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Memory T Cells Constitute a Subset of the Human $CD8^+CD45RA^+$ Pool with Distinct Phenotypic and Migratory Characteristics[1]

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Using HLA class I-viral epitope tetramers to monitor herpes virus-specific $CD8^+$ T cell responses in humans, we have shown that a significant fraction of responding cells revert from a $CD45RO^+$ to a $CD45RA^+$ state after priming. All tetramer-binding $CD45RA^+$ cells, regardless of epitope specificity, expressed a phenotype $LFA\text{-}1^{high}CCR7^{low}$ that was stable for at least 10 years in infectious mononucleosis patients and indefinitely in asymptomatic carriers. $CD8^+CD45RA^+LFA\text{-}1^{high}$ cells were not present in cord blood but in adults account for up to 50% of $CD8^+CD45RA^+$ cells. These $CD45RA^+LFA\text{-}1^{high}$ cells have significantly shorter telomeres than $CD45RA^+LFA\text{-}1^{low}$ cells, suggesting that the latter represent a naive population, while the former are memory cells. $CD45RA^+$ memory cells are a stable population of noncycling cells, but on stimulation they are potent producers of IFN-γ, while naive $CD8^+$ cells produce only IL-2. The chemokine receptor profile and migratory potential of $CD45RA^+$ memory cells is very similar to $CD45RO^+$ cells but different to naive CD8 cells. In accord with this, $CD45RA^+$ memory cells were significantly underrepresented in lymph nodes, but account for virtually all $CD8^+CD45RA^+$ T cells in peripheral tissues of the same individuals. ***The Journal of Immunology*****, 2001, 167: 212–220.**

Memory is a defining characteristic of the specific immune system. The ability to produce a more rapid and vigorous response on secondary Ag challenge can persist for decades. The processes that lead to the generation and persistence of memory B lymphocytes in germinal centers are well understood (1, 2), but the precise nature of memory T cells has remained controversial. In humans, differentially spliced isoforms of the leukocyte common Ag (CD45) have been used to discriminate populations that were thought to represent naive and memory cells (3). The highest m.w. isoform, CD45RA, is expressed on a stable population of cells that divide approximately once every 2 years; they respond poorly to recall Ags and when activated they lose expression of the CD45RA isoform and instead express the low m.w. CD45RO splice variant. The $CD45RO^+$ population responds rapidly to recall Ags, and cells within it divide about once every 2 wk in vivo (4, 5). These characteristics led to the hypothesis that CD45RA expression defines naive cells and CD45RO memory T cells. With each cell division $CD45RO^+$ cells become more susceptible to apoptosis, requiring high levels of survival factors to prevent their death; in vivo they reach clonal senescence within ~3 years of the proliferative cycle (6). This phenomenon is crucial for the maintenance of a homeostatic balance to T cell populations, but also defines a limit to memory maintained by continuously cycling cells. Several lines of evidence have suggested that a population of memory cells exists in the stable $CD45RA^+$ state (7–10), but the lack of markers available to discriminate them from naive cells has prevented this hypothesis from being tested. Primed rat T cells that lack the CD45RC isoform re-express it when introduced into nude rats, suggesting the potential for reversion (7). In humans, $CD4^+CD45RA^+$ T cells contain a high frequency of recall Ag-responsive cells in primed individuals, which can be revealed when adequate costimulation of T cells is provided (8). The concept of a stable, noncycling population of memory T cells is attractive because the naive pool is rapidly depopulated following initial Ag challenge; clonal senescence induced by the progressive increase in susceptibility to apoptosis should consequently lead to loss of response within a few years.

The ability to identify individual Ag-specific $CD8^+$ T cells by staining with MHC class I-peptide tetramers (11, 12) provides a novel way to address these issues. We have used such reagents to track human $CD8^+$ T cell responses to two common herpes viruses: EBV and CMV. These are genetically stable agents that persist at a low level in the healthy immunocompetent host and therefore provide a chronic stimulus to the immune system. Furthermore, primary EBV infection is clinically recognizable as infectious mononucleosis (IM),[4] allowing the $CD8^+$ response to be followed in individual patients from the acute primary phase to memory.

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[4] Abbreviations used in this paper: IM, infectious mononucleosis; ECD, phycoerythrin-Texas Red; MIP, macrophage inflammatory protein; HEV, high endothelial venule.

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Materials and Methods

Subjects studied

Samples were collected from nine subjects (two HLA-A2, six HLA-B8, one both HLA-A2 and -B8) with recent onset acute IM as soon as the diagnosis was confirmed. Chronic-phase samples were collected from the same patients 12 mo later. In three cases, cryopreserved samples were tested from IM patients (HLA-B8) obtained at diagnosis and 1 and 10 years after resolution of symptoms. Samples were also collected from five healthy subjects (two HLA-A2, three HLA-B7) that had tested positive for CMV IgG by latex agglutination at least 3 years previously and from three healthy subjects that had been EBV seropositive for at least 10 years (one HLA-A2, two HLA-B8). Peripheral blood, thoracic lymph nodes, and liver tissue was obtained from healthy organ donors and from individuals suffering from hepatitis C infection.

Cell preparation

Mononuclear cells were isolated from fresh heparinized venous or umbilical cord blood samples using Ficoll-Hypaque density gradient centrifugation (Pharmacia, St. Albans U.K.), according to the manufacturer's instructions. Samples for flow cytometry were immediately cryopreserved. $CD8^+$ lymphocytes were purified from freshly isolated PBMC by negative selection with magnetic Dynabeads (Dynal, Bromborough, Wirral, U.K.) as previously described (13). Briefly, adherent cells were depleted by two rounds of incubation on human serum-coated petri dishes at 37°C for 45 min. An Ab cocktail consisting of RFB7 (CD37), OKM1 (CD11b), RFT4 (CD4) (Royal Free Hospital, London, U.K.), RM052 (CD14), 3G8 (CD16), IMMU510 (TCR$\gamma\delta$) (Beckman Coulter, High Wycombe, U.K.), and GA-R2/HIR2 (glycophorin A) (BD PharMingen, Cowley, Oxford, U.K.) was used to enrich for $CD8^+$ cells. A single round of Dynabead depletion routinely generated samples of 60–80% $CD8^+$ cells. These cells were used for transmigration assays or further purified to $CD8^+CD45RO^+$ or $CD8^+CD45RA^+$ populations by two rounds of bead depletion with the addition of SN130 (CD45RA) or UCHL1 (CD45RO) (Royal Free Hospital). Purities were routinely >95%.

Liver-infiltrating lymphocytes were isolated from tissue removed during routine transplant operations from inflamed (five samples) and noninflamed (three samples) liver tissue as previously described (14). Blood and lymph node cells were isolated from the same individuals; expression of chemokine receptors was not affected by these procedures (14). Approval for these procedures was granted by the University Hospital Trust Local Ethics Committee.

MHC class I tetramers

Soluble PE-labeled peptide-MHC class I tetramers were constructed as previously described (11). EBV tetramers consisted of HLA-A2 with the peptide GLCTLVAML (BMLF1, aa 190–197) (15) or YVLDHLIVV (BRLF1, aa 109–117) (N. Annels, unpublished data), and HLA-B8 with RAKFKQLL (BZLF1, aa 280–288) (15). CMV tetramers consisted of HLA-A2 with NLVPMVATV (pp65, aa 495–503) (16) and HLA-B7 with TPRVTGGGAM (pp65, aa 417–426) (16).

Flow cytometry

Aliquots of PBMC were analyzed by four-color flow cytometry using a panel of surface molecule-specific Abs as follows: CD40L FITC (Ancell, Bayport, MA), CD16 FITC, HLA-DR FITC (Dako, Cambridge, U.K.), CD62L, CD29 FITC (Beckman Coulter), CCR1, CCR5, CCR6, CXCR3, CXCR5, CD49e, CD57 FITC, CD58 FITC, (Serotec, Oxford, U.K.), CD11a FITC, CD18 FITC, CD49d (HP2/1), CD27 FITC, CD28 FITC, CD29, CD61, CD69 FITC (BD PharMingen), CCR2, CXCR1, CXCR4, CD31 (9G11) (R&D Systems, Abingdon, U.K.), CCR7 (Millennium Pharmaceuticals, Cambridge, MA), $\alpha_4\beta_7$ (ACT-1) (a gift from Dr. M. Robinson, Celltech, Slough, U.K.), CD49f (4F10) (Imperial Cancer Research Fund, London, U.K.). Indirect labeling was achieved using an anti-mouse IgG FITC-conjugated Ab (Southern Biotechnology Associates, Birmingham, AL). Free binding sites were blocked with normal mouse serum (Serotec). Cells were colabeled with anti-CD8 phycoerythrin-Texas Red (ECD) (Beckman Coulter) and anti-CD45RA PE-Cy5 (Serotec) and either the appropriate PE-conjugated MHC class I tetramer or anti-CD11a PE (Beckman Coulter).

Intracellular protein expression was detected in PBMC labeled initially with anti-CD11a PE and anti-CD8 ECD Abs. Cells were resuspended in Permeafix (Ortho Diagnostic Systems, Amersham, U.K.) for 40 min at room temperature, before labeling with anti-CD45RA PE-Cy5 and either anti-perforin FITC (Ancell), anti-Bcl-2 FITC (Dako), or anti-Bcl-x_L (Santa Cruz Biotechnology, Santa Cruz, CA). Bcl-x_L binding was detected with a goat anti-rabbit IgG FITC Ab (Southern Biotechnology Associates). Samples were analyzed on a Beckman Coulter Epics XL benchtop flow cytometer (Beckman Coulter).

Telomere length analysis

Purified adult $CD8^+CD45RA^+$ lymphocytes were labeled with Ab-38-biotin (anti-CD11a) (a gift from Prof. N. Hogg, Imperial Cancer Research Fund, London, U.K.), followed by streptavidin Cy5 (Jackson ImmunoResearch, West Grove, PA). Adult $CD8^+CD45RA^+$ cells expressing high or low levels of CD11a were sorted to >95% purity using a Coulter EPICS Elite flow cytometer (Beckman Coulter), as previously described (6). Cord blood mononuclear cells, purified adult $CD8^+CD45RO^+$, and sorted adult $CD8^+CD45RA^+$ $CD11a^{high}$ and $CD11a^{low}$ cells were labeled with a FITC-conjugated peptide nucleic acid probe specific for telomeric repeat sequences (FITC-OO-$(CCCTAA)_3$) (PE Biosystems, Framingham, MA) as previously described (17). Samples were analyzed on the EPICS XL flow cytometer.

Cell cycle analysis

Freshly isolated PBMC were surface labeled with anti-CD11a PE, anti-CD8 ECD, and anti-CD45RA PE-Cy5 Abs before fixing in 70% ethanol for at least 4 h at −20°C. Cells were stained with control IgG1 FITC (Dako) or the Ki-67-specific Ab MIB FITC (Beckman Coulter) for 30 min before analysis on the EPICS XL flow cytometer.

Intracellular cytokine assay

Purified $CD8^+CD45RA^+$ and $CD8^+CD45RO^+$ lymphocytes were labeled with anti-CD11a biotin, followed by streptavidin RED670 (Life Technologies, Paisley, U.K.). Cells were stimulated for 6 h at 37°C, 5% CO_2 in the presence of 200 ng/ml PMA and 500 ng/ml calcium ionophore A23187 (Sigma, Poole, U.K.). Brefeldin A (5 μg/ml, Sigma) was added after 1 h of culture. Cells were labeled with anti-CD8 ECD before incubation in Permeafix, and finally labeled with optimal concentrations of anti-IFN-γ FITC and anti-IL-2 PE (BD PharMingen).

Transmigration assays

Migration of $CD8^+$ lymphocytes was assessed using 6.5-mm diameter, 5.0-μm pore size Transwell inserts (Corning Costar, Cambridge, MA) as previously described (18). Optimal doses (determined by prior titration) of the chemokines macrophage inflammatory protein (MIP)-1β, IL-8, (PeproTech, Rocky Hill, NJ), Epstein-Barr virus induced molecule 1 chemokine, secondary lymphoid tissue chemokine stromal cell-derived factor-1α (SDF-1), RANTES, MIP-1α, or monocyte chemoattractant protein-1 (MCP-1) (R&D Systems) were prepared in 0.5% fraction V BSA (Sigma) in RPMI 1640 (Sigma) supplemented with L-glutamine, benzylpenicillin, and streptomycin (Life Technologies). Chemokines were warmed to 37°C, and 600 μl was added to the lower Transwell chamber. A total of 5×10^5 $CD8^+$ lymphocytes in 100 μl were added to the upper Transwell chamber. After incubation for 18 h at 37°C, 5% CO_2, cells were carefully resuspended from the upper and lower chambers into 250 μl of 0.5% fraction V BSA/RPMI 1640. Control wells containing no chemokine or 100 ng/ml IL-15 (PeproTech) were included in each assay.

Cell counts for transmigration assays were determined by flow cytometry using an aliquot of recovered cells resuspended in a fixed volume of 25 μg/ml propidium iodide (Sigma), 25% FCS in PBS. The number of viable cells present in each sample was determined on the basis of cell size and exclusion of propidium iodide. A further aliquot of cells was labeled with anti-CD11a FITC, anti-CD45RA PE (Beckman Coulter) and anti-CD8 Tricolor (Caltag, Burlingame, CA) for phenotype analysis by flow cytometry. The migration of each subset of cells was determined as follows. Specific cell migration = (number of cells of phenotype in lower chamber)/(total number of cells of phenotype in upper and lower chambers).

Results

Identification of $CD8^+CD45RA^+$ memory cells specific for EBV epitopes in IM

We constructed tetramers specific for three EBV lytic cycle epitopes, GLC and YVL restricted through HLA A2.01 and RAK

restricted through HLA B8. In each case, donors with the appropriate HLA alleles, but EBV uninfected as defined by seronegativity in standard Ab screening assays, never showed significant levels of tetramer staining of CD8$^+$ T cells (Fig. 1*a*). By contrast, in acute IM patients with the appropriate HLA alleles the frequency of cells binding to these lytic epitopes was extremely high; 8–40% of CD8$^+$ T cells were tetramer positive (Fig. 1*b*) but fell to lower values (0.5–10% of CD8$^+$ cells) over the 12 mo following resolution of IM symptoms and establishment of the EBV carrier state. At the time of primary infection, all three EBV epitope-specific responses were largely composed of CD45RO$^+$ cells with few if any cells in the CD45RA$^+$ subset, consistent with their in vivo activation at this time. After resolution of the infection, the distribution of tetramer-positive cells for all three epitopes shifted significantly to include separate CD45RO$^+$ and CD45RA$^+$ subpopulations. Fig. 1, *b* and *c* shows results from a single patient during acute and postconvalescent phases, and Fig. 1*d* summarizes the data from all nine patients studied. We then studied healthy long-term carriers of EBV using the above three tetramers and also healthy carriers of CMV using tetramers specific for two immunodominant epitopes, both derived from the pp65 virion protein and restricted through HLA-A2.01 or -B7. In each case, tetramer staining was restricted to individuals with the relevant HLA allele, and the stained cells were distributed between the CD8$^+$ T cells subpopulations expressing CD45RA and CD45RO. CD8$^+$ T cells that have recently been recruited from the naive pool and then revert to rest can show expression of both CD45RA and CD45RO (7, 9–10). While a small proportion of tetramer-bearing cells were observed to express low levels of both isoforms, CD45RA^{bright} cells were invariably CD45RO$^-$; this population was gated for subsequent experiments (Fig. 1, *e* and *f*).

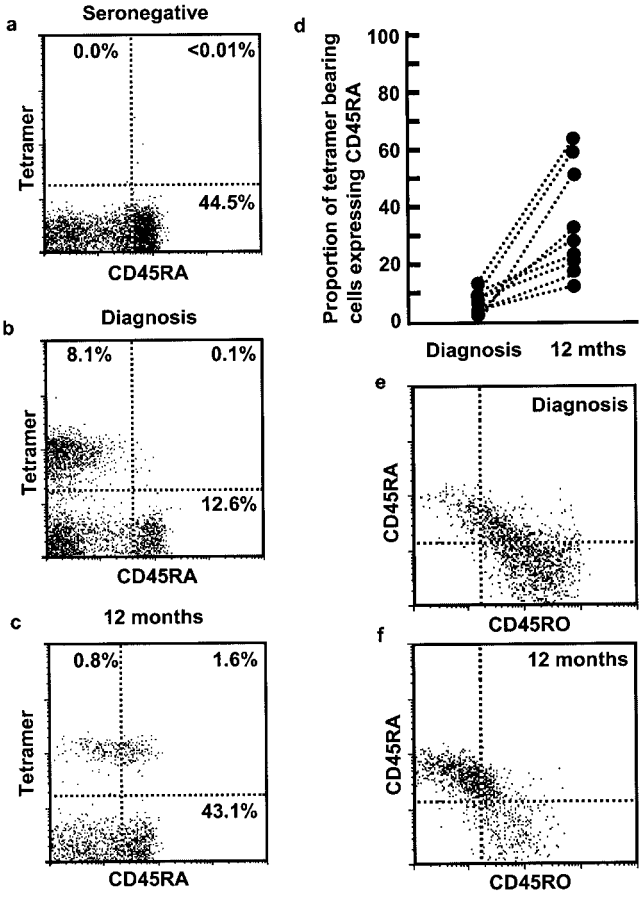

FIGURE 1. Tetramer-binding CD8$^+$ T cells appear in the CD45RA$^+$ population following Ag exposure and increase in number over time. PBMC from EBV-seronegative subjects and EBV-associated IM patients were assessed by four-color flow cytometry to determine the frequency of EBV Ag-specific cells. Cells were stained with CD3 FITC, PE-conjugated tetramer, CD8 ECD, and CD45RA PE-Cy5. All panels shown are gated on CD3$^+$CD8$^+$ cells. *a*, EBV-seronegative HLA-B8 individuals show very low levels of RAK-B8 tetramer binding, exclusively within the CD45RA$^+$ population. Data shown is from one of four individuals tested. *b*, A patient with EBV-associated IM assessed at diagnosis (<1 mo after infection) showed substantial binding of the RAK-B8 tetramer, predominantly in the CD45RO$^+$ population. *c*, The same patient shown in *b* assessed after resolution of disease, 12 mo after diagnosis. A marked shift of tetramer-positive cells to the CD45RA$^+$/CD45RO$^-$ population was observed. *d*, Summary of the proportion of tetramer-binding cells within the CD3$^+$CD8$^+$CD45RA$^+$ population during acute disease (at diagnosis) and the memory phase (12 mo after resolution of symptoms). A similar shift toward the CD45RA$^+$ population was seen in nine individuals with EBV-associated IM studied using three EBV tetramers (GLC-A2, YVL-A2, RAK-B8). *e* and *f*, Relative expression of CD45RA and CD45RO isoforms by RAK-B8 tetramer binding cells from a single patient at diagnosis (*e*) and at 12 mo (*f*).

These data indicate that large numbers of Ag-specific CD8$^+$CD45RA$^+$ T cells exist within the pool of virus-induced memory T cells. These tetramer-binding CD45RA$^+$ T cells cannot represent direct thymic emigrants, because the production of T cell specificities in the thymus is a random process that is unaffected by exposure to Ag. Consequently, they are likely to represent a population of memory cells that have reverted from the CD45RO$^+$-primed state that dominates Ag-specific T cell populations during primary infection.

CD8$^+$CD45RA$^+$ memory cells express high levels of LFA-1

Memory T cells exhibit distinct patterns of migration compared with naive T cells, and these are determined by their expression of specific adhesion molecules and chemokine receptors. For example, naive T cells express high levels of L-selectin and CCR7 that promote their recruitment via high endothelial venule (HEV) into lymph node, whereas memory cells lose these two receptors and instead express high levels of integrins and chemokine receptors that promote recruitment into tissue. Therefore, we used the EBV and CMV epitope tetramers to identify memory cells within the circulating CD8$^+$CD45RA$^+$ T cell pool and analyzed their profile of cell surface integrin and chemokine receptor expression compared with that of the CD8$^+$CD45RA$^+$ T cell pool as a whole. As shown in Fig. 2 by representative data based on the EBV RAK/B8 epitope, all tetramer-staining cells within the CD45RA$^+$ population showed high levels of expression of the LFA-1 adhesion molecule assayed by staining for its constituent chains: CD11a and CD18. In this regard, the tetramer-binding CD45RA$^+$ population was identical with tetramer-binding cells within the CD45RO$^+$ population. However, the total CD8$^+$CD45RA$^+$ pool was biphasic with respect to LFA-1 levels, clearly containing LFA-1^{high} and LFA-1^{low} fractions; this was in contrast to the total CD8$^+$CD45RO$^+$ pool, which was uniformly LFA-1^{high}. Studies with all five tetramers showed the same pattern of results; all tetramer-positive cells showed high levels of LFA-1, whether in the CD45RA$^+$ or the CD45RO$^+$ population and regardless of the time after infection.

Intriguingly, several other adhesion molecules showed elevated levels of expression on CD45RA$^+$ tetramer-binding cells, suggesting that this population may have different migratory properties to the naive CD8 population. Fewer tetramer-binding CD45RA$^+$ cells were CD62L$^+$ compared with tetramer-negative cells and more CD45RA$^+$ cells expressed high levels of CD29 and CD49e

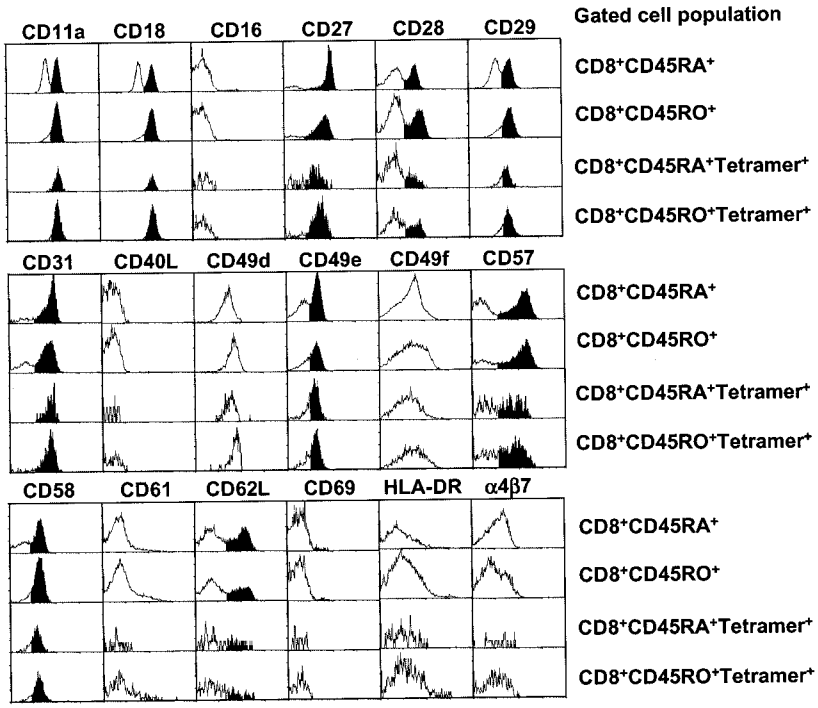

FIGURE 2. $CD8^+CD45RA^+$ tetramer-binding cells express high levels of LFA-1. PBMC from an HLA-A2/B8 individual seropositive for EBV were screened by four-color flow cytometry to assess the phenotype of RAK-B8 tetramer-binding T cells. Cells were stained with a panel of surface marker Abs on FITC, tetramer PE, CD8 ECD, and CD45RA PE-Cy5. Cells were gated to determine the expression of each marker in the indicated populations. Filled areas of histograms indicate the higher-expressing population where a biphasic distribution was observed within the $CD8^+CD45RA^+$ population. Both CD11a and CD18 (the α- and β-chains of LFA-1, respectively) showed an absolute relationship with tetramer-binding cells. These results were consistent in 18 individuals tested using EBV tetramers GLC-A2, YVL-A2, and RAK-B8 or CMV tetramers NLV-A2 and TPR-B7.

(Fig. 2). The $CD62L^{low}CD29^{high}CD49e^{high}$ phenotype was identical with that of the CD45RO population. That $CD45RA^+$ tetramer-binding cells are $LFA\text{-}1^{high}/CD49e^{high}$ and $CD62L^{low}$ has major implications for their migratory pathways.

The LFA-1^{high} phenotype of tetramer-bearing memory T cells is stable over time

In three patients with IM, we were able to study cryopreserved samples taken at diagnosis (~1 mo after infection) and 1 and 10 years after infection. In all three cases, at all time points, the tetramer-binding cells were exclusively $CD11a^{high}$ and $CD18^{high}$ (Fig. 3*a*), irrespective of their CD45 status. This was also true for asymptomatic individuals infected over 20 years previously (data not shown).

$CD45RA^+$ memory T cells have undergone more cell divisions than $CD45RA^+$ naive T cells

These data suggest that all $CD45RA^+$ memory cells are $LFA\text{-}1^{high}$. Intriguingly, this population of $CD45RA^+LFA\text{-}1^{high}$ cells does not exist in neonates (Fig. 3*b*), and previous reports suggest that the frequency of $CD8^+CD45RA^+LFA\text{-}1^{high}$ cells increases with age (19), providing indirect evidence that the $LFA\text{-}1^{high}$ population reflects accumulated memory cells of diverse specificities. Although it is not possible to test this by direct observation of Ag binding, a clear prediction of the hypothesis is that $CD8^+CD45RA^+LFA\text{-}1^{high}$ cells should have undergone significantly more cell divisions than $CD8^+CD45RA^+LFA\text{-}1^{low}$ cells. We tested this prediction by measuring the length of telomeres using an in situ hybridization flow cytometry assay (17) (Fig. 3*c*). Telomeres consist of repeating units at the end of chromosomes that are shortened progressively with each cycle of division. The enzyme telomerase can replace lost telomeres in stem cell populations, but in T cells, telomere length is a good index of the number of divisions that cells have undergone. The population of $CD45RA^+LFA\text{-}1^{high}$ cells had significantly shorter telomeres and had therefore undergone many more cycles of division than $CD45RA^+LFA\text{-}1^{low}$ cells. Furthermore, the telomere length in primed ($CD45RO^+$) cells was indistinguishable from $CD45RA^+LFA\text{-}1^{high}$ cells. These data strongly support the proposal that $CD45RA^+LFA\text{-}1^{high}$ cells represent a population of memory CD8 cells.

$CD45RA^+$ memory cells express a $CCR7^{low}CCR5^{high}$ phenotype

It has been assumed that the majority of $CD45RA^+$ T cells are naive and thus migrate between blood and lymph nodes via the HEV. However, the low levels of CD62L would hinder recruitment of the $CD45RA^+$ tetramer-binding population into lymph nodes. High levels of LFA-1 would promote migration across inflamed endothelium, and high levels of CD49e (the fibronectin receptor) would allow them to interact with extracellular matrix. Taken together, these changes suggest $CD45RA^+$ tetramer-binding cells are similar to $CD45RO^+$ cells and are programmed to migrate into extralymphoid tissues.

We tested this hypothesis further by studying the expression of a range of chemokine receptors on CD8 T cell subsets ($CD45RA^+$ and $CD45RO^+$) in comparison with expression of the α-chain of LFA-1 (CD11a) (Fig. 4). We found marked differences in the expression of several chemokine receptors between the $CD45RA^+LFA\text{-}1^{high}$ (memory) and $CD45RA^+LFA\text{-}1^{low}$ (naive) populations. $CD45RA^+LFA\text{-}1^{high}$ memory cells were $CCR5^{high}$, $CXCR4^{low}$, $CCR7^{low}$, and contained a population of $CXCR1^{high}$ cells. In contrast $CD45RA^+LFA\text{-}1^{low}$ cells were $CXCR1^{low}$, $CCR5^{low}$, $CXCR4^{high}$, and $CCR7^{high}$. Expression of CCR7 by $CD45RA^+$ cells proved to be as effective as LFA-1 expression at discriminating these populations (Fig. 4). This was confirmed by substituting tetramer for the Ab to CD11a in the analysis for the four discriminatory chemokines (Fig. 5). Tetramer-positive cells, both in the $CD45RA^+$ and $CD45RO^+$ populations, were predominantly $CXCR4^{low}CCR7^{low}$.

The chemokine receptor profile of $CD45RA^+LFA\text{-}1^{high}$ memory cells was very similar to primed ($CD45RO^+$) cells (Fig. 4) with three exceptions: CCR6 showed a biphasic distribution in primed cells, but all $CD45RA^+LFA\text{-}1^{high}$ cells were negative.

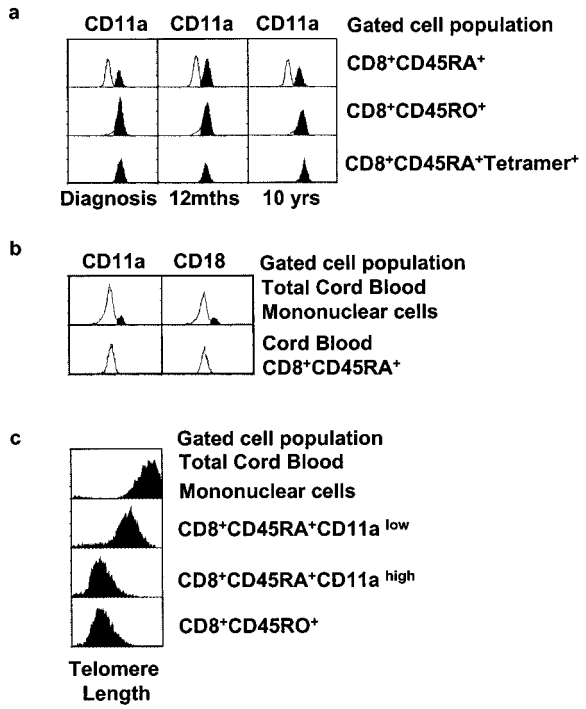

FIGURE 3. Stability of the CD8$^+$CD45RA$^+$LFA-1^{high} phenotype on tetramer-bearing cells. *a*, Cryopreserved PBMC samples from adult patients with EBV-associated IM collected at intervals after infection were stained using CD11a FITC, tetramer PE, CD8 ECD, and CD45RA PE-Cy5 for four-color flow cytometry. The expression of CD11a was examined in the indicated cell populations. All tetramer-binding cells were in the high level population of CD11a expression at diagnosis and 1 and 10 years after infection. Results are from one HLA-B8 individual using RAK-B8 tetramer and are representative of three individuals tested. Filled areas of histograms indicate the CD11a^{high} population. Results for CD18 expression were identical with those for CD11a in each case. *b*, T cells isolated from umbilical veins were stained using CD11a FITC, CD8 ECD, and CD45RA PE-Cy5. The CD8$^+$CD45RA$^+$ fraction showed no cells with high-level expression of CD11a or CD18. Filled areas of histograms indicate the CD11a^{high} population. Representative of three subjects. *c*, Purified adult CD8$^+$ T cell subsets (>95% purity) and whole cord blood mononuclear cells were labeled with a PNA probe specific for telomere repeat sequences and analyzed by flow cytomery. PNA fluorescence in adult CD8$^+$CD45RA$^+$CD11a^{high} cells was significantly less intense than in the CD8$^+$CD45RA$^+$CD11a^{low} cells but was indistinguishable from primed CD8$^+$CD45RO$^+$ cells. Data are representative of three experiments.

CXCR3 was expressed by a significant proportion of CD45RO$^+$ cells, whereas very few CD45RA$^+$LFA-1^{high} memory cells expressed it. CCR7 was also expressed by a small population of primed (CD45RO$^+$) cells, though not as a distinctly separate population. In contrast, all CD45RA$^+$LFA-1^{high} cells were negative for CCR7 expression. The expression of CCR7 has previously been shown to be a prerequisite for entry into lymph nodes across HEV, while CCR5 is associated with entry into inflamed tissues (20), and the clear discordance in expression of these receptors between the naive and memory CD45RA$^+$ populations suggests different migratory potential. The population of CD45RO$^+$-primed cells that coexpress CCR7 has recently been proposed as a "central memory" reservoir population by Sallusto et al. (21), who suggested that such cells may migrate to secondary lymphoid tissue.

In vitro migration of CD8$^+$ T cell subsets

The close similarity in the chemokine receptor profiles of CD45RA$^+$LFA-1^{high} memory cells and CD45RO$^+$-primed cells suggested that the migratory potential of CD45RA$^+$LFA-1^{high} memory cells may resemble that of primed T cells rather than naive CD8 cells. To assess whether the differential chemokine receptor profile induces functional effects on the migration of these subsets, we performed chemotaxis assays using a variety of chemokines. The number of cells from each subset that migrated was expressed as a proportion of the number of those cells in the original population. In all of the specific chemokine migration assays, the results obtained for migration of CD45RA$^+$LFA-1^{high} memory cells were very similar to those for the CD45RO$^+$-primed population. Thus both populations migrated effectively to RANTES, MIP-1α, and MIP-1β, all of which are ligands for CCR5, which is expressed by both populations (Fig. 6*a*). Primed (CD45RO$^+$) and CD45RA$^+$LFA-1^{high} memory cells also migrated in response to IL-8, which binds to CXCR1, again expressed by cells in both populations. However, substantial migration of naive cells was only observed with the CCR7 ligands Epstein-Barr virus-induced molecule 1 ligand chemokine and secondary lymphoid tissue chemokine and with stromal cell-derived factor-1, which is the only known ligand for CXCR4 (Fig. 6*a*). These results closely accord with the chemokine receptor expression (Fig. 4) and suggest that naive cells will preferentially enter lymph nodes via HEV, whereas CD45RA$^+$LFA-1^{high} memory cells are able to respond to inflammatory signals from tissue.

The distribution of CD8$^+$ T cell subsets in vivo

To test whether the CD45RA$^+$ memory population does indeed migrate to different locations in vivo, compared with naive cells, we studied the expression of CD11a and CCR7 on CD8$^+$CD45RA$^+$ T cells derived from peripheral blood, thoracic lymph nodes, and liver tissue in each of eight patients (Fig. 6*b*). In each case, the peripheral blood population displayed the expected biphasic distribution of CD11a, which showed an inverse association with CCR7. In contrast, the lymph nodes tested contained almost entirely CD11a^{low}CCR7^{high} cells, while the liver tissue contained almost exclusively CD11a^{high}CCR7^{low} cells. This was the case in five inflamed samples (hepatitis C) and three healthy donors' liver tissue.

Stability and function of CD8$^+$ T cell subsets

The close relationship between CD45RA$^+$LFA-1^{high} cells and CD45RO$^+$ cells in terms of adhesion molecule expression, chemokine receptor expression, and migration patterns suggests that the function of these cells is likely to be different to CD45RA$^+$LFA-1^{low} naive cells. Primed T cells are known to show a small population in cycle at any given time and also to have increased susceptibility to apoptosis. CD45RO$^+$ cells showed a significant population of Bcl-2^{low} cells in each of the five individuals tested. In contrast, both CD45RA$^+$LFA-1^{high} and CD45RA$^+$LFA-1^{low} cells showed no evidence of a Bcl-2 low population. Intriguingly, CD45RA$^+$LFA-1^{high} cells consistently showed slightly higher levels of Bcl-x_L expression (between 145 and 230% of median fluorescence intensity in each case) than either naive CD45RA$^+$LFA-1^{low} cells or CD45RO$^+$ cells (Fig. 7*a*), suggesting low susceptibility to apoptosis. Failure to express Ki-67 in all individuals tested indicates that CD45RA$^+$LFA-1^{high} cells are not actively cycling, in contrast to the CD45RO$^+$ population. Intriguingly, CD45RA$^+$LFA-1^{high} cells express high levels of the cytotoxin perforin and when stimulated produce IFN-γ; both of these characteristics were shared with CD45RO$^+$ cells, but not CD45RA$^+$LFA-1^{low} cells (Fig. 7*b*).

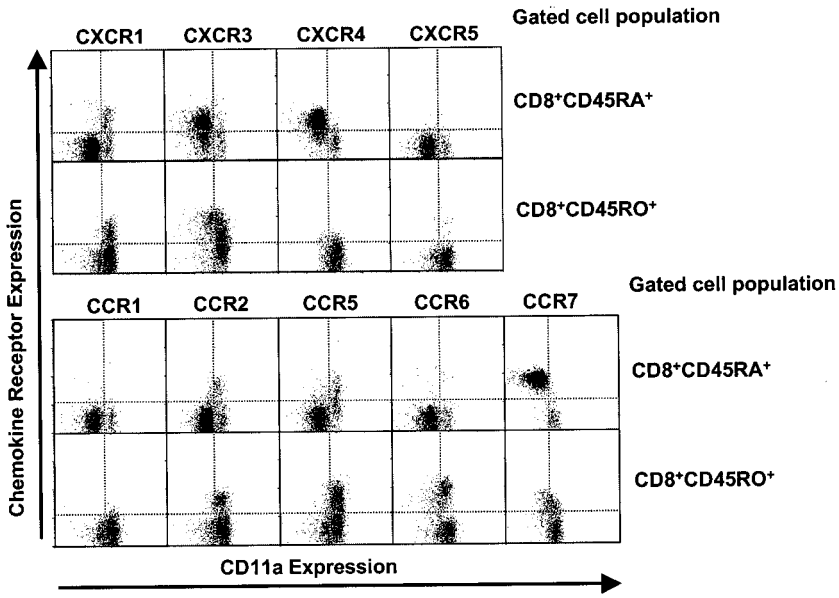

FIGURE 4. Chemokine receptor expression by $CD8^+$ T cell subsets. PBMC from healthy adults were stained for flow cytometry with a panel of antichemokine receptor Abs labeled with FITC, CD11a PE, CD8 ECD, and CD45RA PE-Cy5. Expression of chemokine receptors was examined in $CD8^+CD45RA^+CD11a^{high}$, $CD8^+CD45RA^+CD11a^{low}$, and $CD8^+CD45RO^+$ cells. Expression of chemokine receptors by $CD45RA^+CD11a^{high}$ memory cells closely paralleled that of primed rather than naive $CD8^+$ ($CD45RA^+CD11a^{low}$) cells. Representative of five experiments.

Discussion

The data presented in this report show that a substantial population of Ag-specific $CD8^+$ memory T cells is found in the $CD45RA^+/CD45RO^-$ population after resolution of viral infection. This population is highly stable, expressing high levels of Bcl-2 and Bcl-x_L, and shows no evidence of current activation or proliferative cycle using cell cycle analysis and Ki-67 staining. The $CD45RA^+$ memory cells express high levels of LFA-1 and very low levels of CCR7. These markers are the most characteristic for the population, but they also express relatively high levels of CD49e, CXCR1, and CCR5. The chemokine receptor profile and in vitro migratory responses resemble primed ($CD45RO^+$) $CD8^+$ T cells rather than naive cells. This evidence is supported by the in vivo distribution of the cells. The $CD45RA^+LFA\text{-}1^{high}$ memory population was underrepresented in lymph nodes but present in the liver, accounting for the overwhelming majority of $CD8^+CD45RA^+$ T cells in this tissue. This suggests that the $CD45RA^+$ memory population does not enter lymph nodes via HEV, but instead migrates to extralymphoid tissues. Further evidence for functional similarities with $CD45RO^+$ cells comes from our finding that the $CD45RA^+CD8^+$ memory cells constitutively express high levels of the cytotoxin perforin and produce high levels of IFN-γ on stimulation, characteristics of a secondary response. $CD8^+CD45RA^+CD11a^{high}$ cells expressed high levels of Bcl-2 and Bcl-x_L, virtually indistinguishable from naive $CD8^+CD45RA^+CD11a^{low}$ cells.

A population of $CD8^+CD45RA^+LFA\text{-}1^{high}$ cells has been described previously, though they have not been shown to reflect an accumulated memory population (22, 23). High-level expression of perforin and production of IFN-γ led to the suggestion that these cells are recently activated effector cells (22–24). However, their lack of cell cycle markers and high levels of antiapoptosis markers argues against this. The origin of $CD8^+CD45RA^+LFA\text{-}1^{high}$ cells is not entirely clear. It is possible that these cells represent an entirely distinct pattern of differentiation from $CD45RA^+$ naive precursors that have never expressed CD45RO, but such a differentiation pathway has not been reported previously (25). Furthermore, the very short telomeres expressed by this population suggest a very high level of differentiation, equivalent to the primed $CD45RO^+$ population. Experiments in rats by Bell and Sparshott (7) support the concept of primed T cell reversion, showing that purified primed $CD8^+$ T cells (expressing low m.w. CD45 isoforms) do revert to the resting (high m.w. CD45 isoform-expressing) state in vivo when introduced into either nude or euthymic animals.

In vivo and in vitro studies of viral infection in mouse models have suggested that rapidly proliferating effector CTL precursors are predominantly $CD62L^{low}$, while a smaller population of $CD62L^{high}$ cells require extended exposure to Ag before developing effector function (26, 27). The $CD8^+CD45RA^+LFA\text{-}1^{high}$ cells identified as a distinct memory population in the present study were heterogeneous with respect to CD62L expression, suggesting that this may represent a distinct facet of memory discrimination. Recent data suggest that tetramer-binding cells in mouse models similarly express high levels of LFA-1, which is up-regulated on activation and maintained by memory cells thereafter, entirely consistent with the present study (28).

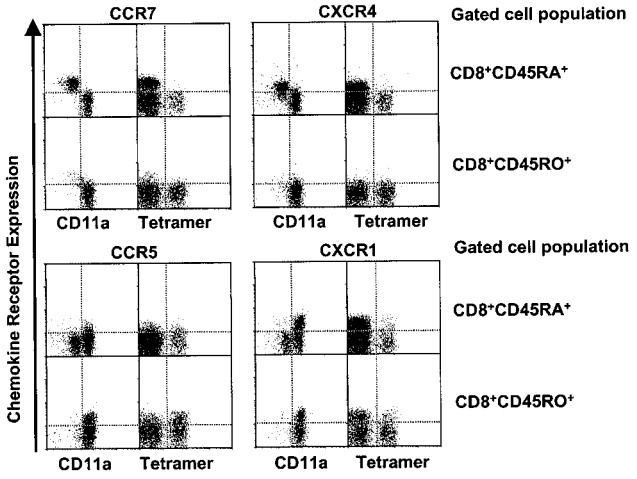

FIGURE 5. Comparison of chemokine receptor expression by tetramer-binding $CD45RA^+$ T cells and $CD45RA^+CD11a^{high}$ cells. PBMC from EBV-seropositive subjects were screened for chemokine receptor expression as described in Fig. 4. In parallel experiments, CD11a PE was replaced by the appropriate PE tetramer. Tetramer-binding $CD8^+CD45RA^+$ T cells show the same profile of chemokine receptor expression as $CD8^+CD45RA^+CD11a^{high}$ cells. Representative of three experiments.

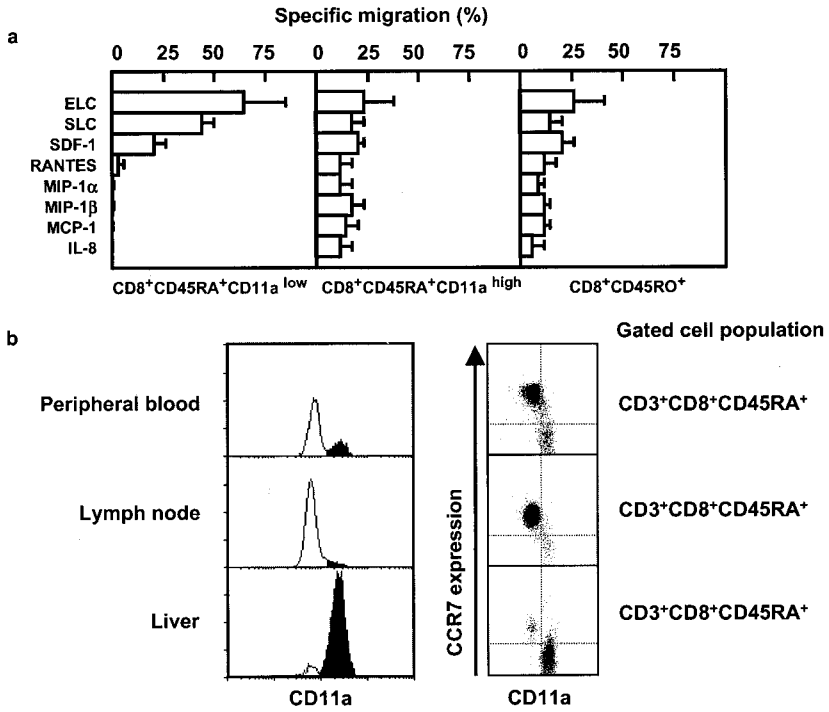

FIGURE 6. CD8$^+$CD45RA$^+$ memory cells migrate similarly to primed T cells. *a*, PBL from adult donors were depleted of adherent cells by culture on human serum-coated plates and enriched for CD8$^+$ lymphocytes (60–80% CD8$^+$) by immunomagnetic bead depletion using the Ab cocktail described in *Materials and Methods*. Overnight migration through 6.5-mm diameter, 5.0-μm Transwells was measured in the presence and absence of chemokines. Cells were resuspended from the Transwell chambers, accurately counted by flow cytometry, and phenotyped by staining with CD11a FITC, CD45RA PE, and CD8 Tricolor. Specific migration of the indicated cell populations was determined by subtracting the values obtained in the absence of chemokine. In vitro migration of CD45RA$^+$CD11a^{high} memory cells closely paralleled that of primed rather than naive CD8$^+$ cells. Mean ± SD of three experiments. *b*, Cells freshly isolated from blood, lymph node, and liver tissue of a single individual were stained with CCR7 FITC, CD11a PE, CD8 ECD, and CD45RA PE-Cy5 for four-color flow cytometry to determine the distribution of CD8$^+$CD45RA$^+$ subpopulations. All histograms were gated to include only CD8$^+$CD45RA$^+$ cells. The reciprocal distribution of CD11a and CCR7 most effectively discriminates naive CD8$^+$CD45RA$^+$CD11a^{low} cells from memory CD8$^+$CD45RA$^+$CD11a^{high} cells. One of eight individuals tested.

In the present study, we used tetramers specific for two viruses, EBV and CMV, to detect Ag-specific cells in patients with active infections and asymptomatic seropositive individuals. Both of these viruses persist in humans after infection, and previous studies have reported that between 0.1 and 10% of the CD8$^+$ T cell population bind specific tetramers in individuals with persistent viral infections (12, 29–31). It is likely that such high levels are maintained by continuous exposure to Ag because tetramer-binding cells decline rapidly after clearance of nonpersistent viruses, frequently falling below the level of detection, but increase rapidly again after rechallenge with Ag (32, 33). Infection with HIV leads to high levels of HIV tetramer-binding cells, but a reduction in viral load after starting antiviral therapy is accompanied by a fall in the number of tetramer-binding T cells (34, 35). Thus the population of CD8$^+$CD45RA$^+$CD11a^{high}CCR7^{low} memory cells we describe may have arisen as an adaptation to control persistent viral infections. However, it is likely that such cells are a facet of memory for all viruses and that the lack of detection in blood in nonpersistent viral infections is because the number of cells involved is much smaller (33). Furthermore, a recent study has shown that stable LFA-1^{high} tetramer-binding memory cells were generated following infection of C57BL/6J mice with the vesicular stomatitis virus (28), which is a transient, nonpersistent virus (36, 37).

Memory cells are believed to recirculate to lymph nodes, where they acquire Ag on subsequent secondary challenge (1). However, the population of cells we describe appears unlikely to enter secondary lymphoid tissue via HEV because they lack the two recep-

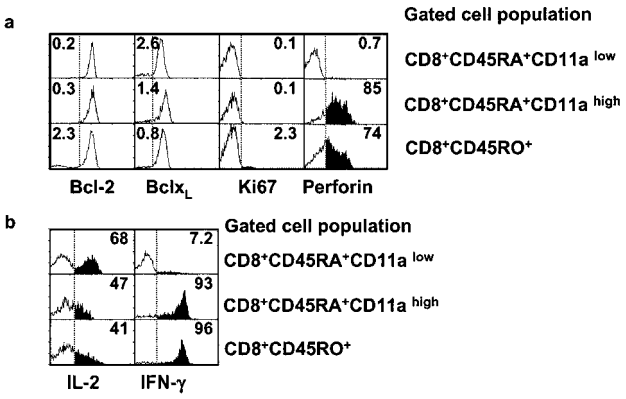

FIGURE 7. Functional characteristics of CD8$^+$ T cells subsets. *a*, Freshly isolated PBMC from adult donors were surface stained with CD11a PE, CD8 ECD, and CD45RA PE-Cy5, fixed, and subsequently stained for the indicated intracellular markers. Cell cycle characteristics (Ki-67) or constitutive expression of perforin, Bcl-2, and Bcl-x$_L$ were determined in CD8$^+$CD45RA$^+$CD11a^{high}, CD8$^+$CD45RA$^+$CD11a^{low}, and CD8$^+$CD45RO$^+$ cells by flow cytometry. Representative of five experiments. *b*, CD8$^+$CD45RA$^+$ and CD8$^+$CD45RO$^+$ cells were purified by negative selection with magnetic beads and stained with CD11a biotin/streptavidin RED670. Both populations were cultured for 6 h in PMA/Ca$^+$ ionophore with brefeldin A added after 1 h. Cells were permeafixed and stained for intracellular cytokine expression. Production of IL-2 and IFN-γ by CD8$^+$CD45RA$^+$CD11a^{high}, CD8$^+$CD45RA$^+$CD11a^{low}, and CD8$^+$CD45RO$^+$ cells was determined by flow cytometry. Representative of five experiments.

tors that are crucial for extravasation across HEV, CCR7 and L-selectin. The tissue homing characteristics of high CCR5 and CXCR1 expression (20) were corroborated by the observation that the $CD45RA^+$ memory population accounts for virtually all of the $CD8^+CD45RA^+$ cells seen in normal and hepatitis C-infected liver tissues. It would seem most likely that these cells reenter secondary lymphoid tissue via the afferent lymphatics, like $CD45RO^+$ cells (38). However, compared with $CD8^+CD45RO^+$ cells, they were underrepresented in secondary lymphoid tissue (approximately half the expected frequency in each of eight individuals tested).

These data suggest that the stable, noncycling population of $CD8^+CD45RA^+LFA\text{-}1^{high}$ T cells migrates to tissues, rather than lymph nodes. The $CD45RO^+CCR7^+$ population described as central memory cells (21) are a primed population, committed to cell division approximately every 2 wk (4). Such central memory cells may represent a precursor population, giving rise either to more highly differentiated effector cells ($CD45RO^+CCR7^-$) or to the $CD45RA^+LFA\text{-}1^{high}CCR7^-$ population of stable memory cells. Our data are consistent with the observations of Masopust et al. (28), who showed that $LFA\text{-}1^{high}$ (effector) memory cells in vesicular stomatitis virus-infected mice either continuously migrate through or reside in nonlymphoid tissues.

The overwhelming fate of primed T cells in any immune response is death by apoptosis, which is essential to prevent a rapid and cumulative increase in the size of the immune system (39). Therefore, it follows that rescue of T cells from apoptosis under these conditions is a prerequisite for immune memory. T cell apoptosis can be inhibited either by IL-2R common γ-chain cytokines (40) or by stromal-derived type I IFN (41). Common γ-chain cytokines induce cell cycle in addition to survival and are probably most important during the course of an active immune response. IFNs inhibit apoptosis but also induce a stable resting (G_0) phenotype, characteristic of memory cells. Type I IFNs in lymph nodes are thought to play a significant role in the inhibition of T cell apoptosis leading to memory. Competition for entry into the stable $CD45RA^+$ population is likely to be considerable. In a stable adult immune system, this population cannot expand significantly over time, though it does develop from nothing to ~50% of the total $CD45RA^+$ population between birth and adulthood. The data presented here together with recent reports (28, 42) suggest that $CD45RA^+LFA\text{-}1^{high}CCR7^-$ memory cells play a significant role in memory for both persistent and transient viral infections. The processes that induce reversion of primed $CD45RO^+$ T cells to a stable $CD45RA^+LFA\text{-}1^{high}CCR7^-$ memory state are not known, but the crucial homeostatic constraints suggest the lymph node as the most plausible site and the $CD45RO^+CCR7^+$ "central memory" population as strong candidates for precursors.

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