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Cutting Edge: Shift in Antigen Dependence by an Antiviral MHC Class Ib-Restricted CD8 T Cell Response during Persistent Viral Infection1

Phillip A. Swanson II, Amelia R. Hofstetter, Jarad J. Wilson, and Aron E. Lukacher2

The requirement for Ag in maintaining memory CD8 T cells often differs between infections that are acutely resolved and those that persist. Using the mouse polyoma virus (PyV) persistent infection model, we recently described a novel CD8 T cell response directed to a PyV peptide presented by Q9, an MHC class Ib molecule. This antiviral Q9-restricted CD8 T cell response is characterized by a 3-mo expansion phase followed by a long-term plateau phase. In this study, we demonstrate that viral Ag is required for this protracted inflation phase but is dispensable for the maintenance of this Q9-restricted CD8 T cell response. Moreover, proliferation by memory T cells, not recruitment of naive PyV-specific T cells, is primarily responsible for Q9-restricted, antipyV CD8 T cell inflation. These data reveal a dynamic shift in Ag dependence by an MHC class Ib-restricted memory CD8 T cell response during a persistent viral infection. *The Journal of Immunology*, 2009, 182: 5198–5202.

The CD8 T cells response to acutely cleared viral infections are characterized by rapid expansion followed by dramatic contraction and differentiation into memory cells that self-renew in a cytokine-dependent, Ag-independent manner (1–5). In contrast, memory CD8 T cells in persistent viral infections may suffer defects in homeostatic proliferation, with the severity of this dysfunction associated with level, duration, and pathogenesis of the infection (6, 7). For example, maintenance of antiviral memory CD8 T cells in high-level systemic chronic lymphocytic choriomeningitis virus (LCMV)3 infection requires cognate Ag but not IL-7 and IL-15 (8). Low-level systemic viral infections, however, appear to inflict a different insult on antiviral CD8 T cell responses. Depending on their epitope specificity, antiviral CD8 T cell numbers in mice infected by murine CMV (MCMV) increase over the course of infection and then stabilize at high frequencies, a phenomenon termed memory inflation (9, 10). Similarly, CMV-specific CD8 T cells in humans accumulate throughout an individual’s lifetime (11). Conventional MHC class Ia-restricted antiviral CD8 T cells in mice persistently infected by polyoma virus (PyV) fail to divide and are gradually lost, with the maintenance of stable numbers of antiviral CD8 T cells requiring ongoing recruitment of virus-specific, naive CD8 T cell progenitors (12).

Using the PyV infection mouse model, we recently uncovered a novel protective MHC class Ib-restricted CD8 T cell response whose expansion profile differs dramatically from that of conventional class Ia-restricted anti-PyV CD8 T cells (13). These unconventional CD8 T cells recognize a peptide derived from aa 139–148 of the PyV VP2 capsid protein (VP2.139) presented by the nonpolymorphic molecule Q9, a member of the Qa-2 family of class Ib molecules. In PyV-infected MHC class Ia-deficient mice, the Q9/VP2.139-specific CD8 T cell response progressively expands for ~12 wk, then enters a long-term plateau phase. In this study, we tested the hypothesis that cognate Ag regulates the inflation of these MHC class Ib-restricted antiviral CD8 T cells.

Materials and Methods

**Mice**

C57BL/6NCr (B6) female mice were purchased from the National Cancer Institute (Frederick, MD). B6.Kb−/−/Db−/−/(Kb−/−/Db−/−) mice (Thy1.2) were obtained from Taconic Farms; Kb−/−/Db−/−/Thy1.1 (14) mice were provided by P. Jensen (University of Utah, Salt Lake City, UT). Mice were bred and housed by the Division of Animal Resources at Emory University (Atlanta, GA) in accordance with the guidelines of the Institutional Animal Care and Use Committee of Emory University. Mice were 6- to 8-wk old at the time of infection.

**Viruses and cell transfers**

Kb−/−/Db−/− and B6 mice were infected s.c. with 1 × 106 PFU of PyV. A recombinant vaccinia virus (VV) carrying the PyV VP2 gene (VV-VP2) (15) was provided by R. Consigli (Kansas State University, Manhattan, KS); Kb−/−/Db−/− mice received 1 × 106 PFU of VV-VP2 i.p. The A2.H145A mutant virus was created as described (13). Splenocytes from PyV-infected

1 Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; MCMV, mouse CMV; p.i., postinfection; PyV, mouse polyomavirus; VV, vaccinia virus.

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FIGURE 1. VP2.139-specific CD8 T cell expansion is associated with persistent viral infection. Percentage of Q9/VP2.139 tetramer + CD8 T cells in the blood of PyV or VV-VP2-infected Kb−/−Db−/− mice ± SEM over time (n = 3 mice). Data are representative of two independent experiments.

Kb−/−Db−/− Thy1.1 mice were B cell depleted, labeled with 5 μm CFSE, and injected i.v. (5 × 10⁶ cells) into infected Kb−/−Db−/− Thy1.2 mice.

Flow cytometry
Anti-CD3ε, anti-CD8α, anti-Thy1.1, anti-Ki67, annexin V, propidium iodide, 7-aminoactinomycin D (7-AAD) and a TCR Vβ Ab panel were purchased from BD Biosciences and used as described (16). Q9/VP2.139 tetramers were constructed using either cloned full-length Q9 cDNA (17) or Q9 cDNA expressing CD3 domain of H-2Db in place of that of Q9. Both tetramers stained equivalent percentages of splenocytes from PyV-infected Kb−/−Db−/− mice with the same mean fluorescence intensity and were used interchangeably as described (13). Samples were acquired on a FACS Calibur flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

Bone marrow chimera
Persistently infected Kb−/−Db−/− mice given 600 μg of busulfan (Busulfex; Otsuka America Pharmaceutical) i.p. were injected i.v. 24 h later with 25 × 10⁶ CD3-depleted bone marrow cells from Kb−/−Db−/− Thy1.1 mice. After CD3 depletion using anti-CD3ε and MACS sorting, only 0.4% of mononuclear cells expressed CD3.

Results and Discussion
Persistent infection is associated with the Q9/VP2.139-specific CD8 T cell inflammatory response
PyV-infected Kb−/−Db−/− mice generate a VP2.139-specific CD8 T cell response that progressively increases over the first 3 mo after infection (13). Inflationary CD8 T cell responses have been observed in several different persistent viral infections, with one report showing that persistent infection is necessary for the prolonged expansion of Ag-specific cells (16). To determine whether persistent viral infection was necessary for the protracted expansion of VP2.139-specific CD8 T cells, we compared the Q9/VP2.139-specific CD8 T cell response longitudinally in the blood of individual Kb−/−Db−/− mice infected by either PyV, which establishes a persistent infection

Kb−/−Db−/− Thy1.1 mice were representative of mice in these panels, which indicate the percentage of Ki67 + cells and numerical values indicate the percentage of cells of that of Q9. Both tetramers stained equivalent percentages of splenocytes from PyV-infected Kb−/−Db−/− mice with the same mean fluorescence intensity and were used interchangeably as described (13). Samples were acquired on a FACS Calibur flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

De novo priming of VP2.139-specific CD8 T cells during persistent infection. Representative dot plots of lymphocytes isolated from the indicated organs of Thy congenic mice 50 days after bone marrow transfer (n = 3–4 mice) are shown. Plots are gated on CD8 T cells and the numerical values indicate the percentage of donor tetramer + cells of the total tetramer + population. Data are representative of two independent experiments.
During persistent PyV infection in wild-type B6 mice, de novo primed CD8 T cells resupply the short-lived MHC class II-restricted CD8 T cells and thereby maintain stable numbers of these antiviral T cells (12). We asked whether naïve Q9/VP2.139-specific CD8 T cells similarly contribute to the inflammatory response of VP2.139-specific CD8 T cells. To do this, Kb\(^{-/-}\)/D\(^{b/-}\) mice underwent minimal myeloablative busulfan conditioning midway through the Q9/VP2.139-specific CD8 T cell expansion phase (day 35 postinfection (p.i.)), followed by an injection of T cell-depleted, Thy congenic Kb\(^{-/-}\)/D\(^{b/-}\) bone marrow. Fifty days after bone marrow transfer (which just precedes the plateau phase), donor-derived Thy1.1\(^+\) Q9/VP2.139 tetramer\(^+\) CD8 T cells were detected, but they accounted for only a small fraction of the total VP2.139-specific CD8 T cell response (Fig. 2). In contrast, virus-specific CD8 T cells recruited in persistently infected wild-type B6 mice constituted 10–14% of the total dominant, PyV epitope-specific, MHC-Ia-restricted CD8 T cell population (18). These results indicate that naïve Q9/VP2.139-specific CD8 T cells are indeed recruited during the protracted expansion phase but that this process does not fully account for the dramatic inflation of this antiviral MHC-Ib-restricted CD8 T cell response.

Expansion phase VP2.139-specific CD8 T cells are highly proliferative

We next investigated the relative contributions of proliferation and survival of VP2.139-specific CD8 T cells over the course of their long-term expansion phase. Previously, we had observed that ~3 mo p.i. VP2.139-specific CD8 T cells no longer expand but are maintained at high numbers (13). We therefore compared inflation phase VP2.139-specific CD8 T cells (1 mo p.i.) to those from the plateau phase (3 mo p.i.) for the expression of molecules marking cell proliferation and survival. A larger fraction of inflation phase VP2.139-specific CD8 T cells expressed Ki67, a cell cycle-related nuclear protein, than those in the plateau phase (Fig. 3, A and C). In contrast, few VP2.139-specific CD8 T cells in either phase of the response stained with annexin V, a marker of apoptosis (Fig. 3, B and C); the anti-apoptotic protein Bcl-2 was expressed by similar frequencies of Q9/VP2.139 tetramer\(^+\) CD8 T cells and at comparable mean fluorescence intensity at 1 and 3 mo p.i. (P. A. Swanson, unpublished observations). These phenotypic data indicate that VP2.139-specific CD8 T cells survive long term in a nonproliferative or low proliferative state without appreciable cell death during the plateau phase.

The strikingly narrow expression of different TCR V\(\beta\) families by Q9/VP2.139 tetramer\(^+\) CD8 T cells in individual

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**FIGURE 4.** Inflation, but not survival, of memory VP2.139-specific CD8 T cells is Ag dependent. A, B cell-depleted spleen cells from wild-type PyV (A2 strain)-infected Kb\(^{-/-}\)/D\(^{b/-}\) Thy1.1 mice at 1 or 3 mo p.i. were transferred to infection-matched Kb\(^{-/-}\)/D\(^{b/-}\) Thy1.2 mice (1 mo \(\rightarrow\) 1 mo; 3 mo \(\rightarrow\) 3 mo) or cells from A2-infected Kb\(^{-/-}\)/D\(^{b/-}\) Thy1.1 mice at 3 mo p.i. were transferred into A2-infected Kb\(^{-/-}\)/D\(^{b/-}\) Thy1.2 mice at 1 mo p.i. (3 mo \(\rightarrow\) 1 mo). PBLs were monitored over time and the numerical values indicate the percentages of donor Q9/VP2.139 tetramer\(^+\) CD8 T cells \(\pm\) SEM normalized for input tetramer\(^+\) cells at day 4 after transfer (\(n = 3–6\) mice). B, As in A except that cells were transferred from A2-infected Kb\(^{-/-}\)/D\(^{b/-}\) mice at 1 mo p.i. to Thy congenic Kb\(^{-/-}\)/D\(^{b/-}\) mice infected 1 mo previously by either A2 PyV or a VP2.139 epitope\(^{-}\)null mutant PyV (A2.H145A). C, Representative CFSE profiles of donor VP2.139-specific CD8 T cell populations at the indicated time points after transfer for the experiments in A and B. Values indicate the percentage of cells in the marked regions. Two independent experiments were performed.
Kb<sup>b</sup>−/−D<sup>b</sup>−/− mice compared with the diverse V<sub>B</sub> family usage by CD8 T cells in uninfected Kb<sup>b</sup>−/−D<sup>b</sup>−/− mice (Fig. 3D) further suggests that a particular public clonotype of Q9/VP2.139-specific CD8 T cells does not preferentially expand and dominate this antiviral T cell population. Interestingly, CD8α<sup>−/−</sup> mice mount an MHC class Ia-restricted, PyV-specific T cell response having a similar dramatic narrowing of V<sub>B</sub> expression, with V<sub>B</sub> usage differing between individual mice (16). A salient feature of the Q9 structure, which is otherwise highly homologous to MHC-Ia molecules, is the deviated orientation of an α3 domain loop that renders CD8<sup>+</sup> subunit binding inefficient (19, 20). Weak to absent (as in CD8α<sup>−/−</sup> mice) CD8 coreceptor engagement may permit only a trickle of MHC-I-restricted thymic emigrants, with a consequent small oligoclonal reserve of naive anti-PyV T cell precursors.

Ag is required for VP2.139-specific CD8 T cell proliferation, but not maintenance

To directly investigate the proliferative state and survival of Q9/VP2.139-specific CD8 T cells during PyV infection, we longitudinally monitored the fate of CFSE-labeled Q9/VP2.139 tetramer<sup>+</sup> CD8 T cells from donor Kb<sup>b</sup>−/−D<sup>b</sup>−/− mice at 1 mo p.i. (inflation phase) or 3 mo p.i. (plateau phase) following transfer into infection-matched Thy congenic Kb<sup>−/−</sup> recipients (Fig. 4A). For the 1 mo p.i. donor-to-recipient adoptive cell transfers, the frequency of donor VP2.139-specific CD8 T cells steadily increased over the 30-day post-transfer observation period (Fig. 4A) and this was accompanied by substantial cell division as indicated by CFSE dilution (Fig. 4C). In contrast, the donor Q9/VP2.139 tetramer<sup>+</sup> CD8 T cells exhibited minimal expansion in the 3 mo p.i. donor-to-recipient adoptive cell transfers (Fig. 4A) and failed to divide (Fig. 4C). To exclude the possibility that VP2.139-specific CD8 T cells from the plateau phase suffer a cell-intrinsic proliferation defect, we transferred CFSE-labeled splenocytes from 3 mo p.i. mice to 1 mo p.i. mice. In this experimental setup, VP2.139-specific CD8 T cells from the plateau phase recapitulated the expansion profile and cell division seen by the inflation phase cells (Fig. 4A). These data further suggest that the failure of the plateau phase cells to proliferate is due to insufficient numbers of Q9/VP2.139 epitope<sup>+</sup> APCs. To test this possibility, splenocytes from Kb<sup>b</sup>−/−D<sup>b</sup>−/− mice infected by wild-type PyV (strain A2) 1 mo earlier were transferred to Thy congenic Kb<sup>b</sup>−/−D<sup>b</sup>−/− mice infected by either the A2 virus or a mutant A2 virus, A2.H145A, in which the dominant Q9-anchoring histidine in the seventh position (from the amino terminus) of the VP2.139 epitope was replaced by alanine. An H145A VP2.139–148 analog synthetic peptide fails to compete with the wild-type VP2.139–148 peptide in Q9 peptide-binding assays (A. R. Hofstetter and P. A. Swanson, unpublished observations), and infection by the A2.H145A mutant virus does not induce a Q9/VP2.139-specific CD8 T cell response in Kb<sup>b</sup>−/−D<sup>b</sup>−/− mice (13). Unlike VP2.139-specific CD8 T cell transfers from donors 1 mo p.i. with A2 to A2-infected recipients, those donor anti-PyV cells transferred to A2.H145A-infected recipients did not proliferate (Fig. 4C) and yet are stably maintained (Fig. 4B). These findings demonstrate that Ag is required for VP2.139-specific CD8 T cell expansion but is dispensable for cell survival.

The phenotype and longevity of expansion phase PyV-specific, MHC class Ib-restricted CD8 T cells differ from that of the inflationary epitope-specific CD8 T cells in MCMV infection. Those epitope-specific CD8 T cells that undergo progressive expansion during persistent MCMV infection are mostly short-lived effector cells that are replenished primarily from memory cells primed during the early stages of infection (21, 22). These MCMV-specific CD8 T cells do not express costimulatory molecules such as CD27 and CD28, nor do they express receptors for the homeostatic cytokines IL-7 and IL-15, which could account for their inability to survive long term (21, 22). In contrast, the inflationary VP2.139-specific CD8 T cells are long lived and express CD127, CD122, and Bcl-2 (Ref. 13 and P. A. Swanson, unpublished observations). Of note, VP2.139-specific CD8 T cells eventually reach stable high frequencies and are maintained in the absence of homeostatic proliferation. The long-term nonproliferative state of these T cells is reminiscent of LCMV-specific memory CD8 T cells that reside in the intestinal epithelium (23). Whether the differences between inflationary VP2.139-specific and MCMV-specific CD8 T cell responses reflect differences at the level of virus-host interaction or MHC class Ia vs Ib presentation remains to be determined.

The mechanism by which Ag controls memory CD8 T cell responses may also differ depending on the level of persistent infection. Ag appears to play a dual role in the CD8 T cell response in high-level LCMV clone 13 infection. High levels of Ag during early stages of LCMV clone 13 infection results in the selective culling of antiviral CD8 T cells of particular specificities (6, 24, 25), whereas CD8 T cells directed to other viral epitopes are maintained by Ag-driven proliferation (8). During a low-level persistent viral infection such as MCMV, stable memory virus-specific CD8 T cell responses do not require Ag for homeostatic proliferation or survival, but those that undergo inflation are highly dependent on Ag for expansion (21). Conventional MHC Ia-restricted, PyV-specific memory CD8 T cells do not homeostatically proliferate and are short lived, with the antiviral response maintained during persistent infection by the recruitment of PyV-specific naive CD8 T cells (12). The Ag-dependent inflation and Ag-independent maintenance of the PyV-specific, MHC class Ib-restricted CD8 T cell response described here reveal a novel pattern of memory CD8 T cell responses to persistent viral infection.

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Disclosures

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