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MHC Class Ib-Restricted CD8 T Cells Differ in Dependence on CD4 T Cell Help and CD28 Costimulation over the Course of Mouse Polyomavirus Infection

Amelia R. Hofstetter,*† Mandy L. Ford,‡ Lucy C. Sullivan,§ Jarad J. Wilson,*† Annette Hadley,* Andrew G. Brooks,§ and Aron E. Lukacher*  

We recently identified a protective MHC class Ib-restricted CD8 T cell response to infection with mouse polyomavirus. These CD8 T cells recognize a peptide from aa 139–147 of the VP2 viral capsid protein bound to the nonpolymorphic H-2Kb molecule, a member of the Qa-2 family of β2m-associated MHC class Ib molecules. Q9:VP2.139-specific CD8 T cells exhibit an unusual inflationary response characterized by a gradual expansion over 3 mo followed by a stable maintenance phase. We previously demonstrated that Q9:VP2.139-specific CD8 T cells are dependent on Ag for expansion, but not for long-term maintenance. In this study, we tested the hypothesis that the expansion and maintenance components of the Q9:VP2.139-specific T cell response are differentially dependent on CD4 T cell help and CD28 costimulation. Depletion of CD4⁺ cells and CD28/CD40L blockade impaired expansion of Q9:VP2.139-specific CD8 T cells, and intrinsic CD28 signaling was sufficient for expansion. In contrast, CD4 T cell insufficiency, but not CD28/CD40L blockade, resulted in a decline in frequency of Q9:VP2.139-specific CD8 T cells during the maintenance phase. These results indicate that the Q9:VP2.139-specific CD8 T cell response to mouse polyomavirus infection depends on CD4 T cell help and CD28 costimulation for inflationary expansion, but only on CD4 T cell help for maintenance. The Journal of Immunology, 2012, 188: 3071–3079.

Onclassical MHC class Ib molecules are generally distinguished from the classical MHC class Ia molecules in being less polymorphic, having limited tissue distribution, and lower cell surface expression levels. Although some class Ib molecules present nonpeptide molecules such as lipids, transferrin, or odorants (1), others structurally resemble class Ia molecules and present oligopeptides to CD8 T cells. Class Ib-restricted CD8 T cell responses to peptides and lipids contribute to both innate and adaptive immunity (2). Most class Ib-restricted T cell responses described so far are to specific peptides, making them difficult to analyze and study. However, Qa-1b-restricted T cells mediate protection against Listeria monocytogenes (3, 4), and H2-M3 presents N-formyl peptides to T cell responses to several bacterial infections, including L. monocytogenes (5) and Mycobacterium tuberculosis (6).  

Sequence homology between the Q9 class Ib molecule and class Ia molecules is closer than for other class Ib molecules (7), with the structures between Q9 and H-2Kb showing close overlap. However, unlike class Ia molecules, Q9 lacks a transmembrane domain and is instead bound to cell membranes by a GPI linkage (8). Q9 is expressed on all somatic cells, although expression levels may be lower than for class Ia molecules (9). The Q9 gene is situated in the murine Qa-2 locus and has no allelic polymorphisms among mice of inbred strains, although in some strains, Q9 exists as a pseudogene (10). Only two dominant residues are critical for anchoring nonameric peptides to Q9 (i.e., histidine at position 7 and hydrophobic residue at position 9), allowing Q9 to bind a diverse peptide repertoire, akin to class Ia molecules (7).

We recently identified a novel population of mouse polyomavirus (MPyV)-specific αβ TCR⁺ CD8 T cells whose ligand consists of Q9 complexed to a nonamer peptide (aa 139–147) of the VP2 capsid protein (11). Using MHC class Ia-deficient (B6. K⁺/−; αβ−/−) mice, we demonstrated that this Q9:VP2.139-specific CD8 T cell response controls MPyV infection. These Q9:VP2.139-specific CD8 T cells exhibit response kinetics and Ag dependence that depart dramatically from those of conventional class Ia-restricted anti-MPyV CD8 T cells, being initially detected 8 d postinfection (p.i.) and then progressively increasing in magnitude for 3 mo. Thereafter, the population is stably maintained, comprising up to 80% of the CD8 T cell compartment with minimal proliferation or apoptosis (12). However, Q9:VP2.139-specific CD8 T cells exhibit a marked defect in cytokine effector activity, with only 20–50% of these cells capable of producing IFN-γ, a dominant anti-MPyV cytokine (13). We recently reported that the Q9:VP2.139-specific CD8 T cell response depends on Ag for its expansion, but not for its maintenance phase (12); however, it is unclear what additional determinants are critical for maintenance of this population.

In this study, we explored roles of CD4 T cell help and CD28/CD40L costimulation as determinants of expansion and maintenance for the Q9:VP2.139-specific CD8 T cell response. In the absence of CD4 T cell help, conventional class Ia-restricted CD8

*Department of Pathology, Emory University School of Medicine, Atlanta, GA 30322; †Graduate Program in Immunology and Molecular Pathogenesis, Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, GA 30322; ‡Department of Surgery, Emory University School of Medicine, Atlanta, GA 30322; and §Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia

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Address correspondence and reprint requests to Dr. Aron E. Lukacher at the current address: Department of Microbiology and Immunology, H107, Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033. E-mail address: alukacher@hmc.psu.edu.

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Abbreviations used in this article: B6, C57BL/6; MFI, mean fluorescence intensity; MPyV, mouse polyomavirus; p.i., postinfection; qPCR, quantitative PCR.
T cells show no deficiency in recruitment to acute MPyV infection, but then suffer massive attrition during persistent infection; this response profile cannot be attributed to elevated viral infection levels (14). We further showed that the class Ia-restricted anti-MPyV CD8 T cell response depends on both CD28 and CD40L costimulation for expansion but is independent of these signals during maintenance (15). We hypothesized that, in parallel with their Ag dependence, the Q9:VP2.139-specific response would be dependent on CD4 T cell help and CD28/CD40L costimulation for expansion, but not for maintenance. However, although inflammatory expansion of these responses was found to be dependent on CD28 costimulation and CD4 T cell help, CD4 T cells were also required for maintenance of Q9:VP2.139-specific CD8 T cells.

### Materials and Methods

#### Mice

B6.Kb−/− D0−/− mice (Thyl.12) were purchased from Taconic Farms (Germantown, NY); B6.Kb−/− D0−/− Thy1.1 mice (16) were originally provided by Peter Jensen (University of Utah, Salt Lake City, UT). CD40−/− mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). B6.BL/6 (B6) mice were purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD). B6.129S2-Cd28tm1Akj/J (CD28−/−) mice were purchased from The Jackson Laboratory. 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. Female mice were 6–12 wk old at time of infection.

#### Viruses

MPyV wild-type virus (strain A2) was prepared as described previously (17). An MPyV mutant with a histidine-to-alanine substitution at aa 145 in VP2 (A2.H145A) was prepared as described previously (11). Mice were inoculated s.c. with 1 × 10⁶ PFU in the hind footpads.

#### Costimulation blockade and CD4⁺ cell deletion

Anti-CD4 (clone GK1.5) was administered i.p. at 250 µg/mouse on days −3, −1, and 1 relative to MPyV infection and then weekly thereafter. CD4⁺ cell deletion was verified by staining blood samples with anti-CD4 clone RM4-5 (eBioscience). Anti-CD40L (clone MR-1; BioXCell) and/or CTLA-4-Ig (Bristol-Meyers Squibb) were administered i.p. at 500 µg/mouse on days 0 and 2 of MPyV infection and then weekly thereafter. After mice were infected for 3 mo, GK1.5, MR-1, and/or CTLA-4 was administered twice in the first 3 d and weekly thereafter using 500 µg/mouse of each protein.

#### Flow cytometry

A total of 1 × 10⁶ RBC-lysed splenocytes, incubated with BD FcBlock for 15 min at 4°C, were stained with a chimeric Q9(Kb)VP2.139 tetramer, generated by swapping the connection, it merits noting that MPyV infection elicits a T cell-dependent, virus-neutralizing IgG response that controls viral replication (20) using B6 or CD28−/− eBioscience and BioLegend). Intracellular cytokine staining was performed as described previously (18). After incubation with and without 1 µM VP2.139 peptide for 5–6 h at 37°C in the presence of GolgiPlug (BD Biosciences), cells were stained intracellularly with Abs to IFN-γ, TNF-α, and IL-2 (BD Pharmingen), and for CD28 and CD40L (eBioscience) and CD69 (Invitrogen). The limit of detection for expansion during maintenance (15). We hypothesized that, in parallel with their Ag dependence, the Q9:VP2.139-specific response would be dependent on CD4 T cell help and CD28/CD40L costimulation for expansion, but not for maintenance. However, although inflammatory expansion of these responses was found to be dependent on CD28 costimulation and CD4 T cell help, CD4 T cells were also required for maintenance of Q9:VP2.139-specific CD8 T cells.

### Generation of Q9:VP2.139-specific TCR retrogenic mice

A Q9:VP2.139-specific CD8 T cell hybridoma (C3K) was derived by fusing the C3-8 clone (described in Ref. 11) with a CD8-transfected BW5147 fusion partner (described in Ref. 19). Generation of replication-defective retroviral producer cell lines was performed as described by Holst and colleagues (20). TCRα- and TCRβ-chains from C3K were cloned by PCR into pGEM using the following primer sequences: α-chain (5α): forward: 5′-GGCCGCAATTCAGATCATCAGAAGGCTGCTGTGCTCTCGTCTGG-3′; β-chain (5′β): forward: 5′-CTGTTAAAGAAGGCGGAGAACGGTAAGAAGAACCCCCGTCCCATGGGCTCATTTTCTACGTGGTCTCGG-3′. The products were then subcloned into the GFP-encoding murine stem cell virus-based retroviral vector pMig at the BamHI restriction site using the following reverse primers to generate a 2A-linked multistrictonic construct: 5′-CTTCCAGCTGTCTCGTTGTTTAAACAGAGAAGGTCGTGCTCGCCGAGACGACGCTACGTCATCAGTAG-3′; 5′-GCTCGTGGAGGGATCTCAGGAAGAATTTTYYTCTTGACATRGC-3′. 293T cells were transiently transfected with three plasmids, separately encoding packaging genes, envelope genes, and the C5K TCR retroviral vector. Retroviruses produced by these 293T cells were used to infect GFP-B6 mice to generate replication-deficient retroviruses. Retrovirus-mediated stem cell gene transfer was carried out as described by Holst and colleagues (20) using B6 or CD28−/− bone marrow and B6 recipients to generate retrogenic donor mice. In brief, bone marrow isolated from B6 or CD28−/− mice was infected by culture with irradiated GP+E86 producer cells, then transferred i.v. into B6 recipients. After 5 wk, retrogenic donor mice were checked for reconstitution by staining with anti-Vp4 and Q9:VP2.139 tetramer, and by GFP expression. In our hands, 80–90% of retrogenic donor mice’s splenocytes express GFP. Of the CD8⁺ T cells in this population, 20–30% stains with Vp4. Splenocytes were FACS sorted for GFP⁺ cells (FACS.Aria; BD Biosciences) and either 1 × 10⁶ or 1 × 10⁶ cells were transferred i.v. Mice received wild-type MPyV or A2.H145A 1 d posttransfer.

#### IL-2 treatment

Recombinant human IL-2 (Amgen) was administered i.p. at 15,000 U/mouse in PBS and 0.1% B6.Kb−/− Db−/− mouse serum every 12 h for 16 d.

#### Statistics

Statistical analyses were performed using Prism software (GraphPad, La Jolla, CA). For blood kinetic studies, data were analyzed using two-way ANOVA with Bonferroni post tests. For phenotyping and quantitative PCR (qPCR) studies, two-tailed Mann–Whitney U tests were used, with the exception of qPCR data for singly administered anti-CD40L or CTLA-4-Ig, for which a Kruskal–Wallis Test was used, with Dunn’s multiple-comparison test.

### Results

#### Expansion of Q9:VP2.139-specific cells is dependent on CD4 T cell help

To investigate whether CD4 T cell help is required for expansion of Q9:VP2.139-specific T cells, MHC class Ia-deficient B6.Kb−/− Db−/− mice were depleted of CD4⁺ cells by administering anti-CD4 (clone GK1.5). Although CD4 T cell insufficiency in B6.Kb−/− Db−/− mice has been shown to foster homeostatic expansion of the CD8 T cell compartment (16), we did not observe an increase in numbers of CD8⁺ cells in the spleens of GK1.5 mAb-treated, MPyV-infected B6.Kb−/− Db−/− mice at 1 mo p.i. (A.R. Hofstetter, unpublished observations). As shown in Fig. 1A and 1B, CD4 insufficiency was associated with severely blunted expansion of Q9:VP2.139-specific T cells, as detected by both tetramer binding and VP2.139 peptide-stimulated intracellular IFN-γ production. Depletion of CD4 cells was associated with a modest loss in viral control that differed between organs examined. As shown in Fig. 1C, MPyV load in the salivary gland was ~6-fold higher in CD4−/− cell-depleted mice, although the MPyV genome copy numbers in kidneys and salivary glands were not significantly different between those in rat IgG-treated control mice. In this connection, it merits noting that MPyV infection elicits a T cell-independent, virus-neutralizing IgG response that controls viral
burden (21). Compared with rat IgG-treated infected mice, at 1 mo p.i., Q9:VP2.139-specific CD8 T cells in GK1.5-treated mice expressed higher cell surface levels of CD27, PD-1, and slightly higher levels of CD122, with lower levels of KLRG-1 (Fig. 1D). This phenotypic profile resembles the effector/effector memory phenotype seen during chronic infection by LCMV with CD4 depletion (22), suggesting that the unhelped Q9:VP2.139-specific cells may be similarly fated for functional exhaustion.

Based on evidence that increased CD27 signaling may suppress effector differentiation (23), we asked whether unhelped Q9:VP2.139-specific cells have less effector cytokine function than helped cells. However, the same frequency (19%) of Q9:VP2.139-specific CD8 T cells elaborated IFN-γ production after ex vivo VP2.139 peptide stimulation as those in rat IgG-treated infected mice (Fig. 1B). Together, these results indicate that CD4 T cell help is important for normal expansion of Q9:VP2.139-specific CD8 T cells during MPyV infection.

Expansion of Q9:VP2.139-specific cells is dependent on CD28 costimulation

CD4 T cell help is thought to act through CD40:CD40L-mediated activation of dendritic cells, leading to CD28:CD80/86 interactions between dendritic cells and CD8 T cells (24). Because we saw a detrimental effect on Q9:VP2.139-specific expansion because of CD4 depletion, we next asked whether expansion of Q9:VP2.139-specific CD8 T cells requires costimulation by CD28 and CD40L. We applied an established combined costimulation blockade regimen using anti-CD40L (clone MR1) and CTLA-4–Ig, which were administered together to MPyV-infected B6. K b−/− D b−/− mice for the first month p.i. As shown in Fig. 2A and 2B, CD28-CD40L costimulation blockade profoundly crippled the expansion of Q9:VP2.139-specific CD8 T cells. By 1 mo p.i., Q9:VP2.139 tetramer+ CD8 T cells had expanded to >9% of the CD8 population in only one third of treated mice. In contrast, Q9:VP2.139 tetramer+ CD8 T cells in all untreated mice ranged from 18–62% of total CD8 T cells. Q9:VP2.139-specific CD8 T cells in the costimulation blockade responders phenotypically resembled those in untreated mice, except for having fewer KLRG-1hi cells and more PD-1hi cells (A.R. Hofstetter, unpublished observations); interestingly, this shift in KLRG-1 and PD-1 expression is similar to that of Q9:VP2.139-specific CD8 T cells in CD4 depletion (24). Therefore, costimulation blockade responders phenotypically resembled those in untreated mice, except for having fewer KLRG-1hi cells and more PD-1hi cells (A.R. Hofstetter, unpublished observations); interestingly, this shift in KLRG-1 and PD-1 expression is similar to that of Q9:VP2.139-specific CD8 T cells in CD4 depletion (24).

Expansion of Q9:VP2.139-specific cells depends on CD28 costimulation

Numbers of MPyV genome copies were determined by qPCR in indicated organs at day 28 p.i. Each point corresponds to an individual mouse, and horizontal lines indicate geometric mean. Data are representative of six mice per group pooled from two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 1. Q9:VP2.139-specific cells depend on CD4 T cell help for expansion. GK1.5 mAb or rat IgG were administered to B6. K b−/− D b−/− mice coincident with MPyV inoculation and then once weekly for 1 mo. (A) Frequency (±SD) of Q9:VP2.139-specific CD8 T cells in blood over time, determined by cell surface Q9:VP2.139 tetramer staining. (B) Number (±SD) of Q9:VP2.139-specific CD8 T cells in spleen detected by cell surface Q9:VP2.139 tetramer binding and VP2.139 peptide-stimulated intracellular IFN-γ production at day 29 p.i. Each point corresponds to an individual mouse, and horizontal lines indicate geometric mean.

FIGURE 2. Expansion of Q9:VP2.139-specific CD8 T cells depends on CD28 and CD40L costimulation. A mixture of MR-1 (anti-CD40L) and CTLA-4–Ig was administered to B6. K b−/− D b−/− mice coincident with MPyV inoculation and then once weekly for 1 mo. Control mice received no Ab. (A) Frequency (±SD) of CD8+ Q9:VP2.139-specific cells in blood over time, determined by cell surface Q9:VP2.139 tetramer staining. (B) Number (±SD) of splenic Q9:VP2.139-specific CD8 T cells at day 28 p.i. was determined by tetramer staining and VP2.139 peptide-stimulated intracellular IFN-γ production. (C) Numbers of MPyV genome copies were determined by qPCR in indicated organs at day 28 p.i. Each point corresponds to an individual mouse, and horizontal lines indicate geometric mean. Data are representative of six mice per group pooled from two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
To determine whether both CD28- and CD40L-mediated co-stimulation were necessary for expansion of Q9:VP2.139-specific CD8 T cells, MPyV-infected B6.Kb−/− Db−/− mice were treated separately with anti-CD40L or CTLA-4-Ig. In the first 2 wk p.i., CTLA-4-Ig-treated mice showed only modest expansion of Q9:VP2.139-specific CD8 T cells; in contrast, Q9:VP2.139-specific CD8 T cell expansion progressed unimpaired in anti-CD40L–treated mice (Fig. 3A). However, after 2 wk, the Q9:VP2.139-specific CD8 T cell population in CTLA-4–Ig–treated mice became detectable and reached magnitudes that were not significantly different from in anti-CD40L–treated and untreated control mice (Fig. 3A, 3B), and virus loads were equivalent (Fig. 3C). Breakthrough T cell responses during costimulation blockade have been observed in mouse skin allograft transplantation, with anti-donor T cell responses overcoming blockade by 21 d after transplantation (21). To examine the Q9:VP2.139-specific CD8 T cell response before breakthrough occurred, we sacrificed mice at 2 wk p.i. At this time point, CD28 blockade was observed to clearly dampen the response (Fig. 3D) and was associated with significantly greater virus levels in the spleen and salivary glands (Fig. 3E). As shown in Fig. 3D, anti-CD40L–treated mice also had a diminished number and frequency (A.R. Hofstetter, unpublished observations) of Q9:VP2.139-specific CD8 T cells in the spleen, but there was no significant difference in viral titers compared with the untreated control group. The frequency of Q9:VP2.139-specific CD8 T cells in the blood of anti-CD40L–treated mice at day 15 was equivalent to that in untreated mice (A.R. Hofstetter, unpublished observations). Impaired cellular trafficking in the presence of CD40L blockade (26, 27) may explain the discord between the frequency of Q9:VP2.139-specific CD8 T cells in the blood and spleen. These results demonstrate that expansion of Q9:VP2.139-specific CD8 T cells is dependent on CD28 costimulation.

Q9:VP2.139-specific CD8 T cells require intrinsic CD28 signaling for expansion

To directly determine whether the dependence on CD28-mediated costimulation for expansion of Q9:VP2.139-specific CD8 T cells is autogenous, we used retrogenic TCR technology to create mice with monoclonal Q9:VP2.139-specific CD8 T cells (20). In brief, lethally irradiated B6 mice were engrafted using bone marrow infected by a recombinant, replication-deficient retrovirus bicistronic for genes for GFP and a TCR from a cloned line of Q9:VP2.139-specific CD8 T cells. To demonstrate Ag specificity, we FACs sorted GFP+ CD8+ cells from spleens of these TCR retrogenic mice and transferred them to naive B6 recipients, which were then infected with either wild-type MPyV or a mutant MPyV in which the codon for the dominant histidine anchor residue in VP2.139 epitope was replaced with one for alanine (A2.H145A). As shown in Fig. 4A, GFP+ (i.e., donor TCR retrogenic) CD8 T cells were recruited in mice infected by wild-type MPyV, but not A2.H145A. It is important to note that expansion of the immunodominant class Ia-restricted CD8 T cell response to MPyV was not affected in recipients of the retrogenic T cells (A.R. Hofstetter, unpublished observations). To investigate whether CD28 expression by Ag-specific CD8 T cells per se was essential for their expansion in MPyV-infected mice, we generated Q9:VP2.139 TCR retrogenic mice using bone marrow from Thy1.1+ wild-type B6 mice (B6.PL) and Thy1.2+ CD28−/− B6 mice. Equal numbers (1 × 10⁵) of FACs-sorted GFP+ CD8+ cells from B6.PL and CD28−/− bone marrow TCR retrogenic mice were cotransferred into naive Thy1.2+ B6 recipients (Fig. 4C), then infected with parental MPyV. As shown in Fig. 4D, CD28-sufficient GFP+ retrogenic cells efficiently expanded in mice infected by wild-type MPyV. In contrast, Thy1.1+ GFP+ (i.e., CD28−/−) cells expanded in only one of seven infected recipients, did so with delayed kinetics, and reached substantially lower frequencies than those of CD28-sufficient donor T cells (Fig. 4E). Importantly, wild-type Q9:VP2.139 TCR retrogenic cells transferred into CD40L−/− mice expanded equivalently to that of wild-type retrogenic cells transferred into wild-type mice p.i. by MPyV (A.R. Hofstetter, unpublished observations). These data demonstrate that CD28 signaling is intrinsically required by Q9:VP2.139-specific CD8 T cells for expansion in MPyV-infected mice.

**FIGURE 3.** Expansion of Q9:VP2.139-specific CD8 T cells depends on CD28 costimulation. MR-1 (anti-CD40L) or CTLA-4–Ig was administered to B6.Kb−/− Db−/− mice before and weekly during MPyV infection. Control mice received no Ab. (A) Frequency (±SD) of CD8+ Q9:VP2.139-specific cells in the blood over time. Significance indicated is the result of comparing CTLA-4–Ig–treated mice with controls. There was no significant difference between anti-CD40L–treated mice and controls. (B) Frequency (±SD) of Q9:VP2.139-specific cells in the spleen was substituted for values below the limit of detection. *Number (±SD) of MPyV genome copies was determined by qPCR in indicated organs at day 29 p.i. (D) Number (±SD) of Q9:VP2.139-specific cells in spleen were determined by tetramer staining and intracellular anti–IFN-γ staining at day 15 p.i. Significance indicated is the result of comparing tetramer+ or IFN-γ+ cell numbers of experimental with control mice. (E) Number of MPyV genome copies was determined by qPCR in indicated organs at day 15 p.i. (C, E). Each point corresponds to an individual mouse, and horizontal lines indicate geometric mean. Data are representative of six mice per group pooled from two independent experiments. Sample is below the limit of detection; to calculate mean for this group, 500 copies/mg organ was substituted for values below the limit of detection. *p < 0.05, **p < 0.01, ***p < 0.001.
Maintenance of Q9:VP2.139-specific cells does not require CD28 and CD40L costimulation

We previously demonstrated that CD28 and CD40L costimulation is dispensable for maintenance of class Ia-restricted antiviral CD8 T cells during persistent MPyV infection (15). Based on this and our prior data showing that maintenance of Q9:VP2.139-specific CD8 T cells in persistently infected mice is Ag independent (12), we hypothesized that CD28 and CD40L costimulation blockade would not impact long-term maintenance of Q9:VP2.139-specific CD8 T cells. To test this possibility, we administered CD28 and CD40L costimulation blockade to B6.Kb2−/− bone marrow donor mice and transferred i.v. into B6 mice, which were inoculated with WT MPyV. (D) Frequency of Q9:VP2.139-specific CD8 T cells in blood over time. Dots represent individual mice. (E) Frequency of Q9:VP2.139-specific CD8 T cells from B6.PL and CD28−/− bone marrow donors in blood over time. Dots represent individual mice. n = 7 mice pooled from two independent experiments. (F) Frequency of Q9:VP2.139-specific cells from B6.PL and CD28−/− bone marrow donors in the splenocytes in recipient mice at day 40 p.i., gated on CD8 T cells. To test this possibility, we administered CD28 and CD40L costimulation blockade to B6.Kb2−/− bone marrow donors in blood over time. Dots represent individual mice. n = 7 mice pooled from two independent experiments.

Maintenance of Q9:VP2.139-specific cells is dependent on CD4 T cell help

Because the Q9:VP2.139-specific CD8 T cell population was independent of Ag and CD28/CD40L costimulation for its maintenance, we hypothesized that CD4 T cell help during this phase would similarly be dispensable. To test this possibility, we administered GK1.5 mAb-mediated CD4+ cell depletion to B6.Kb2−/− mice 3 mo p.i. Unexpectedly, CD4 depletion resulted in a steady attrition of circulating Q9:VP2.139-specific CD8 T cells, whereas rat IgG-treated mice maintained their Q9:VP2.139-specific population (Fig. 6A). The rapid attrition of the Q9:VP2.139 population during CD4+ cell deficiency may be explained by the loss of IL-2 secretion by CD4+ cells. However, administering recombinant human IL-2 to B6.Kb2−/− mice depleted of CD4+ cells at 3 mo p.i. failed to prevent loss of the Q9:VP2.139-specific CD8 T cell population (Supplemental Fig. 1). By 1 mo p.i., GK1.5 mAb-treated mice had 5-fold fewer Q9:VP2.139-specific CD8 T cells in the spleen than rat IgG-treated controls (Fig. 6B). Q9:VP2.139 tetramer+ CD8 T cells from GK1.5 mAb-treated mice, although still CD62Llo−, displayed higher expression of CD127 and CD27 (Fig. 6C), which may indicate that those CD8 T cells with more central memory-like characteristics (28) are less dependent on CD4 help for survival. CD4 cell depletion-mediated attrition of Q9:VP2.139-specific cells did not lead to a change in viral loads after 1 mo (Fig. 6D). We demonstrated earlier that CD4 depletion during expansion had only a modest effect on viral loads, suggesting 10% of the CD8 T cell compartment, composed of Q9:VP2.139-specific cells, can control viral loads. Control would be enhanced during the maintenance phase, because virus levels are lower at this time. Taken together, these data show that the Q9:VP2.139-specific CD8 T cell response depends on CD4 T cell help for both expansion and maintenance, whereas CD28 costimulation is only required for expansion of this MHC class Ia-restricted T cell response.

Discussion

In this study, we provide evidence that the inflationary kinetic profile for an antiviral MHC class Ia-restricted CD8 T cell response varies over time in its dependence on CD28 costimulation but requires continuous CD4 T cell help. After a prolonged period of gradual expansion that requires CD28 costimulation and CD4 T cell help, the Q9:VP2.139-specific CD8 T cell response to MPyV infection plateaus to a CD4 T cell-dependent maintenance phase that is independent of CD28 or CD40L costimulation. The dependence on CD28 signaling is an intrinsic property of the Q9:VP2.139-specific CD8 T cells. Although this expansion-maintenance phase difference in CD28 costimulation for Q9:VP2.139-specific CD8 T cells parallels the dependence of this response on Ag (12), the...
dispensability for viral Ag during maintenance is at odds with the ongoing dependence on CD4 T cell help.

The role of CD4 T cell help for the primary expansion of CD8 T cells has been investigated in numerous models with varied results. Early experiments with noninflammatory cell-based immunization (29) or HSV infection (30) indicated a clear role for CD4 T cell help in generating CD8 T cell responses. In contrast, CD4 T cell-independent primary expansion of pathogen-specific class Ia-restricted CD8 T cells has been documented in several experimental infection systems, including MPyV (14, 31–36). Unlike for these virus-specific, class Ia-restricted CD8 T cell responses, the primary expansion of Q9:VP2.139-specific CD8 T cells is CD4 T cell dependent, as shown by its apparently abbreviated peak magnitude response. However, a similar pattern has been seen for unhelped primary CD8 T cell responses to L. monocytogenes, vaccinia virus (36).

This apparent CD4 T cell independence for naive CD8 T cell responses to MPyV infection, which display dependence on both CD28 and CD40L signals for expansion (15). Thus, CD25 expression on CD8 T cells may similarly be involved in expansion of activated naive Q9:VP2.139-specific CD8 T cells, possibly resulting from CD4 T cell help promoting upregulation of high-affinity IL-2Rs to enable their efficacy and enable them to exert effective control of MPyV infection.

Secretion of IL-2 by CD4 T cells constitutes a well-recognized mechanism of T cell help (40). D’Souza and Lefrancois (41) reported that IL-2R signaling is unnecessary for the initiation of CD8 T cell cycling, but required for sustained expansion. CD4 T cells may similarly be involved in expansion of activated naive Q9:VP2.139-specific CD8 T cells, possibly resulting from upregulation of high-affinity IL-2Rs through CD28/CD80/86 signaling (36). Furthermore, CD25 expression on CD8 T cells has been shown to be independent of CD40 signaling (36), which also holds for expansion of Q9:VP2.139-specific CD8 T cells. This is in contrast with the class Ia-restricted CD8 T cell responses to MPyV infection, which display dependence on both CD28 and CD40L signals for expansion (15). Thus, CD28 signaling may be primarily involved in recruitment of naive Q9:VP2.139-specific CD8 T cells, with CD4 T cell help promoting upregulation of high-affinity IL-2Rs to enable expansion by IL-2. However, we did not observe decreased CD25 expression on Q9:VP2.139-specific cells of CD4-depleted B6.Kb−/−Db−/− mice on day 10 p.i. (A.R. Hofstetter, unpublished observations).

CD28 signaling by Q9-restricted CD8 T cells may be essential for amplifying weak TCR signaling. Data from our laboratory and others support the concept that Q9:VP2.139-specific cells bind weakly with their pMHC ligands. Preliminary surface plasmon resonance data indicate that the Q9:VP2.139 TCR binds to its cognate ligand with lower affinity than that typical for class Ia-restricted TCR (A. Brooks and L. Sullivan, unpublished observations). Furthermore, the orientation of the CD8-binding loop in

**FIGURE 5.** Q9:VP2.139-specific cells do not depend on costimulation for maintenance. B6.Kb−/−Db−/− mice were infected with MPyV and 3 mo p.i. given a mixture of MR-1 (anti-CD40L) and CTLA-4-Ig weekly for 1 mo. Control-infected mice received no Ab. (A) Frequency (±SD) of Q9:VP2.139-specific CD8 T cells in blood over time. Baseline indicates frequency before start of treatment. (B) Number (±SD) of splenic Q9:VP2.139-specific CD8 T cells was determined by tetramer staining and VP2.139 peptide-stimulated intracellular anti–IFN-γ-specific CD8 T cells in blood over time. Baseline indicates frequency before start of treatment. (C) Number of MPyV genome copies was determined by qPCR in indicated organs after 1 mo of treatment. Each point corresponds to an individual mouse, and horizontal lines indicate geometric mean. (D) Expression of indicated surface molecule by CD8+ Q9:VP2.139-specific splenocytes after 1 mo of treatment. (E) CD28 expression on Q9:VP2.139-specific CD8 T cells in MPyV-infected B6.Kb−/−Db−/− mice at the indicated time p.i. Data are representative of six mice per group pooled from two independent experiments. The limit of detection of this assay is 2000 copies of genomic viral DNA. One thousand copies per milligram organ was substituted for values below the limit of detection.
the α3 domain of Q9 suggests that it weakly engages CD8 coreceptors (7). This possibility is supported by our data showing that Q9:VP2.139 tetramers constructed using a chimeric Q9 molecule with a K\(^{b}\) α3 domain, which strongly binds CD8α (42), stains Ag-specific CD8 T cells with higher mean fluorescence intensity (MFI) than Q9 tetramers with native α3 domains (A.R. Hofstetter, unpublished observations). The tissue distribution and expression levels of Q9 are similar to that of MHC class Ia molecules (9); however, Q9 is upregulated by IFN-γ (43), and the defect in IFN-γ effector function by Q9:VP2.139-specific CD8 T cells may impact Q9 expression levels. Suboptimal TCR signaling may also explain the slow inflationary expansion of the Q9:VP2.139-specific cells and their effector function defect. By corollary, high epitope density and/or strong CD28 signaling may compensate for inefficient CD8 coreceptor binding and enable full activation of Q9-restricted CD8 T cells. Because the inflammatory kinetics of the Q9:VP2.139-specific response depend on persistent infection (12), it is possible that continuous TCR stimulation together with CD28 costimulation is needed for Q9:VP2.139-specific CD8 T cells to maximally expand.

As seen in this study for memory MHC class Ia-restricted CD8 T cells to MPyV infection, CD4 T cell help has been shown to maintain memory CD8 T cells in the setting of other persistent infections (14, 31, 32, 44). The nature of the CD4 T cell help for maintaining the MHC class Ia-restricted CD8 T cell population is unknown. CD4 T cells likely provide other forms of help for maintaining the MHC class Ib-restricted CD8 T cell population (12), as lack of TCR signaling precludes a requirement for costimulation (49).

Costimulatory molecules other than CD28 and CD40L may contribute to the expansion and maintenance of Q9:VP2.139-specific CD8 T cells. The kinetics of CD27 expression by Q9:VP2.139-specific CD8 T cells parallel those of CD28 expression (Fig. 5E, Supplemental Fig. 2). The decreased expression of CD27 over the course of MPyV infection would imply declining dependence on CD27-mediated signals. 2B4 is expressed at a steady but low level throughout the response (Supplemental Fig. 2). This receptor can be activating or inhibitory, depending on the level of 2B4 expression, the extent of 2B4 cross-linking, and the relative abundance of signaling lymphocyte activation molecule-associated protein (50). OX-40, 4-1BB, and ICOS are expressed at only low levels by Q9:VP2.139-specific CD8 T cells (Supplemental Fig. 2). In contrast, CD94/NKG2A expression increases after day 12 p.i. and is maintained at high expression levels (Fig. 6D, Supplemental Fig. 2). We previously showed that CD94/NKG2A expression is increased by MHC class Ia-restricted, MPyV-specific CD8 T cells, where it inhibits cytotoxicity whereas increasing IL-2 production and proliferative potential (51, 52). However, the impact of CD94/NKG2A expression varies depending on infection model (53–55). Whether CD27, 2B4, or CD94/NKG2A expression on Q9:VP2.139-specific cells regulate IFN-γ functionality of these cells remains to be determined.
In this study, we explored CD4 T cell and CD28/CD40L costimulation as determinants for the inflammatory kinetic profile of an MHC class Ib-restricted antiviral CD8 T cell population. We demonstrated that CD4 T cell help and intrinsic CD28 costimulation are necessary for expansion of Q9-restricted, MPyV-specific CD8 T cells, and that CD4 T cell help is important for survival of these cells during their Ag-independent maintenance phase. Together with our previous study on the role of Ag in expansion and maintenance of Q9:VP2.139-specific CD8 T cells (12), we propose the following model: Q9:VP2.139-specific CD8 T cells are primed in a CD4 T cell-independent fashion, expand in response to combined signals provided by cognate Ag, CD4 T cell help, and intrinsic CD28 signaling, and are then maintained independent of Ag and CD28/CD40L but dependent on CD4 T cell help. This sustained dependence on CD4 T cell help, which does not involve a CD40L-dependent mechanism or IL-2, suggests a novel mechanism of help for maintaining an anti-MPyV MHC class Ib-restricted CD8 T cell response.

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Disclosures

The authors have no financial conflicts of interest.

References


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