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Investigating the Requirements for Expansion and Maintenance of a Mouse Polyomavirus-specific MHC Class Ib-Restricted CD8 T Cell Response

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# Investigating the Requirements for Expansion and Maintenance of a Mouse Polyomavirus-specific MHC Class Ib-Restricted CD8 T Cell Response

By

Amelia Ruth Hofstetter
B.S., Berry College, 2007

Advisor: Aron E. Lukacher, M.D., Ph.D.

An abstract of
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## Abstract

Investigating the Requirements for Expansion and Maintenance of a Mouse Polyomavirus-specific MHC Class Ib-Restricted CD8 T Cell Response

By Amelia Ruth Hofstetter

Major Histocompatibility Complex (MHC) class I molecules present intracellular peptides to CD8 T cells to mediate pathogen surveillance. In contrast to the highly polymorphic MHC class Ia molecules, the non-classical MHC class Ib proteins are oligomorphic or monomorphic. It is increasingly apparent that MHC class Ib molecules can participate in antigen presentation to CD8 T cells. If CD8 T cells can recognize an identical peptide presented in an MHC class Ib molecule in allogenic hosts, this would allow for safe, cost-effective peptide-based vaccines which could induce CD8 T cell immunity in an outbred population.

We recently identified a class Ib-restricted CD8 T cell response specific for an epitope from the VP2 capsid protein of mouse polyomavirus (MPyV). The VP2.139-147 peptide, presented on Q9, a class Ib Qa-2 family protein, is protective against MPyV in class Ia-deficient mice ($B6.K^{b-/-}D^{b-/-}$). MPyV infection of $B6.K^{b-/-}D^{b-/-}$ mice initiates a 3-month long inflation of Q9:VP2.139-specific αβ CD8 T cells. This is followed by antigen-independent maintenance of the clone, with no defined contraction. It is unclear what factors drive the long inflationary kinetics or the antigen-independent maintenance of the Q9:VP2.139-specific CD8 T cells. We hypothesized that, parallel with the requisite for antigen, the Q9:VP2.139-specific response would depend on CD4 help and CD28/CD40L costimulation for expansion, but not for maintenance. However, we found that although CD4 help and autochthonous CD28 costimulation are required for expansion, CD4 help continues to be required for maintenance.

Because Q9 is non-polymorphic, we hypothesized that a Q9:VP2.139-specific CD8 T cell response could be generated in MHC allotypic $Q9^{+}$ mouse strains. Mice of the $H\text{-}2^{s}$ and $H\text{-}2^{g7}$ haplotypes are $Qa\text{-}2^{+}$. We were able to elicit Q9:VP2.139-specific CD8 T cells in mice of both haplotypes after a prime-boost regimen with VP2.139 peptide. We demonstrate that Q9:VP2.139-specific CD8 T cells are protective against MPyV in MHC class Ia allogenic mice. These results imply that class Ib MHC molecules can contribute to protective immunity in mice of different MHC haplotypes.

# Investigating the Requirements for Expansion and Maintenance of a Mouse Polyomavirus-specific MHC Class Ib-Restricted CD8 T Cell Response

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# Chapter 1

# Introduction

Section III was originally published in Current Opinions in Immunology: Hofstetter, A.R., L.C. Sullivan, A.E. Lukacher, and A.G. Brooks. 2011. *Diverse roles of non-diverse molecules: MHC Class Ib molecules in host defense and control of autoimmunity*. **Curr**. **Opin**. **Immunol**. **23**: 104–110. 
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# I. The role of CD8 T cells in immune surveillance for intracellular pathogens.

The immune system is the network of organs, cells and proteins which guard a multicellular organism from foreign invasion. Bodies are wet, climate-controlled and nutrient-rich, constituting an attractive niche for smaller organisms. Indeed, we play host to up to $10^{12}$ bacteria per gram of intestinal contents, comprised of around 1000 different species (1). Extended to the other surfaces of the body these numbers can reach $10^{14}$ bacteria of 2000 different species (2). These symbionts may exist as commensals or mutualists, but there is a fine balance between hosting these benign colonists and preventing invasion by parasites. Extra individuals of the bacterial, fungal, viral or macroparasitic types are always looking for entry into the body. It is the job of the immune system to protect this balance, and to keep external symbionts from becoming invaders.

### *A. Innate and adaptive immunity for defense against pathogens.*

All life forms have developed strategies for defense against invasion by other organisms or viruses, which can be broadly classified into innate and adaptive immunity. Innate immunity is characterized by the capacity to discern 'self' from 'non-self' (3, 4) or 'benign' from 'danger' (5) via recognition of conserved molecular patterns, and to effect mechanisms to discourage and destroy non-self intruders. The receptors to distinguish non-self molecular structures are germline encoded, and will generally remain unchanged during the lifetime of the organism (4). No previous encounter with a pathogen is needed to activate innate immune effectors. Each subsequent encounter will elicit the same

response. In contrast, vertebrates have developed adaptive immune responses to supplement innate immunity. Adaptive immunity is characterized by the capacity to remember a specific molecular structure that was dangerous in the past, and respond more rapidly and with a greater magnitude of effector responses upon subsequent exposure to that antigen (6). Adaptive immune receptors are originally germline encoded, but germline recombinations and mutations during lymphocyte maturation lead to highly variable receptors that are virtually random. Innate immunity is important for the rapid destruction of invaders by recognition of conserved, known, foreign structures, while adaptive immunity allows recognition of unknown foreign structures which improves over repeated challenges.

Innate immunity employs pattern recognition receptors (PRRs) specific for pathogen-associated molecular patterns (PAMPs) which are shared broadly among types of pathogens, but not found, or inaccessible, in the host (7). For example, humans and mice express toll-like receptor 4 (TLR-4), which recognizes lipopolysaccharide (LPS) (7, 8). LPS is found in the membrane of gram-negative bacteria (7). Pattern recognition is also utilized by unicellular organisms to recognize foreign DNA. Bacterial restriction enzymes break down DNA at specific nucleotide sequences that are masked by methylation in host, but not foreign, DNA (4). The evolution of multicellular organisms required the development of more advanced innate immune mechanisms, including cytotoxic cells and phagocytes (3). Cytotoxic cells secrete extracellular proteins or reactive oxygen species to kill other cells (3, 9). This mechanism of killing is utilized by cells of both the innate and adaptive immune systems. In contrast, phagocytic cells kill by engulfing microbes whole, akin to amoeba. Through the use of PRRs they target

pathogens rather than self, and secrete effector cytokines which alert other cells to the danger (3). PRR-bearing phagocytic cells, in the form of dendritic cells (DCs) and macrophages, are critical components of the vertebrate immune system, mediating the intersection of innate and adaptive immunity. The innate immune system is a front-line of defense that acts with immediate and nonspecific action in response to foreign PAMPs.

In contrast to the recognition of broadly conserved PAMPs, the adaptive immune system is highly specific. The adaptive immune system in mice and humans is mediated by T cells and B cells. The antigen receptors of both T and B cells are randomly generated, so they can recognize a virtually infinite range of antigens. The antigen-binding subunit of the B cell receptor is immunoglobulin (Ig) (10). Mature B cells of the plasma cell variety can secrete Ig into the serum in the form of antibodies. The T cell receptor (TCR) is not secreted. Humans are estimated to have an antibody repertoire exceeding $10^{11}$(11), and the potential diversity of TCRs is higher than that of Igs (11). However, adaptive immune cells have no intrinsic system to determine whether they are bound to a self- or pathogen-antigen. To prevent autoimmunity, naïve adaptive immune cells cannot respond to antigen without exogenous signals. Once they have been activated, lymphocytes undergo multiple rounds of division, and differentiate into effector cells (12). At this point, they have acquired the ability to rapidly respond to antigen stimulation, and they contribute to antigen clearance. After antigen has been cleared from the host, they then contract to a memory population, still significantly higher than the naïve precursor population (12). The memory cells are capable of responding rapidly, with better effector capacity, and with higher magnitude the next time they encounter antigen. Therefore, while the highly specific cells of the adaptive immune

response cannot participate in front-line defenses during the first encounter with an antigen, they act specifically and rapidly upon rechallenge.

### *B. Thymic selection of CD8 T cells*

The antigen receptors of lymphocytes are generated through germline rearrangements. Lymphocytes arise from the hematopoietic lineage in the bone marrow (13). Early thymic progenitors traffic to the thymus (13) driven by expression of the thymic homing marker, CD44 integrin (14) and the CC-chemokine receptor 9 (CCR9) (15). Thymocytes lack expression of either CD4 or CD8 at this stage, and so are known as double negative (DN) cells. As the thymocytes migrate through the distinct microenvironments of the thymus, they gradually become more committed to the T cell lineage, and less plastic. Cells that reach the subcapsular zone of the thymus have downregulated CD44 expression. At this stage the thymocytes, known as DN3 cells, stop proliferating and begin rearranging the TCR genes (15).

The vast diversity of the TCR stems from a process of reassorting germline-encoded fragments of the TCR genes to make a contiguous coding region. In the germline, the genes encoding the α and β chains of the TCR are broken up into a series of variable (V) and joining (J) coding regions upstream of constant (C) regions. The TCR β genes also include an extra diversity (D) region between the series of V and J segments. In a somewhat stochastic process, the recombination activating gene (RAG) complex directs DNA cleavage between a particular V and J, or V, D, and J segment(s). The non-homologous end-joining DNA repair process may add or delete more nucleotides as it re-

anneals the DNA (16). This complex process is the source of the incredible diversity of the TCR chains.

Because there is no guarantee that these germline rearrangements resulted in a functional product, there are multiple checkpoints to test the signaling capacity of the TCR. The TCR β chain rearranges first, and is paired with a germline-encoded pre-TCR-α to stabilize the TCR for surface expression, along with the CD3 proteins to mediate signaling. If the rearranged β chain encodes a product sufficient for surface expression and signaling, the DN3 cells will go through 6-8 cell divisions, before beginning to rearrange the TCR α chain. An α-chain successfully rearranged in-frame will replace the pre-TCR-α. More cell divisions follow, and then both the CD8 and CD4 coreceptors are expressed on the cells (17). $CD8^+$ $CD4^+$, or double positive (DP), thymocytes are tested for reactivity to self antigens (17, 18). T cells that fail to respond to self antigen will ultimately be deleted, because they must be able to sense a self major histocompatibility (MHC) molecule to be effective. MHC molecules are surface proteins that bind exogenous or endogenous peptides and present them for TCR recognition (19). DP thymocytes are exposed to self peptides presented on MHC molecules of the thymic epithelium (17) while in the cortex of the thymus. Ninety percent of DP cells die at this stage from a failure to transmit any signals through the complex of TCR αβ and CD3 (17). The process of TCR gene rearrangement generates many products that are non-functional.

DP cells that do have functioning TCR chains are subjected to positive and negative selection. As outlined in a recent review by Singer et al., it has been proposed that DP cells undergo positive selection simultaneously with CD4/CD8 lineage choice.

Proper coreceptor choice is important because the interaction of the coreceptor with the same peptide in MHC (pMHC) initiates the signaling of the TCR/CD3 complex, as well as increasing the overall avidity of this ternary complex (20). DP cells that receive a strong enough signal through their TCR complex will first downregulate *CD8* gene expression. If the signaling is unabated, it indicates that the CD4 coreceptor is contributing to the signaling of the TCR complex. CD4 binds MHC class II, and in doing so, brings the protein tyrosine kinase Lck to the TCR signaling complex. This initiates a TCR signal, and the cell will commit to the CD4 lineage. If, instead, signaling is abated after downregulation of *CD8*, the IL-7R is permitted to transmit survival signals to the thymocyte, and trigger co-receptor reversal. This turns off *CD4* gene expression and turns on *CD8* expression. CD8 binds MHC class I, and also recruits Lck to the TCR complex. With Lck triggering the TCR signal, the cell will become CD8 lineage-committed (21). In the kinetic signaling model, coreceptor choice is a dynamic process that is concurrent with positive selection.

For the last stage of selection, the CD8- or CD4- single-positive cells migrate to the thymic medulla. Here they interact with thymic dendritic cells, which display co-stimulatory molecules as well as peripheral self antigens (11, 22). In the process of negative selection, T cells that respond too strongly to self antigen will be deleted (17), because they are responding to both the MHC molecule and the self antigen it is presenting, making it a self-reactive T cell (18). Ideally T cells should recognize self MHC, but not self peptides. The process of thymocyte selection has been likened to the classic story of Goldilocks (23), as the T cell with an intermediate affinity for self peptides presented in self MHC is favored for survival. Such T cells are likely to respond

strongly to an unknown foreign peptide presented in self MHC. This extensive selection process ensures that circulating CD8 T cells can distinguish self from foreign peptides presented in self MHC (22)

### *C. The interplay of innate and adaptive immunity in T cell activation*

Despite extensive thymic selection, autoreactive T cells do escape the thymus (24). A series of checks and balances are in place to ensure naïve T cells respond only to non-self antigens. A naïve T cell requires three signals to achieve full activation – simple recognition of pMHC is only the first signal. The other two signals are costimulatory proteins and cytokines, including interleukin (IL)-12 or type I interferon (IFN-α/β) (25, 26). Costimulation and cytokines will only occur with pMHC in the context of an activated antigen presenting cell (APC) (25). A healthy cell should not express costimulatory molecules or secrete activating cytokines. This prevents a naïve T cell from reacting to a pMHC on a normal, uninfected cell.

APCs, particularly DCs, are phagocytic cells that are uniquely equipped to activate the adaptive immune response (27). DCs take up microbes, dead cells, or pathogenic proteins through phagocytosis, pinocytosis, and endocytosis (28). Digested proteins are presented as oligomeric peptides on MHC class I and MHC class II molecules (18). However, a naïve DC is poor at antigen presentation due to low levels of MHC class II and lack of costimulatory molecules (29). DC activation can be triggered by interaction of a PRR with a PAMP (28, 30), or recognition of a danger signal, which may include cell stress or tissue damage (5, 31). DC activation leads to lymph node homing. En route, the cell matures, upregulating MHC class II and costimulatory

molecules (29). CD80 and CD86 are important costimulatory molecules expressed on activated DCs in the mouse (29). DCs arrive in the lymph node with CD80 and 86 associated with MHC class I and class II on the cell surface (30). In the activated state, APCs provide all three activation signals, antigen, costimulation and cytokines (25), that are required to activate a naïve T cell specific for any of the antigens presented on the APC's MHC molecules. Thus, T cells are only activated by APCs that have sensed a danger signal, and are also presenting MHC-bound peptide recognized by their TCR. APCs couple danger signals and antigen presentation to selectively activate pathogen-specific T cells.

Two of the best-characterized costimulatory signals needed to prime CD8 T cells are transmitted by cross-linking CD28 or CD40L on the surface of the CD8 T cell. CD28 cross-linking is considered the strongest costimulatory signal (32). CD28 binds both CD80 and 86, expressed on activated DCs. Ligation of CD28 promotes survival and expansion of CD8 T cells by providing the critical second signal needed for their activation (33). In contrast, CD40 signaling can be a direct or an indirect signal for CD8 T cells (34). The study of CD40:CD40L interactions initially focused on CD40 expressed on DCs and B cells interacting with CD40L on CD4 T cells (34). In this model, CD40:CD40L interactions were only important to CD8 T cells indirectly. DCs interacting with activated CD40L-expressing CD4 T cells could then activate CD8 T cells in a CD40-independent manner. However, recent reports suggest that CD40 can be directly ligated on a CD8 T cell, either by CD4 T cells directly or by CD40L upregulated on a TLR-activated DC (34, 35). Although there are other costimulatory proteins that can

influence CD8 T cell activation, CD28 and CD40L represent the strongest and most well characterized of these molecules.

The power of CD40 and CD28 signaling in activation of cytotoxic T cells (CTL) is such that blockade of these signaling pathways is an important goal in the field of transplant immunology. Co-blockade of CD40L and CD28 during the time of an allogeneic transplant significantly extended the lifespan of the graft (36). CD40L is blocked with the antibody MR1, preventing the ligand from binding CD40 (37). Blocking CD28 signaling proved to be more complicated. Attempts were made with blocking antibodies for CD80 and CD86, but this strategy was never pursued beyond Phase I clinical trials. Phase I clinical trials of a CD28 agonist antibody unfortunately led to the deaths of six healthy volunteers due to hyperactivation and inflammatory cytokine secretion (38). As an alternative strategy, Lenschow et al. took advantage of the fact that the cell-surface protein CTLA-4 binds to the same ligands as CD28, and with 20-fold higher affinity (38-40). Normally this molecule is upregulated on the CD8 T cell's surface after CD28 signaling. CTLA-4 binds the CD80 and CD86 molecules (39) with a higher affinity than CD28, successfully competing with CD28 for its ligands(41), and therefore modulating the signal. CTLA-4 was engineered into a fusion protein with a human Ig molecule by researchers at Bristol-Myers Squibb. CTLA-4-Ig binds to CD80 and CD86 molecules, preventing them from signaling to CD28 (38, 39). A second-generation CD28 inhibitor based off of CTLA-4-Ig, LEA297 or belatacept (42), displayed improved performance in non-human primate models (38). Although MR1 and CTLA-4-Ig blockade have not yet translated to humans (38, 43), these reagents still serve as powerful tools to dissect the signaling requirements of novel CD8 T cell populations.

### *D. The different roles of $CD4^+$ and $CD8^+$ T cells in immunity against pathogens*

The two types of T cells, $CD4^+$ and $CD8^+$, are important in maintaining the sterility of two discrete spaces in the body, the intracellular and extracellular spaces. CD4 selectively binds MHC class II molecules. MHC class II is only expressed on APCs. Through interaction with MHC class II on DCs, macrophages and B cells, activated CD4 T cells direct various arms of immunity. CD4 T cell recognition of antigen on a DC allows for the interaction of CD40L on the CD4 T cell and CD40 on the DC (44-47) . This will activate the DC and enhance CD80 and 86 expression, along with other costimulatory molecules, including CD70, the ligand for CD27 (48). This CD4-dependent mechanism of DC activation is termed 'licensing', and is especially important in situations where the pathogen does not cause inflammation (49). CD4 T cells recognizing antigen on a macrophage can direct the macrophage to kill intracellular bacteria which have invaded the macrophage (11). If B cells present a foreign epitope on MHC class II, it indicates that the BCR has bound up and internalized an antigen. A mature CD4 T cell can activate a naïve B cell if it identifies is cognate pMHC on the B cell surface (11). Through all these mechanisms and more, CD4 T cells help the immune response.

The extracellular space is patrolled by phagocytic cells (50), and bathed in antibodies which can bind up antigens (51), among other defenses. However, these mechanisms of protection are blind to intracellular spaces, because they cannot cross cell membranes. It is primarily the role of CD8 T cells to maintain the sterility of intracellular spaces. CD8 T cells probe the inside of cells by recognition of a peptide presented on MHC class I molecules (18). MHC class I proteins are expressed on every

nucleated cell in the body. These proteins bind 8-11 amino acid peptides in the endoplasmic reticulum (ER) before trafficking to the cell surface (18, 52). Importantly, even uninfected cells will present peptides on MHC class I, digested from the misfolded self proteins which are the result of 30% of the total cell protein biogenesis (53). Peptides are continually generated by digestion of cytosolic proteins in a large protease complex called the proteasome. However, IFN-γ promotes the synthesis of three proteasome subunits, β1i/LMP2, β2i/MECL1 and β5i/LMP7. These subunits alter the cleavage site of the proteasome to promote the generation of pathogen-specific epitopes. IFN-γ also drives expression of the PA28 protein complex, which increases the efficiency substrate exit from the proteasome. With the addition of these IFN-γ-mediated proteins, the complex is called the immunoproteasome. DCs express immunoproteasomes even when naive, although immunoproteasome and PA28 expression are upregulated with DC maturation (54). Peptides from the immunoproteasome or proteasome are shunted back into the ER by the transporter associated with antigen processing (TAP) proteins (54). There, peptides bind newly synthesized MHC class I for transport to the cell surface. Therefore, MHC class I molecules on a cell surface act as a window to display which proteins the cell is making. Constant presentation of self-peptides indicates the cell is actively synthesizing new proteins (53). When a foreign peptide is presented, it reveals that the cell is sick (55). In the case of infection, this means that an invader has taken over the cell's machinery for protein manufacturing, and that the cell needs to be destroyed to halt parasitic growth. By recognition of cellular peptides bound to MHC class I molecules, CD8 T cells are able to monitor intracellular spaces.

Although MHC class I is responsible for presentation of intracellular proteins, the DCs which are required to prime CD8 T cells may not be infected by pathogen that a CD8 T cell must target. However, DCs use a strategy called cross-priming to load exogenous antigens onto MHC class I. Several models have been suggested as to how an antigen from the extracellular space reaches the ER lumen (56). An exogenous protein located in the endosome or phagosome must be degraded and interact with class I molecules. Degradation has been linked to the cytosolic proteasome (57), although in some cases cross-priming is independent of the proteasome (56). It is unclear which transporters transfer endosomal proteins to the cytosol for proteasomal processing, although one group has recently published evidence that heat-shock protein 90 mediates this translocation (58). Exogenous peptides processed by proteasome are likely transported back into the ER by TAP. TAP along with other peptide-loading proteins has been found expressed in phagosomes and endosomes, so it is possible that peptides might be loaded onto MHC recycled from the cell surface in an ER-independent fashion. Multiple mechanisms may operate under different circumstances to load exogenous peptide onto MHC class I (56). Whatever the mechanism, cross-priming allows a DC to prime CD8 T cells with proteins sampled from the extracellular space.

When activated CD8 T cells recognize a foreign peptide bound to MHC class I, they can respond in several ways. CD8 T cells can release cytokines and chemokines which have a number of effects, including recruitment and activation of other cells in the immune system (59), direct antiviral activity (60), and other direct effects on pathogens. To kill a cell they can release cytotoxic granules, or trigger death through cell-surface death receptors. When a CTL degranulates, cytotoxic proteins (perforin, granzyme (59)

and granulysin (9)) are released into the space between the T cell and the infected cell (9). These proteins breach the infected cell and trigger apoptotic cascades (9). For example, granzyme B can directly activate caspase 3, which cleaves DNA , cytoskeleton, or important nuclear membrane proteins. Granzyme B also destroys mitochondrial protein Bid, releasing cytochrome *c* from the mitochondria. Cytosolic cytochrome *c* leads to the formation of apoptosomes and activation of caspase 9, which then contribute to the activation of the downstream caspases, like caspase 3 (9). Alternatively, by upregulating FasLigand on their cell surface, CTL can trigger trimerization of Fas on the surface of infected cells (61). Fas-associated death domain (FADD) proteins are recruited to the cytoplasmic domains of trimerized Fas. Procaspase 8 or 10 are recruited to the FADD proteins, and self-cleave into the active state. The complex of Fas, FADD and caspase 8 forms the death- inducing signaling complex (DISC). DISC formation promotes the proteolysis of procaspases 3, 6 or 7. Caspase 8 also destroys Bid, releasing cytochrome *c*. Thus, both granule release and Fas trimerization trigger caspase-mediated apoptotic cascades, leading to apoptosis of the target cell.

### *E. The formation of a CD8 T cell memory population in response to acute infection.*

During the course of an archetypical immune response to an acute infection, naïve cells must expand 2- to 10-fold (18) from only 10-1200 naïve T cells (62, 63) to as many as $10^7$ CTL (64). Each activated cell can therefore give rise to upwards of $10^4$ progeny (65). A brief exposure to antigen and costimulation triggers a developmental program that drives several rounds of division (66). However, antigen and costimulation need to be available for 24 hours to promote maximal expansion, and cytokine signals need to be available from 20 to 60 hours after activation to promote maximal survival and cytokine

effector function (25). Besides CD27, other tumor necrosis factor receptor (TNFR) family members can promote survival of a CD8 T cell clone, including OX-40 and 4-1BB (67). 4-1BB has been shown to provide maximal support when administered 5 days post-infection, although it inhibits expansion when administered in the first few days p.i. (68). Expansion consists of as many as 19 divisions (12, 62) at 2-8 hours per division (67). At the peak of the CD8 T cell clonal burst, which is around day 8 for the model viral infection lymphocytic choriomeningitis virus (LCMV) (64, 69), virus-specific cells of a single specificity can make up 50% of the splenic CD8+ T cell population.

At the peak of expansion, the antigen-specific CD8 T cell clone is heterogeneous. Cells expressing low levels of the IL-7R (CD127) but high levels of KLRG-1, a marker of division history, are short-lived effector cells (SLECs), and are programmed to contract rapidly after the infection is cleared. The other major population is $CD127^{hi}$ and $KLRG\text{-}1^{lo}$, and these memory precursor cells (MPECs) are programmed to survive through IL-7 and IL-15 signals after antigen clearance (67, 70, 71). Activated CD8 T cells upregulate CXCR3, and downregulate CD62L and CCR7. CXCR3 is a chemokine receptor that drives CTL to move into infected tissue (72). CD62L, or L-selectin, and CCR7 are expressed on naïve T cells and a subset of memory T cells. L-selectin and CCR7 allow T cells to enter lymph nodes and other lymphoid organs (73, 74). Therefore, increasing CXCR3 and decreasing CD62L allows CD8 T cells to move out of the lymphoid organs and into the periphery, to combat infection or malignancy. After antigen clearance, CD8 T cells contract via apoptosis to only 1-10% of their maximum number (6, 18), stabilizing at a memory setpoint at 5-10% of the maximal expansion (75).

For a memory response that peaks at 20 x $10^6$ cells, the memory pool is 1 x $10^6$ cells (64). These cells are known as 'memory cells'.

Memory cells are similarly hetergeneous. Differential expression of CD62L and CCR7 defines two memory populations with different physiological niches. $CD62L^{hi}CCR7^{+}$ cells are called 'central memory' T cells ($T_{CM}$), as they are localized to the lymphoid organs. $CD62L^{lo}CCR7^{-}$ cells are localized to the periphery and are called 'effector memory' T cells ($T_{EM}$). $T_{EM}$ are so called because of their increased ability to immediately lyse a target encountered in the periphery, a function that is unavailable to $T_{CM}$ (75). It has been demonstrated that the activation status of the memory CD8 T cells is a better gauge of recall capacity than homing markers, with cells displaying a less active $CD27^{hi}$ $CD43^{lo}$ phenotype showing improved recall over memory cells with more activated, and in some cases, senescent phenotypes (76). However, in a different model system, CD27 and KLRG-1 phenotype was correlated only with the level of inflammation during priming (77). This is supported by Sarkar et al.'s demonstration that increasing KLRG-1 expression denotes a longer exposure to stimulation, which is inversely correlated to the ability to differentiate into a long-lived memory cell (78). The relevant biomarkers denoting the best memory recall response vary with the type of infection, which is likely due to the vast differences in inflammation length and intensity between models. There continues to be active interest in identifying proteins which denote a 'more fit' memory cell, because expanding this population is the target of vaccinations.

The memory recall capacity of lymphocytes is a vital characteristic of a functional adaptive immune response. Unlike the primary response, the memory CD8 T cell has

already received signals linking its cognate peptide to a danger signal, so no further validation is needed (18). Memory CD8 T cells can proliferate to higher levels after antigen rechallenge and kill infected targets without costimulation (79). For example, in an allogenic tumor challenge model, memory CD8 T cells can respond to a tumor in 12 hours as compared to 5 days for the primary response (80). LCMV-specific memory CD8 T cells require only 3 hours of in vitro stimulation to produce cytokines, as compared to more than 12 hours for naïve CD8 T cells (81). In illustration of the relative autonomy of memory CD8 T cells compared to naïve T cells, there is evidence that memory CD8 T cells can proliferate and secrete cytokines independently of antigen, in response to cytokine signals (82). The increased proliferation and effector functions of CD8 T cells allow for swifter control of a pathogen during repeated exposures.

CD8 T cells are critical effector cells of the immune system. Through recognition of MHC class I, these cells monitor real-time peptide sampling. Because of this, they can quickly recognize foreign protein products and respond by killing the infected cell and/or recruiting other immune effector cells. A naïve CD8 T cell must first see its cognate pMHC in the context of costimulation and cytokine signals, divide and proliferate, and then it can exert effector activities upon infected cells. After the infection is controlled, most of the progeny CD8 T cells will contract to a stable memory population, which is ready to respond quickly to a second challenge.

## II. The Mouse Polyomavirus Model

Polyomaviruses (PyVs) were first discovered in 1953. Two groups (83, 84) published on a filterable agent from mouse leukemic extracts which could cause salivary gland tumors

in mice. After the discovery of Simian Virus 40 (SV40) PyV in 1960 (85) , the small size of the PyV double-stranded genome made it an important model for investigating the replication of double-stranded DNA genomes, as well as certain transcriptional and cell-cycle mechanics (86). PyVs are becoming increasingly relevant in the clinic, with 9 novel human PyVs discovered since 2007 (87). Some of these new PyVs are associated with upper respiratory tract infections, skin diseases and cancer (88). Serological surveys indicate that human PyV infection often occurs in childhood, and 40 to 90% of humans have seroconverted to each of 5 different PyVs by adulthood (89). Furthermore, PyV-associated pathologies affect increasing numbers of patients, due to either immunosuppressive regimens or AIDS (90, 91). These new clinical findings underscore the need to better identify and characterize these prevalent human pathogens.

### *A. PyV Virology*

PyVs are small, non-enveloped viruses, approximately 40-45 nm in diameter (88). Their compact, economical genome consists of around 5 kbp of closed circular double-stranded DNA (85, 86, 88, 92) (Figure 1.1). Both strands of DNA encode multiple proteins in overlapping reading frames (93). Transcription occurs bidirectionally from a single origin of replication. 450 bp of noncoding DNA flanks the origin, which includes early and late promoters (94). It is important to note that this region also encodes enhancers that could theoretically bind up to 30 different cellular transcription factors, allowing the virus to replicate in a wide variety of host tissues (95). In mouse PyV (MPyV), the early promoter drives transcription of a single species of precursor RNA. Differential splicing of this RNA generates transcripts for three nonstructural proteins. These Tumor (T) antigens (85), small T, middle T and large T (94), are so called due to

their expression in PyV-induced tumors (85). The T antigens have multitfarious functions within a cell, including regulation of cell cycle (94) and initiation of late promoter transcription (88).

The late promoter drives transcription of an RNA encoding three structural proteins: viral protein (VP)1, VP2 and VP3. VP1 makes up 80% of the assembled virion protein (86), and has the capacity to self-assemble in vitro into various structures, including the native viral shell (93). In vivo, cellular chaperones, including heat shock protein 70, catalyze the appropriate assembly of 360 copies VP1 in 72 pentamers into an icosahedron (93, 96). The underside of each VP1 pentamer forms a cavity which is occupied by either VP2 or VP3 (96). VP2 and VP3 are translated in frame, but VP2 is lengthened with unique N-terminal sequences including a myristoylation (93, 96). PyV makes economical use of a minimal genome by using overlapping reading frames and differential splicing.

Upon infection of a cell, viral uncoating appears to occur via several degredative steps (97). The outer surface of VP1 from most polyomaviruses binds the α2,3- or α2,6-linked sialic acid from specific gangliosides or glycoproteins on target cells. The preferred ganglioside(s) vary with the virus. At least some PyVs undergo a conformational change after binding sialic acid, which allows them to bind a co-receptor. In the case of MPyV, the co-receptor is α4β1 integrin (97). Once bound to the cell, the virus initiates endocytosis. Endocytosis is usually mediated by caveolae or an alternate cholesterol-dependent pathway. Irrespective of the mechanism of endocytosis, the virus eventually traffics to a caveosome. In a microtubule-dependent manner, long tubular structures carrying the virion extend from the caveosome and fuse with the endoplasmic

reticulum (ER) (97). In SV40, a monkey polyomavirus, VP2 appears to facilitate trafficking of the viral particle to the ER (98). Within the ER, host protein disulfide isomerases destabilize the capid by cleaving the protein tail mediating interactions between VP1 pentamers (93, 97). The virion is then treated as a native misfolded protein, and shunted out of the ER by retrotranslocation mechanisms (97). In SV40, VP3 participates in viral exit from the ER into the cytosol (98). A 1000-fold drop in calcium concentration upon entry to the cytosol is thought to further destabilize the calcium-dependent capsid structure (97, 98). This reveals nuclear localization signals on VP1, VP2 and VP3 which ferry the viral genome to the nucleus through the nuclear pore (99). The PyV structural proteins are critical for delivery of the viral genome, coiled in the histones of its previous host cell (93), to the nucleus.

The activities of the three nonstructural proteins allow efficient takeover of the cell's cycle. Each of the nonstructural proteins has multiple mechanisms to activate and cause division of the cell (95). Although the three proteins have identical N-termini, the differing C-termini serve to direct the localization of each protein (100). Accordingly, Large T (LT) homes to the nucleus, Small T (ST) to the cytosol and Middle T (MT) to lipid bilayers (100, 101). Most MT is found in the plasma membrane, but it can also be found in other membranes including the ER (101). LT acts as a 3'-5' ATP-dependent helicase to unwind the PyV genome (93, 102) However, PyV contributes no other proteins to the viral replication process (94). In order to usurp the host polymerase, among other members of the DNA replication machinery, PyV must drive the cell into the synthesis phase of the cell cycle. LT promotes the G1 to S transition by binding and inactivating Rb, a tumor suppressor protein (92, 93). Binding to Rb is mediated through

a conserved LXCXE motif which binds the pocket domain of Rb. LT also contains a Psycho domain responsible for modulating the activity of Rb (102). Most PyV LT proteins also bind p53 tumor suppressor, which would otherwise trigger apoptosis after the cell is driven into S phase (100). MPyV LT does not bind p53, and it is unknown how apoptosis is avoided (94). The activities of the other two T antigens may override p53 programming (92).

ST contains a serine-threonine protein phosphatase PP2A-binding domain (100, 103), allowing it to replace the β subunit of PP2A (100, 104). The mechanism is unclear, but this leads to activation of the MAP kinase cascade (104). Transcripts of the AP-1 subunits c-fos and c-jun are upregulated by ST. AP-1 binds the enhancer region of PyV and promotes gene transcription and genome replication (103). Presumably through this pathway, ST stimulates both viral gene transcription and DNA replication (103). Middle T shares the PP2A-binding domain of ST, and so shares the functions of ST. However, the elongated C-terminal end of MT contains 3 major and 3 minor tyrosine residues (103). It also binds and activates members of the src tyrosine kinase family, including c-srk, Yes and Fyn. Src family members phosphorylate the 3 major tyrosine residues of MT. Each of these residues allows docking and activation of another kinase, phospholipase, or adaptor protein, leading to expansive downstream signals. One important downstream effect of MT is the activation of Akt, a serine/threonine protein kinase with multiple effects on survival, differentiation and proliferation (100). The actions of Akt may act in MPyV to prevent apoptosis, despite active p53. The combined actions of the T antigens force the cell to stay alive and cycle, giving PyV time and

resources to replicate. The multiple functions of each T antigen allow this small virus to exert control over the cell with a minimum of gene products.

### *B. PyV Immunology*

PyV infection is persistent in immunocompetent hosts (105, 106). Some chronic infections, such as the human immunodeficiency virus, achieve high titers in the host, because the immune system cannot keep up with the pace of viral replication. Herpesviruses escape from the immune system by shutting down to a minimal transcriptional program and quiescing into a latent state. Such viruses may reactivate at a later time, when the host is immunocompromised (106). In contrast to both of these viral lifecycles, MPyV is thought to persist at a low, but relatively constant level. There is no evidence that it enacts a true latency program (107). It instead appears that the virus undergoes constant turnover, at levels that are tightly controlled by the healthy immune system (107).

In the natural host, PyV completes a productive infection, in which both early and late proteins are translated (108) and production of new virions lyses the infected cell. In rat or hamster cells, which allow MPyV entry but are not natural host cells, a nonproductive infection occurs where the virus fails to replicate (93). In a nonproductive infection, the T antigens continue to be expressed without the structural proteins, and the cell becomes transformed (93). Even in the natural host, a disruption to the LT coding region will lead to nonproductive infection because LT drives the switch between the early and late proteins (109). If the PyV genome integrates into the host DNA, stable transformation will be established (101). This is not to be confused with a latency

program, because the mutations and deletions leading to a nonproductive infection indicate that PyV has irrevocably lost its ability to replicate. Furthermore, there is no evidence that PyV genomes can be excised after integration into the host DNA (107).

In the natural host, PyV oncogenesis correlates with a failure to control viral replication. A T cell-independent neutralizing antibody response decreases the viral load and prevents the acute myeloproliferative disease observed in SCID mice, but does not prevent tumor development (110). Effective control of MPyV infection is mediated mainly by CD8 T cells. This was evidenced by the potent oncogenesis of MPyV in athymic mice (111, 112), T cell deficient mice (110), or $\beta_2m^{-/-}$ mice, which have no class I MHC molecules (113). Furthermore, passive tumor immunity results from adoptive transfer of splenocytes from PyV-infected syngeneic mice (114). Strains of mice lacking MPyV-specific TCR are extremely susceptible to MPyV tumors. This became apparent when differing tumor susceptibilities of mice of the H-$2^k$ haplotype were discovered to be inherited as a simple Mendelian trait in an autosomal dominant fashion (115). It was later discerned that susceptible strains carry the integrated genome of the mouse mammary tumor virus. This provirus encodes a surface antigen which binds the constant region of specific TCR Vβ chains, leading to deletion of any thymocyte carrying that chain during thymic education. This so-called superantigen (SAG) culls all Vβ6 and Vβ8.1-bearing thymocytes. Incidentally, these are the primary TCR Vβ chains needed to recognize MPyV-specific pMHC in H-$2^k$ haplotype mice (116, 117). These studies underscore the critical role of PyV-specific CD8 T cells in controlling PyV oncogenesis.

The classical CD8 T cell response to PyV peaks 1 wk p.i., then contracts 6-fold to a stable memory population (118, 119). Despite detectable viral titers long-term (119,

120), memory CD8 T cells during persistence retain most of their effector function (107). However, CD8 T cells primed during the first month p.i. undergo exhaustion (121), and must be replaced by new thymic emigrants (122). The CD8 T cells primed after the acute phase of infection make up the bulk of the durable memory population (121). CTL killing of MPyV infected cells is FasL and perforin mediated, but dispensable for control of virus (123). Production of IFN-γ, but not TNF-α, is important for control of MPyV (123). Constant recruitment of new thymic emmigrants is important to maintain MPyV-specific CTL during persistent infection.

Mice lacking an appropriate CTL response develop a unique pattern of epithelial and mesenchymal tumors (124). The virus name – 'poly' for variety and 'oma' for tumors - describes the 'polyomavirus tumor constellation' that is observed in susceptible mice infected by MPyV (85, 124). MPyV tumors may consist of cells with either high or low viral genome copy numbers (109). It is likely that this variation is determined by both tissue-specific factors and the location of genome deletions. Epithelial tumors display high copy numbers of unintegrated viral DNA, while this is virtually never observed in mesenchymal tumors. Epithelial tumors host genome deletions in the C-terminal region of LT, or in the capsid proteins, but never in ST or MT. In mesenchymal tumors, viral DNA is presumed to be integrated into the host DNA. Lytic infection is impaired if the double-strand break leading to integration of the viral DNA occurs downstream of the end of the MT coding region. Thus, in epithelial tumors it appears that PyV mutations arise in unintegrated DNA, while in mesenchymal tumors the process of integration disrupts LT (109). Despite the varied genome deletions, all tumors express middle T protein, which drives cell cycling, leading to neoplasia. Middle T is the most

important oncogene, and is "necessary and sufficient for transformation and tumor induction" (94, 125). In a study of tumors generated by MPyV in highly susceptible $SAG^{+}$ C3H/BiDa mice, all tumors had detectable middle T antigen, regardless of the expression of the other T antigens (109). Failure to control MPyV replication leads to the accumulation of mutations or DNA integrations, driving the switch from lytic to nonproductive, oncogenic infection.

### *C. PyV Pathogenesis*

PyVs are species-specific viruses. At least 25 PyVs have been identified in bats, monkeys, orangutans, chimpanzees, cattle, sea lions, and various rodents and birds (88). Despite their name, polyomaviruses are typically noncarcinogenic in their natural host, although MPyV and hamster PyV are exceptions (85). Thus far, PyVs have not been definitively linked to cancers in humans, although there is strong evidence that Merkel Cell Carcinoma (MCPyV) is caused by a human PyV (85, 126). Still, the vast majority of these viruses have a much more benign pathology. This may be the result of a long history of coevolution, suggested by the prevalence of PyVs among vertebrates (106). At least nine different PyVs have been isolated from human tissue to date (87, 88, 102), and it is thought that more will be discovered (85).

PyV infection is usually subclinical in immunocompetent hosts. This low-level persistent infection aids the spread of the virus. However, in an immunocompromised host viral replication is unchecked, and can lead to pathology. Two human PyVs are of major clinical relevance in immunocompromised patients. BK virus is the major cause of kidney rejection (127). When a patient is on immunosuppression following a kidney

transplant, this kidney-tropic virus can expand in the transplant and lead to rejection (127, 128). JC virus has been linked to a demyelinating disease of the central nervous system, progressive multifocal leukoencephalopathy (PML), occurring primarily in patients with AIDS, or those receiving monoclonal antibody therapy promoting $CD4^+$ or $CD8^+$ lymphopenia. Therapies associated with PML risk include rituximab, eflaizumab, and natalizumab (91). Furthermore, MCPyV is associated with immunosuppression (126). It is a testament to the efficacy of constant immune surveillance that immunocompetent hosts can harbor multiple subclinical polyomavirus infections simultaneously.

Despite their low-level persistence, PyVs are normally well-controlled by the immune system, and infections are subclinical. However, with the modern era of public health arose new challenges that bring PyV to clinical relevance for many patients. These challenges include a rise in immunosupression, due both to the HIV pandemic and to the increasing prevalence of immunosuppressive drugs. It is difficult to study these typically silent infections in humans. In point of fact, most of the newly identified human PyVs have only been discerned through random PCR amplification (102, 129, 130). As we face these challenges, the highly characterized MPyV infection of mice provides an attractive model of the host-pathogen interaction between humans and their PyVs.

## III. Diverse roles of non-diverse molecule: MHC Class Ib molecules in host defense and control of autoimmunity

The highly polymorphic “classical” or “class Ia” MHC molecules serve an integral role in adaptive immunity by presenting peptides to cytotoxic T cells. However, there are also a number of “non-classical” or “class Ib” MHC molecules that, while structurally similar to class Ia molecules, frequently have quite distinct functions. Class Ib molecules are

typically far less polymorphic than their classical counterparts, can have a more limited pattern of expression and in some cases bind non-peptide ligands (for a review see (131)). MHC class Ib molecules have a diverse range of functions, including in the presentation of lipid antigens (CD1d) for recognition by natural killer T (NKT) cells, as ligands (MR1) for mucosal-associated invariant T (MAIT) cells and serving as dual ligands for both NK cells and αβ T cell receptors (HLA-E and Qa-$1^b$). A rapidly expanding body of literature highlights the diverse roles played by class Ib molecules in pathogen recognition, virus-induced immunopathology, tumor immunosurveillance and regulation of autoimmunity. Here, we summarize recent key work in these areas.

***MHC Class Ib as Innate Pathogen Recognition Molecules***

Specific MHC class Ib molecules serve as ligands for T cells expressing "semi-invariant" T cell receptors (TCR). These cells operate early in the course of an immune response and may modulate the subsequent differentiation of the adaptive response (131, 132). From this perspective, these MHC Ib-restricted T cells constitute components of the cellular innate immune response and their TCRs may arguably be regarded as pattern recognition receptors.

The non-MHC encoded CD1d molecule can present lipid antigens, such the marine sponge derived alpha-galactosylceramide (α-GalCer), to NKT cells. These cells express a semi-invariant TCR consisting of a fixed TCRα-chain (Vα14 in mice, Vα24 in humans) that can pair with a limited number of TCRβ-chains (Vβ11 in humans, Vβ8.2, Vβ7 or Vβ2 in mice). Interestingly, although the CDR3α of the NKT cell TCR is fixed, the CDR3β regions are highly diverse. Recent evidence suggests that variability in the TCRβ chain of NKT cells may play a role in the specificity for distinct lipid ligands (133,

134). For example, while the CDR2β region is critical for recognition of α-GalCer, both the CDR1β and CDR3β also contribute to recognition of isoglobotriaosylceramide (iGb3). Therefore, it appears that although the NKT cell TCR may have a common mode of docking on CD1d, specific beta chain residues may impart distinct fine specificities for different glycolipids.

Recognition of CD1d by NKT cells can result in the production of both Th1 and Th2 cytokines, as well as various chemokines (135). These factors can then orchestrate the activation of other cells of the immune system, including dendritic cells (DCs), natural killer (NK) cells, and lymphocytes. Although many studies and even clinical trials have utilized α-GalCer to efficiently activate NKT cells, the relative importance of more physiological ligands remains a subject of intense interest. In this connection, pathogen-derived glycolipids such as α-galacturonosylceramide (α-GalACer) from *Sphingomonas* and α-galactosyldiacylglycerol (α-GalDAG) from *Borrelia burgdorferi* have been shown to bind to CD1d and activate NKT cells (136). In addition, TLR ligation during bacterial infections can drive the synthesis of endogenous glycosphingolipids (GSLs), such as iGb3 that can also be presented to NKT cells by CD1d (136-138). Moreover, the repertoire of CD1d-bound ligands may be further modulated during infection as TLR signaling through the MyD88 adaptor molecule blocks lysosomal α-galactosidase A–mediated degradation of iGb3 (139). Taken together, these data are consistent with a model in which stimulation of innate pattern recognition receptors by bacterial products leads to upregulation of iGb3, elevated CD1d ligand density, and activation of NKT cells.

The ability of NKT cells to produce both Th1 and Th2 cytokines makes these cells attractive targets for manipulating adaptive immune responses. Several studies have shown that NKT cells vary in the profile of cytokines produced in response to alterations in the structure of α-GalCer (140-145). Im *et al.* (146) recently reported that derivatives of α-GalCer that drive Th2 responses can directly bind CD1d on the cell surface. In contrast, α-GalCer and derivatives that produce mixed Th1/Th2 responses require lipid transfer factors to load them onto CD1d in an endosome. As a result, these CD1d:glycolipid complexes take longer to appear on the cell surface. Thus the endosomal loading process itself may force association of CD1d:glycolipid complexes with molecules that direct them to lipid rafts within the immunological synapse, whereas glycolipids that directly bind cell surface CD1d are excluded from lipid rafts. This difference in cell surface localization of CD1d may be responsible for the distinct cytokine profiles elicited by different glycolipid ligands.

Another specialized T cell population with a semi-invariant TCR utilizes a fixed TCRα (Vα19-Jα33 in mice and Vα7.2-Jα33 in humans) that paired with a limited repertoire of TCRβ chains (147). These cells, simultaneously identified in mice and humans (148), are found in the circulation and intestinal lamina propria (149), and therefore have been termed 'mucosa-associated invariant T cells' or MAIT cells. MAIT cells are largely $CD4^{-}CD8^{-}$ or $CD8\alpha\alpha^{+}$, are selected in the thymus and seed the intestinal mucosa (150), where they expand upon encounter with B cells expressing the MHC class Ib molecule MR1. Gut bacterial flora is an additional essential determinant for the expansion of MAIT cells in intestinal mucosal tissues, as MAIT cells are absent in germ free mice (149). MR1 is highly conserved genetically and functionally in mammals, with

~90% identity in the predicted amino acid sequences of mouse and human α1-α2 domains and evidence for cross-species TCR-MR1 interaction (151, 152). Earlier work suggested that the MR1-associated ligand may be an α-mannosyl ceramide (153). Based on this, Shimamura tested a series of α-mannosyl ceramide derivatives and found that certain sphingosine glycolipids such as (α-Man)2-PI and α-Man-α-GlcNH2-PI preferentially stimulated murine MAIT cells (154). Interestingly, depending on the type of lipid moiety used, MAIT cells, like NKT cells, variably produced IFN- γ and IL-4, raising the possibility that specific glycolipids could be used to direct MAIT cells in order to skew CD4 T cell responses.

Recent investigations into the intracellular pathways for MR1 antigen uptake and processing suggest that MAIT cells contribute to host anti-bacterial immunity. Antigen presentation by mouse MR1 is independent of TAP and the MHC class I peptide-loading complex, but facilitated by the MHC class II chaperone HLA-DM, and dependent on the MHC class II chaperone Ii. Confocal and cryoimmunoelectron microscopy studies have shown MR1 to be present in late endosomes, raising the possibility that antigens including endocytosed microbial products may be acquired from this compartment (155). Circulating MAIT cells in healthy individuals recognize antigens derived from *Mycobacterium tuberculosis* (*Mtb*) in an MR1-dependent manner, providing evidence that microbial antigens are presented by MR1 (156). Moreover, Le Bourhis *et al*. demonstrated that mouse MAIT cells recognize cells infected by unrelated strains of bacteria and yeast in an MR1-dependent manner and that these T cells mediate protection from certain bacterial infections *in vivo* (157). Taken together, current evidence

implicates MAIT cells as important participants in host microbial immunity of the gut and respiratory mucosa.

***Adaptive Immune Recognition of MHC Class Ib Molecules***

Although a number of MHC class Ib molecules are ligands for receptors expressed by innate immunocytes, recent literature points toward an important role for these molecules in adaptive immune responses to pathogens. Class Ib-restricted αβ TCR-expressing CD8 T cells have been identified as participants in the overall adaptive T cell response in a number of mouse disease models (158-160), and have been shown to be protective *in vivo* in the case of Qa-$1^b$-restricted and H2-M3-restricted responses to *Listeria monocytogenes* (161-163), an H2-M3-restricted response to *Mtb* (164), and a class Ia-independent CD8 T cell response to a mouse γ-*herpesvirus* (165). Swanson *et al.* recently defined a protective antiviral CD8 T cell response directed toward an oligopeptide derived from the mouse polyomavirus (MPyV) presented by Q9, a murine Qa-2 family member (166, 167). As Q9 is nonpolymorphic, we have been able to generate Q9-restricted MPyV-specific CD8 T cell responses across MHC class Ia haplotype barriers (ARH and AEL, unpublished data). The finding that class Ib-restricted CD8 T cells mediate protection against a mouse viral infection will hopefully spur efforts to identify additional microbe-specific class Ib-restricted T cell responses in humans that might be effective across a range of different HLA haplotypes.

The existence of viral immunoevasins that impair expression of class Ib molecules also implies a role for these MHC molecules in host defense. Renukaradhya *et al*. provided evidence that the matrix protein of vesicular stomatitis virus (VSV) downregulates mouse CD1d expression in a p38 MAPK-dependent manner, implying

that VSV has devised tactics to offset immunity conferred by NKT cells (168). Similarly vaccinia virus inhibits CD1d expression (169), indicating that this molecule, and possibly its human homolog, are recognized by immune effector cells involved in the surveillance of this viral infection. Although the function of human class Ib molecule HLA-G is unclear, Park *et al.* recently showed that the US10 protein of human cytomegalovirus (HCMV) downregulates its expression (170), suggesting that HLA-G may have a role in anti-HCMV immunity. Taken together, these studies argue that elucidation of class Ib-specific responses to viral infections could provide new insights for viral vaccine development.

***Qa-1$^b$/HLA-E*: *double duty MHC Class Ib molecules***

As ligands for both inhibitory and activating receptors, Qa-1$^b$ and its human ortholog HLA-E have diverse immunological roles (Figure 1.2). The peptide-binding groove of Qa-1$^b$ is predominantly occupied by the highly conserved Qa-1$^b$ determinant modifier (Qdm) 9mer peptide that is derived from the signal sequence of class Ia molecules (171, 172). The Qdm:Qa-1$^b$ complex is the ligand for CD94-NKG2A (173) an inhibitory signaling receptor on NK cells and CD8 T cells, as well as activating receptors such as CD94-NKG2C (174). Similarly, the peptide-binding groove of its human ortholog, HLA-E, is also largely occupied by conserved peptides derived from other HLA-class I molecules (175) and recognition by CD94-NKG2 receptors is acutely dependent on the sequence of such peptides (176). CD94-NKG2A appears to operate as a sensor for the expression of MHC class Ia molecules and the functional integrity of the antigen processing machinery (177, 178) both of which can be impaired in neoplastic cells or targeted by immunoevasins expressed by a variety of pathogens.

While the function of the activating isoforms of the CD94-NKG2 family are only beginning to be understood, it is believed they may have a role in the control of pathogens, particularly HCMV. For example, positive serology for HCMV is strongly associated with high proportions of CD94-NKG2C expressing NK cells (179). Similarly, co-culture of NK cells with HCMV-infected fibroblasts results in the expansion of CD94-NKG2C positive NK cells (180).

Antiviral CD8 effector T cells can also express CD94-NKG2A, which can act to balance antiviral immunity with virus-associated immunopathology. Inhibition of virus-specific cytotoxic effector activities is critical in situations of widespread viral antigen expression by parenchymal cells as well as expression by valuable nonrenewable somatic cells. In the MPyV infection model, high load infection is associated with protracted expression of CD94-NKG2A by antiviral CD8 T cells, which serves to dampen antigen-specific cytotoxicity. For MPyV, the unwitting consequence of this host defense against excessive cell death is a high set point of persistent viral load and the elevated risk for polyomavirus-induced tumors (181). Similarly, Zhou *et al*. (182) showed that Qa-$1^b$:CD94-NKG2A engagement negatively regulates TNF-α production by influenza virus-specific CD8 effector T cells that, in turn, limits pulmonary immunopathology. Similarly, herpes simplex virus-specific CD8 T cells in proximity to latently infected trigeminal ganglion cells express CD94-NKG2A, which engages Qa-$1^b$ expressed by neurons and thereby protects infected neurons from CD8 T cell-mediated cytotoxicity (183). From these models of CD94-NKG2A-mediated inhibition of effector CD8 T cells, it is also apparent that this receptor regulates discrete effector activities and may do so in a manner that controls virus replication without destruction of virus-infected host cells

(184). Because IFN-γ upregulates Qa-$1^b$:Qdm expression (185), it is also possible that IFN-γ produced by activated NK cells and T cells serves to modulate the strength of CD94-NKG2A signaling and the range of effector activities that are inhibited.

In a recent important study, Oliveira *et al.* showed that Qa-$1^b$ also binds a diverse array of peptides derived from improperly processed housekeeping proteins that are generated by impairments in antigen processing (186). These peptides replace Qdm and are immunogenic for a novel population of Qa-$1^b$-restricted CD8 T cells that mediate cytotoxicity against target cells expressing these antigens. Importantly, these Qa-$1^b$-restricted T cells are readily detected in the immune response to tumors deficient in antigen processing. This study raises the exciting possibility that humans may possess a similar population of HLA-E-restricted CD8 T cells in their T cell repertoire that could be harnessed for tumor immunotherapy.

A sizeable body of literature shows that Qa-$1^b$ and HLA-E operate in host microbial adaptive immune responses. In humans, HLA-E-restricted CD8 T cell responses to *Mycobacterium tuberculosis* (*Mtb)*, *Salmonella typhi* and HCMV have been observed (187-189). Interestingly, presentation of Mtb-derived antigens by HLA-E but not class Ia molecules was shown to be largely resistant to cycloheximide treatment of APC, suggesting that HLA-E can capture peptide antigens from distinct intracellular compartments, possibly utilizing recycled HLA-E (190).

The UL40 protein of HCMV encodes a sequence that mimics the leader sequence of most HLA-C alleles. Consequently UL40 may facilitate the interaction between HLA-E and CD94-NKG2A following HCMV-infection. However in individuals in whom the UL40-derived peptide differs from self-encoded HLA-C sequences as a result of HLA

polymorphism, HCMV infection results in a robust UL40-specific, HLA-E restricted T cell response. These responses do not appear to be subdominant to class Ia-restricted HCMV responses and in some individuals comprise in excess of 20% of the CD8 T cell population (175, 191). Such populations in the setting of HLA-C-mismatched transplantation, could negatively impact graft survival as the UL40 sequence is identical to that found in most HLA-C alleles. To this end, we have recently identified expansions of HLA-E restricted T cells in lung transplant recipients at various stages posttransplant (LCS and AGB, unpublished data).

Qa-$1^b$ also serves as a restriction element for a population of CD8 T cells that control autoreactive CD4 T cells. Expression of Qa-$1^b$ is transiently upregulated on activated CD4 T cells, which can be loaded with peptides derived from TCR Vβ chains and recognized by these CD8 regulatory T cells ($T_{reg}$). Because these CD8 $T_{reg}$ may also express CD94-NKG2A, there is an opportunity for dynamic interplay between activating and inhibitory receptors depending on the relative occupancy of Qdm or Vβ peptides by Qa-$1^b$ (Figure 1.2). This dynamic regulation was investigated in an elegant paper by Lu *et al*. (192) utilizing the murine experimental autoimmune encephalomyelitis (EAE) model for multiple sclerosis. By using two different mutations in Qa-$1^b$, D227K, which disrupts binding of Qa-$1^b$ to CD8 (mitigating TCR engagement), and R72A, which disrupts Qa-$1^b$ binding to NKG2A, this group was able to distinguish the impact of these two interactions on CD8 $T_{reg}$ control of autoreactive CD4 T cells. Transgenic mice expressing the D227K mutation exhibited increased susceptibility to EAE, while those transgenic for the R72A mutation were EAE-resistant (193). Kumar and colleagues (194) recently described a population of CD8αα$^+$ TCRα$^+$ $T_{reg}$ cells which recognize Qa-

$1^b$-associated TCR peptides presented by DCs that have ingested apoptotic CD4 T cells. This cross-presentation was driven by inflammatory stimuli. These CD8 $T_{reg}$ were shown to suppress EAE and apparently do so by killing myelin basic protein-reactive CD4 T cells. This study raises the possibility that 'tolerogenic' DCs may be harnessed to ameliorate autoimmune diseases by eliciting such Qa-$1^b$/HLA-E-restricted CD8 $T_{reg}$ effectors.

The identification of a non-Qdm peptide that enables CD8 $T_{reg}$ cells to recognize autoreactive T cells suggests that Qa-$1^b$/HLA-E-restricted responses may be put to therapeutic use to control autoimmunity or enhance vaccine efficiency. In a series of papers exploring this possibility (195-197), the Chess and Jiang group determined that intermediate affinity T cells that expand following exposure to nominal antigens preferentially express Qa-$1^b$ bearing the signal peptide from heat shock protein 60 (Hsp60sp), the ligand for a novel population of CD8 T cells with immunoregulatory capability. Qa-$1^b$:Hsp60sp-specific CD8 T cells appear to constrain the expansion of intermediate affinity T cells, which include those that cross-react with self antigens, while sparing high affinity T cells specific for cognate antigen. Importantly, Hsp60sp peptide vaccination induced these Qa-$1^b$-restricted CD8 T cells that then mediated cross protection in two autoimmune disease models (24). Thus, Qa-$1^b$:Hsp60sp is a common structure expressed by activated T cells having TCRs of intermediate affinity for self, which allows these potentially autoreactive cells to be recognized by a generic population of Qa-$1^b$-restricted regulatory CD8 T cells (Figure 1.2). Although there is evidence that HLA-E can bind an Hsp60-derived peptide (198), whether or not T cells with

intermediate affinity for self antigens express such peptides are recognized by such class Ib-restricted CD8 $T_{reg}$ remains to be determined.

***Conclusions***

In summary, it is increasingly evident that MHC class Ib molecules can act as restricting elements for effector T cells which can limit the spread of pathogens. Consequently, given the limited polymorphism in these genes, particularly in humans, pathogen-derived peptides that bind these molecules may represent attractive vaccine candidates. However, molecules such as CD1d, MR1, HLA-E and Qa-$1^b$ also appear to have distinct roles in immunity through the stimulation of specialized T cell populations that can both orchestrate the quality of the immune response and regulate auto-reactive T cell responses. Further insights into the antigens presented by these molecules, the mechanisms by which they acquire such antigens and the way in which they are recognized by T cells may provide novel approaches to improving pathogen-specific responses and ameliorating autoimmune disease.

# IV. A class Ib-restricted CD8 T cell response protects against MPyV infection across MHC class Ia allotypes

CTL are critical for control of MPyV and MPyV-associated tumors. Mice lacking T cells which recognize MPyV epitopes are susceptible to tumors, including SCID mice, which have no T or B cells, TCR β x δ knockout mice, which have no T cells, $\beta_2m^{-/-}$ mice (113), which have no MHC class I molecules, and $SAG^+$ H-$2^k$ haplotype mice (107). Importantly, B6.$K^{b-/-}D^{b-/-}$ mice, which lack class Ia molecules, and therefore have no class Ia-restricted CD8 T cells, control MPyV infection equivalently to B6 mice (167). This

finding led to the identification of a class Ib-restricted CD8 T cell response which is specific for a nonamer epitope from the VP2 capsid protein. To test the ability of this response to control MPyV infection, the anchor residue of the target epitope, VP2.139-147, was mutated to generate a mutant virus, MPyV.H145A (H145A). Virus levels were higher in the kidney of B6.$K^{b-/-}D^{b-/-}$ mice infected with H145A than wild-type MPyV by day 80 p.i. (167). These findings represent the first identification of a class Ib-restricted CD8 T cell response that controls a virus infection.

Previous studies in the lab (166, 167) indicated that the Q9:VP2.139-specific population exhibits unique kinetics in B6.$K^{b-/-}D^{b-/-}$ mice, including a long inflationary expansion over the first 3 months p.i., and antigen-independent maintenance thereafter. The Q9:VP2.139-specific CD8 T cell response also diverges from the PyV-specific class Ia-restricted CD8 T cell responses in that a much smaller percentage of Q9:VP2.139-specific CD8 T cells respond to VP2.139 peptide stimulation ex vivo than stain with the Q9:VP2.139 tetramer. My dissertation project focused on identifying the mechanisms behind the inflationary kinetics and poor IFN-γ production by the Q9:VP2.139-specific response. Another unique feature of the Q9:VP2.139-specific CD8 T cell response feature is the use of a non-polymorphic MHC molecule to restrict a protective CD8 T cell response. The third aim of my project was to demonstrate that a Q9:VP2.139-restricted response could be generated in allogeneic mice.

**Aim 1: To determine the requirement for CD4 help and costimulation during the Q9:VP2.139-specific T cell response.**

In B6.$K^{b-/-}D^{b-/-}$ mice, the Q9:VP2.139-specific CD8 T cells expand for 3 mo. p.i. and then are maintained at a steady level with no defined contraction. The expansion correlates with persistent antigen, while the plateau is related to the relative absence of antigen (166, 167). We probed the mechanisms driving both the long expansion phase and the maintenance of the Q9:VP2.139-specific cells. Previously, our lab demonstrated that the class Ia-restricted CD8 T cell responses to MPyV expand normally without CD4 help, although the recruitment of new thymic emigrants is impaired during persistent infection, leading to a gradual attrition of the CD8 T cell population (199). Furthermore, class Ia-restricted CD8 T cell responses depend on both CD28 and CD40L costimulation for expansion, but not for maintenance during persistent infection (200). Based on these data, and the shifting dependence on antigen by the Q9:VP2.139-specific CD8 T cells, I hypothesized that CD4 T cell help and costimulation are required for the expansion, but not for the maintenance of Q9:VP2.139-specific CD8 T cells. My investigation of this hypothesis can be found in Chapter 2.

**Aim 2: To determine if the functional defect of Q9:VP2.139-specific T cells is at the level of priming.**

Only a fraction of Q9:VP2.139-specific CD8 T cells produce IFN-γ after ex vivo stimulation with VP2.139 peptide. At day 8 p.i. only ~15% of the Q9:VP2.139-specific cells produce IFN-γ. During the next 3 months this fraction increases to ~25%. After 3 months the frequency of IFN-γ-reactive cells increases, but never approaches 100% (167). This is in contrast to class Ia-restricted CD8 T cell responses to MPyV, for which ex vivo cytokine assays elicit IFN-γ from 70-90% of $tetramer^{+}$ cells ex vivo (118, 167). It is known that the structure of the α3 subunit of Q9 differs from that of H2-$K^{b}$ at critical

residues which are involved in the interaction of H2-$K^b$ and the CD8 coreceptor. Both the AB and CD loops of the H2-$K^b$ α3 subunit interact with CD8. In comparison, the Q9 AB loop has several mutations which orient the loop away from CD8 molecule, and abolish hydrogen bonds (201). Coreceptor interaction improves the interaction of the TCR and p:MHC both through increasing the affinity of the ternary complex and by increasing downstream signaling through recruitment of Lck (20). Therefore we hypothesized that Q9:VP2.139-specific CD8 T cells are insufficiently primed, and so have a deficit of IFN-γ effector function. We hypothesized that a tetramer synthesized with the α3 loop from $K^b$ engineered into the Q9 molecule will bind to Q9:VP2.139-specific TCR with higher avidity. We further hypothesized that methods to increase the epitope density and growth factors available to Q9:VP2.139-specific CD8 T cells during priming will augment priming, and generate more highly functional Q9:VP2.139-specific CD8 T cells. The Q9($K^b$) chimeric tetramer was synthesized by the NIH Tetramer Core, and a comparison of its binding with that of the Q9:VP2.139 tetramer is presented in the text of Chaper 3. However, the other premises of this hypothesis were not formally addressed in this work.

**Aim 3: To generate protective Q9:VP2.139-specific T cells across MHC Class Ia haplotype barriers.**

MHC class Ia molecules are highly polymorphic. This means that the epitope specificities of the CD8 T cell response against a single protein antigen will be different throughout the population. Similarly, between inbred strains of mice, different haplotypes are polymorphic at the MHC class Ia locus. In contrast, class Ib molecules are nonpolymorphic or oligomorphic (131). Epitope specificities restricted by class Ib

molecules are therefore much more likely to be identical between individuals, or between strains of mice. This is important for vaccine design, because it would be cheaper, safer, and easier than current vaccine strategies if the entire population could be effectively vaccinated against a pathogen using a single peptide epitope.

Q9 is a class Ib molecule from the Qa-2 family of H-2 antigens. Four genes encode the serologically identical Qa-2 antigen: Q6, Q7, Q8 and Q9. The even numbered genes, Q6 and Q8, are closely related, as are the odd-numbered genes. Q7 and Q9 differ by only one amino acid, which is not predicted to be in the peptide-binding groove (202). Consequently, the peptide-binding repertoires of Q7 and Q9 overlap (203). Our lab previously demonstrated that Q9, but not Q8, was recognized by Q9:VP2.139-specific CD8 T cell clones (167). It is highly likely that Q9:VP2.139-specific CD8 T cells also recognize Q7:VP2.139, although this has not been formally demonstrated. Because these epitopes are predicted to be identical, I will be referring to both as Q9:VP2.139 throughout this dissertation. The Qa-2 genes are not polymorphic, although they differ both in the number of genes encoded in the locus and in Qa-2 expression level (202). Because Q9 is nonpolymorphic, we hypothesized that a Q9:VP2.139-specific CD8 T cell response can be expanded in a mouse from any MHC haplotype that encodes Q7/Q9. This hypothesis is addressed in Chapter 3.

References

1. Macpherson, A. J., and N. L. Harris. 2004. Interactions between commensal intestinal bacteria and the immune system. *Nat. Rev. Immunol.* 4: 478-485.
2. Moens, E., and M. Veldhoen. 2012. Epithelial barrier biology: good fences make good neighbours. *Immunology* 135: 1-8.
3. Danilova, N. 2006. The evolution of immune mechanisms. *Journal of experimental zoology. Part B, Molecular and developmental evolution* 306: 496-520.
4. Abedon, S. T. 2012. Bacterial 'immunity' against bacteriophages. *Bacteriophage* 2: 50-54.
5. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol* 12: 991-1045.
6. Wakim, L. M., and M. J. Bevan. 2010. From the thymus to longevity in the periphery. *Curr. Opin. Immunol.* 22: 274-278.
7. Kumar, H., T. Kawai, and S. Akira. 2011. Pathogen recognition by the innate immune system. *International reviews of immunology* 30: 16-34.
8. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282: 2085-2088.
9. Chavez-Galan, L., M. C. Arenas-Del Angel, E. Zenteno, R. Chavez, and R. Lascurain. 2009. Cell death mechanisms induced by cytotoxic lymphocytes. *Cell Mol Immunol* 6: 15-25.

10. Weiss, A., and D. R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell* 76: 263-274.

11. Murphy, K. M., Travers, P., Walport, M. 2008. *Janeway's Immunobiology*. Garland Science, New York and London.

12. Kaech, S. M., and R. Ahmed. 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat. Immunol.* 2: 415-422.

13. Naito, T., H. Tanaka, Y. Naoe, and I. Taniuchi. 2011. Transcriptional control of T-cell development. *Int Immunol* 23: 661-668.

14. Rajasagi, M., M. Vitacolonna, B. Benjak, R. Marhaba, and M. Zoller. 2009. CD44 promotes progenitor homing into the thymus and T cell maturation. *J Leukoc Biol* 85: 251-261.

15. Rothenberg, E. V., J. E. Moore, and M. A. Yui. 2008. Launching the T-cell-lineage developmental programme. *Nat. Rev. Immunol.* 8: 9-21.

16. Schatz, D. G., and Y. Ji. 2011. Recombination centres and the orchestration of V(D)J recombination. *Nat. Rev. Immunol.* 11: 251-263.

17. Germain, R. N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat. Rev. Immunol.* 2: 309-322.

18. Goldrath, A. W., and M. J. Bevan. 1999. Selecting and maintaining a diverse T-cell repertoire. *Nature* 402: 255-262.

19. Klein, J., and A. Sato. 2000. The HLA system. First of two parts. *N Engl J Med* 343: 702-709.

20. Thome, M., V. Germain, J. P. DiSanto, and O. Acuto. 1996. The p56lck SH2 domain mediates recruitment of CD8/p56lck to the activated T cell receptor/CD3/zeta complex. *Eur. J. Immunol.* 26: 2093-2100.

21. Singer, A., S. Adoro, and J. H. Park. 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat. Rev. Immunol.* 8: 788-801.

22. Derbinski, J., and B. Kyewski. 2010. How thymic antigen presenting cells sample the body's self-antigens. *Curr. Opin. Immunol.* 22: 592-600.

23. Yun, T. J., and M. J. Bevan. 2001. The Goldilocks conditions applied to T cell development. *Nat. Immunol.* 2: 13-14.

24. Wu, Y., Z. Zheng, Y. Jiang, L. Chess, and H. Jiang. 2009. The specificity of T cell regulation that enables self-nonself discrimination in the periphery. *Proc. Natl. Acad. Sci. USA* 106: 534-539.

25. Curtsinger, J. M., C. M. Johnson, and M. F. Mescher. 2003. CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine. *J. Immunol.* 171: 5165-5171.

26. Mescher, M. F., P. Agarwal, K. A. Casey, C. D. Hammerbeck, Z. Xiao, and J. M. Curtsinger. 2007. Molecular basis for checkpoints in the CD8 T cell response: tolerance versus activation. *Semin Immunol* 19: 153-161.

27. Sprent, J. 1995. Antigen-presenting cells. Professionals and amateurs. *Curr Biol* 5: 1095-1097.

28. Rossi, M., and J. W. Young. 2005. Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. *J. Immunol.* 175: 1373-1381.

29. Pulendran, B. 2004. Modulating vaccine responses with dendritic cells and Toll-like receptors. *Immunol Rev* 199: 227-250.

30. Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena. 2002. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 20: 621-667.

31. Pulendran, B. 2004. Immune activation: death, danger and dendritic cells. *Curr Biol* 14: R30-32.

32. Smith-Garvin, J. E., G. A. Koretzky, and M. S. Jordan. 2009. T cell activation. *Annu Rev Immunol* 27: 591-619.

33. Acuto, O., and F. Michel. 2003. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat. Rev. Immunol.* 3: 939-951.

34. Johnson, S., Y. Zhan, R. M. Sutherland, A. M. Mount, S. Bedoui, J. L. Brady, E. M. Carrington, L. E. Brown, G. T. Belz, W. R. Heath, and A. M. Lew. 2009. Selected Toll-like receptor ligands and viruses promote helper-independent cytotoxic T cell priming by upregulating CD40L on dendritic cells. *Immunity* 30: 218-227.

35. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science* 297: 2060-2063.

36. Larsen, C. P., E. T. Elwood, D. Z. Alexander, S. C. Ritchie, R. Hendrix, C. Tucker-Burden, H. R. Cho, A. Aruffo, D. Hollenbaugh, P. S. Linsley, K. J. Winn,

and T. C. Pearson. 1996. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 381: 434-438.

37. Noelle, R. J., M. Roy, D. M. Shepherd, I. Stamenkovic, J. A. Ledbetter, and A. Aruffo. 1992. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc. Natl. Acad. Sci. USA* 89: 6550-6554.

38. Ford, M. L., and C. P. Larsen. 2009. Translating costimulation blockade to the clinic: lessons learned from three pathways. *Immunol Rev* 229: 294-306.

39. Linsley, P. S., W. Brady, M. Urnes, L. S. Grosmaire, N. K. Damle, and J. A. Ledbetter. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* 174: 561-569.

40. Lenschow, D. J., Y. Zeng, J. R. Thistlethwaite, A. Montag, W. Brady, M. G. Gibson, P. S. Linsley, and J. A. Bluestone. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science* 257: 789-792.

41. Qureshi, O. S., Y. Zheng, K. Nakamura, K. Attridge, C. Manzotti, E. M. Schmidt, J. Baker, L. E. Jeffery, S. Kaur, Z. Briggs, T. Z. Hou, C. E. Futter, G. Anderson, L. S. Walker, and D. M. Sansom. 2011. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* 332: 600-603.

42. Ford, M. L., L. L. Stempora, and C. P. Larsen. 2011. CD28 blockade induces division-dependent downregulation of interleukin-2 receptor alpha. *Transplant immunology* 24: 94-99.

43. Ford, M. L., and C. P. Larsen. 2010. Overcoming the memory barrier in tolerance induction: molecular mimicry and functional heterogeneity among pathogen-

specific T-cell populations. *Current opinion in organ transplantation* 15: 405-410.

44. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393: 478-480.

45. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393: 474-478.

46. Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. 1994. Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med.* 180: 1263-1272.

47. Schoenberger, S. P., R. E. Toes, E. I. van der Voort, R. Offringa, and C. J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393: 480-483.

48. Taraban, V. Y., T. F. Rowley, and A. Al-Shamkhani. 2004. Cutting edge: a critical role for CD70 in CD8 T cell priming by CD40-licensed APCs. *J. Immunol.* 173: 6542-6546.

49. Smith, C. M., N. S. Wilson, J. Waithman, J. A. Villadangos, F. R. Carbone, W. R. Heath, and G. T. Belz. 2004. Cognate CD4(+) T cell licensing of dendritic cells in CD8(+) T cell immunity. *Nat. Immunol.* 5: 1143-1148.

50. Soehnlein, O., and L. Lindbom. 2010. Phagocyte partnership during the onset and resolution of inflammation. *Nat. Rev. Immunol.* 10: 427-439.

51. Tarlinton, D. 2006. B-cell memory: are subsets necessary? *Nat. Rev. Immunol.* 6: 785-790.

52. Yewdell, J., L. C. Anton, I. Bacik, U. Schubert, H. L. Snyder, and J. R. Bennink. 1999. Generating MHC class I ligands from viral gene products. *Immunol Rev* 172: 97-108.

53. Schubert, U., L. C. Anton, J. Gibbs, C. C. Norbury, J. W. Yewdell, and J. R. Bennink. 2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 404: 770-774.

54. Sijts, E. J., and P. M. Kloetzel. 2011. The role of the proteasome in the generation of MHC class I ligands and immune responses. *Cellular and molecular life sciences : CMLS* 68: 1491-1502.

55. Rock, K. L., I. A. York, T. Saric, and A. L. Goldberg. 2002. Protein degradation and the generation of MHC class I-presented peptides. *Adv Immunol* 80: 1-70.

56. Segura, E., and J. A. Villadangos. 2011. A modular and combinatorial view of the antigen cross-presentation pathway in dendritic cells. *Traffic (Copenhagen, Denmark)* 12: 1677-1685.

57. Palmowski, M. J., U. Gileadi, M. Salio, A. Gallimore, M. Millrain, E. James, C. Addey, D. Scott, J. Dyson, E. Simpson, and V. Cerundolo. 2006. Role of immunoproteasomes in cross-presentation. *J. Immunol.* 177: 983-990.

58. Udono, H. 2012. Heat shock protein magic in antigen trafficking within dendritic cells: implications in antigen cross-presentation in immunity. *Acta Med Okayama* 66: 1-6.

59. Harty, J. T., A. R. Tvinnereim, and D. W. White. 2000. CD8+ T cell effector mechanisms in resistance to infection. *Annu Rev Immunol* 18: 275-308.

60. Wilson, J. J., E. Lin, C. D. Pack, E. L. Frost, A. Hadley, A. I. Swimm, J. Wang, Y. Dong, C. P. Breeden, D. Kalman, K. A. Newell, and A. E. Lukacher. 2011. Gamma interferon controls mouse polyomavirus infection in vivo. *J. Virol.* 85: 10126-10134.

61. Hanabuchi, S., M. Koyanagi, A. Kawasaki, N. Shinohara, A. Matsuzawa, Y. Nishimura, Y. Kobayashi, S. Yonehara, H. Yagita, and K. Okumura. 1994. Fas and its ligand in a general mechanism of T-cell-mediated cytotoxicity. *Proc. Natl. Acad. Sci. USA* 91: 4930-4934.

62. Badovinac, V. P., J. S. Haring, and J. T. Harty. 2007. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity* 26: 827-841.

63. Obar, J. J., K. M. Khanna, and L. Lefrancois. 2008. Endogenous naive CD8+ T cell precursor frequency regulates primary and memory responses to infection. *Immunity* 28: 859-869.

64. Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 8: 177-187.

65. Schumacher, T. N., C. Gerlach, and J. W. van Heijst. 2010. Mapping the life histories of T cells. *Nat. Rev. Immunol.* 10: 621-631.

66. Masopust, D., S. M. Kaech, E. J. Wherry, and R. Ahmed. 2004. The role of programming in memory T-cell development. *Curr. Opin. Immunol.* 16: 217-225.

67. Zhang, N., and M. J. Bevan. 2011. CD8(+) T cells: foot soldiers of the immune system. *Immunity* 35: 161-168.

68. Zhang, B., C. H. Maris, J. Foell, J. Whitmire, L. Niu, J. Song, B. S. Kwon, A. T. Vella, R. Ahmed, J. Jacob, and R. S. Mittler. 2007. Immune suppression or enhancement by CD137 T cell costimulation during acute viral infection is time dependent. *J Clin Invest* 117: 3029-3041.

69. Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *J. Exp. Med.* 160: 521-540.

70. Obar, J. J., E. R. Jellison, B. S. Sheridan, D. A. Blair, Q. M. Pham, J. M. Zickovich, and L. Lefrancois. 2011. Pathogen-induced inflammatory environment controls effector and memory CD8+ T cell differentiation. *J. Immunol.* 187: 4967-4978.

71. Surh, C. D., and J. Sprent. 2008. Homeostasis of naive and memory T cells. *Immunity* 29: 848-862.

72. Nakanishi, Y., B. Lu, C. Gerard, and A. Iwasaki. 2009. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature* 462: 510-513.

73. Lefrancois, L. 2006. Development, trafficking, and function of memory T-cell subsets. *Immunol Rev* 211: 93-103.

74. Obar, J. J., M. J. Molloy, E. R. Jellison, T. A. Stoklasek, W. Zhang, E. J. Usherwood, and L. Lefrancois. 2010. CD4+ T cell regulation of CD25 expression

controls development of short-lived effector CD8+ T cells in primary and secondary responses. *Proc. Natl. Acad. Sci. USA* 107: 193-198.

75. Williams, M. A., and M. J. Bevan. 2007. Effector and memory CTL differentiation. *Annu Rev Immunol* 25: 171-192.

76. Hikono, H., J. E. Kohlmeier, S. Takamura, S. T. Wittmer, A. D. Roberts, and D. L. Woodland. 2007. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. *J. Exp. Med.* 204: 1625-1636.

77. Prlic, M., J. A. Sacks, and M. J. Bevan. 2012. Dissociating markers of senescence and protective ability in memory T cells. *PLoS One* 7: e32576.

78. Sarkar, S., V. Kalia, W. N. Haining, B. T. Konieczny, S. Subramaniam, and R. Ahmed. 2008. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J. Exp. Med.* 205: 625-640.

79. Curtsinger, J. M., D. C. Lins, and M. F. Mescher. 1998. CD8+ memory T cells (CD44high, Ly-6C+) are more sensitive than naive cells to (CD44low, Ly-6C-) to TCR/CD8 signaling in response to antigen. *J. Immunol.* 160: 3236-3243.

80. Kedl, R. M., and M. F. Mescher. 1998. Qualitative differences between naive and memory T cells make a major contribution to the more rapid and efficient memory CD8+ T cell response. *J. Immunol.* 161: 674-683.

81. Bachmann, M. F., M. Barner, A. Viola, and M. Kopf. 1999. Distinct kinetics of cytokine production and cytolysis in effector and memory T cells after viral infection. *Eur. J. Immunol.* 29: 291-299.

82. Kambayashi, T., E. Assarsson, A. E. Lukacher, H. G. Ljunggren, and P. E. Jensen. 2003. Memory CD8+ T cells provide an early source of IFN-gamma. *J. Immunol.* 170: 2399-2408.

83. Stewart, S. E. 1953. Leukemia in Mice Produced by a Filterable Agent Present in Akr Leukemic Tissues with Notes on a Sarcoma Produced by the Same Agent. *Anat Rec* 117: 532-532.

84. Gross, L. 1953. A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. *Proc Soc Exp Biol Med* 83: 414-421.

85. zur Hausen, H. 2008. Novel human polyomaviruses--re-emergence of a well known virus family as possible human carcinogens. *Int J Cancer* 123: 247-250.

86. Lukacher, A. E. 2004. Pathogen-host standoff: immunity to polyomavirus infection and neoplasia. *Immunol Res* 29: 139-150.

87. Van Ghelue, M., M. T. Khan, B. Ehlers, and U. Moens. 2012. Genome analysis of the new human polyomaviruses. *Rev Med Virol*.

88. Johne, R., C. B. Buck, T. Allander, W. J. Atwood, R. L. Garcea, M. J. Imperiale, E. O. Major, T. Ramqvist, and L. C. Norkin. 2011. Taxonomical developments in the family Polyomaviridae. *Arch Virol* 156: 1627-1634.

89. Delbue, S., M. Comar, and P. Ferrante. 2012. Review on the Relationship between Human Polyomaviruses-Associated Tumors and Host Immune System. *Clin Dev Immunol* 2012: 542092.

90. Bennett, S. M., N. M. Broekema, and M. J. Imperiale. 2012. BK polyomavirus: emerging pathogen. *Microbes Infect*.

91. Bellizzi, A., C. Nardis, E. Anzivino, D. Rodio, D. Fioriti, M. Mischitelli, F. Chiarini, and V. Pietropaolo. 2012. Human polyomavirus JC reactivation and pathogenetic mechanisms of progressive multifocal leukoencephalopathy and cancer in the era of monoclonal antibody therapies. *Journal of neurovirology* 18: 1-11.

92. Moser, J. M., and A. E. Lukacher. 2001. Immunity to polyoma virus infection and tumorigenesis. *Viral Immunol* 14: 199-216.

93. Benjamin, T. L. 2006. Polyoma Viruses. In *The Mouse In Biomedical Research, 2nd Edition*, 2 ed. J. G. Fox, Davisson, M.T., Quimby, F.W., Barthold, S.W., Newcomer, C.E., & Smith, A.L., ed. Elsevier. 105-139.

94. Fluck, M. M., and S. Z. Haslam. 1996. Mammary tumors induced by polyomavirus. *Breast Cancer Res Treat* 39: 45-56.

95. Benjamin, T. L. 2001. Polyoma virus: old findings and new challenges. *Virology* 289: 167-173.

96. Chen, X. S., T. Stehle, and S. C. Harrison. 1998. Interaction of polyomavirus internal protein VP2 with the major capsid protein VP1 and implications for participation of VP2 in viral entry. *EMBO J* 17: 3233-3240.

97. Neu, U., T. Stehle, and W. J. Atwood. 2009. The Polyomaviridae: Contributions of virus structure to our understanding of virus receptors and infectious entry. *Virology* 384: 389-399.

98. Inoue, T., and B. Tsai. 2011. A large and intact viral particle penetrates the endoplasmic reticulum membrane to reach the cytosol. *PLoS Pathog* 7: e1002037.

99. Kasamatsu, H., and A. Nakanishi. 1998. How do animal DNA viruses get to the nucleus? *Annual review of microbiology* 52: 627-686.

100. Cheng, J., J. A. DeCaprio, M. M. Fluck, and B. S. Schaffhausen. 2009. Cellular transformation by Simian Virus 40 and Murine Polyoma Virus T antigens. *Semin Cancer Biol* 19: 218-228.

101. Kaplan, D. R., D. C. Pallas, W. Morgan, B. Schaffhausen, and T. M. Roberts. 1989. Mechanisms of transformation by polyoma virus middle T antigen. *Biochim Biophys Acta* 948: 345-364.

102. Johnson, E. M. 2010. Structural evaluation of new human polyomaviruses provides clues to pathobiology. *Trends Microbiol* 18: 215-223.

103. Chen, L., X. Wang, and M. M. Fluck. 2006. Independent contributions of polyomavirus middle T and small T to the regulation of early and late gene expression and DNA replication. *J. Virol.* 80: 7295-7307.

104. Rodriguez-Viciana, P., C. Collins, and M. Fried. 2006. Polyoma and SV40 proteins differentially regulate PP2A to activate distinct cellular signaling pathways involved in growth control. *Proc. Natl. Acad. Sci. USA* 103: 19290-19295.

105. McCance, D. J. 1981. Growth and persistence of polyoma early region deletion mutants in mice. *J. Virol.* 39: 958-962.

106. Virgin, H. W., E. J. Wherry, and R. Ahmed. 2009. Redefining chronic viral infection. *Cell* 138: 30-50.

107. Swanson, P. A., 2nd, A. E. Lukacher, and E. Szomolanyi-Tsuda. 2009. Immunity to polyomavirus infection: the polyomavirus-mouse model. *Semin Cancer Biol* 19: 244-251.

108. Sanjuan, N., A. Porras, J. Otero, and S. Perazzo. 2001. Expression of major capsid protein VP-1 in the absence of viral particles in thymomas induced by murine polyomavirus. *J. Virol.* 75: 2891-2899.

109. Talmage, D. A., R. Freund, T. Dubensky, M. Salcedo, P. Gariglio, L. M. Rangel, C. J. Dawe, and T. L. Benjamin. 1992. Heterogeneity in state and expression of viral DNA in polyoma virus-induced tumors of the mouse. *Virology* 187: 734-747.

110. Szomolanyi-Tsuda, E., and R. M. Welsh. 1996. T cell-independent antibody-mediated clearance of polyoma virus in T cell-deficient mice. *J. Exp. Med.* 183: 403-411.

111. Allison, A. C., J. N. Monga, and V. Hammond. 1974. Increased susceptibility to virus oncogenesis of congenitally thymus-deprived nude mice. *Nature* 252: 746-747.

112. Law, L. W. 1966. Immunologic responsiveness and the induction of experimental neoplasms. *Cancer Res* 26: 1121-1134.

113. Drake, D. R., 3rd, and A. E. Lukacher. 1998. Beta 2-microglobulin knockout mice are highly susceptible to polyoma virus tumorigenesis. *Virology* 252: 275-284.

114. Law, L. W., R. C. Ting, and E. Leckband. 1967. Prevention of virus-induced neoplasms in mice through passive transfer of immunity by sensitized syngeneic lymphoid cells. *Proc. Natl. Acad. Sci. USA* 57: 1068-1075.

115. Lukacher, A. E., R. Freund, J. P. Carroll, R. T. Bronson, and T. L. Benjamin. 1993. Pyvs: a dominantly acting gene in C3H/BiDa mice conferring susceptibility to tumor induction by polyoma virus. *Virology* 196: 241-248.

116. Lukacher, A. E., Y. Ma, J. P. Carroll, S. R. Abromson-Leeman, J. C. Laning, M. E. Dorf, and T. L. Benjamin. 1995. Susceptibility to tumors induced by polyoma virus is conferred by an endogenous mouse mammary tumor virus superantigen. *J. Exp. Med.* 181: 1683-1692.

117. Lukacher, A. E., and C. S. Wilson. 1998. Resistance to polyoma virus-induced tumors correlates with CTL recognition of an immunodominant H-2Dk-restricted epitope in the middle T protein. *J. Immunol.* 160: 1724-1734.

118. Lukacher, A. E., J. M. Moser, A. Hadley, and J. D. Altman. 1999. Visualization of polyoma virus-specific CD8+ T cells in vivo during infection and tumor rejection. *J. Immunol.* 163: 3369-3378.

119. Kemball, C. C., E. D. Lee, V. Vezys, T. C. Pearson, C. P. Larsen, and A. E. Lukacher. 2005. Late priming and variability of epitope-specific CD8+ T cell responses during a persistent virus infection. *J. Immunol.* 174: 7950-7960.

120. Han Lee, E. D., C. C. Kemball, J. Wang, Y. Dong, D. C. Stapler, Jr., K. M. Hamby, S. Gangappa, K. A. Newell, T. C. Pearson, A. E. Lukacher, and C. P. Larsen. 2006. A mouse model for polyomavirus-associated nephropathy of kidney transplants. *Am J Transplant* 6: 913-922.

121. Wilson, J. J., C. D. Pack, E. Lin, E. L. Frost, J. A. Albrecht, A. Hadley, A. R. Hofstetter, S. S. Tevethia, T. D. Schell, and A. E. Lukacher. 2012. CD8 T cells

recruited early in mouse polyomavirus infection undergo exhaustion. *J. Immunol.* 188: 4340-4348.

122. Vezys, V., D. Masopust, C. C. Kemball, D. L. Barber, L. A. O'Mara, C. P. Larsen, T. C. Pearson, R. Ahmed, and A. E. Lukacher. 2006. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *J. Exp. Med.* 203: 2263-2269.

123. Byers, A. M., A. Hadley, and A. E. Lukacher. 2007. Protection against polyoma virus-induced tumors is perforin-independent. *Virology* 358: 485-492.

124. Dawe, C. J., R. Freund, G. Mandel, K. Ballmer-Hofer, D. A. Talmage, and T. L. Benjamin. 1987. Variations in polyoma virus genotype in relation to tumor induction in mice. Characterization of wild type strains with widely differing tumor profiles. *Am J Pathol* 127: 243-261.

125. Freund, R., A. Sotnikov, R. T. Bronson, and T. L. Benjamin. 1992. Polyoma virus middle T is essential for virus replication and persistence as well as for tumor induction in mice. *Virology* 191: 716-723.

126. Shuda, M., H. Feng, H. J. Kwun, S. T. Rosen, O. Gjoerup, P. S. Moore, and Y. Chang. 2008. T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus. *Proc. Natl. Acad. Sci. USA* 105: 16272-16277.

127. Ramos, E., C. B. Drachenberg, R. Wali, and H. H. Hirsch. 2009. The decade of polyomavirus BK-associated nephropathy: state of affairs. *Transplantation* 87: 621-630.

128. Albrecht, J. A., Y. Dong, J. Wang, C. Breeden, A. B. Farris, 3rd, A. E. Lukacher, and K. A. Newell. 2012. Adaptive Immunity Rather than Viral Cytopathology Mediates Polyomavirus-Associated Nephropathy in Mice. *Am J Transplant*.

129. Schowalter, R. M., D. V. Pastrana, K. A. Pumphrey, A. L. Moyer, and C. B. Buck. 2010. Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. *Cell Host Microbe* 7: 509-515.

130. Scuda, N., J. Hofmann, S. Calvignac-Spencer, K. Ruprecht, P. Liman, J. Kuhn, H. Hengel, and B. Ehlers. 2011. A novel human polyomavirus closely related to the african green monkey-derived lymphotropic polyomavirus. *J. Virol.* 85: 4586-4590.

131. Rodgers, J. R., and R. G. Cook. 2005. MHC class Ib molecules bridge innate and acquired immunity. *Nat. Rev. Immunol.* 5: 459-471.

132. Hansen, T. H., S. Huang, P. L. Arnold, and D. H. Fremont. 2007. Patterns of nonclassical MHC antigen presentation. *Nat. Immunol.* 8: 563-568.

133. Florence, W. C., C. Xia, L. E. Gordy, W. Chen, Y. Zhang, J. Scott-Browne, Y. Kinjo, K. O. Yu, S. Keshipeddy, D. G. Pellicci, O. Patel, L. Kjer-Nielsen, J. McCluskey, D. I. Godfrey, J. Rossjohn, S. K. Richardson, S. A. Porcelli, A. R. Howell, K. Hayakawa, L. Gapin, D. M. Zajonc, P. G. Wang, and S. Joyce. 2009. Adaptability of the semi-invariant natural killer T-cell receptor towards structurally diverse CD1d-restricted ligands. *EMBO J* 28: 3579-3590.

134. Mallevaey, T., J. P. Scott-Browne, J. L. Matsuda, M. H. Young, D. G. Pellicci, O. Patel, M. Thakur, L. Kjer-Nielsen, S. K. Richardson, V. Cerundolo, A. R. Howell,

J. McCluskey, D. I. Godfrey, J. Rossjohn, P. Marrack, and L. Gapin. 2009. T cell receptor CDR2 beta and CDR3 beta loops collaborate functionally to shape the iNKT cell repertoire. *Immunity* 31: 60-71.

135. Middendorp, S., and E. E. Nieuwenhuis. 2009. NKT cells in mucosal immunity. *Mucosal Immunol* 2: 393-402.

136. Mattner, J., K. L. Debord, N. Ismail, R. D. Goff, C. Cantu, 3rd, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, K. Hoebe, O. Schneewind, D. Walker, B. Beutler, L. Teyton, P. B. Savage, and A. Bendelac. 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 434: 525-529.

137. De Libero, G., A. P. Moran, H. J. Gober, E. Rossy, A. Shamshiev, O. Chelnokova, Z. Mazorra, S. Vendetti, A. Sacchi, M. M. Prendergast, S. Sansano, A. Tonevitsky, R. Landmann, and L. Mori. 2005. Bacterial infections promote T cell recognition of self-glycolipids. *Immunity* 22: 763-772.

138. Paget, C., T. Mallevaey, A. O. Speak, D. Torres, J. Fontaine, K. C. Sheehan, M. Capron, B. Ryffel, C. Faveeuw, M. Leite de Moraes, F. Platt, and F. Trottein. 2007. Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. *Immunity* 27: 597-609.

139. Darmoise, A., S. Teneberg, L. Bouzonville, R. O. Brady, M. Beck, S. H. Kaufmann, and F. Winau. 2010. Lysosomal alpha-galactosidase controls the generation of self lipid antigens for natural killer T cells. *Immunity* 33: 216-228.

140. Fujio, M., D. Wu, R. Garcia-Navarro, D. D. Ho, M. Tsuji, and C. H. Wong. 2006. Structure-based discovery of glycolipids for CD1d-mediated NKT cell activation: tuning the adjuvant versus immunosuppression activity. *J Am Chem Soc* 128: 9022-9023.

141. Yu, K. O., J. S. Im, A. Molano, Y. Dutronc, P. A. Illarionov, C. Forestier, N. Fujiwara, I. Arias, S. Miyake, T. Yamamura, Y. T. Chang, G. S. Besra, and S. A. Porcelli. 2005. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. *Proc. Natl. Acad. Sci. USA* 102: 3383-3388.

142. Goff, R. D., Y. Gao, J. Mattner, D. Zhou, N. Yin, C. Cantu, 3rd, L. Teyton, A. Bendelac, and P. B. Savage. 2004. Effects of lipid chain lengths in alpha-galactosylceramides on cytokine release by natural killer T cells. *J Am Chem Soc* 126: 13602-13603.

143. Schmieg, J., G. Yang, R. W. Franck, and M. Tsuji. 2003. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide. *J. Exp. Med.* 198: 1631-1641.

144. Miyamoto, K., S. Miyake, and T. Yamamura. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* 413: 531-534.

145. Li, X., M. Fujio, M. Imamura, D. Wu, S. Vasan, C. H. Wong, D. D. Ho, and M. Tsuji. 2010. Design of a potent CD1d-binding NKT cell ligand as a vaccine adjuvant. *Proc. Natl. Acad. Sci. USA* 107: 13010-13015.

146. Im, J. S., P. Arora, G. Bricard, A. Molano, M. M. Venkataswamy, I. Baine, E. S. Jerud, M. F. Goldberg, A. Baena, K. O. Yu, R. M. Ndonye, A. R. Howell, W. Yuan, P. Cresswell, Y. T. Chang, P. A. Illarionov, G. S. Besra, and S. A. Porcelli. 2009. Kinetics and cellular site of glycolipid loading control the outcome of natural killer T cell activation. *Immunity* 30: 888-898.

147. Godfrey, D. I., D. G. Pellicci, O. Patel, L. Kjer-Nielsen, J. McCluskey, and J. Rossjohn. 2010. Antigen recognition by CD1d-restricted NKT T cell receptors. *Semin Immunol* 22: 61-67.

148. Tilloy, F., E. Treiner, S. H. Park, C. Garcia, F. Lemonnier, H. de la Salle, A. Bendelac, M. Bonneville, and O. Lantz. 1999. An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. *J. Exp. Med.* 189: 1907-1921.

149. Treiner, E., L. Duban, S. Bahram, M. Radosavljevic, V. Wanner, F. Tilloy, P. Affaticati, S. Gilfillan, and O. Lantz. 2003. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* 422: 164-169.

150. Martin, E., E. Treiner, L. Duban, L. Guerri, H. Laude, C. Toly, V. Premel, A. Devys, I. C. Moura, F. Tilloy, S. Cherif, G. Vera, S. Latour, C. Soudais, and O. Lantz. 2009. Stepwise development of MAIT cells in mouse and human. *PLoS Biol* 7: e54.

151. Riegert, P., V. Wanner, and S. Bahram. 1998. Genomics, isoforms, expression, and phylogeny of the MHC class I-related MR1 gene. *J. Immunol.* 161: 4066-4077.

152. Huang, S., E. Martin, S. Kim, L. Yu, C. Soudais, D. H. Fremont, O. Lantz, and T. H. Hansen. 2009. MR1 antigen presentation to mucosal-associated invariant T cells was highly conserved in evolution. *Proc. Natl. Acad. Sci. USA* 106: 8290-8295.

153. Okamoto, N., O. Kanie, Y. Y. Huang, R. Fujii, H. Watanabe, and M. Shimamura. 2005. Synthetic alpha-mannosyl ceramide as a potent stimulant for an NKT cell repertoire bearing the invariant Valpha19-Jalpha26 TCR alpha chain. *Chem Biol* 12: 677-683.

154. Shimamura, M. 2008. Non-reducing end alpha-mannosylated glycolipids as potent activators for invariant Valpha19 TCR-bearing natural killer T cells. *Carbohydr Res* 343: 2010-2017.

155. Huang, S., S. Gilfillan, S. Kim, B. Thompson, X. Wang, A. J. Sant, D. H. Fremont, O. Lantz, and T. H. Hansen. 2008. MR1 uses an endocytic pathway to activate mucosal-associated invariant T cells. *J. Exp. Med.* 205: 1201-1211.

156. Gold, M. C., S. Cerri, S. Smyk-Pearson, M. E. Cansler, T. M. Vogt, J. Delepine, E. Winata, G. M. Swarbrick, W. J. Chua, Y. Y. Yu, O. Lantz, M. S. Cook, M. D. Null, D. B. Jacoby, M. J. Harriff, D. A. Lewinsohn, T. H. Hansen, and D. M. Lewinsohn. 2010. Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol* 8: e1000407.

157. Le Bourhis, L., E. Martin, I. Peguillet, A. Guihot, N. Froux, M. Core, E. Levy, M. Dusseaux, V. Meyssonnier, V. Premel, C. Ngo, B. Riteau, L. Duban, D. Robert, M. Rottman, C. Soudais, and O. Lantz. 2010. Antimicrobial activity of mucosal-associated invariant T cells. *Nat. Immunol.* 11: 701-708.

158. Lo, W. F., H. Ong, E. S. Metcalf, and M. J. Soloski. 1999. T cell responses to Gram-negative intracellular bacterial pathogens: a role for CD8+ T cells in immunity to Salmonella infection and the involvement of MHC class Ib molecules. *J. Immunol.* 162: 5398-5406.

159. Milligan, G. N., L. Flaherty, V. L. Braciale, and T. J. Braciale. 1991. Nonconventional (TL-encoded) major histocompatibility complex molecules present processed viral antigen to cytotoxic T lymphocytes. *J. Exp. Med.* 174: 133-138.

160. Kerksiek, K. M., D. H. Busch, I. M. Pilip, S. E. Allen, and E. G. Pamer. 1999. H2-M3-restricted T cells in bacterial infection: rapid primary but diminished memory responses. *J. Exp. Med.* 190: 195-204.

161. D'Orazio, S. E., D. G. Halme, H. L. Ploegh, and M. N. Starnbach. 2003. Class Ia MHC-deficient BALB/c mice generate CD8+ T cell-mediated protective immunity against Listeria monocytogenes infection. *J. Immunol.* 171: 291-298.

162. Seaman, M. S., C. R. Wang, and J. Forman. 2000. MHC class Ib-restricted CTL provide protection against primary and secondary Listeria monocytogenes infection. *J. Immunol.* 165: 5192-5201.

163. Xu, H., T. Chun, H. J. Choi, B. Wang, and C. R. Wang. 2006. Impaired response to Listeria in H2-M3-deficient mice reveals a nonredundant role of MHC class Ib-specific T cells in host defense. *J. Exp. Med.* 203: 449-459.

164. Doi, T., H. Yamada, T. Yajima, W. Wajjwalku, T. Hara, and Y. Yoshikai. 2007. H2-M3-restricted CD8+ T cells induced by peptide-pulsed dendritic cells confer protection against Mycobacterium tuberculosis. *J. Immunol.* 178: 3806-3813.

165. Braaten, D. C., J. S. McClellan, I. Messaoudi, S. A. Tibbetts, K. B. McClellan, J. Nikolich-Zugich, and H. W. Virgin. 2006. Effective control of chronic gamma-herpesvirus infection by unconventional MHC Class Ia-independent CD8 T cells. *PLoS Pathog* 2: e37.

166. Swanson, P. A., 2nd, A. R. Hofstetter, J. J. Wilson, and A. E. Lukacher. 2009. Cutting edge: shift in antigen dependence by an antiviral MHC class Ib-restricted CD8 T cell response during persistent viral infection. *J. Immunol.* 182: 5198-5202.

167. Swanson, P. A., 2nd, C. D. Pack, A. Hadley, C. R. Wang, I. Stroynowski, P. E. Jensen, and A. E. Lukacher. 2008. An MHC class Ib-restricted CD8 T cell response confers antiviral immunity. *J. Exp. Med.* 205: 1647-1657.

168. Renukaradhya, G. J., M. A. Khan, D. Shaji, and R. R. Brutkiewicz. 2008. Vesicular stomatitis virus matrix protein impairs CD1d-mediated antigen presentation through activation of the p38 MAPK pathway. *J. Virol.* 82: 12535-12542.

169. Renukaradhya, G. J., T. J. Webb, M. A. Khan, Y. L. Lin, W. Du, J. Gervay-Hague, and R. R. Brutkiewicz. 2005. Virus-induced inhibition of CD1d1-mediated antigen presentation: reciprocal regulation by p38 and ERK. *J. Immunol.* 175: 4301-4308.

170. Park, B., E. Spooner, B. L. Houser, J. L. Strominger, and H. L. Ploegh. 2010. The HCMV membrane glycoprotein US10 selectively targets HLA-G for degradation. *J. Exp. Med.*

171. DeCloux, A., A. S. Woods, R. J. Cotter, M. J. Soloski, and J. Forman. 1997. Dominance of a single peptide bound to the class I(B) molecule, Qa-1b. *J. Immunol.* 158: 2183-2191.

172. Cotterill, L. A., H. J. Stauss, M. M. Millrain, D. J. Pappin, D. Rahman, B. Canas, P. Chandler, A. Stackpoole, E. Simpson, P. J. Robinson, and P. J. Dyson. 1997. Qa-1 interaction and T cell recognition of the Qa-1 determinant modifier peptide. *Eur. J. Immunol.* 27: 2123-2132.

173. Vance, R. E., J. R. Kraft, J. D. Altman, P. E. Jensen, and D. H. Raulet. 1998. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b). *J Exp Med* 188: 1841-1848.

174. Vance, R. E., A. M. Jamieson, and D. H. Raulet. 1999. Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J. Exp. Med.* 190: 1801-1812.

175. Petrie, E. J., C. S. Clements, J. Lin, L. C. Sullivan, D. Johnson, T. Huyton, A. Heroux, H. L. Hoare, T. Beddoe, H. H. Reid, M. C. Wilce, A. G. Brooks, and J. Rossjohn. 2008. CD94-NKG2A recognition of human leukocyte antigen (HLA)-E bound to an HLA class I leader sequence. *J. Exp. Med.* 205: 725-735.

176. Hoare, H. L., L. C. Sullivan, C. S. Clements, L. K. Ely, T. Beddoe, K. N. Henderson, J. Lin, H. H. Reid, A. G. Brooks, and J. Rossjohn. 2008. Subtle changes in peptide conformation profoundly affect recognition of the non-classical MHC class I molecule HLA-E by the CD94-NKG2 natural killer cell receptors. *J Mol Biol* 377: 1297-1303.

177. Bland, F. A., M. K. Lemberg, A. J. McMichael, B. Martoglio, and V. M. Braud. 2003. Requirement of the proteasome for the trimming of signal peptide-derived epitopes presented by the nonclassical major histocompatibility complex class I molecule HLA-E. *J Biol Chem* 278: 33747-33752.

178. Braud, V. M., D. S. Allan, D. Wilson, and A. J. McMichael. 1998. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr Biol* 8: 1-10.

179. Guma, M., A. Angulo, C. Vilches, N. Gomez-Lozano, N. Malats, and M. Lopez-Botet. 2004. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* 104: 3664-3671.

180. Guma, M., M. Budt, A. Saez, T. Brckalo, H. Hengel, A. Angulo, and M. Lopez-Botet. 2006. Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood* 107: 3624-3631.

181. Moser, J. M., J. Gibbs, P. E. Jensen, and A. E. Lukacher. 2002. CD94-NKG2A receptors regulate antiviral CD8(+) T cell responses. *Nat. Immunol.* 3: 189-195.

182. Zhou, J., M. Matsuoka, H. Cantor, R. Homer, and R. I. Enelow. 2008. Cutting edge: engagement of NKG2A on CD8+ effector T cells limits immunopathology in influenza pneumonia. *J. Immunol.* 180: 25-29.

183. Suvas, S., A. K. Azkur, and B. T. Rouse. 2006. Qa-1b and CD94-NKG2a interaction regulate cytolytic activity of herpes simplex virus-specific memory CD8+ T cells in the latently infected trigeminal ganglia. *J. Immunol.* 176: 1703-1711.

184. Divito, S., T. L. Cherpes, and R. L. Hendricks. 2006. A triple entente: virus, neurons, and CD8+ T cells maintain HSV-1 latency. *Immunol Res* 36: 119-126.

185. Ota, T., K. Takeda, H. Akiba, Y. Hayakawa, K. Ogasawara, Y. Ikarashi, S. Miyake, H. Wakasugi, T. Yamamura, M. Kronenberg, D. H. Raulet, K. Kinoshita, H. Yagita, M. J. Smyth, and K. Okumura. 2005. IFN-gamma-mediated negative feedback regulation of NKT-cell function by CD94/NKG2. *Blood* 106: 184-192.

186. Oliveira, C. C., P. A. van Veelen, B. Querido, A. de Ru, M. Sluijter, S. Laban, J. W. Drijfhout, S. H. van der Burg, R. Offringa, and T. van Hall. 2010. The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. *J Exp Med* 207: 207-221.

187. Heinzel, A. S., J. E. Grotzke, R. A. Lines, D. A. Lewinsohn, A. L. McNabb, D. N. Streblow, V. M. Braud, H. J. Grieser, J. T. Belisle, and D. M. Lewinsohn. 2002. HLA-E-dependent presentation of Mtb-derived antigen to human CD8+ T cells. *J. Exp. Med.* 196: 1473-1481.

188. Pietra, G., C. Romagnani, P. Mazzarino, M. Falco, E. Millo, A. Moretta, L. Moretta, and M. C. Mingari. 2003. HLA-E-restricted recognition of cytomegalovirus-derived peptides by human CD8+ cytolytic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 100: 10896-10901.

189. Salerno-Goncalves, R., M. Fernandez-Vina, D. M. Lewinsohn, and M. B. Sztein. 2004. Identification of a human HLA-E-restricted CD8+ T cell subset in volunteers immunized with Salmonella enterica serovar Typhi strain Ty21a typhoid vaccine. *J. Immunol.* 173: 5852-5862.

190. Grotzke, J. E., M. J. Harriff, A. C. Siler, D. Nolt, J. Delepine, D. A. Lewinsohn, and D. M. Lewinsohn. 2009. The Mycobacterium tuberculosis phagosome is a HLA-I processing competent organelle. *PLoS Pathog* 5: e1000374.

191. Hoare, H. L., L. C. Sullivan, G. Pietra, C. S. Clements, E. J. Lee, L. K. Ely, T. Beddoe, M. Falco, L. Kjer-Nielsen, H. H. Reid, J. McCluskey, L. Moretta, J. Rossjohn, and A. G. Brooks. 2006. Structural basis for a major histocompatibility complex class Ib-restricted T cell response. *Nat. Immunol.* 7: 256-264.

192. Lu, L., H. J. Kim, M. B. Werneck, and H. Cantor. 2008. Regulation of CD8+ regulatory T cells: Interruption of the NKG2A-Qa-1 interaction allows robust suppressive activity and resolution of autoimmune disease. *Proc. Natl. Acad. Sci. USA* 105: 19420-19425.

193. Leavenworth, J. W., C. Schellack, H. J. Kim, L. Lu, P. Spee, and H. Cantor. 2010. Analysis of the cellular mechanism underlying inhibition of EAE after treatment with anti-NKG2A F(ab')2. *Proc. Natl. Acad. Sci. USA* 107: 2562-2567.

194. Smith, T. R., X. Tang, I. Maricic, Z. Garcia, S. Fanchiang, and V. Kumar. 2009. Dendritic cells use endocytic pathway for cross-priming class Ib MHC-restricted CD8alphaalpha+TCRalphabeta+ T cells with regulatory properties. *J. Immunol.* 182: 6959-6968.

195. Chen, W., L. Zhang, B. Liang, Y. Saenger, J. Li, L. Chess, and H. Jiang. 2007. Perceiving the avidity of T cell activation can be translated into peripheral T cell regulation. *Proc. Natl. Acad. Sci. USA* 104: 20472-20477.

196. Jiang, H., H. Kashleva, L. X. Xu, J. Forman, L. Flaherty, B. Pernis, N. S. Braunstein, and L. Chess. 1998. T cell vaccination induces T cell receptor Vbeta-

specific Qa-1-restricted regulatory CD8(+) T cells. *Proc. Natl. Acad. Sci. USA* 95: 4533-4537.

197. Jiang, H., Y. Wu, B. Liang, Z. Zheng, G. Tang, J. Kanellopoulos, M. Soloski, R. Winchester, I. Goldstein, and L. Chess. 2005. An affinity/avidity model of peripheral T cell regulation. *J Clin Invest* 115: 302-312.

198. Michaelsson, J., C. Teixeira de Matos, A. Achour, L. L. Lanier, K. Karre, and K. Soderstrom. 2002. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. *J. Exp. Med.* 196: 1403-1414.

199. Kemball, C. C., C. D. Pack, H. M. Guay, Z. N. Li, D. A. Steinhauer, E. Szomolanyi-Tsuda, and A. E. Lukacher. 2007. The antiviral CD8+ T cell response is differentially dependent on CD4+ T cell help over the course of persistent infection. *J. Immunol.* 179: 1113-1121.

200. Kemball, C. C., E. D. Lee, E. Szomolanyi-Tsuda, T. C. Pearson, C. P. Larsen, and A. E. Lukacher. 2006. Costimulation requirements for antiviral CD8+ T cells differ for acute and persistent phases of polyoma virus infection. *J. Immunol.* 176: 1814-1824.

201. He, X., P. Tabaczewski, J. Ho, I. Stroynowski, and K. C. Garcia. 2001. Promiscuous antigen presentation by the nonclassical MHC Ib Qa-2 is enabled by a shallow, hydrophobic groove and self-stabilized peptide conformation. *Structure* 9: 1213-1224.

202. Stroynowski, I., and P. Tabaczewski. 1996. Multiple products of class Ib Qa-2 genes which ones are functional? *Res. Immunol.* 147: 290-301.

203. Tabaczewski, P., E. Chiang, M. Henson, and I. Stroynowski. 1997. Alternative peptide binding motifs of Qa-2 class Ib molecules define rules for binding of self and nonself peptides. *J. Immunol.* 159: 2771-2781.

204. Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsh, and B. E. Griffin. 1980. Coding potential and regulatory signals of the polyoma virus genome. *Nature* 283: 445-453.

**Figure Legends**

**Figure 1.1: MPyV encodes six proteins in overlapping reading frames.** An approximation of the MPyV genome is diagrammed, with the early region genes on the left and the late region genes on the right. The black line represents the 5,292 base pairs of double stranded DNA (204). Numbers represent base pairs from the start of the linearized genome. Colored arrows represent the open reading frames of the six proteins. ORI: origin of replication. VP: Viral Protein. T: Tumor antigen.

**Figure 1.2. Different peptide:Qa-1b ligands trigger varied responses by Qa-1b-specific CD8 T cells.** (**Left**): CD8 T cells expressing TCRs specific for pathogen derived peptide (pAg) bound to Qa-1b are stimulated to employ anti-pathogen effector activities. (**Middle**): Dynamic interplay between TCR activation and CD94-NKG2A inhibition on a CD8 Treg determines the fate of the autoreactive CD4 T cell. Here, TCRs of CD8 Treg cells recognize a peptide from the TCR-Vb (pVb) of an autoreactive CD4 T cell presented by Qa-1b. Qa-1b also presents Qdm peptide that engages inhibitory CD94-

NKG2A receptors. (**Right**): TCR mediates CD8 Treg recognition of a peptide derived from the signal sequence of Hsp60 presented by Qa-1b. The CD8 Treg is signaled to control an intermediate affinity, potentially self-reactive CD4 T cell.

**Figure 1.1**

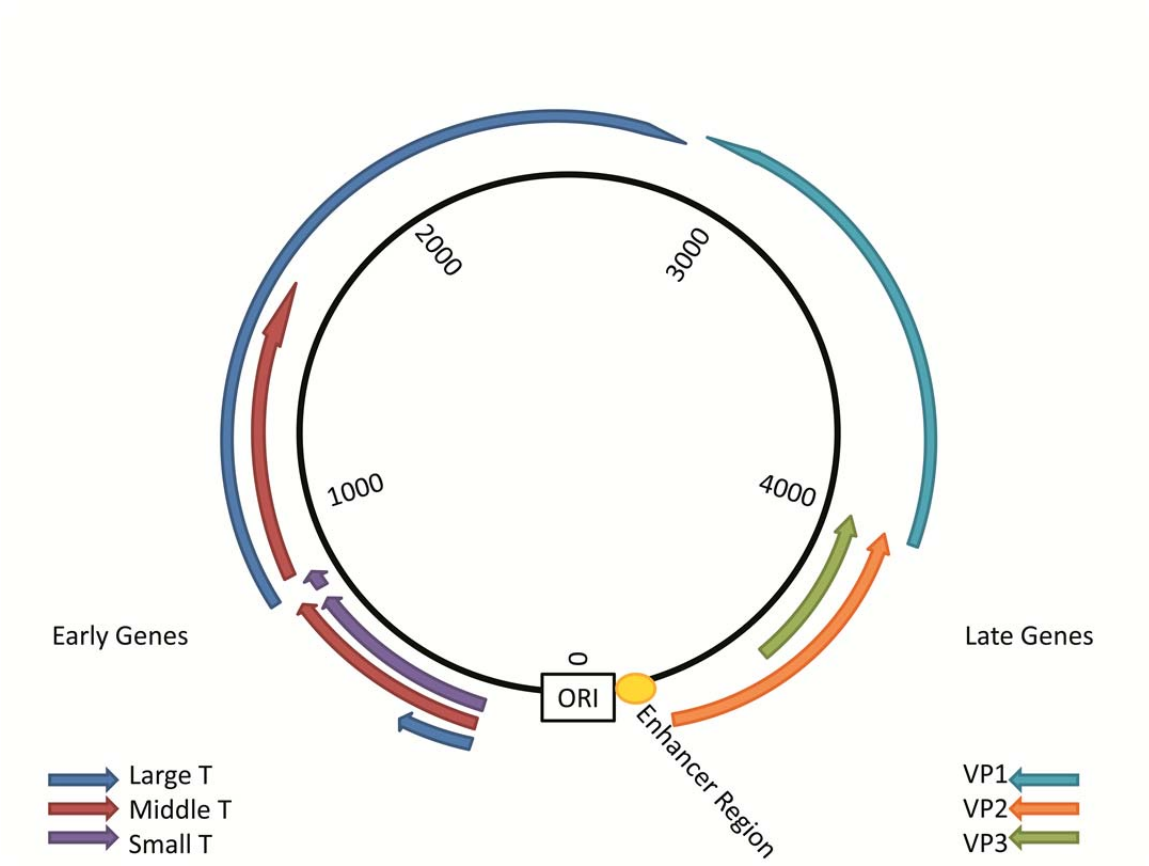
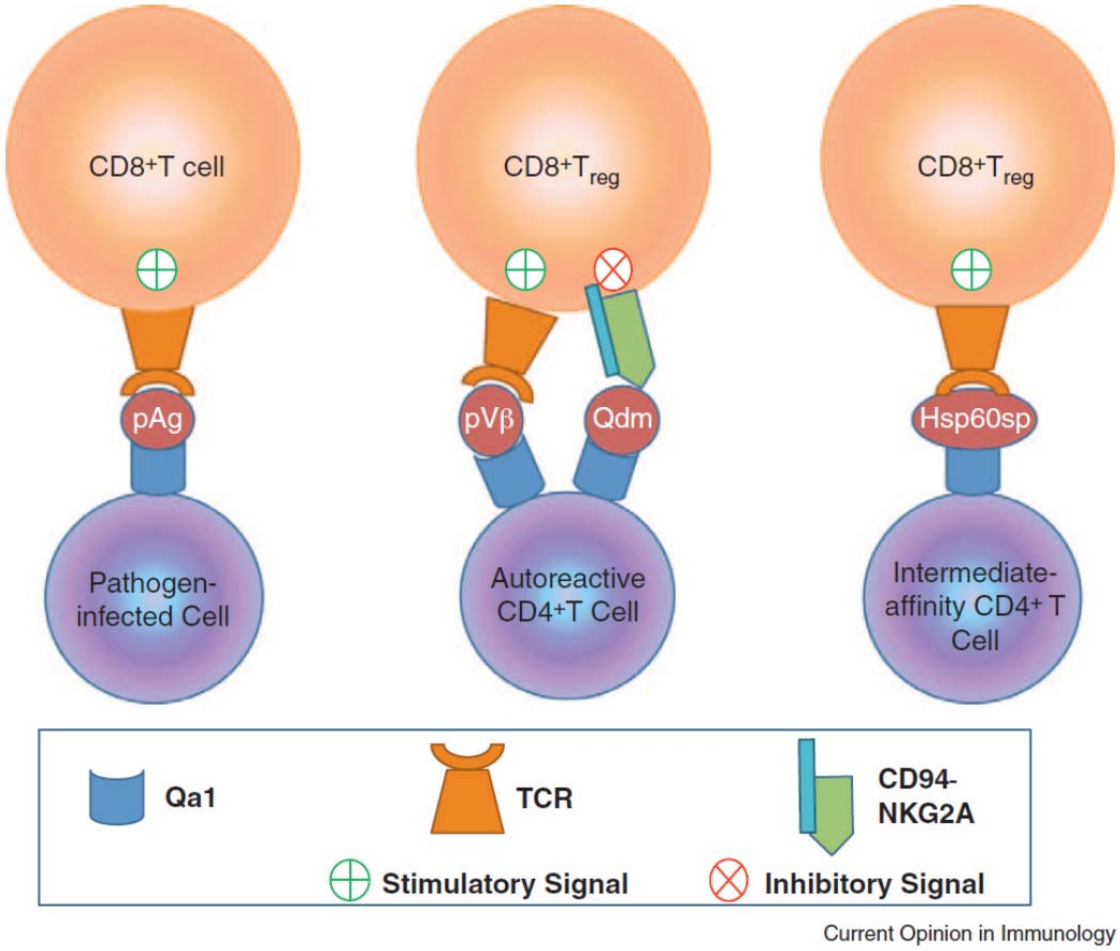

2000
3000
1000
4000
0
ORI
Enhancer Region
Early Genes
Late Genes
Large T
Middle T
Small T
VP1
VP2
VP3

**Figure 1.2**


CD8+T cell
CD8+Treg
CD8+Treg
pAg
pVβ
Qdm
Hsp60sp
Pathogen-infected Cell
Autoreactive CD4+T Cell
Intermediate-affinity CD4+ T Cell
Qa1
TCR
CD94-NKG2A
Stimulatory Signal
Inhibitory Signal
Current Opinion in Immunology

# Chapter 2

# MHC class Ib-restricted CD8 T cells differ in dependence on CD4 T cell help and CD28 costimulation over the course of mouse polyomavirus infection

All figures and tables in Chapter 2 are based on data generated by the Ph.D. candidate.

Data presented in Figures 2.2 and 2.3 were generated in collaboration with Dr. Mandy L. Ford, Department of Surgery, Emory University, Atlanta GA.

Data presented in Figure 2.4 was generated in collaboration with Dr. Lucy C. Sullivan and Dr. Andrew G. Brooks, Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia.

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**Abstract**

We recently identified a protective MHC class Ib-restricted CD8 T cell response to infection with mouse polyomavirus. These CD8 T cells recognize a peptide from aa 139-147 of the VP2 viral capsid protein bound to the nonpolymorphic H-2Q9 molecule, a member of the Qa-2 family of $\beta_2$m-associated MHC-Ib molecules. Q9:VP2.139-specific CD8 T cells exhibit an unusual inflationary response characterized by a gradual expansion over 3 months followed by a stable maintenance phase. We previously demonstrated that Q9:VP2.139-specific CD8 T cells are dependent on Ag for expansion, but not for the long-term maintenance. In this study, we tested the hypothesis that the expansion and maintenance components of the Q9:VP2.139-specific T cell response are differentially dependent on CD4 T cell help and CD28 costimulation. Depletion of $CD4^+$ cells and CD28/CD40L blockade impaired expansion of Q9:VP2.139-specific CD8 T cells, and intrinsic CD28 signaling was sufficient for expansion. In contrast, CD4 T cell-insufficiency, but not CD28/CD40L blockade, resulted in a decline in frequency of Q9:VP2.139-specific CD8 T cells during the maintenance phase. These results indicate that the Q9:VP2.139-specific CD8 T cell response to mouse polyomavirus infection depends on CD4 T cell help and CD28 costimulation for inflationary expansion, but only on CD4 T cell help for maintenance.

## Introduction

Nonclassical MHC class Ib molecules are generally distinguished from the classical MHC class Ia molecules in being less polymorphic, having limited tissue distribution, and lower cell surface expression levels. Although some class Ib molecules present non-peptide molecules such as lipids, transferrin, or odorants (1), others structurally resemble class Ia molecules and present oligopeptides to CD8 T cells. Class Ib-restricted CD8 T cell responses to peptides and lipids contribute to both innate and adaptive immunity (2). Most class Ib-restricted T cell responses described to date participate in anti-bacterial host defense. For example, Qa-$1^b$-restricted T cells mediate protection to infection by *Listeria monocytogenes* (3, 4), and H2-M3 presents *N*-formyl peptides to T cell responses to several bacterial infections, including *L. monocytogenes* (5) *and Mycobacterium tuberculosis* (6).

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

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

In this study, we explored roles of CD4 T cell help and CD28/CD40L costimulation as determinants of expansion and maintenance for the Q9:VP2.139-specific CD8 T cell response. In the absence of CD4 T cell help, conventional class Ia-restricted CD8 T cells show no deficiency in recruitment to acute MPyV infection, but then suffer massive attrition during persistent infection; this response profile cannot be attributed to elevated viral infection levels (14). We further showed that the class Ia-restricted anti-MPyV CD8 T cell response depends on both CD28 and CD40L costimulation for expansion, but is independent of these signals during maintenance (15). We hypothesized

that, in parallel with their Ag dependence, the Q9:VP2.139-specific response would be dependent on CD4 T cell help and CD28/CD40L costimulation for expansion, but not for maintenance. However, although inflationary expansion of these cells was found to be dependent on CD28 costimulation and CD4 T cell help, CD4 T cells were also required for maintenance of Q9:VP2.139-specific CD8 T cells.

## Materials and Methods

### *Mice*

B6.$K^{b-/-}D^{b-/-}$ mice (Thy1.2) were purchased from Taconic Farms (Germantown, NY); B6.$K^{b-/-}D^{b-/-}$ Thy1.1 mice (16) were originally provided by Peter Jensen (University of Utah, Salt Lake City, UT). $CD40^{-/-}$ mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 (B6) mice were purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD). B6.129S2-$Cd28^{tm1Mak}$/J ($CD28^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were bred and housed by the Division of Animal Resources at Emory University (Atlanta, GA) in accordance with the guidelines of the Institutional Animal Care and Use Committee of Emory University. Female mice were 6-12-wk old at time of infection.

### *Viruses*

MPyV wild-type virus (strain A2) was prepared as described previously (17). An MPyV mutant with a histidine to alanine substitution at aa 145 in VP2 (A2.H145A) was prepared as described previously (11). Mice were inoculated s.c. with 1 x $10^6$ PFU in the hind footpads.

### *Costimulation blockade and $CD4^+$ cell depletion*

Anti-CD4 (clone GK1.5) was administered i.p. at 250 μg/mouse on days -3, -1, and 1 relative to MPyV infection, and then weekly thereafter. $CD4^+$ cell depletion was verified by staining blood samples with anti-CD4 clone RM4-5 (eBioscience). Anti-CD40 ligand

(clone MR-1; BioXCell) and/or CTLA-4-Ig (Bristol-Meyers Squibb) were administered i.p. at 500 μg/mouse on days 0 and 2 of MPyV infection, and then weekly thereafter. After mice were infected for 3 mo, GK1.5, MR-1, and/or CTLA-4 was administered twice in the first 3 d and weekly thereafter using 500 μg/mouse of each protein.

*Flow cytometry*

1 x $10^6$ RBC-lysed splenocytes, incubated with BD FcBlock for 15 min at 4°C, were stained with a chimeric Q9(Kb)VP2.139 tetramer, generated by swapping the α3 chain of H-$2K^b$ with that of Q9 (National Institutes of Health Tetramer Core Facility). This tetramer binds Q9:VP2.139-specific cells at a 4-fold higher dilution than wild-type Q9 tetramers (A.R. Hofstetter, unpublished observations). Cells were also stained with Abs against the following molecules: CD44, CD62L, CD25, NKG2A/C/E (Clone 20d5), 2B4, 4-1BB and Thy1.1 (BD Pharmingen), CD4, CD127, CD122 , CD27, PD-1, CD28, NKG2A (Clone 16a11), ICOS, OX-40, rat IgG2a and rat IgG2b (ebioscience), CD69 (Invitrogen), CD43 (BioLegend), KLRG-1 (SouthernBiotech), and CD8α (BD Pharmingen and BioLegend). Intracellular cytokine staining was performed as described (18). After incubation with and without 1μM VP2.139 peptide for 5-6 h at 37°C in the presence of GolgiPlug (BDBiosciences), cells were stained intracellularly with antibodies to IFN-γ, TNF-α, and IL-2 (BD Pharmingen), and for CD40L (eBioscience) and CD69 (Invitrogen). Samples were acquired on a FACSCalibur (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

*Quantitation of MPyV genomes*

Tissue samples were taken from spleen, kidney, salivary gland, and heart and snap-frozen. DNA isolation and TaqMan-based quantitative real-time PCR were performed as previously described (18). The limit of detection of this assay is 1000 copies of genomic viral DNA unless specified elsewhere.

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

A Q9:VP2.139-specific CD8 T cell hybridoma (C3K) was derived by fusing the C3-8 clone (described in Ref. (11)) with a CD8-transfected BW5147 fusion partner (described in Ref. (19)). Generation of replication-defective retroviral producer cell lines was performed as described BY Holst and colleagues (20). TCRα- and TCRβ- chains from C3K were cloned by PCR into pGEM using the following primer sequences: α chain (vα10) forward: 5' GCGCCAGAATTCAGATCTACCATGAAGAGGCTGCTGTGCTCTCTGCTGG 3'; β chain (vβ4) forward: 5' CTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCCATGGGCTCC ATTTTCCTCAGTTGCCT 3'. The products were then subcloned into the GFP-encoding murine stem cell virus-based retroviral vector pMIG at the BamHI site using the following reverse primers to generate a 2A-linked multicistronic construct: P2A-Cα reverse: 5' CTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCTCCGGAGCCG GACCACAGCCTCAGCGTCATGAG 3'

P2A-Cβ reverse: 5' GCGTCGCTCGAGGGATCCTCAGGAATTTTTTYTCTTGACCATRGC 3'. 293T

cells were transiently transfected with three plasmids, separately encoding packaging genes, envelope genes, and the C3K TCR retroviral vector. Retroviruses produced by these 293T cells were used to infect GP+E86 cells to generate replication-deficient retroviruses. Retrovirus-mediated stem cell gene transfer was carried out as described by Holst and colleagues (20) using B6 or $CD28^{-/-}$ bone marrow and B6 recipients to generate retrogenic donor mice. In brief, bone marrow isolated from B6 or $CD28^{-/-}$ mice was infected by culture with irradiated GP+E86 producer cells, then transferred i.v. into B6 recipients. After 5 wk, retrogenic donor mice were checked for reconstitution by staining with anti-Vβ4 and Q9:VP2.139 tetramer, and by GFP expression. In our hands, 80-90% of retrogenic donor mice's splenocytes express GFP. Of the $CD8^+$ T cells in this population 20-30% stain with Vβ4. Splenocytes were FACS-sorted for $GFP^+$ cells (FACSAria, BD Biosciences) and either $1 \times 10^5$ or $1 \times 10^6$ cells were transferred i.v. Mice received wild-type MPyV or A2.H145A 1 d post-transfer.

*IL-2 treatment*

Recombinant human IL-2 (Amgen) was administered i.p. at 15,000 U/mouse in PBS and 0.1% $B6.K^{b-/-}D^{b-/-}$ mouse serum every 12 h for 16 d.

*Statistics*

Statistical analyses were performed using Prism software (GraphPad, La Jolla, CA). For blood kinetic studies, data were analyzed using 2-way ANOVA with Bonferroni post tests. For phenotyping and quantitative PCR (qPCR) studies, two-tailed Mann-Whitney *t* tests were used, with the exception of qPCR data for singly administered anti-CD40L or

CTLA-4Ig, for which a Kruskal-Wallis Test was used, with Dunn's Multiple Comparison Test.

## Results

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

To investigate whether CD4 T cell help is required for expansion of Q9:VP2.139-specific T cells, MHC class Ia-deficient B6.$K^{b-/-}D^{b-/-}$ mice were depleted of $CD4^+$ cells by administering anti-CD4 (clone GK1.5). Although CD4 T cell insufficiency in B6.$K^{b-/-}D^{b-/-}$ mice has been shown to foster homeostatic expansion of the CD8 T cell compartment (16), we did not observe an increase in numbers of $CD8^+$ cells in the spleens of GK1.5 mAb-treated, MPyV-infected B6.$K^{b-/-}D^{b-/-}$ mice at 1 mo p.i. (A.R. Hofstetter, unpublished observations). As shown in Fig. 2.1A and 2.1B, CD4-insufficiency was associated with severely blunted expansion of Q9:VP2.139-specific T cells, as detected by both tetramer binding and VP2.139 peptide-stimulated intracellular IFN-γ production. Depletion of CD4 cells was associated with a modest loss in viral control that differed between organs examined. As shown in Fig. 2.1C, MPyV load in the salivary gland was ~6-fold higher in $CD4^+$ cell-depleted mice, although the MPyV genome copy numbers in kidneys and salivary glands were not significantly different from those in rat IgG-treated control mice. In this connection, it merits noting that MPyV infection elicits a T cell-independent virus-neutralizing IgG response response that controls viral burden (21). Compared with rat IgG-treated infected mice, at 1 mo p.i., Q9:VP2.139-specific CD8 T cells in GK1.5-treated mice expressed higher cell surface levels of CD27, PD-1, and slightly higher levels of CD122, with lower levels of KLRG-1 (Fig. 2.1D). This phenotypic profile resembles the phenotype seen during chronic infection with LCMV with CD4 depletion (22), suggesting that the unhelped Q9:VP2.139-specific cells may be similarly fated for functional exhaustion.

Based on evidence that increased CD27 signaling may suppress effector differentiation (23), we asked whether unhelped Q9:VP2.139-specific cells have less effector cytokine function than helped cells. However, the same frequency (~19%) of Q9:VP2.139-specific CD8 T cells elaborated IFN-γ production after ex vivo VP2.139 peptide stimulation as those in rat IgG-treated infected mice (Fig. 2.1B). Together, these results indicate that CD4 T cell help is important for normal expansion of Q9:VP2.139-specific CD8 T cells during MPyV infection.

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

CD4 T cell help is thought to act through CD40:CD40L-mediated activation of dendritic cells, leading to CD28:CD80/86 interactions between dendritic cells and CD8 T cells (24). Because we saw a detrimental effect on Q9:VP2.139-specific expansion because of CD4 depletion, we next asked whether expansion of Q9:VP2.139-specific CD8 T cells requires costimulation by CD28 and CD40L. We applied an established combined costimulation blockade regimen using anti-CD40L (clone MR1) and CTLA-4-Ig, which were administered together to MPyV-infected B6.$K^{b-/-}D^{b-/-}$ mice for the first month p.i. As shown in Fig. 2.2A and 2.2B, CD28-CD40L costimulation blockade profoundly crippled the expansion of Q9:VP2.139-specific CD8 T cells. By 1 mo p.i., Q9:VP2.139 tetramer$^{+}$ CD8 T cells had expanded to >9% of the CD8 population in only one third of treated mice. In contrast, Q9:VP2.139 tetramer$^{+}$ CD8 T cells in all untreated mice ranged from 18-62% of total CD8 T cells. Q9:VP2.139-specific CD8 T cells in the costimulation blockade responders phenotypically resembled those in untreated mice, except for having fewer KLRG-$1^{hi}$ cells and more PD-$1^{hi}$ cells (A.R. Hofstetter, unpublished observations);

interestingly, this shift in KLRG-1 and PD-1 expression is similar to that of Q9:VP2.139-specific CD8 T cells in $CD4^+$ cell-depleted mice (Fig. 2.1D). Mirroring its deleterious effect on the magnitude of the Q9:VP2.139-specific CD8 T cell response, CD28-CD40L costimulation blockade also resulted in a greater loss of viral control than in $CD4^+$ cell-depleted mice (Fig. 2.2C).

To determine whether both CD28- and CD40L- mediated costimulation were necessary for expansion of Q9:VP2.139-specific CD8 T cells, MPyV-infected $B6.K^{b-/-}D^{b-/-}$ mice were treated separately with anti-CD40L or CTLA-4-Ig. In the first 2 wk p.i., CTLA-4-Ig-treated mice showed only modest expansion of Q9:VP2.139-specific CD8 T cells; in contrast, Q9:VP2.139-specific CD8 T cell expansion progressed unimpaired in anti-CD40L-treated mice (Fig. 2.3A). However, after 2 wk, the Q9:VP2.139-specific CD8 T cell population in CTLA-4-Ig-treated mice became detectable and reached magnitudes that were not significantly different from in anti-CD40L-treated and untreated control mice (Fig. 2.3A, 2.3B), and virus loads were equivalent (Fig. 2.3C). Breakthrough T cell responses during costimulation blockade have been observed in mouse skin allograft transplantation, with anti-donor T cell responses overcoming blockade by 21 d after transplantation (25). To examine the Q9:VP2.139-specific CD8 T cell response before breakthrough occurs, we sacrificed mice at 2 wk p.i. At this time point, CD28 blockade was observed to clearly dampen the response (Fig. 2.3D), and was associated with significantly greater virus levels in the spleen and salivary glands (Fig. 2.3E). As shown in Fig. 2.3D, anti-CD40L-treated mice also had a diminished number and frequency (A.R. Hofstetter, unpublished observations) of Q9:VP2.139-specific CD8 T cells in the spleen, but there was no significant difference in viral titers compared with

the untreated control group. The frequency of Q9:VP2.139-specific CD8 T cells in the blood of anti-CD40L-treated mice at day 15 was equivalent to that in untreated mice (A.R. Hofstetter, unpublished observations). Impaired cellular trafficking in the presence of CD40L blockade (26, 27) may explain the discord between the frequency of Q9:VP2.139-specific CD8 T cells in the blood and spleen. These results demonstrate that expansion of Q9:VP2.139-specific CD8 T cells is dependent upon CD28 costimulation.

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

To directly determine whether the dependence on CD28-mediated costimulation for expansion of Q9:VP2.139-specific CD8 T cells is autochthonous, we used retrogenic TCR technology to create mice with monoclonal Q9:VP2.139-specific CD8 T cells (20). In brief, lethally irradiated B6 mice were engrafted using bone marrow infected by a recombinant, replication-deficient retrovirus bicistronic for genes for GFP and a TCR from a cloned line of Q9:VP2.139-specific CD8 T cells. To demonstrate Ag specificity, we FACS sorted $GFP^+$ $CD8^+$ cells were from spleens of these TCR retrogenic mice and transferred to naïve B6 recipients, which were then infected with either wild-type MPyV or a mutant MPyV in which the codon for the dominant histidine anchor residue in VP2.139 epitope was replaced with one for alanine (A2.H145A). As shown in Fig. 2.4A, $GFP^+$ (i.e., donor TCR retrogenic) CD8 T cells were recruited in mice infected by wild-type MPyV but not A2.H145A. It is important to note that expansion of the immunodominant class Ia-restricted CD8 T cell response to MPyV was not affected in recipients of the retrogenic T cells (A.R. Hofstetter, unpublished observations). To investigate whether CD28 expression by Ag-specific CD8 T cells per se was essential for

their expansion in MPyV-infected mice, we generated Q9:VP2.139 TCR retrogenic mice using bone marrow from Thy1.1$^{+}$ wild-type B6 mice (B6.PL) and Thy1.2$^{+}$ CD28$^{-/-}$ B6 mice. Equal numbers (1 x $10^{5}$) of FACS-sorted GFP$^{+}$ CD8$^{+}$ cells from B6.PL and CD28$^{-/-}$ bone marrow TCR retrogenic mice were co-transferred into naïve Thy 1.2$^{+}$ B6 recipients (Fig. 2.4C), then infected with parental MPyV. As shown in Fig. 2.4D, CD28-sufficient GFP$^{+}$ retrogenic cells efficiently expanded in mice infected by wild-type MPyV. In contrast, Thy1.1$^{+}$ GFP$^{+}$ (i.e., CD28$^{-/-}$) cells only expanded in one of seven infected recipients, did so with delayed kinetics, and reached substantially lower frequencies than those of CD28-sufficient donor T cells (Fig. 2.4E). Importantly, wild-type Q9:VP2.139 TCR retrogenic cells transferred into CD40L$^{-/-}$ mice expanded equivalently to that of wild-type retrogenic cells transferred into wild-type mice p.i. by MPyV (A.R. Hofstetter, unpublished observations). These data demonstrate that CD28 signaling is intrinsically required by Q9:VP2.139-specific CD8 T cells for expansion in MPyV-infected mice.

*Maintenance of Q9:VP2.139-specific cells does not require CD28 and CD40L costimulation*

We previously demonstrated that CD28 and CD40L costimulation is dispensible for maintenance of class Ia-restricted antiviral CD8 T cells during persistent MPyV infection (15). Based on this and our prior data showing that maintenance of Q9:VP2.139-specific CD8 T cells in persistently infected mice is Ag independent (12), we hypothesized that CD28 and CD40L costimulation blockade would not impact long-term maintenance of Q9:VP2.139-specific CD8 T cells. To test this possibility, we administered CD28 and CD40L costimulation blockade to B6.K$^{b-/-}$D$^{b-/-}$ mice for 1 mo beginning at 3 mo p.i. As

shown in Fig. 2.5A, the frequency of circulating Q9:VP2.139-specific CD8 T cells in mice given anti-CD40L and CTLA-4-Ig was maintained at the same levels as in untreated, infected B6.$K^{b-/-}D^{b-/-}$ mice. Moreover, after 1 mo p.i., direct ex vivo analyses showed minimal differences in frequency, magnitude, cytokine effector function (Fig. 2.5B), and surface phenotype (Fig. 2.5D) of Q9:VP2.139-specific CD8 T cells in untreated mice and mice receiving costimulation blockade. Consistent with these findings, MPyV loads were similar in the kidney, spleen, salivary glands, and heart in both groups (Fig. 2.5C). Interestingly, CD28 expression by Q9:VP2.139-specific CD8 T cells in B6.$K^{b-/-}D^{b-/-}$ mice declined between d 12 and 1 mo p.i. and remained low long-term (Fig. 2.5E), a finding that may correlate with the differential impact of CD28 blockade during expansion and maintenance phases of this T cell response.

*Maintenance of Q9:VP2.139-specific CD8 T cells is dependent on CD4 T cell help*

Because the Q9:VP2.139-specific CD8 T cell population was independent of Ag and CD28/CD40L costimulation for its maintenance, we hypothesized that CD4 T cell help during this phase would similarly be dispensable. To test this possibility, we administered GK1.5 mAb-mediated $CD4^+$ cell depletion to B6.$K^{b-/-}D^{b-/-}$ mice 3 mo p.i. Unexpectedly, CD4 depletion resulted in a steady attrition of circulating Q9:VP2.139-specific CD8 T cells, whereas rat IgG-treated mice maintained their Q9:VP2.139-specific population (Fig. 2.6A). The rapid attrition of the Q9:VP2.139 population during $CD4^+$ cell deficiency may be explained by the loss of IL-2 secretion by $CD4^+$ cells. However, administering recombinant human IL-2 to B6.$K^{b-/-}D^{b-/-}$ mice depleted of $CD4^+$ cells at 3 mo p.i. failed to prevent loss of the Q9:VP2.139-specific CD8 T cell population (Fig 2.7).

By 1 mo p.i., GK1.5 mAb treated mice had 5-fold fewer Q9:VP2.139-specific CD8 T cells in the spleen than rat IgG-treated controls (Fig. 2.6B). Q9:VP2.139 tetramer$^{+}$ CD8 T cells from GK1.5 mAb-treated mice, although still CD62L$^{lo}$, displayed higher expression of CD127 and CD27 (Fig. 2.6C), which may indicate that those CD8 T cells with more central memory-like characteristics (28) are less dependent on CD4 help for survival. CD4 cell depletion-mediated attrition of Q9:VP2.139-specific cells did not lead to a change in viral loads after 1 mo (Fig. 2.6D). We demonstrated earlier that CD4 depletion during expansion had only a modest effect on viral loads, suggesting 10% of the CD8 T cell compartment, composed of Q9:VP2.139-specific cells, can control viral loads. Control would be enhanced during the maintenance phase, because virus levels are lower at this time. Taken together, these data show that the Q9:VP2.139-specific CD8 T cell response depends on CD4 T cell help for both expansion and maintenance, whereas CD28 costimulation is only required for expansion of this MHC class Ib-restricted T cell response.

**Discussion**

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

The role of CD4 T cell help for the primary expansion of CD8 T cells has been investigated in numerous models, with varied results. Early experiments with non-inflammatory cell-based immunization (29) or HSV infection (30) indicated a clear role for CD4 T cell help in generating CD8 T cell responses. In contrast, CD4 T cell-independent primary expansion of pathogen-specific class Ia-restricted CD8 T cells has been documented in several experimental infection systems, including MPyV (14, 31-36). Unlike for these virus-specific, class Ia-restricted CD8 T cell responses, the primary expansion of Q9:VP2.139-specific CD8 T cells is CD4 T cell dependent as shown by its apparently abbreviated peak magnitude response. However, a similar pattern has been seen for unhelped primary CD8 T cell responses to *Plasmodium yoelii* (37, 38), *L. monocytogenes,* and vaccinia virus (36). This apparent CD4 T cell independence for

naïve CD8 T cell priming, but dependence on CD4 T cells for sustained proliferation, was predicted for pathogens triggering intermediate levels of danger signals (39). The CD4 T cell independence of the MPyV-specific, class Ia-restricted CD8 T cell response to acute infection, however, argues against inflammation level as a major determinant of expansion by these unhelped MHC class Ib-restricted CD8 T cells.

Irrespective of the availability of CD4 T cell help, there is a similar dissociation between tetramer-binding and IFN-γ production by Q9:VP2.139-specific CD8 T cells. We previously demonstrated that IFN-γ has direct anti-MPyV activity and is important for controlling MPyV infection (13). Although CD4 T cell depletion reduced expansion of the Q9:VP2.139-specific CD8 T cell response, viral load was kept in check in most of the organs examined. It is interesting to speculate that the high magnitude Q9:VP2.139-specific CD8 T cell response in $K^{b-/-}D^{b-/-}$ mice may compensate for their depressed effector functionality and enable them to exert effective control of MPyV infection.

Secretion of IL-2 by CD4 T cells constitutes a well-recognized mechanism of T cell help (40). D'Souza and Lefrancois (41) reported that IL-2R signaling is unnecessary for the initiation of CD8 T cell cycling, but required for sustained expansion. CD4 T cells may similarly be involved in expansion of activated naïve Q9:VP2.139-specific CD8 T cells, possibly resulting from upregulation of high-affinity IL-2Rs through CD28:CD80/86 signaling (36). Furthermore, CD25 expression on CD8 T cells has been shown to be independent of CD40 signaling (36), which also holds for expansion of Q9:VP2.139-specific CD8 T cells. This is in contrast with the class Ia-restricted CD8 T cell responses to MPyV infection, which display dependence on both CD28 and CD40L signals for expansion (15). Thus, CD28 signaling may be primarily involved in

recruitment of naïve Q9:VP2.139-specific CD8 T cells, with CD4 T cell help promoting upregulation of high-affinity IL-2Rs to enable expansion by IL-2. However, we did not observed decreased CD25 expression on Q9:VP2.139-specific cells CD4-depleted B6.$K^{b-/-}D^{b-/-}$ mice on day 10 p.i. (A.R. Hofstetter, unpublished observations).

CD28 signaling by Q9-restricted CD8 T cells may be essential for amplifying weak TCR signaling. Data from our lab and others support the concept that Q9:VP2.139-specific cells bind weakly with their pMHC ligands. Preliminary surface plasmon resonance data indicates that the Q9:VP2.139 TCR binds to its cognate ligand with lower affinity than that typical for class Ia-restricted TCR (A. Brooks and L. Sullivan, unpublished observations). Furthermore, the orientation of the CD8-binding loop in the α3 domain of Q9 suggests that it weakly engages CD8 coreceptors (7). This possibility is supported by our data showing that Q9:VP2.139 tetramers constructed using a chimeric Q9 molecule with a $K^b$ α3 domain, which strongly binds CD8α (42), stains Ag-specific CD8 T cells with higher mean fluorescence intensity (MFI) than Q9 tetramers with native α3 domains (A.R. Hofstetter, unpublished observations). The tissue distribution and expression levels of Q9 are similar to that of MHC class Ia molecules (9); however, Q9 is upregulated by IFN-γ (43) and the defect in IFN-γ effector function by Q9:VP2.139-specific CD8 T cells may impact Q9 expression levels. Suboptimal TCR signaling may also explain the slow inflationary expansion of the Q9:VP2.139-specific cells and their effector function defect. By corollary, high epitope density and/or strong CD28 signaling may compensate for inefficient CD8 coreceptor binding and enable full activation of Q9-restricted CD8 T cells. Because the inflationary kinetics of the Q9:VP2.139-specific response depend on persistent infection (12), it is possible that continuous TCR

stimulation together with CD28 costimulation is needed for Q9:VP2.139-specific CD8 T cells to maximally expand.

As seen in this study for memory MHC class Ib-restricted CD8 T cells to MPyV infection, CD4 T cell help has been shown to maintain memory CD8 T cells in the setting of other persistent infections (14, 31, 32, 44). The nature of the CD4 T cell help for maintaining the MHC class Ib-restricted CD8 T cell population is unknown. CD4 T cells likely provide other forms of help aside from IL-2, because exogenous addition of IL-2 during the maintenance phase did not prevent decay of the Q9:VP2.139 population after $CD4^+$ cell depletion. We have recently demonstrated that MPyV-specific CD4 T cells, in addition to producing IL-2, produce IFN-γ, TNF-α, IL-10 and IL-21 (45). IL-21 has been demonstrated to sustain CD8 T cells during chronic infection (46-48), and it is tempting to speculate that IL-21 signaling may also support the sustained expansion and maintenance of Q9:VP2.139-specific CD8 T cells. In contrast with CD4 help, CD28 and CD40L costimulation are not required during the maintenance phase. This is consistent with Ag independence of the Q9:VP2.139-specific CD8 T cell population at this time (12), as lack of TCR signaling precludes a requirement for costimulation (49).

Costimulatory molecules other than CD28 and CD40L may contribute to the expansion and maintenance of Q9:VP2.139-specific CD8 T cells. The kinetics of CD27 expression by Q9:VP2.139-specific CD8 T cells parallel those of CD28 expression (Fig. 2.5E, Fig. 2.8). The decreased expression of CD27 over the course of MPyV infection would imply declining dependence on CD27-mediated signals. 2B4 is expressed at a steady but low level throughout the response (Fig. 2.8). This receptor can be activating or inhibitory, depending on the level of 2B4 expression, the extent of 2B4 cross-linking, and

the relative abundance of signaling lymphocyte activation molecule-associated protein (50). OX-40, 4-1BB, and ICOS are expressed at only low levels by Q9:VP.139-specific CD8 T cells (Fig. 2.8). In contrast, CD94/NKG2A expression increases after d 12 p.i. and is maintained at high expression levels (Fig. 2.6D and Fig. 2.8). We previously showed that CD94/NKG2A is expressed by MHC class Ia-restricted, MPyV-specific CD8 T cells, where it inhibits cytotoxicity whereas increasing IL-2 production and proliferative potential (51, 52). However, the impact of CD94/NKG2A expression varies depending on infection model (53-55). Whether CD27, 2B4 or CD94/NKG2A expression on Q9:VP2.139-specific cells regulate IFN-γ functionality of these cells remains to be determined.

In this study, we explored CD4 T cell and CD28/CD40L costimulation as determinants for the inflationary kinetic profile of an MHC class Ib-restricted antiviral CD8 T cell population. We demonstrated that CD4 T cell help and intrinsic CD28 costimulation are necessary for expansion of Q9-restricted, MPyV-specific CD8 T cells, and that CD4 T cell help is important for survival of these cells during their Ag-independent maintenance phase. Together with our previous study on the role of Ag in expansion and maintenance of Q9:VP2.139-specific CD8 T cells (12), we propose the following model: Q9:VP2.139-specific CD8 T cells are primed in a CD4 T cell-independent fashion, expand in response to combined signals provided by cognate Ag, CD4 T cell help and intrinsic CD28 signaling, and are then maintained independent of Ag and CD28/CD40L but dependent on CD4 T cell help. This sustained dependence on CD4 T cell help, which does not involve a CD40L-dependent mechanism or IL-2,

suggests a novel mechanism of help for maintaining anti-MPyV MHC class Ib-restricted CD8 T cell response.

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References

1. Rodgers, J. R., and R. G. Cook. 2005. MHC class Ib molecules bridge innate and acquired immunity. *Nat. Rev. Immunol.* 5: 459-471.

2. Hofstetter, A. R., L. C. Sullivan, A. E. Lukacher, and A. G. Brooks. 2011. Diverse roles of non-diverse molecules: MHC class Ib molecules in host defense and control of autoimmunity. *Curr. Opin. Immunol.* 23: 104-110.

3. Bouwer, H. G., M. S. Seaman, J. Forman, and D. J. Hinrichs. 1997. MHC class Ib-restricted cells contribute to antilisterial immunity: evidence for Qa-1b as a key restricting element for Listeria-specific CTLs. *J. Immunol.* 159: 2795-2801.

4. Seaman, M. S., B. Perarnau, K. F. Lindahl, F. A. Lemonnier, and J. Forman. 1999. Response to Listeria monocytogenes in mice lacking MHC class Ia molecules. *J. Immunol.* 162: 5429-5436.

5. Xu, H., T. Chun, H. J. Choi, B. Wang, and C. R. Wang. 2006. Impaired response to Listeria in H2-M3-deficient mice reveals a nonredundant role of MHC class Ib-specific T cells in host defense. *J. Exp. Med.* 203: 449-459.

6. Doi, T., H. Yamada, T. Yajima, W. Wajjwalku, T. Hara, and Y. Yoshikai. 2007. H2-M3-restricted CD8+ T cells induced by peptide-pulsed dendritic cells confer protection against Mycobacterium tuberculosis. *J. Immunol.* 178: 3806-3813.

7. He, X., P. Tabaczewski, J. Ho, I. Stroynowski, and K. C. Garcia. 2001. Promiscuous antigen presentation by the nonclassical MHC Ib Qa-2 is enabled by a shallow, hydrophobic groove and self-stabilized peptide conformation. *Structure* 9: 1213-1224.

8. Stroynowski, I., M. Soloski, M. G. Low, and L. Hood. 1987. A single gene encodes soluble and membrane-bound forms of the major histocompatibility Qa-2 antigen: anchoring of the product by a phospholipid tail. *Cell* 50: 759-768.

9. Ungchusri, T., E. Y. Chiang, G. Brown, M. Chen, P. Tabaczewski, L. Timares, and I. Stroynowski. 2001. Widespread expression of the nonclassical class I Qa-2 antigens in hemopoietic and nonhemopoietic cells. *Immunogenetics* 53: 455-467.

10. Stroynowski, I., and P. Tabaczewski. 1996. Multiple products of class Ib Qa-2 genes which ones are functional? *Res. Immunol.* 147: 290-301.

11. Swanson, P. A., 2nd, C. D. Pack, A. Hadley, C. R. Wang, I. Stroynowski, P. E. Jensen, and A. E. Lukacher. 2008. An MHC class Ib-restricted CD8 T cell response confers antiviral immunity. *J. Exp. Med.* 205: 1647-1657.

12. Swanson, P. A., 2nd, A. R. Hofstetter, J. J. Wilson, and A. E. Lukacher. 2009. Cutting edge: shift in antigen dependence by an antiviral MHC class Ib-restricted CD8 T cell response during persistent viral infection. *J. Immunol.* 182: 5198-5202.

13. Wilson, J. J., E. Lin, C. D. Pack, E. L. Frost, A. Hadley, A. I. Swimm, J. Wang, Y. Dong, C. P. Breeden, D. Kalman, K. A. Newell, and A. E. Lukacher. 2011. Gamma interferon controls mouse polyomavirus infection in vivo. *J. Virol.* 85: 10126-10134.

14. Kemball, C. C., C. D. Pack, H. M. Guay, Z. N. Li, D. A. Steinhauer, E. Szomolanyi-Tsuda, and A. E. Lukacher. 2007. The antiviral CD8+ T cell response is differentially dependent on CD4+ T cell help over the course of persistent infection. *J. Immunol.* 179: 1113-1121.

15. Kemball, C. C., E. D. Lee, E. Szomolanyi-Tsuda, T. C. Pearson, C. P. Larsen, and A. E. Lukacher. 2006. Costimulation requirements for antiviral CD8+ T cells differ for acute and persistent phases of polyoma virus infection. *J. Immunol.* 176: 1814-1824.

16. Jay, D. C., L. M. Reed-Loisel, and P. E. Jensen. 2008. Polyclonal MHC Ib-restricted CD8+ T cells undergo homeostatic expansion in the absence of conventional MHC-restricted T cells. *J. Immunol.* 180: 2805-2814.

17. Andrews, N. P., C. D. Pack, V. Vezys, G. N. Barber, and A. E. Lukacher. 2007. Early virus-associated bystander events affect the fitness of the CD8 T cell response to persistent virus infection. *J. Immunol.* 178: 7267-7275.

18. Kemball, C. C., E. D. Lee, V. Vezys, T. C. Pearson, C. P. Larsen, and A. E. Lukacher. 2005. Late priming and variability of epitope-specific CD8+ T cell responses during a persistent virus infection. *J. Immunol.* 174: 7950-7960.

19. Gu, J. J., and P. D. Gottlieb. 1992. Inducible functions in hybrids of a Lyt-2+ BW5147 transfectant and the 2C CTL line. *Immunogenetics* 36: 283-293.

20. Holst, J., A. L. Szymczak-Workman, K. M. Vignali, A. R. Burton, C. J. Workman, and D. A. Vignali. 2006. Generation of T-cell receptor retrogenic mice. *Nat. Protoc.* 1: 406-417.

21. Szomolanyi-Tsuda, E., and R. M. Welsh. 1996. T cell-independent antibody-mediated clearance of polyoma virus in T cell-deficient mice. *J. Exp. Med.* 183: 403-411.

22. Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular

signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27: 670-684.

23. Carr, J. M., M. J. Carrasco, J. E. Thaventhiran, P. J. Bambrough, M. Kraman, A. D. Edwards, A. Al-Shamkhani, and D. T. Fearon. 2006. CD27 mediates interleukin-2-independent clonal expansion of the CD8+ T cell without effector differentiation. *Proc. Natl. Acad. Sci. USA* 103: 19454-19459.

24. Shedlock, D. J., J. K. Whitmire, J. Tan, A. S. MacDonald, R. Ahmed, and H. Shen. 2003. Role of CD4 T cell help and costimulation in CD8 T cell responses during Listeria monocytogenes infection. *J. Immunol.* 170: 2053-2063.

25. Coley, S. M., M. L. Ford, S. C. Hanna, M. E. Wagener, A. D. Kirk, and C. P. Larsen. 2009. IFN-gamma dictates allograft fate via opposing effects on the graft and on recipient CD8 T cell responses. *J. Immunol.* 182: 225-233.

26. Meng, L., Z. Wu, Y. Wang, C. Lassman, R. W. Busuttil, Y. Zhai, and J. W. Kupiec-Weglinski. 2008. Differential impact of CD154 costimulation blockade on alloreactive effector and regulatory T cells in murine renal transplant recipients. *Transplantation* 85: 1332-1338.

27. Sitati, E., E. E. McCandless, R. S. Klein, and M. S. Diamond. 2007. CD40-CD40 ligand interactions promote trafficking of CD8+ T cells into the brain and protection against West Nile virus encephalitis. *J. Virol.* 81: 9801-9811.

28. Wherry, E. J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. *J. Virol.* 78: 5535-5545.

29. Keene, J. A., and J. Forman. 1982. Helper activity is required for the in vivo generation of cytotoxic T lymphocytes. *J. Exp. Med.* 155: 768-782.

30. Jennings, S. R., R. H. Bonneau, P. M. Smith, R. M. Wolcott, and R. Chervenak. 1991. CD4-positive T lymphocytes are required for the generation of the primary but not the secondary CD8-positive cytolytic T lymphocyte response to herpes simplex virus in C57BL/6 mice. *Cell. Immunol.* 133: 234-252.

31. Matloubian, M., R. J. Concepcion, and R. Ahmed. 1994. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* 68: 8056-8063.

32. Cardin, R. D., J. W. Brooks, S. R. Sarawar, and P. C. Doherty. 1996. Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells. *J. Exp. Med.* 184: 863-871.

33. Stevenson, P. G., G. T. Belz, J. D. Altman, and P. C. Doherty. 1998. Virus-specific CD8(+) T cell numbers are maintained during gamma-herpesvirus reactivation in CD4-deficient mice. *Proc. Natl. Acad. Sci. USA* 95: 15565-15570.

34. Sun, J. C., and M. J. Bevan. 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* 300: 339-342.

35. Agnellini, P., M. Wiesel, K. Schwarz, P. Wolint, M. F. Bachmann, and A. Oxenius. 2008. Kinetic and mechanistic requirements for helping CD8 T cells. *J. Immunol.* 180: 1517-1525.

36. Obar, J. J., M. J. Molloy, E. R. Jellison, T. A. Stoklasek, W. Zhang, E. J. Usherwood, and L. Lefrancois. 2010. CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. *Proc. Natl. Acad. Sci. USA* 107: 193-198.

37. Overstreet, M. G., Y. C. Chen, I. A. Cockburn, S. W. Tse, and F. Zavala. 2011. CD4+ T cells modulate expansion and survival but not functional properties of effector and memory CD8+ T cells induced by malaria sporozoites. *PLoS One* 6: e15948.

38. Carvalho, L. H., G. Sano, J. C. Hafalla, A. Morrot, M. A. Curotto de Lafaille, and F. Zavala. 2002. IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. *Nat. Med.* 8: 166-170.

39. Bevan, M. J. 2004. Helping the CD8(+) T-cell response. *Nat. Rev. Immunol.* 4: 595-602.

40. Williams, M. A., A. J. Tyznik, and M. J. Bevan. 2006. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* 441: 890-893.

41. D'Souza, W. N., and L. Lefrancois. 2003. IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion. *J. Immunol.* 171: 5727-5735.

42. Moody, A. M., Y. Xiong, H. C. Chang, and E. L. Reinherz. 2001. The CD8alphabeta co-receptor on double-positive thymocytes binds with differing affinities to the products of distinct class I MHC loci. *Eur. J. Immunol.* 31: 2791-2799.

43. Wu, L., G. E. Exley, and C. M. Warner. 1998. Differential expression of Ped gene candidates in preimplantation mouse embryos. *Biol. Reprod.* 59: 941-952.

44. Battegay, M., D. Moskophidis, A. Rahemtulla, H. Hengartner, T. W. Mak, and R. M. Zinkernagel. 1994. Enhanced establishment of a virus carrier state in adult CD4+ T-cell-deficient mice. *J. Virol.* 68: 4700-4704.

45. Lin, E., C. C. Kemball, A. Hadley, J. J. Wilson, A. R. Hofstetter, C. D. Pack, and A. E. Lukacher. 2010. Heterogeneity among viral antigen-specific CD4+ T cells and their de novo recruitment during persistent polyomavirus infection. *J. Immunol.* 185: 1692-1700.

46. Elsaesser, H., K. Sauer, and D. G. Brooks. 2009. IL-21 is required to control chronic viral infection. *Science* 324: 1569-1572.

47. Frohlich, A., J. Kisielow, I. Schmitz, S. Freigang, A. T. Shamshiev, J. Weber, B. J. Marsland, A. Oxenius, and M. Kopf. 2009. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science* 324: 1576-1580.

48. Yi, J. S., M. Du, and A. J. Zajac. 2009. A vital role for interleukin-21 in the control of a chronic viral infection. *Science* 324: 1572-1576.

49. Janeway, C. A., Jr., and K. Bottomly. 1994. Signals and signs for lymphocyte responses. *Cell* 76: 275-285.

50. Chlewicki, L. K., C. A. Velikovsky, V. Balakrishnan, R. A. Mariuzza, and V. Kumar. 2008. Molecular basis of the dual functions of 2B4 (CD244). *J. Immunol.* 180: 8159-8167.

51. Moser, J. M., J. Gibbs, P. E. Jensen, and A. E. Lukacher. 2002. CD94-NKG2A receptors regulate antiviral CD8(+) T cell responses. *Nat. Immunol.* 3: 189-195.

52. Byers, A. M., N. P. Andrews, and A. E. Lukacher. 2006. CD94/NKG2A expression is associated with proliferative potential of CD8 T cells during persistent polyoma virus infection. *J. Immunol.* 176: 6121-6129.

53. Zhou, J., M. Matsuoka, H. Cantor, R. Homer, and R. I. Enelow. 2008. Cutting edge: engagement of NKG2A on CD8+ effector T cells limits immunopathology in influenza pneumonia. *J. Immunol.* 180: 25-29.

54. Suvas, S., A. K. Azkur, and B. T. Rouse. 2006. Qa-1b and CD94-NKG2a interaction regulate cytolytic activity of herpes simplex virus-specific memory CD8+ T cells in the latently infected trigeminal ganglia. *J. Immunol.* 176: 1703-1711.

55. Cush, S. S., and E. Flano. 2011. KLRG1+NKG2A+ CD8 T cells mediate protection and participate in memory responses during gamma-herpesvirus infection. *J. Immunol.* 186: 4051-4058.

**Figure Legends**

**Figure 2.1: Q9:VP2.139-specific cells depend on CD4 T cell help for expansion.** GK1.5 mAb or rat IgG were administered to B6.$K^{b-/-}D^{b-/-}$ mice coincident with MPyV inoculation and then once weekly for 1 mo. (**A**) Frequency (±SD) of Q9:VP2.139-specific CD8 T cells in blood over time, determined by cell surface Q9:VP2.139 tetramer staining. (**B**) Number (±SD) of Q9:VP2.139-specific CD8 T cells in spleen detected by cell surface Q9:VP2.139 tetramer binding and VP2.139 peptide-stimulated intracellular IFN-γ production at day 29 p.i. (**C**) Numbers of MPyV genome copies as determined by qPCR in indicated organs at day 29 p.i. Each point corresponds to an individual mouse, and horizontal lines indicate geometric mean. (**D**) Expression of indicated surface molecules by splenic Q9:VP2-139–specific CD8 T cells on day 29 p.i. Average geometric MFI values are indicated in the top right corner of molecules for which a significant difference was observed between experimental and control groups. Data are representative of six mice per group from two independent experiments. *$p < 0.05$, **$p < 0.01$.

**Figure 2.2: Expansion of Q9:VP2.139-specific CD8 T cells depends on CD28 and CD40L costimulation.** A mixture ofMR-1 (anti-CD40L) and CTLA-4–Ig was administered to B6.$K^{b-/-}D^{b-/-}$ mice coincident with MPyV inoculation and then once weekly for 1 mo. Control mice received no Ab. (**A**) Frequency (±SD) of CD8+ Q9:VP2.139-specific cells in blood over time, determined by cell surface Q9:VP2.139 tetramer staining. (**B**) Number (±SD) of splenic Q9:VP2.139-specific CD8 T cells at day 28 p.i. determined by tetramer staining and VP2.139 peptide-stimulated intracellular IFN-

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

**Figure 2.3: Expansion of Q9:VP2.139-specific CD8 T cells depends on CD28 costimulation.** MR-1 (anti-CD40L) or CTLA-4–Ig were administered to B6.$K^{b-/-}D^{b-/-}$ mice before and weekly during MPyV infection. Control mice received no Ab. (**A**) Frequency (±SD) of $CD8^+$ Q9:VP2.139-specific cellsin the blood over time. Significance indicated is the result of comparing CTLA-4–Ig-treated mice with controls. There was no significant difference betweenanti-CD40L–treated mice and controls. (**B**) Number (±SD) of Q9:VP2.139-specific cells in spleen were determined by tetramer staining and intracellular anti–IFN-γ staining at day 29 p.i. (**C**) Number (±SD) of MPyV genome copies was determined by qPCR in indicated organs at day 29 p.i. (**D**) Number (±SD) of Q9:VP2.139-specific cells in spleen was determined by tetramer surface and intracellular anti–IFN-γ staining at day 15 p.i. Significance indicated is the result of comparing $tetramer^+$ or IFN-$\gamma^+$ cell numbers of experimental with control mice. (**E**) Number of MPyV genome copies was determined by qPCR in indicated organs at day 15 p.i. (**C**, **E**). Each point corresponds to an individual mouse, and horizontal lines indicate geometric mean. Data are representative of six mice per group pooled from two independent experiments. [§]Sample is below the limit of detection; to calculate mean for this group, 500 copies/mg organ was substituted for values below the limit of detection. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

**Figure 2.4: Q9:VP2.139-specific cells depend on intrinsic CD28 costimulation for expansion.** (**A** and **B**) A total of 1 3 106 Q9:VP2.139 TCR retrogenic $GFP^+$ cells were transferred i.v. into B6 mice, which were subsequently infected with wild-type (WT) MPyVor A2.H145A. (**A**) Representative dot plots of splenocytes in recipient mice at day 40 p.i., gated on viable lymphocytes. (**B**) Frequency (±SD) of $GFP^+$ Q9:VP2.139-specific CD8 T cells in blood over time. *n* = 3 mice. (**C**) Experimental design for (**D**) and (**E**): Q9:VP2.139 TCR retrogenic CD8 T cells derived from Thy1.1 B6.PL and B6.CD282/2 bone marrow were mixed 1:1 and transferred to B6 mice, which were inoculated with WT MPyV. (**D**) Frequency of Q9:VP2.139-specific CD8 T cells from B6.PL and $CD28^{-/-}$ bone marrow donors in blood over time. Dots represent individual mice. *n* = 7 mice pooled from two independent experiments. (**E**) Frequency of Q9:VP2.139-specific cells from B6.PL bone marrow donors compared with cells from $CD28^{-/-}$ bone marrow donors in the blood over time after a cotransfer (± SD). *n* = 3 mice pooled from two independent experiments.

**Figure 2.5: Q9:VP2.139-specific cells do not depend on costimulation for maintenance.** $B6.K^{b-/-}D^{b-/-}$ mice were infected with MPyV and 3 mo p.i.given a mixture of MR-1 (anti-CD40L) and CTLA-4–Ig weekly for 1 mo. Control-infected mice received no Ab. (**A**) Frequency (6SD) of Q9:VP2.139 specific CD8 T cells in blood over time. Baseline indicates frequency before start of treatment. (**B**) Number (±SD) of splenic Q9:VP2.139-specific CD8 T cells was determined by tetramer staining and VP2.139 peptide-stimulated intracellular anti–IFN-γ staining after 1 mo of treatment. (**C**) Number

of MPyV genome copies was determined by qPCR in indicated organs after 1 mo of treatment. Each point corresponds to an individual mouse, and horizontal lines indicate geometric mean. (**D**) Expression of indicated surface molecule by $CD8^+$ Q9:VP2.139-specific splenocytes after 1 mo of treatment. (**E**) CD28 expression on Q9:VP2.139-specific CD8 T cells in MPyV-infected $B6.K^{b-/-}D^{b-/-}$ mice at the indicated time p.i. Data are representative of six mice per group pooled from two independent experiments. The limit of detection of this assay is 2000 copies of genomic viral DNA. One thousand copies per milligram organ was substituted for values below the limit of detection.

**Figure 2.6: Q9:VP2.139-specific cells depend on CD4 help for maintenance.** $B6.K^{b-/-}D^{b-/-}$ mice were infected with MPyV for 3 mo before the start of GK1.5 or rat IgG treatment. (**A**) Frequency (± SD) of Q9:VP2.139 $tetramer^+$ CD8 T cells in blood over time. (**B**) After 1 mo of treatment, the number (± SD) of splenic Q9:VP2.139-specific CD8 T cells [same mice as in (**A)**] was determined by tetramer staining and intracellular anti-IFN-γ staining (white bars) after ex vivo VP2.139 peptide stimulation. (**C**) Expression of indicated surface molecules by Q9:VP2-139 $tetramer^+$ $CD8^+$ splenocytes after 1 mo of treatment are indicated in (*top right corner of panels*) for molecules in which a significant difference was observed between experimental and control groups. Data are representative of six mice per group pooled from two independent experiments. (**D**) Number of MPyV genome copies was determined by qPCR in indicated organs after 1 mo of treatment. Each point corresponds to an individual mouse, and horizontal lines indicate geometric mean. The limit of detection of this assay is 2000 copies of genomic viral DNA. $^{\dagger}$, mouse with salivary gland tumor. $^{\S\S}$, sample is below the limit of detection.

To calculate mean for this group, 1000 copies/mg organ was substituted for values below the limit of detection. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

**Figure 2.7. Exogenous IL-2 therapy does not prevent loss of the Q9:VP2.139-specific population following maintenance-phase CD4 depletion.** B6.$K^{b-/-}D^{b-/-}$ mice were infected with MPyV for 3 mo. Before administering anti-CD4 (clone GK1.5), peripheral blood lymphocytes were surface stained with Q9:VP2.139 tetramers, anti-CD8α, and anti-CD44 (designated "baseline"). Synchronistic with the start of anti-CD4 i.p. injections, 3 of 6 mice received 15,000 units/mouse recombinant human IL-2 i.p. every 12 h. Anti-CD4 was administered on d 0, d2 and d 9. Mice were bled weekly. Values indicate the frequency of Q9:VP2.139-specific CD8 T cells in the blood over time after initiation of IL-2 treatment.

**Figure 2.8. Phenotypic characterization of Q9:VP2.139-specific CD8 T cells in B6.$K^{b-/-}D^{b-/-}$ mice during expansion and maintenance phases.** (**A**) Expression of the indicated molecules by Q9:VP2.139 tetramer$^+$ CD8 T cells at d 12 and d 41 p.i., with isotype controls. (**B**) Expression of the indicated molecules by Q9:VP2.139 tetramer$^+$ CD8 T cells at 3 mo p.i., with isotype controls. Histograms are representative of 6 mice from 2 independent experiments.

**Figure 2.1**

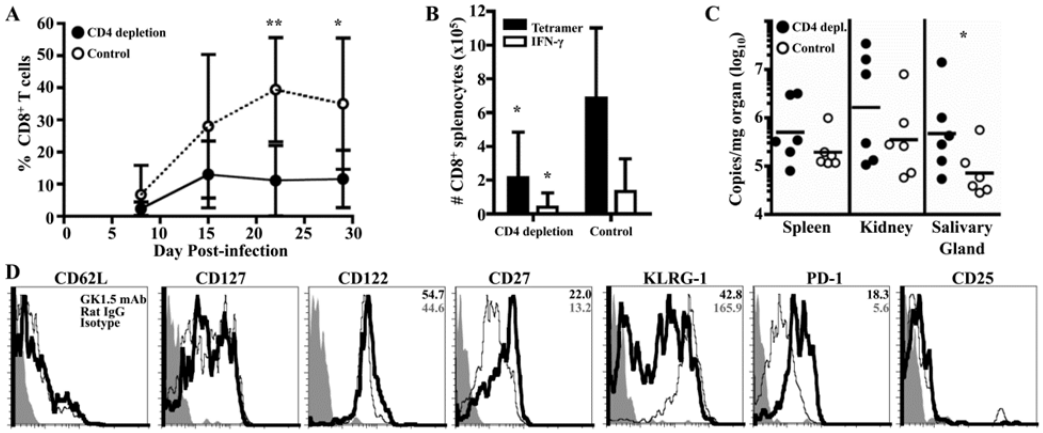

A
CD4 depletion
Control
% CD8+ T cells
Day Post-infection
B
Tetramer
IFN-γ
# CD8+ splenocytes (x10^6)
CD4 depletion
Control
C
CD4 depl.
Control
Copies/mg organ (log10)
Spleen
Kidney
Salivary Gland
D
CD62L
GK1.5 mAb
Rat IgG
Isotype
CD127
CD122
54.7
44.6
CD27
22.0
13.2
KLRG-1
42.8
165.9
PD-1
18.3
5.6
CD25

**Figure 2.2**

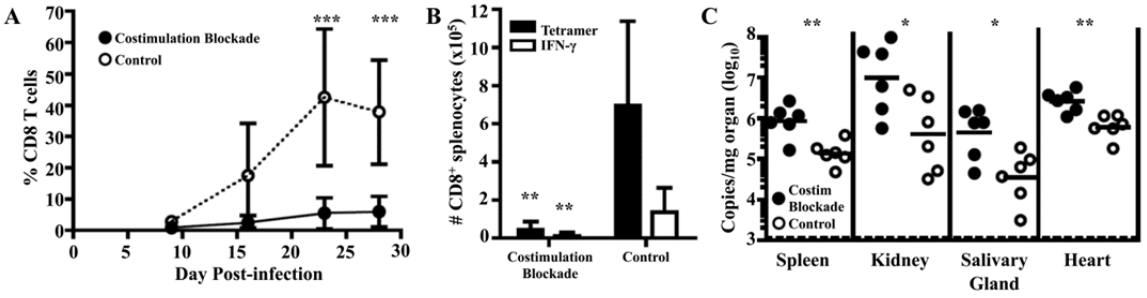

A
Costimulation Blockade
Control
% CD8 T cells
Day Post-infection
***
***
B
Tetramer
IFN-γ
# CD8+ splenocytes (x10^5)
**
**
Costimulation Blockade
Control
C
Copies/mg organ (log10)
Costim Blockade
Control
**
*
*
**
Spleen
Kidney
Salivary Gland
Heart

**Figure 2.3**

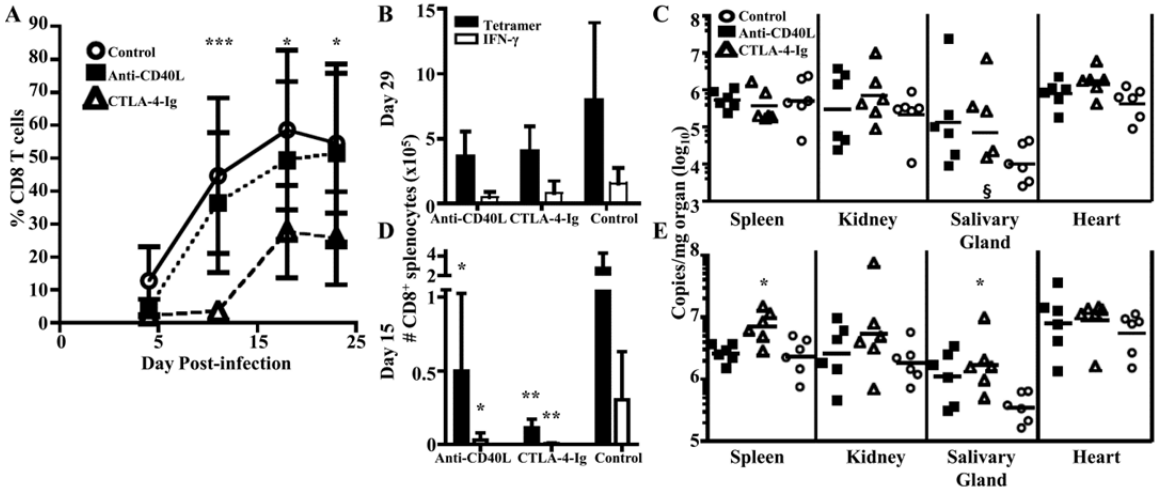

A
% CD8 T cells
Day Post-infection
Control
Anti-CD40L
CTLA-4-Ig
***
*
*
B
Day 29
# CD8+ splenocytes (x10^5)
Tetramer
IFN-γ
Anti-CD40L
CTLA-4-Ig
Control
C
Copies/mg organ (log10)
Spleen
Kidney
Salivary Gland
Heart
§
D
Day 15
# CD8+ splenocytes (x10^5)
E
Copies/mg organ (log10)

**Figure 2.4**

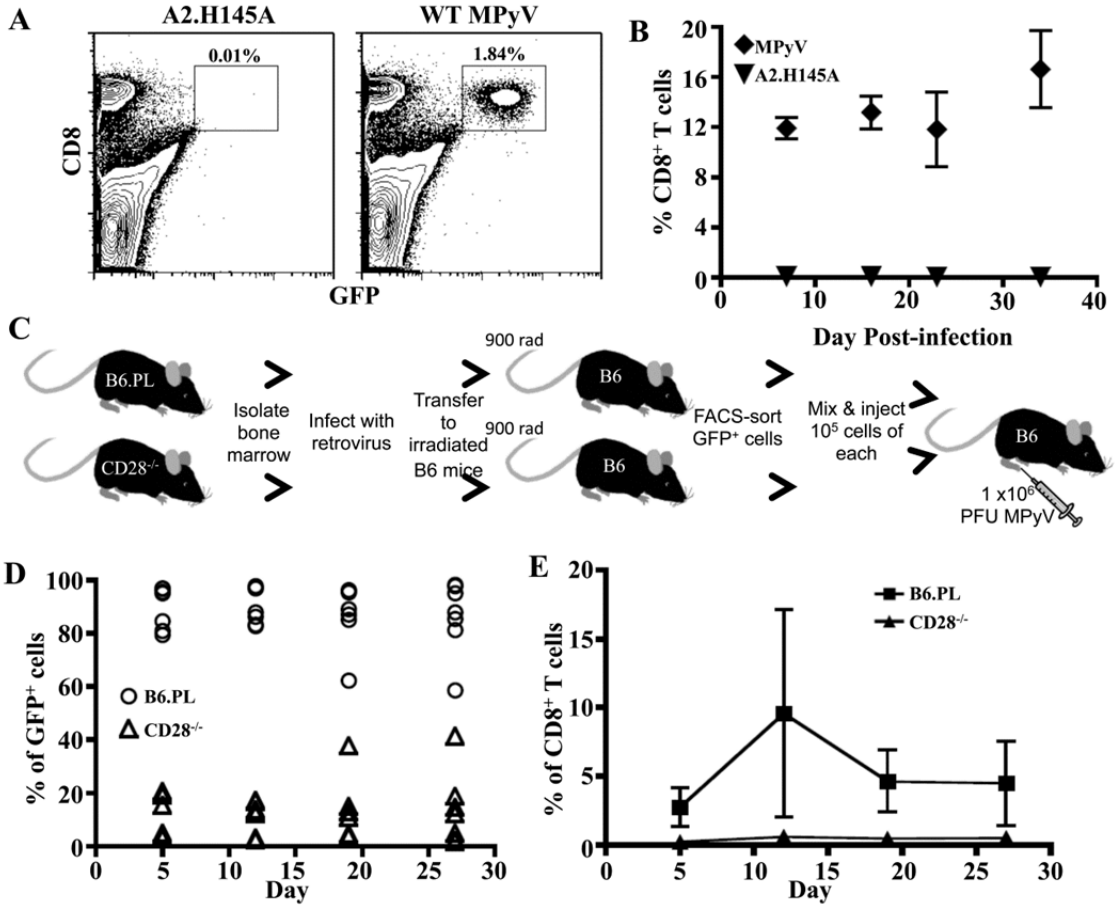

A
A2.H145A
WT MPyV
0.01%
1.84%
CD8
GFP
B
MPyV
A2.H145A
% CD8+ T cells
Day Post-infection
C
B6.PL
CD28-/-
Isolate bone marrow
Infect with retrovirus
Transfer to irradiated B6 mice
900 rad
900 rad
B6
B6
FACS-sort GFP+ cells
Mix & inject 10^5 cells of each
B6
1 x10^6 PFU MPyV
D
% of GFP+ cells
B6.PL
CD28-/-
Day
E
% of CD8+ T cells
B6.PL
CD28-/-
Day

**Figure 2.5**

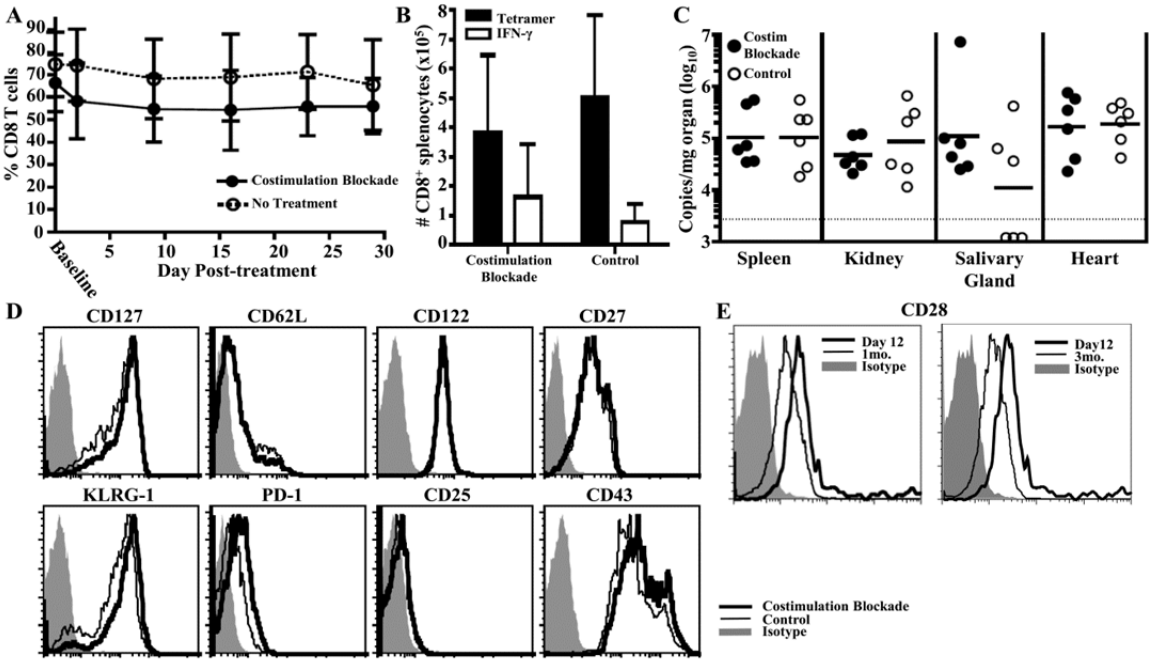

A
% CD8 T cells
Day Post-treatment
Baseline
Costimulation Blockade
No Treatment
B
Tetramer
IFN-γ
# CD8+ splenocytes (x10^5)
Costimulation Blockade
Control
C
Costim Blockade
Control
Copies/mg organ (log10)
Spleen
Kidney
Salivary Gland
Heart
D
CD127
CD62L
CD122
CD27
KLRG-1
PD-1
CD25
CD43
E
CD28
Day 12
1mo.
Isotype
Day12
3mo.
Isotype
Costimulation Blockade
Control
Isotype

**Figure 2.6**

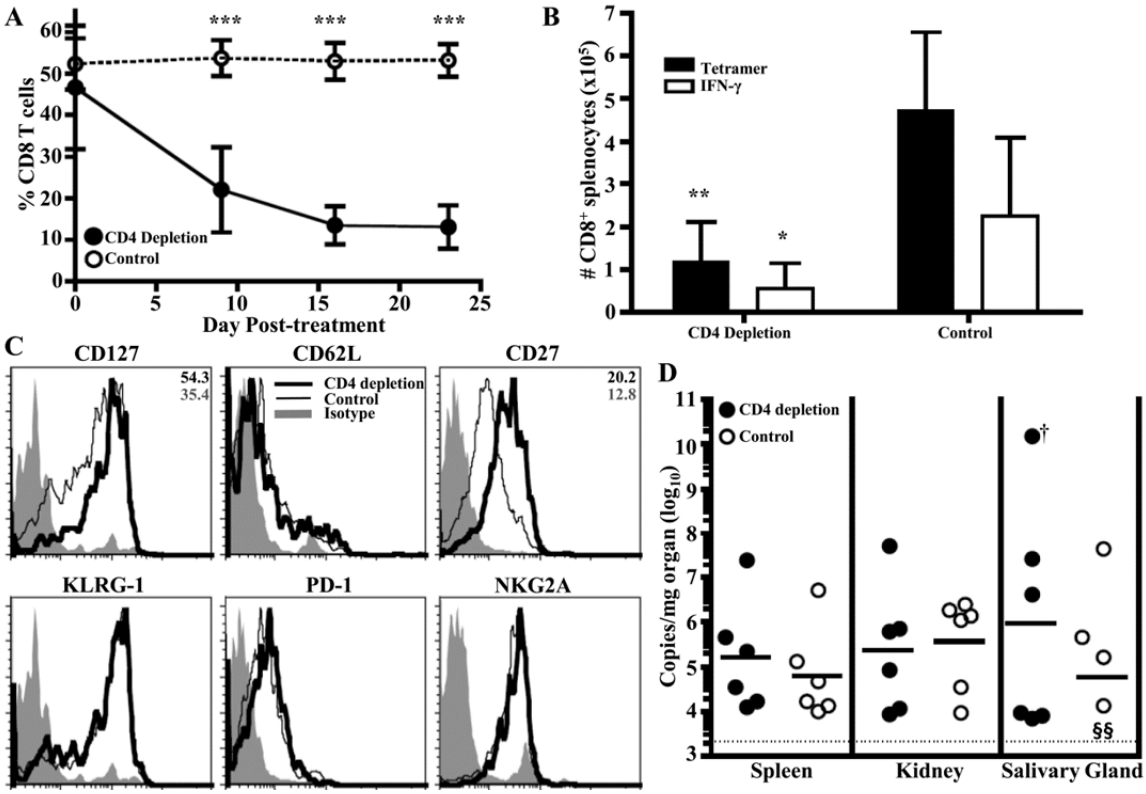

A
% CD8 T cells
Day Post-treatment
CD4 Depletion
Control
***
B
# CD8+ splenocytes (x10^5)
Tetramer
IFN-γ
**
*
CD4 Depletion
Control
C
CD127
CD62L
CD27
54.3
35.4
20.2
12.8
CD4 depletion
Control
Isotype
KLRG-1
PD-1
NKG2A
D
Copies/mg organ (log10)
CD4 depletion
Control
†
§§
Spleen
Kidney
Salivary Gland

**Figure 2.7**

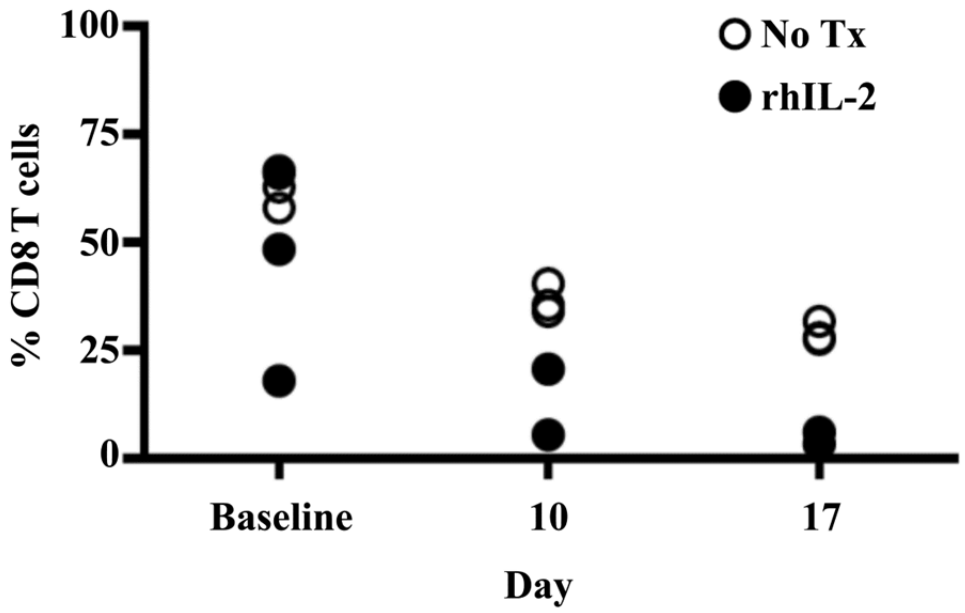
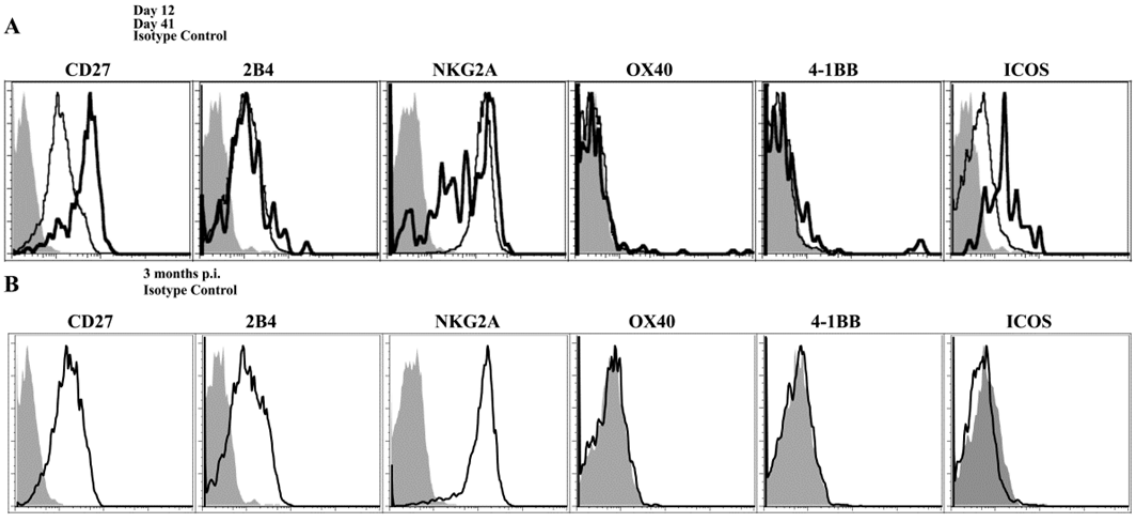


**Figure 2.8**


A
Day 12
Day 41
Isotype Control
CD27
2B4
NKG2A
OX40
4-1BB
ICOS
B
3 months p.i.
Isotype Control
CD27
2B4
NKG2A
OX40
4-1BB
ICOS

# Chapter 3

## Polyomavirus-specific class Ib-restricted CD8 T cells are protective across MHC class Ia haplotype barriers.

All figures and tables in Chapter 3 are based on data generated by the Ph.D. candidate.

**Abstract**

Unlike the polymorphic MHC class Ia molecules, MHC class Ib molecules are oligomorphic or nonpolymorphic. We recently discovered a protective CD8 T cell response to mouse polyomavirus (MPyV) in H-$2^b$ haplotype mice that is restricted by H2-Q9, a member of the Qa-2 MHC class Ib family. Here, we demonstrate that immunization with a peptide corresponding to a virus capsid-derived peptide presented by Q9 also elicits MHC class Ib-restricted MPyV-specific CD8 T cells in mice of H-$2^s$ and H-$2^{g7}$ strains. Expansion of these CD8 T cells is associated with decreased MPyV levels. These findings support the concept that immunization with a single MHC class Ib-restricted peptide can protect against viral infection in MHC class Ia allogeneic hosts.

Conventional αβ T cell receptor (TCR)-expressing CD8 T cells recognize antigens presented by polymorphic MHC class Ia molecules. This presents a challenge for peptide-based vaccine strategies to elicit pathogen-specific CD8 T cells; i.e., any single peptide will only bind a fraction of the MHC molecules in an outbred population. However, it is now well-established that αβ TCR CD8 T cells also recognize microbial antigens in the context of MHC class Ib molecules. The limited polymorphism of MHC class Ib molecules raises the possibility that the same peptide can be used as an immunogen to recruit protective anti-pathogen CD8 T cells across MHC class Ia allogeneic hosts.

Polyomaviruses are ubiquitous viruses that persist in a smoldering infectious state in many vertebrate species, including humans (1-3). In healthy hosts, polyomavirus infection is clinically silent (4). However,.immunosuppression resulting from HIV/AIDS, aging, and immunodulatory agents may result in unchecked viral replication with life-threatening complications, such as nephropathy in kidney transplant patients, central nervous system demyelination, and cutaneous malignancies (5-10).

Using MHC class Ia-deficient C57BL/6 mice (B6.$K^{b-/-}D^{b-/-}$), we recently discovered a class Ib-restricted CD8 T cell response that confers protection against MPyV infection (11). A peptide derived from amino acids 139-147 of the VP2 capsid protein (VP2.139; HALNVVHDW) binds Q9, a $\beta_2$m-associated MHC class I molecule encoded in the mouse Qa-2 locus. *H2-Q9* is non-polymorphic in mice (12). In addition, H2-Q9 shares the peptide binding specificity of the nearly identical Qa-2 family member H2-Q7 (13). In this study, we tested the hypothesis that immunization with the VP2.139 peptide will generate Q9:VP2.139-specific CD8 T cells in mice of non-$H\text{-}2^b$ haplotypes

that express Q9 and/or Q7. H-2$^{s}$ and H-2$^{g7}$ strains express Qa-2 (14, 15). We confirmed Qa-2 expression in SJL (H-2$^{s}$) and NOD (H-2$^{g7}$) mice by positive staining of splenocytes with Qa-2 antibody (clone 1-1-2, BD Biosciences) which recognizes the Qa-2 antigen expressed by Q6, Q7, Q8 and Q9 (16, 17) (data not shown).

Adult (6-13 wk) female SJL and male NOD mice were injected in a hindfootpad with 100 μg VP2.139 peptide emulsified in Complete Freund's Adjuvant (CFA) containing 1 mg/mL heat-killed *Mycobacterium tuberculosis*. Control mice received phosphate buffered saline (PBS) emulsified in CFA. Male NOD mice were used because their incidence of diabetes is lower than in NOD females (18). Two weeks later, mice were boosted with 50 μg VP2.139 peptide emulsified in Incomplete Freund's Adjuvant (IFA) injected subcutaneously (s.c.) at the tail base. Control mice received PBS emulsified in IFA. As shown in Fig. 3.1, this prime-boost regimen elicited a Q9:VP2.139-specific response in the majority of SJL and NOD mice. Two-3 weeks after the IFA boost, mice were infected with 2 x $10^5$ PFU of MPyV (strain A2) intraperitoneally (i.p.). Six days later, samples of spleen, kidney, salivary gland and heart were snap-frozen. DNA was isolated from these organs and Taqman-based quantitative (q)PCR performed as previously described (19). Single cell suspensions of RBC-lysed splenocytes prepared from each of these mice were treated with FcBlock (BD Biosciences) then co-stained with tetramers (described below) and antibodies as previously described (20). Samples were run on BD FACSVerse (BD Biosciences). FACS data were analyzed using FlowJo (Tree Star) and statistical analyses were performed using Prism (GraphPad).

Engagement of CD8 coreceptors with Q9 is likely handicapped by an unusual structure and orientation of a CD8-binding loop of the Q9 α3 domain (21). Therefore, a

novel Q9 tetramer having the α3 domain of H-2K$^b$, which efficiently binds CD8 (22), was constructed by The NIH Tetramer Core Facility. This chimeric Q9(K$^b$):VP2.139 tetramer stains CD8 T cells from MPyV-infected B6.K$^{b-/-}$D$^{b-/-}$ mice with approximately 2-fold higher mean fluorescence intensity than Q9:VP2.139 tetramers (data not shown). To control for nonspecific tetramer binding, a chimeric Q9(K$^b$) tetramer containing a Q9-binding peptide from the L19 ribosomal protein (23) was also constructed by the NIH Tetramer Core Facility.

As shown in Fig. 3.1A-D, Q9:VP2.139-specific CD8 T cells are readily detected in VP2.139-immunized mice at day 6 p.i. PBS-immunized mice showed no tetramer binding above background staining by Q9:L19 tetramers. The Q9:VP2.139-specific CD8 T cell response often reached higher frequencies in NOD mice than in SJL mice; because of this difference, we used the NOD mice for further analyses. Moreover, it is important to note that Q9:VP2.139-specific CD8 T cells are detected in only a third of C57BL/6 mice infected with MPyV, with Q9:VP2.139 tetramer$^+$ CD8 T cells first detected at day 8 p.i. (11). In this experimental setup, most VP2.129 peptide-immunized mice produced a Q9:VP2.139-specific CD8 T cells by 6 days after MPyV inoculation.

Q9:VP2.139-specific CD8 T cells express a phenotypic profile suggestive of a memory recall response after initial priming under high inflammatory conditions. The Q9:VP2.139-specific CD8 T cells were predominantly CD62L$^{lo}$, as expected for a secondary effector response (Fig. 3.1E). Most of the Q9:VP2.139 tetramer$^+$ CD8 T cells expressed CD127, which may be a consequence of CFA-based immunization (24). In addition, nearly 50% of the Q9:VP2.139-specific CD8 T cells were KLRG-1$^+$, which may similarly reflect the highly inflammatory CFA priming environment (25, 26). The

majority of the tetramer$^{+}$ cells were CD27$^{hi}$CD43$^{hi}$, which has been associated with potential for antigenic recall (24). The Q9:VP2.139-specific CD8 T cells were predominantly CD28$^{+}$, as expected for activated T cells (27). Approximately half of the tetramer$^{+}$ cells expressed CD94-NKG2A/C/E (clone 20d5). In B6 mice, NKG2A is the predominant form expressed by this heterodimeric receptor (28), and has been interpreted as a T cell activation marker (29-31). These data are consistent with our previous observations that NKG2A expression increases while CD28 expression decreases on Q9:VP2.139-specific CD8 T cells over the course of MPyV infection (20).

Importantly, viral titers were significantly lower in the spleens of VP2.139 peptide-immunized NOD mice having Q9:VP2.139 CD8 T cell responses (Fig. 3.2). This indicates that the Q9:VP2.139 specific response elicited by VP2.139 + CFA immunization is able to decrease the early viral burden. That a difference in viral titers is only seen in the spleen may be due to the route of infection, as i.p.-delivered virus may initiate infection in the spleen before the other organs examined here.

We consistently observed a less robust Q9:VP2.139-specific response in SJL mice than NOD mice. The diversity and level of expression of Qa-2 MHC class Ib molecules varies among inbred mouse strains due to differences in their germline representation of H-2Q6, Q7, Q8, and Q9 (32). In addition, H2-Q7 and -Q9 differ by only a single amino acid outside their peptide-binding grooves and have overlapping peptide-binding repertoires (13, 23, 33). In contrast to B6 mice, which express both Q7 and Q9 (34), NODs express either Q7 or Q9 (14). There is also evidence that SJLs express less surface Qa-2 than B6 mice (35). It is possible that differences between SJL and NOD in the composition of Qa-2 genes may affect the level of Q9:VP2.139 epitope density and

explain the differences in magnitude of antigen-specific CD8 T cells observed between these mouse strains.

In summary, evidence is presented here that immunization with an antigenic determinant presented by an MHC class Ib molecule can elicit a cross-MHC haplotype CD8 T cell response. Importantly, peptide-elicited MHC class Ib-restricted CD8 T cells can limit the extent of a primary virus infection. These data support the concept that vaccines based on single antigenic polypeptides may confer protection against microbial infection.

**Acknowledgements**

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References

1. Swanson, P. A., 2nd, A. E. Lukacher, and E. Szomolanyi-Tsuda. 2009. Immunity to polyomavirus infection: the polyomavirus-mouse model. *Semin Cancer Biol* 19: 244-251.

2. Johne, R., C. B. Buck, T. Allander, W. J. Atwood, R. L. Garcea, M. J. Imperiale, E. O. Major, T. Ramqvist, and L. C. Norkin. 2011. Taxonomical developments in the family Polyomaviridae. *Arch Virol* 156: 1627-1634.

3. Van Ghelue, M., M. T. Khan, B. Ehlers, and U. Moens. 2012. Genome analysis of the new human polyomaviruses. *Rev Med Virol*.

4. Delbue, S., M. Comar, and P. Ferrante. 2012. Review on the Relationship between Human Polyomaviruses-Associated Tumors and Host Immune System. *Clin Dev Immunol* 2012: 542092.

5. Bennett, S. M., N. M. Broekema, and M. J. Imperiale. 2012. BK polyomavirus: emerging pathogen. *Microbes Infect*.

6. Bellizzi, A., C. Nardis, E. Anzivino, D. Rodio, D. Fioriti, M. Mischitelli, F. Chiarini, and V. Pietropaolo. 2012. Human polyomavirus JC reactivation and pathogenetic mechanisms of progressive multifocal leukoencephalopathy and cancer in the era of monoclonal antibody therapies. *Journal of neurovirology* 18: 1-11.

7. Schrama, D., S. Ugurel, and J. C. Becker. 2012. Merkel cell carcinoma: recent insights and new treatment options. *Current opinion in oncology* 24: 141-149.

8. Bloomgren, G., S. Richman, C. Hotermans, M. Subramanyam, S. Goelz, A. Natarajan, S. Lee, T. Plavina, J. V. Scanlon, A. Sandrock, and C. Bozic. 2012.

Risk of natalizumab-associated progressive multifocal leukoencephalopathy. *N Engl J Med* 366: 1870-1880.

9. Siguier, M., P. Sellier, and J. F. Bergmann. 2012. BK-virus infections: A literature review. *Medecine et maladies infectieuses*.

10. Wanat, K. A., P. D. Holler, T. Dentchev, K. Simbiri, E. Robertson, J. T. Seykora, and M. Rosenbach. 2012. Viral-associated trichodysplasia: characterization of a novel polyomavirus infection with therapeutic insights. *Archives of dermatology* 148: 219-223.

11. Swanson, P. A., 2nd, C. D. Pack, A. Hadley, C. R. Wang, I. Stroynowski, P. E. Jensen, and A. E. Lukacher. 2008. An MHC class Ib-restricted CD8 T cell response confers antiviral immunity. *J. Exp. Med.* 205: 1647-1657.

12. Stroynowski, I., and P. Tabaczewski. 1996. Multiple products of class Ib Qa-2 genes which ones are functional? *Res. Immunol.* 147: 290-301.

13. Soloski, M. J., L. Hood, and I. Stroynowski. 1988. Qa-region class I gene expression: identification of a second class I gene, Q9, encoding a Qa-2 polypeptide. *Proc. Natl. Acad. Sci. USA* 85: 3100-3104.

14. Lund, T., E. Simpson, and A. Cooke. 1990. Restriction fragment length polymorphisms in the major histocompatibility complex of the non-obese diabetic mouse. *Journal of autoimmunity* 3: 289-298.

15. Sandrin, M. S., P. M. Hogarth, and I. F. McKenzie. 1983. Two "Qa" specificities: Qa-m7 and Qa-m8 defined by monoclonal antibodies. *J. Immunol.* 131: 546-547.

16. Sharrow, S. O., J. S. Arn, I. Stroynowski, L. Hood, and D. H. Sachs. 1989. Epitope clusters of Qa-2 antigens defined by a panel of new monoclonal antibodies. *J. Immunol.* 142: 3495-3502.

17. Devlin, J. J., E. H. Weiss, M. Paulson, and R. A. Flavell. 1985. Duplicated gene pairs and alleles of class I genes in the Qa2 region of the murine major histocompatibility complex: a comparison. *EMBO J* 4: 3203-3207.

18. Anderson, M. S., and J. A. Bluestone. 2005. The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* 23: 447-485.

19. Kemball, C. C., E. D. Lee, V. Vezys, T. C. Pearson, C. P. Larsen, and A. E. Lukacher. 2005. Late priming and variability of epitope-specific CD8+ T cell responses during a persistent virus infection. *J. Immunol.* 174: 7950-7960.

20. Hofstetter, A. R., M. L. Ford, L. C. Sullivan, J. J. Wilson, A. Hadley, A. G. Brooks, and A. E. Lukacher. 2012. MHC Class Ib-Restricted CD8 T Cells Differ in Dependence on CD4 T Cell Help and CD28 Costimulation over the Course of Mouse Polyomavirus Infection. *J. Immunol.* 188: 3071-3079.

21. He, X., P. Tabaczewski, J. Ho, I. Stroynowski, and K. C. Garcia. 2001. Promiscuous antigen presentation by the nonclassical MHC Ib Qa-2 is enabled by a shallow, hydrophobic groove and self-stabilized peptide conformation. *Structure* 9: 1213-1224.

22. Moody, A. M., Y. Xiong, H. C. Chang, and E. L. Reinherz. 2001. The CD8alphabeta co-receptor on double-positive thymocytes binds with differing affinities to the products of distinct class I MHC loci. *Eur. J. Immunol.* 31: 2791-2799.

23. Joyce, S., P. Tabaczewski, R. H. Angeletti, S. G. Nathenson, and I. Stroynowski. 1994. A nonpolymorphic major histocompatibility complex class Ib molecule binds a large array of diverse self-peptides. *J. Exp. Med.* 179: 579-588.

24. Hikono, H., J. E. Kohlmeier, S. Takamura, S. T. Wittmer, A. D. Roberts, and D. L. Woodland. 2007. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. *J. Exp. Med.* 204: 1625-1636.

25. Prlic, M., J. A. Sacks, and M. J. Bevan. 2012. Dissociating markers of senescence and protective ability in memory T cells. *PLoS One* 7: e32576.

26. Obar, J. J., E. R. Jellison, B. S. Sheridan, D. A. Blair, Q. M. Pham, J. M. Zickovich, and L. Lefrancois. 2011. Pathogen-induced inflammatory environment controls effector and memory CD8+ T cell differentiation. *J. Immunol.* 187: 4967-4978.

27. Turka, L. A., J. A. Ledbetter, K. Lee, C. H. June, and C. B. Thompson. 1990. CD28 is an inducible T cell surface antigen that transduces a proliferative signal in CD3+ mature thymocytes. *J. Immunol.* 144: 1646-1653.

28. Vance, R. E., A. M. Jamieson, D. Cado, and D. H. Raulet. 2002. Implications of CD94 deficiency and monoallelic NKG2A expression for natural killer cell development and repertoire formation. *Proc. Natl. Acad. Sci. USA* 99: 868-873.

29. Moser, J. M., A. M. Byers, and A. E. Lukacher. 2002. NK cell receptors in antiviral immunity. *Curr. Opin. Immunol.* 14: 509-516.

30. Moser, J. M., J. Gibbs, P. E. Jensen, and A. E. Lukacher. 2002. CD94-NKG2A receptors regulate antiviral CD8(+) T cell responses. *Nat. Immunol.* 3: 189-195.

31. Maris, C. H., J. D. Miller, J. D. Altman, and J. Jacob. 2003. A transgenic mouse model genetically tags all activated CD8 T cells. *J. Immunol.* 171: 2393-2401.

32. Chiang, E. Y., and I. Stroynowski. 2006. The role of structurally conserved class I MHC in tumor rejection: contribution of the Q8 locus. *J. Immunol.* 177: 2123-2130.

33. Tabaczewski, P., E. Chiang, M. Henson, and I. Stroynowski. 1997. Alternative peptide binding motifs of Qa-2 class Ib molecules define rules for binding of self and nonself peptides. *J. Immunol.* 159: 2771-2781.

34. Wu, L., G. E. Exley, and C. M. Warner. 1998. Differential expression of Ped gene candidates in preimplantation mouse embryos. *Biol. Reprod.* 59: 941-952.

35. Rucker, J., M. Horowitz, E. A. Lerner, and D. B. Murphy. 1983. Monoclonal antibody reveals H-2-linked quantitative and qualitative variation in the expression of a Qa-2 region determinant. *Immunogenetics* 17: 303-316.

**Figure Legends**

**Figure 3.1: VP2.139 peptide-immunized NOD and SJL mice generate Q9:VP2.139-specific CD8 T cell responses. A and B:** Representative Q9:VP2.139 tetramer staining of splenocytes from VP2.139 peptide-immunized NOD (**A**) or SJL (**B**) mice at day 6 p.i. by MPyV. "VP2.139" indicates mice immunized with CFA + peptide. "No Peptide" indicates control mice receiving CFA +PBS. Q9:L19 is the control tetramer for Q9:VP2.139. Numbers indicate the frequency of tetramer$^+$ CD8$^+$ lymphocytes. Data are representative of (**A**) 6 mice or (**B**) 10 mice per group pooled from two separate experiments. **C and D:** Frequency of Q9:VP2.139-specific CD8$^+$ lymphocytes, with Q9:L19 tetramer staining subtracted, in VP2.139 peptide-immunized NOD (**C**) and SJL (**D**) mice at day 6 p.i. Horizontal lines indicate geometric mean. Dots represent individual mice. † indicates a mouse with no Q9:VP2.139 tetramer staining; because this mouse lacked a VP2.139-specific CD8 T cell response, it was not included in the analysis of MPyV levels (Fig. 3.2). **E.** Phenotyping of Q9:VP2.139-specific NOD splenocytes. Q9:VP2.139 tetramer$^+$ CD8 T cells were surface-stained with antibodies to the indicated molecules. Data are clustered by costained markers, as indicated in the y axis. **+** indicates cells which are positive for the indicated molecule, and **–** indicates cells which are negative for the indicated molecule. Data are representative of 5 mice pooled from two separate experiments.

**Figure 3.2: Q9:VP2.139-specific CD8 T cells protect against MPyV infection in peptide-immunized NOD mice.** MPyV genome copies in the indicated organs at day 6

p.i. by MPyV were determined by Taqman-based qPCR. Open circles: VP2.139 peptide-immunized NOD mice. Closed circles: PBS control NOD mice. Horizontal lines represent geometric mean of each group of samples. Dots represent individual mice, pooled from two separate experiments. *p* value was calculated using a two-tailed Mann-Whitney *t* test.

**Figure 3.1**

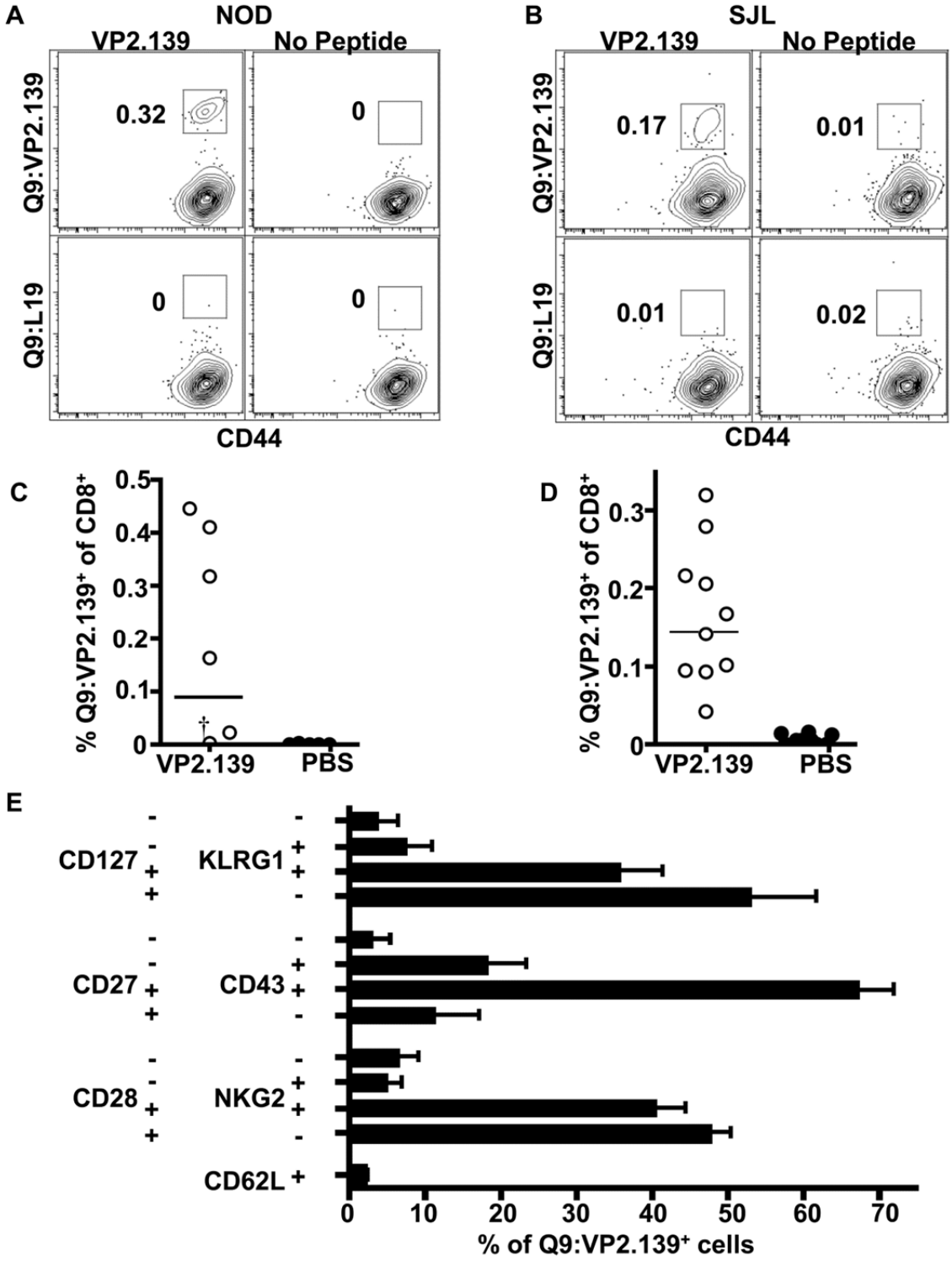
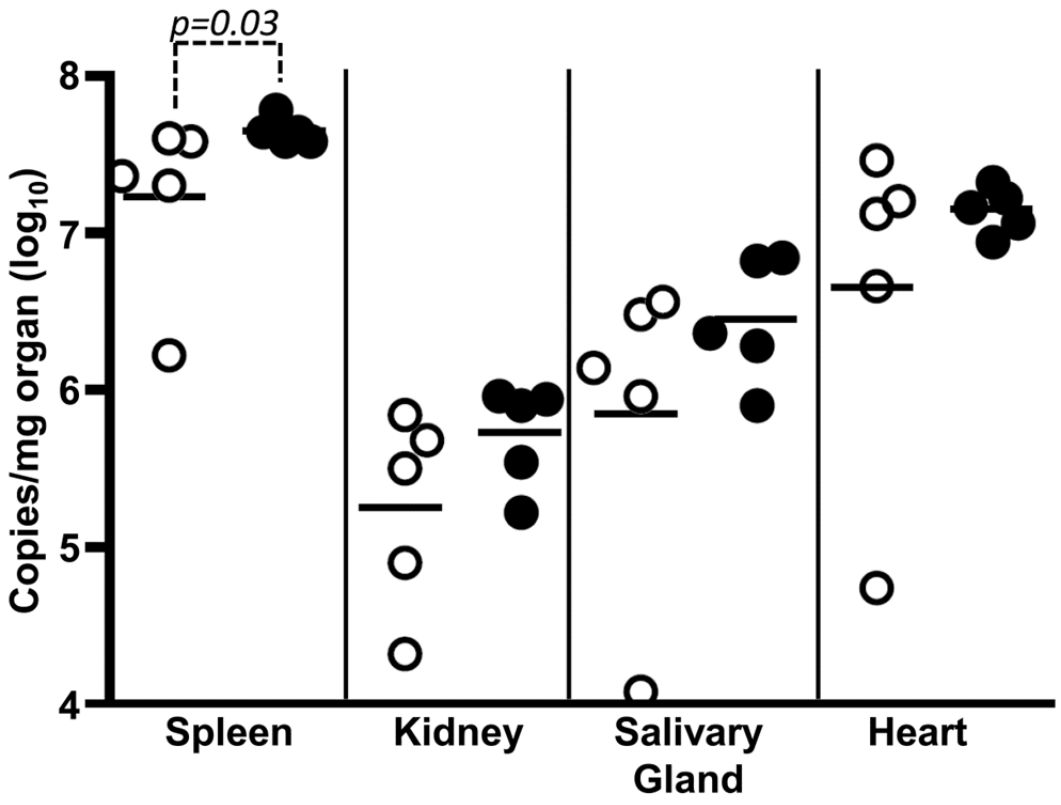

A
NOD
VP2.139
No Peptide
Q9:VP2.139
Q9:L19
0.32
0
0
0
CD44
B
SJL
VP2.139
No Peptide
Q9:VP2.139
Q9:L19
0.17
0.01
0.01
0.02
CD44
C
% Q9:VP2.139+ of CD8+
0.5
0.4
0.3
0.2
0.1
0
VP2.139
PBS
D
% Q9:VP2.139+ of CD8+
0.3
0.2
0.1
0
VP2.139
PBS
E
CD127
KLRG1
CD27
CD43
CD28
NKG2
CD62L
0
10
20
30
40
50
60
70
% of Q9:VP2.139+ cells

**Figure 3.2**


p=0.03
8
7
6
5
4
Copies/mg organ (log10)
Spleen
Kidney
Salivary Gland
Heart

# Chapter 4

# Discussion

CD8 T cells safeguard the body from intracellular infection and malignancy. Vaccines prime the exquisite sensitivity and killing mechanisms of CD8 T cells, among other components of the adaptive immune system. It has recently become clear that MHC class Ib molecules can present peptides to CD8 T cells. In contrast to MHC class Ia molecules, MHC class Ib molecules are largely non-polymorphic. Therefore, it is possible that a protective CD8 T cell response restricted by a peptide bound to a class Ib MHC molecule could be elicited in individuals who have otherwise dissimilar MHC haplotypes. It is important to better characterize the role of class Ib MHC molecules in the immune response. This knowledge may allow us to exploit class Ib-binding molecules in vaccines to generate consistent, protective CD8 T cell responses.

Peptide-based vaccines are an attractive alternative to killed or live-attenuated vaccines. Advantages to peptide-based vaccines include safety, stability and cost effectiveness (1-4). It is cheap to mass produce a defined peptide determinant and no cold-chain is needed for its distribution (5). In comparison to constructs where a peptide or protein antigen is engineered into a vector, there is never a concern about prior or subsequent immunity to the vector (5). In comparison to attenuated pathogens, there is no concern about reversion to a virulent strain (5). The ability to identify potentially immunologically relevant MHC-binding peptides was improved by the use of mass spectrometry to identify the sequences of peptides bound to HLA class I and II. Successful use of this method increased between 1990 and 2006 (2). The appeal of peptide-based vaccines makes identification of relevant HLA-binding epitopes increasingly important.

However, there are also important disadvantages to the use of peptide-based vaccines. In terms of efficacy, a peptide moiety is typically non-immunogenic. Some type of adjuvant must be used to trigger the adaptive immune response. This is especially important in the case of class I-directed peptides, as cross-priming by APCs is necessary to display exogenously-delivered antigens on the cell surface. Furthermore, such a potent trigger as a targeted peptide can lead to unforeseen consequences, such as induction of tolerance or autoimmunity, rather than immunity (5). Further challenges include degradation of the peptides by proteolysis (1), and the inability of these vaccines to protect against viral escape mutants (5).

One important disadvantage of peptide-based vaccines is a consequence of the exquisite specificity of the MHC peptide-binding groove (6). Any peptide found to bind a particular HLA-A,-B or -C molecule is not likely to cross-react with other alleles of the HLA molecule. Therefore, an individual lacking expression of the correct MHC allele to bind a given peptide would be unprotected by this peptide (4). In humans, over 1000 HLA variants have been identified to date (7). For example, among a single Dutch population, more than 14 HLA-A antigens can be detected by serological methods among 2400 individuals. Vaccines must offer a range of antigens, increasing the odds that at least one peptide will bind appropriately to one of an individual's MHC class Ia molecules. For the Dutch population study, it was estimated that peptides binding a minimum of 5 different HLA-A molecules would be required to achieve 95% coverage of this population. However, Shipper et al. note that HLA-A is the least polymorphic of the human class Ia MHC types (4). Furthermore, HLA molecules should be typed at the DNA level, rather than serologically, because differences that are undetectable by

antibodies can still affect peptide binding (4). This was verified by Longmate et al. (6). Fourteen molecularly-typed alleles were estimated necessary for 90% coverage of an ethnically diverse population, with 17 alleles needed to guarantee that all three of the Asian-, African- and Caucasian-American groups in the study would have 90% coverage. A caveat to these studies is that HLA supertypes have been identified, where multiple HLA alleles have overlapping peptide repertoires (2, 6). However, immunodominance and response magnitude do not necessarily correlate simply with expression of a peptide-binding MHC molecule. Longmate et al. demonstrate that a theoretical 90% coverage panel derived from CMV proteins only generates a response in vitro from 75% of CMV-positive blood donors (6). The specificity that makes class Ia MHC-TCR interactions so sensitive also stands as a barrier to peptide-based vaccines.

In contrast, class Ib molecules display little to no polymorphism. Only two or three proteins, which differ by single amino acid exchanges, are encoded by 11 HLA-E alleles in the human population (8-12). Importantly, many of the alleles encode silent nucleotide substitutions. When a nucleotide substitution encodes an amino acid difference, the two resulting products are allelic isoproteins (13). The most consistently reported isoproteins of HLA-E differ by an arginine to glycine substitution in a connecting loop of the protein, not involved in peptide binding (8, 14). A third reported allele codes for a protein which differs by an arginine to glycine substitution in the α2 domain of HLA-E, but oriented such that the side chain faces away from the amino acid binding groove (14). However, this allele has not been consistently observed (9, 12). Other human MHC class Ib genes that exhibit limited polymorphism include HLA-F, with 4 isoproteins, HLA-G, with 15 isoproteins, and HLA-H, which has 9 isoproteins (10,

11). In addition, HLA-G encodes 2 null alleles, which encode no protein (11, 15). The mouse functional equivalent to HLA-E, Qa-1, has 4 alleles (16), and no functional differences have been found between them (17). It has been suggested that HLA-G is the functional homolog of Qa-2, akin to the HLA-E:Qa-1 relationship (18). It is important to note that while HLA-E/Qa-1 and HLA-G/Qa-2 appear to be functional homologs, they are not orthologs. The convergent evolution of these proteins underscores the importance of their respective functions (19). That these proteins maintain limited polymorphism in the outbred human population might be interpreted as further evidence that polymorphism of these proteins is not beneficial.

The limited polymorphisms of class Ib molecules might be useful for peptide-based vaccine development. Class Ib molecules have a bias towards TCR interaction, as evidenced by their structural homology with the class Ia molecules (20). This makes class Ib molecules prime suspects for antigen presentation, even if such function has not yet been discovered. Furthermore, several class Ib molecules have been observed bearing pathogen antigens in mouse models and in humans (21). Our group demonstrated that the Q9 molecule can present a peptide from MPyV that primes a protective CD8 T cell response against MPyV. Importantly, this response is protective across MHC class Ia haplotype barriers. It is therefore conceivable that human class Ib molecules could prime protective CD8 T cell memory responses in humans.

Recently, Swanson et al. identified the specific peptide and class Ib molecule restricting a class Ia-independent CD8 T cell response against MPyV. The VP2.139 peptide, derived from the VP2 capsid protein, is presented by Q9. Most importantly, Swanson et al. demonstrated this response was able to decrease viral loads in B6.$K^{b-/-}D^{b-/-}$

mice infected with wild-type virus, as compared to H145A, an engineered mutant virus which does not present the VP2.139 peptide. Only a fraction of Q9:VP2.139-specific CD8 T cells produce IFN-γ in response to peptide in a 5 hour ex vivo ICCS. The Q9:VP2.139-specific CD8 T cell response displays unique inflationary kinetics in B6.$K^{b-/-}D^{b-/-}$ mice. The Q9:VP2.139-specific CD8 T cells are undetectable until ~1 wk p.i. After that, they expand for three months, followed by a plateau phase. Although the Q9:VP2.139-specific population expands in class Ia-sufficient B6 mice, priming was inconsistent (22). Swanson et al. went on to explore the unique inflationary kinetics of the Q9:VP2.139-specific response in $K^{b-/-}D^{b-/-}$ mice, demonstrating that persistent MPyV infection drives long-term expansion of the T cell clone. Curiously, only one or two T Q9:VP2.139-specific CD8 T cell clones expand in any individual mouse, but the Vβ clonotypes differ between mice. After antigen levels decrease, the Q9:VP2.139-specific cells cease proliferation. Thereafter, the cells are maintained at a high level, without appreciable death or turnover. This plateau can be attributed to a lack of VP2.139-presenting APCs (23). The identification and characterization of the Q9:VP2.139-specific CD8 T cell response was the foundation of my dissertation project.

Several characteristics distinguish the Q9:VP2.139-specific CD8 T cell response from classical class Ia-restricted responses. First, the use of the nonclassical class Ib MHC molecule for peptide presentation. Second, the slow, inflationary kinetics of expansion of the Q9:VP2.139-specific CD8 T cells, and their failure to appreciably contract. Third, the discordance between tetramer-binding cells and IFN-γ-producing cells, indicative of a deficit in IFN-γ production. For my dissertation project I explored possible mechanisms for the unique kinetics and IFN-γ deficiency of the Q9:VP2.139-

specific CD8 T cell response. I also investigated whether or not Q9:VP2.139-specific cells, which respond to a nonpolymorphic MHC molecule, might be protective across MHC class Ia haplotype barriers.

Expanding on Swanson et al.'s investigation into the kinetics of the Q9:VP2.139-specific response, I showed that Q9:VP2.139-specific CD8 T cells in B6.$K^{b-/-}D^{b-/-}$ mice require CD4 help and autochthonous CD28 costimulation, as well as antigen, for their initial expansion. During the plateau phase, I determined that CD28 and CD40L are dispensable for maintenance. However, CD4 help is necessary for survival at this stage.

It is unclear how CD4 T cells promote survival of the Q9:VP2.139-specific CD8 T cells during the plateau phase. A preliminary experiment revealed that twice-daily administration of recombinant human IL-2 did not rescue plateau-phase Q9:VP2.139-specific CD8 T cells from decline in the face of CD4 depletion (Figure 2.7). These data deviate from what has been seen before with prototypical Th-dependent and Th-independent antiviral CTL responses. Janssen et al. demonstrated that CD4 help is needed to program CD8 T cells to expand upon restimulation, but addition of exogenous IL-2 could rescue the memory capacity of unhelped CTL (24). Previously in our lab, Kemball et al. showed that MPyV-specific class Ia-restricted CD8 T cell responses require CD4 help during persistence to enable new recruitment of CD8 T cells (25). However, the data from Swanson et al. does not support a large role for new naïve T cell recruitment due to the relative lack of death or proliferation during the plateau phase (23). The ability of certain $CD4^+$ subsets expressing the high-affinity IL-2R to act as an IL-2 sink is debated (26, 27). It is possible that removal of CD4 T cells leads to a glut of IL-2 in B6.$K^{b-/-}D^{b-/-}$ mice, which could program the cells for apoptosis (28-30), although this

role for IL-2 is also debated (31). However, CD4-depleted mice treated with IL-2 appeared to have a more rapid decline of Q9:VP2.139-specific CD8 T cells (Figure 2.7), which would support an IL-2-mediated apoptotic effect on the CD8 T cell population.

Alternatively, other CD4 T cell-produced cytokines could promote the survival of the Q9:VP2.139-specific population. One likely candidate is IL-21, which has been shown to support CD8 T cell survival and effector function during chronic infection (32-34). MPyV-specific CD4 T cells produce IL-21 (35). The importance of IL-21 in maintaining the Q9:VP2.139-specific CD8 T cell responses could be directly assessed by generating Q9:VP2.139-specific retrogenic CD8 T cells in an IL-21R$^{-/-}$ mouse. The retrogenic cells would be co-transferred with retrogenic cells from a wild-type mouse into a B6 mouse, which was then infected by MPyV. If the retrogenic CD8 T cells from IL-21R$^{-/-}$ mice declined, while those from WT mice reached the plateau phase, it would indicate a dependence on IL-21 for survival. Alternatively, dependence could also be tested by administration of an IL-21R-blocking antibody, or IL-21R fused onto the Fc portion of murine IgG (36). Either of these reagents administered during the maintenance phase would phenocopy CD4 depletion in B6.K$^{b-/-}$D$^{b-/-}$ mice if IL-21 signals are critical for the survival of the Q9:VP2.139-specific CD8 T cell population.

CD4 T cells may be providing help to the Q9:VP2.139-specific CD8 T cells more directly, through the ligation of costimulatory molecules. Although CD40L and CD28 proved unnecessary for the maintenance of the Q9:VP2.139-specific CD8 T cells, other costimulatory molecules may be important. As shown in Figure 2.8, CD27, 2B4, and ICOS are costimulatory molecules that are expressed at day 12 of the Q9:VP2.139-specific response in B6.K$^{b-/-}$D$^{b-/-}$ mice. Surface expression of ICOS and CD27 declines

after day 12, although expression is maintained out to 3 mo. p.i. NKG2A is also expressed at day 12, although expression of this inhibitory protein is higher thereafter. CD27 and ICOS are known costimulatory molecules (37, 38). 2B4 has been reported to have inhibitory or costimulatory functions depending on the experimental model (39). It would be revealing to attempt antibody blockade of these molecules individually, or simultaneously, during the maintenance phase of the Q9:VP2.139-specific CD8 T cell response. However, CD27 and ICOS are expressed at a higher level early in the response rather than later, suggesting that they are of limited importance during maintenance.

Antibody blockade of the CD27 or ICOS during the expansion phase of the Q9:VP2.139-specific response could allow us to discern whether these costimulatory molecules contribute to the expansion and survival of the Q9:VP2.139-specific CD8 T cell response. We could then attempt to increase the frequency of the Q9:VP2.139-specific CD8 T cell response in vivo through antibody-mediated agonism of the relevant receptors. For example, even though 4-1BB appears to be expressed only on a small fraction of the Q9:VP2.139-specific population at day 12 p.i., (Figure 2.8), Phillip A. Swanson II was able to increase the frequency of Q9:VP2.139 tetramer$^{+}$ B6 mice by a single administration of anti-4-1BB at day 5 post-immunization (P.A. Swanson II, unpublished observations). In conjunction with this observation, the one instance when we observed the expansion of a Q9:VP2.139-specific CD8 T cell response in a SJL mouse immunized with psoralen-cross-linked VV.VP2.139 (described in more detail below) corresponded to a mouse that received 4-1BB 5 days post immunization (A.R. Hofstetter, unpublished observations). Better understanding of the costimulatory

molecules that drive proliferation and survival of the Q9:VP2.139-specific CD8 T cell population could facilitate the study of this population in a wild-type host.

When B6 mice are infected with a typical dose ($10^6$ pfu) of MPyV, there is no detectable Q9:VP2.139-specific CD8 T cell response (22). In order to expand the Q9:VP2.139-specific CD8 T cell population without an immunization regimen, it is necessary to use a low-dose infection ($10^3$ pfu), which decreases early bystander inflammation (40). Even then, only one-third of B6 mice will have a detectable Q9:VP2.139-specific response. Therefore, to consistently study the Q9:VP2.139-specific population in class Ia-competent mice, we turned to the retrogenic T cell model developed by Dario Vignali's lab (41). We collaborated with Dr. Andrew Brooks and Dr. Lucy Sullivan from the University of Melbourne to develop the TCR retrogenic mice. This gave us access to a large quantity of naive Q9:VP2.139-specific retrogenic CD8 T cells for adoptive transfer into class Ia-competent mice. While this protocol circumvents the time that would be needed to develop a transgenic mouse, it is an inefficient method. There is a virtually 1:1:1 ratio of bone marrow donor mice, bone marrow recipient mice, and retrogenic T cell recipient mice, depending on the number of retrogenic T cells that are needed for the experiment. Also, the process is expensive, due to the cost of the mice needed and cytokines for culture. Finally, it takes 7 weeks from the infection of the bone marrow to when transgenic cells are available for adoptive transfer. Due to these constraints, I was unable to take full advantage of this protocol. There are a number of experiments I would like to have done, given more time. Most importantly, I would have liked to titrate down the number of retrogenic cells as far as possible. It is important to determine the minimum number of Q9:VP2.139-specific CD8 T cells needed to

consistently expand a detectable response to MPyV. This would then give a slight overestimate of the endogenous precursor frequency of Q9:VP2.139-specific CD8 T cells. The restricted Vβ repertoires of the Q9:VP2.139-specific response suggests that the endogenous precursor frequency is very close to one cell per mouse, which may contribute to the difficulty of expanding such clones in competition with class Ia-restricted CD8 T cell populations. Knowing the minimum precursor frequency required to consistently observe a detectable Q9:VP2.139-specific CD8 T cell population would stretch the retrogenic mice further, so more experiments could be done per retrogenic mouse developed. This would also allow us to get better understanding of the early kinetics of the Q9:VP2.139-specific response in direct competition with the class Ia-restricted response. Repeating CD4 depletion and costimulation blockade experiments from my first paper in a retrogenic B6 model system would allow me to validate my results in the wildtype mouse. The retrogenic cells would also allow us to conduct a head-to-head comparison of the impact of costimulation blockade or CD4 depletion on the Q9:VP2.139-specific response versus the class Ia-restricted response. These experiments would be an elegant follow-up to both my manuscript and that of Christopher Kemball, who published the CD4 help and costimulation requirements of the MPyV-specific class Ia-restricted CD8 T cell responses (25, 42).

Q9:VP2.139-specific CD8 T cells expand during the first three months p.i. in B6.$K^{b-/-}D^{b-/-}$ mice, marked by increasing numbers and frequencies in lung and spleen (22), as well as expression of Ki67, indicating cell cycling (23). However, when B6.$K^{b-/-}$ $D^{b-/-}$ mice are infected with an acute virus (vaccinia) expressing VP2.139 (23), or when a Q9:VP2.139-specific CD8 T cell response is observed in B6 mice due to either low-dose

infection or retrogenic cell transfer ((22) and Figure 2.4), the early kinetics change. In these cases, the Q9:VP2.139-specific CD8 T cells exhibit an expansion and contraction typical of a classical class Ia-restricted CD8 T cell response to an acute antigen. That is, they expand to peak between 11 days and 2 weeks p.i., then contract to a memory setpoint by day 25 (Figure 2.4 and (22, 23)). As demonstrated by Swanson et al., the ability to avoid contraction correlates with a persistent availability of antigen (23). However, this does not explain why the Q9:VP2.139-specific population contracts in B6 mice infected with MPyV. It is possible that in B6 mice, class Ia-restricted CD8 T cells constrain virus to a titer that is lower than is attained by the class Ib-restricted CD8 T cells alone in B6.$K^{b-/-}D^{b-/-}$ mice. However, MPyV genome copies are equivalent between B6 mice and B6.$K^{b-/-}D^{b-/-}$ mice at days 8 and 35 p.i. (22). These results suggest that both the relatively empty T cell niche of the B6.$K^{b-/-}D^{b-/-}$ mice and persistent infection are required to avoid contraction of the Q9:VP2.139-specific population.

One way to determine whether competition from the class Ia-specific CD8 T cells prevents the inflationary expansion of the Q9:VP2.139-specific CD8 T cells in B6 mice would be to mutate the three immunodominant class Ia-restricted epitopes in MPyV (43). We could then compare the kinetics of the Q9:VP2.139-specific CD8 T cell response as it expands in response to the triple epitope knockout MPyV in B6 and B6.$K^{b-/-}D^{b-/-}$ mice. I predict that in B6 mice infected with a triple epitope knockout MPyV, Q9:VP2.139-specific CD8 T cells will expand to peak at around day 11 or before, as is observed when B6 mice are infected with low-dose MPyV (22). However, I would expect the maximal expansion to be larger due to the lack of competition with the class Ia MHC-restricted response for antigen or growth factors. If both antigen persistence and a

perturbed T cell pool are required to avoid contraction, a defined contraction would be observed immediately following the maximal expansion of the Q9:VP2.139-specific population in B6 mice. However, if the triple epitope knockout virus drives inflationary expansion of the Q9:VP2.139-specific response in B6 mice, then the contraction observed with wild-type MPyV in B6 mice could be attributed to competition with the class Ia-restricted CD8 T cell response.

Availability of a class Ia-restricted epitope knockout MPyV might help elucidate other CD8 T cell responses to MPyV. It is well established that deleting a viral epitope allows immunodominance of less dominant epitope-restricted responses (44-46). Overlapping peptide libraries revealed three class Ia-restricted CD8 T cell responses to MPyV in acutely infected B6 mice (43). It is possible that there are further class Ia-restricted CD8 T cell clones available in the repertoire that never expand due to the efficient clearance by the three dominant responses. More importantly, P. A. Swanson II observed that the MPyV-derived peptide MT264-272 (LQQIHPHIL) binds Q9 in vitro (unpublished observations). We were able to demonstrate by ELISA assay that this peptide will compete with VP2.139 for binding to Q9 (P.A. Swanson and A.R. Hofstetter, unpublished observations). However, we have not been able to consistently demonstrate a Q9:MT246-specific CD8 T cell response in vivo. A class Ia epitope knockout MPyV would replicate to high viral titers, possibly expanding Q9:MT264-specific CD8 T cells. Such a virus would be an important tool to better understand the contribution of MHC class Ib-restricted CD8 T cells to the anti-MPyV CD8 T cell response.

T cells specific for peptides in the context of Q9 may be less likely to pass the selection mechanisms in the thymus. It has been demonstrated that GPI-linked MHC

molecules fail to appropriately educate cytotoxic CTL in the thymus. Both negative selection and positive selection for $D^b$-specific TCR are broken when the only $D^b$ molecules expressed in the thymus are GPI-linked. $D^b$-specific CTL are not completely deleted, although they are tolerant to a $D^b$-expressing skin graft. P14 transgenic mice expressing a GPI-linked $D^b$ molecule fail to positively select P14 cells (47). It has been demonstrated that GPI-linked MHCs bind exogenous antigen more readily than endogenous antigens, the latter being critical for thymic education (48). These data suggest that thymic Q9 expression is a poor selection determinant for developing thymocytes. Q9-specific CD8 T cells might only escape the thymus rarely, as stochastic error. This is reinforced by Swanson's et al's demonstration that the Q9:VP2.139-specific CD8 T cell population has a severely limited Vβ repertoire that varies between mice (23). Deficient thymic education would also explain the slow expansion kinetics for Q9:VP2.139-specific CD8 T cells in B6.$K^{b-/-}D^{b-/-}$ mice, as an abnormally small precursor frequency must expand to the point of tetramer recognition. The GPI tail of H2-Q9 may be a structural detriment for the education of Q9-specific CD8 T cells.

Although the GPI tail of Q9 may lead to less efficient signal transduction, retrogenic Q9:VP2.139-specific CD8 T cells do mature in mice receiving retrovirus-transformed bone marrow (Figure 2.4). This suggests that there is a highly restricted repertoire of TCRs that can efficiently signal upon engagement of Q9. Use of the TCRαβ encoded in the retrovirus avoids the random processes of gene rearrangement that might or might not generate a Q9-specific TCR. Interestingly, Holst et al. report that greater than 50% of the $GFP^+$ retrogenic T cells in retrogenic BM-recipient mice should express the proper TCR. In contrast, we only observed 20-30% of the $GFP^+$ $CD8a^+$ retrogenic T

cells from retrogenic bone marrow recipient mice with the correct Vβ chain (49). It is possible that weak signaling through the retrogenic TCR due to weak TCR:Q9 interactions may allow the developing T cells to rearrange and express an endogenous TCR. Simultaneous expression of two TCR Vβ chains occurs for ~1% of T cells naturally (50). Because transduced TCRs must compete for endogenous TCRs for binding to CD3 proteins, a retrogenic TCR may not be expressed on the cell surface despite translation of the retrogenic α and β chains (51). The low frequency of Q9:VP2.139-specific CD8 T cells that exit the thymus of retroviral bone marrow-recipient mice suggests that Q9:VP2.139-specific TCRs are less fit to signal through CD3 than the average endogenous TCR.

Outside the thymus, Q9-specific T cells may receive poor-quality signals from their cognate MHC. Two structural abnormalities of Q9 make it less fit for engaging TCR than classical class Ia MHC molecules. The GPI tail increases the binding of MHC molecules to exogenous peptides, which has been attributed to inefficient endogenous peptide loading in the endoplasmic reticulum (48). It also makes the TCR:pMHC interaction less efficient due to the high mobility of a GPI-linked protein (48). With equal levels of peptide loading, CTL lyse cells with transmembrane MHC molecules with several hundredfold greater efficiency than cells expressing GPI-linked MHC molecules (48). This is supported by Swanson et al.'s observation that a longer (12 h rather than 4 h) in vivo CTL assay was necessary to observe killing by Q9:VP2.139-specific CD8 T cells than for MHC class Ia-restricted MPyV-specific CTL (22, 52).

The second structural abnormality is the orientation of one of the CD8α-binding loops in the α3 domain of the Q9 molecule. The α3 domain of H-2K$^b$ includes two loops

which are important for binding CD8αα (and presumably CD8αβ): the AB and CD loop. The CD loop is the most important structure determining the interaction of H-2K$^b$ and CD8αα (53). A mutation of amino acid 227 within the CD loop of H-2D$^d$ abrogates binding of H-2D$^d$ to CD8αα (54). In H-2K$^b$ amino acid 227 is an asparagine residue that forms a hydrogen bond with CD8αα (53). Four other hydrogen bonds stabilize the CD loop within a pocket formed by the dimerization of the two CD8α proteins. In contrast, the AB loop only contributes two hydrogen bonds to the interaction with CD8αα (53). He et al. (55) demonstrated by protein crystallography that while the CD loop is relatively normal, the AB loop is turned away from the CD8αα molecule. The two residues mediating hydrogen bonding are mutated in Q9 from their long-chain hydrophilic counterparts in H2-K$^b$, making them less favorable for interaction with CD8α (55). In fact, Kern et al. predicted that Qa-2 would not bind to CD8 based on these AB loop mutations, three years before He et al. solved the Q9 crystal structure (53, 55). Whether or not Q9 binds CD8 has not been determined. However, it seems likely that the interaction of Q9 with the CD8 coreceptor is considerably less efficient than for H2-K$^b$.

We were able to indirectly verify that the structure of the Q9 α3 domain is important for TCR binding through collaboration with the NIH Tetramer Core Facility. The Tetramer Core substituted the α3 domain of H2-K$^b$ into the structure of Q9, and constructed tetramers with this chimeric molecule. We observed that the chimeric tetramers bound Q9:VP2.139-specific CD8 T cells with 2-fold higher affinity than native-conformation Q9 tetramers (Chapter 3). However, we hoped to generate more direct evidence that the higher affinity could be attributed to increased interactions with CD8. I attempted to block the site of interaction between the chimeric Q9:VP2.139 tetramers and

the CD8 molecule on Q9:VP2.139-specific CD8 T cells in vitro by pre-incubation of splenocytes from MPyV-infected B6.$K^{b-/-}D^{b-/-}$ mice with an antibody known to block this interaction. The CT-CD8 antibody has been shown to decrease tetramer-CD8α interactions, and thereby decrease tetramer binding (56). We hypothesized that blocking CD8α would decrease the binding of Q9($K^b$):VP2.139 tetramer to the level of the Q9:VP2.139 tetramer, while the latter would be unaffected. However, pre-incubation of MPyV-infected B6.$K^{b-/-}D^{b-/-}$ splenocytes with CT-CD8 equally abrogated binding of both the Q9:VP2.139 and Q9($K^b$):VP2.139 tetramers. Schott and Ploegh observed a similar impact of a CD8β antibody on tetramers of H2-$K^b$ and $K^b$D227N mutant tetramers (57). Titrating down the concentration of CT-CD8 did not show a differential impact on binding of either tetramer. This result makes it unclear how addition of the α3 loop from H2-$K^b$ increased the affinity of the Q9:VP2.139 tetramer.

It is possible that steric hindrance causes the CT-CD8 antibody to occlude the binding of tetramers to the TCR. This scenario seems unlikely, because the CD8 molecules are not recruited into direct proximity of the TCR until at least 5-10 minutes after TCR stimulation (58). However, it would be straightforward to attempt this blocking experiment using Fab fragments of CT-CD8. Fab fragments would introduce less protein to the cell surface, while still blocking the CD8α molecule. It is possible that this would reveal a difference in dependence on CD8α-binding between Q9:VP2.139 and Q9($K^b$):VP2.139. An alternative explanation for my results is that binding of CT-CD8 to the CD8 coreceptor might cause a conformational change that disrupts the binding of CD8 to either the CD or the AB loops. However, Schott and Ploegh suggest an alternative scenario where an anti-CD8 antibody alters the ability of CD8 to associate

with the TCR (57). In this model, tetramer binding is weaker in the presence of an anti-CD8 antibody because CD8 cannot localize the TCR to lipid rafts. According to the model, TCRs are constitutively associated with Lck-independent CD8 molecules, outside of lipid rafts. Lck-bound CD8 localizes to the lipid rafts. Poor binding of CD8 to the MHC means that the TCR will not be recruited to the Lck-rich lipid rafts, leading to weaker signaling. This model is supported by data from Cawthon et al. who demonstrate that disruption of lipid rafts decreases the functional avidity of normally high-avidity CTL (59). Therefore, Q9 may be inefficiently recruited into Lck-rich lipid rafts, decreasing both the length of the interaction with the APC and the quality of downstream signals.

Antibody-mediated blocking studies may not be the best method to test the contribution of the AB loop to the interaction of Q9 and CD8. Mutating the H2-$K^b$ α3 loop within the context of the Q9 tetramer may afford better insight as to how the addition of this loop improves affinity. For example, making point mutations to the residues within the α3 loop to disrupt CD8α binding would reveal whether the effect of the α3 loop is related to its CD8α-binding capacity or to some other mechanism. One strategy would be to use differentially labeled Q9 and chimeric Q9($K^b$) tetramers in a competition assay, as demonstrated by Schott and Plouegh (57). Using chimeric tetramers bearing point mutations at critical CD8-binding residues in such competition assays might better reveal the characteristics of the H2-$K^b$ α3 domain which improve binding of Q9 to CD8.

In addition to the GPI linkage and misaligned CD8 binding loop, the interaction of the TCR with Q9:VP2.139 may be inherently weak. Unpublished Surface Plasmon

Resonance data from Andrew Brooks' lab at the University of Melbourne indicates that the interaction of the TCR from a Q9:VP2.139-specific clone with its cognate pMHC is ten-fold weaker than that of P14 cells with their cognate p:MHC (60). Multiple structural abnormalities along with a weak TCR:pMHC interaction suggest a poor prognosis for the ability of Q9:VP2.139 to bind to TCRs.

It is tempting to speculate that the different factors negatively affecting the Q9:VP2.139-TCR binding avidity might be responsible for the low frequency of IFN-γ-reactive Q9:VP2.139-specific CD8 T cells. The high mobility of a GPI-linked MHC, the poor TCR:pMHC affinity, and the lack of coreceptor binding might together lead to lower-quality downstream signals. To quote R. Germain (NIAID), "suboptimal binding does not simply result in a smaller quantity of the same signals, but in a different quality of proximal signaling" (61). In Q9:VP2.139-specific CD8 T cells, the signaling quality may be enough to drive clonal expansion, yet only under some circumstances (prolonged contact with pMHC, for example) would the quality of signals be enough to promote IFN-γ production. If this is true, then the longer the Q9:VP2.139-specific CD8 T cells are exposed to antigen, the larger the percentage of the clone producing IFN-γ should be. One important experiment would be increasing the length of the ICCS assay, from the typical 5-6 hours to 12 hours. Swanson et al (22) observed that a longer timecourse for an in vivo CTL assay was needed to observe killing; similarly, a longer time may be necessary to see optimal IFN-γ production in vitro. However, such long exposure to BFA could be cytotoxic (62, 63). Adding the BFA only after an overnight stimulation would give as many cells as possible the chance to respond. Increasing the duration of the ICCS

might be the best way to observe the true cytokine effector capacity of the Q9:VP2.139-specific CD8 T cells.

Importantly, increasing the concentration of VP2.139 peptide from 1 uM to 50 uM during a 6 hour ICS leads to increasing frequencies of IFN-γ-producing CD8 T cells (Figure 4.1). This supports the hypothesis that IFN-γ functionality determined by a 5 hour ICCS with 1 or 10 uM peptide might underestimate the functionality of the Q9:VP2.139-specific CD8 T cells. High concentrations of peptide may be necessary to sufficiently coat APCs in vitro at the level that an infected cell would be producing antigen in vivo. It is possible that a high level of surface Q9:VP2.139 is necessary to trigger the appropriate downstream signaling that elaborates IFN-γ production.

I was able to observe that the frequency cells producing IFN-γ in a 5 hour ICCS relative to Q9:VP2.139-specific cells increased over the course of infection (Figure 4.1). Memory cells are in part defined by their ability to more rapidly respond to antigen stimulation (64). It is possible that the Q9:VP2.139-specific CD8 T cell clone gradually acquires more memory characteristics over the course of its long kinetic expansion. In other words, a Q9:VP2.139-specific CD8 T cell at 3 mo. p.i. would be a 'better' memory cell than a Q9:VP2.139-specific CD8 T cell at 1 mo. p.i. This is supported by the phenotype of Q9:VP2.139-specific CD8 T cells, which increase in CD127 and Granzyme B expression between 1 and 6 months p.i., while decreasing in CD43, KLRG-1 and CD62L expression (22). T cell responses have been observed to increase in affinity as cells with the highest affinity TCRs are preferentially expanded (65). However, the limited clonality of the Q9:VP2.139-specific response (23) makes it unlikely that TCR affinity plays role in the increasing IFN-γ production of this population.

Poor effector cytokine function could also be related to the expression of inhibitory receptors on the surface of Q9:VP2.139-specific CD8 T cells. Wirth et al. demonstrated that MPyV replication decreases with the age of the mouse (66). It is possible that the Q9:VP2.139-specific population is under some form of repression which declines as viral inflammation decreases. We never observe much PD-1 expression on Q9:VP2.139-specific CD8 T cells (21, 22, 49). The inhibitory receptors LAG-3 and Tim-3 (67) are expressed on Q9:VP2.139-specific CD8 T cells from B6.$K^{b-/-}D^{b-/-}$ mice at day 35 p.i. – their expression has not been monitored long-term (unpublished observations). I never observed a significant decline of NKG2A over the course of MPyV persistence. Upregulation of CD94/NKG2A has been associated with decreased cytotoxic and cytokine effector functions (68, 69), although Cush et al. report that in a γ-Herpesvirus model, the effector function of KLRG-$1^+$ $NKG2A^+$ CD8 T cells is not impaired, and that this phenotype may in fact correlate with more protective CD8 T cells (70). Expression of CD94/NKG2A on Q9:VP2.139-specific CD8 T cells increases after day 12 p.i., and is thereafter maintained at a high level of expression (Figure 2.8). These are the same kinetics of NKG2A expression that have been observed for the class Ia-restricted CD8 T cell response to MPyV (68). Upregulation of CD94/NKG2A has been demonstrated to reduce immunopathology associated with influenza virus (69). CD94/NKG2A expression by MPyV-specific CD8 T cells may similarly be critical to prevent immunopathology. Blockade of Qa-$1^b$ can relieve CD94/NKG2A-mediated inhibition (68), suggesting that Qa-$1^b$ treatment of B6.$K^{b-/-}D^{b-/-}$ mice might increase the cytotoxic or cytokine effector function of Q9:VP2.139-specific CD8 T cells in vivo. It is possible that

as viral titers and inflammation decrease, the Q9:VP2.139-specific CD8 T cells decrease expression of inhibitory receptors, leading to more rapid cytokine production.

Alternatively, the Q9:VP2.139-specific CD8 T cells could predominantly produce a cytokine besides IFN-γ. I never observed a large population of IL-10 or IL-17-producing Q9:VP2.139-specific CD8 T cells (unpublished observations). However, a multiplex cytokine assay might better elucidate the cytokine effector function, or lack of function, of the Q9:VP2.139-specific CD8 T cells. It remains unclear whether the low frequency of cytokine-producing Q9:VP2.139-specific CD8 T cells is due to structural characteristics of the Q9 molecule, or an incomplete examination of the parameters needed to observe the effector function of these unique cells.

The Q9:VP2.139-specific CD8 T cell response protects against MPyV infection in vivo. This has been demonstrated by comparing viral loads between mice infected with MPyV and mice infected H145A. B6.$K^{b-/-}D^{b-/-}$ mice infected with MPyV have over a log lower viral titers in their kidneys at day 80 p.i. than mice infected H145A. Importantly, adoptive transfer of $20 \times 10^6$ splenocytes from spleens of MPyV infected mice into naïve mice at the time of infection leads to significantly lower viral titers in the spleen, kidney and salivary glands, in mice infected with MPyV as compared to H145A. This underscores the necessity for a high frequency of Q9:VP2.139-specific CD8 T cells to exert detectable protection. Wilson et al. (71) recently demonstrated that IFN-γ is a major antiviral effector molecule during MPyV infection. A previous study indicated that protection against MPyV infection is independent of perforin, fas, and TNFα (72). If we assume for a moment that, like the class Ia-restricted CD8 T cell responses, the Q9:VP2.139-specific CD8 T cells rely on IFN-γ to kill infected cells, the following

model would fit our observations. Due to the abnormalities of Q9, the Q9:VP2.139-TCR interaction has a low avidity. This low avidity decreases the probability of a stable interaction between the effectors and target cells. When a stable interaction is established, IFN-γ-mediated killing occurs. In scenarios where there are very few Q9:VP2.139-specific CD8 T cells, protection is not observed due to the infrequency of these stable interactions. Similarly, when the Q9:VP2.139-specific CD8 T cells must compete for antigen with class Ia-restricted CD8 T cells, the class Ia-restricted cells control the infection before the class Ib-restricted cell(s) in the precursor pool achieve enough stimulation to begin division.

It was clear from our attempts to expand Q9:VP2.139-specific CD8 T cells in B6 mice that class Ia wild-type mice would be intractable to expansion of this class Ib-restricted response. However, it was important to demonstrate that the lack of Q9 polymorphism meant the Q9:VP2.139 epitope could be recognized across allogeneic MHC class Ia haplotypes. P. A. Swanson screened various strains of mice for Qa-2 expression. Importantly, the Qa-2-specific 1-1-2 monoclonal antibody (MAb) does not discriminate between Q6, Q7, Q8 and Q9 (73). He discerned that mice of the H2-Q, H2-S, and H2-V haplotypes expressed Qa-2 (P.A. Swanson, unpublished observations). This is in agreement with the published allele expression from the Jackson Catalog (74). The MAb 1-1-2 staining of the SJL splenocytes most closely resembled the staining pattern seen in B6 splenocytes; I speculate that this is why he chose to attempt vaccination in the SJL strain first. We first attempted to vaccinate SJL mice with psoralen-crosslinked VV.VP2.139. Upon exposure to UV light, psoralen cross-links the DNA of vaccinia virus. Random crosslinking will more likely occur in the large native vaccinia genes. In

contrast, a small epitope-encoding minigene is relatively spared. This strategy, proposed by Christopher Norbury (75) is intended to increase transcription of an epitope of interest during immunization with the vaccinia vector. We were primarily unsuccessful. Importantly, one SJL mouse, immunized with psoralen-VV.VP2.139, and treated with anti-4-1BB five days later, expanded a detectable Q9:VP2.139-specific CD8 T cell response. 0.55% of the CD8 T cells were Q9:VP2.139$^{+}$. This was the first evidence that the Q9:VP2.139-specific CD8 T cell response could be elicited in SJL mice.

It was encouraging to have evidence that the Q9:VP2.139-specific CD8 T cell response could expand in SJL mice, but it was critical that we be able to consistently repeat this observation. Therefore, we turned to a very strong immunization regimen. CFA was emulsified with the VP2.139 peptide in PBS and injected into the footpads of SJL mice. Two weeks later, mice were boosted with VP2.139 emulsified in IFA, injected into the base of the tail. Two weeks after the boost, mice were challenged with MPyV. Using this regimen, I was able to detect a Q9:VP2.139-specific response in at least 80% of immunized mice (Figure 3.1). To compare the capacity of the primed Q9:VP2.139-specific CD8 T cells to protect against MPyV infection, I set up a controlled experiment. Half of the naïve mice received CFA + peptide and IFA + peptide, the other half were primed with CFA emulsified with PBS alone, and boosted with IFA emulsified with PBS alone. Then all mice were challenged with 2 x $10^5$ p.f.u. MPyV. The viral loads and tetramer levels in the mice were observed at day 6 p.i (Figure 3.1). To my disappointment, there was no significant difference in viral titers in the spleen, kidney, salivary gland or heart in SJL mice that had been primed and boosted with Q9:VP2.139-specific CD8 T cells (A.R. Hofstetter, unpublished observations). Furthermore, although

over 90% of the VP2.139-primed and –boosted mice expanded a detectable Q9:VP2.139-specific CD8 T cell response, I never observed more than 0.4% of the CD8 T cells to be Q9:VP2.139 tetramer-positive.

While the SJL experiments were underway, I had, at the suggestion of Dr. Brian D. Evavold, started the same prime-boost regimen in NOD mice. NOD mice are haplotype H2-g7, which is Qa-$2^{+}$ (74). I verified Qa-2 expression on NOD splenocytes with MAb 1-1-2 (A.R. Hofstetter, unpublished observation). Male NOD mice were readily available, and a better option than females due to the diabetogenic phenotype observed in females (76). In contrast to the SJL mice, I was able to discern a significant decrease in MPyV titers in the spleens of VP2.139-primed and –boosted mice at day 6 p.i. compared with PBS-primed and –boosted controls (Figure 3.2). The NOD mice that demonstrated a detectable Q9:VP2.139-specific response tended to have a more robust expansion of this clone than observed in SJL mice. Importantly, the two NOD mice with the highest frequency of Q9:VP2.139-specific CD8 T cells – over 0.4% of the CD8 T cell population - were the two mice with the lowest MPyV viral titers in the spleen. This suggests that no protection was observed in the SJL mice because the Q9:VP2.139-specific population was not large enough to make a discernable difference.

In retrospect, neither the priming regimen nor the mouse choices were ideal to efficiently address the hypothesis. CFA is not an ideal priming regimen because it is highly inflammatory. Hikono et al. demonstrated that Sendai-specific peptide + CFA-immunized mice developed relatively poor immune responses to Sendai virus as compared to mice previously exposed to Sendai virus. In response to a CFA + Sendai peptide immunization, the CD8 T cell population was predominated by $CD127^{hi}$ cells,

and a $CD27^{lo}$ $CD43^{hi}$ population that was never seen after normal Sendai infection. The CFA-elicited Sendai-specific memory response was quantitatively and qualitatively poorer than the memory response to Sendai infection (77). I observed many of the same phenotypic aberrations observed by Hikono et al. in the CD8 T cells from CFA-immunized mice (Figure 3.1E). Therefore, a less inflammatory priming regimen might lead to the expansion of a more functional memory recall response.

Our immunization attempts with vaccinia were largely unsuccessful. Besides the VV-VP2.139 + psoralen strategy, we also used the VV-VP2 virus, which expresses the entire VP2 protein from MPyV. Swanson et al. demonstrated that this elicits a Q9:VP2.139-specific CD8 T cell response in $B6.K^{b-/-}D^{b-/-}$ mice (23), but efforts to immunize B6 mice were unsuccessful. We hypothesize that this is due to the large class Ia-restricted CD8 T cell response generated against the vaccinia epitopes, which out-compete any Q9:VP2.139-specific clone for antigen and growth factors.

In line with my speculation about the need for prolonged exposure to high concentrations of antigen, an immunization regimen that deposited VP2.139 peptide, and no other peptide antigens, for an extended period of time, would be the best method to expand a robust Q9:VP2.139-specific CD8 T cell population. An oil-in-water adjuvant without killed bacteria, such as IFA, can generate a short-term antigen depot and deliver a peptide to DCs (78). Supplementing the emulsion with CpG, a potent TLR-9 adjuvant, helps increase the IFN-γ producing phenotype of the responding CD8 T cells (79). Alternatively, our lab previously demonstrated that mouse dendritic cells permit MPyV infection (80). DCs infected with a class Ia epitope knockout PyV could be used as a DC-based vaccine strategy. It could be beneficial to supplement any new priming

regimens with coadministration of recombinant human (rh)-IL-7 and -IL-15, as these have been shown to boost the memory responses to vaccination, especially to subdominant epitopes (81). These examples allude to the extensive variety of immunization strategies that have been published, and different methods could be tested alone or in combination to generate the most robust Q9:VP2.139-specific CD8 T cell response.

The prime-boost immunization studies could also be improved by optimizing the timing and quantity of the viral challenge. The lowered viral dose ($10^5$ rather than $10^6$ pfu MPyV) and early timing of the experiment (day 6 p.i.) were chosen in order to allow as much time as possible for the Q9:VP2.139-specific recall response to expand, while delaying the class Ia-specific CD8 T cell response through a lower-dose infection (40). Andrews et al. observed that the expansion of class Ia-restricted CD8 T cell responses to MPyV was delayed after decreasing the dose of MPyV 1000-fold, from $10^6$ to $10^3$ pfu A2 (40). However, when B6 mice treated with the VP2.139 prime-boost regimen were infected with $10^5$ pfu MPyV, the immunodominant class Ia-restricted CD8 T cell response had undergone significant expansion by day 6 p.i. (A.R. Hofstetter, unpublished observations). This indicates that a 10-fold decrease in virus did not sufficiently delay the expansion of the class Ia-restricted CD8 T cell responses. While the class Ia-restricted CD8 T cell responses to MPyV in H-$2^s$ and H-$2^{g7}$ haplotypes are not known, it is likely that they expand with similar kinetics as in B6 mice. Therefore, decreasing the dose of the viral challenge to $10^3$ pfu A2 might allow the memory Q9:VP2.139-specific CD8 T cell response to expand more robustly before the delayed proliferation of the class

Ia-restricted responses. This might allow us to see a larger difference in viral titers between VP2.139-immunized and PBS-control mice.

In addition to the immunization and infection regimens, alternative mouse choices could have led to better expansion of Q9:VP2.139-specific CD8 T cells. Although SJL and NOD mice express Qa-2 on the surface of their splenocytes, this is not indicative of the actual expression of Q7 and Q9. Lund et al. published evidence that NOD mice express either Q7 or Q9, but not both (82). One Qa-2 reactive antibody clone induces less complement-mediated lysis of SJL lymphocytes than B6 lymphocytes (83) which is in agreement with the MAb 1-1-2 staining of B6 and SJL splenocytes (P.A. Swanson, unpublished data). Qa-2 expression is thought to vary with the number of genes encoding Qa-2 (84, 85). Therefore it is possible that SJLs express fewer Qa-2 genes than B6 mice, but more direct evidence is needed. Of the Qa-2 genes, the Q6/Q8 and Q7/Q9 gene pairs differ significantly at the amino acid level (86). Q7 and Q9 differ by only a single amino acid, outside of the predicted amino acid binding groove (87). Importantly, Q9:VP2.139-specific CD8 T cell clones recognize VP2.139-pulsed Q9-expressing cells, but not VP2.139-pulsed Q8-expressing cells (22). Therefore, a Qa-$2^{+}$ mouse that expressed only a Q6 and/or Q8 gene product would not present to Q9:VP2.139-specific CD8 T cells. It appears that we were lucky in choosing the mouse haplotypes that express either Q7 or Q9, because they do expand a Q9/Q7:VP2.139-specific CD8 T cell response. The lower levels of Q9:VP2.139-specific CD8 T cells observed in the SJL mice as compared to the NOD mice may be due to differences in either the number of genes or the gene expression levels between these strains of mice (88).

I used the NOD mice because they were readily available in the lab. However, NOD mice are known for developing spontaneous diabetes. This phenotype is seen more often in females than males (76). I did not perform any tests to determine if the NOD males were diabetic during the course of my experiments. Both the NOR mice and ICR mice are H2-g7, but do not exhibit the diabetogenic phenotype (74). A non-diabetic H2-g7 strain would have been a better choice, to eliminate the confounding immunologic variables of diabetes.

I would also like to test other strains of mice in the future. H2-V and –Q haplotypes appear to be Qa-2 positive, as indicated by P.A. Swanson's unpublished observations and the Jackson catalog (74). It would streamline immunization studies to first test novel strains of mice for expression of Q7 and/or Q9. Detection would need to be performed by molecular genotyping, because serological reagents cannot differentiate between the Qa-2 proteins (89).

Optimizing the immunization strategy and mouse model for eliciting Q9-restricted CD8 T cell responses would allow me to test whether other pathogens with putative Q9-binding epitopes could drive a protective Q9-restricted CD8 T cell response. Tabaczewski et al. published 17 intracellular pathogen-derived peptides that fit the binding motif of Q9-binding peptides, all derived from infections for which a mouse model is readily available. Of these, five demonstrated strong stabilization of Q9 in an ELISA-based peptide stabilization assay; these epitopes were derived from rabies virus, rotavirus and adenovirus (87). It would be fascinating to challenge Q9-expressing mice with these peptides, and determine if clones specific for these peptides bound by Q9 exist in the naïve T cell pool. The existence of such clones would be an indication that Q9-

specific CD8 T cells could contribute to protection in adenovirus, rabies or rotavirus infections in mice. We could test this hypothesis using the optimized prime-boost regimen, followed by virus challenge. These studies would be an elegant test of the breadth of the immune responses contributed to by Q9.

The studies presented in this work demonstrate the role of a class Ib-restricted CD8 T cell response to MPyV. Humans do not have a Q9 ortholog, and although HLA-G has been suggested as a functional homolog (18), these genes map to different sections of the overlaid human and mouse MHC locus (90). Therefore, these studies serve as a proof-of-principle: nonclassical class Ib MHC molecules can restrict protective CD8 T cell responses to viral infections in allogeneic hosts. The Q9:VP2.139-specific CD8 T cell response exhibits weak protection against MPyV, which is likely to be associated with its poor IFN-γ production. It appears likely that structural abnormalities make Q9 a poor ligand for inducing strong CTL responses. However, these characteristics need not be the rule for class Ib-restricted CD8 T cells. Neither the native structure or function of HLA-F is known, but this molecule may not bind peptides (19). HLA-G binds a limited repertoire of peptides, but many lines of evidence tie this molecule to tolerogenic roles, indicating that the ability of HLA-G to bind several immunoreceptor tyrosine-based inhibitory receptor-associated molecules may be more important than its ability to bind peptide (15). In contrast, HLA-E binds peptides, and while it is usually occupied by the leader sequences of other HLA molecules (91), HLA-E restricted CD8 T cell responses to *M. tuberculosis*, *Salmonella typhi* and cytomegalovirus have been reported (92-94). Seredipitously, HLA-E is the human class Ib molecule with the fewest polymorphisms, having functionally only three alleles (11). Therefore, HLA-E is a promising target for a

universal peptide vaccine formulated with no more than three pathogen-derived peptides. It would be of interest to screen protein databanks for intracellular pathogen-derived peptides fitting the binding pattern for HLA-E, to determine the prevalence of such motifs.

In summary, I have investigated the requirements to prime and sustain a class Ib-restricted CD8 T cell response that is protective against PyV infection in allogeneic hosts. In Chapter 2 I determined the dependence of the Q9:VP2.139-specific CD8 T cell response on CD4 help and CD28/CD40L costimulation during two discreet phases of the response in B6.$K^{b-/-}D^{b-/-}$ mice. During the first month p.i., the start of the long inflationary expansion of the Q9:VP2.139-specific CD8 T cell population, CD4 help and CD28 costimulation are necessary for the clone to proliferate. After three months p.i., when the cells are maintainted at a high level, independent of antigen, I determined that CD4 T cells are critical for the survival of the Q9:VP2.139-specific population in a costimulation- and IL-2-independent manner. In Chapter 3 I present the results of my attempts to prime a Q9:VP2.139-specific CD8 T cell response, which we first identified in the H-$2^b$ haplotype, in mice of other haplotypes. I demonstrated that a CFA + peptide prime and IFA + peptide boost consistently elicited this population H-$2^k$ and H-$2^{g7}$ mouse strains, and that the Q9:VP2.139-specific cells were protective against MPyV infection in H-$2^{g7}$ mice. These studies demonstrate that MHC class Ib-restricted CD8 T cells can protect against intracellular infections in allogeneic hosts. It is my hope that these findings will increase interest in studying the antigen-presenting capacity of human class Ib MHC molecules. Such investigations could reveal novel MHC class Ib-restricted epitope targets for peptide-based vaccines.

References

1. Ada, G. 2001. Vaccines and vaccination. *N Engl J Med* 345: 1042-1053.

2. Ovsyannikova, I. G., K. L. Johnson, H. R. Bergen, 3rd, and G. A. Poland. 2007. Mass spectrometry and peptide-based vaccine development. *Clinical pharmacology and therapeutics* 82: 644-652.

3. Fujita, Y., and H. Taguchi. 2011. Current status of multiple antigen-presenting peptide vaccine systems: Application of organic and inorganic nanoparticles. *Chemistry Central journal* 5: 48.

4. Schipper, R. F., C. A. van Els, J. D'Amaro, and M. Oudshoorn. 1996. Minimal phenotype panels. A method for achieving maximum population coverage with a minimum of HLA antigens. *Hum Immunol* 51: 95-98.

5. Hunziker, I. P., R. Zurbriggen, R. Glueck, O. B. Engler, J. Reichen, W. J. Dai, W. J. Pichler, and A. Cerny. 2001. Perspectives: towards a peptide-based vaccine against hepatitis C virus. *Mol Immunol* 38: 475-484.

6. Longmate, J., J. York, C. La Rosa, R. Krishnan, M. Zhang, D. Senitzer, and D. J. Diamond. 2001. Population coverage by HLA class-I restricted cytotoxic T-lymphocyte epitopes. *Immunogenetics* 52: 165-173.

7. Bui, H. H., J. Sidney, K. Dinh, S. Southwood, M. J. Newman, and A. Sette. 2006. Predicting population coverage of T-cell epitope-based diagnostics and vaccines. *BMC bioinformatics* 7: 153.

8. Grimsley, C., and C. Ober. 1997. Population genetic studies of HLA-E: evidence for selection. *Hum Immunol* 52: 33-40.

9. Pyo, C. W., L. M. Williams, Y. Moore, H. Hyodo, S. S. Li, L. P. Zhao, N. Sageshima, A. Ishitani, and D. E. Geraghty. 2006. HLA-E, HLA-F, and HLA-G polymorphism: genomic sequence defines haplotype structure and variation spanning the nonclassical class I genes. *Immunogenetics* 58: 241-251.

10. Marsh, S. G., E. D. Albert, W. F. Bodmer, R. E. Bontrop, B. Dupont, H. A. Erlich, M. Fernandez-Vina, D. E. Geraghty, R. Holdsworth, C. K. Hurley, M. Lau, K. W. Lee, B. Mach, M. Maiers, W. R. Mayr, C. R. Muller, P. Parham, E. W. Petersdorf, T. Sasazuki, J. L. Strominger, A. Svejgaard, P. I. Terasaki, J. M. Tiercy, and J. Trowsdale. 2010. Nomenclature for factors of the HLA system, 2010. *Tissue Antigens* 75: 291-455.

11. Robinson, J., K. Mistry, H. McWilliam, R. Lopez, P. Parham, and S. G. Marsh. 2011. The IMGT/HLA database. *Nucleic Acids Res* 39: D1171-1176.

12. Grimsley, C., A. Kawasaki, C. Gassner, N. Sageshima, Y. Nose, K. Hatake, D. E. Geraghty, and A. Ishitani. 2002. Definitive high resolution typing of HLA-E allelic polymorphisms: Identifying potential errors in existing allele data. *Tissue Antigens* 60: 206-212.

13. Milner-White, E. J. 1984. Isoenzymes, isoproteins and introns. *Trends in Biochemical Sciences* 9: 517-519.

14. Ohya, K., K. Kondo, and S. Mizuno. 1990. Polymorphism in the human class I MHC locus HLA-E in Japanese. *Immunogenetics* 32: 205-209.

15. Donadi, E. A., E. C. Castelli, A. Arnaiz-Villena, M. Roger, D. Rey, and P. Moreau. 2011. Implications of the polymorphism of HLA-G on its function,

regulation, evolution and disease association. *Cellular and molecular life sciences : CMLS* 68: 369-395.

16. Doolittle, D. P., Davisson, M.T., Guidi, J.N., and Green, M.C. 1996. Catalog of mutant genes and polymorphic loci. In *Genetic Variants and Strains of the Laboratory Mouse*, 3rd ed. Oxford University Press, Oxford; New York; Tokyo. 17-854.

17. Rodgers, J. R., and R. G. Cook. 2005. MHC class Ib molecules bridge innate and acquired immunity. *Nat. Rev. Immunol.* 5: 459-471.

18. Comiskey, M., C. Y. Goldstein, S. R. De Fazio, M. Mammolenti, J. A. Newmark, and C. M. Warner. 2003. Evidence that HLA-G is the functional homolog of mouse Qa-2, the Ped gene product. *Hum Immunol* 64: 999-1004.

19. Goodridge, J. P., A. Burian, N. Lee, and D. E. Geraghty. 2010. HLA-F complex without peptide binds to MHC class I protein in the open conformer form. *J. Immunol.* 184: 6199-6208.

20. Sullivan, L. C., H. L. Hoare, J. McCluskey, J. Rossjohn, and A. G. Brooks. 2006. A structural perspective on MHC class Ib molecules in adaptive immunity. *Trends Immunol* 27: 413-420.

21. Hofstetter, A. R., L. C. Sullivan, A. E. Lukacher, and A. G. Brooks. 2011. Diverse roles of non-diverse molecules: MHC class Ib molecules in host defense and control of autoimmunity. *Curr. Opin. Immunol.* 23: 104-110.

22. Swanson, P. A., 2nd, C. D. Pack, A. Hadley, C. R. Wang, I. Stroynowski, P. E. Jensen, and A. E. Lukacher. 2008. An MHC class Ib-restricted CD8 T cell response confers antiviral immunity. *J. Exp. Med.* 205: 1647-1657.

23. Swanson, P. A., 2nd, A. R. Hofstetter, J. J. Wilson, and A. E. Lukacher. 2009. Cutting edge: shift in antigen dependence by an antiviral MHC class Ib-restricted CD8 T cell response during persistent viral infection. *J. Immunol.* 182: 5198-5202.

24. Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421: 852-856.

25. Kemball, C. C., C. D. Pack, H. M. Guay, Z. N. Li, D. A. Steinhauer, E. Szomolanyi-Tsuda, and A. E. Lukacher. 2007. The antiviral CD8+ T cell response is differentially dependent on CD4+ T cell help over the course of persistent infection. *J. Immunol.* 179: 1113-1121.

26. Barthlott, T., H. Moncrieffe, M. Veldhoen, C. J. Atkins, J. Christensen, A. O'Garra, and B. Stockinger. 2005. CD25+ CD4+ T cells compete with naive CD4+ T cells for IL-2 and exploit it for the induction of IL-10 production. *Int Immunol* 17: 279-288.

27. Schmidt, A., N. Oberle, and P. H. Krammer. 2012. Molecular mechanisms of treg-mediated T cell suppression. *Frontiers in immunology* 3: 51.

28. Lenardo, M. J. 1991. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature* 353: 858-861.

29. Wei, C. H., H. Yagita, M. G. Masucci, and V. Levitsky. 2001. Different programs of activation-induced cell death are triggered in mature activated CTL by immunogenic and partially agonistic peptide ligands. *J. Immunol.* 166: 989-995.

30. Pipkin, M. E., J. A. Sacks, F. Cruz-Guilloty, M. G. Lichtenheld, M. J. Bevan, and A. Rao. 2010. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* 32: 79-90.

31. Cheng, L. E., C. Ohlen, B. H. Nelson, and P. D. Greenberg. 2002. Enhanced signaling through the IL-2 receptor in CD8+ T cells regulated by antigen recognition results in preferential proliferation and expansion of responding CD8+ T cells rather than promotion of cell death. *Proc. Natl. Acad. Sci. USA* 99: 3001-3006.

32. Elsaesser, H., K. Sauer, and D. G. Brooks. 2009. IL-21 is required to control chronic viral infection. *Science* 324: 1569-1572.

33. Frohlich, A., J. Kisielow, I. Schmitz, S. Freigang, A. T. Shamshiev, J. Weber, B. J. Marsland, A. Oxenius, and M. Kopf. 2009. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science* 324: 1576-1580.

34. Yi, J. S., M. Du, and A. J. Zajac. 2009. A vital role for interleukin-21 in the control of a chronic viral infection. *Science* 324: 1572-1576.

35. Lin, E., C. C. Kemball, A. Hadley, J. J. Wilson, A. R. Hofstetter, C. D. Pack, and A. E. Lukacher. 2010. Heterogeneity among viral antigen-specific CD4+ T cells and their de novo recruitment during persistent polyomavirus infection. *J. Immunol.* 185: 1692-1700.

36. Vugmeyster, Y., H. Guay, P. Szklut, M. D. Qian, M. Jin, A. Widom, V. Spaulding, F. Bennett, L. Lowe, T. Andreyeva, D. Lowe, S. Lane, G. Thom, V.

Valge-Archer, D. Gill, D. Young, and L. Bloom. 2010. In vitro potency, pharmacokinetic profiles, and pharmacological activity of optimized anti-IL-21R antibodies in a mouse model of lupus. *mAbs* 2: 335-346.

37. Hendriks, J., Y. Xiao, and J. Borst. 2003. CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool. *J. Exp. Med.* 198: 1369-1380.

38. Nurieva, R. I., X. Liu, and C. Dong. 2009. Yin-Yang of costimulation: crucial controls of immune tolerance and function. *Immunol Rev* 229: 88-100.

39. Chlewicki, L. K., C. A. Velikovsky, V. Balakrishnan, R. A. Mariuzza, and V. Kumar. 2008. Molecular basis of the dual functions of 2B4 (CD244). *J. Immunol.* 180: 8159-8167.

40. Andrews, N. P., C. D. Pack, V. Vezys, G. N. Barber, and A. E. Lukacher. 2007. Early virus-associated bystander events affect the fitness of the CD8 T cell response to persistent virus infection. *J. Immunol.* 178: 7267-7275.

41. Holst, J., A. L. Szymczak-Workman, K. M. Vignali, A. R. Burton, C. J. Workman, and D. A. Vignali. 2006. Generation of T-cell receptor retrogenic mice. *Nat. Protoc.* 1: 406-417.

42. Kemball, C. C., E. D. Lee, E. Szomolanyi-Tsuda, T. C. Pearson, C. P. Larsen, and A. E. Lukacher. 2006. Costimulation requirements for antiviral CD8+ T cells differ for acute and persistent phases of polyoma virus infection. *J. Immunol.* 176: 1814-1824.

43. Kemball, C. C., E. D. Lee, V. Vezys, T. C. Pearson, C. P. Larsen, and A. E. Lukacher. 2005. Late priming and variability of epitope-specific CD8+ T cell responses during a persistent virus infection. *J. Immunol.* 174: 7950-7960.

44. Tanaka, Y., R. W. Anderson, W. L. Maloy, and S. S. Tevethia. 1989. Localization of an immunorecessive epitope on SV40 T antigen by H-2Db-restricted cytotoxic T-lymphocyte clones and a synthetic peptide. *Virology* 171: 205-213.

45. Webby, R. J., S. Andreansky, J. Stambas, J. E. Rehg, R. G. Webster, P. C. Doherty, and S. J. Turner. 2003. Protection and compensation in the influenza virus-specific CD8+ T cell response. *Proc. Natl. Acad. Sci. USA* 100: 7235-7240.

46. Weidt, G., O. Utermohlen, J. Heukeshoven, F. Lehmann-Grube, and W. Deppert. 1998. Relationship among immunodominance of single CD8+ T cell epitopes, virus load, and kinetics of primary antiviral CTL response. *J. Immunol.* 160: 2923-2931.

47. Simpson, E., P. J. Robinson, P. Chandler, M. M. Millrain, H. P. Pircher, D. Brandle, P. Tomlinson, J. Antoniou, and A. Mellor. 1994. Separation of thymic education from antigen presenting functions of major histocompatibility complex class I molecules. *Immunology* 81: 132-136.

48. Segura, J. M., P. Guillaume, S. Mark, D. Dojcinovic, A. Johannsen, G. Bosshard, G. Angelov, D. F. Legler, H. Vogel, and I. F. Luescher. 2008. Increased mobility of major histocompatibility complex I-peptide complexes decreases the sensitivity of antigen recognition. *J Biol Chem* 283: 24254-24263.

49. Hofstetter, A. R., M. L. Ford, L. C. Sullivan, J. J. Wilson, A. Hadley, A. G. Brooks, and A. E. Lukacher. 2012. MHC Class Ib-Restricted CD8 T Cells Differ

in Dependence on CD4 T Cell Help and CD28 Costimulation over the Course of Mouse Polyomavirus Infection. *J. Immunol.* 188: 3071-3079.

50. Blichfeldt, E., L. A. Munthe, J. S. Rotnes, and B. Bogen. 1996. Dual T cell receptor T cells have a decreased sensitivity to physiological ligands due to reduced density of each T cell receptor. *Eur. J. Immunol.* 26: 2876-2884.

51. Heemskerk, M. H., R. S. Hagedoorn, M. A. van der Hoorn, L. T. van der Veken, M. Hoogeboom, M. G. Kester, R. Willemze, and J. H. Falkenburg. 2007. Efficiency of T-cell receptor expression in dual-specific T cells is controlled by the intrinsic qualities of the TCR chains within the TCR-CD3 complex. *Blood* 109: 235-243.

52. Byers, A. M., C. C. Kemball, J. M. Moser, and A. E. Lukacher. 2003. Cutting edge: rapid in vivo CTL activity by polyoma virus-specific effector and memory CD8+ T cells. *J. Immunol.* 171: 17-21.

53. Kern, P. S., M. K. Teng, A. Smolyar, J. H. Liu, J. Liu, R. E. Hussey, R. Spoerl, H. C. Chang, E. L. Reinherz, and J. H. Wang. 1998. Structural basis of CD8 coreceptor function revealed by crystallographic analysis of a murine CD8alphaalpha ectodomain fragment in complex with H-2Kb. *Immunity* 9: 519-530.

54. Potter, T. A., T. V. Rajan, R. F. Dick, 2nd, and J. A. Bluestone. 1989. Substitution at residue 227 of H-2 class I molecules abrogates recognition by CD8-dependent, but not CD8-independent, cytotoxic T lymphocytes. *Nature* 337: 73-75.

55. He, X., P. Tabaczewski, J. Ho, I. Stroynowski, and K. C. Garcia. 2001. Promiscuous antigen presentation by the nonclassical MHC Ib Qa-2 is enabled by

a shallow, hydrophobic groove and self-stabilized peptide conformation. *Structure* 9: 1213-1224.

56. Holman, P. O., E. R. Walsh, and S. C. Jameson. 2005. Characterizing the impact of CD8 antibodies on class I MHC multimer binding. *J. Immunol.* 174: 3986-3991.

57. Schott, E., and H. L. Ploegh. 2002. Mouse MHC class I tetramers that are unable to bind to CD8 reveal the need for CD8 engagement in order to activate naive CD8 T cells. *Eur. J. Immunol.* 32: 3425-3434.

58. Yachi, P. P., J. Ampudia, T. Zal, and N. R. Gascoigne. 2006. Altered peptide ligands induce delayed CD8-T cell receptor interaction--a role for CD8 in distinguishing antigen quality. *Immunity* 25: 203-211.

59. Cawthon, A. G., and M. A. Alexander-Miller. 2002. Optimal colocalization of TCR and CD8 as a novel mechanism for the control of functional avidity. *J. Immunol.* 169: 3492-3498.

60. Boulter, J. M., N. Schmitz, A. K. Sewell, A. J. Godkin, M. F. Bachmann, and A. M. Gallimore. 2007. Potent T cell agonism mediated by a very rapid TCR/pMHC interaction. *Eur. J. Immunol.* 37: 798-806.

61. Germain, R. N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat. Rev. Immunol.* 2: 309-322.

62. Torii, S., T. Banno, T. Watanabe, Y. Ikehara, K. Murakami, and K. Nakayama. 1995. Cytotoxicity of brefeldin A correlates with its inhibitory effect on membrane binding of COP coat proteins. *J Biol Chem* 270: 11574-11580.

63. Larsson, D. E., M. Wickstrom, S. Hassan, K. Oberg, and D. Granberg. 2010. The cytotoxic agents NSC-95397, brefeldin A, bortezomib and sanguinarine induce apoptosis in neuroendocrine tumors in vitro. *Anticancer Res* 30: 149-156.

64. Whitmire, J. K., B. Eam, and J. L. Whitton. 2008. Tentative T cells: memory cells are quick to respond, but slow to divide. *PLoS Pathog* 4: e1000041.

65. Wakim, L. M., and M. J. Bevan. 2010. From the thymus to longevity in the periphery. *Curr. Opin. Immunol.* 22: 274-278.

66. Wirth, J. J., A. Amalfitano, R. Gross, M. B. Oldstone, and M. M. Fluck. 1992. Organ- and age-specific replication of polyomavirus in mice. *J. Virol.* 66: 3278-3286.

67. Richter, K., P. Agnellini, and A. Oxenius. 2010. On the role of the inhibitory receptor LAG-3 in acute and chronic LCMV infection. *Int Immunol* 22: 13-23.

68. Moser, J. M., J. Gibbs, P. E. Jensen, and A. E. Lukacher. 2002. CD94-NKG2A receptors regulate antiviral CD8(+) T cell responses. *Nat. Immunol.* 3: 189-195.

69. Zhou, J., M. Matsuoka, H. Cantor, R. Homer, and R. I. Enelow. 2008. Cutting edge: engagement of NKG2A on CD8+ effector T cells limits immunopathology in influenza pneumonia. *J. Immunol.* 180: 25-29.

70. Cush, S. S., and E. Flano. 2011. KLRG1+NKG2A+ CD8 T cells mediate protection and participate in memory responses during gamma-herpesvirus infection. *J. Immunol.* 186: 4051-4058.

71. Wilson, J. J., E. Lin, C. D. Pack, E. L. Frost, A. Hadley, A. I. Swimm, J. Wang, Y. Dong, C. P. Breeden, D. Kalman, K. A. Newell, and A. E. Lukacher. 2011.

Gamma interferon controls mouse polyomavirus infection in vivo. *J. Virol.* 85: 10126-10134.

72. Byers, A. M., A. Hadley, and A. E. Lukacher. 2007. Protection against polyoma virus-induced tumors is perforin-independent. *Virology* 358: 485-492.

73. Sharrow, S. O., J. S. Arn, I. Stroynowski, L. Hood, and D. H. Sachs. 1989. Epitope clusters of Qa-2 antigens defined by a panel of new monoclonal antibodies. *J. Immunol.* 142: 3495-3502.

74. Jackson. 2008. Appendix 2: MHC *H2* Haplotypes.In *Appendix 2: MHC H2 Haplotypes*. The Jackston Laboratory.

75. Fischer, M. A., D. C. Tscharke, K. B. Donohue, M. E. Truckenmiller, and C. C. Norbury. 2007. Reduction of vector gene expression increases foreign antigen-specific CD8+ T-cell priming. *J Gen Virol* 88: 2378-2386.

76. Anderson, M. S., and J. A. Bluestone. 2005. The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* 23: 447-485.

77. Hikono, H., J. E. Kohlmeier, S. Takamura, S. T. Wittmer, A. D. Roberts, and D. L. Woodland. 2007. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. *J. Exp. Med.* 204: 1625-1636.

78. Cox, J. C., and A. R. Coulter. 1997. Adjuvants--a classification and review of their modes of action. *Vaccine* 15: 248-256.

79. Schijns, V. E., and E. C. Lavelle. 2011. Trends in vaccine adjuvants. *Expert review of vaccines* 10: 539-550.

80. Drake, D. R., 3rd, J. M. Moser, A. Hadley, J. D. Altman, C. Maliszewski, E. Butz, and A. E. Lukacher. 2000. Polyomavirus-infected dendritic cells induce antiviral CD8(+) T lymphocytes. *J. Virol.* 74: 4093-4101.

81. Melchionda, F., T. J. Fry, M. J. Milliron, M. A. McKirdy, Y. Tagaya, and C. L. Mackall. 2005. Adjuvant IL-7 or IL-15 overcomes immunodominance and improves survival of the CD8+ memory cell pool. *J Clin Invest* 115: 1177-1187.

82. Lund, T., E. Simpson, and A. Cooke. 1990. Restriction fragment length polymorphisms in the major histocompatibility complex of the non-obese diabetic mouse. *Journal of autoimmunity* 3: 289-298.

83. Rucker, J., M. Horowitz, E. A. Lerner, and D. B. Murphy. 1983. Monoclonal antibody reveals H-2-linked quantitative and qualitative variation in the expression of a Qa-2 region determinant. *Immunogenetics* 17: 303-316.

84. Kumanovics, A., A. Madan, S. Qin, L. Rowen, L. Hood, and K. Fischer Lindahl. 2002. QUOD ERAT FACIENDUM: sequence analysis of the H2-D and H2-Q regions of 129/SvJ mice. *Immunogenetics* 54: 479-489.

85. Tine, J. A., A. Walsh, D. Rathbun, L. Leonard, E. K. Wakeland, R. Dilwith, and L. Flaherty. 1990. Genetic polymorphisms of Q region genes from wild-derived mice: implications for Q region evolution. *Immunogenetics* 31: 315-325.

86. Chiang, E. Y., and I. Stroynowski. 2006. The role of structurally conserved class I MHC in tumor rejection: contribution of the Q8 locus. *J. Immunol.* 177: 2123-2130.

87. Tabaczewski, P., E. Chiang, M. Henson, and I. Stroynowski. 1997. Alternative peptide binding motifs of Qa-2 class Ib molecules define rules for binding of self and nonself peptides. *J. Immunol.* 159: 2771-2781.

88. Soloski, M. J., L. Hood, and I. Stroynowski. 1988. Qa-region class I gene expression: identification of a second class I gene, Q9, encoding a Qa-2 polypeptide. *Proc. Natl. Acad. Sci. USA* 85: 3100-3104.

89. Stroynowski, I., and P. Tabaczewski. 1996. Multiple products of class Ib Qa-2 genes which ones are functional? *Res. Immunol.* 147: 290-301.

90. Kumanovics, A. 2007. Genomic Organization of the Mouse Major Histocompatibility Complex. In *The Mouse in Biomedical Research*, 2nd ed. J. G. Fox, M. T. Davisson, F. W. Quimby, S. W. Barthold, C. E. Newcomer, and A. L. Smith, eds. Academic Press; Elsevier, Amsterdam; Boston; Heidelberg; London; New York; Oxford; Paris; San Diego; San Francisco; Singapore; Sydney; Tokyo. 119-134.

91. Petrie, E. J., C. S. Clements, J. Lin, L. C. Sullivan, D. Johnson, T. Huyton, A. Heroux, H. L. Hoare, T. Beddoe, H. H. Reid, M. C. Wilce, A. G. Brooks, and J. Rossjohn. 2008. CD94-NKG2A recognition of human leukocyte antigen (HLA)-E bound to an HLA class I leader sequence. *J. Exp. Med.* 205: 725-735.

92. Pietra, G., C. Romagnani, P. Mazzarino, M. Falco, E. Millo, A. Moretta, L. Moretta, and M. C. Mingari. 2003. HLA-E-restricted recognition of cytomegalovirus-derived peptides by human CD8+ cytolytic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 100: 10896-10901.

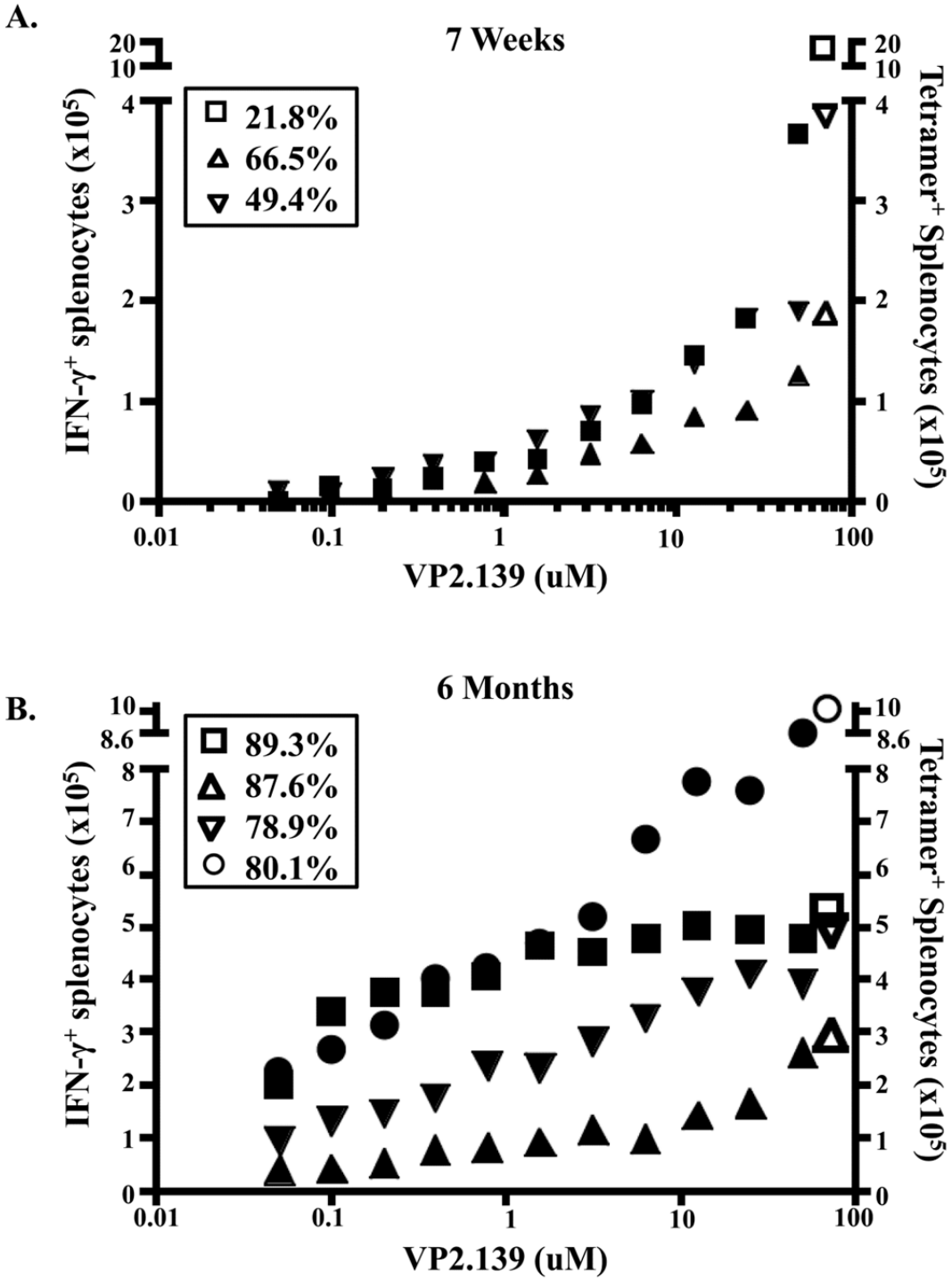
93. Heinzel, A. S., J. E. Grotzke, R. A. Lines, D. A. Lewinsohn, A. L. McNabb, D. N. Streblow, V. M. Braud, H. J. Grieser, J. T. Belisle, and D. M. Lewinsohn. 2002. HLA-E-dependent presentation of Mtb-derived antigen to human CD8+ T cells. *J. Exp. Med.* 196: 1473-1481.

94. Salerno-Goncalves, R., M. Fernandez-Vina, D. M. Lewinsohn, and M. B. Sztein. 2004. Identification of a human HLA-E-restricted CD8+ T cell subset in volunteers immunized with Salmonella enterica serovar Typhi strain Ty21a typhoid vaccine. *J. Immunol.* 173: 5852-5862.

## Figure Legends

**Figure 4.1: IFN-γ production by Q9:VP2.139-specific CD8 T cells increases with peptide dose and with time p.i.** Splenocytes from B6.$K^{b-/-}D^{b-/-}$ mice infected with MPyV for (**A**) 7 weeks or (**B**) 6 months were stained with Q9($K^b$):VP2.139 tetramer at 1:100 (right axis, open symbols) or stimulated with a gradient of VP2.139 peptide for 5 hours in the presence of Brefeldin A. Stimulated cells were stained for CD8α, then treated with BD Cytofix/Cytoperm and stained for intracellular IFN-γ. Cells incubated with no peptide were used to control for background IFN-γ signal. Closed symbols represent the number of splenocytes producing IFN-γ at each concentration of VP2.139 peptide. Each symbol represents one mouse. In the figure legend, percentages indicate the relative frequency of $tetramer^+$ cells that produce IFN-γ at 50 μM VP2.139.

**Figure 4.1**


A.
7 Weeks
21.8%
66.5%
49.4%
IFN-γ+ splenocytes (x10^5)
Tetramer+ Splenocytes (x10^5)
VP2.139 (uM)
B.
6 Months
89.3%
87.6%
78.9%
80.1%
IFN-γ+ splenocytes (x10^5)
Tetramer+ Splenocytes (x10^5)
VP2.139 (uM)