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Late Priming and Variability of Epitope-Specific CD8\(^+\) T Cell Responses during a Persistent Virus Infection\(^1\)

Christopher C. Kemball,* Eun D. Han Lee,† Vaiva Vezys,* Thomas C. Pearson,† Christian P. Larsen,* and Aron E. Lukacher*\(^2\)

Control of persistently infecting viruses requires that antiviral CD8\(^+\) T cells sustain their numbers and effector function. In this study, we monitored epitope-specific CD8\(^+\) T cells during acute and persistent phases of infection by polyoma virus, a mouse pathogen that is capable of potent oncogenicity. We identified several novel polyoma-specific CD8\(^+\) T cell epitopes in C57BL/6 mice, a mouse strain highly resistant to polyoma virus-induced tumors. Each of these epitopes is derived from the viral T proteins, nonstructural proteins produced by both productively and nonproductively (and potentially transformed) infected cells. In contrast to CD8\(^+\) T cell responses described in other microbial infection mouse models, we found substantial variability between epitope-specific CD8\(^+\) T cell responses in their kinetics of expansion and contraction during acute infection, maintenance during persistent infection, as well as their expression of cytokine receptors and cytokine profiles. This epitope-dependent variability also extended to differences in maturation of functional avidity from acute to persistent infection, despite a narrowing in TCR repertoire across all three specificities. Using a novel minimal myeloblastation-bone marrow chimera approach, we visualized priming of epitope-specific CD8\(^+\) T cells during persistent virus infection. Interestingly, epitope-specific CD8\(^+\) T cells differed in CD62L-selectin expression profiles when primed in acute or persistent phases of infection, indicating that the context of priming affects CD8\(^+\) T cell heterogeneity. In summary, persistent polyoma virus infection both quantitatively and qualitatively shapes the antiviral CD8\(^+\) T cell response. The Journal of Immunology, 2005, 174: 7950–7960.

Continuous surveillance by virus-specific CD8\(^+\) T cells is essential for keeping persistent viral infections in check. Understanding how antiviral CD8\(^+\) T cells maintain effector competence in the face of chronic exposure to viral Ags is of paramount importance for preventing diseases linked to persistently infecting viruses. Variability in replication, life cycle, and tropism among persistent viruses likely translates into differences in the fate and function of virus-specific CD8\(^+\) T cells responding to infection by different persistent viruses (1). CD8\(^+\) T cell control of latent (e.g., HSV, varicella zoster virus, EBV) and high-level persistent (e.g., hepatitis B virus, hepatitis C virus, HIV) human viral infections has been examined in the analogous murine models of gammaherpesvirus (γHV)\(^6\) 68 and lymphochytic choriomeningitis virus (LCMV) clone 13 strain infection, respectively (2, 3).

The human polyomaviruses (PyV) (BK and JC) and CMV arguably represent a third class of persistent viral infection, the low-level “smoldering” infection (4, 5). How antiviral CD8\(^+\) T cells contend with these low-level persistent viral infections remains largely unexplored.

Virus-specific CD8\(^+\) T cell responses evolve during the course of persistent infection. For a number of viral infections in humans and mice, the immunodominance hierarchy for antiviral CD8\(^+\) T cell responses has been shown to shift between acute and persistent phases of infection (2, 3, 6–8). In addition, CD8\(^+\) T cells directed toward different viral epitopes may vary phenotypically and functionally as persistent infection becomes established (1, 6, 7, 9, 10). In the case of a high-level replicating persistent viral infection, such as that caused by LCMV clone 13, there is a viral epitope-dependent functional deterioration of antiviral CD8\(^+\) T cells that may culminate in clonal deletion (3). The rate of functional loss by epitope-specific CD8\(^+\) T cells may vary as a function of the frequency of Ag encounter, APC type, and TCR avidity (4, 8, 10). How low-level systemic persistent infection impacts the function and phenotype of antiviral CD8\(^+\) T cells has not been extensively examined.

PyV represents a family of oncogenic DNA viruses that establish persistent infection in humans and mice. BK and JC viruses are ubiquitous human pathogens that persist as lifelong silent infections in healthy individuals. Upon immunosuppression, however, JC virus, which persists in oligodendrocytes, may progress to a fatal CNS demyelinating disease (5). In addition, BK virus, JC virus, and the rhesus monkey PyV SV40 have been linked to a number of human malignancies (11). Mouse PyV can cause a multitude of different epithelial and mesenchymal lineage tumors when inoculated into immunocompromised adult mice and newborn mice of particular inbred strains (11). Of the six PyV proteins, those encoded within the early region of the PyV genome are essential for tumor formation; in particular, constitutive expression of middle T (MT) is required for cellular transformation and tumor formation, while large T (LT) disables cell cycle arrest machinery (11). Even in mice of tumor-resistant inbred strains, polyoma DNA and mRNA persist long term in multiple organs (12).

\(^1\) Abbreviations used in this paper: γHV-68, gammaherpesvirus 68; LCMV, lymphochytic choriomeningitis virus; LT, large T protein; MT, middle T protein; PyV, polyoma virus; ICCS, intracellular cytokine staining; CD62L, CDL-selectin.
PyV-specific antiviral CD8+ T cells are required for protection against PyV-induced tumors. C57BL/6 (B6) mice are highly resistant to PyV-induced tumors, but CD8-deficient mice on a B6 background have increased susceptibility to PyV tumorigenesis (12). In H-2b haplotype mice, the magnitude and functional integrity of the PyV-specific CD8+ T cell response is tightly associated with resistance to PyV-induced tumors (13, 14). In this haplotype, anti-PyV CD8+ T cells are almost entirely directed toward a single epitope in MT (14). As a result, this virus-haplotype combination is not amenable to exploring epitope-specific differences during the course of PyV infection.

In this study, we identified three CD8+ T cell epitopes to PyV in B6 mice and tracked the epitope-specific CD8+ T cell responses during acute and persistent phases of infection. We found that epitope-specific CD8+ T cell populations varied in rates of expansion and contraction, hierarchy, phenotype, and cytokine profile in acute and persistent infection. Using a persistently infected congenic bone marrow chimera model, we further demonstrate that PyV-specific CD8+ T cells are primed in the face of a preexisting PyV-specific CD8+ T cell memory population. The implications of these findings for maintaining CD8+ T cell-mediated resistance to persistent viral infections are discussed.

Materials and Methods

Mice

C57BL/6Ncr female mice were purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD). B6.SJL-Pipc/Boa1Tac (C57Dj1) and C57BL/6J-K11d2 N12 female mice were kindly provided by Dr. P. E. Jensen (Department of Pathology, Emory University, Atlanta, GA). Mice were housed and bred in accordance with the guidelines of the Institutional Animal Care and Use Committee, and the Department of Animal Resources at Emory University.

Viruses and virus inoculation

PyV strain A2 was molecularly cloned and plaque purified, and virus stocks were prepared on primary baby mouse kidney cells as previously described (14). Each mouse was inoculated s.c. in each hind footpad with 2 × 10^6 PFU of virus. Mice were infected at 6–12 wk of age.

Synthetic peptides

LT360–368, MT245–253, MT243–252, and LT638–646 peptides were synthesized by the solid-phase method using F-moc chemistries. HPLC analysis showed that peptides were 90–95% pure. Except for synthetic peptide LT638–646, peptide stock solutions were prepared in water and stored at −20°C. Peptides were diluted in assay medium immediately before use. The MT and LT peptide libraries consisted of 17-mer peptides overlapping by 12 aa and were prepared as PepSets by Chiron Mimotopes, and were generously provided by Dr. J. Altman (Emory Vaccine Center, Atlanta, GA); the VP1 and VP2 peptide libraries consisted of 15-mer peptides overlapping by 10 aa and were prepared on a Symphony/Multiplex Peptide Synthesizer (Rainin).

RMA/S class I MHC-peptide stabilization assay

RMA/S cells were cultured overnight at 27°C, then incubated with peptide at 23°C for 1 h. After incubation at 37°C for 2 h, cells were stained with PE-conjugated anti-Kb (AF6-88.5; BD Pharmingen) or anti-Dd (28-14-8; eBioscience) and analyzed by flow cytometry to determine mean fluorescence intensity.

Preparation of H-2b tetramers

Escherichia coli strain BL21 (DE3) transformed with plasmids encoding H-2Db or H-2Kb H chains with C-terminal biotinylation sequence peptide were kindly provided by Dr. J. Altman. Human β2-microglobulin inclusion bodies were kindly provided by Dr. P. E. Jensen. Inclusion body preparation, protein refolding, purification, and biotinylation were performed according to the National Institutes of Health Tetramer Core Facility protocols (http://www.yerkes.emory.edu/TETRAMER/protocol.html). Tetramers were made by mixing biotinylated peptide-class I MHC monomers with allophycocyanin-conjugated streptavidin (Molecular Probes) in a 4:1 molar ratio. Class I MHC molecules were refolded with synthetic peptides corresponding to the wild-type viral peptide sequence listed in Table I, with the exception of the LT360 peptide, in which the cysteine residue at position 6 was replaced with α-aminoxybutyric acid, a non-natural cysteine analogue, to prevent interpeptide disulfide bonding and peptide dimerization during the protein refolding reaction (15, 16). This peptide, LT360C6Abu, stimulated IFN-γ production by CD8+ T cells from the spleens of PyV-infected B6 mice equivalent to the wild-type peptide (data not shown).

Isolation of lymphocyte populations from blood and lungs

Heparinized blood samples were treated with ACK buffer (0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM Na2EDTA, pH 7.0) to lyse RBC and washed before use. For lung lymphocyte isolation, anesthetized mice were perfused with PBS containing 100 U/ml heparin (Elkins-Sinn). The lungs were minced and incubated in HBSS (Mediatech) containing 1.3 mM Na2EDTA (pH 8.0) in a 37°C shaker for 30 min. This was followed by treatment with collagenase (100 U/ml; Worthington Biochemical) in RPMI 1640 containing 1 mM MgCl2, 1 mM CaCl2, and 5% FBS in a 37°C shaker for 1 h. The cell suspension was washed through a 70-μm nylon cell strainer (BD Biosciences), resuspended in 44% Percoll (Amersham Biosciences), underlaid with 67% Percoll, and centrifuged at ~600 × g for 20 min. Cells were removed at the gradient interface and washed before use.

Flow cytometry

Cells were stained in PBS containing 2% FBS and 0.1% sodium azide (FACS buffer) for 45 min at 4°C or room temperature, followed by two washes in FACS buffer and fixation in freshly prepared PBS containing 1% paraformaldehyde. Cells were stained with allophycocyanin-conjugated tetramers, PE- or PerCP-conjugated anti-CD8α (BD Pharmingen), and FITC-conjugated anti-CD25, CD69, CD122 (BD Pharmingen), or anti-CD127 (eBioscience). For Vβ repertoire analyses, cells were stained with allophycocyanin-conjugated tetramers, PE-conjugated anti-CD8α, and a panel of FITC-conjugated Vβ mAbs (BD Pharmingen). For bone marrow chimera phenotypic analyses, cells were stained with allophycocyanin-conjugated tetramers, PerCP-conjugated anti-CD8α, PE-conjugated anti-CD45.1 (BD Pharmingen), and FITC-conjugated anti-CD11a or anti-CD62L-selectin ligand (CD62L; Caltag Laboratories). Samples were acquired on a FACSCalibur (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences).

Intracellular IFN-γ staining

Cells were cultured for 5 h in 96-well round-bottom microtiter plates (Costar) in 0.2 ml/well IMDM (Invitrogen) containing 10% FBS, 50 μM 2-ME, penicillin/streptomycin, and supplemented with 1 μg/ml brefeldin A (Sigma-Aldrich) and synthetic peptides. Cells were then surface stained with PE-conjugated anti-CD8α (CT-CD8α; Caltag Laboratories); FITC-conjugated CD45.1 mAb was included for bone marrow chimera experiments. After washing, cells were permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen) and stained for intracellular IFN-γ with allophycocyanin-conjugated anti-IFN-γ (XM1G1.2; BD Pharmingen). For multi-color cytokine analysis, cells were stained intracellularly with FITC or allophycocyanin-conjugated anti-IFN-γ, and allophycocyanin-conjugated anti-IL-2 (BD Pharmingen) or PE-conjugated anti-TNF-α (Caltag Laboratories).

IFN-γ ELISPOT assay

The single-cell ELISPOT assay was performed as described previously (17). Briefly, 96-well filtration plates (Millipore) were coated overnight with anti-IFN-γ (R4-6A2; BD Pharmingen). Splenocytes from PyV-infected B6 mice on day 8 after infection were incubated for 36 h at 37°C with individual overlapping peptides encompassing sequences for PyV T cell epitopes in C57BL6 mice

### Table I. PyV-specific CD8+ T cell epitopes in C57BL6 mice

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Source Viral Protein</th>
<th>Amino Acid Sequence</th>
<th>Restriction Element</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT245–253</td>
<td>MT</td>
<td>LSNNPTEYSVM</td>
<td>Kb/Dd</td>
</tr>
<tr>
<td>MT246–253</td>
<td>MT</td>
<td>SNNPTEYSVM</td>
<td>Kb</td>
</tr>
<tr>
<td>MT243–252</td>
<td>MT</td>
<td>SSSNPSYTV</td>
<td>Dd</td>
</tr>
<tr>
<td>LT360–368</td>
<td>LT</td>
<td>AVENYCSKL</td>
<td>Dd</td>
</tr>
<tr>
<td>LT638–646</td>
<td>LT</td>
<td>MVANVDDNL</td>
<td>Kb</td>
</tr>
</tbody>
</table>

*CD8+ T cells recognizing this epitope are only detected in acutely infected mice.
MT, LT, VP1, and VP2 proteins, or no peptide. The final concentration of each peptide was 10 µM. After the incubation, plates were washed and incubated with biotinylated anti-IFN-γ (XMGI.2; BD Pharmingen). Wells were then incubated with HRP avidin D (Vector Laboratories), washed, and developed with freshly prepared substrate buffer (0.03% (w/v) 3-amino-9-ethyl-carbazole and 0.015% (v/v) 3% H2O2 in 0.1 M sodium acetate, pH 5).

**TaqMan real-time PCR to quantitate PyV DNA**

DNA was extracted from whole blood or snap-frozen tissue using the QIAamp DNA Mini kit (QiaGen) according to the manufacturer’s instructions. For TaqMan real-time PCR, the following primers and TaqMan probe were used: forward primer 5'-CCG ACA TAC TGC TGG AAG AAG A-T corresponding to nt 1040–1061 of the PyV A2 strain genomic sequence (18); reverse primer 5’-TCT GGG CTT CTT GCA TAC AG-3’ corresponding to nt 1120–1142; and TaqMan probe 5’-ATC CTT GTG TTG AGC CAG ATG A-3’ corresponding to nt 1066–1090. PCR amplification was performed in a 20-µl reaction containing 1 ng of genomic DNA derived from tissue (or 1 µl of a 1/10 dilution of the DNA stock solution extracted from 100 µl of whole blood), primers (0.75 µM forward, 1.0 µM reverse), 100 nM TaqMan probe, and 10 µl of TaqMan Master Mix (Roche). The reaction was incubated at 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, and 60°C for 1 min in an ABI PRISM 7500 sequence detection system (Applied Biosystems). The PyV DNA quantity is expressed in genome copies per milligram of tissue and is calculated based on a standard curve of known PyV genome copy number vs threshold cycle of detection. The detection limit with this assay is 10 copies of genomic viral DNA.

**Generation of CD45 congenic bone marrow chimeras**

A recently reported minimal myeloablation and bone marrow transplantation protocol was performed as described elsewhere (19), with the following modifications. Naïve or persistently PyV-infected B6 mice were given 600 µg busulfan i.p. (BusulfeX; Orphan Medical). Twenty-four hours later, these mice received 20 x 106 nucleated cells i.v. from the bone marrow of naïve B6.SJL mice. Establishment of chimerism was confirmed by flow cytometric analysis of whole blood cells for CD45.1 expression (data not shown). Blood, spleen, and lungs of chimeric mice were analyzed by flow cytometry >43 days after bone marrow transplantation.

**Results**

**Noncoordinated evolution of epitope-specific CD8+ T cell responses in PyV-infected B6 mice**

PyV-specific antiviral CD8+ T cells are required for protection against PyV-induced tumors in B6 mice, as CD8-deficient mice are more susceptible than B6 mice to PyV tumorigenesis (12). To map H-2Kb-restricted PyV-specific CD8+ T cell epitopes, we screened overlapping synthetic peptide libraries encompassing sequences for PyV MT, LT, and the VP1 and VP2 capsid proteins for their capacity to stimulate IFN-γ production by spleen cells from acutely infected B6 mice. By IFN-γ ELISPOT and intracellular cytokine staining (ICCS) assays, we identified three CD8+ T cell epitopes encoded in the early region of PyV based on peptide library sequence overlap and peptide binding motifs of H-2Kb and H-2Dd (20) (Table I). MT245–253 is derived from the MT protein while LT360–368 and LT638–646 are derived from the LT protein.

No CD8+ T cell epitopes were identified in the late-region VP1 or VP2 proteins. Interestingly, the dominant antipyV-specific CD8+ T cell response in C3H (H-2d) mice is similarly directed to an epitope from an early-region PyV protein (14), raising the possibility that the antipolyoma CD8+ T cell response is preferentially focused on epitopes of viral proteins produced in both lytically infected and transformed cells.

To confirm the predicted class I MHC-restricting molecule for each epitope, splenocytes from acutely infected B6 mice or B6 mice lacking Kd or Dd were stimulated with LT360, MT245, or LT638 peptides and analyzed for intracellular IFN-γ production. CD8+ T cell responses specific for LT360 and LT638 were detectable in Kd+/− mice but not in Dd+/− mice, clearly demonstrating that LT360 and LT638 are Dd-restricted epitopes (Fig. 1A). Interestingly, the frequency of LT638-specific CD8+ T cells was 2-fold greater in the spleens of Kd+/− mice than in wild-type mice. Possible explanations include differences between wild-type and Kd+/− mice in numbers of PyV-specific CD8+ T cell precursors, T cell repertoire, numbers of infected APCs, or emergence of otherwise undetectable epitope-specific CD8+ T cells in Kd+/− mice (21). MHC restriction mapping of the MT245-specific CD8+ T cell response was less clear-cut. CD8+ T cell responses specific for MT245 were detectable in both Kd+/− and Dd+/− mice, indicating that overlapping Dd- and Kd-restricted epitopes were contained within the MT245–253 sequence (Fig. 1A).

**To quantitate epitope-specific CD8+ T cells directly ex vivo during acute and persistent phases of PyV infection, we used Dd tetramers complexed to LT360 or LT638 peptides and, to include both Dd- and Kd-restricted responses, IFN-γ ICCS for the MT245-specific population. Comparable numbers of LT360- and LT638-specific CD8+ T cells in the spleens were observed using either tetramers or IFN-γ ICCS (data not shown). As shown in Fig. 2A, these epitopes fall into the following dominance hierarchy: LT360 > MT245 > LT638. However, the LT638-specific CD8+ T cell response differed from that directed to LT360 and MT245 in both expansion and contraction phases during acute infection and maintenance during persistent infection. The expansion-contraction profile of the LT638-specific CD8+ T cell response was shifted 2 days earlier than the LT360- and MT245-specific CD8+ T cell responses, which behaved similarly to each other. Because LT360 and LT638 are derived from the same viral protein, differences in their CD8+ T cell profiles cannot be ascribed to temporal differences in viral protein production. Through late (>300 days) postinfection time points, LT360- and MT245-specific CD8+ T cells were each maintained at fairly constant numbers, with only small differences between individual mice. In contrast, there was considerable variation between individual mice at late time points in the magnitude of the LT638-specific CD8+ T cell response, with numbers often exceeding those seen at the peak response during acute infection. As a result, although the same immunodominance hierarchy held throughout infection, it tended to narrow by late-phase infection.

We constructed class I MHC H-2Kd tetramers complexed to either the MT245–253 or MT246–253 peptides. Because the Kr MT245 and Kd MT246 tetramers showed equivalent levels of staining of splenic CD8+ T cells from acutely infected mice, for simplicity, we refer to these tetramers as Kr MT245 tetramers. The frequency of splenic tetramer “CD8+ T cells was found to closely match the frequency of IFN-γ “CD8+ T cells in PyV-infected memory (>day 30 postinfection) mice; however, ~2-fold more IFN-γ “CD8+ T cells than tetramer “CD8+ T cells were detected during the acute CD8+ T cell response (data not shown). These data suggest that the MT245-specific CD8+ T cell response in acutely infected B6 mice is restricted to both Kr and Dd, while only the Kr-restricted population is present in persistently infected animals.

We next asked whether the expansion of PyV-specific CD8+ T cells is coordinate with PyV clearance. Using a highly sensitive TaqMan real-time PCR assay to quantitate PyV genomes, we found an inverse association between PyV DNA copy number and PyV-specific CD8+ T cell numbers in the spleen (Fig. 2B). In agreement with our earlier findings using conventional PCR (12), PyV DNA was detectable in the spleen >40 days postinfection and persisted for as long as 400 days after infection in some animals. However, even in mice lacking detectable PyV DNA in the spleen, we routinely detected PyV DNA persisting in sites such as the
incubated with MT peptides in a class I MHC peptide-stabilization assay to determine the binding affinity of each peptide for Db and Kb. Cells were stained
responses during acute and persistent infection, we performed V
rowing of the TCR repertoire for antipolyoma CD8
intracellular IFN-
specific CD8
a change in functional avidity for either LT360-specific or LT638-
within the peptide concentration range examined (Fig. 3
B, RMA/S cells were incubated with MT peptides in a class I MHC peptide-stabilization assay to determine the binding affinity of each peptide for D\textsuperscript{b} and K\textsuperscript{b}. Cells were stained with D\textsuperscript{b} or K\textsuperscript{b} mAbs and analyzed by flow cytometry to measure surface expression of MHC, expressed as mean fluorescence intensity (MFI).

kidney, heart, cartilage, and salivary gland (data not shown). Thus, viral DNA persists in the face of functionally competent antiviral CD8\textsuperscript{T} T cells.

Epitope-specific CD8\textsuperscript{T} T cell maturation during PyV infection

To compare TCR diversity among epitope-specific CD8\textsuperscript{T} T cell responses during acute and persistent infection, we performed V\textbeta repertoire analysis on splenocytes isolated from B6 mice on days 8 and 111 postinfection, respectively. During acute infection, each of the three epitope-specific CD8\textsuperscript{T} T cell responses showed extensive diversity in V\textbeta family expression, with a common preference for V\textbeta 8.1/8.2 (Fig. 3A). In the persistent phase of infection, the V\textbeta expression profile for CD8\textsuperscript{T} T cells recognizing each epitope became markedly less diverse (Fig. 3A). It is worth noting that these shifts in V\textbeta usage by PyV-specific CD8\textsuperscript{T} T cells from acute to persistent infection could not be detected in C3H (H-2\textsuperscript{k}) mice, where up to 70% of the dominant epitope-specific CD8\textsuperscript{T} T response expressed V\textbeta6 and V\textbeta8.1 during acute PyV infection (22). Thus, V\textbeta usage analysis in infected B6 mice revealed narrowing of the TCR repertoire for antipolyoma CD8\textsuperscript{T} T cells between acute and persistent infection, a phenomenon also observed for virus-specific CD8\textsuperscript{T} T cell responses to EBV and murine CMV (7, 23).

Interestingly, CD8\textsuperscript{T} T cells to these three epitopes differ in their functional avidity over the course of PyV infection. Peptide dose-response curves for intracellular IFN-\gamma production failed to reveal a change in functional avidity for either LT360-specific or LT638-specific CD8\textsuperscript{T} T cells between acute and persistent infection within the peptide concentration range examined (Fig. 3B). However, it is possible that a change in functional avidity for LT638-specific CD8\textsuperscript{T} T cells might be revealed at peptide concentrations <10\textsuperscript{-9} M. In marked contrast, a clear increase in sensitivity was seen in CD8\textsuperscript{T} T cell responsiveness to the K\textsuperscript{b}-restricted MT246 peptide in persistently vs acutely infected mice (Fig. 3B). Although the same trend was detected using the MT245 peptide, the difference between acutely and persistently infected mice was masked by the contribution of the D\textsuperscript{b}-restricted MT243 epitope during acute infection (data not shown). The responsiveness to MT243 paradoxically drops in persistently infected mice, such that IFN-\gamma production can only be elicited by a nonphysiologically high peptide concentration (10\textsuperscript{-7} M). At this concentration, it is possible that some MT246-specific CD8\textsuperscript{T} T cells can be activated. This finding is in line with the concordance in CD8\textsuperscript{T} T cell numbers detected by K\textsuperscript{b} MT245 tetramers and MT245 peptide-stimulated IFN-\gamma production in persistent but not acute infection.

Phenotypic and functional heterogeneity of epitope-specific anti-PyV CD8\textsuperscript{T} T cell responses

The variable kinetics of the PyV-specific CD8\textsuperscript{T} T cell response between the three epitope-specific populations led us to ask whether they expressed phenotypic differences during PyV infection. A detailed phenotypic analysis of all three populations during acute PyV infection revealed distinct expression patterns for CD25 (IL-2R\alpha) (Fig. 4A), CD69 (Fig. 4B), and CD127 (IL-7R\alpha) (Fig. 4D). Of note, the frequency of CD69\textsuperscript{+} K\textsuperscript{b} MT245 and D\textsuperscript{b} LT638 tetramer\textsuperscript{+} CD8\textsuperscript{T} T cells was markedly higher than that of the dominant LT360 CD8\textsuperscript{T} T cell response at early infection time points, but a higher fraction of the K\textsuperscript{b} MT245 CD8\textsuperscript{T} T cells expressed CD69 postcontraction (Fig. 4B). Because CD69 expression is tightly linked to TCR stimulation (24, 25), these findings suggest
that there is a more long-lived duration of epitope encounter by Kb MT245 CD8+ T cells; few CD69+ CD8+ T cells were found in persistently infected mice (data not shown). CD122 (IL-2R/IL-15R common β-chain) was expressed at similar frequencies by CD8+ T cells of each specificity in acutely (Fig. 4C) and persistently infected mice (~20% CD122+, day 295 postinfection). Because very few epitope-specific CD8+ T cells also remained CD25+ (data not shown), these data suggest that only a small fraction of these CD8+ T cells express the IL-15R. In this connection, IL-15-independent maintenance of memory CD8+ T cells to γHV-68 infection has been recently reported (26). It is notable that, compared with Dα LT360- and Kβ MT245-specific CD8+ T cells, Dα LT638-specific CD8+ T cells nearly entirely lost CD127 expression by day 8 postinfection (Fig. 4D). Because IL-7R expression has been reported to tag memory T cell precursors during acute infection, this finding implies that there are vanishingly few memory-fated LT638-specific CD8+ T cells. Despite these differences during acute infection, the majority (60%) of each PyV epitope-specific CD8+ T cell population expressed CD127 by 300 days after infection (data not shown). It is interesting to note here that >90% of memory CD8+ T cells in resolved LCMV infection are IL-7Rα+ by 40 days after infection (27).

TNF-α and IL-2 production by CD8+ T cells has been used to reveal memory CD8+ T cell heterogeneity and effector-to-memory CD8+ T cell differentiation (3, 28). We asked whether cytokine expression differed among CD8+ T cells that recognize different epitopes. During the acute phase (days 5–13) of infection, a markedly higher frequency of MT245-specific CD8+ T cells coproduced IFN-γ and TNF-α than LT360- and LT638-specific CD8+ T cells (Fig. 5A). Similar results were obtained for IFN-γ and IL-2 coproduction (Fig. 5B). Unexpectedly, the dominant LT360-specific CD8+ T cells showed a more limited range of cytokine production than the subdominant MT245-specific CD8+ T cells during acute infection. An inversion of cytokine capabilities occurred during persistent (>300 days) infection, where LT360-specific CD8 T cells now were clearly the superior producers of each of the three tested cytokines (Fig. 5). However, in contrast to the concordance in IFN-γ and TNF-α production by antiviral CD8+ T cells to resolved LCMV infection (28), no >50% of any of the epitope-specific CD8+ T cells in persistent PyV infection were coproducers of IFN-γ and TNF-α.

PyV-specific CD8+ T cell priming in persistently infected congenic bone marrow chimeric mice

Because persistent virus infection would be expected to present viral epitopes to T cells long term, we next asked whether PyV-specific CD8+ T cell priming occurs during persistent infection. To do this, we modified a novel microchimerism protocol to allow us to visualize expansion of PyV-specific CD8+ T cells during the persistent phase of infection. A minimally myeloablative dose of busulfan, a cytotoxic DNA alkylating agent, was administered to persistently infected B6 (CD45.2) mice, followed 24 h later with an i.v. transfer of 20 × 10⁶ bone marrow cells from naïve B6.SJL

were stained directly ex vivo with anti-CD8α and Dα LT360 or Dα LT638 tetramers and analyzed by flow cytometry. The magnitude of the MT245-specific CD8+ T cell response was determined by IFN-γ intracellular cytokine staining after a 5-h incubation of splenocytes with 10 μM peptide. Each point indicates the number of epitope-specific splenic CD8+ T cells from an individual mouse. B, The amount of PyV DNA in the spleen on the indicated day after infection was quantitated by real-time PCR. Each point indicates the number of genome copies per milligram tissue from an individual mouse.
FIGURE 3. Epitope-specific CD8⁺ T cell maturation during PyV infection. A, Vβ TCR repertoire analysis of PyV epitope-specific CD8⁺ T cell responses in acutely and persistently infected mice. Splenocytes from B6 mice on day 8 or day 111 after infection were triple stained with anti-CD8α, class I MHC tetramer, and the indicated Vβ TCR mAb and analyzed by flow cytometry. The frequency of D^b LT360-, K^b MT245-, and D^b LT638-specific CD8⁺ T cells that express a particular Vβ for each of three mice (■, □, and △) are shown. B, Splenocytes from B6 mice on day 8 (●) or day 168 (○) after infection were stimulated in a peptide dose-response IFN-γ ICCS assay with LT360, LT638, MT243, and MT246 peptides. The frequency of CD8⁺ T cells that produced IFN-γ when stimulated with 10 μM peptide is designated as the maximum (100%) response. Values represent the mean ± SEM of three mice.
(CD45.1) congenic mice. Using this persistently infected bone marrow chimeric host, we could distinguish host-derived (CD45.1-negative) recipient PyV-specific CD8 T cells from donor-derived (CD45.1-positive) PyV-specific CD8 T cells. The busulfan regimen alone did not impact preexisting Db LT360-specific CD8 T cells (Fig. 6A). In addition, there was no significant difference in the number of PyV genomes per milligram of tissue between mice that received busulfan alone or busulfan and bone marrow (data not shown). Forty-nine days after bone marrow transplant (113 days postinfection), we detected newly generated CD45.1 Db LT360-specific cells in the blood, spleen, and lungs of the chimeric mice (Fig. 6A). Both host and donor populations were functional, because each made intracellular IFN-γ after a 5-h ex vivo peptide stimulation (data not shown). Peptide dose-response curves for intracellular IFN-γ production did not reveal differences in functional avidity between host- and donor-specific cells (data not shown). Db LT360-specific cells derived from the host or donor were Ag experienced, as both uniformly up-regulated CD11a expression (Fig. 6B). Thus, PyV-specific CD8 T cells undergo priming and expansion during persistent infection.

Intriguingly, most of the donor-derived Db LT360-specific cells were CD62L<sup>high</sup>, whereas >95% of host-derived Db LT360-specific cells were CD62L<sup>low</sup>, in both the spleen and lungs (Fig. 6B). In contrast, >90% of Db LT360-specific cells isolated from the spleen of persistently infected B6 mice are CD62L<sup>low</sup>. To confirm that this phenotype was unique to CD8<sup>+</sup> T cells newly generated...
During persistent infection, we analyzed the PyV-specific CD8^+ T cell response in mice rendered chimeric before PyV infection. Congenic bone marrow chimeras created using the same busulfan and bone marrow transplant regimen were infected 6 wk posttransplant, when microchimerism was fully established (data not shown). We detected both host (CD45.1^-) and donor (CD45.1^-)-derived D^b LT360-specific CD8^+ T cells in the blood, spleen, and lungs of chimeras during acute (day 8) and persistent (day 43) infection (Fig. 7A). Both host and donor populations made IFN-γ after a 5-h ex vivo peptide stimulation (data not shown). D^b LT360-specific cells derived from the host or donor were uniformly CD11ahigh in both the spleen and lungs on days 8 and 43 postinfection (Fig. 7B). Unlike the discrepant CD62L expression levels seen in chimerism induced during persistent infection, >90% of host- and donor-derived D^b LT360-specific cells were now CD62Llow by day 43 postinfection in either the spleen or lungs. These findings suggest that D^b LT360-specific cells primed during persistent infection may be programmed differently than cells primed during acute infection, resulting in an altered cell surface phenotype.

**Discussion**

In this study, we tracked the evolution of epitope-specific CD8^+ T cell responses from acute to persistent infection by mouse PyV. We identified a strongly dominant (LT360) and two subdominant (MT245 and LT638) MHC class I-restricted viral epitopes in B6 mice. These three epitope-specific CD8^+ T cell responses were noncoordinately regulated in their expansion and contraction during acute infection, maturation in functional avidity in persistent infection, and functional competency. Interestingly, the MT245 response was found to represent an amalgam of responses to a K^b^- and D^b^-restricted epitope that each varied in magnitude and avidity in acutely and persistently infected mice. Finally, by establishing congeneric bone marrow chimerism in persistently infected mice, we found that PyV-specific CD8^+ T cells undergo priming and expansion during persistent infection.

The variability in expansion and contraction of epitope-specific CD8^+ T cells during acute PyV infection is markedly different from the coordinate regulation in CD8^+ T cell responses to acute infection seen in other microbial infection mouse models (17, 29). During the expansion phase, LT360- and LT638-specific cells expanded more rapidly than MT245-specific cells. Both LT360- and MT245-specific cells peaked in number at day 8 after infection, by which time the contraction of LT638-specific cells was well underway (Fig. 2A). Multiple factors are known to contribute to differences in T cell response kinetics and immunodominance, including biases in the host’s T cell repertoire (30), T cell competition for access to APCs (31), immunodomination, and efficiency of Ag presentation (32, 33). During PyV infection, alternative splicing of a common parental transcript gives rise to individual transcripts encoding the MT and LT proteins; as a result, differences in transcriptional efficiency cannot account for the difference in CD8^+ T cell kinetics between MT245 and the two LT CD8^+ T cell responses. Differences between these antigenic peptides in binding affinity to MHC class I molecules (17, 29) also failed to reveal an association with immunodominance hierarchy or acute infection expansion kinetics. Although differential Ag processing among PyV-specific CD8^+ T cell epitopes is a likely suspect, another possibility is that the profile of CD8^+ T cell epitope display varies among discrete populations of APCs, as reported for CD8^+ T cell responses to γHV-68 viral infection (2, 34). A theme emerging from these studies is that the microbial context for antigenic stimulation shapes the timing and magnitude of Ag-specific CD8^+ T cell responses.

Differences in cytokine profiles among the PyV epitope-specific CD8^+ T cells is reminiscent of epitope-specific cytokine profile
diversity reported during acute murine influenza virus and LCMV infection (35, 36). It has been suggested that differences in cytokine expression profiles of CD8\(^+\) T cells may be determined by differences in epitope density, TCR avidity, and frequency of epitope encounter (37). An expanded range of cytokine competency by a given Ag-specific T cell population could also indicate a differentiation state more akin to that of effector-memory than effector T cells (28). In acute PyV infection, a greater proportion of MT245-specific CD8\(^+\) T cells coproduced IFN-\(\gamma\) and TNF-\(\alpha\) or IL-2 and maintained CD25 and CD69 expression than did LT360- or LT638-specific CD8\(^+\) T cells. This phenotypic-functional correlation argues that the MT245-specific CD8\(^+\) T cell response is being driven more forcefully toward memory T cell differentiation than CD8\(^+\) T cells recognizing the other PyV specificities.

Persistent PyV infection impacts the TCR repertoire and functional avidity of antiviral CD8\(^+\) T cells, but with unexpected differences between epitope-specific populations. First, irrespective of its standing in the immunodominance hierarchy, each Ag-specific CD8\(^+\) T cell response exhibited a profound narrowing in V\(\beta\) domain usage from acute to persistent PyV infection. Second, this narrowing in TCR usage was not invariably associated with an increase in functional avidity. Third, of the two CD8\(^+\) T cell populations recognizing each of the overlapping epitopes contained within the MT245 sequence, only the K\(^b\)-restricted MT246 epitope was selectively maintained in persistent infection; this population alone showed maturation in functional avidity. It is of interest to note that tyrosine 250 within the MT246 epitope is not only a dominant anchor for antigenic peptide binding to K\(^b\), but also constitutes a phosphotyrosine-based binding site for the Src homology 2 domains of ShcA and the p85 subunit of PI3K (38); MT binding to ShcA and PI3K is essential for MT-induced cellular transformation and affects tumor induction by PyV (39, 40). From a teleological perspective, it is advantageous to the host to maintain a high avidity CD8\(^+\) T cell population directed toward a viral domain essential for cellular transformation. Although the relative importance of the different antipolyoma-specific CD8\(^+\) T cells for controlling persistent infection and eliminating nascently neoplastic cells is unknown, dissociation between CD8\(^+\) T cell immunodominance and TCR functional avidity is well documented (41). Fourth, there was a stochastic expansion of LT638-specific CD8\(^+\) T cells in persistent infection, with the number of LT638-specific CD8\(^+\) T cells in individual mice often rivaling that present at the peak of expansion during acute infection. This phenomenon is distinct from “memory inflation,” the progressive accumulation of virus-specific memory CD8\(^+\) T cells in all mice in the persistent phase of murine CMV infection (7). Given the selective expansion of the LT638-specific T cells, its high functional avidity, and the high D\(^b\)-binding affinity of the LT638 peptide, one possibility is that this subdominant population may expand to compensate for a relative decrease in number or function of another antiviral CD8\(^+\)
T cell population, and that this occurs on an individual basis. Alternatively, the activation threshold for LT638-specific T cells may be sufficiently low to enable it to expand and control transient small upsurges in PyV replication in persistently infected mice.

IL-7Rα expression marks memory progenitors within the pool of effector CD8+ T cells (27). During the acute CD8+ T cell response to PyV infection, the proportion of anti-PyV CD8+ T cells that expressed IL-7Rα varied among epitope-specific populations (Fig. 4D). Although IL-7Rα was maintained on a subset of LT630- and MT245-specific CD8+ T cells throughout the expansion and contraction phases, LT638-specific cells rapidly lost IL-7Rα expression during acute infection, such that nearly all LT638-specific cells were IL-7Rα− by day 8 after infection. The early contraction of LT638-specific CD8+ T cells beginning on days 6–7 after infection may be linked to the rapid loss of IL-7Rα expression. If few memory-fated LT638-specific T cells exist postcontraction, then LT638-specific cells that expand in the persistent phase of infection would be predicted to be highly oligoclonal. The “expansion” of LT638-specific CD8+ T cells during persistent infection (Fig. 2A) may indicate either preferential proliferation of memory CD8+ T cells to this specificity or, as we favor based on the chimeric bone marrow studies (see below), efficient recruitment of naive LT638-specific CD8+ T cells into the response. Given that the vast majority of memory CD8+ T cells express IL-7Rα in mice infected by a nonpersistent strain of LCMV (27), it is also noteworthy that only 60% of PyV-specific CD8+ T cells were IL-7Rα+ by late-phase persistent infection. Moreover, these T cells expressed high IL-7Rα levels comparable to that of effector T cells at the peak of acute PyV infection (data not shown); in contrast, high-level persistent LCMV infection is associated with reduced IL-7Rα levels (42).

A central finding in this study is that naive virus-specific CD8+ T cells precursors are recruited during persistent PyV infection. The novel chimeric bone marrow approach used here enabled simultaneous visualization of the preexisting virus-specific T cell population in persistently infected mice and emergence of endogenously derived, but temporally displaced, antiviral CD8+ T cells. Moreover, these data confirm findings in adoptive transfer models that in vivo competition between memory and naive CD8+ T cells to a common epitope occurs in vivo (43). The absence of central tolerance for LT360-specific thymocytes fits with the lack of PCR-detectable PyV DNA in the thymus of persistently infected mice (data not shown). The “young” donor and “old” host PyV-specific CD8+ T cells are functionally competent, as assessed by peptide-stimulated intracellular IFN-γ production, and have similar functional avidity (data not shown). However, PyV-specific CD8+ T cells elicited during persistent infection have a priming history distinct from those that are generated during acute infection, both in terms of viral Ag load and proinflammatory microenvironment. This distinction appears to be revealed by differences in CD62L expression levels between the host and donor antiviral CD8+ T cells when chimerism is established in persistently infected mice, but not when it is established before infection (Figs. 6 and 7). Given the substantially lower viral load in persistent than acute infection, LT360-specific CD8+ T cells primed during persistent infection receive weaker or shorter duration TCR stimulation, which, in turn, may translate into only a temporary down-regulation of cell surface CD62L expression. An inverse association between CD62L expression levels by memory CD8+ T cells and the duration of Ag stimulation has been described in other persistent viral infection models (44, 45). Interestingly, a similar dichotomy in CD62L expression patterns is also seen between late-emerging latent epitope-specific CD8+ T cells and early-emerging lytic epitope-specific CD8+ T cells in mouse γHV-68 infection (9). The finding that acute infection-primed, but not persistent infection-primed, LT360-specific cells remain CD62L+ further implies that the priming environment imprints a particular differentiation program on antiviral CD8+ T cells. Understanding how this programming impacts the trafficking and effector function of virus-specific CD8+ T cells arising at different stages of persistent infection and the nature of this programming will be important in designing therapeutic interventions to sustain immunosurveillance for persistently infected cells.

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References


