An MHC class Ib–restricted CD8 T cell response confers antiviral immunity

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Although immunity against intracellular pathogens is primarily provided by CD8 T lymphocytes that recognize pathogen-derived peptides presented by major histocompatibility complex (MHC) class Ia molecules, MHC class Ib–restricted CD8 T cells have been implicated in antiviral immunity. Using mouse polyoma virus (PyV), we found that MHC class Ia–deficient ($K^{b-/-}D^{b-/-}$) mice efficiently control this persistently infecting mouse pathogen. CD8 T cell depletion mitigates clearance of PyV in $K^{b-/-}D^{b-/-}$ mice. We identified the ligand for PyV-specific CD8 T cells in $K^{b-/-}D^{b-/-}$ mice as a nonamer peptide from the VP2 capsid protein presented by Q9, a member of the β_2 microglobulin–associated Qa-2 family. Using Q9-VP2 tetramers, we monitored delayed but progressive expansion of these antigen-specific CD8$\alpha\beta$ T cells in $K^{b-/-}D^{b-/-}$ mice. Importantly, we demonstrate that Q9-VP2–specific CD8 T cells more effectively clear wild-type PyV than a VP2 epitope^{null} mutant PyV. Finally, we show that wild-type mice also generate Q9-restricted VP2 epitope–specific CD8 T cells to PyV infection. To our knowledge, this is the first evidence for a defined MHC class Ib–restricted antiviral CD8 T cell response that contributes to host defense. This study motivates efforts to uncover MHC class Ib–restricted CD8 T cell responses in other viral infections, and given the limited polymorphism of MHC class Ib molecules, it raises the possibility of developing peptide-based viral vaccines having broad coverage across MHC haplotypes.

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Abbreviations used: β_2m, β_2 microglobulin; KLRG1, killer cell lectin-like receptor subfamily G, member 1; LT, large T antigen; LT359, aa 359–368 of LT; MT, middle T antigen; p.i., postinfection; PyV, mouse polyoma virus; VP2.139, aa 139–147 of the VP2 PyV capsid protein.

Immunosurveillance for intracellular pathogens is primarily mediated by conventional TCR$\alpha\beta$ CD8 T lymphocytes that recognize pathogen-derived oligopeptides presented by "classical" MHC class Ia molecules (the H-2K, D, and L molecules in the mouse). Yet, a sizeable number of H-2D distal genes in the Q, T, and M regions of the mouse MHC and the non–MHC-linked *CD1* and *MR1* genes encode β_2 microglobulin (β_2m)–associated class I glycoproteins. Compared with MHC class Ia molecules, these "nonclassical" class Ib molecules have limited polymorphism, low-level ubiquitous or tissue-specific expression, truncated cytoplasmic/transmembrane domains, and/or alternative splicing patterns (1). Several class Ib molecules have been shown to mediate immunoregulatory as well as nonimmunological functions (2–6).

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CD8 T cells restricted by MHC class Ib molecules have also been implicated in microbial defense. In particular, the mouse class Ib molecules H2-M3, CD1d (and human CD1), and Qa-1 (and its human orthologue HLA-E) serve as TCR restriction elements for CD8 T cells that provide immunity against intracellular bacteria, including *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Salmonella typhimurium*, respectively (7–9). H2-M3 preferentially presents *N*-formyl peptides, and CD1 presents mycobacterial lipid antigens to nonconventional T cells implicated in immunity to *M. tuberculosis* infection. Other than Qa-1 and HLA-E, which present the GroEL nonamer peptide from *S. typhi*, there are few examples of class Ib molecules that present "class Ia–like" peptides and none that serve as ligands for CD8 T cells that fight nonbacterial pathogens (1).

Limited evidence suggests that class Ib–restricted CD8 T cells may also contribute to

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viral immunity. Early studies identified a nonformylated peptide derived from the influenza virus hemagglutinin that induced an in vitro primary expansion of H2-M3–restricted CD8 CTLs, but these CTLs were unable to kill influenza virus–infected cells (10, 11). In addition, HLA-E–restricted TCRαβ CD8 CTL cloned lines recognizing a nonamer peptide derived from the UL40 protein of human CMV have been previously described (12). Recently, Braaten et al. demonstrated that β_2m-dependent TCRαβ CD8 T cells in mice selectively lacking MHC class Ia molecules ($K^{b-/-}D^{b-/-}$ mice) controlled chronic infection by rodent γ-herpesvirus 68, although the class Ib–restricting molecule and viral epitope remain to be identified (13).

Polyomaviruses are highly prevalent vertebrate pathogens that generally cause a self-limited acute infection followed by long-term asymptomatic persistent infection in their natural host reservoir (14). In the immunosuppressed population, human polyomaviruses JC and BK can reactivate with severely debilitating consequences, including a typically fatal central nervous system demyelinating disease and allograft nephropathy, respectively (15, 16). Recently, two new human polyomaviruses have been identified in respiratory tract infections, and another has been associated with the cutaneous malignancy Merkel cell carcinoma (17–19). Mouse polyomavirus (PyV) establishes low-level systemic persistent infection and causes tumors in immunocompromised mice and neonatally infected mice of certain inbred strains (20). The ~5-kb double-stranded PyV genome encodes only six proteins, three nonstructural proteins (small T, middle T [MT], and large T [LT] antigens) and three capsid proteins (VP1, VP2, and VP3). Interestingly, only peptides derived from the T antigens have been defined as epitopes for MHC class Ia–restricted PyV-specific CD8 T cells (14, 21).

In this study, we investigated whether PyV infection elicits virus-specific CD8 T cells restricted by MHC class Ib molecules. We previously showed that $\beta_2m^{-/-}$ mice inefficiently control PyV infection, as indicated by the realization of this virus's oncogenic potential (22). Unexpectedly, $K^{b-/-}D^{b-/-}$ mice are highly resistant to tumor formation and efficiently control PyV infection, implicating MHC class Ia–independent CD8 T cells in surveillance for PyV-infected cells. We identified the ligand for the dominant PyV-specific CD8 T cell response in $K^{b-/-}D^{b-/-}$ mice as a VP2-derived nonameric peptide presented by Q9, a member of the Qa-2 family of Q region–encoded MHC class Ib molecules. Importantly, PyV infection also elicits Q9-restricted VP2-specific CD8 T cells in wild-type mice. Our findings provide the first formal demonstration of an antigen-specific class Ib–restricted CD8 T cell response that confers immunity to a viral infection.

RESULTS

MHC class Ia–deficient mice control PyV infection

In contrast to $\beta_2m^{-/-}$ mice, which are both MHC class Ia– and class Ib–deficient and highly susceptible to PyV tumorigenesis (22), we observed that PyV-infected MHC class Ia–deficient $K^{b-/-}D^{b-/-}$ mice retain the resistance to PyV-induced tumors of B6 mice (3 out of 17 $K^{b-/-}D^{b-/-}$ mice developed tumors at 7 mo postinfection [p.i.]). Consistent with their tumor resistance, $K^{b-/-}D^{b-/-}$ mice clear acute PyV infection and check persistent viral replication with an efficiency similar to B6 mice (Fig. 1 A). Compared with B6 mice, however, $K^{b-/-}D^{b-/-}$ mice had a significantly higher viral load in the kidney (and a higher, but not significant, viral load in the spleen) at day 8 p.i. It is interesting to note that with this apparent lag in control of acute PyV infection, CD8 T cell numbers in $K^{b-/-}D^{b-/-}$ mice do not appreciably change until 25 d p.i. but then progressively increase well into the persistent phase of infection (Fig. 1 B). $K^{b-/-}D^{b-/-}$ mice depleted of CD8 T cells manifested significantly higher viral loads than those mice given control rat IgG (Fig. 1 C). Collectively, these data implicate non–MHC class Ia–restricted CD8αβ T cells in anti-PyV immunity in $K^{b-/-}D^{b-/-}$ mice.

Identification of the ligand for MHC class Ib–restricted PyV-specific CD8 T cells

Because of the small CD8 T cell compartment in $K^{b-/-}D^{b-/-}$ mice (~2% of splenic CD8 T cells in B6 mice), we were unable to reliably and consistently detect PyV-specific CD8 T cell responses in $K^{b-/-}D^{b-/-}$ mice ex vivo by IFN-γ intracellular staining assays. To circumvent this obstacle, we isolated and established continuous cultures of T cell clones from individual $K^{b-/-}D^{b-/-}$ mice that specifically recognized PyV-infected APCs (Fig. 2 A). These CD8αβ (unpublished data) T cell clones provided the cellular reagents to define the ligands for the anti-PyV CD8 T cells in $K^{b-/-}D^{b-/-}$ mice.

PyV-infected spleen cells from mice lacking all β_2m-associated class I molecules ($K^{b-/-}D^{b-/-}\beta_2m^{-/-}$) failed to stimulate these $K^{b-/-}D^{b-/-}$ mouse–derived CD8 T cell clones, narrowing the candidate restriction elements to a β_2m-associated class I molecule (Fig. 2 B). Infected splenic APCs from B6 mice with targeted disruption of *H2-M3*, *CD1d* (Fig. 2 B), or *Qa-1* (unpublished data) genes were recognized by the T cell clones, excluding these molecules as MHC class Ib–restricting elements. In contrast, the T cell clones did not produce IFN-γ when co-cultured with infected APCs from B6.K1 mice, a congenic B6 mouse strain null for the *Qa-2* locus, implicating a Qa-2 gene product as the MHC class Ib–restricting element.

Concomitant with these restriction mapping studies, we used libraries of 15-17mer overlapping peptides covering all six PyV proteins in an MHC-unbiased approach to define viral epitopes recognized by the $K^{b-/-}D^{b-/-}$ PyV-specific T cell clones. By testing the antigenicity of individual peptides in IFN-γ ELISPOT assays, we found that only one 15mer peptide corresponding to aa 136–150 within the VP2 minor capsid protein, designated peptide 28 (Table I), stimulated each of two cloned lines isolated from different $K^{b-/-}D^{b-/-}$ mice (unpublished data). Coincidentally, this peptide contained sequences that satisfied the MHC anchor consensus motif for 8-9mer peptides bound to Qa-2 molecules: histidine at position 7 (numbered from the amino end), and a hydrophobic carboxy terminus. Of three 9mers

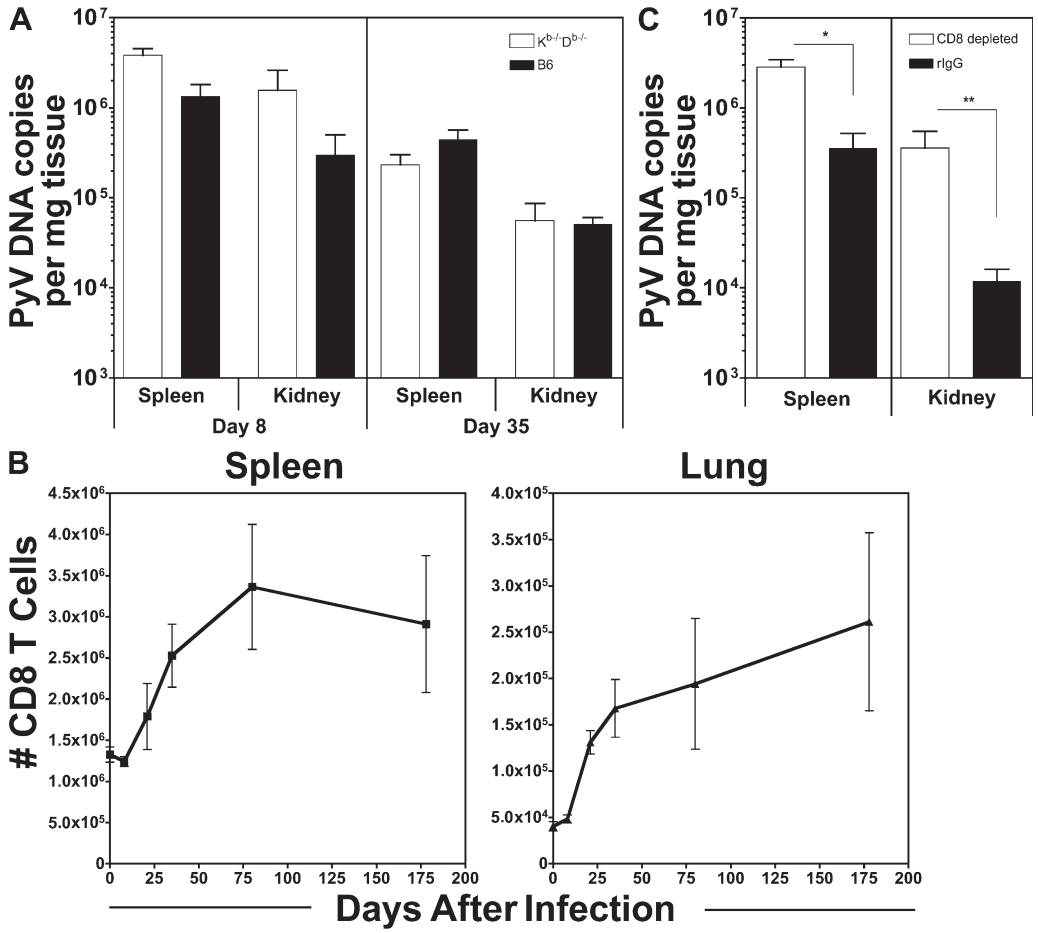

Figure 1. $K^{b-/-}D^{b-/-}$ mice control PyV replication. (A) Levels of PyV DNA (±SEM) in organs at the indicated days p.i. (B) Number of splenic and lung CD8 T cells (±SD) in PyV-infected $K^{b-/-}D^{b-/-}$ mice over time. (C) $K^{b-/-}D^{b-/-}$ mice received either CD8-depleting mAb or an isotype control rat IgG, and the PyV genome copy numbers (±SEM) in the indicated organs were assayed on day 35 p.i. Data are the mean of three to five mice per group and are representative of two independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$.

having a histidine at P7 within the VP2 aa sequence 133–151 (Table II), only 139–147 of the VP2 PyV capsid protein (VP2.139) stimulated intracellular IFN-γ production by all of the $K^{b-/-}D^{b-/-}$ mouse–derived CD8 T cell clones (Fig. 2 C). Single residue truncations of the VP2.139 peptide at the carboxy or amino terminus resulted in peptides that did not stimulate any of the clones, indicating that aa 139–147 of VP2 represent the minimum Qa-2–restricted epitope (unpublished data). Interestingly, the carboxy terminus of VP2.139 is occupied by tryptophan, a residue not detected at this position among endogenous peptides eluted from Qa-2 molecules (23); however, the Qa-2 F pocket is relatively spacious and can accommodate large side chains (24).

The Qa-2 family is composed of several members, of which Q9 is among the most thoroughly characterized mouse class Ib proteins. Q9 and another Qa-2 family member, Q8, share largely overlapping peptide-binding motifs, despite having >20 aa differences in the α1 and α2 domains (25). To determine whether Q8 and/or Q9 present the VP2.139 epitope to the $K^{b-/-}D^{b-/-}$ PyV-specific CD8 T cell clones, we used TAP2-deficient, MHC class Ia–negative B78H1 lines stably transfected with TAP2 and Q8 (H1Q8TAP.11), TAP2 and Q9 (H1Q9TAP11), or TAP2 and empty vector (H1TAP.11) as target cells in cytotoxicity assays (26). As shown in Fig. 2 D, only VP2.139 peptide–loaded cells expressing Q9 were recognized by these CD8 T cell clones. Collectively, these results define Q9-VP2.139 as the ligand for TCRs expressed by the PyV-specific CD8 T cell clone lines isolated from $K^{b-/-}D^{b-/-}$ mice.

Visualization of Q9-VP2.139–specific CD8 T cells in $K^{b-/-}D^{b-/-}$ mice

We constructed Q9 tetramers complexed to the VP2.139 peptide to monitor the evolution of these novel antigen-specific CD8 T cells through the course of PyV infection. To confirm the specificity of the Q9-VP2.139 tetramers, we determined that tetramer$^+$ CD8 T cells were readily detectable in spleens of $K^{b-/-}D^{b-/-}$ mice infected by wild-type PyV, but not in uninfected mice (Fig. 3 A). Moreover, the Q9-VP2.139 tetramer did not stain CD8 T cells in mice infected by a mutant

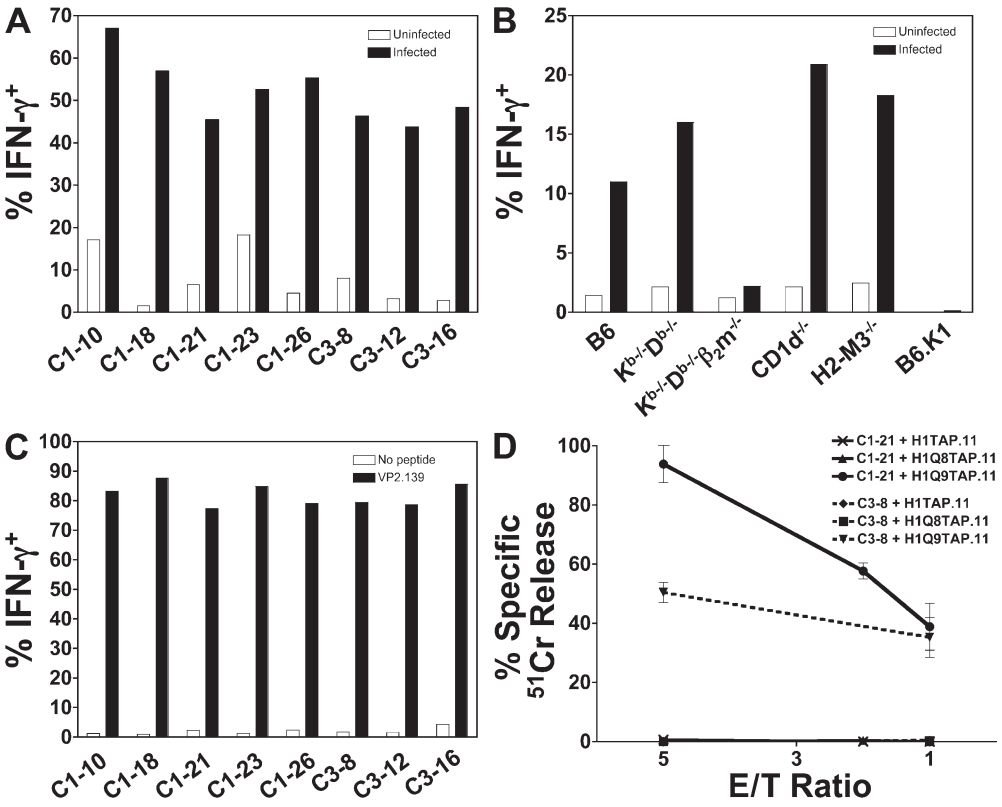

Figure 2. Q9-VP2.139 is the ligand for PyV-specific $K^{b-/-}D^{b-/-}$ CD8 T cell clones. CD8 T cell clones isolated from PyV-infected $K^{b-/-}D^{b-/-}$ mice were stimulated by PyV-infected or uninfected $K^{b-/-}D^{b-/-}$ splenocytes (A) or PyV-infected or uninfected splenocytes from mice of the indicated strains (B) for 5 h, and analyzed for intracellular IFN-γ. Data in B show the frequency of IFN-γ–producing clone C3-8 cells; the same response pattern is shown by clone C1-21 (unpublished data). (C) Frequency of T cell clones stained intracellularly by anti–IFN-γ after stimulation by $K^{b-/-}D^{b-/-}$ splenocytes in the presence of VP2.139 peptide or no added peptide. (D) Cytotoxic activity (±SD) by C1-21 and C3-8 T cell clones against VP2.139 peptide–coated control target cells (H1TAP.11) and target cells expressing Q8 (H1Q8TAP.11) or Q9 (H1Q9TAP.11). Without peptide, no specific lysis was seen by either cloned line against any of these target cells (unpublished data).

PyV in which the Q9-anchoring histidine in the VP2.139 epitope was replaced by alanine (designated A2.H145A). Direct ex vivo Q9-VP2.139 tetramer staining showed that PyV-infected $K^{b-/-}D^{b-/-}$ mice did not generate a detectable antigen-specific CD8 T cell response in either spleen or lung until day 8 of infection, at which point VP2.139 epitope–specific CD8 T cells steadily increased in both frequency and magnitude into persistent infection (Fig. 3 B). Notably, by 80 d p.i., VP2.139-specific CD8 T cells represented nearly 80% of CD8 T cells in the lung and blood (unpublished data), and 40% of splenic CD8 T cells. This T cell response profile differs considerably from that of MHC class Ia–restricted anti-PyV CD8 T cell responses, where tetramer$^+$ CD8 T cells are typically detected by 5 d p.i., peak in magnitude by 8 d p.i., and then quickly contract to a lower long-term steady-state level (14, 27).

Chronic encounter by cognate antigen typically directs MHC class Ia–restricted CD8 T cells toward an effector differentiation state having curtailed effector competency. To determine whether persistent PyV infection similarly affected this antiviral CD8 T cell response in $K^{b-/-}D^{b-/-}$ mice, we phenotypically and functionally characterized VP2.139-specific CD8 T cells during their expansion (day 35 p.i.) and long-term maintenance (day 178 p.i.) phases. At day 35 p.i., Q9-VP2.139 tetramer$^+$ CD8 T cells displayed an overall effector-like phenotype ($CD44^{hi}$, CD94/$NKG2^{hi}$, and $CD122^{lo}$; unpublished data), but with a mixed CD43 (clone 1B11 epitope), CD62L-selectin, and IL-7Rα (CD127) expression pattern, and elevated expression of killer cell lectin-like receptor subfamily G, member 1 (KLRG1), a marker of short-lived effector CD8 T cells (Fig. 3 C) (28, 29). By day 178 p.i., however, the VP2.139-specific

Table I. Overlapping VP2 peptides used to map the Qa-2–restricted PyV-specific T cell epitope

Peptide no.																										
Peptide 27	F	P	G	V	N	Q	F	A	H	A	L	N	V	V	H											
Peptide 28						Q	F	A	H	A	L	N	V	V	H	D	W	G	H	G						
Peptide 29											L	N	V	V	H	D	W	G	H	G	L	L	H	S	V

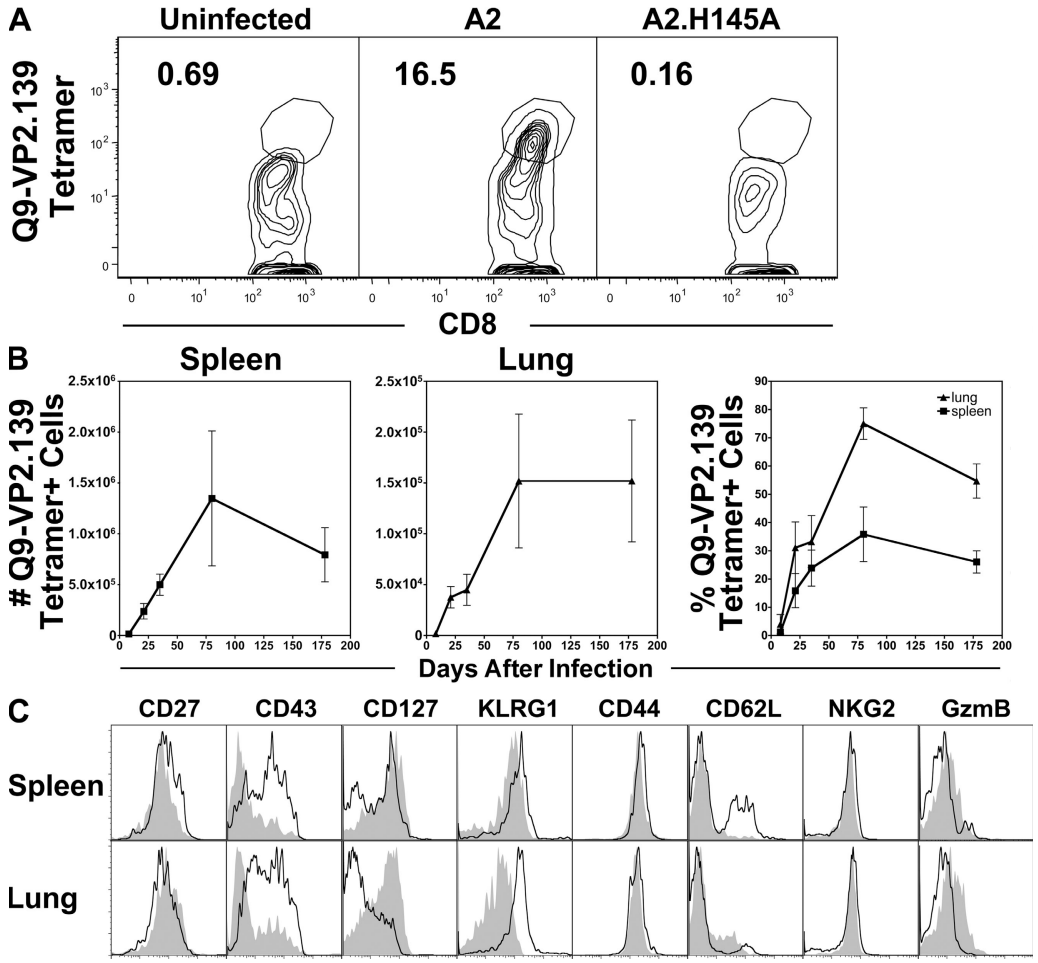

Figure 3. Evolution of the Q9-VP2.139–specific CD8 T cell response in $K^{b-/-}D^{b-/-}$ mice. (A) Splenic Q9-VP2.139 tetramer$^+$ CD8 T cells in uninfected, wild-type A2 PyV-infected, or VP2.139 epitope^{null} A2.H145A mutant PyV-infected $K^{b-/-}D^{b-/-}$ mice at day 40 p.i. Plots are gated on CD8$^+$ cells, and values represent the percentage of cells within the indicated gate. (B) Numbers (left and middle) and frequency (right) of splenic and lung Q9-VP2.139 tetramer$^+$ CD8 T cells (±SEM) over time (n = 3–9 mice at each of the indicated time points). (C) Splenic and lung tetramer$^+$ cells were analyzed at days 35 (open) and 178 (shaded) p.i. (plots are representative of four to nine mice at each time point). Plots are gated on CD8$^+$ Q9-VP2.139 tetramer$^+$ cells.

CD8 T cells assumed an unusual phenotype overlapping both effector and memory phenotypic profiles, consistent with repetitive antigenic stimulation of memory CD8 T cells (30, 31). Although nearly all of the maintenance-phase Q9-VP2.139 tetramer$^+$ CD8 T cells were CD62L^{lo}, they had reduced expression of CD27 and CD43, and increased levels of surface IL-7Rα. This CD27^{lo}CD43^{lo}CD127^{hi} profile has been recently ascribed to CD8 T cells having strong recall response capability (32). Notably, VP2.139-specific CD8 T cells at days 35 and 178 p.i. did not express the inhibitory programmed death 1 receptor (unpublished data), which is often present on the surface of functionally impaired MHC class Ia–restricted antiviral CD8 T cells maintained in the setting of persistent viral infections, including those responding to PyV infection (33).

VP2.139-specific CD8 T cells in $K^{b-/-}D^{b-/-}$ mice exhibited pronounced functional impairment. Throughout the course of PyV infection, <50% of VP2.139-specific CD8 T cells detected by tetramers produced intracellular IFN-γ after VP2.139 peptide stimulation (Fig. 4 A). Interestingly, a fraction of the VP2.139-specific CD8 T cells acquired the ability to coproduce IFN-γ and TNF-α at a late time point, when persistent infection viral loads had fallen (Fig. 1 A and Fig. 4 B). To assay antigen-specific cytotoxic activity in vivo,

Table II. Peptides fitting the Q9 peptide-binding motif

VP2 sequence																				
133–141	G	V	N	Q	F	A	H	A	L											
139–147							H	A	L	N	V	V	H	D	W					
143–151												V	V	H	D	W	G	H	G	L

$K^{b-/-}D^{b-/-}$ mice infected 35 d earlier were injected with equal proportions of naive $K^{b-/-}D^{b-/-}$ splenocytes pulsed with VP2.139 peptide ($CFSE^{hi}$ labeled) or left untreated ($CFSE^{lo}$ labeled). No specific elimination of peptide-coated target cells was seen 4 h after injection (unpublished data), but after 12 h, infected $K^{b-/-}D^{b-/-}$ mice demonstrated substantial specific clearance of VP2.139-coated targets (Fig. 4 C); the longer assay timeframe may enable colocalization of target cells with the low numbers of VP2.139-specific CD8 T cells, which, given their cytokine effector dysfunction, may also suffer reduced cytotoxic effector capability.

VP2.139-specific CD8 T cells confer antiviral immunity

We used two approaches to directly determine whether VP2.139-specific CD8 T cells operated to protect the host from viral infection. We first asked whether $K^{b-/-}D^{b-/-}$ mice were able to control infection by the VP2.139 $epitope^{null}$ mutant PyV, A2.H145A. As shown in Fig. 5 A, at day 5 p.i. no differences were seen in viral load between the parental and mutant viruses in multiple organs (spleen and kidney are shown). Thus, this single mutation at aa 145 in VP2 did not alter the capacity of PyV to replicate and disseminate in vivo at a time point preceding the emergence of a detectable CD8 T cell response (Fig. 1 B and Fig. 3 B). During persistent infection (day 80 p.i.), however, $K^{b-/-}D^{b-/-}$ mice infected by the A2.H145A virus had 100-fold more viral DNA in their kidneys than the parental A2-infected animals. This difference was not seen in the spleen or salivary glands (unpublished data), possibly pointing toward the importance of immune surveillance in an organ prone to harboring viruses that persistently infect their hosts (e.g., BK virus, JC virus, and human CMV).

Second, we used an adoptive transfer approach to ask whether VP2.139-specific CD8 T cells could protect $K^{b-/-}D^{b-/-}$ mice against a primary PyV infection. Spleen cells from PyV-infected (day 35 p.i.) $K^{b-/-}D^{b-/-}$ mice were transferred into naive $K^{b-/-}D^{b-/-}$ mice that were simultaneously infected either by A2 or A2.H145A PyVs. On day 5 p.i., virus levels were significantly lower in recipients of the parental PyV than the VP2.139 $epitope^{null}$ mutant PyV (Fig. 5 B). Collectively, these studies indicate that VP2.139-specific CD8 T cells mediate viral clearance in $K^{b-/-}D^{b-/-}$ mice.

B6 mice generate Q9-VP2.139–specific CD8 T cells

To determine whether PyV infection elicits Q9-restricted VP2.139-specific CD8 T cells in B6 mice, we initially used our standard inoculation dose of 10^6 PFU and monitored blood and spleen over the course of infection for CD8 T cells that bound Q9-VP2.139 tetramers. Under these conditions, we were unable to detect VP2.139-specific CD8 T cells (unpublished data). Based on our recent finding that antigen-nonspecific bystander inflammation associated with PyV infection impairs the antiviral CD8 T cell response (33), we reduced the viral inoculation dose 1,000-fold. As shown in Fig. 6 A, Q9-VP2.139 tetramers now stained CD8 T cells in approximately one third of infected B6 mice at day 11 p.i., the

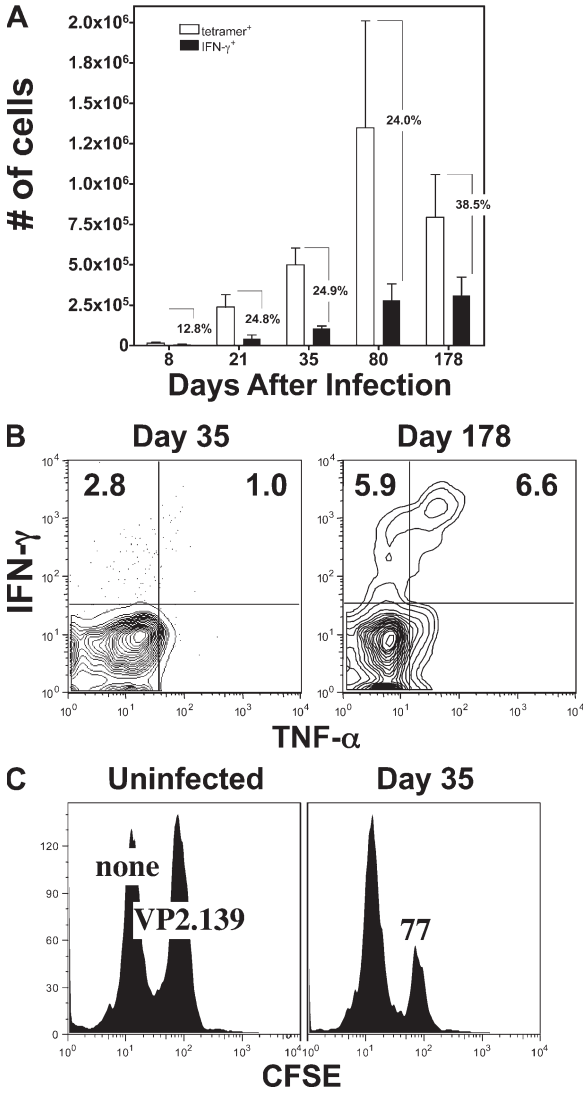

Figure 4. VP2.139-specific CD8 T cells have reduced effector function. (A) Number of Q9-VP2.139 tetramer+ and VP2.139 peptide–stimulated IFN-γ+ CD8 T cells (±SEM) in spleens of $K^{b-/-}D^{b-/-}$ mice at the indicated time points p.i. Values indicate the percentages of IFN-γ+/tetramer+ CD8 T cells. (B) VP2.139-stimulated spleen cells from $K^{b-/-}D^{b-/-}$ mice stained for intracellular IFN-γ and TNF-α at days 35 and 178 p.i. Plots are gated on CD8. (C) Naive $K^{b-/-}D^{b-/-}$ splenocytes coated with VP2.139 peptide ($CFSE^{hi}$) or left uncoated ($CFSE^{lo}$) were injected into naive or PyV-infected $K^{b-/-}D^{b-/-}$ mice ($n = 3$) at day 35 p.i. 12 h later, splenocytes were harvested and analyzed by flow cytometry. Value indicates the percentage loss of peptide-coated target cells and is representative of two independent experiments.

peak magnitude of the VP2.139-specific CD8 T cell response (unpublished data). The Q9-VP2.139–specific CD8 T cells constitute 0.5–2% of splenic CD8 T cells in B6 responders to this epitope, compared with the dominant D^b–aa 359–368 of LT (LT359) CD8 T cell response, which occupied ~16% of the splenic CD8 T cell compartment in each infected B6 mouse (Fig. 6 B). As was seen in the $K^{b-/-}D^{b-/-}$ mice, only a fraction of the Q9-VP2.139–specific CD8 T cells in B6 mice produced IFN-γ after peptide stimulation; however,

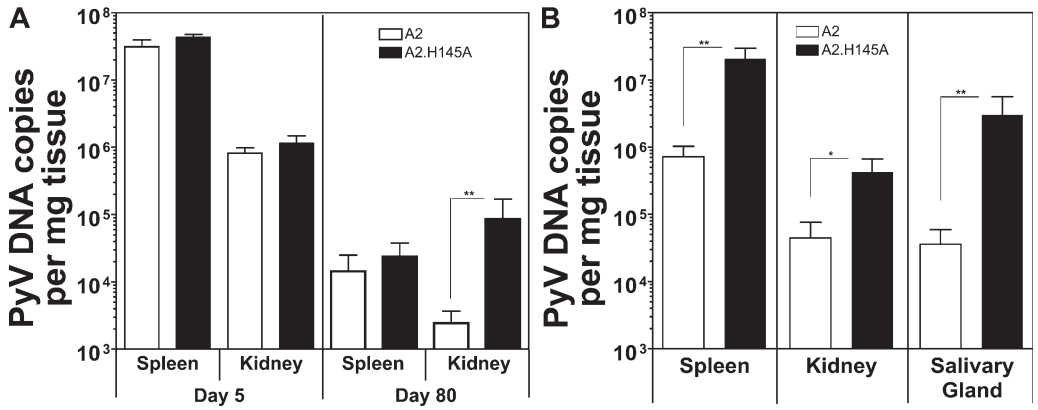

Figure 5. VP2.139-specific CD8 T cells are protective. (A) $K^{b-/-}D^{b-/-}$ mice were infected by parental A2 PyV or the VP2.139 epitope^{null} A2.H145A mutant PyV. Levels of PyV DNA (±SEM) in the spleen and kidney were determined by quantitative PCR at the indicated time points p.i. (B) $K^{b-/-}D^{b-/-}$ mice were infected by A2 or A2.H145A PyVs (10^6 PFU) and simultaneously received 20×10^6 splenocytes from A2 virus–infected (day 35 p.i.) $K^{b-/-}D^{b-/-}$ splenocytes. Virus levels in the indicated organs were assayed as in A at days 5–6 p.i. of five to six mice pooled from two independent experiments. **, $P < 0.0001$; *, $P < 0.001$.

most of the D^b-restricted LT359-specific CD8 T cells detected by tetramer staining produced IFN-γ (Fig. 6 C). Coincident with this functional deficit, Q9-VP2.139 CD8 T cells express a higher level of surface KLRG1 and a lower level of CD127 compared with D^b-LT359 CD8 T cells (Fig. 6 D). Thus, although MHC class Ib–restricted antiviral CD8 T cells are indeed recruited into the PyV-specific CD8 T cell response in acutely infected B6 mice, these T cells appear to be more terminally differentiated at this time point than the MHC class Ia–restricted antiviral CD8 T cells.

DISCUSSION

In this study, we identified a novel MHC class Ib peptide ligand for virus-specific CD8 T cells and demonstrated that these antiviral T cells confer protection against infection. We found that the MHC class Ia–deficient $K^{b-/-}D^{b-/-}$ mice efficiently control infection by PyV and mount an antiviral CD8 T cell response that is predominantly directed toward a viral capsid–derived nonamer peptide bound to Q9, a member of the Qa-2 family of MHC class Ib molecules. Of the β_2m-associated MHC class I products encoded in the Qa-2 locus, only Q9-restricted T cell responses have been previously shown to contribute to host immunity. An early report showed that expression of Q9 correlated with resistance to mouse cysticercosis (34). More recently, it has been demonstrated that immunization by Q9-expressing tumor cells elicits Q9-restricted CTLs, which are capable of cross-protecting against histologically disparate $Q9^+$ tumors (35, 36). A role for Q9-restricted CD8 T cells in antiviral immunity has been postulated and is supported by evidence that synthetic peptides from several viral proteins are capable of binding to Q9 molecules (37). To our knowledge, however, the data presented in this paper represent the first identification of a Qa-2–restricted T cell epitope and the first description of a MHC class Ib–restricted virus-specific CD8 T cell response in vivo.

An unusual feature of the Q9-restricted CD8 T cell response in $K^{b-/-}D^{b-/-}$ mice is its steady accumulation during persistent infection. Naive virus-specific CD8 T cells primed de novo during persistent infection are primarily responsible for maintaining stable numbers of conventional PyV-specific CD8 T cells (38) and are also a source of cells supplying the inflationary viral epitope–specific CD8 T cell populations in mouse CMV infection (Snyder, C., K.S. Cho, and A.B. Hill, personal communication). Although antigen-driven proliferation of virus-specific CD8 T cells has been shown to maintain CD8 T cell numbers during persistent lymphocytic choriomeningitis virus infection (39), PyV-specific MHC class Ia–restricted T cells undergo gradual attrition and fail to divide in persistently infected wild-type mice (38, 40). Whether de novo priming of naive T cells or self-renewal of effector/memory T cells predominantly accounts for the accumulation of VP2.139-specific CD8 T cells in persistently infected $K^{b-/-}D^{b-/-}$ mice is currently under investigation. The progressive increase in Q9-VP2.139–specific CD8 T cell numbers may also suggest that viral capsid proteins are expressed during persistent PyV infection. In support of this possibility, VP1 capsid proteins are expressed in PyV-induced tumors (41) and in the kidneys and lungs of persistently infected $\beta_2m^{-/-}$ mice (22). Interestingly, HLA-A2–restricted CD8 T cells directed to peptides derived from VP1 represent dominant specificities of human polyomavirus–specific CD8 T cells responses in healthy individuals (42, 43). Whether structural proteins are synthesized continuously during low-level "smoldering" infection by polyomavirus or are transiently resurrected from a dormant state is currently unknown.

A central finding of this study is that the peripheral T cell compartments of both H-$2K^{b-/-}D^{b-/-}$ and parental B6 mice contain a reservoir of virus-specific Q9-restricted CD8 T cells. The character of this response, however, differs depending on the level of MHC class Ia sufficiency. The Q9-VP2.139–specific CD8 T cell response is strikingly immunodominant in $K^{b-/-}D^{b-/-}$ mice but constitutes a subdominant, variable response in acutely infected B6 mice. A straightforward explanation is that MHC class Ib–restricted T cells in $K^{b-/-}D^{b-/-}$ mice

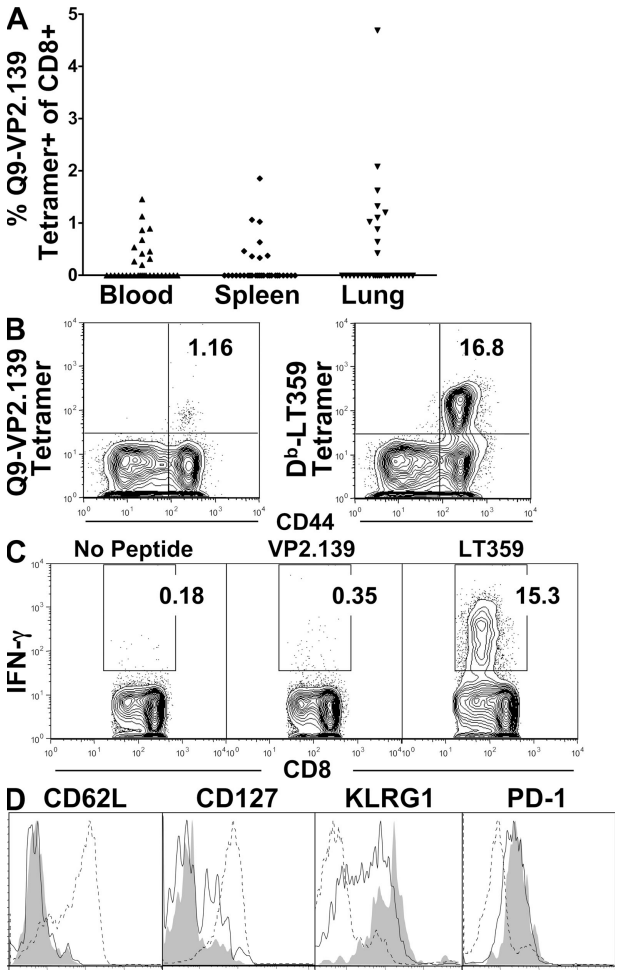

Figure 6. PyV-infected B6 mice generate a Q9-VP2.139–specific CD8 T cell response. (A) Frequency of Q9-VP2.139$^+$ cells of CD8 T cells in the blood, spleen, and lung of PyV-infected (day 11 p.i.) B6 mice ($n = 32$). (B) Representative dot plots of splenic Q9-VP2.139 tetramer$^+$ and D^b-LT359 tetramer$^+$ CD8 T cells from B6 mice at day 11 p.i. Plots are gated on CD8 T cells. Values indicate percentages of CD8 T cells that are tetramer$^+$. (C) VP2.139 and LT359 peptide–stimulated spleen cells from the same mice shown in B were analyzed for intracellular IFN-γ. Plots are gated on CD8 T cells. Values indicate percentages of CD8 T cells that are IFN-γ$^+$. (D) Expression of the indicated surface molecules by splenic CD8 T cells from naive mice (dashed line) and Q9-VP2.139 tetramer$^+$ (shaded) and D^b-LT359 tetramer$^+$ CD8 T cells (continuous line) from infected (day 11 p.i.) B6 mice.

would not encounter immunodomination by MHC class Ia–restricted T cells, as described for the H2-M3–restricted memory CD8 T cell response to *L. monocytogenes* (44). Thus, in $K^{b-/-}D^{b-/-}$ mice, VP2.139-specific CD8 T cells would have unfettered access to viral epitope–expressing cells, as well as to mitogenic and survival cytokines. A related factor may be that Q9 surface expression (and presumably Q9 epitope density) is higher in $K^{b-/-}D^{b-/-}$ mice than in MHC class Ia–sufficient mice (45), possibly because of a lack of competition for β_2m and MHC class I chaperone molecules. It is also worth noting that to date we have identified anti-PyV CD8 T cell epitopes only from the nonstructural LTs and MTs (14, 21), which are encoded by an early region transcript and are expressed by both productively and nonproductively infected cells. In contrast, the VP2.139 epitope is derived from a late-region viral capsid protein, whose synthesis is limited to productively infected cells. One possibility is that the Q9-VP2.139 CD8 T cell response may be at a disadvantage compared with MHC class Ia–restricted LT/MT-specific CD8 T cell responses both in timing of expression of the VP2 protein during the PyV lifecycle and the number and range of cells presenting the VP2.139 epitope. Alternatively, Q9 cell-surface expression, like MHC class Ia molecules, is up-regulated by certain inflammatory cytokines, such as IFN-γ (46), and may thus be subject to modulation in the context of virus infection. Thus, viral capsid–specific CD8 T cells may preferentially operate during persistent infection to contain pockets of active viral infection, a possibility supported by evidence that the VP2.139-specific CD8 T cells from persistently infected mice control PyV in acutely infected recipients (Fig. 5 B).

The inability of Q9 to efficiently use CD8 coreceptors (47) may contribute to the reduced magnitude and effector capability of CD8 T cells restricted by this class Ib molecule. Mice transgenic for a recombinant MHC class Ia molecule whose α3 domain was exchanged for that of Q7 (which is identical to Q9) (48) show profound inefficiency in the selection of MHC class I alloreactive CD8 CTLs. The recently solved Q9 crystal structure reveals that the AB loop of Q9's α3 domain points away from where it would normally bind CD8 in MHC class Ia molecules (24). A small pool of VP2.139-specific naive T cells may explain the delay in expansion of PyV-specific CD8 T cells in $K^{b-/-}D^{b-/-}$ mice. Weak CD8 engagement may also raise T cell activation thresholds for Q9-restricted T cells, requiring elevated epitope densities to achieve full effector potential and possibly altering the differentiation of T cells recruited into the antiviral response (49, 50). In this connection, it is interesting to note that $CD8\alpha^{-/-}$ mice mount a delayed, cytokine effector function–handicapped, H-$2D^b$–restricted PyV-specific T cell response (40).

The central question is to what extent MHC class Ib–restricted T cells contribute to viral immunity. MHC class Ib–restricted CD8 T cells may provide a contingency for the host against viral immune evasion. For example, human CMV encodes a mimic MHC class Ia leader peptide, UL40, which binds to HLA-E and engages inhibitory CD94/NKG2A receptors on NK cells (51). Yet, HLA-E/UL40–specific CD8 T cells with cytokine and cytotoxic effector capabilities have been isolated from CMV-seropositive individuals (12). From a general perspective, MHC class Ib–restricted CD8 T cells expand epitope diversity of the antiviral immune response and protect against immune selection of epitope-escape viral variants. More specifically, because the dominant antiviral CD8 T cell response is directed toward MHC class Ia–presented epitopes, MHC class Ib–restricted T cells may assume importance as a failsafe antiviral defense strategy. From the surface phenotype of the VP2.139-specific CD8 T cells, which suggests recall-competent effector/effector–memory cells (Fig. 3 C), it is tempting to speculate that MHC class Ib–restricted

T cells may be play a substantial role in limiting persistent viral infection.

Because MHC class Ib molecules have few polymorphisms, identification of MHC class Ib–restricted epitopes would offer much more comprehensive coverage across a population for peptide-based CD8 T cell–mediated immunotherapy than can be achieved by immunization using MHC class Ia–restricted peptides. Although Q9 is a mouse-only MHC class Ib molecule, HLA-G has been proposed to constitute its functional human homologue (52). It is also notable that PyV is unique among avian and mammalian polyomaviruses in possessing a VP2.139 sequence available for binding to Q9 (53). This apparent example of host–pathogen coevolution lends additional support toward uncovering MHC class Ib–restricted antiviral CD8 T cell responses in humans, with the prospect of developing seemingly "non–MHC-restricted" epitope-based vaccinations for viral infections.

MATERIALS AND METHODS

Mice. C57BL/6NCr (B6) female mice were purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute. H2-$K^{b-/-}D^{b-/-}$ ($K^{b-/-}D^{b-/-}$) mice (45) were obtained from Taconic. H2-$M3^{-/-}$ (54), $CD1d^{-/-}$ (55), Qa-$1b^{-/-}$ (56), and B6.K1 (57) mice have been previously described. Spleens from Qa-$1b^{-/-}$ mice were provided by H. Cantor (Dana-Farber Cancer Institute, Boston, MA). $K^{b-/-}D^{b-/-}$ mice were bred and housed by the Department of Animal Resources at Emory University in accordance with the guidelines of the university's Institutional Animal Care and Use Committee. All mice were between 6 and 8 wk of age at the time of infection.

Cell lines. The derivation and maintenance of the B78H1 transfectants expressing TAP2 (designated H1TAP.11), TAP2 and Q8 (designated H1Q8TAP.11), or TAP2 and Q9 (designated H1Q9TAP.11) have been previously described (25).

Viruses and cell transfers. $K^{b-/-}D^{b-/-}$ and B6 mice were infected s.c. by 10^6 and 10^3 PFU of PyV (A2 strain), respectively, in hind footpads. The A2.H145A mutant virus was created using PCR-based site-directed mutagenesis of A2 genomic DNA (forward primer, 5′-GCTCTAAATGTAGTAGCTGATTGGGGCCATGGC-3′; reverse primer, 5′-GCCATGGCCCCAATCAGCTACTACATTTAGAGC-3′; mutation nucleotides are underlined) to change the codon for aa 145 in the VP2 protein from histidine to alanine. A VP2.139-147 (145A) analogue peptide did not stimulate spleen cells from PyV-infected $K^{b-/-}D^{b-/-}$ mice, and A2.H145A-infected $K^{b-/-}D^{b-/-}$ mice did not generate VP2.139-specific CD8 T cells (unpublished data). 20×10^6 spleen cells from $K^{b-/-}D^{b-/-}$ mice at day 35 p.i. by wild-type PyV were transferred i.v. to naive $K^{b-/-}D^{b-/-}$ mice, which were inoculated immediately after transfer with 10^6 PFU of parental A2 or mutant A2.H145A PyV.

Quantitation of PyV genomes. DNA isolation and Taqman-based real-time PCR were performed as previously described (14). 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 numbers versus threshold cycle of detection. The detection limit with this assay is 10 copies of genomic viral DNA.

Synthetic peptides. VP2.133-141, VP2.139-147, and VP2.143-151 peptides (Table II) and LT359-368Abu peptide (33) were synthesized by the solid-phase method using F-moc chemistries. Peptide stocks were solubilized in water or DMSO and stored at −20°C. Peptides were diluted in assay medium immediately before use. The MT and LT peptide libraries, consisting of 17mer peptides overlapping by 12 aa, were prepared as PepSets by Chiron and were provided by J. Altman (Emory Vaccine Center, Atlanta, GA); the VP1 and VP2 peptide libraries, consisting of 15mer peptides overlapping by 10 aa, were prepared on a peptide synthesizer (Symphony/Multiplex; Rainin). The MT peptide library includes all of the small T antigen except for its four carboxy-terminal aa, and the VP2 peptide library covers the entire VP3 sequence.

Establishment of PyV-specific CD8 T cell cloned lines. PyV-specific CD8 T cells were cloned by limiting dilution from in vitro secondary cultures of popliteal lymph node cells from PyV-immune (2–3 wk p.i.) $K^{b-/-}D^{b-/-}$ mice using infected, γ-irradiated $K^{b-/-}D^{b-/-}$ splenocytes, as previously described (58).

Cell isolation and flow cytometry. Single-cell suspensions were made of popliteal lymph node cells and RBC-lysed spleens. For lung lymphocyte isolation, mice were perfused with PBS containing 100 U/ml heparin, and lungs were digested in vitro by collagenase and centrifuged on Percoll step gradients, as previously described (59). Antibodies to CD8α, CD43, CD44, CD62L, and NKG2A/C/E (clone 20d5) were purchased from BD Biosciences and used according to the manufacturer's specifications. Anti-CD27, anti-CD127, and anti–programmed death 1 were purchased from eBioscience. Anti-KLRG1 was purchased from SouthernBiotech. Q9-VP2.139 tetramers were constructed by the National Institutes of Health Tetramer Core Facility at Emory University using cloned full-length Q9 cDNA (60). Tetramer staining was performed, along with antibody surface staining for 35 min at room temperature in PBS containing 2% FBS and 0.1% sodium azide. Samples were acquired on a FACSCalibur (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Inc.).

IFN-γ ELISPOT assay. Single-cell ELISPOT assays were performed as previously described (17). In brief, 96-well filtration plates (Millipore) were coated overnight with anti–IFN-γ (R4-6A2; BD Biosciences). 2 wk after stimulation by infected, irradiated $K^{b-/-}D^{b-/-}$ splenocytes, PyV-specific $K^{b-/-}D^{b-/-}$ CD8 T cell cloned lines were incubated with naive $K^{b-/-}D^{b-/-}$ splenocytes for 36 h at 37°C with individual overlapping peptides encompassing sequences for MT, LT, VP1, and VP2 PyV proteins. The final concentration of each peptide was ∼10 μM. After the incubation, plates were washed and incubated with biotinylated anti–IFN-γ (XMG1.2; BD Biosciences). Wells were incubated with horseradish peroxidase–avidin D (Vector Laboratories), washed, and developed with freshly prepared substrate buffer (0.03% [wt/vol] 3-amino-9-ethyl-carbazole and 0.015% [vol/vol] H_2O_2 in 0.1 M sodium acetate, pH 5).

Intracellular antibody staining. Cells were stimulated directly ex vivo with 1 μM VP2.139 peptide for 5 h in the presence of brefeldin A, and were then stained for surface CD8 and intracellular cytokines, as previously described (14). For assays using CD8 T cell clones, the clones were labeled with CFSE to distinguish them from splenic stimulator cells. mAbs to IFN-γ and TNF-α were purchased from BD Biosciences. For granzyme B staining, spleen cells were surface stained directly ex vivo with anti-CD8α and Q9-VP2.139 tetramers, permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), and stained with anti–human granzyme B or the isotype control antibody (Invitrogen).

In vivo CD8 T cell depletion. CD8β mAb (clone H35; provided by P. Allen, Washington University, St. Louis, MO) (61) was prepared in flasks (INTEGRA Celline CL1000; Integra Biosciences) using defined IgG-free medium (Invitrogen), according to the manufacturer's instructions. 1 mg anti-CD8β or ChromPure rat IgG (Jackson ImmunoResearch Laboratories) was administered i.p. on days −3, −1, and +1 from the day of PyV infection, with antibodies given every 5 d up to day 30 p.i. No CD8α-expressing $CD3^+$ T cells were detected in spleens of CD8β mAb–treated mice at day 35 p.i. (unpublished data).

In vivo cytotoxicity assay. RBC-lysed, single-cell spleen suspensions from naive $K^{b-/-}D^{b-/-}$ mice were pulsed at 10^7 cells/ml with 10 μM VP2.139 peptide or without peptide in DMEM containing 10% FBS for 30 min at 37°C. Each cell population was stained at 10^7 cells/ml in HBSS (Invitrogen) with 2 mM CellTrace Far Red DDAO-SE (Invitrogen) at

room temperature for 10 min, with labeling stopped by addition of an equal volume of FBS for 1 min. Each cell population was labeled with a different concentration of CFSE (1 or 0.1 mM) at 2×10^7 cells/ml in HBSS, with labeling stopped by addition of an equal volume of FBS for 1 min. 5×10^6 no-peptide and VP2.139 peptide–pulsed cells were mixed together and injected i.v. into infected and uninfected $K^{b-/-}D^{b-/-}$ mice. Specific elimination of donor cells was determined at 4 and 12 h after transfer, as previously described (62).

^{51}Cr release assay. Adherent H1TAP.11, H1Q8TAP.11, and H1Q9TAP.11 target cells were radiolabeled with 150–200 μCi of $Na_2{}^{51}CrO_4$ (400–1,200 Ci/g; PerkinElmer) and pulsed with 10 μM VP2.139 peptide at 37°C for 1.5 h, as described previously (58). Target cells were aliquotted at 5,000 cells per well into U-bottom 96-well microtiter plates and co-cultured at 37°C for 5 h with PyV-specific CD8 T cell cloned lines at the effector/target (E:T) ratios indicated in the figures in quadruplicate wells, and the percentage of specific ^{51}Cr release was determined as previously described (58).

Statistical analysis. Statistics were performed using statistical software (Prism; GraphPad Software, Inc.). All analyses were unpaired Student's *t* tests. $P \leq 0.05$ was considered statistically significant.

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