peptides are abbreviated to the first three amino acids throughout this text. The peptides were purchased from Alta Biosciences (University of Birmingham, Birmingham, U.K.) and diluted in DMEM (Fisher Chemicals, Loughborough, U.K.) to a concentration of 5 mg/ml and diluted to appropriate concentrations in RPMI 1640 immediately before use.

HLA class I/peptide tetramers were synthesized as described previously (15). Briefly, HLA-A2 recombinant heavy chains containing the Bir A biotinylation motif were refolded with either of the peptides GLC, YVL, or CLG, and HLA-B8 recombinant heavy chains were refolded in the presence of either FLR, QAK, or RAK peptide. After biotinylation with Bir A (Avidity, Denver, CO) the refolded heavy chains were purified on a Superdex G75 HiLoad 26/60 prep grade FPLC column (Amersham Pharma- cia Biotech, Uppsala, Sweden) followed by further purification on a Mono Q anion exchange FPLC column (Amersham Pharmacia Biotech). The purified monomers were tetramered by addition of streptavidin RPhycoerythrin (Molecular Probes, Eugene, OR) at a molar ratio of 4:1, respectively.

Donors

Healthy laboratory donors that were HLA-A2- and/or HLA B8-positive as judged by sequence-specific primer PCR were recruited for this study. All donors were identified as either EBV-seropositive (i.e., virus carriers) or EBV-seronegative (i.e., noninfected) by standard serum Ab assays for virus capsid Ags.

Cell preparation

Donors were bled 120 ml into heparin, and their PBMCs were isolated by centrifugation on a lymphoprep gradient (Nycomed Pharma, Oslo, Norway) as per the manufacturer’s instructions. PBMCs were enriched for CD8+ cells by incubation of PBMCs with mAbs specific for CD4 (clone RF74), CD19 (clone RFB19), γδ TCR, CD14, and CD16 (Immunotech, Marseilles, France), as well as Ab specific for glycoporin (BD PharMingen, San Diego, CA) to remove contaminating erythrocytes. The Ab-coated cells were depleted by multiple rounds of incubation with sheep anti-mouse Ab-coated Dynabeads M-450 (Dynal, Oslo, Norway) as per the manufacturer’s instructions.

Subsets of CD8 cells were additionally enriched by depletion of cells with specific markers. CD8+ CD45RA+ cells were selected by depletion of CD45RO+ cells through incubation of the enriched CD8+ population with an anti-CD45RO mAb (clone UCHL1), whereas CD8+ CD54+ cells were enriched by incubation of the CD8+ population with an anti-CD54 mAb (clone SN130), followed by magnetic bead depletion.

CD62L-negative cells were enriched by depletion of CD62L-expressing cells from the CD8+ population. CD8+ cells were incubated with an anti-CD62L mAb (BD Biosciences, San Jose, CA) followed by incubation with goat anti-mouse Dynabeads M450 and magnetic separation.

CD28-negative cells were enriched for by FACS. CD8+−cells were enriched with an anti-CD28 mAb (BD Phamingen) followed by incubation with goat anti-mouse heavy-light chain-specific FITC-labeled Abs (Southern Biotechnology Associates, Birmingham, AL). Nonstained cells were separated from CD28-expressing cells by using a FACSVantage (BD Biosciences).

Tetramer staining assays

Staining of lymphocytes was undertaken by incubating the cells with a pretitrated concentration (~0.5 µg/ml) of tetramer at 37°C for 15 min. The cells then were stained for surface markers by incubation on ice with saturating amounts of anti-human CD8 conjugated to Tricolor (Caltag Laboratories, Burlingame, CA) and CD45RA-FITC (Beckman Coulter, Fullerton, CA), CD45RO-FITC (Dako, Ely, U.K.), or CD28-FITC (BD PharMingen). To detect CCR7 expression, tetramer-stained cells were stained with anti-CCR7 Abs as described above. These cells then were stained with an Epics flow cytometer (Beckman Coulter). Note that color compensation between the different fluorochromes was set with single fluorochrome-stained cells from the same test population as described previously (29).

For four-color flow cytometric analysis, cells were stained with tetramer and anti-CCR7 Abs as described above. These cells then were stained with anti-human CD8-PerCP (Becton Dickinson) in combination with allophycocyanin (APC)-labeled Abs specific for either CD54RA, CD45RO, CD62L, or CD8 (BD Pharmingen). These cells then were analyzed with a FACSCalibur flow cytometer (BD Biosciences).

Cytokine secretion assays

IFN-γ production by cells was assayed either by ELISPOT analysis or by intracellular cytokine staining, and TNF-α production was assayed by intracellular cytokine staining.

For ELISPOT analysis, serial dilutions of cells were plated on MultiScreen Immobilon-P filtration plates (Millipore, Bedford, MA) that had been precoated with IFN-γ-specific mAbs from an IFN-γ ELISPOT kit.
used as per the manufacturer’s instructions (Mabtech, Nacka, Sweden). The cells were incubated overnight with peptide at a concentration of 20 μg/ml or an equivalent dilution of DMSO. IFN-γ production by the cells was shown by using a human IFN-γ ELISPOT kit (Mabtech) and regions of secreted IFN-γ revealed by using an alkaline phosphatase chromogenic substrate kit (Bio-Rad, Hercules, CA). These regions then were counted with a dissecting microscope.

Intracellular cytokines produced by CD8+ lymphocytes were detected by initially incubating PBMCs at room temperature for 30 min with a phycoerythrin-labeled HLA-A2 GLC or an HLA-B8 RAK tetramer in RPMI 1640 supplemented with 10% FCS. The cells were washed and incubated in RPMI 1640 10% FCS in the presence or absence of GLC or RAK peptide, respectively, at a final concentration of 10 μM, in V-bottom tubes, at 37°C in the presence of 5% CO2 for 6 h. Brefeldin A (Sigma-Aldrich, Poole, U.K.) was added to the cultures at a final concentration of 5 μg/ml after the first hour of incubation. The cells then were stained with saturating amounts of anti-CD8-PerCP (BD Biosciences), washed in PBS, and fixed in 4% formaldehyde. The cells then were washed twice in a PBS buffer containing 0.1% saponin and 1% FCS (permeabilization buffer), and stained with either 0.5 μg of anti-IFN-γ-APC Ab (BD Biosciences) or 0.5 μg of anti-TNF-α-APC Ab (BD Biosciences) or 0.5 μg of anti-IgG-APC-negative control Ab, diluted in 100 μl of permeabilization buffer for 60 min on ice. The cells then were washed, resuspended in PBS, and analyzed on a FACScalibur flow cytometer (BD Biosciences). In experiments to analyze the phenotype of the T cells that expressed cytokines, an additional staining step was performed after the 6-h incubation step whereby the cells were stained with one of the following Abs: anti-CD45RA-FITC (BD Biosciences), anti-CD62L-FITC (BD Biosciences), or anti-CCR7-FITC (made in the laboratory of H. Hasegawa; Ref. 30). Control experiments were performed to show that the stimulation protocol did not alter expression of CD45RA, CD45RO, CD28, or CCR7 on the CD8+ T cells.

**Results**

**Epitope-specific cytotoxicity by memory CD8+ T cells**

We initially screened eight healthy EBV-seropositive donors with the HLA-A2.1 and/or HLA-B8 allele by staining fresh PBMC preparations with HLA class I/peptide tetramers refolded with one of three A2.1-restricted epitopes (the lytic epitopes GLC and YVL and the latent epitope CLG) or one of three B8-restricted epitopes (the lytic epitope RAK and the latent epitopes FLR and QAK). To explore whether these freshly isolated cells might display immediate cytotoxic function, we enriched CD8+ T cells from PBMCs by negative selection for CD19+, CD14+, CD16+, CD4+, and γδ+ populations and then assayed these CD8-enriched effectors on autologous target cells loaded with the relevant epitope peptide. Fig. 1A shows representative results from such assays involving three different donors. Clear evidence of epitope-specific lysis was detectable, whether using CD8+ effectors containing 1–3% cells staining with the A2/GLC or B8/RAK tetramer or using CD8+ effectors where the frequency of B8/FLR-staining cells was only 0.2%. Similar results were obtained in assays involving all eight donors tested on the six different epitopes. In many cases, significant lysis could be detected at E:T ratios as low as 0.2 tetramer-positive cells per 1 target cell in the assay. The specificity of these results was confirmed in parallel studies with EBV-seronegative donors carrying the A2.1 or B8 allele. Staining assays on PBMCs from seronegative donors never identified significant numbers of CD8+ T cells binding the relevant tetramers, and enriched CD8+ preparations from these donors never gave lysis above background when assayed on epitope-labeled targets. Representative results from control donors, again looking for evidence of A2.1/GLC, B8/RAK or B8/FLR reactivities by tetramer staining and by ex vivo cytotoxicity assays, are shown in Fig. 1B.

We also screened the same panel of EBV-seropositive and EBV-seronegative donors for evidence of peptide-induced cytokine production with two previously established assays: 1) ELISPOT assays of overnight IFN-γ release; and 2) intracellular staining for IFN-γ and of TNF-α production within 6 h of peptide stimulation. With the same set of EBV-epitopes, both assays clearly showed specific responses that were restricted to EBV-seropositive donors with the appropriate A2.1 or B8 allele (Ref. 21 and data not shown).

**Phenotype of EBV epitope-specific memory populations**

In the next series of experiments, we analyzed the phenotype of epitope-specific memory cells by staining PBMCs from the above EBV-seropositive donors with a PE-labeled tetramer, a tricolor-labeled anti-CD8 mAb, and a FITC-labeled mAb against one of a
series of potentially relevant cell surface differentiation markers. Certain markers were either uniformly absent or uniformly present on the tetramer-positive population irrespective of the epitope being analyzed. Fig. 2 presents relevant cytometric staining profiles (gated on the CD8+ T cells) for the same PBMC populations from EBV-seropositive donors JL, DW, and YS used as the source of effectors for the ex vivo cytotoxicity assays in Fig. 1A. The tetramer-positive cells within these populations were uniformly negative for the T cell activation marker CD69 (Fig. 2, top) and for a second activation marker CD38 (data not shown). The cells also were negative for CD56 (Fig. 2, middle), a marker that is usually associated with NK cells but that also has been proposed as identifying a CD8+ T cell subset with direct cytotoxic function in assays of anti-CD3-directed cytotoxicity (31). The same tetramer-positive cells were almost entirely positive for CD27, a marker the loss of which has been proposed to mark the acquisition of effector function in redirected killing assays (7, 8, 10).

By contrast, other phenotypic markers of potential interest did reveal heterogeneity within certain epitope-specific (i.e., tetramer-staining) populations. Fig. 3 shows representative results from one of the eight EBV-seropositive donors analyzed, DW. This donor is HLA-A2.1, B8-positive and so was screened by using two A2.1-restricted lytic cycle epitopes, GLC and YVL, the B8-restricted lytic cycle epitope RAK, and two B8-restricted latent cycle epitopes, FLR and QAK. The lytic epitope-specific populations, which accounted for 1% (GLC) and 3% (RAK) of circulating CD8+ T cells, were clearly heterogeneous in terms of CD45 isoforms. In both cases, some 30–35% of the tetramer-staining cells were CD45RAhigh, and some 65–70% were CD45ROLow. In earlier work, double staining for CD45RA and RO has clearly shown an inverse relationship between cell surface expression levels for the two markers both within the CD8+ T cell population as a whole (32) and within tetramer-positive populations (9, 32). Therefore, we interpret the illustrated data for GLC- and RAK-specific memory in donor DW as denoting the existence of distinct CD45RAhighROlow and CD45RAlowROhigh populations, with a CD45RAhighROlow intermediate population. The YVL lytic epitope-specific population followed the same distribution pattern as these two epitope specificities (data not shown). Interestingly, the smaller numbers of CD8+ T cells in donor DW staining with the FLR- and QAK-latent epitope-tetramers did not show this heterogeneity. These memory populations were almost uniformly CD45ROhigh, with little or no cells of a CD45RAhighROlow phenotype detected.

A similar distinction between lytic and latent epitope-specific responses could be drawn from CD28 staining. In the GLC-, RAK-, and YVL-specific populations, CD28+ cells outnumbered CD28− cells, whereas the FLR-specific and QAK-specific populations were predominantly CD28−. When the analysis was extended to two other markers, CD62L and CCR7, which are often coexpressed on cells in the CD8+ lineage, both showed heterogeneity within tetramer-staining populations. There was no clear-cut distinction between lytic and latent epitope-specific phenotypes with respect to these two markers, although there was a suggestion that a higher proportion of latent epitope-specific memory was CCR7+ compared with lytic epitope memory.

Table I presents the overall data from the eight EBV-seropositive donors analyzed. The results, presented so as to distinguish between lytic and latent epitope-specific populations, make clear that the trends illustrated in Fig. 3 are consistent across donors. Thus, the latent epitope-specific response was strongly polarized toward a CD45RA−, CD45ROLow, CD28− phenotype. In contrast CD8+ memory to lytic epitopes showed a significant degree of CD45RO loss and acquisition of a CD45RAhigh phenotype and a significant percentage of cells that had lost CD28. Interestingly, the extent of CD45RA positivity and CD28 negativity among lytic epitope-specific T cells differed significantly between individual donors but was similar when responses to different lytic epitopes were compared within an individual. In particular, we noted that where detectable in memory, the YVL response phenotype resembled that of the GLC response despite differences in the size of the two populations. We also noted that latent epitope-specific populations tended to show higher proportions of CCR7+ and of CD62L+ cells than did lytic epitope populations.

Memory CD8+ T cell phenotype and cytotoxic function

We then sought to analyze the relationship between memory T cell phenotype and the epitope-specific cytotoxic activity seen earlier in CD8-enriched effector populations. For this purpose, PBMCs were first CD8-enriched as before with CD19, CD14, CD16, CD4, and CD62L counter selection, and then aliquots further depleted either of CD45RO+ cells or of CD45RA+ cells.

Fig. 4 shows representative flow cytometric profiles of the three effector populations produced in one of several experiments of this kind. Here, the CD8-enriched preparation from the B8-positive donor CW contained a population of B8/RAK tetramer-staining cells that was 25% CD45RA− and 82% CD45ROLow. Depletion of CD45RO− cells gave a preparation in which the tetramer-positive cells were 100% CD45RA−, of which the great majority were CD45RAhigh and CD45ROLow but a small minority (<10%) were...
CD45RO$^{low}$. Likewise, depletion of CD45RA$^+$ cells gave a preparation in which the tetramer-positive cells were 100% CD45RO$^{low}$ of which the great majority were CD45RO$^{high}$ and CD45RA$^-$, but with <10% showing low but detectable CD45RA expression. Epitope-specific lysis was observed with the CD8-enriched, CD45RA-enriched, and CD45RO-enriched effector cell preparations. Similar results were observed in additional experiments of this kind screening for GLC-specific lysis by total CD8, CD45RO-depleted, and CD45RA-depleted effector preparations from the A2.1-positive donor DCC (data not shown). Throughout these experiments, we again usually detected significant epitope-specific lysis (~15% $^{51}$Cr release above low background levels) at tetramer-positive E:T ratios down to 0.25:1. These studies clearly show that when CD8$^+$ effector function is measured against the cognate target in ex vivo assays, cytolysis is not restricted to the CD45RA$^{high}$ subset but is present also in the CD45RO$^{low}$ population. This could be confirmed in several assays on latent epitope-loaded target cells with CD8-enriched effector preparations where relevant tetramer-staining population was naturally polarized to be 100% CD45RO$^{-}$RA$^-$; significant levels of killing were reproducibly observed (e.g., Fig. 1A, B8/FLR-specific killing by effectors from B8-positive donor YS).

Another series of experiments examined the relationship between CD28 status and cytolytic function. Fig. 5A shows the results of one such experiment with effector populations from donor DW. Here, B8/RAK tetramer-staining cells in the CD8$^+$ pool were 35% CD28$^+$ and 65% CD28$^-$. Depletion of a CD8-enriched preparation for CD28$^-$. Depletion of a CD8-enriched preparation for CD28$^+$ cells produced an effector population in which >93% tetramer-positive cells were CD28$^+$; these effectors clearly mediated significant levels of RAK-specific lysis. The converse experiment with CD28-enriched preparations was not possible because 1) there is no counter marker allowing CD28$^+$ cells to be

**FIGURE 3.** Analysis of EBV epitope-specific CD8$^+$ T cells for CD45RA, CD45RO, CD28, CD62L, and CCR7 status. PBMCs from EBV-seropositive donor DW (HLA-2.1$^+$, -B8$^+$) were stained with the appropriate tetramer followed by Abs to CD8 and to the third marker in question. Assays were conducted with A2/GLC, B8/RAK, B8/FLR and B8/QAK tetr...
enriched by negative selection; and 2) in preliminary experiments, we noticed that treatment with anti-CD28 increased levels of killing by PBMC effectors, strongly implying that CD28 ligation could amplify effector function. Therefore, we again took advantage of the fact that many latent epitope-specific CD8 T cell responses being seen in CD45RO-enriched, CD45RA-enriched, CD28–, CD28+, CD62L− and CCR7− populations (data not shown). We subsequently used immunofluorescence assays in which PBMCs were first stained with the tetramer, then exposed to the epitope peptide and cultured for 6 h (in the presence of brefeldin A to prevent cytokine release), stained for CD8 and the additional surface marker of choice, and then permeabilized and stained for intracytoplasmic IFN-γ or TNF-α. This allowed the cytokine response to epitope peptide stimulation to be visualized within tetramer-positive cells of defined phenotype.

In the donors studied we found that between 30 and 90% of the GLC-specific T cells expressed IFN-γ after stimulation in vitro. Fig. 5B shows the results of an ex vivo assay with CD8-enriched effectors from donor CW, in which 93% of the B8/FLR tetramer-staining cells are CD28+. Strong lysis of FLR-loaded target cells was observed. These findings strongly suggest that both CD28+ and CD28− components of EBV-specific CD8 memory have immediate epitope-specific cytolytic capacity.

The two other markers of potential interest, CD62L and CCR7, always showed heterogeneous expression across both lytic and latent epitope-specific populations. Therefore, by using the approach of negative selection, we were only able to examine the cytolytic potential of the CD62L− and CCR7− components of epitope-specific memory. In both cases, we observed significant levels of ex vivo lysis on epitope-loaded targets (data not shown).

### Memory CD8+ T cell phenotype and cytokine release

In parallel experiments, we studied the relationship between memory cell phenotype and the ability to secrete cytokines in response to epitope-peptide stimulation. Initial studies with the ELISPOT assay as a measure of overnight IFN-γ release gave a pattern of results that exactly mirrored the cytotoxicity assays, positive responses being seen in CD45RO-enriched, CD45RA-enriched, CD28+, CD28−, CD62L+ and CCR7− populations (data not shown). We subsequently used immunofluorescence assays in which PBMCs were first stained with the tetramer, then exposed to the epitope peptide and cultured for 6 h (in the presence of brefeldin A to prevent cytokine release), stained for CD8 and the additional surface marker of choice, and then permeabilized and stained for intracytoplasmic IFN-γ or TNF-α. This allowed the cytokine response to epitope peptide stimulation to be visualized within tetramer-positive cells of defined phenotype.

In the donors studied we found that between 30 and 90% of the GLC-specific T cells expressed IFN-γ after stimulation in vitro. Fig. 6 displays the results obtained from a representative HLA-A2-positive donor whose GLC epitope-specific memory population was heterogeneous for CD45RO, CD45RA, and CD28 expression. Freshly isolated PBMCs from this donor were analyzed for IFN-γ production after 6 h either in culture medium as a control or in presence of the GLC epitope peptide. The flow cytometric profiles are obtained by gating on the tetramer-positive, CD8+ cells in the PBMC population and plotting IFN-γ staining against expression of the phenotypic marker of choice (y-axis). As can be seen from the three profiles shown for unstimulated cells, there were very few tetramer-positive cells spontaneously producing the cytokine. Fig. 6 (top) shows the response in relation to CD45RO status. Both the CD45RO+ fraction and the CD45RO− fraction contained responsive cells after peptide stimulation, such that slightly below half of the cells in each fraction became positive for cytoplasmic IFN-γ. Likewise, responses were observed in CD45RA+ and CD45RA− fractions (Fig. 6, middle) and in CD28+ and CD28− fractions (Fig. 6, bottom) in parallel assays. A similar pattern of results was obtained in assays on B8-positive donors with the B8-restricted RAK lytic cycle epitope and also in assays that used TNF-α instead of IFN-γ as the index cytokine (data not shown). Note that in such assays the overall profile of CD45RO, CD45RA, and CD28 staining among tetramer-positive cells was not altered as a result of the peptide stimulation.

This approach then was extended to look at the responsiveness of tetramer-positive CD8+ memory cells in relation to CD62L and CCR7 status. Fig. 7 shows representative data from an experiment that again focused on the GLC-specific memory population of an

### Table I. Phenotypic analysis of lytic and latent epitope-specific populations

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*a Lytic epitopes are shown in the top half of the table, latent epitopes are shown in the bottom half.

*b nd, Not done.
A2-positive donor. When analyzed with respect to CD62L (Fig. 7, top), the IFN-γ response was almost entirely restricted to those tetramer-positive cells that were CD62L−. However, in this type of experiment, exposure to the epitope peptide reproducibly induced a shift in the distribution of tetramer-positive cells so that post-stimulation more cells scored as CD62L−. Therefore, although much of the cytokine response to peptide stimulation appears to be mediated by the original CD62L+ cells or of CD45RA+ cells or left undepleted. Cells from each preparation were stained with tetramer followed by Abs to CD8 and either to CD45RA (left) or CD45RO (middle); percentage values on staining profiles refer to the percentage of tetramer-positive cells that stained for the CD45RA or CD45RO marker. Other cells from each preparation were tested as effectors in cytotoxicity assays and the results expressed as in Fig. 1.

Given the apparent relationship between CCR7 status and effector capacity, we went on to analyze the phenotype of the CCR7+ and CCR7− populations of tetramer-positive cells in terms of the phenotypic markers, CD45RO, CD28, and CD62L+. Fig. 8 (left) shows the results of four-color staining of PBMCs from an A2+, B8+ donor with the PE-labeled B8/RAK tetramer, PerCP-labeled anti-CD8, FITC-labeled anti-CCR7, and an APC-labeled mAb against the fourth marker of choice. The first profile, gated only on CD8+ cells, shows tetramer staining in relation to CCR7 status. Note that (as was also apparent in Fig. 3) those tetramer-positive cells that are CCR7+ show levels of CCR7 staining significantly lower than shown by the CD8− CCR7+ population as a whole (see Discussion). The other profiles in Fig. 8 (left), gated on tetramer-positive CD8+ cells, show that most if not all of the CCR7+ fraction are CD45ROhigh, CD28+ and CD62L+. By contrast, the CCR7− fraction of B8/RAK tetramer-positive cells are heterogeneous for all three of the above markers and contained significant numbers of cells that are CD45RO−, CD28− and CD62L−. Fig. 8 (right) shows the corresponding data for CD8 memory to a latent cycle epitope B8/FLR, in the same donor. Again, the tetramer-positive population could be divided into low CCR7+ and CCR7− fractions. In this case, where epitope-specific memory is polarized toward a CD45ROhigh CD28− phenotype, then not surprisingly, both CCR7+ and CCR7− fractions are uniformly positive for both the CD45RO and CD28 markers. However, latent epitope-specific memory is known to be heterogeneous in terms of CD62L status. Here, just as seen earlier for the lytic epitope response, the CCR7− fraction of FLR-specific memory is uniformly CD62L− whereas the CCR7+ fraction is composed of CD62L+ and CD62L− cells.

**Discussion**

The original study of EBV-specific CD8+ responses with HLA class I tetramers was the first to show definitively that Ag-experienced CD8+ T cells in man could be phenotypically heterogeneous in terms of CD45RO vs RA expression and in terms of CD28 status (3). The present work was prompted by our observation that these tetramer-staining CD8+ populations also appeared functionally heterogeneous, in that only 20–40% of tetramer-positive cell numbers were detected in ELISPOT assays of peptide-induced IFN-γ release (21). Our aim was to realign the much
discussed relationship between CD8+ T cell phenotype, type I cytokine production, and cytotoxic capacity (7, 8, 10–12, 31) with assays specific for the cognate epitope rather than the nonspecific assays (PMA/ionomycin stimulation and anti-CD3 redirected cytotoxicity respectively) that had been used to date. As others have reported (22, 33), we found that staining for the tetramer, CD8, and a third surface marker of choice could be combined with intracellular staining for epitope-induced IFN-\gamma or TNF-\alpha release, allowing the phenotype of cytokine-producing cells to be analyzed (Figs. 6 and 7). We also found that EBV-specific CD8+ T cells in the blood of healthy virus carriers were capable of lysing epitope-loaded target cells at significant levels in standard 5-h cytotoxicity assays, providing another indicator of specific effector function (Fig. 1). Although EBV epitope-specific lysis has been detected in ex vivo assays using primary effector populations from IM patients (19, 20), we had not expected the much smaller numbers of epitope-specific CD8+ T cells in healthy donors to mediate detectable levels of lysis. However, the result was observed on numerous occasions at tetramer-positive target cell ratios as low as 0.2:1. Direct comparisons of the efficiency of EBV-specific lysis by primary vs memory CD8+ cells at equivalent E:T ratios remain to be conducted. It may be that the levels of killing seen to date in ex vivo assays with T cells from IM patients are underestimates of the true lytic potential because the assays have been conducted only with cryopreserved effectors and the activated T cells present in IM are highly susceptible to apoptosis during such manipulations (34).

The present results make clear that these two epitope-specific rapid effector functions, IFN-\gamma/TNF-\alpha synthesis and target cell lysis, are present within CD8+ populations in which the tetramer-positive cells are uniformly negative for conventional markers of activation such as CD69 or CD38 (Fig. 2 and data not shown); they

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**FIGURE 5.** A, RAK epitope-specific cytotoxic function of CD28+ and CD28− preparations of CD8 effectors. PBMCs from EBV-seropositive donor DW (HLA-B8+) were first enriched for CD8+ cells as in Fig. 4, then aliquots of this preparation were either further depleted of CD28+ cells or left undepleted. Some cells from each preparation were stained with tetramer followed by Abs to CD8 and to CD28; percentage values on staining profiles refer to the percentage of tetramer-positive cells which stain from the CD28 marker. Other cells from each preparation were tested as effectors in cytotoxicity assays and the results expressed in Fig. 1. B, FLR epitope-specific cytotoxic function of CD8+ enriched PBMC preparations from the EBV-seropositive donor CW (HLA-B8+). Results are presented as in A.

**FIGURE 6.** Cytoplasmic IFN-\gamma production by tetramer-staining CD8+ T cells in relation to CD45RO, CD45RA, and CD28 status. PBMCs from EBV-seropositive donors (HLA-A2.1+) were stained with the A2.1/GLC tetramer, incubated with the GLC peptide (or with no peptide as a control) followed by a 6 h incubation in the presence of brefeldin A, then stained for CD8 and for the phenotypic marker of choice before fixation, permeabilization, and staining for cytoplasmic IFN-\gamma. Results were obtained by gating on the tetramer-positive CD8+ population; left, staining profiles refer to unstimulated cells, right, staining profiles refer to GLC-stimulated cells. Percentage values show the proportion of cells in each of the four quadrants.

**FIGURE 7.** Cytoplasmic IFN-\gamma production by tetramer-staining CD8+ T cells in relation to CD62L. and CCR7 status. PBMCs from an EBV-seropositive donor (HLA-A2.1+) were treated as in Fig. 6 with CD62L or CCR7 as the phenotypic marker of choice. Results are expressed as in Fig. 6.
Wherever the cytokine synthesis and cytotoxicity assays could be applied to the same subpopulation of CD8+ T cells, concordant results were observed. Hence, in the experiment described in Fig. 6 we found that a significant proportion (30–50%) of tetramer-positive cells responded to epitope stimulation by IFN-γ synthesis whether these cells lay within the CD45RA+, CD45RO+, CD28+, or CD28− subpopulation (Fig. 6). These findings are in line with those of an earlier report focussing entirely the CD8+ memory response to the A2-restricted lytic cycle epitope GLC (33). That report also analyzed the IFN-γ response in relation to CD62L status and found that all positive responses appeared to map within the CD62L− fraction. Essentially similar results were observed in the present work; however, they need to be interpreted with caution because, as we show, some responses may be mediated by cells that originally lie within the CD62L− fraction but that down-regulate the marker during the assay period. More importantly, we showed that CCR7 status was a better discriminator of competence in the cytokine assay. Thus, there were essentially no IFN-γ-responsive cells within the CCR7−/H11001 × 1027 fraction of the tetramer-staining population, whereas the majority of (but not all) CCR7+ cells were responsive (Fig. 7).

These findings strengthen the view that during CD8+ T cell differentiation, loss of CCR7 (subsequently followed by loss of CD62L) denotes the transition from a “central memory” compartment of cells that naturally home to lymph nodes and lack immediate effector function to an “effector memory” compartment of cells that can migrate to tissues and do have immediate effector function (14). We also noted that CCR7 levels in tetramer-positive CCR7+ cells were always lower than on the CD8+ CCR7+ population as a whole. Because the latter population is predominantly made up of naive CD8+ T cells, recognized by their CD45RAhigh LFA1low phenotype (9, 14), we postulate that the transition from naive to “central memory” compartments is associated with a reduction in CCR7 surface levels. All EBV epitope-specific memory cells identifiable by tetramer staining contained both CCR7+ and CCR7− fractions; we found that the CCR7+ fraction of this memory population is uniformly CD45RO+, CD28+, and CD62L−, whereas the CCR7− fraction can be heterogeneous for all three markers (Fig. 8). This is consistent with a view of CD8+ T cell differentiation where, after loss of CCR7, Ag-experienced cells can lose CD62L and/or CD28, and (irrespective of CD62L or CD28 status) may revert to become CD45RA+ (7, 9, 12). However, based on the assays used in the present work, there is as yet no clear functional difference between these phenotypically distinct subsets of the effector memory (CCR7+) pool.

Finally, the present study highlights a hitherto unnoticed feature of EBV-specific CD8+ memory cells that may help to explain the circumstances under which heterogeneity is generated in “effector memory” populations. There is a clear relationship between the phenotype of CD8 memory and the identity of the EBV epitope. Thus, latent epitope-specific T cells in the blood of healthy virus carriers are strongly polarized toward a CD45ROhigh CD45RA−, CD28+ phenotype. Such findings are consistent both with early work showing that EBV-specific memory T cells responsive to LCL stimulation in vitro (i.e., latent Ag-specific reactivities) were concentrated within the CD45ROhigh subpopulation (35), and also with more recent studies that used TCR rearrangement to locate FLR-specific memory clonotypes in the CD45RO+ but not in the CD45RA− fraction of circulating CD8+ T cells (36). Others also have used EBV-latent epitope peptides as inducers of IFN-γ release and have found responses only within the CD28− CD8+ subset (37). By contrast, a significant proportion (2–66%, mean 25%) of lytic epitope-specific T cells in the donors studied here

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**FIGURE 8.** Analysis of the CCR7+ and CCR7− subsets of tetramer-stained CD8+ T cells for expression of the CD28, CD45RA, and CD62L markers. PBMCs from an EBV-seropositive donor (HLA-B8+) were stained with B8/RAK tetramer (left) or with the B8/FLR tetramer (right), followed by Abs to CCR7, to CD8, and to the fourth phenotypic marker of choice. The upper staining profiles were obtained by gating the analysis on CD8+ T cells; percentage values refer to the percentage of tetramer staining cells that were CCR7+. The other staining profiles were obtained by gating the analysis on tetramer-staining CD8+ cells; percentage values show the proportion of cells in each of the four quadrants.

are also negative for the cell cycle marker Ki67 (9). This contrasts with the situation in IM where the primary EBV-specific CD8+ T cells are actively cycling and express activation markers (3–5). Therefore, it seems unlikely that the functions being detected in the CD8+ pool of virus carriers reflect the reactivation of a small fraction of the circulating EBV-specific memory pool to a highly activated lymphoblastoid state akin to that shown by primary CD8+ effectors in IM blood.

Our results also show that epitope-specific cytolytic function is not restricted to the particular CD8+ subpopulations that have been proposed as “effectors” on the basis of their capacity for anti-CR3 redirected killing of Fc receptor-positive targets. Such “effectors” have been variously identified with the CD45RAhigh CD11a+ (8), CD45RA+ CD27− (7, 10), CD28− (11, 12), and CD56− HLA-DR+ (13, 31) subpopulations of circulating CD8+ T cells. However, we detected epitope-specific lysis within both the CD45RA+ and CD45RO+ subpopulations, and within the CD28+ and CD28− subpopulations; furthermore, lysis was regularly observed despite the fact that the tetramer-positive component of the CD8+ T cell pool was uniformly CD27+ and CD56− (Figs. 1, 2, 4, and 5).
had a CD45RA+ phenotype, and many had also lost the CD28 marker (Fig. 3 and Table 1).

This difference is interesting in view of what is known about the magnitude of the relevant responses. Although lytic epitope specificity in CD8+ memory cells usually outnumber those against latent epitopes, the contrast is much more dramatic during primary infection. There the expansion of lytic epitope responses is at least 10-fold greater than that of latent responses (3), and this is even true for the primary response to a lytic cycle epitope such as YVL, of which the subsequent representation in memory can be quite low (N. Annels and A. Hislop, manuscript in preparation).

Indeed, some clonotypes within the highly amplified primary response to the lytic cycle epitope GLC can apparently be driven to the point of clonal exhaustion (29), whereas immunodominant responses to the lytic cycle epitope FLR are not (38, 39). These differences in the degree to which responses are amplified may reflect the higher levels of Ag load produced by lytic EBV replication in vivo compared with that in latently infected cells. We suggest that this amplification of the lytic Ag-induced response over time drives some cells within the “effector memory” to differentiate further to a CD45RAhigh and/or CD28− phenotype. In this context, several studies of infection with human CMV, a β-herpesvirus that appears to elicit even higher levels of CD8 immunity than does EBV, report that CMV-specific memory populations are predominantly CD45RA−, CD28−, CD27− (32, 40–42). This appears to reflect an even greater level of virus-driven CD8+ T cell differentiation in vivo to a point where the CD27 marker also is lost. This might also explain the finding that in HIV-positive patients, where EBV loads can become very high, EBV-specific CD8 memory cells may themselves acquire a CD27− phenotype (D. van Baarle, personal communication). In the present work, it was interesting to note that although there were differences between individuals in the degree to which lytic epitope-specific effector memory populations became CD45RAhigh and/or CD28−, within any one individual all lytic epitope-specific populations analyzed showed similar degrees of phenotypic progression. Further studies will be needed to determine whether the prevailing level of chronic EBV replication occurring in virus carriers influences the memory phenotype or whether these differences between individuals are established early on in the immediate aftermath of the primary infection.

Acknowledgments

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References


