Discovery and Characterization of a Novel Antiviral CD8 T cell Response

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B.S., University of Illinois, 2002

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An abstract of
A dissertation submitted to the Faculty of the Graduate School of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Science
Immunology and Molecular Pathogenesis
2009

Abstract

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Immunity to viral infection is primarily provided by CD8 T cells that recognize pathogen-derived peptides presented by MHC class Ia molecules, however little attention has been devoted to whether MHC class Ib molecules also have a role in the antiviral CD8 T cell response. Using the persistent mouse pathogen polyoma virus (PyV) model, we identified an MHC class Ib-restricted antiviral CD8 T cell response in infected MHC class Ia-deficient ($K^{b-/-}D^{b-/-}$) mice. PyV-specific CD8 T cells in infected $K^{b-/-}D^{b-/-}$ mice recognize a viral peptide derived from the VP2 capsid protein (VP2.139) presented by the class Ib molecule Q9. The Q9/VP2.139-specific CD8 T cells are both protective and long-lived in PyV-infected $K^{b-/-}D^{b-/-}$ mice. Importantly, these cells are also detected in both acute and persistent PyV-infected wild type mice. Although the Q9-restricted anti-PyV CD8 T cell response represents the first identification and characterization of an endogenous class Ib-restricted antiviral CD8 T cell response, the ubiquitous tissue expression and peptide-binding capabilities of several different class Ib molecules indicate that they could be involved in a diverse array of antiviral responses. From a therapeutic standpoint, CD8 T cell vaccines targeting class Ia-restricted viral epitopes only provide partial protection across a population due to the high polymorphism associated with these molecules. Vaccines using nonpolymorphic class Ib-restricted CD8 T cell epitopes could provide broad protection across haplotype barriers.

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Chapter 1: Introduction

CD8 T cell antiviral immunity

For millions of years viruses and other pathogens have played a cat and mouse game with their hosts, which has driven the evolution of increasingly complex and sophisticated defense systems within targeted species. Humans, along with other jawed vertebrates, use the innate immune system of their early ancestors, characterized by general pattern recognition, in combination with a highly evolved adaptive immune response. The two central features of adaptive immunity are 1) high specificity to a wide array of pathogens and 2) the ability to remember and rapidly respond to reencounter with pathogens. Together, the innate and adaptive immune systems function in harmony to stave off infection and prevent disease.

CD8 T cells are critical to the adaptive immune response to viruses. Healthy individuals are equipped with thousands of CD8 T cells, each of which is specialized to recognize a different viral-derived antigen (Ag). Upon recognition of Ag, CD8 T cells clonally expand, disseminate throughout the body, and kill virus-infected targets. After the resolution of infection, a population of the virus-specific CD8 T cells, termed memory cells, remain and protect the host against reinfection. However, CD8 T cell function can be altered in situations where viral infection is not resolved. Persistent viral infections, which last for an extended period of time, are usually associated with immune dysfunction. In circumstances where viruses persist at high levels, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV)-infections, virus-specific CD8 T cells are associated with a lack of antiviral effector functions; a state termed exhaustion (1). Additionally, low-level persistent viral

infections have been shown to detrimentally affect antiviral CD8 T cell responses (2). Only by fully understanding the processes that regulate CD8 T cell function during both acutely resolved and persistent viral infections, can we hope to harness the therapeutic potential of these cells in an effort to prevent virus-induced disease.

CD8 T cell responses to many different acute viral infections have been well characterized. Expression of lymphoid homing receptors CCR7 and CD62L allows circulating naïve CD8 T cells to enter lymph nodes where they can engage and scan Ag-bearing DCs. If they do not recognize any Ag, they quickly exit through the efferent ducts and reenter the circulation. However, during viral infection, virus-specific CD8 T cells that encounter cognate Ag are brought to a halt and begin to proliferate and differentiate into effector cells. One study using adoptive transfer of TCR transgenic CD8 T cells demonstrated that Ag recognition and proliferation can happen as early as 30 hours post infection (p.i.) (3). Additional experiments have shown that only a brief encounter with Ag is needed to drive CD8 T cell expansion (4-7). This expansion continued even after separation from Ag, which has led to the idea that the CD8 T cell response is programmed after stimulation with Ag. CD8 T cells continue to divide every 6 hours after encountering Ag, accumulating up to a 100,000 fold in number at the peak of the response (8). CD8 T cell expansion is accompanied by a differentiation pattern whereby cells acquire effector molecules important for sustaining clonal expansion (IL-2) (9) and eliminating virus infected cells (perforin, granzyme, IFN-γ, and TNF-α) (10-12). These cells then disseminate throughout the body in order to fight infection (13, 14).

Following the peak of CD8 T cell expansion, between 90 and 95% of antiviral CD8 T cells are eliminated, leaving behind a small stable population of memory CD8 T

cells. Although the level of CD8 T cell expansion is dependent upon the initial amount of Ag, Badovinac et al. demonstrated that CD8 T cell contraction following viral infection is not (15). In fact, these experiments showed that Ag-specific CD8 T cell contraction still occurs even when viral antigen persists. The remaining memory CD8 T cells are long-lived and have the capacity to rapidly respond to reinfection (16, 17). Memory CD8 T cells are maintained by a process of slow turnover called homeostatic proliferation, which is dependent upon IL-7 and IL-15 cytokines (18-21).

Exactly how memory cells are established is not well understood. Mouse studies have generally supported the idea that memory CD8 T cells arise from the pool of effector CD8 T cells (11, 22, 23), In fact, effector CD8 T cells expressing an IL-7Rα^+KLRG1$^-$ phenotype, have been shown to preferentially survive and become long-lived memory cells (24, 25). However, how or when the switch from effector to memory CD8 T cell occurs has not been determined. Experiments support several different models for this transition ranging from a linear differentiation pattern dependent on the amount of antigen stimulus received by effector CD8 T cells (24, 26) to a model where asymmetric division of effector CD8 T cells could lead to distinct states of differentiation (27). Additionally, cell extrinsic factors such as IFN-γ and IL-12 as well as CD4-dependent IL-2 production have also been shown to regulate memory cell transition (25, 28-31).

During chronic viral infection, CD8 T cell responses deviate from those in acutely cleared viral infections. Chronic viral infections with high viremia such as HIV, HCV, and HBV are associated with dysfunctional CD8 T cell responses (32-35). In mice, LCMV clone 13 is the best studied model for high level chronic viral infection. Unlike

the acutely cleared Armstrong strain of LCMV, clone 13 can be detected in the serum of mice for up to 2 months p.i. (36). Clone 13 infection is associated with a gradual loss of CD8 T cell effector functions including proliferation, cytokine production, and cytotoxicity (36-39). In addition, the two dominant CD8 T cell responses during LCMV Armstrong infection (D^b/NP396 and K^b/GP34) are selectively deleted during clone13 infection, shifting the immunodominance to two normally minor epitopes (36). The loss of the two immunodominant CD8 T cell responses correlates with increasing levels of Ag presentation. Thus, it has been hypothesized that the loss of the immunodominant cells represents the most severe form of exhaustion. Changes in CD8 T cell immunodominance have also been associated with increasing levels of viremia during HIV infection (40, 41).

In addition to loss of effector functions, LCMV-speicific CD8 T cells in clone 13-infected mice do not express receptors for the homeostatic cytokines IL-7 and IL-15 (42-44). Instead, Ag seems to play a dual role in the regulation of memory CD8 T cells during clone 13 infection. While early overexposure to Ag is detrimental for the CD8 T cell response, a recent study has shown that LCMV-specific CD8 T cells become dependent upon Ag for their survival in clone 13-infected mice (44). This is in contrast to memory CD8 T cells in acutely cleared viral infections, which do not require Ag for their long-term survival (45).

Several extrinisic factors have been shown to regulate CD8 T cell function during high level chronic viral infection. CD4 T cell help is essential for eventually clearing clone 13 infection. CD4-depleted, clone 13-infected mice become viremic for life with high virus levels in all peripheral tissues (37, 46). Additionally, CD8 T cell exhaustion is

much more severe in CD4-depleted, clone 13-infected mice (39). However, considering that CD4 depletion eliminates the antibody response which in turn leads to increased viremia, it is unclear whether CD4 T cells directly or indirectly affect CD8 T cell exhaustion. Systemic increases of the T cell inhibiting cytokine IL-10 have been detected during HCV, HBV, and HIV infections (47). Moreover, IL-10 has also been shown to regulate LCMV-specific CD8 T cells during chronic infection, and *in vivo* blockade of IL-10 receptors leads to rapid clearance of clone 13 infection (48, 49).

Several inhibitory receptors have been shown to negatively regulate CD8 T cell function. CTLA-4, a member of the CD28 superfamily, competes with the costimulatory molecule CD28 for binding to CD80 and CD86 molecules on APCs. CTLA-4 expression is induced by TCR signaling (50), but is quickly degraded in activated T cells (51). The importance of CTLA-4-mediated inhibition of T cells was demonstrated by the fact that CTLA-4$^{-/-}$ mice die within 3-4 weeks of birth from a spontaneous autoimmune lymphoproliferative disorder (52, 53). During chronic LCMV infection, CTLA-4 genes are upregulated in exhausted CD8 T cells (54), however antibody blockade of CLTA-4 did not lead to a recovery of function (39). On the other hand, CTLA-4 blockade in SIV-infected mice enhanced CD8 T cell responses (55), suggesting variability in the impact of CTLA-4 signaling depending on the infectious agent.

PD-1 is another inhibitory molecule whose expression is correlated with CD8 T cell dysfunction during high level chronic viral infections. Like CTLA-4, PD-1 expression is upregulated on activated CD8 T cells, but quickly downregulated after clearance of infection (39). However during high level chronic viral infections such as HIV, HCV, HBV, and LCMV clone 13, PD-1 expression is maintained, especially on

exhausted CD8 T cells (39, 56-58). The ligands for PD-1 are PD-L1, which is expressed

ubiquitously, and PD-L2, which is more limited in its tissue distribution (59). Blockade

of PD-1 signaling by anti-PD-L1 antibodies during chronic LCMV infection has been

shown to dramatically increase exhausted CD8 T cell effector functions including

proliferation, cytokine production, and cytotoxic capabilities leading to a decrease in viral

load (39). Additionally, *in vitro* blockade of PD-1 on exhausted CD8 T cells from HIV,

HCV, or HBV-infected individuals leads to a recovery of effector functions (56-58, 60-

62). Recent data has demonstrated that *in vivo* blockade of PD-1 during SIV infection

also leads to increased CD8 T cell function and reduction of viral loads (63). Although

blockade of PD-1 signaling is highly effective at restoring CD8 T cell function during

chronic viral infection, combination treatments targeting multiple inhibitory pathways

have been shown to be more effective at reducing viral load, implicating non-redundant

pathways of CD8 T cell inhibition (46, 64).

In contrast to high level persistent viral infections, low level persistent viral

infections such as CMV and EBV establish silent infections in immune-competent hosts.

In fact, nearly 70% of adults in the western world are infected by CMV (65) and over

90% are infected by EBV (66). The importance of the immune system in controlling

these infections is revealed by the CMV and EBV-associated pathologies in those

infected individuals who become immune-suppressed, whether through treatment after

transplantation or cancer, or as a result of other infections such as HIV (67, 68). The

virus-specific CD8 T cells within individuals infected by EBV and/or CMV constitute a

substantial fraction of the overall CD8 T cell repertoire and are fully functional, unlike

antiviral CD8 T cells in high-level chronic infections (2, 66, 69). Studies with mouse

CMV (MCMV) have shown that virus-specific CD8 T cells progressively expand during persistent infection, a process termed "memory inflation" (70, 71). This process could account for the substantial population of CMV-specific CD8 T cells in seropositive adults. However, whether the steady accumulation of antiviral CD8 T cells during low-level persistent viral infection is an example of immune dysregulation or a response to pockets of active viral replication remains to be determined.

The deleterious effects of high-level chronic viral infections (e.g., HIV, HCV in humans; LCMV clone 13 in mice) on the host's antiviral immunity are well documented, however we know surprisingly little how healthy individuals maintain long-term control of viruses that establish low-level persistent infection. Additional model systems are needed to understand the role of CD8 T cells in controlling the diverse array of low-level persistent viral infections. Moreover, because low-level persistent viral infections have evolved with the human immune system for over millions of years, understanding the immune response to these persistent pathogens not only sheds light on the pathogenesis of these viruses, but also provides insight into how the normal immune system functions.

PyV as a model system for studying persistent viral infection

Over 90% of the human population worldwide is infected lifelong with both the BK virus (BKV) and JC virus (JCV) polyomaviruses. Like CMV and EBV, these viruses establish silent low-level persistent infections that may become viremic in immunosuppressed individuals. BKV-associated nephropathy is the leading cause of graft loss in renal transplant patients (72), and JCV is the etiologic angent for Progressive Multifocal Leukoencephalopathy (PML), a frequent and often fatal demyelinating disease

in the AIDS population (73-75). CD8 T cells have been implicated in early viral control for both BKV and JC infections (76, 77). Yet, the mechanisms used by CD8 T cells to maintain virus control during persistent infection remain unclear.

Like human polyoma viruses, mouse polyomavirus (PyV), establishes a ubiquitous silent persistent infection in its natural host. PyV infection induces a robust CD8 T cell response that can be tracked well into persistent infection. Additionally, the small size of the PyV genome readily lends itself to mutagenesis, which has permitted fine dissection of the viral determinants for PyV pathogenesis both at the level of interaction with host cells as well as systemically at the level of the host's antiviral immune response. These features, along with the fact that mice are one of the most genetically tractable animal models, make mouse polyomavirus (PyV) an excellent experimental tool for studying CD8 T cell responses to persistent viral infection.

The PyV 5kb double-stranded covalently closed DNA genome is divided into early and late regions based on temporal expression of early and late region transcripts relative to the time of DNA replication. Both early and late transcripts extend in opposite directions around the circular genome from a start site within a single origin of DNA replication. Early region primary RNA transcripts are alternatively spliced to generate RNAs for the three nonstructural proteins: small T (ST), middle T (MT), and large T (LT), which are essential for subverting host signaling molecules and driving host cells into cell cycle (78, 79). Late region transcripts, which accumulate after the onset of DNA replication, are alternatively spliced to generate RNAs for the 3 structural proteins VP1, VP2, and VP3, which assemble to form an icosahedral capsid shell around the PyV genome. Because the cellular receptors for VP1, the major viral capsid protein, are

sialylated glycoproteins and glycolipids (80, 81), PyV infects a wide array of cell types (82).

One of the salient features of PyV is its ability to induce tumors when inoculated into immunocompromised mice or newborn mice of particular strains. The importance of immune surveillance for PyV-induced tumors has long been appreciated. Early studies using neonatally thymectomized mice, congenically athymic mice, and adoptive transfer of splenocytes from PyV-infected mice demonstrated that T cells prevent tumors induced by PyV infection (83-85). Later studies showed that CD8 T cells are the primary immunocyte population required for protection against PyV-induced tumors, as evidenced by the inability of mice subjected to antibody-mediated CD8 T cell depletion to reject a syngeneic tumor cell challenge, the ability of a synthetic class I MHC-binding viral peptide to confer protection against a polyoma tumor cell challenge, and the increased tumor susceptibility of mice incapable of MHC class I-restricted Ag presentation (i.e., $\beta_2 m^{-/-}$ mice) (86-88). In this connection, it is interesting to note that $CD8^{-/-}$ mice are PyV tumor-resistant (88, 89), a surprising phenotype that may be explained by the capacity of these mice to mount an MHC class I-restricted $CD4^{-/-}CD8^{-/-}$ anti-PyV T cell response (90). Additionally, MHC class Ia-deficient $K^{b-/-}D^{b-/-}$ mice are also highly resistant to PyV-induced tumors, implicating a role for a novel subset of CD8 T cells in anti-PyV immunity (Chapter 2), the subject of this dissertation.

Anti-PyV CD8 T cell epitopes have been defined for inbred mouse strains of the $H-2^k$ and $H-2^b$ haplotypes, and their fate and function of epitope-specific CD8 T cells monitored during acute and persistent phases of infection (91, 92). Each of these MHC class Ia (i.e., H-2D and H-2K)-restricted CD8 T cells are directed to peptides derived

from a nonstructural T Ag. In general, the anti-PyV CD8 T cell response peaks around one week after virus inoculation, then undergoes a 6-fold contraction to a small population that is stably maintained throughout persistent infection (92, 93). In H-2^b mice, epitope-specific CD8 T cell responses exhibit noncoordinate regulation in their expansion and contraction during acute infection, expression of activation and differentiation cell surface markers, and cytokine effector profiles (92). Many factors may contribute to this variability in kinetics, magnitude, and function of T cell directed to different viral epitopes, including competition for infected cells, efficiency in epitope processing and presentation by MHC class I molecules, and the number of precursor naïve T cells of a given specificity in the host's T cell repertoire (94). While control of acute PyV infection parallels the expansion of the antiviral CD8 T cell response, it is important to point out that PyV DNA is detected long-term in the face of functionally competent antiviral CD8 T cells (92, 95).

As described previously, memory T cells generated in the context of persistent infection are qualitatively distinct from those generated in infections that are completely resolved. "Acute memory" T cells exhibit heterogeneity in the lymph node homing receptors CD62L-selectin and CCR7 (25). Differential expression in CD62L and CCR7 is commonly used to demarcate memory T cells into central-memory (CD62L^{high}CCR7^{high}) and effector-memory (CD62L^{low}CCR7^{low}) subsets; these memory subpopulations differ in anatomic location, kinetics of effector expression, and homeostatic proliferation (96). PyV-specific CD8 T cells isolated during the persistent phase of infection are primarily CD127^{int}CD62L^{lo}CD44^{hi}, consistent with an effector-memory phenotype (92). This effector-memory T cell bias, which deviates from that of

"acute memory", is also seen in low-level persistent virus infections in humans and other experimental infection models (65). Additionally, PyV-specific memory CD8 T cells, like those of most persistent low-level viral infections, generally retain most of their effector functions (70, 92, 97). Perforin mediated killing seems to be particularly important during persistent infection as perforin-deficient mice are associated with higher viral loads and have a reduced ability to kill viral Ag-loaded targets (98).

Although the memory CD8 T cell population is stable in PyV-infected mice, PyV-specific memory CD8 T cells adoptively transferred into infection-matched hosts do not proliferate and undergo rapid attrition (99). These perplexing data were resolved by experiments showing that naïve CD8 T cells are primed during persistent infection (92, 99). Thus, during persistent PyV infection, the memory CD8 T cell pool is constantly replenished by newly primed cells. Additionally, this process of *de novo* CD8 T cell priming during persistent PyV infection is dependent upon CD4 T cell help (100). Experiments using mixed bone marrow chimeras in persistent PyV-infected mice demonstrated that mice that received CD4-deficient bone marrow had significantly fewer newly primed CD8 T cells than those that received wild type bone marrow. Because *de novo* CD8 T cell priming during persistent PyV infection is responsible for maintaining the memory CD8 T cell pool, these data were consistent with experiments demonstrating that CD4-deficient mice have significantly fewer anti-PyV memory CD8 T cells than wild type mice (100).

In contrast to anti-PyV CD8 T cells primed during acute infection, those generated during the persistent phase of infection are largely $CD62L^{high}$ and $CD27^+$, indicating that these "late-primed" cells share signature central-memory T cell

phenotypic markers (101). Recent data from the Lukacher laboratory using adoptive

transfers of naïve PyV-specific TCR transgenic CD8 T cells into persistent PyV-infected

mice indicate that the central-memory phenotype becomes more pronounced when

priming occurs at later stages of persistent infection (C.D. Pack and A.E. Lukacher,

personal communication). Thus, priming history may affect the differentiation state of

antiviral CD8 T cells. In this manner, virus-associated bystander inflammation has been

shown to be detrimental to the quality of the PyV-specific CD8 T cell response (102).

Thus, one could imagine that reduced Ag load or virus-associated inflammation during

persistent infection would create an environment conducive for generating authentic

memory T cells which are capable of self-renewal.

Costimulation requirements for PyV-specific CD8 T cell expansion are also

affected by the priming environment. CD28 and CD40L costimulation are particularly

important for PyV-specific CD8 T cell expansion during the acute phase of infection, as

blockade of these pathways acts additively to profoundly inhibit the magnitude of these

cells. However, costimulation requirements shift during the persistent phase of infection

as indicated by data showing that combined CD28/CD40L blockade has no effect on

memory or de novo primed PyV-specific CD8 T cells (101). Phenotypic analysis of

PyV-specific CD8 T cells in persistently infected mice shows that most of these cells

express the costimulatory molecule CD27. In fact, PyV-specific CD8 T cells primed

during persistent infection exhibit a markedly higher CD27 expression level than pre-

existing host anti-PyV CD8 T cells. Expression of this costimulatory molecule during the

persistent phase of infection is necessary, as blockade of CD27, in conjunction with

CD28 blockade, significantly reduces PyV-specific CD8 T cell numbers. Thus, antiviral

CD8 T cell costimulation requirements during PyV-infection swings from being CD28- and CD40L-dependent during the acute phase to CD27- and CD28-dependent in persistently infected mice.

These preliminary studies have provided much insight into the antiviral CD8 T cell response during PyV infection. However, questions remain. Because PyV-infected MHC class Ia-deficient mice are tumor resistant, is the class Ia-restricted CD8 T cell response dispensable for controlling PyV infection? If so, what compensates for it? Do MHC class Ib-restricted CD8 T cells contribute to anti-PyV immunity? These questions will be addressed in Chapters 2 and 3.

MHC class Ib molecules in immunity

CD8 T cell activation occurs through T cell receptor (TCR)-mediated recognition of cognate Ag presented by MHC class I molecules. In uninfected cells, host-derived cytosolic peptides are fed into the endoplasmic reticulum by the heterodimeric Transporter Associated with Ag-Processing (TAP) molecules, where they are loaded onto MHC class I molecules and directed to the cell surface. During viral infection, host cells alert CD8 T cells by displaying TAP-dependent, virus-derived peptides on MHC class I molecules. Activated CD8 T cells with TCRs specific for the viral peptide-MHC complex bind and induce apoptosis in infected cells in order to prevent infection of neighboring cells.

The MHC locus, on chromosome 6 in humans and chromosome 17 in mice, encodes 2 different sets of MHC class I molecules. Most CD8 T cells are specific for the highly polymorphic class Ia (classical) MHC molecules (103). The human class Ia

molecules HLA-A, B, and C, and the mouse class Ia molecules, H-2D, K, and L are heterotrimeric molecules that associate with the beta-2-microglobulin (β_2m) (104). A groove formed in-between the alpha helices of the $\alpha1$ and $\alpha2$ domains of class Ia molecules binds 8-10 amino acid (aa) long peptides, an essential process for folding and maintaining the stability of class Ia molecules. The primary function of class Ia molecules is to alert CD8 T cells to infection by binding and presenting virus- or intracellular bacteria-derived peptides.

The second set of MHC class I molecules, termed class Ib (nonclassical), are more numerous and more diverse in both structure and function than class Ia molecules. The human class Ib genes are interspersed among the class Ia genes within the MHC locus, while the 40-plus mouse class Ib genes are located in distinct regions labeled Q, T, and M, telomeric to the class Ia genes (105). Many of the class Ib molecules are evolutionarily older than the class Ia molecules and have limited or no roles in immunity. Human class Ib molecules MHC class I polypeptide-related sequence (MIC) A and MICB, which do not bind β_2m or peptide, signal cell stress by activating the NKG2D receptors on NK cells (106). Another human class Ib molecule, HFE (formerly HLA-H) binds to the transferrin receptor and is involved in iron transport by intestinal epithelial cells (107). Other class Ib molecules such as HLA-G in humans and Q10 in mice are structurally similar to class Ia molecules and bind peptides, but their restricted tissue distribution, placenta and liver, respectively, most likely precludes them from having a significant role in CD8 T cell antimicrobial immunity (108, 109).

On the other hand, some class Ib molecules do bind peptides and have widespread tissue distribution. One example is the Qa-2 family of class Ib molecules in mice. The

Qa-2 molecules, encoded by two nearly perfect duplicate gene pairs *Q6/Q8* and *Q7/Q9*

(110), are more similar at the protein sequence level to class Ia molecules than any other

class Ib molecule (111). Although Qa-2 molecules associate with β_2m and bind peptides,

they are structurally divergent from class Ia molecules in two distinct ways. First, some

Qa-2 molecules are attached to cell membranes through a glycosylphosphatidylinositol

(GPI)-linkage rather than the transmembrane domain possessed by class Ia molecules.

Second, in addition to membrane-bound forms, alternative spliced forms of Qa-2

transcripts encode secreted Qa-2 molecules (112).

Originally noted for their role in preimplantation embryo development (113), Qa-

2 molecules have also been implicated in regulation of anti-tumor immunity. Most

studies have focused on the Qa-2 family member Q9. Transfection of melanoma cell

lines with Q9 confers resistance to NK cell-mediated lysis *in vitro* (114), and injection of

Q9-transfected melanoma cells has been associated with reduced tumor incidence *in vivo*

compared to untransfected melanoma cells (115). *In vivo* rejection of Q9-expressing

melanoma cells was found to be dependent on both CD8 T cells and Q9 (116). These

Q9-restricted anti-tumor CTLs also formed memory cells that were capable of rejecting

less immunogenic tumor variants. Q9-restricted anti-tumor CTLs also recognize Q8-

bearing tumor cells even though Q9 and Q8 differ from each other at over 20 aa in the α1

and α2 domains (117).

Qa-2 molecules have yet to be implicated in antimicrobial immunity, however the

promiscuity of peptide binding to Q9 (the only preferences being 9 aa-long peptides with

a histadine at position 7 and a hydrophobic residue at position 9 (112)) makes Q9 well

suited for binding pathogen-derived peptides. In fact, a search of protein databases using

the Q9 binding motif revealed three rabies virus-derived peptides to be strong Q9 binders (112). The combination of class Ia structural homology, diverse peptide-binding capabilities, and widespread tissue distribution point to a role for Q9 in CD8 T cell responses to pathogens (Chapter 2). In support of this, one report demonstrated that Qa-2 transgenic mice are associated with resistance to the parasite *Taenia crassiceps* (118).

A few peptide-binding MHC molecules have already been identified for their active role in immune defense. HLA-E and its mouse homolog Qa-1 are important for alerting NK cells during viral infection. The peptide ligands for HLA-E and Qa-1 are generated from the signal peptides of class Ia molecules and are referred to as Qa-1 determinant modifier peptides (Qdm). HLA-E and Qa-1 bind Qdm peptides in a TAP-dependent manner (103) and are displayed on the cell surface where they serve as the ligand for the NK cell inhibitory receptor CD94-NKG2A (119). Virus-mediated immune evasion mechanisms such as MHC class Ia molecule down-regulation or disruption of TAP function leads to reduced levels of host cell surface expression of HLA-E or Qa-1 rendering cells susceptible to NK cell-mediated lysis.

The peptide binding specificities for HLA-E and Qa-1 molecules have been well defined (120, 121) and indicate potential for binding a diverse array of peptides, including those derived from pathogens. In support of this, HLA-E restricted CD8 T cells have been identified in both *Mycobactrium tuberculosis* and *Salmonella enterica*-infected patients (122, 123). Qa-1-restricted CD8 T cells specific for peptides from the highly conserved bacterial protein GroEL have been identified in *Salmonella typhimurium* and *Listeria monocytogenes*-infected mice (124-127). Because the Qa-1 binding GroEL peptide sequence is highly conserved among many different bacterial

species (125), Qa-1 restricted anti-bacterial CD8 T cell responses could be more prevalent than what has been reported to date.

CD1 is another class Ib molecule that presents pathogen-derived Ags to CD8 T cells. While humans encode five different CD1 isoforms, CD1a, CD1b, CD1c, CD1d, and CD1e, mice only have the CD1d isoform (128). Although CD1 molecules are structurally similar to class Ia molecules including association with β_2m, the CD1 genes are not linked to the MHC locus. In addition, the hydrophobic binding groove of CD1 molecules is uniquely suited for binding lipids rather than peptides. CD1a, b, and c molecules have been shown to bind *Mycobacterium tuberculosis* (Mtb) lipids and induce activation in a clonally diverse array of CD8 T cells (128). Like class Ia-restricted CD8 T cells, *in vitro*-expanded Mtb-specific CD1-restricted TCR$\alpha\beta^+$CD8$^+$ T cells have both IFN-γ and cytotoxic capabilities (129). CD1d molecules are recognized by natural killer T (NKT) cells. A subset of NKT cells with an invariant TCRα chain, called iNKT cells, were initially found to recognize a synthetic marine sponge-derived glycolipid, α-galactosylceramide (αGalCer) presented by CD1d. *Borrelia burgdorferi*, the etiological agent of lyme disease, as well as *Leishmania donovani* are the only pathogens known to stimulate CD1d-restricted NKT cells (130, 131).

Perhaps the best characterized class Ib molecule involved in antimicrobial immunity is H2-M3. Only found in mice, H2-M3 has a unique preference for binding *N*-formylated peptides. Because protein synthesis in bacteria is initiated with *N*-formylmethionine, H2-M3 molecules are specifically suited for participation in anti-bacterial responses (132, 133). H2-M3-restricted CD8 T cell epitopes have been identified for both *Listeria monocytogenes* (LM) and Mtb (134, 135). During LM

infection, the H2-M3-restricted CD8 T cell response peaks around 5 days after infection, while the dominant the class Ia-restricted response peaks around day 7 p.i. (136). Several theories have been proposed to account for the early expansion of H2-M3-restricted CD8 T cells. Due to the paucity of endogenous *N*-formylated epitopes, there should be relatively little competition with bacterial-derived peptides for H2-M3 binding during infection (137). This could lead to a rapid display of bacterial epitopes on H2-M3 in contrast to the class Ia-restricted Ags which would have to compete with all the endogenous peptides for MHC binding (136). Alternatively, H2-M3-restricted CD8 T cells could have been previously primed by commensal bacteria (138). Therefore, the rapid response of these cells during LM infection could be due to the fact that they are memory CD8 T cells (136). Harty and colleagues have proposed the concept that H2-M3-restricted CD8 T cells serve to bridge innate and adaptive immunity to LM infection.

Although a stable population of H2-M3-restricted memory CD8 T cells are maintained after LM infection, they fail to expand upon secondary infection. In an elegant series of experiments, Hamilton et al. demonstrated that during a secondary infection, H2-M3-restricted CD8 T cells are inhibited by the rapid expansion of class Ia-restricted memory CD8 T cells (139). However, despite poor secondary responses, primary H2-M3 CD8 T cell responses are protective against LM infection. MHC class Ia-deficient mice control LM infection as well as wild-type mice (126). Additionally, transfer of LM-immune splenocytes into MHC class Ia-deficient mice protects them from primary LM challenge in a CD8-dependent fashion (126). Finally, in contrast to wild type mice, H2-M3-deficient mice succumb to high titer LM infection (140). These data

demonstrate that H2-M3-restricted CD8 T cells are an integral component in the immune response to LM.

Little is known about the ability of class Ib molecules to participate in CD8 T cell-mediated antiviral immunity. One early study showed that *in vitro* cultured CD3$^+$CD8$^+$TCR$\alpha\beta^+$ splenocytes could recognize a synthetic, nonformylated influenza peptide in an H2-M3-restricted fashion (141, 142). These H2-M3-restricted CD8 T cells were able to specifically kill peptide-coated targets *in vitro*, but not virus-infected cells (141). Considering that H2-M3 preferentially binds *N*-formylated peptides, it was highly surprising that a virus-derived peptide could bind H2-M3. The fact that the influenza epitope did not fit the traditional H2-M3 binding motif most likely accounted for the high level of peptide needed to stimulate Ag-specific CD8 T cells. Additionally, the inability of the influenza peptide-specific H2-M3-restricted CD8 T cells to kill influenza infected targets, casts doubt as to whether these cells are generated *in vivo* during influenza infection.

Recently Braaten et al. described an "unconventional" MHC class Ia-independent CD8 T cell response to γHV68 (143). Control of γHV68 infection was demonstrated to be both MHC class I- and CD8 T cell-dependent. However, K$^{b-/-}$D$^{b-/-}$ mice were able to control infection as well as wild-type mice, implicating a role for class Ib-restricted CD8 T cell immunity. Further proof for this came from the fact that γHV68-infected K$^{b-/-}$D$^{b-/-}$ mice generated a massive expansion of activated CD8 T cells compared to naïve mice. Additionally, nearly all of the activated CD8 T cells in infected mice expressed a single TCR Vβ, unlike naïve mice which had a diverse CD8 TCR Vβ repertoire. However,

neither the restriction element, nor the viral epitope recognized by these "unconventional" CD8 T cells were identified.

HLA-E restricted CD8 T cells have been identified in some CMV seropositive individuals. The CMV protein gpUS6 inhibits TAP function, which would normally leave infected cells susceptible to NK cell mediated killing (144). However, because this virus is adept at evading host immunity, the leader sequence of the CMV protein UL40 serves as a TAP-independent surrogate Qdm peptide, ensuring that HLA-E is presented on the cell surface (145). Small numbers of oligoclonal $CD8^+TCR\alpha\beta^+$ T cells called NK-CTLs, have been identified among the peripheral blood leukocytes of some CMV seropostive donors (144). Once isolated and expanded in culture, NK-CTLs specifically lyse allogeneic cells in a HLA-E/UL40-restricted manner. Although the endogenous function of these cells remains undefined, HLA-E-restricted NK-CTLs could be another layer of defense against virus-infected cells that escape NK cell and class Ia-restricted CD8 T cell detection. Additionally, it's possible that HLA-E restricted CTL responses are generated to other viruses such as EBV, hepatitis C, and influenza, all of which have peptides that bind to HLA-E (103, 146). However, more studies are needed to resolve whether HLA-E restricted antiviral CD8 T cell responses actually occur *in vivo*.

In this dissertation, I describe my studies identifying a novel MHC class Ib-restricted CD8 T cell response, using the PyV infection mouse model. These antiviral CD8 T cells are restricted by Q9, which presents a peptide from the viral capsid VP2 protein. These studies provide the first evidence for a protective antiviral CD8 T cell response restricted by a class Ib molecule.

Chapter 2: An MHC class Ib-restricted CD8 T cell response confers antiviral immunity

ABSTRACT

Although immunity against intracellular pathogens is primarily provided by CD8 T lymphocytes that recognize pathogen-derived peptides presented by 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 murine 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_2m-associated Qa-2 family. Using Q9-VP2 tetramers, we monitored delayed but progressive expansion of these Ag-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 demonstrate 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, raises the possibility for developing peptide-based viral vaccines having broad coverage across MHC haplotypes.

INTRODUCTION

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 murine MHC and the non-MHC-linked *CD1* and *MR1* genes encode β_2 microglobulin (β_2m)-associated class I glycoproteins. Compared to 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 (103). Several class Ib molecules have been shown to mediate immunoregulatory as well and non-immunological functions (147-151).

CD8 T cells restricted by MHC class Ib molecules have also been implicated in microbial defense. In particular, the murine class Ib molecules H2-M3, CD1d (and human CD1), and Qa-1 (and its human ortholog 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 (125, 132, 152). H2-M3 preferentially presents N-formyl peptides and CD1 presents mycobacterial lipid Ags 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 non-bacterial pathogens (103).

Limited evidence suggests that class Ib-restricted CD8 T cells may also contribute to 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 cytotoxic T lymphocytes, but these CTLs were unable to kill influenza virus-infected cells (141, 142). In addition, HLA-E-restricted TCR$\alpha\beta$ CD8 CTL cloned lines recognizing a nonamer peptide derived from the UL40 protein of human cytomegalovirus have been described (153). Recently, Virgin and coworkers demonstrated that β_2m-dependent TCR$\alpha\beta$ 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 (143).

Polyomaviruses are highly prevalent vertebrate pathogens, which generally cause a self-limited acute infection followed by long-term asymptomatic persistent infection in their natural host reservoir (92). 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 (154, 155). Recently, two new human polyomaviruses have been identified in respiratory tract infections, and another has been associated with the cutaneous malignancy, Merkel cell carcinoma (156-158). Mouse polyomavirus (PyV) establishes low-level systemic persistent infection and causes tumors in immunocompromised mice and neonatally infected mice of certain inbred strains (159). The ~5-kb double-stranded PyV genome encodes only six proteins, three nonstructural proteins (small T, middle T and large T Ags) and three capsid proteins (VP1, VP2 and

VP3). Interestingly, only peptides derived from the T Ags have been defined as epitopes for MHC class Ia-restricted PyV-specific CD8 T cells (92, 160).

In this study, we investigated whether PyV infection elicits virus-specific CD8 T cells restricted by MHC class Ib molecules. We previously showed that $\beta_2 m^{-/-}$ mice inefficiently control PyV infection, as evidenced by the realization of this virus' oncogenic potential (88). 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 Ag-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 (88), 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 of 17 $K^{b-/-}D^{b-/-}$ mice developed tumors at 7 months post-infection (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. 1A). Compared to 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 days p.i., but then progressively increase well into the persistent phase of infection (Fig. 1B). $K^{b-/-}D^{b-/-}$ mice depleted of CD8 T cells manifested significantly higher viral loads than those mice given control rat IgG (Fig. 1C). Taken together, 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 (ICS) 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. 2A).

These CD8αβ (unpublished data) T cell clones provided the cellular reagents to define

the ligand(s) 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 element(s) to a β_2m-associated class I molecule (Fig.

2B). Infected splenic APCs from B6 mice with targeted disruption of *H2-M3*, *CD1d*

(Fig. 2B), 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 cocultured 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 epitope(s) 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 1A), 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 carboxyl terminus. Of three 9mers having a histidine at P7 within the VP2

aa sequence 133-151 (Table 1B), only the $VP2_{139-147}$ peptide (referred to as VP2.139)

stimulated intracellular IFN-γ production by all of the $K^{b-/-}D^{b-/-}$ mouse-derived CD8 T cell

clones (Fig. 2C). Single residue truncations of the VP2.139 peptide at the carboxyl 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 carboxyl terminus of VP2.139 is occupied by tryptophan, a residue not detected at this position among endogenous peptides eluted from Qa-2 molecules (161); the Qa-2 F pocket, though, is relatively spacious and can accommodate large side chains (111).

The Qa-2 family is comprised of several members, of which Q9 is among the most thoroughly characterized murine class Ib proteins. Q9 and another Qa-2 family member, Q8, share largely overlapping peptide-binding motifs, despite having over twenty aa differences in the $\alpha 1$ and $\alpha 2$ domains (117). 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 (115). As shown in Fig. 2D, only VP2.139 peptide-loaded cells expressing Q9 were recognized by these CD8 T cell clones. Taken together, these results define Q9-VP2.139 as the ligand for TCRs expressed by the PyV-specific CD8 T cell cloned 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 Ag-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. 3A). Moreover, the Q9-VP2.139 tetramer did not stain CD8 T cells in mice infected by a mutant 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 Ag-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. 3B). Notably, by 80 days 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 days p.i., peak in magnitude by 8 days p.i., then quickly contract to a lower long-term steady-state level (92, 93).

Chronic encounter by cognate Ag 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 phase (day 35 p.i.) and long-term maintenance phase (d 178 p.i.). 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 KLRG1, a marker of short-lived effector CD8 T cells (162, 163) (Fig. 3C). By day 178 p.i., however, the

VP2.139-specific CD8 T cells assumed an unusual phenotype overlapping both effector and memory phenotypic profiles, consistent with repetitive antigenic stimulation of memory CD8 T cells (164, 165). While 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 recently be ascribed to CD8 T cells having strong recall response capability (166). Notably, VP2.139-specific CD8 T cells at days 35 and 178 p.i. did not express the inhibitory PD-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 (102).

VP2.139-specific CD8 T cells in K$^{b-/-}$D$^{b-/-}$ mice exhibited pronounced functional impairment. Throughout the course of PyV infection, fewer than 50% of VP2.139-specific CD8 T cells detected by tetramers produced intracellular IFN-γ after VP2.139 peptide stimulation (Fig. 4A). Interestingly, a fraction of the VP2.139-specific CD8 T cells acquired the ability to coproduce IFN-γ and TNF-α at a late timepoint, when persistent infection viral loads had fallen (Fig. 4B and Fig. 1A). To assay Ag-specific cytotoxic activity in vivo, K$^{b-/-}$D$^{b-/-}$ mice infected 35 days previously were injected with equal proportions of naïve 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. 4C); 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. 5A, at day 5 p.i. no differences were seen in viral load between the parental and mutant viruses in multiple organs (spleen and kidney 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 timepoint preceding the emergence of a detectable CD8 T cell response (Fig. 1B & 3B). During persistent infection (day 80 p.i.), however, $K^{b-/-}D^{b-/-}$ mice infected by 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 cytomegalovirus).

Secondly, 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 naïve $K^{b-/-}D^{b-/-}$ 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. 5B). Taken together, 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 1×10^6 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 Ag-nonspecific bystander inflammation associated with PyV infection impairs the antiviral CD8 T cell response (102), we reduced the viral inoculation dose 1,000-fold. As shown in Fig. 6A, Q9-VP2.139 tetramers now stained CD8 T cells in approximately a third of infected B6 mice at day 11 p.i., the 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 to the dominant D^b-LT359 CD8 T cell response, which occupied approximately 16% of the splenic CD8 T cell compartment in each infected B6 mouse (Fig 6B). 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, most of the D^b-restricted LT359-specific CD8 T cells detected by tetramer staining produced IFN-γ (Fig. 6C). 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 to D^b-LT359 CD8 T cells (Fig. 6D). Thus, while 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 timepoint 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 murine cysticercosis (118). More recently, it has been demonstrated that immunization

by Q9-expressing tumor cells elicits Q9-restricted CTL, which are capable of cross-

protecting against histologically disparate $Q9^+$ tumors (116, 167). 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 (112). To our knowledge, however, the data presented here represents 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. Naïve 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 (99) and are also the major source of

cells supplying the inflationary viral epitope-specific CD8 T cell populations in mouse

cytomegalovirus infection (C. Snyder, K.S. Cho, and A.B. Hill, personal

communication). Although Ag-driven proliferation of virus-specific CD8 T cells has been shown to maintain CD8 T cell numbers during persistent lymphocytic choriomeningitis virus infection (44), PyV-specific MHC class Ia-restricted T cells undergo gradual attrition and fail to divide in persistently infected wild type mice (90, 99). Whether *de novo* priming of naïve 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 (168) and in the kidneys and lungs of persistently infected $\beta_2 m^{-/-}$ mice (88). 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 (169, 170). 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-2$K^{b-/-}D^{b-/-}$ mice 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 would not encounter immunodomination by MHC class Ia-

restricted T cells, as described for the H2-M3-restricted memory CD8 T cell response to *Listeria monocytogenes* (139). 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 (171), possibly due to 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 LT and MT Ags (92, 160), 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 to 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 upregulated by certain inflammatory cytokines, such as IFN-γ (110), 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. 5B).

The inability of Q9 to efficiently utilize CD8 coreceptors (172) 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) (173) show profound inefficiency in selection of MHC class I alloreactive CD8 CTL. 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 (174). A small pool of VP2.139-specific naïve 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 differentiation of T cells recruited into the antiviral response (175, 176). In this connection, it is interesting to note that $CD8\alpha^{-/-}$ mice mount a delayed, cytokine effector function handicapped, $H\text{-}2D^{b}$-restricted PyV-specific T cell response (90).

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 cytomegalovirus (CMV) encodes a mimic MHC class Ia leader peptide, UL40, which binds to HLA-E and engages inhibitory CD94/NKG2A receptors on NK cells (177). Yet, HLA-E/UL40-specific CD8 T cells having cytokine and cytotoxic effector capabilities have been isolated from CMV seropositive individuals (153). 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. 3C), 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 murine-only MHC class Ib molecule, HLA-G has been proposed to constitute its functional human homolog (Comisky, M. et al. 2003. Human Immunol. 64:999-1004). It is also notable that PyV is unique among avian and mammalian polyomaviruses in possessing a VP2.139 sequence available for binding to Q9 (178). This apparent example of host-pathogen co-evolution 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 (Frederick, MD). H2-$K^{b-/-}D^{b-/-}$ ($K^{b-/-}D^{b-/-}$) mice (171) were obtained from Taconic Farms. H2-M3$^{-/-}$ (140), CD1d$^{-/-}$ (179), Qa-1b$^{-/-}$ (180), and B6.K1 (181) mice have been described. $K^{b-/-}D^{b-/-}$ mice were bred and housed by the Department of Animal Resources at Emory University in accordance with the guidelines of the Institutional Animal Care and Use Committee of Emory University. All mice were between 6 and 8 wks of age at the time of infection.

Cell lines. 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 (117).

Viruses and cell transfers. $K^{b-/-}D^{b-/-}$ and B6 mice were infected by 1 x 10^6 and 1 x 10^3 PFU of PyV (A2 strain), respectively, s.c. in hindfootpads. The A2.H145A mutant virus was created using PCR-based site directed mutagenesis of A2 genomic DNA [forward primer, 5'-GCTCTAAATGTAGTA<u>GC</u>TGATTGGGGCCATGGC-3'; reverse primer, 5'-GCCATGGCCCCAATCA<u>GC</u>TACTACATTTAGAGC-3' (mutation nucleotides underlined)] to change the codon for aa 145 in the VP2 protein from histidine to alanine. A VP2.139-147 [145A] analog 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). Twenty x 10^6 spleen cells from $K^{b-/-}D^{b-/-}$ mice at day 35

p.i. by wild type PyV were transferred i.v. to naïve $K^{b-/-}D^{b-/-}$ mice, which were inoculated

immediately after transfer with 1 x 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 described (92). PyV DNA quantity is expressed in genome copies per mg

of tissue and is calculated based on a standard curve of known PyV genome copy

numbers vs. 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 1)

and LT359-368Abu peptide (102) 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 consisted of 17-mer peptides overlapping by 12 aa and were prepared as

PepSets by Chiron Mimotopes and were provided by Dr. John Altman (Emory Vaccine

Center, Atlanta, GA); the VP1 and VP2 peptide libraries consisted of 15-mer peptides

overlapping by 10 aa and were prepared on a Symphony/Multiplex peptide Synthesizer

(Rainin). The MT peptide library includes all of ST except for its 4 carboxyl 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 described (91).

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, lungs were digested in vitro by collagenase, then centrifuged on Percoll step gradients, as described (13). 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-PD-1 were purchased from eBioscience. Anti-KLRG1 was purchased from Southern Biotechnology Associates. Q9-VP2.139 tetramers were constructed by the NIH Tetramer Core Facility (Emory University) using cloned full-length Q9 cDNA (182). 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 analyzed using FlowJo software (Tree Star, Inc.).

IFN-γ ELISPOT assay. Single-cell ELISPOT assays were performed as previously described (17). Briefly, 96-well filtration plates (Millipore) were coated overnight with anti-IFN-γ (R4-6A2; BD Pharmingen). PyV-specific $K^{b-/-}D^{b-/-}$ CD8 T cell cloned lines, 2 wks after stimulation by infected, irradiated $K^{b-/-}D^{b-/-}$ splenocytes, were incubated with naïve $K^{b-/-}D^{b-/-}$ 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 Pharmingen). Wells were then

incubated with HRP avidin D (Vector Laboratories), washed, and developed with freshly

prepared substrate buffer [0.03% (w/v) 3-amino-9-ethyl-carbazole and 0.015% (v/v) 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, or PMA (500 ng/ml) and ionomycin (50 ng/ml), for 5 h in the presence

of brefeldin A, then stained for surface CD8 and intracellular cytokines, as described

(92). 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 Pharmingen. For granzyme B (GzmB) staining, spleen cells were

surface stained directly *ex vivo* with anti-CD8α and Q9-VP2.139 tetramers,

permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen), and then stained with

anti-human granzyme B or the isotype control Ab (Caltag Laboratories).

***In vivo* CD8 T cell depletion.** CD8β (clone H35) mAb (183) was prepared in INTEGRA

Celline CL1000 flasks (Integra Biosciences) using defined IgG-free medium (Invitrogen),

according to manufacturer's instructions. One mg of anti-CD8β or ChromPure rat IgG

(Jackson ImmunoResearch Laboratories) was administered i.p. on days –3, -1, and +1 of

the day of PyV infection, with antibodies given every 5 days 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 1 x 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 1 x 10^7 cells/ml in Hank's Buffered Salt Solution (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 then labeled with a different concentration of CFSE (1 mM, 0.1 mM) at 2 x 10^7 cells/ml in HBSS, with labeling stopped by addition of an equal volume of FBS for 1 min. Five x 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 h and 12 h after transfer, as previously described (16).

^{51}Cr release assay. Adherent H1TAP.11, H1Q8TAP.11 and H1Q9TAP.11 target cells were radiolabeled with 150 to 200 μCi of $Na_2{}^{51}CrO_4$ (400–1200 Ci/g; Perkin Elmer) and pulsed with 10 μM VP2.139 peptide at 37°C for 1.5 h, as described previously (91). Target cells were aliquotted at 5,000 cells/well into U-bottom 96-well microtiter plates, cocultured at 37°C for 5 h with PyV-specific CD8 T cell cloned lines at the indicated E:T ratios in quadruplicate wells, and percent specific ^{51}Cr release determined as described (91).

Statistical analysis. Statistics were performed using Prism statistical software

(GraphPad). All analyses were unpaired Student's t tests. A *p* value ≤0.05 was

considered statistically significant.

ACKNOWLEDGEMENTS

Figure 1. K^b-/-D^b-/- mice control PyV replication. (A) Levels of PyV DNA ± SEM in organs at 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 PyV genome copy number ± SEM in the indicated organ were assayed on day 35 p.i. Data are the mean of 3-5 mice per group and are representative of two independent experiments. *$p \leq 0.05$. ** $p \leq 0.01$.
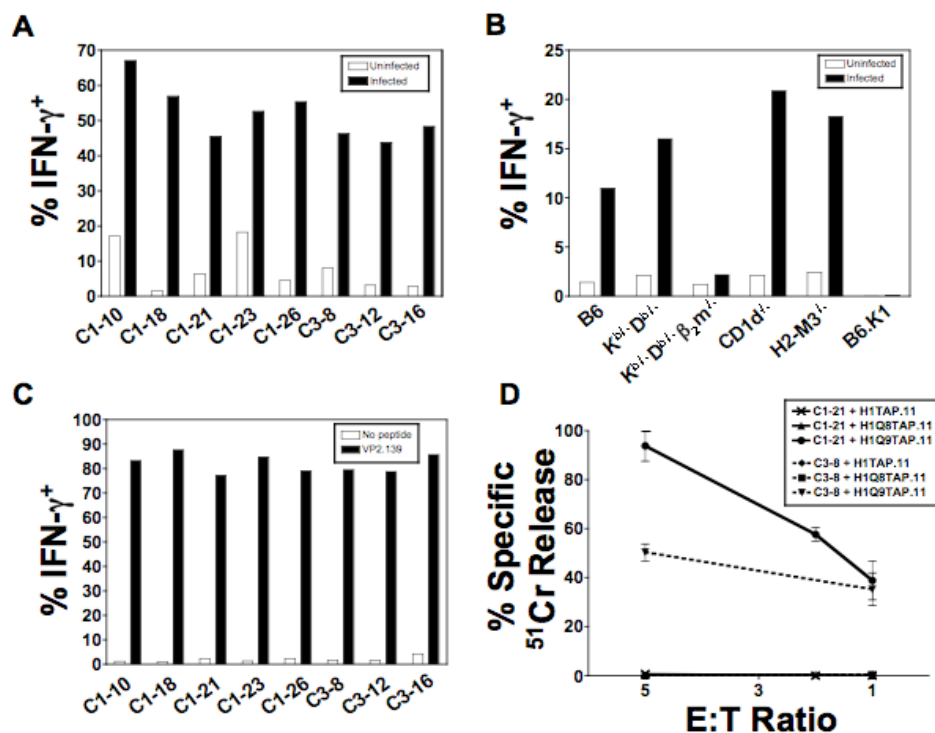
Figure 2. Q9/VP2.139 is the ligand for PyV-specific $K^{b-/-}D^{b-/-}$ CD8 T cell clones. CD8 T cell clones isolated from PyV-infected $K^{b-/-}D^{b-/-}$ mice were stimulated by PyV-infected or uninfected $K^{b-/-}D^{b-/-}$ splenocytes (A) or PyV-infected or uninfected splenocytes from mice of the indicated strains (B) for 5 h, then analyzed for intracellular IFN-g. Data shown in (B) shows the frequency of IFN-g-producing clone C3-8 cells; the same response pattern shown by clone C1-21 (unpublished data). (C) Frequency of T cell clones stained intracellularly by anti-IFN-g following stimulation by $K^{b-/-}D^{b-/-}$ splenocytes in the presence of VP2.139 peptide or no added peptide. (D) Cytotoxic activity 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).

Table 1. Synthetic peptides used to map Qa-2-restricted PyV-specific T cell epitope

A. Overlapping VP2 peptides

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
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

B. Peptides fitting the Q9 peptide-binding motif

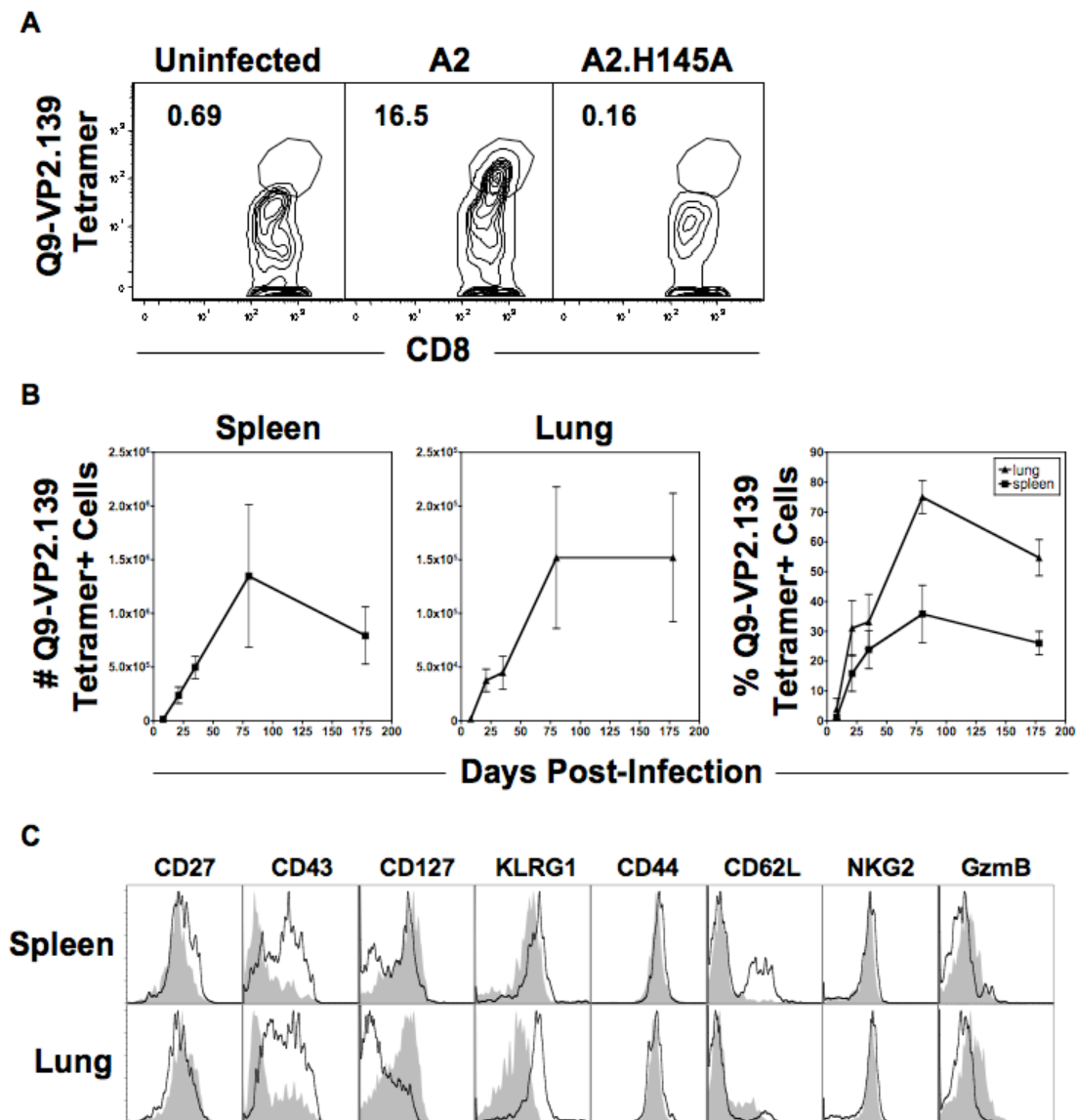|	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
VP2 (133-141)			G	V	N	Q	F	A	H	A	L										
VP2 (139-147)									H	A	L	N	V	V	H	D	W				
VP2 (143-151)													V	V	H	D	W	G	H	G	L

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 at day 40 p.i. $K^{b-/-}D^{b-/-}$ mice. Plots are gated on CD8^{+} cells and values represent the percentage of cells within the indicated gate. (B) Numbers (left and middle panels) and frequency (right panel) of splenic and lung Q9-VP2.139 tetramer[+] CD8 T cells ± SEM over time (n = 3-9 mice at each of the indicated timepoints). (C) Splenic and lung tetramer^{+} cells were analyzed 35 (open) and 178 (shaded) days p.i. (plots are representative of 4-9 mice at each timepoint). Plots are gated on CD8^{+} Q9-VP2.139 tetramer^{+} cells.
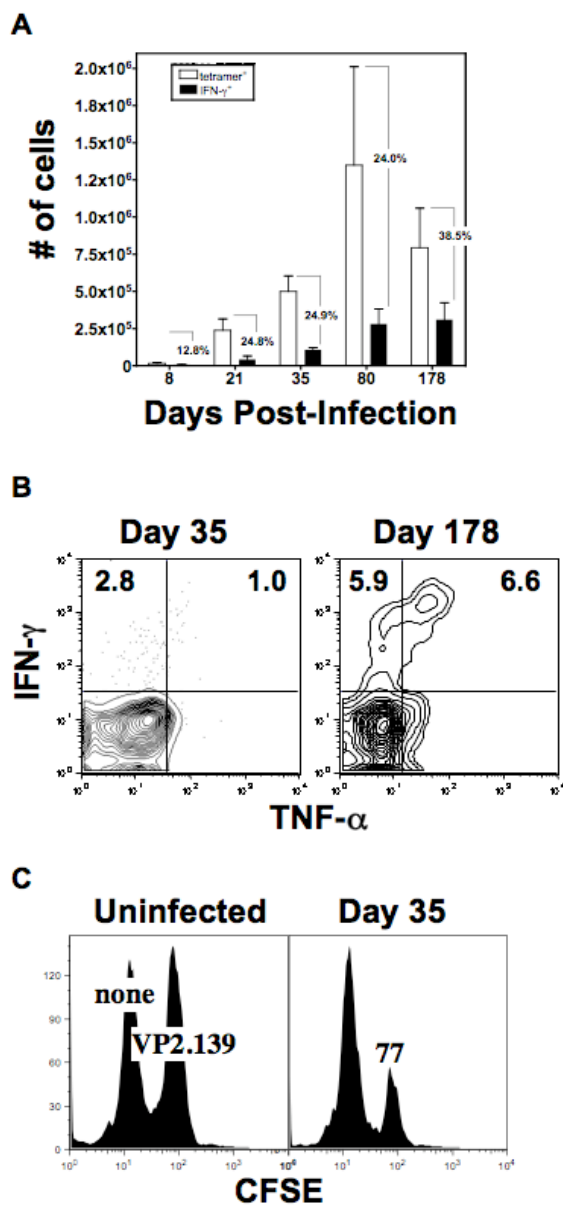
Figure 4. VP2.139-specific CD8 T cells have reduced effector function. (A) Number of Q9-VP2.139 tetramer[+] and VP2.139 peptide-stimulated IFN-γ CD8 T cells in spleens of $K^{b-/-}D^{b-/-}$ mice at the indicated timepoints p.i. Values indicate percentage of IFN-γ[+]/tetramer[+] CD8 T cells. (B) VP2.139-stimulated spleen cells from $K^{b-/-}D^{b-/-}$ mice stained for intracellular IFN-γ and TNF-α at day35 and day 178 p.i. Plots are gated on CD8. (C) Naïve $K^{b-/-}D^{b-/-}$ splenocytes coated with VP2.139 peptide (CFSE[hi]) or left uncoated (CFSE[lo]) were injected into naïve or PyV-infected $K^{b-/-}D^{b-/-}$ mice (n = 3 mice) at day 35 p.i. Twelve hours later, splenocytes were harvested and analyzed by flow cytometry. Value indicates percentage loss of peptide-coated target cells, and is representative of two independent experiments.
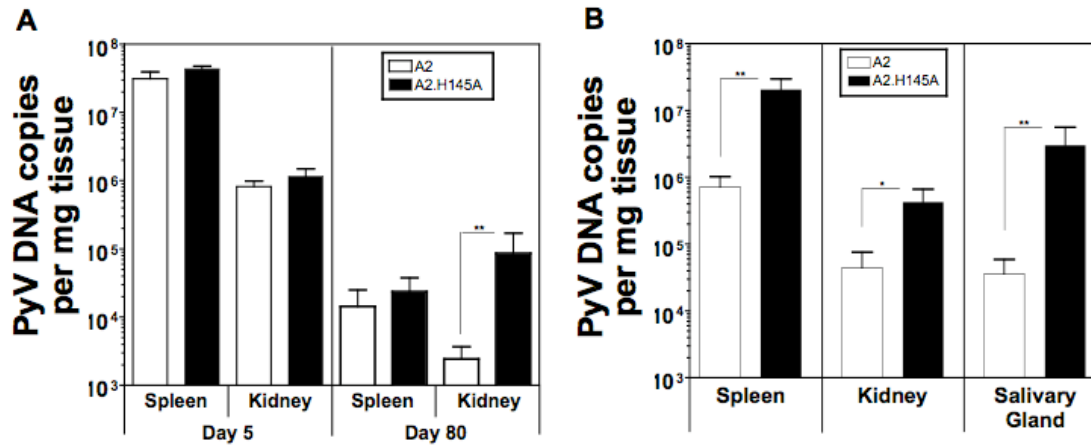
Figure 5. VP2.139-specific CD8 T cells are protective. (A) $K^{b-/-}D^{b-/-}$ mice were infected by parental A2 PyV or the VP2.139 epitope[null] A2.H145A mutant PyV. Levels of PyV DNA ± SEM in spleen and kidney were determined by quantitative PCR at the indicated timepoints p.i. (B) $K^{b-/-}D^{b-/-}$ mice were infected by A2 or A2.H145A PyVs (1 x 10^6 PFU) and simultaneously received 20 x 10^6 splenocytes from A2 virus-infected (day 35 p.i) $K^{b-/-}D^{b-/-}$ splenocytes. Virus levels were assayed as in (A) in the indicated organs at day 5-6 p.i. of 5-6 mice pooled from two independent experiments. **$p < 0.0001$, *$p < 0.001$.
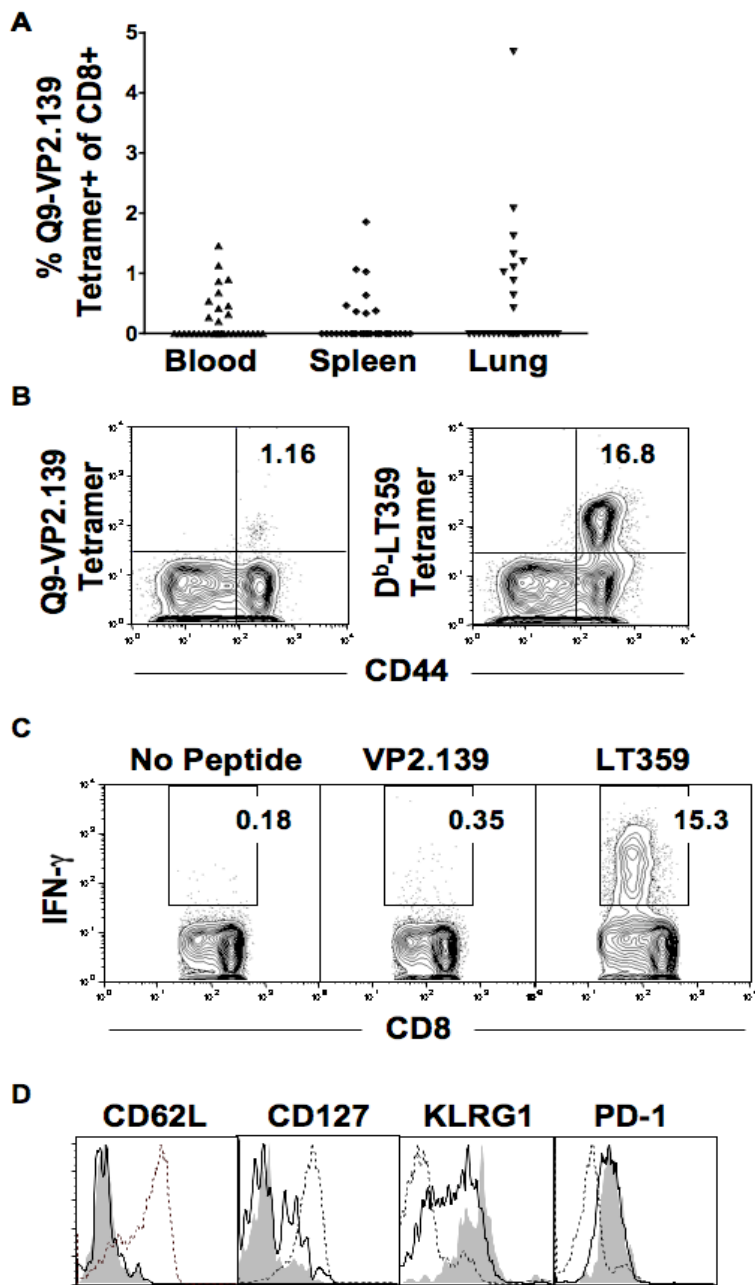
Figure 6. PyV-infected B6 mice generate a Q9/VP2.139-specific CD8 T cell response. (A) Frequency of Q9-VP2.139+ cells of CD8 T cells in the blood, spleen and lung of PyV-infected (day 11 p.i.) B6 mice (n = 32). (B) Representative dot plots of splenic Q9-VP2.139 tetramer+ and Db-LT359 tetramer+ CD8 T cells from B6 mice at day 11 p.i. Plots are gated on CD8 T cells. Values indicate percentage of CD8 T cells that are tetramer+. (C) VP2.139 peptide and LT359 peptide-stimulated spleen cells from the same mice shown in (B) were analyzed for intracellular IFN-γ. Plots gated on CD8 T cells. Values indicate percentage of CD8 T cells that are IFN-γ+. (D) Expression of the indicated surface molecules by splenic CD8 T cells from naïve mice (dotted line), and Q9-VP2.139 tetramer+ (shaded) and Db-LT359 tetramer+ CD8 T cells (solid line) from infected (day 11 p.i.) B6 mice.

Chapter 3: Shift in Antigen Dependence by an Antiviral MHC Class Ib-restricted CD8 T Cell Response During Persistent Viral Infection

Abstract

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-month 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 naïve PyV-specific T cells, is primarily responsible for Q9-restricted anti-PyV 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.

Introduction

CD8 T cell responses 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, antigen-independent manner (10, 18, 19, 21, 45). 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 (1, 36). For example, maintenance of antiviral

memory CD8 T cells in high-level systemic chronic lymphocytic choriomeningitis virus

(LCMV) infection requires cognate antigen but not IL-7 and IL-15 (44). 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 cells in mice

infected by murine CMV (MCMV) continuously expand over the course of infection, a

phenomenon termed memory inflation (70, 71). Similarly, CMV-specific CD8 T cells in

humans accumulate throughout an individual's lifetime (69). 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 maintenance of stable numbers of antiviral CD8

T cells requiring ongoing recruitment of virus-specific naïve CD8 T cell progenitors (99).

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 (184).

These unconventional CD8 T cells recognize a peptide derived from amino acids 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

approximately 12 wks, then enters a long-term plateau phase. In this study, we tested the

hypothesis that cognate antigen regulates the expansion of these MHC class Ib-restricted

antiviral CD8 T cells.

Materials and Methods

Mice

C57BL/6NCr (B6) female mice were purchased from the Frederick Cancer Research and

Development Center of the National Cancer Institute (Frederick, MD). B6.$K^{b-/-}D^{b-/-}$ ($K^{b-/-}$

$D^{b-/-}$) mice (Thy1.2) were obtained from Taconic Farms; $K^{b-/-}D^{b-/-}$Thy1.1 (185) mice were

kindly provided by Peter Jensen (University of Utah, Salt Lake City, UT). Mice were

bred and housed by the Department of Animal Resources at Emory University in

accordance with the guidelines of the Institutional Animal Care and Use Committee of

Emory University. All mice were between 6 and 8 wks of age at the time of infection.

Viruses and cell transfers

$K^{b-/-}D^{b-/-}$ and B6 mice were infected by 1 x 10^6 PFU PyV s.c. in hindfootpads. A

recombinant vaccinia virus carrying the PyV VP2 gene (VV-VP2) was generated as

described (186) and kindly provided by Richard Consigli (Kansas State University,

Manhattan, KS); $K^{b-/-}D^{b-/-}$ mice received 1 x 10^6 PFU VV-VP2 i.p. The A2.H145A

mutant virus was isolated as described (184). RBC-lysed splenocytes from PyV-infected

$K^{b-/-}D^{b-/-}$ mice were B cell-depleted, labeled with 5μm CFSE, and injected i.v. (50 x 10^6

cells) into infected $K^{b-/-}D^{b-/-}$Thy1.1 mice.

Cell isolation and flow cytometry

Single cell suspensions were made of RBC-lysed spleens. Abs to CD3ε, CD8α, Thy1.1,

Ki67, and Annexin V, as well as propidium iodide (PI) and 7-AAD were purchased from

BD Biosciences and used according to manufacturer's instructions. The TCR Vβ

antibody panel was purchased from BD Biosciences and staining was performed as described (90). Q9/VP2.139 tetramers were constructed by the NIH Tetramer Core Facility using either cloned full-length Q9 cDNA (182) or Q9 cDNA encoding the α3 domain of H-2D^b in place of that of Q9. Both tetramers stained equivalent percentages of splenocytes from PyV-infected K$^{b-/-}$D$^{b-/-}$ mice with the same mean fluorescence intensity and were used interchangeably. Tetramer staining was performed as described (184). Samples were acquired on a FACSCalibur (BD Biosciences) and data analyzed using FlowJo software (Tree Star, Inc.).

Bone marrow chimerism induction

Persistently infected K$^{b-/-}$D$^{b-/-}$ mice given 600 mg busulfan (Busulfex; Otsuka America Pharmaceutical, Inc., Rockville, MD) i.p. were injected i.v. 24 h later with 25 x 10^6 CD3-depleted bone marrow cells from K$^{b-/-}$D$^{b-/-}$Thy1.1 mice. CD3 depletion was performed using anti-CD3ε and MACS sorting.

Results and Discussion

Persistent infection is associated with the Q9/VP2.139-specific CD8 T cell inflationary response

PyV-infected $K^{b-/-}D^{b-/-}$ mice generate a VP2.139-specific CD8 T cell response that progressively increases over the first three months (mos) after infection (184)). 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 antigen specific cells (90). 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 $K^{b-/-}D^{b-/-}$ mice infected by either PyV, which establishes a persistent infection (184), or a recombinant vaccinia virus expressing the PyV VP2 protein (VV-VP2), which is cleared after acute infection (unpublished observations). Both viruses generated a VP2.139-specific CD8 T cell response, but after two weeks this Ag-specific T cell response contracted in VV-VP2-infected mice and was maintained at a low level, whereas, in PyV-infected mice, the frequency of Q9/VP2.139 tetramer^{+} CD8 T cells progressively increased over the 48-day period of observation (Fig. 1). These data indicate that persistent PyV infection is associated with the inflationary Q9/VP2.139-specific CD8 T cell response.

Naïve Q9/VP2.139-specific CD8 T cell priming during persistent PyV infection

During persistent PyV infection in wild type B6 mice, *de novo* primed CD8 T cells resupply the short-lived MHC class Ia-restricted CD8 T cells and thereby maintain stable

numbers of these antiviral T cells (99). We asked whether naive Q9/VP2.139-specific

CD8 T cells similarly contribute to the inflationary response of VP2.139-specific CD8 T

cells. To do this, $K^{b-/-}D^{b-/-}$ mice underwent minimal myeloablative busulfan conditioning

midway in the Q9/VP2.139-specific CD8 T cell expansion phase (day 35 p.i.), followed

by injection of T cell-depleted, Thy congenic $K^{b-/-}D^{b-/-}$ bone marrow (Fig. 2*A*). Fifty days

after bone marrow transfer, 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*B*). 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 around 3 months p.i. VP2.139-specific CD8 T cells no longer expand

but are maintained at high numbers (184). We therefore compared expansion phase

VP2.139-specific CD8 T cells (1month p.i.) to those from the plateau phase (3 months

p.i.) for expression of molecules marking cell proliferation and survival. A larger

fraction of expansion phase VP2.139-specific CD8 T cells expressed Ki67, a cell cycle

related nuclear protein, than those in the plateau phase (Figs. 3*A*, 3*C*). In contrast, few

VP2.139-specific CD8 T cells in either phase of the response stained with Annexin V, a

marker of apoptosis (Figs. 3*B*, 3*C*); the anti-apoptotic protein Bcl-2 stained similar

numbers of Q9/VP2.139 tetramer[+] CD8 T cells at comparable MFI at one and three mos

p.i. (unpublished observations). The strikingly narrow expression of TCR Vβ elements

by Q9/VP2.139 tetramer[+] CD8 T cells in individual $K^{b-/-}D^{b-/-}$ mice compared to the

diverse Vβ usage by CD8 T cells in uninfected $K^{b-/-}D^{b-/-}$ mice (Fig. 3D) 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. Taken together, these phenotypic

data indicate that VP2.139-specific CD8 T cell proliferation exceeds cell death during the

expansion phase and that during the plateau phase VP2.139-specific CD8 T cells survive

long-term in a nonproliferative state without appreciable cell death.

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[+] CD8 T cells from donor $K^{b-/-}D^{b-/-}$ mice at 1 mo p.i. (expansion

phase) or 3 mo p.i. (plateau phase) following transfer into infection-matched Thy1

congenic $K^{b-/-}D^{b-/-}$ 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[+] 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

expansion phase cells (Fig. 4A). This data further suggests that the failure of the plateau

phase cells to proliferate is due to insufficient numbers of Q9/VP2.139 epitope$^+$ APCs.

To test this possibility, splenocytes from $K^{b-/-}D^{b-/-}$ mice infected by wild-type PyV (strain

A2) 1 mo earlier were transferred to congenic $K^{b-/-}D^{b-/-}$ mice infected by either 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 (unpublished observations), and

infection by the A2.H145A mutant virus does not induce a Q9/VP2.139-specific CD8 T

cell response in $K^{b-/-}D^{b-/-}$ mice (184). Unlike VP2.139-specific CD8 T cell transfers from

1 mo A2 p.i. donors to A2-infected recipients, those donor anti-PyV cells transferred to

A2.H145A-infected recipients do not proliferate (Fig. 4C), yet are stably maintained (Fig.

4B). These findings demonstrate that Ag is required for VP2.139-specific CD8 T cell

expansion, but it is dispensable for cell survival.

Several characteristics of the Q9/VP2.139-specific CD8 T cell response parallel

those of an MHC class Ia-restricted PyV-specific T cell response seen in $CD8\alpha^{-/-}$ mice

(90). First, both anti-PyV T cell responses became detectable only after an

approximately one week lag. Second, both responses progressively expand and lack an

apparent contraction phase. Third, Vβ mAb co-staining of MHC-I tetramer$^+$ cells

revealed dramatic narrowing of Vβ expression, with Vβ usage differing between

individual mice. 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 CD8α subunit binding inefficient (111, 172, 173). Weak to absent (as in CD8α$^{-/-}$ mice) CD8 coreceptor engagement may permit only a trickle of MHC-I-restricted thymic emigrants, with the consequent small oligoclonal reserve of naïve anti-PyV T cell precursors requiring substantial expansion or ongoing thymic output to achieve detection.

The phenotype and longevity of expansion phase PyV-specific MHC class Ib-restricted CD8 T cells differ from 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, which are continuously replenished from cells primed during acute infection (97, 187). These MCMV-specific CD8 T cells do not express costimulatory molecules such as CD27 and CD28 nor do they express the homeostatic cytokines IL-7 and IL-15, which could account for their inability to survive long-term (97, 187). In contrast, the inflationary VP2.139-specific CD8 T cells are long-lived and express CD127 (184), CD122 (unpublished observations) and bcl-2 (unpublished observations). Yet, VP2.139-specific CD8 T cells do not undergo homeostatic proliferation. This low-turnover, long-lived, effector-like phenotype is reminiscent of LCMV-specific memory CD8 T cells that reside in the intestine (188). 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 Ag presentation remain 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

the early stages of LCMV clone 13 infection lead to the selective culling of antiviral CD8 T cells of particular specificities (36, 37, 39), while CD8 T cells directed to other viral epitopes are maintained by Ag-driven proliferation (44).   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 (97). PyV-specific, MHC Ia-restricted memory CD8 T cells are short-lived and the antiviral response is maintained by priming of naïve CD8 T cell during persistent infection (99). 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.

Acknowledgements

We thank the NIH Tetramer Core Facility (Emory Vaccine Center) for constructing the

Q9/VP2.139 tetramers. We also thank Annette Hadley for expert technical assistance.

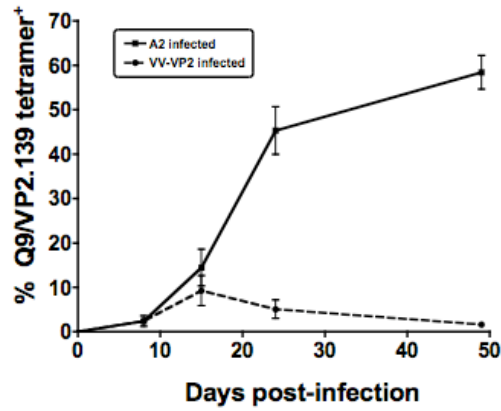**FIGURE 1.** VP2.139-specific CD8 T cell expansion is associated with persistent viral infection. Percentage of Q9/VP2.139 tetramer$^+$ CD8 T cells in the blood of PyV or VV-VP2-infected $K^{b-/-}D^{b-/-}$ mice ± SEM over time (n = 3 mice). Data are representative of two independent experiments.tetramer$^+$ (shaded) and D^b-LT359 tetramer$^+$ CD8 T cells (solid line) from infected (day 11 p.i.) B6 mice.
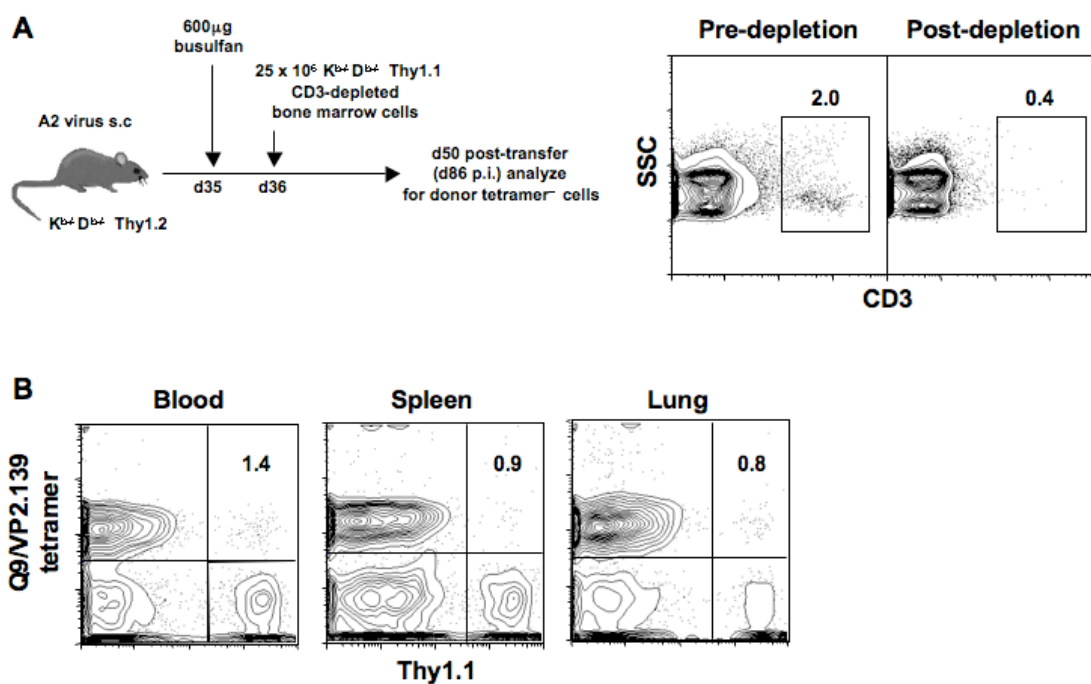
FIGURE 2. *De novo* priming of VP2.139-specific CD8 T cells during persistent infection. *A*, Experimental setup and representative dot plots of $K^{b-/-}D^{b-/-}$ Thy1.1 donor bone marrow cells before and after anti-CD3 mediated depletion. Plots are gated on live mononuclear cells and values indicate the percentage of $CD3^+$ cells. *B*, Representative dot plots of lymphocytes isolated from indicated organs of mice 50 days after congenic bone marrow transfer. Plots are gated on CD8 T cells and values indicate the percentage of donor tetramer$^+$ cells of total tetramer$^+$ population. Data is representative of two independent experiments.
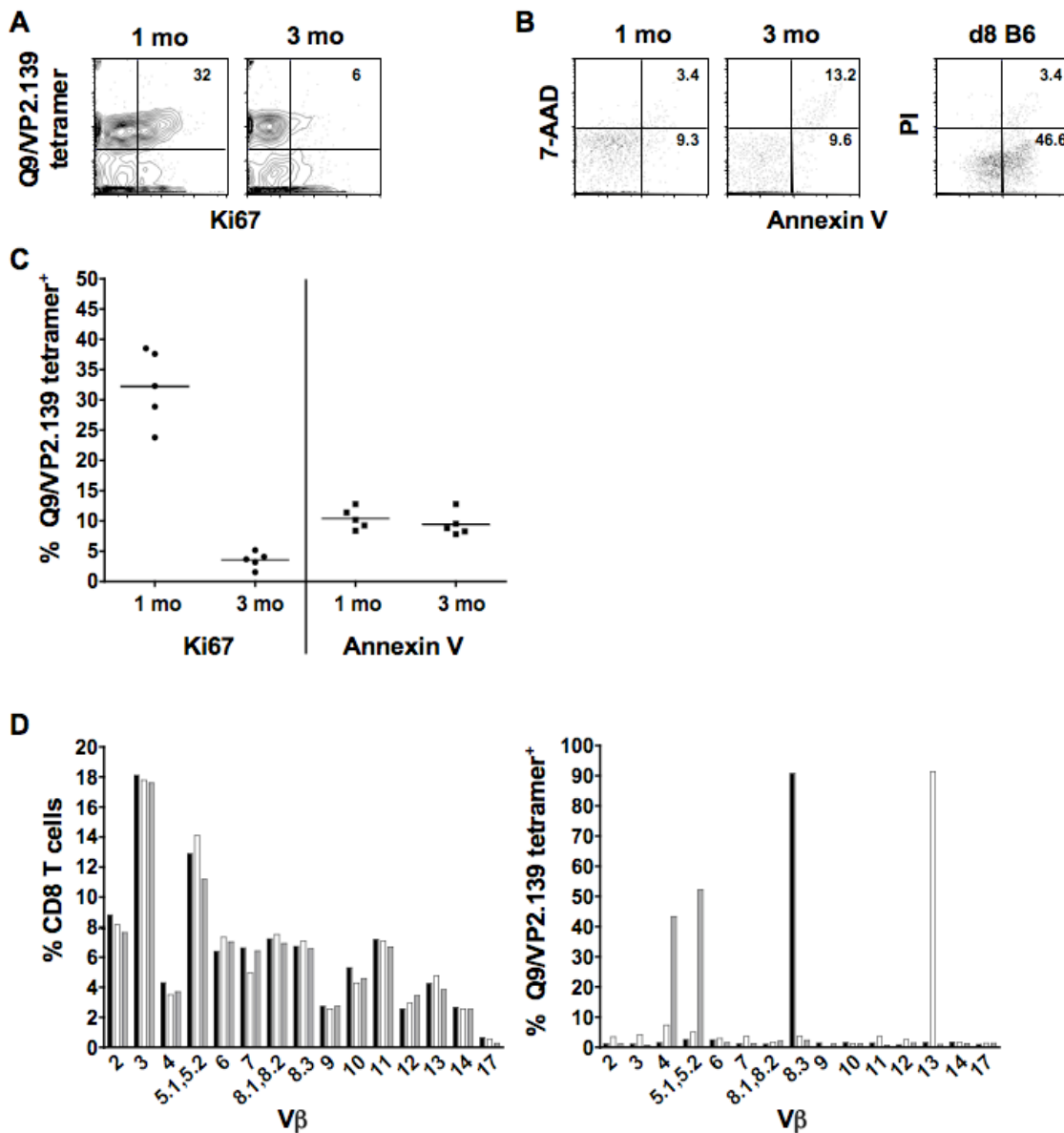
FIGURE 3. Dynamic phenotype of VP2.139-specific cells over the course of persistent PyV infection. Splenic Q9/VP2.139 tetramer^{+} cells from PyV-infected $K^{b-/-}D^{b-/-}$ mice were analyzed for (*A*) Ki67 or (*B*) Annexin V and 7-AAD coexpression at 1 mo and 3 mos p.i. *A*, Plots are gated on CD8^{+} cells and values indicate the percentage Ki67^{+}tetramer^{+} cells. *B*, Plots are gated on tetramer^{+} cells and values indicate the percentage of cells in the indicated quadrant. Splenic D^{b}/LT359 tetramer^{+} cells from B6 mice at day 8 p.i. were analyzed for Annexin V and propidium iodide (PI) staining as a positive control. Dot plots in (*A*) and (*B*) are representatives of mice in (*C*), which indicates the percentage of Ki67^{+} or Annexin V[+]7-AAD[-] of tetramer[+] CD8 T cells at the indicated timepoints (n = 5). (*D*) TCR Vβ expression by splenic CD8 T cells from uninfected $K^{b-/-}D^{b-/-}$ mice (left panel) and by Q9/VP2.139 tetramer[+] CD8 T cells from $K^{b-/-}D^{b-/-}$ mice day 35 p.i. (right panel). Each bar pattern represents an individual mouse. Two independent experiments were performed.
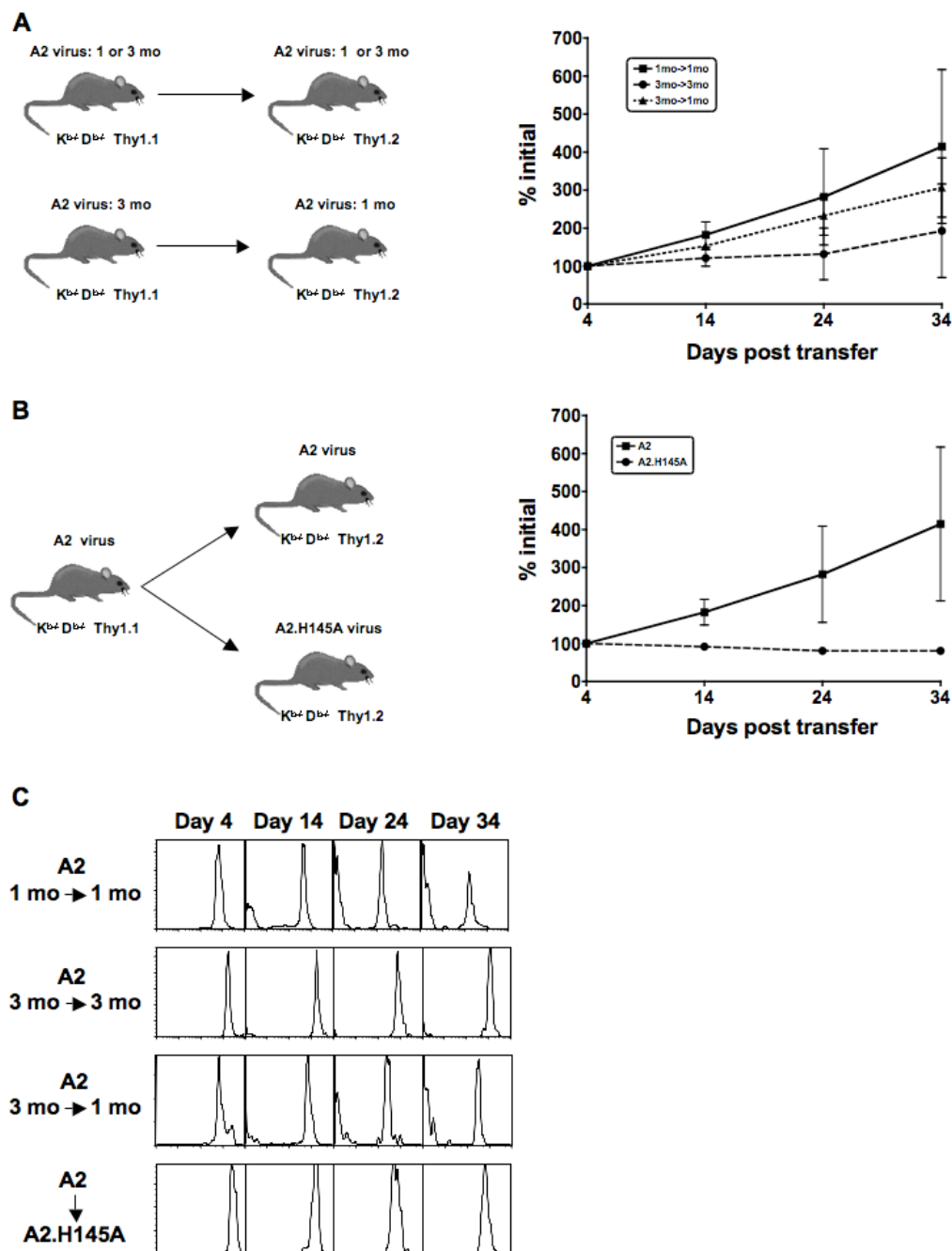
FIGURE 4. Inflation, but not survival, of memory VP2.139-specific CD8 T cells is Ag-dependent. *A*, B cell-depleted spleen cells from wild type PyV (A2 strain)-infected $K^{b-/-}D^{b-/-}$ Thy1.1 mice at 1 mos or 3 mos p.i. were transferred to infection-matched $K^{b-/-}D^{b-/-}$ Thy1.2 mice (1 mo to 1 mo; 3 mo to 3 mo) or cells from A2-infected $K^{b-/-}D^{b-/-}$ Thy1.1 mice at 3 mo p.i were transferred into A2-infected $K^{b-/-}D^{b-/-}$ Thy1.2 mice at 1 mo p.i. (3 mo to 3 mo). PBLs were monitored over time and values indicate the percentage of donor Q9/VP2.139 tetramer[+] CD8 T cells ± 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 $K^{b-/-}D^{b-/-}$ mice at 1 mo p.i. to Thy congenic $K^{b-/-}D^{b-/-}$ 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 timepoints after transfer for experiments in (*A*) and (*B*). Two independent experiments were performed.

Chapter 4: Discussion

MHC class Ib-restricted CD8 T cell immunity is an underappreciated area of immunology research. Many properties of class Ib molecules including the structural homology to class Ia molecules, ubiquitous tissue expression, and peptide-binding capabilities implicate a role for these molecules in immune defense. In support of this, a growing body of evidence from both human and animal research has shown that class Ib molecules do in fact serve as ligands for pathogen-specific CD8 T cells. The studies in this dissertation represent the first description of an endogenous MHC class Ib-restricted antiviral CD8 T cell response. PyV-infected mice generate a Q9-restricted CD8 T cell response directed towards 9mer peptide eptiope derived from the VP2 capsid protein (VP2.139). Q9/VP2.139-specific CD8 T cells are characterized by both their longevity and their ability to protect mice against viral infection. Most importantly, VP2.139-specific cells are found in wild type B6 mice where they could potentially form another layer of immunity against PyV infection.

IFN-γ is a CD8 T cell effector cytokine important for controlling viral infections, such as LCMV. Unpublished observations from our lab show that IFN-γ has direct antiviral properties on PyV *in vitro*. It is curious that even though VP2.139-specific CD8 T cells are protective against primary PyV infection, I have been unable to detect IFN-γ production (as well as TNF or IL-2) from most of these cells using ICS assays. There are several possible explanations for this. The first is that VP2.139-specific CD8 T cells could make IFN-γ *in vivo*, but we are unable to recapitulate the priming conditions that allow for IFN-γ production *in vitro*. Considering that Q9 expression is ubiquitous and the 1μm quantity of peptide used in the ICS assays probably surpasses physiological

amounts of endogenous Ag during PyV infection, it is difficult to imagine that standard *in vitro* culture conditions would be less suitable for priming than those *in vivo*. However, I have not tested whether VP2.139-specific CD8 T cells kill each other during this assay. Additionally I could modify our ICS protocol and attempt to capture IFN-γ production by VP2.139-specific CD8 T cells as it's happening *in vivo*. A procedure developed by Lindsay Whitton's lab (189) involves i.v. injecting infected mice with the protein transport inhibitor brefeldin A at various timepoints during acute viral infection and then analyzing cells directly *ex vivo* for IFN-γ production without the 5 hour stimulation period. This model has been highly effective for detecting IFN-γ-producing effector CD8 T cells after infection with LCMV (189).

Another possible explanation for the lack of IFN-γ production by VP2.139-specific cells is that these cells were at one time able produce IFN-γ *in vivo* but lost the ability to do it at some point after infection. One example of this would be VP2.139-specific CD8 T cell exhaustion. Although Q9 and class Ia molecules share significant structural homology, the α3 domain of Q9 has two amino acid differences that decreases its binding efficiency to the CD8 coreceptor (111). When CD8 T cells engage Ag-bearing cells, CD8 binding to MHC class I molecules is important for stabilizing the TCR-MHC-peptide interaction and for recruitment of the proximal TCR signaling molecule p56[Lck], which is associated with the cytoplasmic domain of CD8 (175, 190). Weaker TCR signaling as a result of the inability of CD8 to bind Q9 could drastically reduce the output of VP2.139-specific CD8 T cells during thymic selection. An extremely small Q9/VP2.139-specific naïve CD8 T cell repertoire would have to go through numerous rounds of division during PyV-infection to be able to be detected by

tetramer staining. By that point, any number of CD8 T cell functions, such as IFN-γ

production, could be exhausted. It is important to note the VP2.139-specific CD8 T cells

do not express PD-1, which is usually associated with T cell exhaustion (39). In order to

determine the size of the naïve VP2.139-specific CD8 T cell repertoire, I could use a

naïve Ag-specific cell-enriching method described by Mark Jenkin's and Leo Lefrancois'

groups (191, 192). Pooled cells from LNs and spleens are stained with antibodies to CD8

as well as two identical tetramers, labeled with two different fluorochromes, in order to

gate out cells that have non-specifically bound to one or the other tetramer. Cells are

then labeled with magnetic beads specific for one of the tetramer fluorochromes, purified

on a cell separation column, and then stained with a panel of antibodies specific for

CD11b, MHC class II, CD19, and CD4 to use as a dump gate. This method has been

highly successful for identifying the precursor frequencies of a number of virus specific

CD8 T cells in B6 mice (192). Comparing the naïve Q9/VP2.139 CD8 T cell repertoire

in $K^{b-/-}D^{b-/-}$ mice to the size of the D^b/LT3359 CD8 T cell repertoire in B6 mice could shed

light onto whether thymic selection of VP2.139-specific cells is abnormally low.

      VP2.139-specific CD8 T cells can not be detected by single tetramer staining until

about a week post-infection, and by that time, most of these cells are already deficient for

IFN-γ production. If VP2.139-specific CD8 T cell IFN-γ production is lost at some point

during the first week of infection, artificially boosting the naïve VP2.139-specific

precursor frequency would make it easier to detect early IFN-γ-producing cells.

Adoptively transferring pooled purified naïve CD8 T cells from $K^{b-/-}D^{b-/-}$ mice into naïve

$K^{b-/-}D^{b-/-}$ mice before PyV infection could allow for detection of VP2.139-specific cells

earlier in infection when they might be capable of making IFN-γ. Currently, we are

developing Q9/VP2.139-specific CD8 T cell TCR transgenic mice in collaboration with Peter Jensen's lab at the University of Utah. Adoptive transfer of naïve VP2.139-specific TCR transgenic cells into PyV-infected $K^{b-/-}D^{b-/-}$ mice at different timepoints post-infection would be a much easier way to determine when/if VP2.139-specific CD8 T cell IFN-γ production is lost.

IFN-γ production by CD8 T cells could also be inhibited *in vivo*. VP2.139-specific CD8 T cells can account for up to 40% and 70% of the total CD8 T cell repertoire in the spleen and lungs of PyV-infected $K^{b-/-}D^{b-/-}$ mice, respectively (Chapter 2). Because such a large frequency of CD8 T cells are activated, inhibition of CD8 T cell IFN-γ production could be a way for the host to protect itself from overwhelming collateral tissue damage. In support of this, $K^{b-/-}D^{b-/-}$ mice infected by a recombinant vaccinia virus expressing the VP2.139 epitope (VV-VP2.139) die by 10 days post-infection (unpublished observations). However, $K^{b-/-}D^{b-/-}$ mice depleted of CD8 T cells by anti-CD8β antibodies show no mortality or morbidity. Therefore, in order to test whether CD8 T cells are being inhibited during PyV infection, CD8 T cells could be purified from bulk spleen cells of PyV-infected $K^{b-/-}D^{b-/-}$ mice and then stimulated on naïve, peptide-coated splenocytes. Additionally, if VP2.139-specific cells are being inhibited *in vivo*, T regulatory cells are likely sources of the inhibition. Consequently, CD4-depletion of PyV-infected mice during persistent infection could increase the frequency of IFN-γ producing VP2.139-specific CD8 T cells.

A third hypothesis for the lack of detectable IFN-γ production from VP2.139-specific CD8 T cells is that only a fraction of Q9/VP2.139-specific cells are ever able to make IFN-γ during PyV infection. The inefficient binding of CD8 to Q9 may only lead

to weak TCR signaling during priming of VP2.139-specific CD8 T cells. This could cause cells to differentiate into effectors with limited function (proliferation and cytotoxicity, but no cytokine production). In support of this, the majority of D^b/LT359-specific CD8 T cells in PyV-infected CD8-deficient mice are also unable to produce IFN-γ (90). Perhaps the few VP2.139-specific cells in PyV-infected $K^{b-/-}D^{b-/-}$ mice that are able to develop a full range of effector functions, including IFN-γ production, are those that have the highest avidity TCRs. Experiments have shown that sustained TCR signaling is required for CD8 T cell mediated cytokine production, while much less Ag can induce cytotoxic capabilities (193). Increasing epitope density in ICS assays with VP2.139-specific cells (albeit far beyond physiological conditions) does lead to a higher frequency of IFN-γ producing cells (unpublished observations). However, this only partially overcomes weak TCR signaling as the increase in frequency of IFN-γ producing cells eventually tops out with only half of the Q9/VP2.139 tetramer$^+$ cells producing IFN-γ.

Our working hypothesis is that the VP2.139-specific CD8 T cell IFN-γ deficit is a consequence of restriction by Q9. Several observations support this possibility. That PyV establishes a persistent infection is inconsequential to the IFN-γ defect because the majority of VP2.139-specific CD8 T cells in VV-VP2-infected $K^{b-/-}D^{b-/-}$ mice are also IFN-γ deficient (unpublished observations). Additionally, the inability of VP2.139-specific CD8 T cells to make IFN-γ is not due to a global defect in IFN-γ production by CD8 T cells in $K^{b-/-}D^{b-/-}$ mice. Additionally, the majority of CD8 T cells from either naïve or PyV-infected $K^{b-/-}D^{b-/-}$ mice stimulated with PMA and ionomycin produce IFN-γ, and

H2-M3-restricted CD8 T cells from *Listeria monocytogenes*-infected $K^{b-/-}D^{b-/-}$ mice produce IFN-γ.

Interestingly, the kinetics of the Q9/VP2.139-specific CD8 T cell response deviate from what has been observed with the class Ia-restricted anti-PyV response. While class Ia-restricted antiviral CD8 T cells rapidly expand and peak about a week after infection, the VP2.139-specific CD8 T cell response in $K^{b-/-}D^{b-/-}$ mice is barely detectable over a week post-infection and then gradually builds over the next three months. A simple explanation for the delay in VP2.139-specific CD8 T cell expansion could be that the VP2.139 epitope is inefficiently processed in infected cells early during infection. Decreased Ag on the surface of cells would make it difficult for naïve VP2.139-specific CD8 T cells to colocalize with VP2.139 epitope$^+$ APCs. There are several experiments that could be performed to test whether there is enough Ag present early in infection to stimulate VP2.139-specific CD8 T cells. The simplest experiment would be to track proliferation of CFSE-labeled Q9/VP2.139-specific TCR transgenic T cells adoptively transferred into infected $K^{b-/-}D^{b-/-}$ mice at various timepoints during the first week of infection. As VP2.139-specific TCR transgenic mice are not yet available, the same experiment could be performed using CFSE-labeled VP2.139 CD8 T cell clones (Chapter 2). As an alternative, splenocytes from infected $K^{b-/-}D^{b-/-}$ mice could be isolated at different timepoints during the first week of infection and used as APCs to test their ability to stimulate proliferation or IFN-γ production from the VP2.139-specific CD8 T cell clones *in vitro*.

However, because a delay in the appearance of PyV-specific CD8 T cells also occurs in PyV-infected CD8-deficient mice (90), it is more likely that weak TCR

signaling in VP2.139-specific CD8 T cells is responsible for the delayed kinetics. As mentioned above, the inefficient binding of CD8 to Q9 could reduce the efficiency of positive selection of VP2.139-specific CD8 T cells thus creating a tiny naïve repertoire. Therefore, during PyV infection, it could take up to a week for VP2.139-specific CD8 T cells to even find Ag, let alone divide enough times to become detectable by Q9/VP2.139 tetramer staining. On the other hand, even if the naïve VP2.139-specific CD8 T cell repertoire is of normal size, weak TCR signaling could extend the amount of time needed for VP2.139-specific CD8 T cells to divide and fully differentiate into effectors.

Although VP2.139-specific CD8 T cells are detectable in PyV-infected B6 mice, the response is much smaller than the VP2.139-specific CD8 T cell response in $K^{b-/-}D^{b-/-}$ mice. During the acute phase of PyV infection, VP2.139-specific CD8 T cells can only be detected in about a third of wild type B6 mice and represent a tiny fraction of the overall anti-PyV CD8 T cell response (Chapter 2). Additionally, acute phase VP2.129-specific CD8 T cells can not be detected by tetramer staining in B6 mice that are infected by our standard high dose of PyV (10^6 pfu). Only by reducing the PyV inoculum dose to 10^3-10^5 pfu, do VP2.139-specific CD8 T cells become detectable in 33% of infected B6 mice. Several questions arise from these early observations of VP2.139-specific CD8 T cells in PyV-infected B6 mice. Why does a low dose PyV infection selectively favor generation of VP2.139-specific CD8 T cells during the acute phase of infection? Why do only 33% of low dose PyV-infected B6 mice generate a VP2.139-specific CD8 T cell response? And in mice that do generate a VP2.139-specific CD8 T cell response, why do these cells make up such a small portion of the total anti-PyV CD8 T cell response?

In wild type mice, Q9 molecules compete with class Ia molecules as well as other class Ib molecules for peptides and binding to β_2m. Presumably this is the reason that Q9 expression on splenocytes is lower in B6 mice than in $K^{b-/-}D^{b-/-}$ mice (171). Evidence for this competition in our system comes from the fact that VP2.139-specific clones produce much less IFN-γ when stimulated with PyV-infected B6 splenocytes as opposed to PyV-infected $K^{b-/-}D^{b-/-}$ splenocytes (Chapter 2). Theoretically, B6 mice could be at a disadvantage for generating a VP2.139-specific CD8 T cell response right from the start. Reduced Q9 expression as well as weak TCR signaling (mentioned above) could decrease thymic output of naïve Q9/VP2.139-specific CD8 T cells to levels even lower than those in $K^{b-/-}D^{b-/-}$ mice. The fact that only 33% of low dose PyV-infected B6 mice generate a VP2.139-specific response could indicate that most mice don't even have Q9/VP2.139-specific CD8 T cells in their naïve repertoire (although this is unlikely based on data below). An easy way to resolve this would be to use the dual Q9/VP2.139 tetramer staining method, described above, to compare the naïve VP2.139-specific CD8 T cell repertoires of B6 and $K^{b-/-}D^{b-/-}$ mice. Attempts to boost the VP2.139-specific CD8 T cell response in B6 mice (described below) may also shed light on the frequency of mice that have these cells in their naïve repertioire.

Q9/VP2.139-specific CD8 T cells also have to compete with PyV-specific class Ia-restricted CD8 T cells for access to APCs as well as cytokines. It's possible that during a high dose PyV infection the class Ia-restricted CD8 T cell response clears Ag-bearing APCs in the lymph nodes before VP2.139-specific CD8 T cells have a chance to become activated. In support of this, John Harty's group has shown that class Ia-restricted CD8 T cells prevent the secondary expansion of the H2-M3-restricted response

to *Listeria* by killing Ag-presenting DCs (139). During PyV infection, the kinetics of PyV expansion in both high and low dose-infected B6 mice are the same, however the dominant class Ia-restricted anti-PyV CD8 T cell response is delayed by a couple of days in low dose-infected mice (102). This could give the VP2.139-specific CD8 T cells time to locate Ag-loaded APCs and proliferate. Adoptive transfer experiments using VP2.139-specitic CD8 TCR transgenic cells or clones could be used to determine the kinetics of VP2.139 Ag presentation during high dose PyV infection in B6 mice. On the other hand, perhaps the delay in class Ia-restricted response during low dose PyV infection is beneficial to the VP2.139-specific CD8 T cells because weak TCR signaling necessitates a requirement for prolonged TCR stimulation in order for VP2.139-specific cells to proliferate and differentiate into effector cells.

Recent data from our lab has shown that bystander inflammation during PyV infection negatively impacts the fitness of PyV-specific CD8 T cells (102). PyV-specific class Ia-restricted CD8 T cells generated after low dose PyV infection produced more IFN-γ on a per cell basis and differentiated into longer-lived memory cells than those primed during high dose PyV infection. It's possible that the environment created during a low-dose infection is more conducive for VP2.139-specific cell activation and proliferation. Using peptide-loaded DCs to induce a primary VP2.139-specific CD8 T cell response could be another way to avoid the detrimental affects of inflammation as well as avoiding competition with class Ia-restricted CD8 T cells.

One of the fundamental goals of these studies is to understand what role Q9/VP2.139-speicific CD8 T cells have in the overall immune response to PyV in wild type mice. Although these cells are not detected during the acute phase of the response in

high dose PyV-infected B6 mice, I have identified large frequencies of VP2.139-specific

CD8 T cells in some high dose PyV-infected mice at 8-12 months p.i. (unpublished

observations). Because VP2.139-specific cells specifically recognize an epitope derived

from a PyV capsid protein, perhaps their role is to contain active PyV replication during

persistent PyV infection. PyV VP1 protein transcripts can be detected in persistently

infected mice indicating that productive viral infection most likely still occurs at late

timepoints (88). Considering that VP2.139 is the only CD8 T cell epitope derived from a

PyV structural protein that we have identified, VP2.139-specific cells could be very

important for containing PyV during persistent infection. It will be interesting to

examine whether mice that mount a VP2.139-specific CD8 T cell response during

persistent infection have lower virus levels compared to mice lacking these cells.

Additionally, I have only looked for VP2.139-specific CD8 T cells in the blood, spleen,

and lungs of persistently infected B6 mice. Perhaps there are higher frequencies of these

cells in other tissues that harbor high levels of persistent PyV such as the kidneys or

salivary gland. Finally, comparing virus levels in wild type PyV (A2)-infected B6 mice

to VP2.139 epitope-null PyV (A2.H145A)-infected B6 mice during persistent infection

could reveal the importance of VP2.139-specific CD8 T cells in these mice, as A2.H145-

infected $K^{b-/-}D^{b-/-}$ mice have higher virus levels in the kidneys compared to $K^{b-/-}D^{b-/-}$ mice

infected by A2 virus.

Because Q9 is a nonpolymophic molecule, VP2.139-specific CD8 T cells could

theoretically be generated in all PyV-infected mice that express Q9, regardless of their

MHC haplotype. From this standpoint, one could envision developing a VP2.139

epitope-based vaccine that could protect mice from PyV infection across haplotype

barriers. However, because only 33% of B6 mice generate a Q9/VP2.139-specific CD8 T cell response, increasing the number of responding mice is essential to developing a protective vaccine. I have taken advantage of two recent reports to develop a potentially successful strategy for boosting the frequency of B6 mice that generate a VP2.139-specific CD8 T cell response. First, Chris Norbury's group has shown that treatment of a recombinant vaccinia virus containing a novel CD8 T cell epitope with a combination of psoralen and UV irradiation before infection can shift the immunodominance of the CD8 T cell response away from the vaccinia proteins and towards the inserted epitope (194). This is because psoralen randomly integrates into DNA and induces basepair crosslinking when activated by UV irradiation. Because of their larger size, full protein genes will be at more risk for crosslinking that inserted transgenes. A second report has shown that treatment of both LCMV and influenza-infected mice with an agonist antibody to the CD8 T cell costimulatory molecule 4-1BB is highly effective at boosting the number of antiviral CD8 T cells (195). Putting these two methods together, I infected B6 mice with psoralen-UV treated VV-VP2.139 virus and then treated the mice with α4-1BB antibody. Although I was unable to detect VP2.139-specific CD8 T cells after this treatment, when the mice were rechallenged with high dose PyV one month later, 3 of 3 mice generated detectable VP2.139-specific CD8 T cell responses (unpublished observations). Not only do these data indicate that we may have a useful strategy for increasing the number of mice that generate VP2.139-specific CD8 T cells, but they also demonstrate that VP2.139-specific cells exist in the naïve CD8 T cell repertoire of most mice. Additionally, identifying which costimulatory molecules are expressed by VP2.139-specific cells, could be useful for developing alternative boosting strategies.   Moreover,

these results warrant experiments with other CD8 T cell boosting methods, such as recombinant IL-7 and IL-15 treatment which has been shown to selectively increase subdominant CD8 T cell responses (196).

Although Q9 is a mouse-only molecule, these studies provide a framework for class Ib-restricted CD8 T cell antiviral immunity in humans, who have several peptide-binding MHC class Ib molecules that can engage TCRs (103). The human class Ib molecule HLA-E has been shown to bind a number of microbial peptides as well as serve as ligands for several different pathogen-specific CD8 T cells (144). HLA-E-restricted, CMV-specific CD8 T cells are readily isolated from PBLs of seropositive patients. Additionally, when PBLs of some patients latently infected with TB are expanded in culture, the majority of the anti-bacterial CD8 T cells are HLA-E restricted (122). These studies merit a search for HLA-E or other class Ib-restricted epitopes in other viral or bacterial infections. Considering their limited polymorphism, a vaccine targeting class Ib-restricted CD8 T cells could theoretically protect more people than a vaccine targeting CD8 T cells restricted by the highly polymorphic class Ia molecules. Therefore, the studies presented in this dissertation not only implicate a novel subset of CD8 T cells in antiviral immunity, but open doors for new strategies in CD8 T cell vaccine design.

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