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**Characterization of the CD4$^+$ T Cell Response to Mouse Polyomavirus Infection**

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# Characterization of the $CD4^+$ T Cell Response to Mouse Polyomavirus Infection

By

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B.A., Boston University, 2000

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

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## Abstract

### Characterization of the CD4$^+$ T Cell Response to Mouse Polyomavirus Infection

**By Eugene Lin**

Virus-specific CD4$^+$ T cells optimize anti-viral responses by providing help for anti-viral humoral responses and CD8$^+$ T cells. However, little is known about the impact of low-level persistent infections on the fate and function of virus-specific CD4$^+$ T cells. Using a mouse polyomavirus (MPyV) infection model, we identified two dominant CD4$^+$ T cell populations specific for epitopes derived from the nonstructural LT antigen and VP1, the major viral capsid protein. These MPyV-specific CD4$^+$ T cells vary in terms of their magnitude, functional profile, and phenotype during acute and persistent phases of infection. Employing a minimally myeloablative mixed bone marrow chimerism approach, we further show that naïve virus-specific CD4$^+$ T cells are primed de novo during persistent virus infection.

After clearance of an acute viral infection, memory T cells develop that survive in the absence of antigen and can mount robust recall responses against challenge infection. During chronic high load infections, however, virus-specific CD8$^+$ T cells become dependent on antigen for their survival. Whether T cells formed during low-level persistent infections require antigen for their survival is less clear. In this study, we developed a novel approach to ablate MPyV replication during persistent infection. We generated a recombinant MPyV in which genes critical for replication are floxed (A2.Flx). Cre-mediated deletion of A2.Flx leads to reductions in viral levels both in vitro and in mice expressing Cre constitutively. Utilizing mice expressing a tamoxifen-inducible Cre, we found that reduction of viral replication during persistent A2.Flx infection had a variable impact on virus-specific CD4$^+$ and CD8$^+$ T cells. While some MPyV-specific T cell populations were notably reduced, others were maintained but adopted a more central memory T cell phenotype. Our data suggests that antigen is not necessarily required for survival of MPyV-specific memory T cells and those cells can be redirected toward a phenotype associated with antigen-independent survival following reductions in viral antigen.

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## Chapter 1: Introduction

### I. Anti-viral immunity and $CD4^+$ T cells

Viruses are one of the most prevalent threats faced by mammals. Consisting of either a DNA or RNA genome enclosed within a protein shell that may than be further surrounded by an additional outer lipid envelope, viruses are unable to replicate themselves but must exploit a cell's own DNA/RNA synthesis and protein production machinery to do so. The disruption of normal cell homeostasis and potential tissue destruction can lead to severe and debilitating disease. Thus, it is critical that mammalian hosts are able to defend themselves against this threat. Following initial detection of a viral invasion, the host immune response mounts a sophisticated multi-pronged assault with the objectives of limiting the extent of infection and then launching a counterattack to clear it. As with any combined arms assault, improper coordination and regulation of the immune response can have deleterious consequences for the host including immunopathology and failure to eliminate the targeted pathogen. $CD4^+$ T cells play a key role in this command and control, closely interacting with cells of both innate and adaptive arms of immunity.

The innate immune system comprises the front line defense against infection. Dendritic cells (DCs) and macrophages express pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I) like receptors (RLRs), which recognize invariant pathogen associated molecular patterns (PAMPs) and provide early warning of infection (1). Engagement of PRRs induces release of proinflammatory cytokines (e.g., IL-1β) and type I interferons (IFN-α/β), which can induce anti-viral activity in cells at the site of infection and activate incoming natural killer (NK) cells (2, 3). At the same time, PRRs also induce maturation of DCs into antigen presenting cells (APCs) (4). DCs take up viral proteins exogenously by phagocytosis or receptor-mediated endocytosis and endogenously from the cytoplasm if infected themselves, process the proteins into peptides, and load peptides onto either Major Histocompatibility Complex (MHC) class I or MHC class II molecules. In addition, mature

DCs upregulate several surface costimulatory molecules and produce cytokines. They then migrate to secondary lymphoid organs such as the spleen and lymph nodes, where they encounter T cells. DCs are critical for initiation of the adaptive immune response.

The adaptive immune response arises a few days after the initial innate response. B cells and αβ T cells mount responses that are highly specific for a wide variety of antigens. In addition, a major hallmark of adaptive immunity is the generation of memory B and T cells that are able to rapidly respond to reinfection. Both B and αβ T cells reside in spleens and lymph nodes as inactive naïve precursors. Following recognition of their cognate antigens, both cell types undergo massive clonal expansion and differentiate into effector cells. B cells are activated after binding specific antigen, either soluble or presented by DCs, macrophages, and follicular dendritic cells (5), through their surface B cell receptor (BCR) and produce antibodies that can neutralize viruses and block infection of new cells. Activation of T cells, however, requires not only recognition of cognate peptide: MHC (pMHC) complexes on DCs by the T cell receptor (TCR) (pMHC I for $CD8^+$ T cells and pMHC II for $CD4^+$), but also a second signal provided through engagement of costimulatory molecules present on DCs (e.g., CD80, CD86) with receptors on T cells (e.g., CD28) (6). In addition, DC-produced cytokines like IL-12, IFN-α/β, and IL-1 provide a "signal 3", which optimizes differentiation and survival (7). $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTLs) that traffic to the periphery and secrete the cytotoxic contents of their lytic granules (e.g., perforin and granzyme) to kill infected cells expressing the cognate pMHC I complex or release cytokines like IFN-γ and TNF-α, which may act noncytolytically to control viral infection (8). $CD4^+$ T cells can also develop into direct effectors, killing or secreting anti-viral effector cytokines directed against infected MHC class $II^+$ cells (9-12), but they play by far their most vital role in shaping and providing help to optimize the ongoing immune response.

The importance of $CD4^+$ T cells for immune protection is vividly illustrated in individuals with acquired immunodeficiency syndrome (AIDS), who are severely deficient in $CD4^+$ T cells and highly susceptible to opportunistic infections. $CD4^+$ T cell "help" appears to be especially critical for sustaining function of anti-viral $CD8^+$ T cells during chronic infections. For instance, $CD8^+$ T cell function progressively deteriorates amidst declining levels of $CD4^+$ T cells in HIV/SIV infections progressing to AIDS (13). In mouse models of chronic lymphocytic choriomeningitis virus (LCMV), $CD4^+$ T cells are required for sustaining optimal anti-viral $CD8^+$ T cell responses and eventual control of infection (14-16). $CD4^+$ T cells are also critical for development and/or maintenance of memory $CD8^+$ T cells arising after acutely resolved infections, endowing memory $CD8^+$ T cells with the ability to mount robust recall responses (17). Furthermore, $CD4^+$ T cell "help" for activated germinal center B cells (GC B cells) is required for them to differentiate into memory B cells and plasma cells that can produce affinity maturated, class-switched antibodies (18). $CD4^+$ T cell help may also be provided at peripheral sites of infection: in a mouse model of HSV infection, $CD4^+$ T cells induce secretion of chemokines CXCL9 and CXCL10 required to recruit anti-viral $CD8^+$ T cells to the local site of infection (vaginal mucosa) (19); influenza-specific memory $CD4^+$ T cells induced production of innate inflammatory cytokines/chemokines in the lung of infected mice that aid early viral control (20).

*What is $CD4^+$ T cell help?*

Help from $CD4^+$ T cells is delivered through a multitude of potential surface molecules and secreted cytokines. Engagement of the costimulatory molecule CD154 (CD40L) on activated $CD4^+$ T cells with its ligand CD40 plays a critical role in "licensing" (i.e. activating) DCs to prime naïve $CD8^+$ T cells (21-23) and is required for formation as well as maintenance of germinal centers, where B cell proliferation and differentiation occur (24). The cytokine IL-2, which drives proliferation of T cells, is produced in abundance by activated $CD4^+$ T cells and is critical for programming functional memory $CD8^+$ T cells (25); a recent study, however, suggests

that primed $CD8^+$ T cells are the source of their own IL-2 and that $CD4^+$ T cell help is primarily provided via CD40L (26). $CD4^+$ T cells are also the predominant producers of IL-21, which appears to be critical for maintaining the function of virus-specific $CD8^+$ T cells and viral control during chronic LCMV infection (27-29). IL-21 also plays major roles in driving optimal GC B cell proliferation and plasma cell differentiation (30-32). Furthermore, the $CD4^+$ T cell-produced cytokines impact the antibody isotype produced, which has functional consequences (33): in the mouse, IL-4 induces IgG1 and IgE, IFN-γ induces IgG2a, and IL-21 induces IgG1.

This is only a small survey of the possible mechanisms of $CD4^+$ T cell help. One of the challenges for future investigations is to determine which mechanisms are critical and operative in a given scenario. This is often complicated by the fact that $CD4^+$ T cells may not be the only expressers of some of these molecules; as mentioned above, $CD8^+$ T cells can produce their own IL-2, and CD40L can be expressed by DCs, monocytes, NK cells, and B cells as well (24). An additional complication is that $CD4^+$ T cells are phenotypically and functionally diverse, an aspect with implications for the overall immune response.

*$CD4^+$ T cells come in a variety of flavors…*

Effector $CD4^+$ T cells are highly heterogeneous and differentiate into lineages or subsets of helper T ($T_H$) cells distinguished by their effector cytokine profiles, surface molecules, and functions (34). $T_H1$ cells producing IFN-γ, TNF-α, and IL-2 are critical for control of intracellular infections such as those caused by viruses. $T_H2$ cells, which produce IL-4, IL-5, and IL-13, control extracellular parasites such as helminthes, while IL-17 producing $T_H17$ cells are required for defense against fungal infections. In addition, effector $CD4^+$ T cells can differentiate into immunosuppressive inducible regulatory T cells (iTregs), which help control ongoing responses and prevent immunopathology. Recently, follicular helper T cells ($T_{FH}$) have emerged as the subset that provides help for B cells to generate long-lived antibody responses; these cells

predominantly produce IL-21 and IL-4 (some also IFN-γ and IL-17) and express surface molecules like CXCR5, PD-1, and SAP that are critical for their function and localization (33). The predominant subset induced can potentially impact the effectiveness of the anti-microbial immune response. For example, a hurdle to controlling respiratory syncytial virus (RSV) infection, especially after initial immunization with the formalin-inactivated RSV vaccine, is the tendency to generate a $T_H2$ response, which is marked by severe lung immunopathology associated with eosinophilia and fails to clear virus (35). Likewise, generation of iTregs could limit immune collateral damage (36), but it may also lead to suppression of a desired response. Thus, the regulation of $T_H$ subset differentiation has been an area of intense interest ever since the initial descriptions of $T_H1$ and $T_H2$ in 1986 by Mosmann and Coffman (37).

$T_H$ fate is determined by conditions encountered during priming, leading to the expression of "master regulator" transcription factors that, in conjunction with a network of accessory factors, impart distinct differentiation programs (34). For example, expression of T-bet commits a $CD4^+$ T cell to a $T_H1$ phenotype but inhibits $T_H2$ differentiation, which requires GATA-3. Bcl-6, a transcriptional repressor, was shown to be the master regulator of $T_{FH}$ cells and counteracts Blimp-1, which is expressed by other $T_H$ subsets ($T_H1$, $T_H2$, $T_H17$) (33). The cytokine milieu is a predominant factor in differentiation of $T_H$ cells (34). IL-12 and IFN-γ drive $T_H1$ differentiation in contrast to IL-4 and IL-2, which polarize cells toward a $T_H2$ phenotype. TGF-β in the presence of IL-2 induces iTregs but yields $T_H17$ cells instead when combined with the proinflammatory cytokine IL-6. TCR signaling strength, which is co-determined by affinity for antigen and antigen dose, also has an impact even without cytokines: weak TCR signals induce $T_H2$ cells while strong signals lead to $T_H1$ (38, 39) and $T_{FH}$ differentiation (40). Costimulation is yet another regulator of $T_H$ fate as shown by the acquisition of a $T_{FH}$ phenotype ($CXCR5^{hi}PD\text{-}1^{hi}Bcl\text{-}6^{hi}Blimp\text{-}1^{lo}$) early after activation that required interaction with ICOSL on DCs (41).

Mounting evidence suggests that there is plasticity in $T_H$ fate commitment (42). $T_{FH}$ cells, for instance, can produce modest levels of IL-4, IFN-γ, and IL-17, which impact B cell class switching, typical of $T_H1$, $T_H2$, or $T_H17$ cells while expressing a low level of T-bet/GATA-3/RORγt. This plasticity has led some to question defining $T_{FH}$ as a separate lineage (33). LCMV-specific $T_H2$ cells generated in vitro can be reprogrammed toward a mixed $T_H2+1$ profile (GATA-$3^+$T-$bet^+$/ IFN-$\gamma^+$IL-$4^+$) when challenged in vivo with virus, a strong $T_H1$-polarizing regimen (43). $T_H1$ memory cells expressing IL-4 following rechallenge under $T_H2$ conditions has also been observed (44). The current debate about $T_H$ stability versus plasticity depends on elucidation of the epigenetic mechanisms that dictate $CD4^+$ T cell differentiation (42). Understanding $CD4^+$ T cell differentiation will allow rationale design of vaccines and therapeutics to shape and manipulate ongoing immune responses for optimal effectiveness.

*Why our understanding of virus-specific $CD4^+$ T cell responses lags behind that of $CD8^+$ T cells*

Efforts to understand virus-specific $CD4^+$ T cell responses have been hampered by technical hurdles, some of which may stem from intrinsic differences between $CD4^+$ and $CD8^+$ T cells (45). $CD8^+$ T cells only require a short period of stimulation before proliferating and embarking on an autonomous differentiation program. $CD4^+$ T cells require a more prolonged period of stimulation (46) but also appear to proliferate far less (47), which may contribute to the much smaller magnitude of response (10-100-fold < $CD8^+$ T cells) often observed. It is perhaps not surprising then that a fairly limited range of $CD4^+$ T cell-recognized epitopes have been identified. pMHC I tetramers have been useful tools in tracking antigen-specific $CD8^+$ T cells, but pMHC II tetramers have proven to be more difficult to construct and use (48). Recent advances in MHC class II tetramer construction have allowed for the tracking of more antigen-specific $CD4^+$ T cell populations immediately ex vivo (49).

In Chapter 2, I describe the identification of two MHC class II-restricted epitopes in mouse polyomavirus, a model of low-level persistent infection, recognized by $CD4^+$ T cells in C57BL/6 mice. Utilizing MHC class II tetramers complexed to these peptides and intracellular cytokine assays, we were able to follow the evolution of the $CD4^+$ T cell response both phenotypically and functionally. Most importantly, we were able to begin to investigate the mechanisms that regulate and maintain virus-specific $CD4^+$ T cell populations in the face of persistent antigen.

**II. <u>Persistent infection impacts T cell memory and function</u>**

Following activation by viral antigen, $CD4^+$ and $CD8^+$ T cells undergo expansion and differentiation into effectors that control infection through multiple mechanisms. Recently, the quality of the T cell response, a reference to the breadth of effector functions displayed, has been found to correlate with effective control of infection (50). Development of polyfunctional $CD8^+$ and $CD4^+$ T cells is seen in acutely resolving infections in which virus is completely cleared, such as in vaccinia (smallpox vaccine) (51) and yellow fever virus vaccinated humans (52) and in mice infected with influenza (53) and acutely infecting strains of LCMV (14, 54). After clearance, most effector T cells die and the survivors develop into long-lived memory T cells that can respond rapidly to antigen reencounter (55, 56). However, some viruses have developed mechanisms of immune evasion (57) or actively mitigate anti-viral host immune responses. Failure by the immune system to clear pockets of viral resistance leads to stalemate and persistence of the virus at some level. The situation of continual virus antigen exposure inherent in persistent infection has profound consequences for the fate of responding T cells and their development into long-term protective memory cells (58).

Although polyfunctional T cells initially emerge during persistent infections, they can progressively lose functionality as antigen load and duration of antigen exposure increase. This process is termed exhaustion (58). Exhaustion has been demonstrated in virus-specific $CD8^+$ T cells during high load chronic LCMV infection of mice (e.g. DOCILE (59) and strain clone 13),

where effector functions are lost in a hierarchical manner starting with IL-2 production, proliferation, and cytolytic function, then TNF-α production, and finally loss of IFN-γ prior to possible physical deletion (16, 54). Functional impairment is also seen for human anti-viral $CD8^+$ T cells in chronic HCV and HIV (58). $CD4^+$ T cells too appear to suffer exhaustion during the course of chronic high load infections. In HIV progressors, virus-specific $CD4^+$ T cells continue to produce IFN-γ but not IL-2 and do not proliferate (60). Early in infection of mice with the clone 13 strain of LCMV (day 9 post-infection), virus-specific $CD4^+$ T cells produce diminished IL-2 and TNF-α compared to their counterparts at the same time point in acute Armstrong strain infected mice (61). The diminished amount of $CD4^+$ T cell help may exacerbate $CD8^+$ T cell exhaustion (58). However, $CD4^+$ T cells appear to be more plastic and heterogeneous than $CD8^+$ T cells, and they may respond differently to chronic antigen. Recent reports indicate that virus-specific $CD4^+$ T cells during chronic LCMV switch to production of IL-21, which is critical for sustaining $CD8^+$ T cells and B cells (27-29), and increasingly acquire a $CXCR5^+ICOS^{hi}Bcl\text{-}6^{hi}$ $T_{FH}$ phenotype (62).

The loss of function by exhausted T cells is accompanied by the sustained expression of inhibitory receptors such as PD-1 (63-66), CTLA-4 (67), LAG-3 (68), and Tim-3 (69); the level of individual receptors and co-expression of multiple receptors correlates with more severe impairment and higher antigen loads (68). Blockade of these inhibitory receptors, either alone or in combination with PD-1 blockade, leads to a restoration of virus-specific $CD8^+$ and $CD4^+$ T cell functions in chronic LCMV (63, 68) and HIV (64, 65, 67) infections, demonstrating that exhaustion is a reversible state. In contrast to high load infections, during low-level persistent viral infections such as with CMV (mouse and human) (9, 70, 71), polyomaviruses (vide infra) (72), and HIV long-term nonprogressors (LTNP) (73), $CD4^+$ and $CD8^+$ T cells maintain polyfunctionality and may exhibit few obvious signs of dysfunction/exhaustion (74, 75).

In addition to negatively impacting T cell function, persistent viral infections also alter the development and maintenance of T cell memory (58). Memory $CD4^+$ and $CD8^+$ T cells that develop after resolution of acute infections ("acute memory T cells") are largely stably maintained long-term and appear not to need antigen to survive (55, 56); instead, survival and self-renewal is driven by the homeostatic cytokines IL-7 and IL-15. In contrast, during chronic viral infection, $CD8^+$ T cells that survive into persistence ("chronic memory T cells") become dependent on antigen (i.e. "antigen addicted") for their maintenance (58). Virus-specific memory $CD8^+$ T cells from mice persistently infected with LCMV clone 13 undergo extensive antigen-driven proliferation when transferred into infection-matched mice but not mice chronically infected with a variant lacking their cognate T cell epitope or when transferred into naïve mice (76, 77); these cells exhibit defects in homeostatic proliferation to IL-7 and IL-15 as a result of lower CD127 and CD122 expression (77).

The continued presence of viral antigen could also drive maintenance of memory T cell pools in another way. Mouse polyomavirus (MPyV)-specific $CD8^+$ T cells are defective in self-renewal even in the presence of antigen, yet population levels are stably maintained (78). By generating partial bone marrow chimerism in persistently infected mice, Vezys et al. and Kemball et al. demonstrated priming of donor-derived MPyV-specific $CD8^+$ T cells (72, 78). Thus, persistent antigen-driven priming of naïve T cells may replenish a continuously deteriorating pool of memory T cells. But how much does de novo recruitment of T cells contribute to "chronic memory"? Thymectomy experiments showed a modest reduction of MPyV-specific $CD8^+$ T cells (78) but only a transient impact on chronic LCMV-specific $CD8^+$ T cell levels (79), indicating de novo priming is not required for maintenance. In addition, using an elegant reporter mouse model where granzyme B-expressing effector $CD8^+$ T cells are indelibly marked by YFP, Bannard et al. showed that virus-specific $CD8^+$ T cells primed during initial infection persisted and continue to proliferate during the latent phase of infection by a mouse γ-herpesvirus (80). More than likely,

maintenance of virus-specific $CD8^+$ T cells during persistent infections involves a combination of de novo recruitment of naïve T cells and some antigen-drive self-renewal of existing memory T cells, as suggested by a recent study from Ann Hill's group analyzing epitope-specific memory $CD8^+$ T cell inflation in mouse CMV (81).

Memory T cells are heterogeneous in terms of their survival, recall proliferation, functions exhibited, and tissue localization, where local cues may differentially impact memory T cells (82). Based on expression of lymph node homing receptors CD62L and CCR7, memory T cells were originally divided into $CD62L^{lo}CCR7^{lo}$ effector memory ($T_{EM}$) cells residing primarily in nonlymphoid tissues and spleen or $CD62L^{hi}CCR7^{hi}$ central memory ($T_{CM}$) cells found mostly in spleen and lymph nodes (83). Although the initial study suggested that $T_{EM}$ expressed immediate effector function, subsequent comparisons of both $CD4^+$ and $CD8^+$ $T_{EM}$ and $T_{CM}$ demonstrated an equal capacity to display effector functions (IFN-γ, TNF-α, cytolysis) after challenge (84, 85); instead, the key differences were in the degree of proliferation and IL-2 production by $T_{CM}$ (83, 85). Studies comparing the protective capacity of virus-specific $CD8^+$ $T_{EM}$ and $T_{CM}$ suggested that the larger proliferative burst post-challenge of $T_{CM}$ ultimately makes them more protective on a per-cell basis (85). $T_{CM}$ cells also have the advantage of being longer lived than $T_{EM}$ cells as a result of increased homeostatic turnover in response to IL-7 and IL-15 (55). Indeed, in the case of acutely resolving infections, there appears to be a slow shift toward a largely $T_{CM}$ phenotype perhaps involving conversion of some $T_{EM}$ cells into $T_{CM}$ (55). On the other hand, repetitive antigen stimulation, by either repeated infection or repeated DC immunization, progressively drives memory $CD4^+$ and $CD8^+$ T cells toward a more $T_{EM}$ phenotype (86, 87). Not surprisingly then, far more $T_{EM}$ than $T_{CM}$ cells have been observed in chronic viral infections (55), consistent with the poor response to IL-7 and IL-15 displayed by "chronic memory" T cells.

The environment of persistent infection is dynamic. Changes in epitope availability and inflammation shape the functional and phenotypic diversity of antigen-specific T cells. In several models of acutely cleared antigen, latecomer $CD4^+$ and $CD8^+$ T cells primed to low levels of residual cognate antigen (and presumably inflammation) underwent intermediate differentiation and developed into $CD62L^{hi}$ $T_{CM}$-like memory T cells (88-90). Consistent with this, higher frequencies of virus-specific $CD8^+$ T cells recruited de novo during low-level persistent mouse polyomavirus infection are $CD27^{hi}CD62L^{hi}$, characteristics associated with $T_{CM}$, the later they are primed; a gradual increase in the proportion of $CD27^{hi}CD62L^{hi}$ cells within the bulk virus-specific $CD8^+$ T cell population over time was also observed (78). It remains to be seen whether these late generated antigen-specific $CD8^+$ T cells truly behave like "acute memory" T cells.

We are beginning to better understand the behavior and fate of T cells during persistent viral infections. Much of the work has involved models of chronic high load viral infections, exemplified by chronic LCMV, and has provided insights into T cell exhaustion and its molecular regulation. These findings have profound implications for development of effective therapies to combat rapidly disseminating and replicating chronic viral infections in humans like HIV, HCV, and HBV, which cause severe disease and are associated with high morbidity. However, the majority of persisting viruses are clinically silent, primarily causing disease in immunocompromised individuals (57). The continuing scourge of AIDS worldwide along with the increasing effectiveness of immunosuppressive drugs given to transplant/transfusion recipients and sufferers of autoimmune diseases such as multiple sclerosis will assure that we may see ever-increasing incidents of active disease from these pathogens. Thus, it is imperative that we understand how T cells control low-level persistent viral infections under normal circumstances of an intact immune system. The critical role played by $CD8^+$ T cells in eliminating viruses, their much larger numbers, and the maturity of the tools to study antigen-specific $CD8^+$ T cells ex vivo have made them the focus of many investigations. As their help is

critical for optimal immune responses, it will be important to understand how virus-specific $CD4^+$ T cells are impacted by and cope with low-level persistent viral infections. Improvements in development of reagents to study antigen-specific $CD4^+$ T cells should make this ever more feasible. While it appears that virus-specific $CD4^+$ T cells responding to low-level persistent infections are not functionally exhausted, a critical question is how persistent antigen impacts $CD4^+$ T cell differentiation. It also remains to be determined if "chronic memory" $CD4^+$ T cell populations are maintained by the same mechanisms $CD8^+$ T cells are, namely antigen-driven self-renewal and/or de novo priming. Above all, although T cells with a $T_{CM}$ phenotype can develop and survive despite continual antigen in low-level persistent viral infections, there remains the issue of whether or not these cells are truly like bona-fide memory T cells we see after resolution of acute infections; that is, will they survive if their antigen were removed?

## III. Polyomavirus pathogenesis

*Polyomaviridae* are a family of ubiquitous DNA viruses found in a wide variety of animal species. The founding member of the family was discovered in 1953 when Polish-American physician-scientist Ludwik Gross (1904-1999) discovered that a filterable agent in cell free extracts from leukemic mice induced a constellation of solid tissue tumors in infected neonatal mice (91). Dubbed polyomavirus, mouse polyomavirus (MPyV) was one of the earliest demonstrated links between viruses and cancer. A few years later, a second polyomavirus was discovered in rhesus monkey kidney cells, simian virus 40 (SV40) (92). In 1971, two human polyomaviruses (HuPyV), BKV and JCV, were isolated. BKV was recovered from the urine of a kidney transplant recipient suffering from renal failure, and JCV was isolated from the brain of a patient with progressive multifocal leukoencephalopathy (93, 94). More recently, modern molecular biology screening technology has led to the identification of three new HuPyVs (95-97), including one associated

with Merkel cell carcinomas (MCV), rare but highly aggressive skin cancers. Initial HuPyV infection appears to occur during childhood/early adolescence and persists lifelong. 72-98% of the adult human population is seropositive for JCV and BKV (98); a recent study suggests that seropositivity for the new human polyomaviruses (WUPyV, KIPyV, and MCPyV) in adults is as high as about 50% (99). Although polyomavirus infections are typically subclinical and asymptomatic, they are associated with diseases in immunocompromised individuals. "Reactivation" of BKV in kidney transplant recipients leads to hemorrhagic cystitis and polyomavirus-associated nephropathy (PVAN) then graft rejection (100). Neurotropic JCV is the etiologic agent of progressive multifocal leukoencephalopathy, a typically fatal demyelinating disease with symptoms similar to multiple sclerosis (MS), seen in about 5% of AIDS patients as well as in some individuals taking Natalizumab (anti-VLA-4) or Efalizumab (anti-LFA-1), which inhibit T cell priming and/or trafficking into peripheral tissues, to treat autoimmune diseases such as multiple sclerosis and Crohn's disease (101). Unfortunately, little is known regarding the mechanisms of pathogenesis and immune control of polyomaviruses. Given the difficulties associated with studying infections in humans and the stringent species specificity of polyomaviruses (98), models utilizing the originally discovered MPyV offers a system to study pathogenesis by this family of viruses in a natural host (102).

MPyV is a nonenveloped virus comprised of a 45 nm icosahedral capsid composed of 3 viral capsid proteins (VP1-3) with VP1 forming the outer capsid; binding of VP1 to cell surface sialic acid enables the virus to potentially infect a variety of host cell types. The virus' ~5 kb double-stranded DNA plasmid genome encodes 6 genes in two transcripts that are alternatively spliced to generate three mature mRNAs each: the nonstructural small, middle, and large T-antigens, which are expressed in all infected cells, and the 3 capsid proteins expressed only in productively infected cells. Due to its relative simplicity, the MPyV genome is highly amenable to genetic manipulations such as mutagenesis and insertion of new sequences, which we have

taken advantage of to generate recombinant MPyVs encoding epitope tags or model T cell epitopes recognized by TCR transgenic cells (103, 104). Although cytopathic, MPyV establishes a lifelong, silent persistent infection in immunocompetent laboratory mice and is ubiquitous in feral mice (105).

No evidence exists that MPyV is capable of entering a state of latency like herpesviruses do, although some in the literature use the term "reactivating" to denote apparent bursts in viral replication. Instead, MPyV leads to a "smoldering" persistent infection, where viral replication is continuous but below the limit of detection for most viral titer assays. Infectious virus can be readily detected in the urine, saliva, and feces of carrier mice. In addition, the MPyV genome, being an example of packing maximal information into minimal space, does not encode immune evasion genes. Therefore, MPyV is an ideal model for studying the impact of low load persistent viral antigen on generation of an effective immune response.

## IV. Adaptive Immunity to mouse polyomavirus

MPyV can cause diverse tumors in some inbred strains of mice when inoculated as newborns as well as immunocompromised mice. $\beta 2m^{-/-}$ mice, which lack functioning $CD8^+$ T cells, but not MHC class $II^{-/-}$ mice, which lack $CD4^+$ T cells, infected with MPyV are highly susceptible to virus-induced tumors (106, 107). Furthermore, $H\text{-}2^k$ mice expressing an endogenous mouse mammary tumor virus (MMTV) superantigen are devoid of T cells expressing the Vβ6 and Vβ8.1 TCRs including most of the MPyV-specific $CD8^+$ T cells, and thus they too are impaired in protection against virus-induced tumors (108). Infection also induces early T-independent as well as later T-dependent production of neutralizing IgG directed against the VP1 major capsid protein (102). However, although antibody may play a critical role in limiting the spread of infection and reducing viral load, the B cell response by itself is insufficient to combat

development of tumors (102). Therefore, virus-specific $CD8^+$ T cells are required for effective control of MPyV and resistance to virus-induced disease. Not surprisingly, $CD8^+$ T cell responses during MPyV infection have been the focus of our previous studies.

MPyV-tumor resistant C57BL/6 (B6, H-$2^b$) mice mount a non-coordinately regulated classical MHC class Ia-restricted virus-specific $CD8^+$ T cell response directed against epitopes in the nonstructural large and middle T antigens (MT and LT) (72). The epitope-specific $CD8^+$ T cell populations displayed notable heterogeneity in magnitude, kinetics of expansion and contraction during acute infection, activation/differentiation phenotype, effector cytokine profile, and functional avidity maturation during persistent infection. Despite these differences, they retained polyfunctionality and did not become exhausted. Furthermore, the expansion and contraction of MPyV-specific $CD8^+$ T cells closely parallels the changes in viral replication, with MPyV-specific $CD8^+$ T cell populations being stably maintained despite virus persistence. It was previously suggested that persistent antigen is the driving factor behind self-renewal and survival of virus-specific $CD8^+$ T cell memory during chronic viral infection (76, 77). However, MPyV-specific $CD8^+$ T cells from persistently infected mice exhibit minimal proliferation and survived poorly when transferred into infection-matched recipients (78). Hypothesizing that persistent antigen was instead driving recruitment of new naïve antigen-specific T cells, Lukacher and colleagues developed a novel minimally myeloablative mixed bone marrow chimera approach to demonstrate that de novo priming of virus-specific $CD8^+$ T cells occurs during persistent MPyV infection. The critical role of continued recent thymic emigrant priming in MPyV-specific $CD8^+$ memory was further emphasized by a modest but statistically significant drop in number of virus-specific cells in thymectomized mice versus shams. "Late-primed" antigen-specific $CD8^+$ T cells displayed a $CD62L^{hi}CD27^{hi}$ central memory phenotype that contrasts with the effector/effector-memory $CD62L^{lo}$ phenotype of the bulk population, further demonstrating the impact the level of antigen and inflammation has on T cell differentiation (78). Interestingly, MPyV-infected mice

lacking classical MHC class Ia molecules ($K^{b-/-}D^{b-/-}$) can control virus and resist virus-induced tumors. Swanson et al. identified a virus specific $CD8^+$ T cell population specific for an epitope in the minor capsid protein VP2 presented on Q9, a non-classical MHC class Ib molecule (109). These virus-specific $CD8^+$ T cells appear to be partially dysfunctional though they have a phenotype consistent with recall-competent memory T cells. They also share a kinetic profile similar to inflationary epitope-specific $CD8^+$ T cells in persistent MCMV infection (70) but can survive despite persistent antigen, which is required for their expansion but not maintenance (110). Capsid protein-specific $CD8^+$ T cells could help keep persistent virus in check, providing critical backup should the more immunodominant T-antigen specific MHC-Ia-restricted $CD8^+$ T cells drive selection of viral epitope escape mutants (109). Altogether, MPyV-specific $CD8^+$ T cells provide multi-faceted and highly flexible protection.

Given the critical role played by $CD4^+$ T cell help for optimal $CD8^+$ T cell and B cell responses (17, 18), Kemball et al. asked what role $CD4^+$ T cells might play in a low-level systemic persistent infection such as ours using MHC class II-deficient mice, which lack $CD4^+$ T cells (107). Similar to acute resolved infections, the primary MPyV-specific $CD8^+$ T cell response was unimpaired in $CD4^+$ T cell-deficient I-$A^{b-/-}$ mice but was marked by a significant decline in virus-specific $CD8^+$ T cell populations during the transition to persistent infection compared to wildtype B6 control mice. However, unlike in $CD4^+$ T cell-deficient mice chronically infected with LCMV (14, 16), the surviving MPyV-specific $CD8^+$ T cells were not functionally exhausted. Bone marrow experiments demonstrated that de novo recruitment of MPyV-specific $CD8^+$ T cells was defective in persistently infected CD4-deficient mice. In addition to diminished virus-specific $CD8^+$ T cell levels, significantly lower titers of anti-viral IgG and few antibody-secreting plasma cells in bone marrow were detected in persistently infected MHC class II-deficient mice compared to controls (107). $CD4^+$ T cell help is also critical for both the expansion and maintenance of MHC class Ib-restricted MPyV-specific $CD8^+$ T cells (A.R. Hofstetter, manuscript in preparation). These findings suggest then that MPyV induces a functionally

competent and vital virus-specific $CD4^+$ T cell response. However, little has been done so far to identify $CD4^+$ T cell epitopes in MPyV, and the tools to track endogenous antigen-specific $CD4^+$ T cells ex vivo have been lacking until recently. In Chapter 2, I will describe my efforts to identify MHC class II-restricted MPyV epitopes in B6 mice ($I\text{-}A^b$) and to follow the evolution of the virus-specific $CD4^+$ T cell response over the course of persistent infection. As $CD4^+$ T cells can differentiate into multiple $T_H$ subsets, which can impact the immune response to a given type of pathogen, a key question is what the functional profile of MPyV-specific $CD4^+$ T cells, including effector cytokines produced, is and whether it differs during persistent and acute phases of infection (i.e. is it plastic?). I also asked if virus-specific $CD4^+$ T cells are stably maintained in the face of persistent MPyV, and if so, whether this is achieved in part by de novo recruitment of naïve $CD4^+$ T cells.

Another outstanding and unresolved issue in systemic low-level persistent infection with MPyV concerns the role of persistent viral antigen on differentiation and maintenance of virus-specific memory-like T cell pools. Our previous studies suggest a "conveyor belt" scenario, where continual de novo recruitment of naïve antigen-specific $CD8^+$ T cells is required to replenish a deteriorating pool of virus-specific T cells, but as viral load/inflammation wanes, recently primed cells could acquire a more antigen-independent self-renewing central memory phenotype and assume a larger role in sustaining virus-specific T cell memory (102). However, thymectomy experiments (78) and the persistence of a residual anti-viral $CD8^+$ T cell population in $CD4^+$ T cell-deficient mice, in which de novo recruitment is impaired (107), indicate other mechanisms perhaps including chronic antigen stimulation also contribute to maintenance of "chronic memory" to MPyV.

To answer the question of whether MPyV antigen-independent memory T cells can emerge or if some require chronic antigen stimulation, we need to develop a system in which virus antigen does not persist. Most studies of virus-specific T cells during persistent infection attempt to compare them with those responding to acutely cleared strains. The LCMV mouse

model has benefitted from the availability of both acute and persistent strains, exemplified by the Armstrong (acute) and the clone 13 (chronic) strains. These differ only by two amino acids yet retain the same T cell epitopes (54). Thus, one can transfer virus-specific T cells from persistently infected mice into recipients infected with the acute strain and observe their fates. As acute strains of MPyV have not been identified, this cannot be done with MPyV-specific T cells. In addition, there are no effective direct anti-virals against MPyV, which precludes artificially reducing pathogen loads and observing the reaction of existent anti-microbial T cells. The Cre/lox system has been a useful tool to inducibly delete genes, whose outright deletion would otherwise be embryonically lethal (111). In this system, two 34 bp loxP sequences are inserted in the same orientation flanking the gene of interest; Cre recombinase, which recognizes these sites, deletes the intervening region. Making use of MPyV's ease of genetic manipulation, two loxP sites were inserted flanking the T-antigen genes, which are required for viral replication and persistence (112). Cre-mediated deletion is predicted to terminate viral replication and remove a source of MPyV antigen. In Chapter 3, I will cover the effort to generate and validate this floxed MPyV before unveiling some of our initial findings about what happens to MPyV-specific T cell populations, both $CD4^+$ and $CD8^+$, when we remove viral antigen during persistent infection.

**References**

1. Takeuchi, O., and S. Akira. 2009. Innate immunity to virus infection. Immunol. Rev. 227:75-86.

2. O'Neill, L. A., and A. G. Bowie. 2010. Sensing and signaling in antiviral innate immunity. Curr. Biol. 20:R328-33.

3. Garcia-Sastre, A., and C. A. Biron. 2006. Type 1 interferons and the virus-host relationship: a lesson in detente. Science 312:879-82.

4. Iwasaki, A., and R. Medzhitov. 2010. Regulation of adaptive immunity by the innate immune system. Science 327:291-5.

5. Harwood, N. E., and F. D. Batista. 2010. Early events in B cell activation. Annu. Rev. Immunol. 28:185-210.

6. Alegre, M. L., K. A. Frauwirth, and C. B. Thompson. 2001. T-cell regulation by CD28 and CTLA-4. Nat. Rev. Immunol. 1:220-8.

7. Curtsinger, J. M., and M. F. Mescher. 2010. Inflammatory cytokines as a third signal for T cell activation. Curr. Opin. Immunol. 22:333-40.

8. Guidotti, L. G., and F. V. Chisari. 2001. Noncytolytic control of viral infections by the innate and adaptive immune response. Annu. Rev. Immunol. 19:65-91.

9. Casazza, J. P., M. R. Betts, D. A. Price, M. L. Precopio, L. E. Ruff, J. M. Brenchley, B. J. Hill, M. Roederer, D. C. Douek, and R. A. Koup. 2006. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. J. Exp. Med. 203:2865-77.

10. Brown, D. M., A. M. Dilzer, D. L. Meents, and S. L. Swain. 2006. CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. J. Immunol. 177:2888-98.

11. Iijima, N., M. M. Linehan, M. Zamora, D. Butkus, R. Dunn, M. R. Kehry, T. M. Laufer, and A. Iwasaki. 2008. Dendritic cells and B cells maximize mucosal Th1 memory response to herpes simplex virus. J. Exp. Med. 205:3041-52.

12. Brien, J. D., J. L. Uhrlaub, and J. Nikolich-Zugich. 2008. West Nile virus-specific CD4 T cells exhibit direct antiviral cytokine secretion and cytotoxicity and are sufficient for antiviral protection. J. Immunol. 181:8568-75.

13. Altfeld, M., and E. S. Rosenberg. 2000. The role of CD4(+) T helper cells in the cytotoxic T lymphocyte response to HIV-1. Curr. Opin. Immunol. 12:375-80.

14. Fuller, M. J., A. Khanolkar, A. E. Tebo, and A. J. Zajac. 2004. Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. J. Immunol. 172:4204-14.

15. 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. 68:8056-63.

16. Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of activated T cells without effector function. J. Exp. Med. 188:2205-13.

17. Williams, M. A., B. J. Holmes, J. C. Sun, and M. J. Bevan. 2006. Developing and maintaining protective CD8+ memory T cells. Immunol. Rev. 211:146-53.

18. McHeyzer-Williams, L. J., and M. G. McHeyzer-Williams. 2005. Antigen-specific memory B cell development. Annu. Rev. Immunol. 23:487-513.

19. Nakanishi, Y., B. Lu, C. Gerard, and A. Iwasaki. 2009. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. Nature 462:510-3.

20. Strutt, T. M., K. K. McKinstry, J. P. Dibble, C. Winchell, Y. Kuang, J. D. Curtis, G. Huston, R. W. Dutton, and S. L. Swain. 2010. Memory CD4+ T cells induce innate responses independently of pathogen. Nat. Med. 16:558-64, 1p following 564.

21. 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 393:478-80.

22. 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 393:480-3.

23. 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 393:474-8.

24. Elgueta, R., M. J. Benson, V. C. de Vries, A. Wasiuk, Y. Guo, and R. J. Noelle. 2009. Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunol. Rev. 229:152-72.

25. 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 441:890-3.

26. Feau, S., R. Arens, S. Togher, and S. P. Schoenberger. 2011. Autocrine IL-2 is required for secondary population expansion of CD8(+) memory T cells. Nat. Immunol. 12:908-13.

27. Elsaesser, H., K. Sauer, and D. G. Brooks. 2009. IL-21 is required to control chronic viral infection. Science 324:1569-72.

28. 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 324:1576-80.

29. 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 324:1572-6.

30. Ettinger, R., S. Kuchen, and P. E. Lipsky. 2008. The role of IL-21 in regulating B-cell function in health and disease. Immunol. Rev. 223:60-86.

31. Linterman, M. A., L. Beaton, D. Yu, R. R. Ramiscal, M. Srivastava, J. J. Hogan, N. K. Verma, M. J. Smyth, R. J. Rigby, and C. G. Vinuesa. 2010. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. J. Exp. Med. 207:353-63.

32. Zotos, D., J. M. Coquet, Y. Zhang, A. Light, K. D'Costa, A. Kallies, L. M. Corcoran, D. I. Godfrey, K. M. Toellner, M. J. Smyth, S. L. Nutt, and D. M. Tarlinton. 2010. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. J. Exp. Med. 207:365-78.

33. Crotty, S. 2011. Follicular helper CD4 T cells (TFH). Annu. Rev. Immunol. 29:621-63.

34. Zhu, J., and W. E. Paul. 2010. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. Immunol. Rev. 238:247-62.

35. Castilow, E. M., M. R. Olson, and S. M. Varga. 2007. Understanding respiratory syncytial virus (RSV) vaccine-enhanced disease. Immunol. Res. 39:225-39.

36. Curotto de Lafaille, M. A., and J. J. Lafaille. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? Immunity 30:626-35.

37. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136:2348-57.

38. Constant, S., C. Pfeiffer, A. Woodard, T. Pasqualini, and K. Bottomly. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells. J. Exp. Med. 182:1591-6.

39. Pfeiffer, C., J. Stein, S. Southwood, H. Ketelaar, A. Sette, and K. Bottomly. 1995. Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. J. Exp. Med. 181:1569-74.

40. Fazilleau, N., L. J. McHeyzer-Williams, H. Rosen, and M. G. McHeyzer-Williams. 2009. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nat. Immunol. 10:375-84.

41. Choi, Y. S., R. Kageyama, D. Eto, T. C. Escobar, R. J. Johnston, L. Monticelli, C. Lao, and S. Crotty. 2011. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity 34:932-46.

42. O'Shea, J. J., and W. E. Paul. 2010. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. Science 327:1098-102.

43. Hegazy, A. N., M. Peine, C. Helmstetter, I. Panse, A. Frohlich, A. Bergthaler, L. Flatz, D. D. Pinschewer, A. Radbruch, and M. Lohning. 2010. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. Immunity 32:116-28.

44. Krawczyk, C. M., H. Shen, and E. J. Pearce. 2007. Functional plasticity in memory T helper cell responses. J. Immunol. 178:4080-8.

45. Seder, R. A., and R. Ahmed. 2003. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat. Immunol. 4:835-42.

46. Obst, R., H. M. van Santen, D. Mathis, and C. Benoist. 2005. Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. J. Exp. Med. 201:1555-65.

47. Foulds, K. E., L. A. Zenewicz, D. J. Shedlock, J. Jiang, A. E. Troy, and H. Shen. 2002. Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. J. Immunol. 168:1528-32.

48. Wooldridge, L., A. Lissina, D. K. Cole, H. A. van den Berg, D. A. Price, and A. K. Sewell. 2009. Tricks with tetramers: how to get the most from multimeric peptide-MHC. Immunology 126:147-64.

49. Vollers, S. S., and L. J. Stern. 2008. Class II major histocompatibility complex tetramer staining: progress, problems, and prospects. Immunology 123:305-13.

50. Seder, R. A., P. A. Darrah, and M. Roederer. 2008. T-cell quality in memory and protection: implications for vaccine design. Nat. Rev. Immunol. 8:247-58.

51. Hammarlund, E., M. W. Lewis, S. G. Hansen, L. I. Strelow, J. A. Nelson, G. J. Sexton, J. M. Hanifin, and M. K. Slifka. 2003. Duration of antiviral immunity after smallpox vaccination. Nat. Med. 9:1131-7.

52. Ahmed, R., and R. S. Akondy. 2011. Insights into human CD8(+) T-cell memory using the yellow fever and smallpox vaccines. Immunol. Cell Biol. 89:340-5.

53. La Gruta, N. L., S. J. Turner, and P. C. Doherty. 2004. Hierarchies in cytokine expression profiles for acute and resolving influenza virus-specific CD8+ T cell responses: correlation of cytokine profile and TCR avidity. J. Immunol. 172:5553-60.

54. Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J. Virol. 77:4911-27.

55. Kaech, S. M., and E. J. Wherry. 2007. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity 27:393-405.

56. van Leeuwen, E. M., J. Sprent, and C. D. Surh. 2009. Generation and maintenance of memory CD4(+) T Cells. Curr. Opin. Immunol. 21:167-72.

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

58. Wherry, E. J. 2011. T cell exhaustion. Nat. Immunol. 12:492-9.

59. Moskophidis, D., F. Lechner, H. Pircher, and R. M. Zinkernagel. 1993. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. Nature 362:758-61.

60. Younes, S. A., B. Yassine-Diab, A. R. Dumont, M. R. Boulassel, Z. Grossman, J. P. Routy, and R. P. Sekaly. 2003. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. J. Exp. Med. 198:1909-22.

61. Brooks, D. G., L. Teyton, M. B. Oldstone, and D. B. McGavern. 2005. Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. J. Virol. 79:10514-27.

62. Fahey, L. M., E. B. Wilson, H. Elsaesser, C. D. Fistonich, D. B. McGavern, and D. G. Brooks. 2011. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. J. Exp. Med. 208:987-99.

63. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 439:682-7.

64. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature 443:350-4.

65. D'Souza, M., A. P. Fontenot, D. G. Mack, C. Lozupone, S. Dillon, A. Meditz, C. C. Wilson, E. Connick, and B. E. Palmer. 2007. Programmed death 1 expression on HIV-specific CD4+ T cells is driven by viral replication and associated with T cell dysfunction. J. Immunol. 179:1979-87.

66. Petrovas, C., J. P. Casazza, J. M. Brenchley, D. A. Price, E. Gostick, W. C. Adams, M. L. Precopio, T. Schacker, M. Roederer, D. C. Douek, and R. A. Koup. 2006. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. J. Exp. Med. 203:2281-92.

67. Kaufmann, D. E., D. G. Kavanagh, F. Pereyra, J. J. Zaunders, E. W. Mackey, T. Miura, S. Palmer, M. Brockman, A. Rathod, A. Piechocka-Trocha, B. Baker, B. Zhu, S. Le Gall, M. T. Waring, R. Ahern, K. Moss, A. D. Kelleher, J. M. Coffin, G. J. Freeman, E. S. Rosenberg, and B. D. Walker. 2007. Upregulation of CTLA-4 by HIV-specific CD4(+) T

cells correlates with disease progression and defines a reversible immune dysfunction. Nat. Immunol. 8:1246-54.

68. Blackburn, S. D., H. Shin, W. N. Haining, T. Zou, C. J. Workman, A. Polley, M. R. Betts, G. J. Freeman, D. A. Vignali, and E. J. Wherry. 2009. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat. Immunol. 10:29-37.

69. Jones, R. B., L. C. Ndhlovu, J. D. Barbour, P. M. Sheth, A. R. Jha, B. R. Long, J. C. Wong, M. Satkunarajah, M. Schweneker, J. M. Chapman, G. Gyenes, B. Vali, M. D. Hyrcza, F. Y. Yue, C. Kovacs, A. Sassi, M. Loutfy, R. Halpenny, D. Persad, G. Spotts, F. M. Hecht, T. W. Chun, J. M. McCune, R. Kaul, J. M. Rini, D. F. Nixon, and M. A. Ostrowski. 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. J. Exp. Med. 205:2763-79.

70. Munks, M. W., K. S. Cho, A. K. Pinto, S. Sierro, P. Klenerman, and A. B. Hill. 2006. Four distinct patterns of memory CD8 T cell responses to chronic murine cytomegalovirus infection. J. Immunol. 177:450-8.

71. Arens, R., P. Wang, J. Sidney, A. Loewendorf, A. Sette, S. P. Schoenberger, B. Peters, and C. A. Benedict. 2008. Cutting edge: murine cytomegalovirus induces a polyfunctional CD4 T cell response. J. Immunol. 180:6472-6.

72. 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. 174:7950-60.

73. Betts, M. R., M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer, and R. A. Koup. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood 107:4781-9.

74. Harari, A., F. Vallelian, P. R. Meylan, and G. Pantaleo. 2005. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. J. Immunol. 174:1037-45.

75. Klenerman, P., and A. Hill. 2005. T cells and viral persistence: lessons from diverse infections. Nat. Immunol. 6:873-9.

76. Shin, H., S. D. Blackburn, J. N. Blattman, and E. J. Wherry. 2007. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. J. Exp. Med. 204:941-9.

77. Wherry, E. J., D. L. Barber, S. M. Kaech, J. N. Blattman, and R. Ahmed. 2004. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. Proc. Natl. Acad. Sci. USA 101:16004-9.

78. 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. 203:2263-9.

79. Miller, N. E., J. R. Bonczyk, Y. Nakayama, and M. Suresh. 2005. Role of thymic output in regulating CD8 T-cell homeostasis during acute and chronic viral infection. J. Virol. 79:9419-29.

80. Bannard, O., M. Kraman, and D. T. Fearon. 2010. Cutting edge: Virus-specific CD8+ T cell clones and the maintenance of replicative function during a persistent viral infection. J. Immunol. 185:7141-5.

81. Snyder, C. M., K. S. Cho, E. L. Bonnett, S. van Dommelen, G. R. Shellam, and A. B. Hill. 2008. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. Immunity 29:650-9.

82. Woodland, D. L., and J. E. Kohlmeier. 2009. Migration, maintenance and recall of memory T cells in peripheral tissues. Nat. Rev. Immunol. 9:153-61.

83. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401:708-12.

84. Stubbe, M., N. Vanderheyde, M. Goldman, and A. Marchant. 2006. Antigen-specific central memory CD4+ T lymphocytes produce multiple cytokines and proliferate in vivo in humans. J. Immunol. 177:8185-90.

85. Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat. Immunol. 4:225-34.

86. Jelley-Gibbs, D. M., J. P. Dibble, S. Filipson, L. Haynes, R. A. Kemp, and S. L. Swain. 2005. Repeated stimulation of CD4 effector T cells can limit their protective function. J. Exp. Med. 201:1101-12.

87. Masopust, D., S. J. Ha, V. Vezys, and R. Ahmed. 2006. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. J. Immunol. 177:831-9.

88. Catron, D. M., L. K. Rusch, J. Hataye, A. A. Itano, and M. K. Jenkins. 2006. CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. J. Exp. Med. 203:1045-54.

89. D'Souza, W. N., and S. M. Hedrick. 2006. Cutting edge: latecomer CD8 T cells are imprinted with a unique differentiation program. J. Immunol. 177:777-81.

90. Jelley-Gibbs, D. M., D. M. Brown, J. P. Dibble, L. Haynes, S. M. Eaton, and S. L. Swain. 2005. Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. J. Exp. Med. 202:697-706.

91. Gross, L. 1953. A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. Proc. Soc. Exp. Biol. Med. 83:414-21.

92. Sweet, B. H., and M. R. Hilleman. 1960. The vacuolating virus, S.V. 40. Proc. Soc. Exp. Biol. Med. 105:420-7.

93. Gardner, S. D., A. M. Field, D. V. Coleman, and B. Hulme. 1971. New human papovavirus (B.K.) isolated from urine after renal transplantation. Lancet 1:1253-7.

94. Padgett, B. L., D. L. Walker, G. M. ZuRhein, R. J. Eckroade, and B. H. Dessel. 1971. Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy. Lancet 1:1257-60.

95. Allander, T., K. Andreasson, S. Gupta, A. Bjerkner, G. Bogdanovic, M. A. Persson, T. Dalianis, T. Ramqvist, and B. Andersson. 2007. Identification of a third human polyomavirus. J. Virol. 81:4130-6.

96. Feng, H., M. Shuda, Y. Chang, and P. S. Moore. 2008. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science 319:1096-100.

97. Gaynor, A. M., M. D. Nissen, D. M. Whiley, I. M. Mackay, S. B. Lambert, G. Wu, D. C. Brennan, G. A. Storch, T. P. Sloots, and D. Wang. 2007. Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog. 3:e64.

98. zur Hausen, H. 2008. Novel human polyomaviruses--re-emergence of a well known virus family as possible human carcinogens. Int. J. Cancer 123:247-50.

99. Kean, J. M., S. Rao, M. Wang, and R. L. Garcea. 2009. Seroepidemiology of human polyomaviruses. PLoS Pathog. 5:e1000363.

100. Ramos, E., C. B. Drachenberg, R. Wali, and H. H. Hirsch. 2009. The decade of polyomavirus BK-associated nephropathy: state of affairs. Transplantation 87:621-30.

101. Tan, C. S., and I. J. Koralnik. 2010. Progressive multifocal leukoencephalopathy and other disorders caused by JC virus: clinical features and pathogenesis. Lancet Neurol. 9:425-37.

102. Swanson, P. A., 2nd, A. E. Lukacher, and E. Szomolanyi-Tsuda. 2009. Immunity to polyomavirus infection: the polyomavirus-mouse model. Semin. Cancer Biol. 19:244-51.

103. Andrews, N. P., C. D. Pack, and A. E. Lukacher. 2008. Generation of antiviral major histocompatibility complex class I-restricted T cells in the absence of CD8 coreceptors. J. Virol. 82:4697-705.

104. 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. 85:10126-34.

105. Carroll, J., D. Dey, L. Kreisman, P. Velupillai, J. Dahl, S. Telford, R. Bronson, and T. Benjamin. 2007. Receptor-binding and oncogenic properties of polyoma viruses isolated from feral mice. PLoS Pathog. 3:e179.

106. Drake, D. R., 3rd, and A. E. Lukacher. 1998. Beta 2-microglobulin knockout mice are highly susceptible to polyoma virus tumorigenesis. Virology 252:275-84.

107. 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. 179:1113-21.

108. 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. 181:1683-92.

109. 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. 205:1647-57.

110. 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. 182:5198-202.

111. Sauer, B. 1998. Inducible gene targeting in mice using the Cre/lox system. Methods 14:381-92.

112. Benjamin, T. L. 2006. Polyoma Viruses, p. 105-139, The Mouse in Biomedical Research, 2nd ed. Elsevier, San Diego, CA.

# Chapter 2

## Heterogeneity among viral antigen-specific $CD4^+$ T cells and their de novo recruitment during persistent polyomavirus infection

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

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# Heterogeneity among viral antigen-specific $CD4^+$ T cells and their de novo recruitment during persistent polyomavirus infection

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**Abstract**

Virus-specific $CD4^+$ T cells optimize anti-viral responses by providing help for anti-viral humoral responses and $CD8^+$ T cell differentiation. Although $CD4^+$ T cell responses to viral infections that undergo complete clearance have been studied extensively, less is known about virus-specific $CD4^+$ T cell responses to viruses that persistently infect their hosts. Using a mouse polyomavirus (MPyV) infection model, we previously demonstrated that $CD4^+$ T cells are essential for recruiting naïve MPyV-specific $CD8^+$ T cells in persistently infected mice. In this study, we defined two dominant MPyV-specific $CD4^+$ T cell populations, one directed toward an epitope derived from the nonstructural large T antigen and the other from the major viral capsid protein of MPyV. These MPyV-specific $CD4^+$ T cells vary in terms of their magnitude, functional profile, and phenotype during acute and persistent phases of infection. Using a minimally myeloablative mixed bone marrow chimerism approach, we further show that naïve virus-specific $CD4^+$ T cells, like anti-MPyV $CD8^+$ T cells, are primed de novo during persistent virus infection. In summary, these findings reveal quantitative and qualitative differences in the $CD4^+$ T cell response to a persistent virus infection and demonstrate that naïve antiviral $CD4^+$ T cells are recruited during chronic polyomavirus infection.

**Introduction**

$CD4^+$ T cells shape and regulate host immune responses to microbial pathogens (1). Virus-specific $CD4^+$ T cells drive B cell differentiation to memory B cells and plasma cells bearing isotype-switched, affinity matured antibodies (2, 3). A sizeable body of literature documents the critical contribution of $CD4^+$ T cell help for generating memory $CD8^+$ T cells capable of mounting strong anamnestic responses (4). $CD4^+$ T cells may also elaborate antiviral cytokines (5-7) or directly kill infected MHC class II-expressing cells (8-10). In the setting of acute viral infections that are completely resolved, virus-specific $CD4^+$ T cells, like their antiviral $CD8^+$ T cell counterparts, give rise to phenotypically and functionally heterogeneous memory T cells that are maintained by Ag-independent self-renewal and are capable of robust recall responses to challenge infection (1, 11, 12).

In the face of high-level persistent virus infection, however, $CD4^+$ T cells are handicapped in their effector activities. In viremic individuals, HIV-specific $CD4^+$ T cells fail to proliferate or produce IL-2 (13) but upregulate inhibitory receptors that mitigate their functional integrity (14, 15). Using the lymphocytic choriomeningitis virus (LCMV) clone 13 chronic infection mouse model, Brooks et al. recently showed that virus-specific $CD4^+$ T cells acquire functional defects early in the course of infection (16). In contrast, antiviral $CD4^+$ T cells maintained in the setting of low-level persistent virus infections (e.g., human and mouse CMV and EBV) generally retain their functionality (17-20).

Maintenance of memory virus-specific $CD8^+$ T cells in persistent infection, as in acute infection, depends on a functional virus-specific $CD4^+$ T cell response, which is affected by the magnitude and duration of infection, and likely, cell types harboring the infection. For LCMV clone 13 infection, functional deterioration and deletion of virus-specific $CD8^+$ T cells are exacerbated by the absence of $CD4^+$ T cells, with recovery of $CD8^+$ T cell numbers and function

associated with resolution of viremia (21-23). For low-level persistent infections, $CD4^+$ T cell deficiency likewise dramatically affects the size of the memory antiviral $CD8^+$ T cell compartment. Maintenance of stable virus-specific $CD8^+$ T cell numbers during persistent viral infection depends on extensive proliferation of memory $CD8^+$ T cells and/or de novo priming of naïve virus-specific $CD8^+$ T cells (24-26). For persistent mouse polyomavirus (MPyV) infection, virus-specific $CD8^+$ T cells fail to divide and suffer rapid attrition, putting the onus for their maintenance on naïve Ag-specific $CD8^+$ T cell recruitment (25, 27). Moreover, we recently demonstrated that $CD4^+$ T cell help is required for recruitment of naïve antiviral $CD8^+$ T cells during persistent MPyV infection (28). Whether MPyV-specific $CD4^+$ T cells conserve their effector activities, are stably maintained, and are resupplied via naïve $CD4^+$ T cell recruitment during persistent infection are not known.

Efforts to characterize $CD4^+$ T cells in persistent infections have been hampered by the small numbers of defined epitopes, the low magnitude of their response, and until recently, few reagents to track Ag-specific populations compared to $CD8^+$ T cells. To assess the evolution of the antiviral $CD4^+$ T cell response through acute and persistent phases of MPyV infection, we have identified two MPyV-specific $CD4^+$ T cell populations in B6 mice, one directed toward an epitope derived from the nonstructural large T (LT) antigen and the other from the major viral capsid protein of MPyV (VP1). These epitope-specific $CD4^+$ T cells differ in magnitude, phenotype, functional profile, and TCR repertoire. However, $CD4^+$ T cells of both specificities are stably maintained during persistent infection despite exhibiting minimal proliferation. We show in this study that naïve progenitors of each specificity are primed de novo in persistently infected mice. These findings highlight the heterogeneity of the $CD4^+$ T cell response during this low-level systemic persistent viral infection and suggest that virus-specific $CD4^+$ T cell populations are maintained long-term by recruitment of naive virus-specific T cells.

**Materials and Methods**

*Mice*

C57BL/6NCr (B6) and B6/CD45.1 female mice were purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD). Mice were housed and bred in accordance with the guidelines from the Institutional Animal Care and Use Committee and the Department of Animal Resources of Emory University (Atlanta, GA).

*MPyV preparation and inoculation*

MPyV strain A2 was molecularly cloned and plaque purified, and virus stocks were prepared on primary baby mouse kidney cells as described previously (29). Each mouse received $2 \times 10^6$ PFU of virus s.c. Mice were inoculated at 6-12 weeks of age.

*Cell isolation* and *flow cytometry*

Spleens were digested in a solution of 125 U/mL collagenase type XI, 60 U/mL hyaluronidase type I-s, and 60 U/mL of Dnase I in RPMI supplemented with 5% FCS plus $Ca^{2+}$ and $Mg^{2+}$ for 1 h at 37°C, then passed through cell strainers followed by RBC lysis.

Allophycocyanin-conjugated I-$A^b$/$LT_{678-690}$ and I-$A^b$/$VP1_{221-235}$ tetramers were constructed by the National Institutes of Health Tetramer Core Facility at Emory University (Atlanta, GA). Tetramer staining was performed at 37°C for 1.5 h in RPMI 1640 containing 2% FCS at a concentration of 8 μg/mL per 1 x $10^6$ cells followed by surface staining for CD4, CD44, and other indicated surface markers at 4°C for 30 min. In some experiments, cells were stained with 7-aminoactinomycin D (7-AAD, BD Pharmingen, San Diego, CA) per manufacturer's

instructions. For CTLA-4, Bcl-2, Ki-67, and TCR Vβ, cells were permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen) after I-$A^b$ tetramer and surface staining then stained intracellularly for the indicated marker. For Annexin V staining, I-$A^b$ tetramer and surface marker-stained cells were stained with FITC Annexin V (BD Pharmingen) followed by 7-AAD.

The following mAbs were purchased from BD Pharmingen: FITC-conjugated anti-CD44 and anti-Ki-67; allophycocyanin-conjugated anti-IFN-γ; PE-conjugated anti-CD62L, Bcl-2, IL-2, TNF-α (TN3-19.12), IL-10, IL-17, IL-4; V450-conjugated anti-CD44; and isotype controls; Pacific Blue-conjugated anti-CD4 (RM4-5). PerCP-Cy5.5-conjugated mAb to CD45.1 and CD4 (RM4-5), PE-conjugated mAb to CD27 (LG.7F9), CD127 (A7R34), programmed death 1 (PD-1) (RMP1-30), LAG-3, TIM-3 (RMT3-23), CD154, CTLA-4, ICOS (7E.17G9), OX-40, CD28, and isotype controls were purchased from eBioscience (San Diego, CA). Samples were acquired either on a FACSCalibur or LSR-II (both from BD Biosciences, San Jose, CA). Data were analyzed using FloJo software (Treestar, Inc., Ashland, OR).

*BrdU incorporation assay*

MPyV-infected B6 mice were administered 1 mg of BrdU (Sigma-Aldrich, St. Louis, MO) i.p. once daily for 7 days. On the day after the last injection of BrdU, mice were sacrificed, and splenocytes were stained with I-$A^b$/$LT_{678}$ or I-$A^b$/$VP1_{221}$ tetramers followed by surface staining for CD4 and CD44. BrdU incorporation was then assessed using the FITC BrdU flow kit (BD Biosciences) per manufacturer's instructions.

*Intracellular cytokine staining*

Cells were stimulated with peptide (10-50 μM) for 5.5 h in the presence of GolgiPlug (brefeldin A; BD Pharmingen) or GolgiStop (monensin; BD Pharmingen), then surface stained with PerCP-Cy5.5-conjugated anti-CD4 (RM4-5). After washing, cells were permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen) and stained for intracellular IFN-γ and CD154. For multi-cytokine analysis, cells were stained intracellularly with anti-IFN-γ and PE-conjugated antibodies to IL-2, TNF-α, IL-10, IL-17, or IL-4. Intracellular staining for IL-21 was performed as described by Suto et al. (30).

*In vivo programmed death ligand 1 blockade*

B6 mice persistently infected by MPyV (35-50 days postinfection [p.i.]) received 200 μg of rat anti–mouse programmed death ligand 1 (PD-L1) antibody (10F.9G2) or rat IgG control i.p. every 3 days over 2 weeks.

*Quantification of MPyV genomes*

DNA isolation and TaqMan PCR were performed as described previously (27). MPyV DNA quantity is expressed in genome copies per milligram of tissue and is calculated based on a standard curve of known MPyV genome copy number versus threshold cycle of detection. The detection limit with this assay is 10 copies of genomic viral DNA.

*Generation of CD45 congenic bone marrow chimeras*

A minimal myeloablation and bone marrow transplantation protocol was performed as previously described, with the following modifications (25, 27). Naïve or persistently MPyV-infected B6

mice were given 600 μg busulfan i.p. (Busulfex; Otsuka America Pharmaceutical, Rockville, MD). 24 h later, these mice received 20 x $10^6$ nucleated cells i.v. from the bone marrow of naïve B6/CD45.1 mice. Establishment of chimerism was confirmed by flow cytometric analysis of whole blood cells for CD45.1 expression (data not shown). We have previously shown that the minimally myeloablative dose of busulfan that we use (25 mg/kg) does not impact the number of MPyV genomes per mg tissue (27). Spleens of chimeric mice were analyzed by flow cytometry > 90 days after bone marrow transplantation.

*IFN-γ ELISPOT assay*

The single-cell ELISPOT assay was performed as described previously (27).

*Statistics*

Statistical significance was determined by a two-tailed nonparametric Mann-Whitney *U* test using Prism software (GraphPad Software, La Jolla, CA). A $p$-value < 0.05 was considered statistically significant.

## Results

### *Identification of MPyV-specific CD4$^+$ T cell epitopes*

As a first step to empirically map MPyV-specific CD4$^+$ T cell epitopes, we used IFN-γ ELISPOT assays to screen overlapping synthetic peptides covering sequences for each of the six MPyV proteins for their ability to stimulate spleen cells from acutely infected B6 mice. Overlapping peptides yielding positive wells were then assayed by IFN-γ intracellular staining and CD4 surface staining, and minimum length peptides stimulating optimal responses were determined. From these analyses, one 13-mer and one 15-mer CD4$^+$ T cell epitope for MPyV were identified: $LT_{678\text{-}690}$ (NEYLLPQTVWARF) from the nonstructural LT antigen; and $VP1_{221\text{-}235}$ (GMYPVEIWHPDPAKN) from the VP1 major capsid protein. Fig. 1A shows a representative example of $LT_{678}$- and $VP1_{221}$-induced intracellular IFN-γ production by splenic CD4$^+$ T cells from B6 mice at day 8 p.i. To facilitate detection of Ag-reactive CD4$^+$ T cells, we performed intracellular co-staining for CD154 (CD40L), which is transiently expressed by CD4$^+$ T cells following Ag stimulation (31); nearly all of the IFN-γ$^+$ cells co-expressed CD154.

To enumerate and phenotype MPyV-specific CD4$^+$ T cells directly ex vivo, we used MHC class II tetramers complexed to each of these antigenic peptides. As shown in Fig. 1B, I-$A^b$/$LT_{678}$ and I-$A^b$/$VP1_{221}$ tetramers bound Ag-experienced (i.e., CD44$^{hi}$) splenic CD4$^+$ T cells, which were not stained by control I-$A^b$/hCLIP tetramers; in addition, I-$A^b$/$LT_{678}$ and I-$A^b$/$VP1_{221}$ tetramers did not stain CD4$^+$ T cells from uninfected B6 mice. Using these tetramers, we found that >2-fold more $VP1_{221}$-specific CD4$^+$ T cells than $LT_{678}$-specific cells were detected by tetramers in spleens of day 8-infected mice (Fig. 1C). The larger number of I-$A^b$/$VP1_{221}$ tetramer$^+$ cells was evident as early as 6 days p.i. and continued well into the persistent phase of infection. Notably, both CD4$^+$ T cell populations underwent a relatively small contraction and were then maintained at steady levels, response profiles that stand in contrast to the progressive

attrition of memory $CD4^+$ T cell populations seen in acute LCMV and *Listeria monocytogenes* infections (32-34).

*$VP1_{221}$- and $LT_{678}$-specific $CD4^+$ T cells differ in functional competence*

In Fig. 1A we noted that fewer $VP1_{221}$-specific $CD4^+$ T cells at day 8 p.i. produced IFN-γ than were stained by I-$A^b$/$VP1_{221}$ tetramers, a difference not seen with the $LT_{678}$-specific $CD4^+$ T cells. By peptide titration, we first determined that 50 μM peptide stimulated maximal intracellular IFN-γ production by both epitope-specific $CD4^+$ T cell populations and did not resolve the apparent absence of IFN-γ production by $VP1_{221}$-specific $CD4^+$ T cells (data not shown). I-$A^b$/$VP1_{221}$ and I-$A^b$/ $LT_{678}$ tetramer$^+$ cells were also found to express equivalent levels of surface CD3 and slightly higher levels of CD4 than naïve T cells (data not shown), as previously reported for Ag-stimulated $CD4^+$ T cells (35). In addition, the frequency of $CD4^+$ T cells induced to produce IFN-γ by $VP1_{221}$ peptide stimulation closely approximates those that upregulate CD40L (Fig. 1A and data not shown), suggesting that a proportion of the $VP1_{221}$-specific $CD4^+$ T cell population is refractory to T cell activation. We then asked whether this early decline in functionality by $VP1_{221}$-specific $CD4^+$ T cells was a transient defect that resolved over time and whether $LT_{678}$-specific $CD4^+$ T cells preserved their cytokine effector function during persistent MPyV infection. As shown in Fig. 2A, only 40-50% of the I-$A^b$/$VP1_{221}$ tetramer$^+$ $CD4^+$ T cells produce IFN-γ at all acute and persistent infection time points examined, whereas $LT_{678}$-specific $CD4^+$ T cells retained function well into persistent infection. Moreover, when one takes into account this selective dysfunction by the $VP1_{221}$-specific $CD4^+$ T cells, the numerical advantage of these cells over the $LT_{678}$-specific $CD4^+$ T cells nearly vanishes during acute infection and narrows by approximately half of that based on tetramer binding during persistent infection (Fig. 2B). Finally, these analyses reveal that this IFN-γ deficiency by the $VP1_{221}$-specific $CD4^+$ T cells

is evident as early as day 6 p.i., suggesting that factors early in acute infection affect the cytokine effector profile or long-term fitness of the $CD4^+$ T cell response.

We next asked whether this lack of IFN-γ production by nearly half of the $VP1_{221}$-specific $CD4^+$ T cells extended to other effector functions. Using spleen cells from acutely (day 8 p.i.) and persistently (day 60 p.i.) infected mice, we determined the frequency of IFN-$\gamma^+$ cells that co-produced either TNF-α or IL-2 after stimulation by $VP1_{221}$ or $LT_{678}$ peptides. The data in Fig. 3A show that only a fraction of either the $VP1_{221}$-specific or $LT_{678}$-specific $CD4^+$ T cell populations in acutely infected mice co-produce TNF-α and IL-2 along with IFN-γ, although the inability to produce these effector cytokines is greater for the $VP1_{221}$-specific $CD4^+$ T cells (especially when taking into account their lack of IFN-γ-producing capability). Moreover, the deficiency in co-producing these cytokines was maintained into the persistent phase of MPyV infection.

We then investigated the possibility that these MPyV-specific $CD4^+$ T cells may have modulated their effector cytokine profiles. Although neither the $VP1_{221}$- nor $LT_{678}$-specific $CD4^+$ T cell populations produced IL-4 or IL-17 (data not shown), we observed that a fraction of IFN-$\gamma^+$ cells of each specificity co-produced IL-21 or IL-10 (Fig. 3B). Moreover, in contrast to the stability of IFN-γ/TNF-α/IL-2 co-producers over the course of MPyV infection, for both $VP1_{221}$- and $LT_{678}$-specific $CD4^+$ T cells. There was a doubling of those making IL-21 but a halving of those making IL-10 between acute and persistent stages of infection. In addition, <5% of either $VP1_{221}$- or $LT_{678}$-specific $CD4^+$ T cells express the regulatory T cell transcription factor Foxp3 in either acutely or persistently infected mice (data not shown). As recently reported for influenza virus-specific $CD8^+$ T cells (36), it is tempting to speculate that these virus-specific $CD4^+$ T cells may adjust secretion of IL-10 to counter immune-mediated pathology, the potential for which would be higher in acute than persistent infection. The shift toward IL-21 production by

persistent infection-phase MPyV-specific $CD4^+$ T cells also fits with recent reports showing that this cytokine contributes to maintenance of virus-specific $CD8^+$ T cells during chronic LCMV infection (37-39).

*TCR repertoire and phenotypic heterogeneity of MPyV epitope-specific $CD4^+$ T cells*

Differences in efficiency of T cell activation by Ag in vivo have been correlated with changes in the level of clonotypic diversity by the responding T cells (40). We thus asked whether the $VP1_{221}$-specific and $LT_{678}$-specific $CD4^+$ T cells might exhibit differences in TCR repertoire diversity early in the course of MPyV infection. By co-staining I-$A^b$ $tetramer^+$ $CD4^+$ T cells from individual acutely infected (day 8 p.i.) mice with a panel of Vβ mAbs, we observed that the $LT_{678}$-specific T cells express a diverse, and mouse-to-mouse variable, Vβ usage profile. In sharp contrast, 80-90% of the $VP1_{221}$-specific $CD4^+$ T cells in individual B6 mice predominantly express TCRs using Vβ8.1/8.2 and Vβ14 domains (Fig. 4). This constrained Vβ expression profile by the $VP1_{221}$-specific $CD4^+$ T cells during acute infection is in line with the interpretation that only a subset of the naïve T cells population directed to this epitope is recruited.

T cells maintained in the setting of persistent Ag often express surface receptors that dampen their effector function (15, 41). Recent studies using the LCMV infection model have shown that the extent of T cell dysfunction parallels both the level of expression and diversity of inhibitory receptors expressed by virus-specific $CD8^+$ T cells, which correlates with the severity and duration of persistent infection (42). We therefore asked whether differences in cytokine effector function between $LT_{678}$- and $VP1_{221}$-specific $CD4^+$ T cells were reflected in differences in the profile and levels of cell surface inhibitory receptors. Of the inhibitory receptors examined (PD-1, CTLA-4, LAG-3, CD160, and TIM-3), only PD-1 and CTLA-4 were expressed by these MPyV-specific $CD4^+$ T cells. Moreover, the I-$A^b$/$VP1_{221\text{-}235}$ $tetramer^+$ $CD4^+$ T cells displayed a

higher geometric mean fluorescence intensity (gMFI) for PD-1 (Fig. 5A) in both acutely and persistently infected mice, with an increase in PD-1 expression between acute and persistent phases of infection; in contrast, CTLA-4 expression levels by $CD4^+$ T cells of both specificities declined in persistently infected mice. The higher levels of PD-1 expression on $VP1_{221}$-specific $CD4^+$ T cells suggest that these cells more frequently encounter Ag than $LT_{678}$-specific $CD4^+$ T cells and that PD-1 receptors might play a role in their partial dysfunction.

To address the possible role of PD-1 in impairing the function of $VP1_{221}$-specific $CD4^+$ T cells, we performed in vivo blockade of the PD-1:PD-L1 pathway in persistently infected mice (d35-50 p.i.). Anti-PD-L1-treated B6 mice (n = 3 per each of 2 experiments) showed only a modest increase in the numbers of I-$A^b/LT_{678}$ tetramer$^+$ $CD4^+$ T cells ($1.1x10^4 \pm 5.8x10^3$ for control rat IgG-treated vs. $1.7x10^4 \pm 4.1x10^3$ for anti-PD-L1-treated mice; $p = 0.065$) and I-$A^b/VP1_{221}$ tetramer$^+$ $CD4^+$ T cells ($5.9x10^4 \pm 7.9x10^3$ for controls vs. $9.0x10^4 \pm 3.6x10^4$ for anti-PD-L1-treated mice; $p = 0.065$). There was a concomitant small increase in the number of IFN-$\gamma^+$ $VP1_{221}$-specific $CD4^+$ T cells ($1.7x10^4 \pm 3.2x10^3$ for controls vs. $3.0x10^4 \pm 1.0x10^4$ for anti-PD-L1-treated mice; $p = 0.065$) and $LT_{678}$-specific $CD4^+$ T cells ($9.5x10^3 \pm 3.5x10^3$ for controls vs. $1.4x10^4 \pm 3.1x10^3$ for anti-PD-L1-treated mice; $p = 0.041$). However, as a fraction of tetramer-binding cells, $VP1_{221}$- and $LT_{678}$-specific $CD4^+$ T cell functionality was similar between anti-PD-L1-treated and control mice. In addition, no differences between these groups were seen in the dominant $D^b/LT_{359}$-specific $CD8^+$ T cell response or in MPyV viral levels (data not shown). Although PD-1 on its own does not appear to play a role in limiting the function of $VP1_{221}$-specific $CD4^+$ T cells, other inhibitory mechanisms may contribute. Thus, it will be of interest in future studies to see whether CTLA-4 blockade singly or in combination with PD-1 blockade bolsters the expansion and function of MPyV-specific T cells and results in a drop of the persistent MPyV infection set point.

The likelihood these two MPyV-specific $CD4^+$ T cell populations differentially engage Ag is further supported by other phenotypic differences. Although $VP1_{221}$-specific and $LT_{678}$-specific $CD4^+$ T cell populations in persistently infected mice are $CD62L^{lo}$ and express decreased levels of Bcl-2 compared to $CD44^{lo}CD4^+$ T cells, indicative of an effector/effector-memory differentiation state, all of the $LT_{678}$-specific $CD4^+$ T cells and most of the $VP1_{221}$-specific $CD4^+$ T cells are $CD127^{hi}$ and $CD27^{hi}$ (Fig. 5B, 5C). An explanation for this mixed effector-memory/central-memory phenotype may lie in the slower pace for re-expression of surface CD62L than CD127 and CD27 by T cells when cognate Ag is limiting (43). Yet, $VP1_{221}$-specific $CD4^+$ T cells also have sizeable $CD127^{lo}$ and PD-$1^{hi}$ populations, whereas $LT_{678}$-specific $CD4^+$ T cells are uniformly $CD127^{hi}$ and PD-$1^{int}$ (Fig. 5A, 5B), suggesting that $VP1_{221}$-specific $CD4^+$ T cells are more chronically stimulated.

*Recruitment of MPyV-specific $CD4^+$ T cells during persistent infection*

We previously demonstrated by adoptive transfer that MHC class Ia-restricted MPyV-specific $CD8^+$ T cells during persistent infection are short-lived (25). However, because of the small frequency of MPyV-specific $CD4^+$ T cells in persistently infected mice, we were unable to detect them by tetramer staining and follow their fate after adoptive transfer to infection-matched recipients. Instead, we assessed expression of both the prosurvival molecule Bcl-2 and the apoptotic marker AnnexinV on $LT_{678}$- and $VP1_{221}$-specific $CD4^+$ T cells in persistently infected mice (day 60 p.i.). Compared with $CD44^{Lo}$ $CD4^+$ cells, both MPyV-specific $CD4^+$ T cell populations expressed lower levels of Bcl-2 and higher levels of surface Annexin V staining (Fig. 5C). These data suggest that MPyV-specific $CD4^+$ T cells are short-lived.

Because the $LT_{678}$- and $VP1_{221}$-specific $CD4^+$ T cell populations are stably maintained during persistent infection (Figs. 1C, 2B), we then asked whether these T cells were capable of

self-renewal and/or were resupplied by de novo priming of naïve Ag-specific T cells. We monitored levels of DNA synthesis of persistent infection-phase $LT_{678}$- and $VP1_{221}$-specific $CD4^+$ T cells based on their incorporation of the thymidine analog BrdU. We observed little anti-BrdU co-staining of either I-$A^b$ tetramer$^+$ $CD4^+$ T cell population in contrast to the strong BrdU co-staining of these cells in acutely infected mice (Fig. 5D). Consistent with this finding, few splenic I-$A^b$ tetramer$^+$ $CD4^+$ cells from persistently infected mice expressed the nuclear proliferation marker Ki-67 (data not shown).

To explore the possibility that Ag-specific naïve $CD4^+$ T cells were primed de novo during persistent MPyV infection, we administered a minimally myeloablative dose of the cytotoxic DNA alkylating drug busulfan to B6 ($CD45.2^+$) mice at day 40 p.i., and 24 h later, infused them with 2 x $10^7$ bone marrow cells from naïve $CD45.1^+$ congenic donors. Ninety days post-transfer (day 130 p.i.), I-$A^b$ tetramer staining detected both $CD45.1^+$ donor-derived $LT_{678}$-specific and $VP1_{221}$-specific $CD4^+$ T cells (Fig. 6A). Notably, despite having similar levels of chimerism, individual mice showed variability in the frequency of I-$A^b$ tetramer$^+$ $CD4^+$ T cells that are donor-derived (Fig. 6B). This mouse-to-mouse variability may be a reflection of differences in frequency of encounter by naïve virus-specific $CD4^+$ T cells with low numbers of epitope-expressing MHC-$II^+$ cells in persistent MPyV-infected mice; moreover, it is conceivable that the range of viral epitopes presented by APCs during persistent infection may vary among individual mice as a function of those that are productively (i.e., expressing both structural and nonstructural viral proteins) and nonproductively (i.e., expressing only nonstructural viral proteins) infected. Taken together, these data demonstrate that MPyV-specific $CD4^+$ T cells are recruited during persistent infection.

**Discussion**

The natural MPyV provides a valuable model for studying T cell responses to viruses that establish low-level systemic persistent infection. In this study, we defined two I-$A^b$-restricted viral protein epitopes, one recognized by $CD4^+$ T cells directed toward the nonstructural LT antigen and the other toward the VP1 capsid protein. Using MHC class II tetramers and intracellular cytokine assays, we found that virus-specific $CD4^+$ T cells to these epitopes differed quantitatively and qualitatively over the course of infection. Most notably, the VP1-specific $CD4^+$ T cell response dominated that directed toward LT but differed from the latter in terms of its profound early and sustained effector cytokine dysfunction, which was not offset by blockade of the PD-1:PD-L1 pathway. Despite this difference, the LT-specific and VP1-specific $CD4^+$ T cells populations are stably maintained during persistent infection. Data presented in this paper showing that MPyV-specific $CD4^+$ T cell populations exhibit little proliferation in persistently infected hosts with a substantial number suffering apoptosis, coupled with the finding that naïve $CD4^+$ T cells of both specificities are primed in persistent infection, suggest that de novo recruitment contributes to maintenance of MPyV-specific $CD4^+$ T cells in chronically infected hosts.

$CD4^+$ T-cell expansion and differentiation are governed by multiple factors, including level and duration of epitope availability, sustained colocalization with APCs, costimulation, and proinflammatory cytokines (44). For persistent viruses that comprise a host's "virome" additional factors merit consideration, such as viral tropism, kinetics of viral gene expression, state of the viral genome during persistent infection, and tactic(s) orchestrated by the virus to evade host surveillance (45). Characterization of MPyV-specific $CD4^+$ T cell responses to epitopes from a nonstructural and structural viral protein may offer insights into the relationship between polyomaviruses and their hosts. For example, although MPyV DNA is detectable long term at low levels in select tissues, whether it is maintained latently in an episomal state or if

infectious virus is continuously produced at low yield by semi-permissive cells remains an open question. As VP1 protein production is generally taken as evidence of productive infection, PD-1 and CTLA-4 upregulation by $VP1_{221}$-specific $CD4^+$ T cells in persistently infected mice, suggestive of repetitive Ag stimulation, favors the infectious state scenario. This possibility is further supported by evidence in this paper that naïve VP1-specific $CD4^+$ T cells are primed de novo during persistent infection.

Differences in functional competence by the $LT_{678}$-specific and the $VP1_{221}$-specific $CD4^+$ T cells could stem from differences in availability of MHC class II-restricted epitopes derived from nonstructural and structural viral proteins. Extending from studies showing a positive association between Ag levels and $CD8^+$ T cell dysfunction (46, 47), the effector cytokine defect of $VP1_{221}$-specific, but not $LT_{678}$-specific, $CD4^+$ T cells suggests more frequent TCR engagement by the former. This functional disparity may lie in differences in the range of potential APCs and the ability of these viral proteins to access the MHC class II processing machinery. Because polyomaviruses bind to sialyated glycolipids and glycoproteins, virions adsorb to a broad array of cells, irrespective of their capacity to support infection (48). Bound virions will thus undergo receptor-mediated endocytosis, which would facilitate shunting of capsid proteins into degradative and MHC class II peptide-loading compartments. In addition, the ratio of infectious to physical particles for MPyV in vitro is ~1:100, raising the possibility that defective virions (including empty capsids) could also contribute VP1-derived epitopes for MHC class II presentation. In contrast, as expression of nonstructural viral T proteins requires synthesis by infected cells, access to MHC class II compartments would most likely be relegated to less efficient nonreceptor-mediated uptake of protein debris from lytically infected cells.

Recent reports detailing Ag-specific $CD4^+$ T cell expansion, maintenance, and function to different experimental viral infections highlight variability, but also commonalities, among these responses. For example, infection by acutely resolved LCMV-Armstrong gives rise to virus-

specific memory $CD4^+$ T cells that then suffer progressive attrition, but without appreciable functional impairment (32). In contrast, virus-specific $CD4^+$ T cells to the persistently infecting mouse CMV are stably maintained during latent infection and retain polyfunctionality (17, 20). Examination of the I-$A^b$/$GP_{61-80}$-specific $CD4^+$ T cell response to LCMV clone 13, which establishes high-level persistent infection, revealed functional inactivation of these T cells as early as day 9 of infection (16). Analysis of the MPyV-specific $CD4^+$ T cell response yielded a composite of these quantitative and qualitative behaviors, with stable maintenance of virus-specific $CD4^+$ T cell populations during persistent infection yet with variations in functional integrity among epitope-specific responses. Reminiscent of the $GP_{61-80}$-specific $CD4^+$ T cell response to LCMV clone 13 infection, dysfunction of the MPyV $VP1_{221}$-specific $CD4^+$ T cell response was also evident during acute infection. Thus, events at the level of priming (e.g., epitope presentation by non-professional APCs) or soon thereafter (e.g., high epitope density and high TCR avidity) may handicap effector differentiation or force premature senescence, respectively, of a sizeable fraction of the $VP1_{221}$-specific $CD4^+$ T cell population.

Early dysfunction by the $VP1_{221}$-specific $CD4^+$ T cells may also be a consequence of the MPyV life cycle. Because expression of viral capsid proteins is a harbinger for virus-induced cell death, the duration of antigenic stimulation for capsid epitope-specific T cells may be shorter than for T cells recognizing epitopes from nonstructural T antigens, because these are also expressed by abortively infected cells. As a result, naïve $VP1_{221}$-specific $CD4^+$ T cells may be suboptimally primed by productively infected APCs, whereas naive $LT_{678}$-specific $CD4^+$ T cells may continue to receive stimulation by nonproductively infected APCs. This alternative scenario may account not only for the early functional deficiencies of the $VP1_{221}$-specific $CD4^+$ T cells, but also for their restricted TCR Vβ usage if only a subset of the naïve T cells having TCRs of sufficiently high avidity are recruited (40). Differences in MHC class II tetramer MFI at optimal concentrations have been taken as a measure of differential TCR binding affinity (49, 50). Thus,

in support of the above possibility, we found that I-$A^b$/$VP1_{221}$ tetramers stain splenic $CD4^+$ T cells from day 8 p.i. mice with significantly higher gMFI than $A^b$/$LT_{678}$ tetramers, with each population expressing equivalent TCR levels by surface anti-CD3ε staining (data not shown). Stronger TCR signaling by higher affinity $VP1_{221}$-specific $CD4^+$ T cells may also account for their higher level of expression of PD-1 and CTLA-4 receptors.

A central finding of this study is that naïve MPyV-specific $CD4^+$ T cells are recruited in persistently infected mice. This result is in line with recent reports demonstrating emergence of novel clonotypes of virus-specific $CD4^+$ T cells during persistent CMV infection in humans and rhesus macaques and evidence for de novo generation of virus-specific $CD4^+$ T cells in baboons persistently infected by baboon CMV and an EBV-like virus (51-53). Given the variability in priming conditions and history of antigenic exposure of individual virus-specific T cells over the course of a persistent infection, it is not surprising that a phenotypic snapshot of these cells in chronically infected hosts reveals heterogeneity in markers indicative of effector/effector-memory/central memory differentiation, as seen for MPyV-specific $CD4^+$ and $CD8^+$ T cells (Fig. 5) (25). Kinetic profiling of the MPyV-specific $CD4^+$ T cell response (Fig. 1C) is notable for its exceedingly shallow contraction phase, suggesting that naïve antiviral $CD4^+$ T cells may be vigorously recruited even at this early time point. Because more $LT_{678}$-specific than $VP1_{221}$-specific $CD4^+$ T cells bind the Annexin V marker of apoptosis (Fig. 5C), although both populations maintain stable numbers, it is also tempting to speculate that the efficiency for recruitment during persistent infection is higher for the $LT_{678}$-specific $CD4^+$ T cells. In addition, our data offers a possible explanation for the deterioration of the memory $CD4^+$ T cell pool in mice infected by LCMV-Armstrong (i.e., lack of I-$A^b$/$GP_{61-80}$ Ag for priming naïve $CD4^+$ T cells to replenish the virus-specific memory $CD4^+$ T cell compartment).

As we recently reported, $CD4^+$ T cells, although dispensable for expansion of MPyV-specific $CD8^+$ T cells during acute infection, are essential for de novo priming of antiviral naïve

$CD8^+$ T cells in persistently infected hosts (28). The low-level inflammatory milieu in persistent infection may shift the burden to $CD4^+$ T cells for licensing APCs to drive naïve virus-specific $CD8^+$ T cell differentiation. Collectively, our data favor the concept that ongoing recruitment of naïve virus-specific $CD4^+$ T cells is linked to priming of naïve antiviral $CD8^+$ T cells during persistent infection.

In summary, we have found marked differences between two viral epitope-specific $CD4^+$ T cell responses to MPyV infection. Interestingly, despite overall differences in magnitude, which is conventionally used to denote level of immunodominance, the $LT_{678}$ and $VP1_{221}$-specific $CD4^+$ T cell responses are nearly co-dominant based on IFN-γ function. Thus, at least with respect to persistent infections, functional rather than numerical attributes may be a more meaningful guide to positioning epitope-specific T cell populations within immunodominance hierarchies. It is also interesting to note that $CD4^+$ T cells directed to epitopes in LT and VP1 of the human BK polyomavirus are also detected in seropositive healthy individuals (54). Infections by BK and the related human polyomavirus, JC, are associated, respectively, with kidney transplant rejection and progressive multifocal leukoencephalopathy, a demyelinating disease seen in patients immunocompromised by HIV/AIDS or by agents that block T cell entry into the CNS (55, 56). Another implication of our data, then, is that immunomodulatory regimens designed to interdict T cell priming may interfere with recruitment of virus-specific naive T cells, shrinking the antiviral effector/memory T cell pool needed to keep otherwise silent persistent viral infections in check.

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**FOOTNOTES**

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[4] Abbreviations used in this paper: 7-AAD, 7-aminoactinomycin D; B6, C57BL/6; ICS, intracellular cytokine staining; LCMV, lymphocytic choriomeningitis virus; LT, large T antigen; gMFI, geometric mean fluorescence intensity; MPyV, mouse polyomavirus; PD-1, programmed death 1; PD-L1, PD-1 ligand 1; p.i., postinfection; PD-1, programmed death receptor-1; VP1, major capsid protein of MPyV.

**Figure Legends**

**FIGURE 1.** Identification of MPyV epitope-specific $CD4^+$ T cells. A, Splenic $LT_{678}$- and $VP1_{221}$-specific $CD4^+$ T cells from B6 mice at day 8 p.i. are detected by intracellular IFN-γ staining assays. Splenocytes were stimulated with the indicated peptide (50 μM) for 5.5 h, then surface-stained for CD4 and co-stained intracellularly for IFN-γ and CD154. Plots are gated on $CD4^+$ cells. B, Splenic $LT_{678}$- and $VP1_{221}$-specific $CD4^+$ T cells from B6 mice are detected by I-$A^b$ tetramer staining at day 8 p.i. but not in uninfected mice. Splenocytes were stained with the indicated I-$A^b$ tetramers then stained for CD4 and CD44. Tetramer plots are gated on $CD4^+$ 7-$AAD^-$ cells. Values indicate percentage of gated cells that co-stain with IFN-γ and CD154 (A) or I-$A^b$ tetramer and CD44 (B). Plots are representative of 3 experiments with 3 mice apiece. C, Time course of MPyV-specific $CD4^+$ T cell numbers in spleen as assessed by I-$A^b$/$LT_{678}$ and I-$A^b$/$VP1_{221}$ tetramer staining, with background staining by I-$A^b$/hCLIP tetramers subtracted. Values for each time point represent the mean ± SD of 6-9 mice from 2-3 independent experiments.

**FIGURE 2.** Differences in functional competence between $LT_{678}$-specific and $VP1_{221}$-specific $CD4^+$ T cells. A, Comparison of the frequency of I-$A^b$ tetramer versus intracellular IFN-γ staining of $LT_{678}$- and $VP1_{221}$-specific $CD4^+$ T cells over time p.i. At each time point, splenocytes were stained directly ex vivo with I-$A^b$/$LT_{678}$ and I-$A^b$/$VP1_{221}$ tetramers or stimulated with the indicated peptides and stained intracellularly for IFN-γ as in Fig. 1A. Differences between frequency of IFN-$\gamma^+$ and I-$A^b$ $tetramer^+$ $CD4^+$ T cells is statistically significant by the nonparametric Mann-Whitney U test ($p < 0.05$) at each time point for $VP1_{221}$-specific $CD4^+$ T cells and days 10 and 100 p.i. for $LT_{678}$-specific cells. B, Enumeration of splenic $LT_{678}$- and $VP1_{221}$- stimulated IFN-$\gamma^+$ $CD4^+$ T cells (with background intracellular IFN-γ staining by $CD4^+$ T

cells in absence of peptide subtracted) over the course of MPyV infection. Numbers and frequencies are of $CD4^+$ T cells that are I-$A^b$ $tetramer^+CD44^{hi}$ or IFN-$\gamma^+CD154^+$. Values for each time point represent the mean ± SD of 6-9 mice from 2-3 independent experiments.

**FIGURE 3.** Variations in effector cytokine profiles of $LT_{678}$-specific and $VP1_{221}$-specific $CD4^+$ T cells over the course of MPyV infection. At acute (day 8 p.i.) and persistent (day 60 p.i.) infection time points, splenocytes were stimulated with $LT_{678}$ or $VP1_{221}$ peptides for 5.5 h then stained for surface CD4 followed by co-staining for intracellular IFN-γ and TNF-α, IL-2, IL-10 or IL-21. A, Representative plots of IFN-γ/TNF-α or IFN-γ/IL-2 (*left panels*) and frequency of IFN-$\gamma^+$ $CD4^+$ cells that express TNF-α or IL-2 (*right graphs*). B, Frequency of IFN-$\gamma^+$ $CD4^+$ cells that express IL-10 or IL-21. Data in all graphs represent mean ± SD of 6 mice in 2 independent experiments. The *p* values were calculated using the nonparametic Mann-Whitney U test.

**FIGURE 4.** $LT_{678}$-specific and $VP1_{221}$-specific $CD4^+$ T cells differ in TCR repertoire. Vβ TCR chain repertoire analysis of $LT_{678}$- and $VP1_{221}$-specific $CD4^+$ T cells at day 8 p.i. (*upper* and *lower graphs*, respectively). Splenocytes were stained with either I-$A^b$/$LT_{678}$ or I-$A^b$/$VP1_{221}$ tetramers and then intracellularly with indicated Vβ TCR mAb. Each bar of same pattern shows the percentage of $tetramer^+$ cells from an individual mouse expressing the indicated Vβ TCR.

**FIGURE 5.** Phenotypic differences between $LT_{678}$-specific and $VP1_{221}$-specific $CD4^+$ T cells during persistent MPyV-infection. A, Representative histograms showing PD-1 and CTLA-4 expression by I-$A^b$/$LT_{678}$ and I-$A^b$/$VP1_{221}$ $tetramer^+$ $CD4^+$ T cells (black line) compared with naïve $CD44^{lo}$ $CD4^+$ cells (gray filled) from spleens of day 8 and 60 p.i. mice (*upper panels*) and

gMFI of staining by Abs for each of these receptors on tetramer$^+$ cells from individual mice at each of these time points (*lower panels*). CD62L, CD127, and CD27 (B), and Bcl-2 and Annexin V (C, gray line, isotype control) expression on I-A$^b$/LT$_{678}$ and I-A$^b$/VP1$_{221}$ tetramer$^+$ CD4$^+$ T cells (black line) from spleens of day 60 p.i. mice compared with CD44$^{lo}$ CD4$^+$ cells (gray filled). For Annexin V, plots are gated on live (7AAD$^-$) CD4$^+$ I-A$^b$ tetramer$^+$ CD44$^{hi}$ cells. D, Comparison of BrdU incorporation by LT$_{678}$- and VP1$_{221}$-specific CD4$^+$ T cells given daily i.p. injections of BrdU from days 33-40 p.i. (*upper panels*) or from days 0-7 p.i. (*lower panels*). Black line, tetramer$^+$ CD44$^{hi}$ CD4$^+$ T cells; gray filled, CD44$^{lo}$ CD4$^+$ T cells. Data are representative of 2 independent experiments of 3 mice each.

**FIGURE 6**. Naïve MPyV-specific CD4$^+$ T cells are primed de novo during persistent infection. Persistently infected (day 40 p.i.) busulfan-treated B6 (CD45.2$^+$) mice received 2 x 10$^7$ bone marrow cells from naïve B6/CD45.1 mice. A, At 90 days post-transplant (d130 p.i.), splenocytes were analyzed by I-A$^b$ tetramer staining. Donor (CD45.1$^+$)- and host (CD45.1$^-$)-derived I-A$^b$/VP1$_{221}$ (*top row*) and I-A$^b$/LT$_{678}$ (*middle row*) tetramer$^+$ CD4$^+$ T cells. Control I-A$^b$/CLIP tetramer staining of donor and host CD4$^+$ T cells (*bottom row*). All plots are gated on CD4$^+$CD45.1$^+$ (donor) or CD4$^+$CD45.1$^-$ (host) cells and numbers represent the percentage of gated cells that are tetramer$^+$CD44$^{hi}$. Data are representative of 2 experiments with 2-3 mice each. A total of 63.3 ± 24.0 % (mean ± SD) of splenic CD4$^+$ T cells were of donor origin (CD45.1$^+$). B, Frequency of I-A$^b$/LT$_{678}$ tetramer$^+$ or I-A$^b$/VP1$_{221}$ tetramer$^+$ CD4$^+$ T cells that are donor-derived.

**Figure 1**

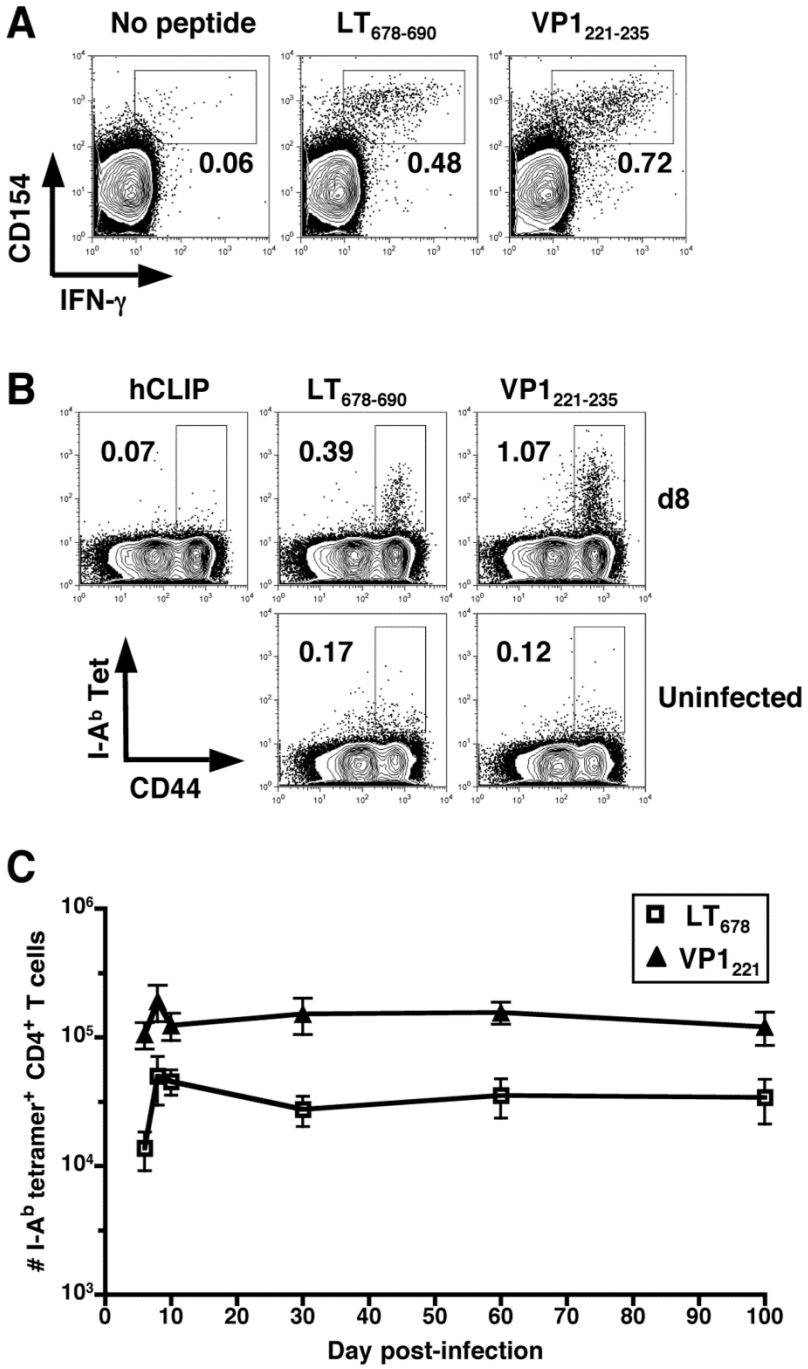

A
No peptide
LT678-690
VP1221-235
0.06
0.48
0.72
CD154
IFN-γ
B
hCLIP
LT678-690
VP1221-235
0.07
0.39
1.07
d8
0.17
0.12
Uninfected
I-Ab Tet
CD44
C
LT678
VP1221
# I-Ab tetramer+ CD4+ T cells
Day post-infection

**Figure 2**

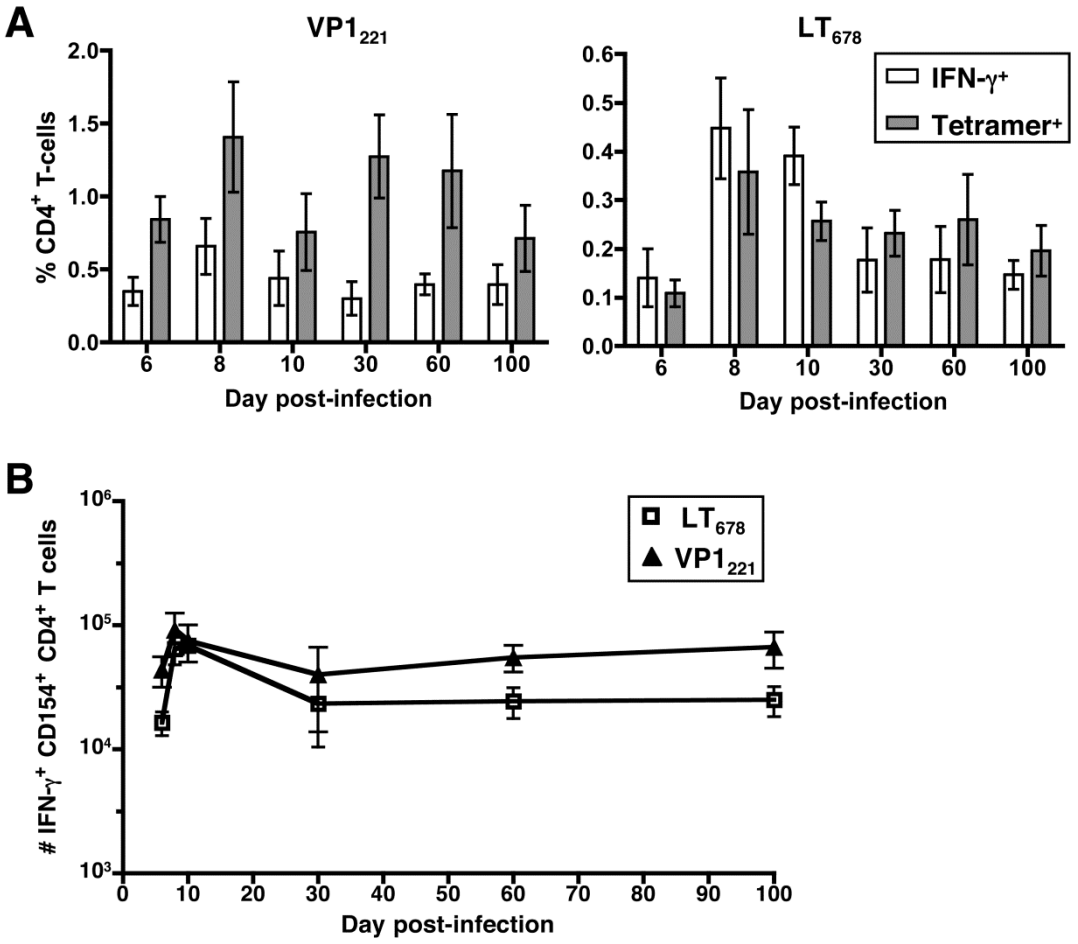
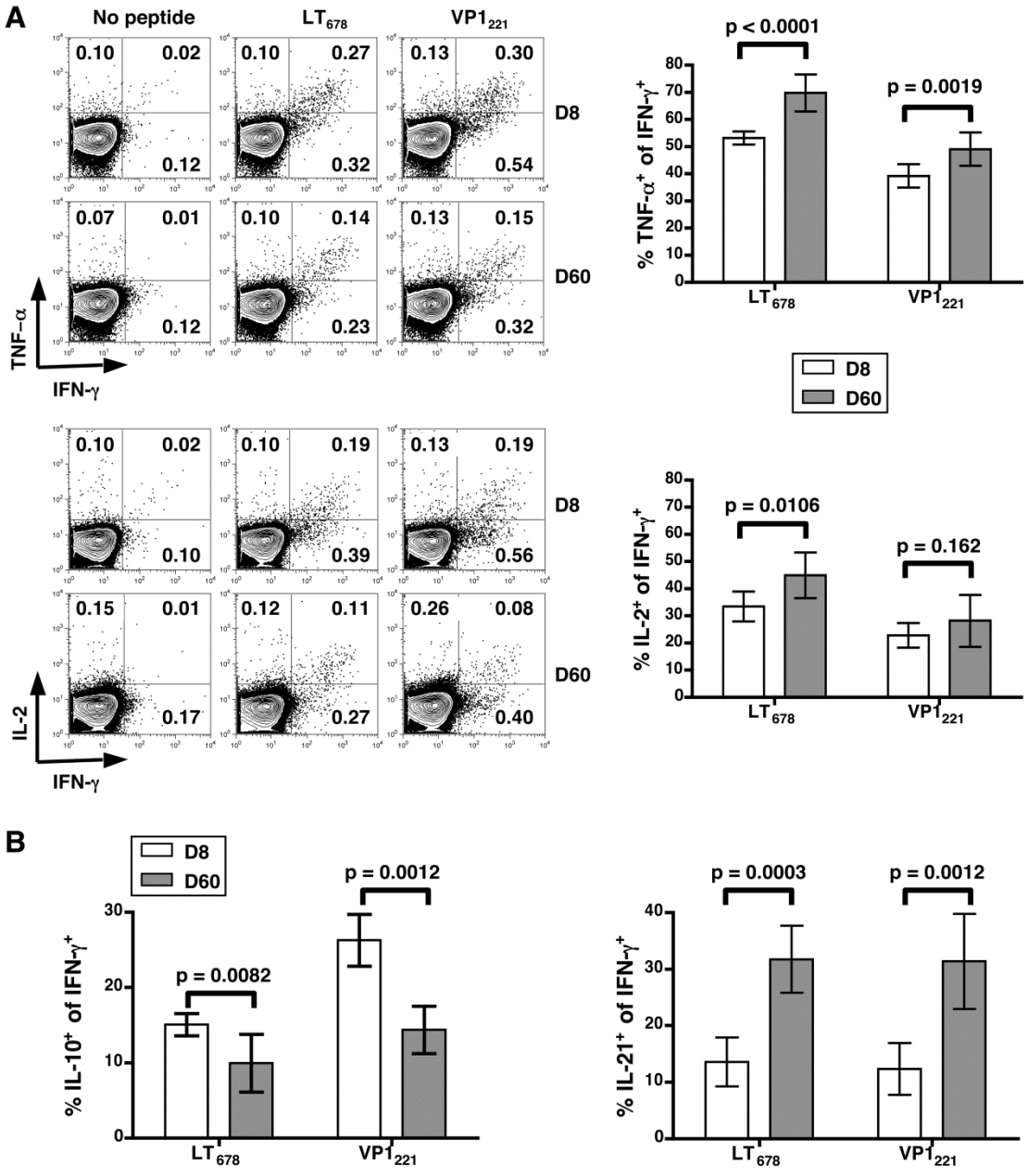

A
VP1221
LT678
IFN-γ+
Tetramer+
% CD4+ T-cells
Day post-infection
B
LT678
VP1221
# IFN-γ+ CD154+ CD4+ T cells
Day post-infection

**Figure 3**


A
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VP1221
0.10
0.02
0.10
0.27
0.13
0.30
D8
0.12
0.32
0.54
0.07
0.01
0.10
0.14
0.13
0.15
D60
0.12
0.23
0.32
TNF-α
IFN-γ
0.10
0.02
0.10
0.19
0.13
0.19
D8
0.10
0.39
0.56
0.15
0.01
0.12
0.11
0.26
0.08
D60
0.17
0.27
0.40
IL-2
IFN-γ
p < 0.0001
p = 0.0019
% TNF-α+ of IFN-γ+
LT678
VP1221
D8
D60
p = 0.0106
p = 0.162
% IL-2+ of IFN-γ+
LT678
VP1221
B
D8
D60
p = 0.0012
p = 0.0082
% IL-10+ of IFN-γ+
LT678
VP1221
p = 0.0003
p = 0.0012
% IL-21+ of IFN-γ+
LT678
VP1221

**Figure 4**

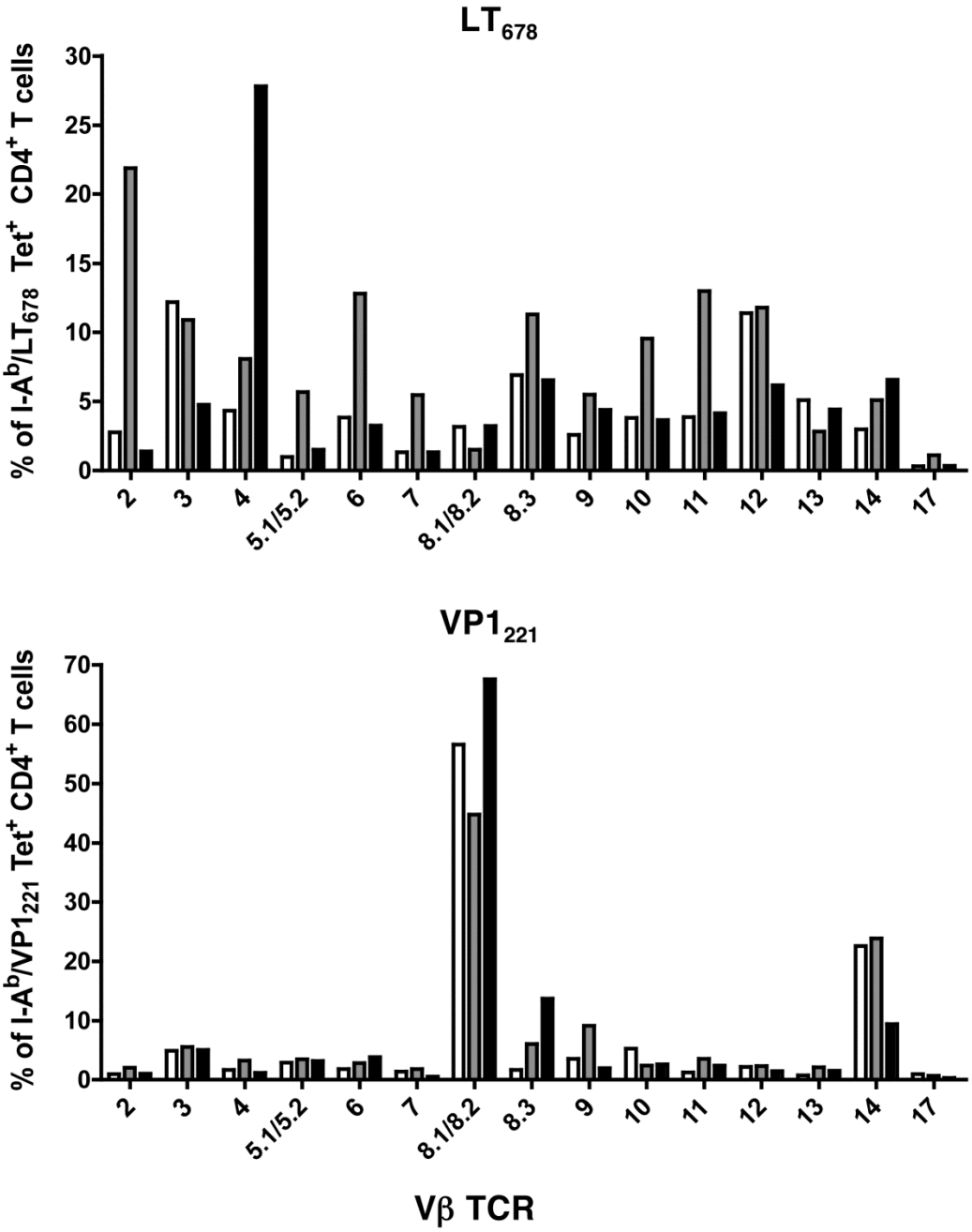

LT678
% of I-Ab/LT678 Tet+ CD4+ T cells
30
25
20
15
10
5
0
2
3
4
5.1/5.2
6
7
8.1/8.2
8.3
9
10
11
12
13
14
17
VP1221
% of I-Ab/VP1221 Tet+ CD4+ T cells
70
60
50
40
30
20
10
0
2
3
4
5.1/5.2
6
7
8.1/8.2
8.3
9
10
11
12
13
14
17
Vβ TCR

**Figure 5**

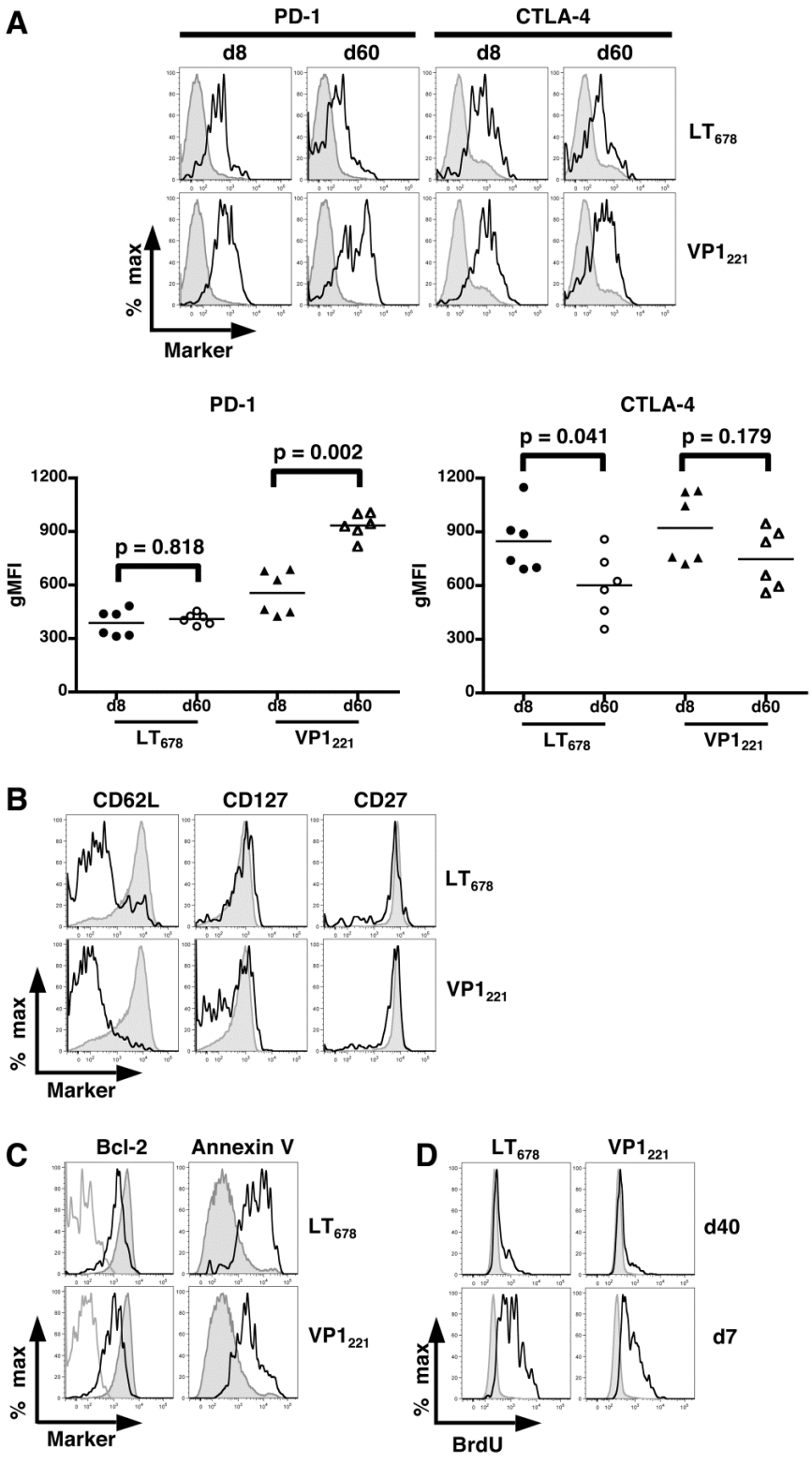
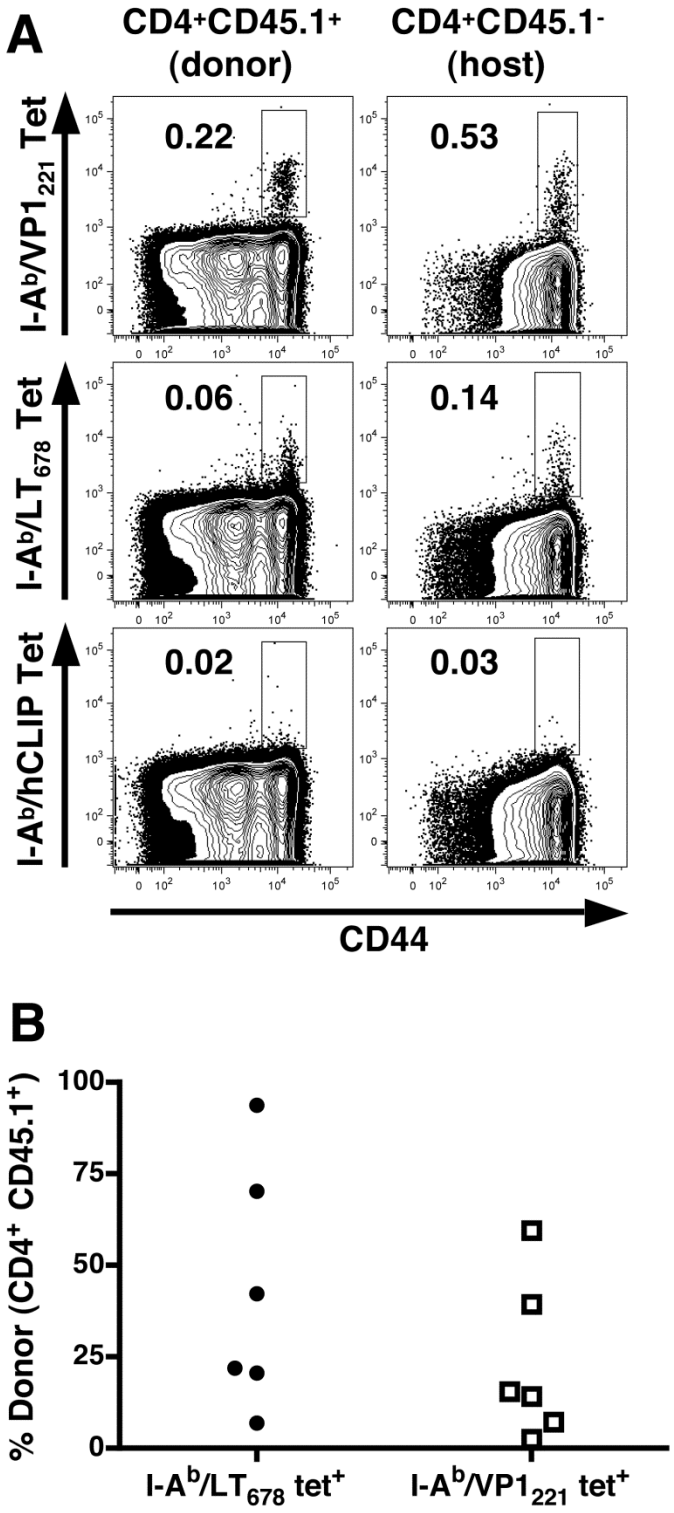

A
PD-1
CTLA-4
d8
d60
d8
d60
LT678
VP1221
% max
Marker
PD-1
CTLA-4
p = 0.002
p = 0.818
p = 0.041
p = 0.179
gMFI
1200
900
600
300
0
d8
d60
d8
d60
LT678
VP1221
B
CD62L
CD127
CD27
LT678
VP1221
% max
Marker
C
Bcl-2
Annexin V
LT678
VP1221
% max
Marker
D
LT678
VP1221
d40
d7
% max
BrdU

**Figure 6**


A
CD4+CD45.1+ (donor)
CD4+CD45.1- (host)
I-Ab/VP1221 Tet
I-Ab/LT678 Tet
I-Ab/hCLIP Tet
0.22
0.53
0.06
0.14
0.02
0.03
CD44
B
% Donor (CD4+ CD45.1+)
100
75
50
25
0
I-Ab/LT678 tet+
I-Ab/VP1221 tet+

**References**

1. Swain, S. L., J. N. Agrewala, D. M. Brown, D. M. Jelley-Gibbs, S. Golech, G. Huston, S. C. Jones, C. Kamperschroer, W. H. Lee, K. K. McKinstry, E. Roman, T. Strutt, and N. P. Weng. 2006. CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza. Immunol. Rev. 211:8-22.
2. Fazilleau, N., L. Mark, L. J. McHeyzer-Williams, and M. G. McHeyzer-Williams. 2009. Follicular helper T cells: lineage and location. Immunity 30:324-35.
3. Sette, A., M. Moutaftsi, J. Moyron-Quiroz, M. M. McCausland, D. H. Davies, R. J. Johnston, B. Peters, M. Rafii-El-Idrissi Benhnia, J. Hoffmann, H. P. Su, K. Singh, D. N. Garboczi, S. Head, H. Grey, P. L. Felgner, and S. Crotty. 2008. Selective CD4+ T cell help for antibody responses to a large viral pathogen: deterministic linkage of specificities. Immunity 28:847-58.
4. Castellino, F., and R. N. Germain. 2006. Cooperation between CD4+ and CD8+ T cells: when, where, and how. Annu. Rev. Immunol. 24:519-40.
5. Iijima, N., M. M. Linehan, M. Zamora, D. Butkus, R. Dunn, M. R. Kehry, T. M. Laufer, and A. Iwasaki. 2008. Dendritic cells and B cells maximize mucosal Th1 memory response to herpes simplex virus. J. Exp. Med. 205:3041-52.
6. Sparks-Thissen, R. L., D. C. Braaten, K. Hildner, T. L. Murphy, K. M. Murphy, and H. W. t. Virgin. 2005. CD4 T cell control of acute and latent murine gammaherpesvirus infection requires IFNgamma. Virology 338:201-8.
7. Stohlman, S. A., D. R. Hinton, B. Parra, R. Atkinson, and C. C. Bergmann. 2008. CD4 T cells contribute to virus control and pathology following central nervous system infection with neurotropic mouse hepatitis virus. J. Virol. 82:2130-9.

8. Brown, D. M., A. M. Dilzer, D. L. Meents, and S. L. Swain. 2006. CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. J. Immunol. 177:2888-98.

9. Jellison, E. R., S. K. Kim, and R. M. Welsh. 2005. Cutting edge: MHC class II-restricted killing in vivo during viral infection. J. Immunol. 174:614-8.

10. van de Berg, P. J., E. M. van Leeuwen, I. J. ten Berge, and R. van Lier. 2008. Cytotoxic human CD4(+) T cells. Curr. Opin.lmmunol. 20:339-43.

11. Harrington, L. E., K. M. Janowski, J. R. Oliver, A. J. Zajac, and C. T. Weaver. 2008. Memory CD4 T cells emerge from effector T-cell progenitors. Nature 452:356-60.

12. Lohning, M., A. N. Hegazy, D. D. Pinschewer, D. Busse, K. S. Lang, T. Hofer, A. Radbruch, R. M. Zinkernagel, and H. Hengartner. 2008. Long-lived virus-reactive memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors. J. Exp. Med. 205:53-61.

13. Younes, S. A., B. Yassine-Diab, A. R. Dumont, M. R. Boulassel, Z. Grossman, J. P. Routy, and R. P. Sekaly. 2003. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. J. Exp. Med. 198:1909-22.

14. Jones, R. B., L. C. Ndhlovu, J. D. Barbour, P. M. Sheth, A. R. Jha, B. R. Long, J. C. Wong, M. Satkunarajah, M. Schweneker, J. M. Chapman, G. Gyenes, B. Vali, M. D. Hyrcza, F. Y. Yue, C. Kovacs, A. Sassi, M. Loutfy, R. Halpenny, D. Persad, G. Spotts, F. M. Hecht, T. W. Chun, J. M. McCune, R. Kaul, J. M. Rini, D. F. Nixon, and M. A. Ostrowski. 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. J. Exp. Med. 205:2763-79.

15. Kaufmann, D. E., and B. D. Walker. 2009. PD-1 and CTLA-4 inhibitory cosignaling pathways in HIV infection and the potential for therapeutic intervention. J. Immunol. 182:5891-7.

16. Brooks, D. G., L. Teyton, M. B. Oldstone, and D. B. McGavern. 2005. Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. J. Virol. 79:10514-27.

17. Arens, R., P. Wang, J. Sidney, A. Loewendorf, A. Sette, S. P. Schoenberger, B. Peters, and C. A. Benedict. 2008. Cutting edge: murine cytomegalovirus induces a polyfunctional CD4 T cell response. J. Immunol. 180:6472-6.

18. Casazza, J. P., M. R. Betts, D. A. Price, M. L. Precopio, L. E. Ruff, J. M. Brenchley, B. J. Hill, M. Roederer, D. C. Douek, and R. A. Koup. 2006. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. J. Exp. Med. 203:2865-77.

19. Harari, A., F. Vallelian, P. R. Meylan, and G. Pantaleo. 2005. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. J. Immunol. 174:1037-45.

20. Walton, S. M., P. Wyrsch, M. W. Munks, A. Zimmermann, H. Hengel, A. B. Hill, and A. Oxenius. 2008. The dynamics of mouse cytomegalovirus-specific CD4 T cell responses during acute and latent infection. J. Immunol. 181:1128-34.

21. Fuller, M. J., A. Khanolkar, A. E. Tebo, and A. J. Zajac. 2004. Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. J. Immunol. 172:4204-14.

22. 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. 68:8056-63.

23. Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of activated T cells without effector function. J. Exp. Med. 188:2205-13.

24. Shin, H., S. D. Blackburn, J. N. Blattman, and E. J. Wherry. 2007. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. J. Exp. Med. 204:941-9.

25. 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. 203:2263-9.

26. Snyder, C. M., K. S. Cho, E. L. Bonnett, S. van Dommelen, G. R. Shellam, and A. B. Hill. 2008. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. Immunity 29:650-9.

27. 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. 174:7950-60.

28. 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. 179:1113-21.

29. 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. 160:1724-34.

30. Suto, A., D. Kashiwakuma, S. Kagami, K. Hirose, N. Watanabe, K. Yokote, Y. Saito, T. Nakayama, M. J. Grusby, I. Iwamoto, and H. Nakajima. 2008. Development and characterization of IL-21-producing CD4+ T cells. J. Exp. Med. 205:1369-79.

31. Frentsch, M., O. Arbach, D. Kirchhoff, B. Moewes, M. Worm, M. Rothe, A. Scheffold, and A. Thiel. 2005. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. Nat. Med. 11:1118-24.

32. Homann, D., L. Teyton, and M. B. Oldstone. 2001. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. Nat. Med. 7:913-9.

33. Pepper, M., J. L. Linehan, A. J. Pagan, T. Zell, T. Dileepan, P. P. Cleary, and M. K. Jenkins. 2009. Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. Nat. Immunol. 11:83-9.

34. Schiemann, M., V. Busch, K. Linkemann, K. M. Huster, and D. H. Busch. 2003. Differences in maintenance of CD8+ and CD4+ bacteria-specific effector-memory T cell populations. Eur. J. Immunol. 33:2875-85.

35. Ridgway, W., M. Fasso, and C. G. Fathman. 1998. Following antigen challenge, T cells up-regulate cell surface expression of CD4 in vitro and in vivo. J. Immunol. 161:714-20.

36. Sun, J., R. Madan, C. L. Karp, and T. J. Braciale. 2009. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. Nat. Med. 15:277-84.

37. Elsaesser, H., K. Sauer, and D. G. Brooks. 2009. IL-21 is required to control chronic viral infection. Science 324:1569-72.

38. 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 324:1576-80.

39. 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 324:1572-6.

40. Baumgartner, C. K., A. Ferrante, M. Nagaoka, J. Gorski, and L. P. Malherbe. 2010. Peptide-MHC class II complex stability governs CD4 T cell clonal selection. J. Immunol. 184:573-81.

41. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 439:682-7.

42. Blackburn, S. D., H. Shin, W. N. Haining, T. Zou, C. J. Workman, A. Polley, M. R. Betts, G. J. Freeman, D. A. Vignali, and E. J. Wherry. 2009. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat. Immunol. 10:29-37.

43. Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat. Immunol. 4:225-34.

44. Stockinger, B., C. Bourgeois, and G. Kassiotis. 2006. CD4+ memory T cells: functional differentiation and homeostasis. Immunol. Rev. 211:39-48.

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

46. Mueller, S. N., and R. Ahmed. 2009. High antigen levels are the cause of T cell exhaustion during chronic viral infection. Proc. Natl. Acad. Sci. USA 106:8623-8.

47. Wherry, E. J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. J. Virol. 78:5535-45.

48. 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 384:389-99.

49. Reichstetter, S., R. A. Ettinger, A. W. Liu, J. A. Gebe, G. T. Nepom, and W. W. Kwok. 2000. Distinct T cell interactions with HLA class II tetramers characterize a spectrum of TCR affinities in the human antigen-specific T cell response. J. Immunol. 165:6994-8.

50. Fazilleau, N., L. J. McHeyzer-Williams, H. Rosen, and M. G. McHeyzer-Williams. 2009. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nat. Immunol. 10:375-84.

51. Kalina, T., H. Lu, Z. Zhao, E. Blewett, D. P. Dittmer, J. Randolph-Habecker, D. G. Maloney, R. G. Andrews, H. P. Kiem, and J. Storek. 2005. De novo generation of CD4 T cells against viruses present in the host during immune reconstitution. Blood 105:2410-4.

52. Price, D. A., A. D. Bitmansour, J. B. Edgar, J. M. Walker, M. K. Axthelm, D. C. Douek, and L. J. Picker. 2008. Induction and evolution of cytomegalovirus-specific CD4+ T cell clonotypes in rhesus macaques. J. Immunol. 180:269-80.

53. van Leeuwen, E. M., E. B. Remmerswaal, M. H. Heemskerk, I. J. ten Berge, and R. A. van Lier. 2006. Strong selection of virus-specific cytotoxic CD4+ T-cell clones during primary human cytomegalovirus infection. Blood 108:3121-7.

54. Zhou, W., M. Sharma, J. Martinez, T. Srivastava, D. J. Diamond, W. Knowles, and S. F. Lacey. 2007. Functional characterization of BK virus-specific CD4+ T cells with cytotoxic potential in seropositive adults. Viral. Immunol. 20:379-88.

55. Major, E. O. 2009. Reemergence of PML in natalizumab-treated patients--new cases, same concerns. N. Engl. J. Med. 361:1041-3.

56. Ramos, E., C. B. Drachenberg, R. Wali, and H. H. Hirsch. 2009. The decade of polyomavirus BK-associated nephropathy: state of affairs. Transplantation 87:621-30.

# Chapter 3

## Assessing the impact of persistent infection on polyomavirus-specific T cells

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

**Abstract**

After clearance of an acute viral infection, stable memory T cell populations develop that are maintained independently of antigen by cytokine-driven homeostatic turnover. In contrast, memory $CD8^+$ T cells during chronic high load infections require persistent antigen for their self-renewal and fail to respond to homeostatic cytokines. Whether memory T cells formed during low-level persistent infections require antigen for their survival is less clear. Here we describe the development of a novel approach to ablating virus replication during persistent infection with mouse polyomavirus (MPyV). We generated a recombinant MPyV in which genes critical for replication are floxed (A2.Flx). Viral levels are reduced following Cre-mediated deletion in vitro and in mice expressing constitutive Cre. Utilizing mice expressing a tamoxifen-inducible Cre, we found that reduction of viral replication during persistent A2.Flx infection has an epitope-dependent effect on virus-specific $CD4^+$ and $CD8^+$ T cells. While major viral capsid protein VP1–specific $CD4^+$ T cells and nonstructural large T protein (LT)-specific $CD8^+$ T cells significantly decreased, LT-specific $CD4^+$ T cells displayed a shift toward $CD62L^{hi}$ central memory phenotype cells with less apparent loss. Our data suggests that antigen is not necessarily required for survival of MPyV-specific memory T cells and that cells can be redirected toward a phenotype associated with antigen-independent central memory T cells following reduction in replication.

**Introduction**

Following complete resolution of an acute viral infection, highly functional memory T cells gradually emerge from effector T cells that have survived the contraction phase (1, 2). Both $CD4^+$ and $CD8^+$ memory T cells are maintained long-term independently of antigen via slow homeostatic turnover driven by the cytokines IL-7 and IL-15 (3). Upon rechallenge infection, memory T cells mount rapid and robust recall responses marked by immediate elicitation of effector function and enhanced accumulation of antigen-specific secondary effectors compared to primary naïve responses (1, 2). However, during persistent viral infections, memory T cell homeostasis is greatly altered, and the memory-like T cell populations depend on persistent antigen for survival and maintenance (4). Virus-specific $CD8^+$ T cells during chronic lymphocytic choriomeningitis virus (LCMV) infection do not respond to IL-7 or IL-15 as they express low levels of the receptors CD127 (IL-7Rα) and CD122 (IL-15Rβ) (5). Instead these "chronic memory" cells are maintained by antigen-driven proliferation and do not survive in an antigen-free environment be it in naïve mice or mice chronically infected with an epitope variant (5, 6). Further support for antigen-dependent self-renewal comes from an elegant study that tracked virus-specific effector $CD8^+$ T cells indelibly marked by YFP during acute infection with murine herpesvirus 68 (MHV68), a model of latent infection, and showed that these cells persisted and continued to proliferate for several months (7). By contrast, in persistent mouse polyomavirus (MPyV) infection, virus-specific chronic memory $CD8^+$ T cells exhibit minimal proliferation and appear to survive poorly (8). Partial bone marrow chimera experiments showed that MPyV-specific $CD8^+$ T cells could be primed de novo during persistent infection (8, 9). Thus, de novo recruitment of virus-specific T cells could be a mechanism to replenish a continually deteriorating memory T cell population. The relative importance of de novo recruitment to chronic memory T cell maintenance compared to antigen-driven turnover remains unclear. However, as shown with mouse cytomegalovirus (MCMV)-specific memory T cell inflation, where populations display

short half-lives despite intermittent antigen-driven proliferation, both mechanisms can contribute (10).

In addition to alterations in maintenance of memory T cell populations, persistent viral infection also leads to changes in memory cell heterogeneity. Based on expression of lymphoid homing receptors CCR7 and CD62L, memory T cells can be divided into $CCR7^{hi}CD62L^{hi}$ central memory T ($T_{CM}$) cells, which tend to reside in lymph nodes as well as spleen, and $CCR7^{lo}CD62L^{lo}$ effector memory T ($T_{EM}$) cells that reside in the spleen and nonlymphoid tissues such as lung or gut (11). Although both are capable of making effector cytokines like IFN-γ and TNF-α upon restimulation (12, 13), $T_{CM}$ produce more IL-2, proliferate more robustly to antigen, and express higher levels of CD127, which correlate with increased homeostatic proliferation in response to IL-7 and improved survival, than $T_{EM}$, which are more terminally differentiated (14). Accumulating evidence suggests that $T_{CM}$ cells ultimately correlate with superior protection compared to $T_{EM}$ against infections and tumors (1). The memory T cell population generated after acutely resolved infections gradually but progressively shifts toward more $T_{CM}$ cells (1). In contrast, chronic memory T cell populations tend to display a $CD62L^{lo}$ $T_{EM}$ phenotype consistent with repetitive antigen stimulation (15, 16). Despite this, $CD62L^{hi}CD127^{hi}$ $T_{CM}$-like cells can emerge during some persistent viral infections by MPyV and MHV68 (8, 17). Development and survival of these cells may reflect the stimulation conditions that they encountered during priming or later as persistent infection progresses.

The level/strength of antigen:TCR signals and inflammation along with costimulation during priming critically influence the activation/differentiation of T cells. $CD4^+$ T cells require prolonged stimulation for their optimal expansion and differentiation (18). Increasing intraclonal competition for antigen and cytokines like IFN-γ by transferring larger numbers of antigen-specific $CD4^+$ T cell receptor (TCR) transgenic cells results in development of partially dysfunctional effector $CD4^+$ T cells and impaired memory formation or survival (19-21). Even in

cases in which intraclonal competition is not an issue, stronger TCR signaling during the primary response, as might be provided with greater antigen availability and/or higher TCR avidity, appears critical for optimal differentiation into $CD4^+$ memory T cells that survive long-term after resolution of acute infection (22). On the other hand, $CD8^+$ T cells can embark on expansion and an autonomous differentiation program with a shorter period of stimulation. Reducing the amount of stimulation to $CD8^+$ T cells, either by curtailing infection or increasing intraclonal competition, diminishes expansion but accelerates memory $CD8^+$ T cell generation, with the balance tilted toward more $CD62L^{hi}CD127^{hi}$ $T_{CM}$ cells (23-25). However, excess antigen and/or inflammatory signals leads to development of short-lived terminally differentiated effector T cells. Increased exposure to the proinflammatory cytokine IL-12, for instance, pushes LCMV-specific $CD8^+$ T cells to develop into KLRG-$1^{hi}CD127^{lo}$T-$bet^{hi}$ short-lived effector cells (SLECs) rather than KLRG-$1^{lo}CD127^{hi}$T-$bet^{lo}$ memory precursor effector cells (MPECs) (26) . Additional evidence that the level of antigen or inflammation impacts memory T cell differentiation comes from studies of latecomer $CD4^+$ and $CD8^+$ T cells, which encounter less antigen and inflammation than cells primed earlier, expand less but differentiate into memory T cells with an intermediately-differentiated $T_{CM}$ phenotype (27-30). During persistent infections, antigen and inflammatory signals continue to impact virus-specific T cell differentiation, leading to altered memory development and degradation of T cell functionality with increasing viral/antigen loads (4). A major question is whether fates of chronic memory T cells are fixed or if the cells retain some plasticity so that removal of persistent viral antigen leads to a restoration of the bona-fide memory T cell characteristics of antigen-independent, homeostatic cytokine-dependent maintenance.

We asked this question utilizing a model of systemic low-level persistent infection with MPyV. In our previous studies, we demonstrated that MPyV-specific $CD8^+$ and $CD4^+$ T cells mount polyfunctional responses and form stable chronic memory populations during persistent

infection (8, 9, 31). MPyV-specific chronic memory $CD4^+$ and $CD8^+$ T cells exhibited defects in self-renewal and survival, but de novo recruitment of naïve antigen-specific $CD4^+$ and $CD8^+$ T cells could be a critical mechanism for sustaining continually deteriorating chronic memory T cell pools. However, we have not fully ruled out the possibility that T cells primed earlier during the acute phase of infection could contribute through antigen-driven self-renewal. In addition, the different stimulation histories of cells primed earlier and more recently could account for the observed phenotypic heterogeneity of MPyV-specific $CD8^+$ T cells, including the emergence of $T_{CM}$-like cells late during the persistent phase of infection (8). We have now developed a novel recombinant MPyV in which genes critical for viral replication and persistence are "floxed". This floxed MPyV (A2.Flx) allows us to temporally ablate viral replication and reduce antigen load following Cre recombinase induction. Utilizing Cre/Esr mice, which expressing a tamoxifen-inducible Cre, we test the hypothesis that persistent MPyV antigen is required for the maintenance of virus-specific $CD4^+$ and $CD8^+$ T cell memory.

**Materials and Methods**

*Generation and propagation of A2.Flx*

The genome of the mouse polyomavirus (MPyV) strain A2 was used as the backbone for construction of A2.Flx. Recombinant A2.Flx was generated by insertion of loxP sequences at a unique BglI site in the noncoding region upstream of the T antigen start site and at the unique BlpI site in frame with the large T antigen (LT Ag). LoxP was inserted at the BlpI site by PCR with high-fidelity *Taq* polymerase (Invitrogen, Carlsbad, CA) using an overhanging forward primer that incorporated a BlpI recognition site (5'-GTGTTGCTGAGCCC***ATCACTTCGTATAATGTATGCTATACGAAGTTAT***ATGACAGCATATC-3') and a reverse primer encoding an EcoRI site (5'-TCAGAATTCGGGCCTGAACTTCC-3') (restriction sites underlined and loxP in bold italics). In order to retain the reading frame of LT, we made a single base pair substitution replacing a G in LT with the final T in loxP. The resulting PCR product was cloned into the BlpI/EcoRI restriction site of A2 SF-pUC19b, where the small fragment of BamHI/EcoRI-digested MPyV A2 genome was subcloned into a modified pUC19 vector in which the BglI site has been mutated. This clone, MPyV llox SF, was used as template for a second PCR to insert loxP at the BglI position using a forward primer incorporating the BglI site (5'-TGGCGCCTTGGAGGC***ATCACTTCGTATAATGTATGCTATACGAAGTTAT***GCTGTGGGGCCAC-3') and the same reverse primer used above. This PCR product was then cloned into the BglI/EcoRI site of A2 SF-pUC19b to generate A2.Flx SF-pUC19b. A2.Flx SF was excised from pUC19b by BamHI/EcoRI digestion and ligated with the large fragment generated by BamHI/EcoRI digestion of the MPyV A2 genome. The ligation product was then cloned into BamHI-digested pUC19 to generate full length A2.Flx cloned into pUC19. Following DNA sequencing verification, A2.Flx was excised by BamHI digestion and the ends religated to generate full length circular MPyV DNA, which was used for transfections of Balb/c A31

fibroblast cell lines by Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The resulting A2.Flx virus was subsequently propagated in primary baby mouse kidney cells and titered as previously described (32) .

*In vitro assay of Cre-mediated deletion of A2.Flx*

Subconfluent layers of nMuMG mouse mammary epithelial cell lines (ATCC, Manassas, VA) were infected with replication-deficient adenovirus expressing Cre recombinase (AdCre), a kind gift from Dr. Daniel Kalman (Emory University), at an MOI of 10 followed by infection 24 hrs later with wildtype MPyV strain A2 or A2.Flx at an MOI of 5. Forty-eight hours later, DNA was isolated according to the cell culture isolation protocol for the QIAamp DNA mini kit (Qiagen, Valencia, CA). Cre-mediated deletion of A2.Flx was assessed by PCR using A2floxed-F (5'-TCTCCACCCAGGCCTAGAATGTTT-3') and A2floxed R (5'-TGGCGTTGCATTGAATGAGCTCTG-3') primers, which flank the region that is floxed.

*Mice*

C57BL/6NCr (B6) female mice were obtained from the National Cancer Institute's Frederick Cancer Research and Development Center (Frederick, MD). B6.C-Tg (CMV-Cre) 1 Cgn/J (CMV-Cre) mice were purchased from Jackson Laboratories (Bar Harbor, ME). B6.Cg-Tg (CAG-Cre/Esr1*) 5 Amc/J (Cre/Esr) mice are originally from Jackson Laboratories and were obtained from Dr. David G. Harrison (Emory University). Cre/Esr mice express a fusion protein of Cre recombinase and a mutated ligand binding domain from mouse estrogen receptor (CreER$^{TM}$) under the control of a chimeric promoter consisting of a modified chicken β-actin promoter/enhancer and cytomegalovirus (CMV) immediate-early enhancer (33). CreER$^{TM}$

translocates to the nucleus in the presence of tamoxifen, thereby allowing Cre-mediated recombination. Cre/Esr mice were genotyped by PCR of DNA from tail clippings isolated according to manufacturer's protocol using the E.Z.N.A. Tissue DNA Kit (Omega-Biotek, Norcross, GA). PCR for Cre was performed using primers Cre For (5'-GCGGTCTGGCAGTAAAAACTATC-3') and Cre Rev (5'-GTGAAACAGCATTGCTGTACTT-3'). All mice were housed and bred in accordance with the guidelines of the Institutional Animal Care and Use Committee, and the Department of Animal Resources of Emory University.

*Mouse infections and tamoxifen administration*

Mice at ages 7-12 weeks received 3-6 x $10^4$ PFU of A2.Flx intraperitoneally (i.p.) or subcutaneously (s.c.) in the hind footpads. For experiments involving tamoxifen injection, tamoxifen free base (Sigma-Aldrich, St. Louis, MO) was dissolved in a 10% ethanol in corn oil solution at a concentration of 10 mg/mL. 2 mg of tamoxifen per 30 g mouse was injected i.p. once daily for 5-10 consecutive days. Cre negative littermates or B6 mice given tamoxifen were used as controls.

*Cell isolation and flow cytometry*

Splenocytes were isolated as previously described (31). In brief, spleens were digested in a solution of collagenase, hyaluronidase, and Dnase I prior to passage through cell strainers and red blood cell lysis.

Staining with allophycocyanin (APC)-conjugated $D^b/LT_{359-368}$, $I\text{-}A^b/LT_{678-690}$, and $I\text{-}A^b/VP1_{221-235}$ tetramers was performed as previously described (9, 31). All tetramers were

constructed by the National Institutes of Health Tetramer Core Facility at Emory University (Atlanta, GA). In some experiments, 7-actinomycin D (7-AAD, BD Pharmingen, San Diego, CA) was used to stain cells after I-$A^b$ tetramer and surface marker staining. For CTLA-4 and Bcl-2 staining, cells were first stained with tetramers and for surface markers CD8α or CD4 and CD44. They were then permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen) prior to staining with antibodies for CTLA-4 or Bcl-2. For intracellular cytokine staining assays, cells were stimulated for 5.5 hr with 10 μM $LT_{678-690}$, 50 μM $VP1_{221-235}$, or 1 μM $LT_{359-368}$ peptide in the presence of brefeldin A (Golgi-Plug, BD Pharmingen) prior to staining as described in earlier work (9, 31). $LT_{678-690}$ and $VP1_{221-235}$ stimulated cells were stained for CD4, IFN-γ, IL-2, and CD154, whereas $LT_{359-368}$ stimulated cells were stained for CD8, IFN-γ, and IL-2.

All mAbs and isotype controls used for staining were purchased from BD Pharmingen or Ebiosciences (San Diego, CA) unless otherwise indicated: FITC-conjugated anti-CD44 (IM7), CD4 (RM4-5), CD8α (53-6.7), CD62L, CD27 (LG.7F9), IFN-γ; PE-conjugated anti-CD127 (A7R34), PD-1 (RMP1-30), CD154, CTLA-4, Bcl-2, CD43 (Biolegend, San Diego, CA), and KLRG-1 (Southern Biotech, Birmingham, AL); Per-CP-conjugated mAb for CD8α; PerCP-Cy5.5-conjugated anti-CD4 and IL-2; V450-conjugated mAb for CD44; and V500-conjugated anti-CD4. Samples were acquired using a BD Biosciences LSR-II, and data analyzed using FloJo software (Treestar, Ashland, OR).

*Quantification of MPyV genomes*

DNA isolation and TaqMan quantitative real-time PCR (qPCR) were performed as described (9) except for a modified TaqMan probe. The TaqMan probe 5'-AGACGAAATCCTTGTGTTGCTGAGC-3' corresponds to nt 1061-1085 of wildtype MPyV strain A2 (Genbank Acc. # J02288.1) and lies immediately upstream of the BlpI restriction site.

MPyV DNA quantity is expressed in genome copies per milligram of tissue and is calculated based on a standard curve of known MPyV genome copy number vs. threshold cycle of detection. The detection limit with this assay is 10 copies of genomic viral DNA.

*Statistics*

Statistical significance was determined by a two-tailed nonparametric Mann-Whitney *U* test using Prism software (GraphPad Software, La Jolla, CA). A $p$-value $< 0.05$ was considered statistically significant.

## Results

### *Development of A2.Flx*

As no approved effective direct anti-MPyV drugs are available, we created a novel recombinant MPyV that would allow us to make use of the Cre/lox system (34) to temporally ablate viral replication and reduce viral antigen. The genome of MPyV consists of a 5 kb circular double-stranded DNA plasmid that has been fully mapped (35-37), attributes permitting use of standard molecular DNA techniques to generate mutants and insert short sequences such as epitopes. The nonstructural T-antigens small, middle, and large T (ST, MT, LT), which are encoded within a single alternatively-spliced mRNA transcript, are critical for viral replication and persistence (38). Utilizing PCR, we inserted two directly repeated 34 bp loxP sites into the genome of wildtype MPyV strain A2, one at the BglI site in the noncoding region upstream of the T-antigen start site and a second at the BlpI site, which was inserted in frame with LT through a single base pair substitution (Fig 1A). The BlpI restriction site is located within sequence shared by MT and LT. This region was shown to tolerate a 66 bp deletion without impacting viral pathogenesis in vitro (39). We thus expected that loxP sequences inserted at these sites would minimally impact virus replication. Permissive A31 mouse fibroblast cell lines were transfected with DNA for this floxed MPyV (A2.Flx) and an initial batch of virus isolated. The stock of A2.Flx was further amplified in primary baby mouse kidney (BMKs) cells. Despite serial rounds of amplification, we observed titers that were ~2 logs less than typically seen for wildtype virus (data not shown), indicating that this virus is attenuated.

Nonetheless, we first tested whether these loxP sites were functional in vitro. NMuMG mouse mammary gland cell lines were infected with A2.Flx or wildtype MPyV in the presence or absence of co-infection with a replication-deficient adenovirus expressing Cre recombinase (AdCre). Cre-mediated deletion was assessed by PCR using primers flanking the floxed region

(Figure 1A). As shown in Figure 1B, the floxed region in A2.Flx was deleted only in cells co-infected with AdCre, whereas wildtype MPyV was unaffected by its presence. Yet there still remained residual DNA corresponding to intact MPyV from the A2.Flx/AdCre co-infected cells, which was similar in size to that detected from cells infected with A2.Flx alone. That band in turn is clearly larger than the band from wildtype-infected cells. Furthermore, sequencing of the A2.Flx stock used and DNA from cells infected with that virus alone revealed the presence of only DNA from A2.Flx. Thus, we believe that the residual intact MPyV band does not indicate contamination by wildtype MPyV and instead corresponds with intact A2.Flx that was not targeted perhaps because of insufficient amounts of Cre or inefficient recombination. In line with the Cre-mediated deletion results, measuring viral genome copies by quantitative real-time PCR (qPCR) of LT showed a nearly two-fold drop in MPyV levels in cells co-infected with A2.Flx and AdCre compared to those infected with just A2.Flx (Figure 1C). These in vitro experiments demonstrate the feasibility of using the Cre-mediated recombination approach to intentionally ablate MPyV replication.

*A2.Flx induces MPyV-specific $CD8^+$ and $CD4^+$T cell responses despite constitutive Cre expression*

To ensure that A2.Flx could induce an anti-viral immune response, we infected wildtype B6 mice. B6 background CMV-Cre mice, which constitutively express Cre under the control of a human CMV minimal promoter (40), were also infected with A2.Flx. We hypothesized that expression of Cre would prevent establishment of an infection and generation of a T cell response as the viral genome would be immediately targeted upon entry into the nucleus of an infected cell. Contrary to expectations, A2.Flx was still able to establish an infection in CMV-Cre mice. However, assessment of viral levels by qPCR at d20 post-infection (p.i.) showed nearly half as many copies

of MPyV DNA in the spleens of CMV-Cre mice compared with B6 (Figure 2A). Thus, it appears that we can also blunt A2.Flx replication in vivo in Cre-expressing mice.

Despite the decrease in viral levels, the impact on the H-$2^b$-restricted MPyV-specific T cell response varied by epitope (Figure 2B and C). While the frequency of $D^b$-restricted $LT_{359}$-specific $CD8^+$ T cells and I-$A^b$-restricted $LT_{678}$-specific $CD4^+$ T cells showed no change between B6 and CMV-Cre mice, there were nearly two-fold less $VP1_{221}$-specific $CD4^+$ T cells in CMV-Cre than B6 control mice regardless if detected by tetramer or IFN- γ production (Figure 2C). We previously reported that $VP1_{221}$-specific $CD4^+$ T cells appear to be partly dysfunctional as there were only ~50% as many IFN-γ-producing as $tetramer^+$ cells; the frequency of cells upregulating CD154 (CD40L), which is transiently upregulated by recently antigen-activated $CD4^+$ T cells (41), was similar to IFN-γ (31). However, we detected no shift in the overall ratio of IFN-$\gamma^+$ or $CD154^+$ $VP1_{221}$-responding cells to I-$A^b$/$VP1_{221}$ $tetramer^+$ cells (Figure 2C and data not shown). As viral levels were only reduced by half in CMV-Cre mice, it is possible that the residual VP1 antigen may still be high enough to impair T cell function. Alternatively, the dysfunction may be an effect of factors during initial priming and not a reaction to continued antigen stimulation. Nonetheless, as VP1 expression is associated with productive replication, the decrease in $VP1_{221}$-specific $CD4^+$ T cells in the $Cre^+$ compared with B6 mice could be interpreted as an indicator of reductions in viral replication.

*Persistent viral antigen is critical for maintenance of MPyV-specific chronic memory T cells*

To determine the impact of depleting MPyV antigen during persistent infection on virus-specific $CD8^+$ and $CD4^+$ chronic memory T cell populations, we infected Cre/Esr mice, which express tamoxifen-inducible Cre recombinase, and transgene negative littermate controls with A2.Flx then waited until persistent infection (~d35 p.i.) before administering tamoxifen (Figure 3A). We

reasoned that the lower level of virus during persistent infection would make Cre deletion more efficient. As these mice are backcrossed onto a C57BL/6 background for >5 generations, we were able to monitor the fate of $D^b$-restricted $LT_{359}$-specific $CD8^+$ T cells as well as I-$A^b$-restricted $LT_{678}$- and $VP1_{221}$-specific $CD4^+$ T cells in these mice. One month after the last dose of tamoxifen, we detected a modest approximately two-fold decrease in the number of tetramer$^+$ $LT_{359}$-specific $CD8^+$ T cells and $VP1_{221}$-specific $CD4^+$ T cells in Cre/Esr mice compared with littermates, although the difference in $LT_{678}$-specific $CD4^+$ T cells is less evident (Figure 3B). Similar epitope-specific decreases were evident when comparing the antigen-specific $CD4^+$ and $CD8^+$ T cells by IFN-γ production (Figure 4A). Despite the diminished number of virus-specific $CD4^=$ T cells, the immunodominance hierarchy was not altered and remained $VP1_{221}$>$LT_{678}$ based on tetramer. Unfortunately, no discernible differences in viral levels as assessed by qPCR for LT was observed in the spleens, kidneys, hearts, or lungs of Cre/Esr versus littermate control mice, with several mice in both groups having nearly undetectable MPyV genome copies (Figure 3C and data not shown). However, extending from the finding of reduced viral levels and numbers of VP1-specific $CD4^+$ T cells in the CMV-Cre compared with B6 mice, the significant decrease in $VP1_{221}$-specific $CD4^+$ T cells in Cre/Esr mice compared with littermate controls suggests that there may indeed be less MPyV replication and antigen in the Cre/Esr mice.

*Epitope variable impact of persistent antigen reduction on MPyV-specific T cell phenotype and function*

As progressive functional exhaustion of T cells, often starting with a diminished ability to produce IL-2, is associated with increasing levels of persistent antigen (4), we asked whether the presumed diminishment of MPyV antigen led to improvements in functional competence of virus-specific T cells. As shown in Figure 4A, the functional competence of $VP1_{221}$-specific

$CD4^+$ T cells did not improve in mice with activated Cre; functionality of $LT_{359}$-specific $CD8^+$ T cells and $LT_{678}$-specific $CD4^+$ T cells remained unaltered as well. In addition, there was no change in the overall ability of any of these MPyV-specific T cells to co-produce IL-2 with IFN-γ (Fig 4B). Measurement of functional avidity of $LT_{359}$-specific $CD8^+$ T cells also failed to uncover any differences (data not shown).

We also wondered if there might be an accelerated shift toward development of virus-specific T cells with a central memory T cell ($T_{CM}$) phenotype as opposed to that of the more prevalent effector memory T cells ($T_{EM}$). While the relative proportion of $CD62L^{hi}CD127^{hi}$ $T_{CM}$, $CD62L^{lo}CD127^{hi}$ $T_{EM}$, and $CD62L^{lo}CD127^{lo}$ effector T ($T_{Eff}$) cells was similar in Cre/Esr mice and littermates for the $VP1_{221}$-specific $CD4^+$ T cells, there appeared to be a significant increase in the proportion of $T_{CM}$ phenotype $LT_{678}$-specific $CD4^+$ T cells and a significant drop in the frequency of antigen-specific $T_{EM}$ cells in the mice with tamoxifen-induced Cre (Figure 5A). The proportion of $T_{CM}$ cells making up the $D^b/LT_{359}$ $tetramer^+$ $CD8^+$ T cell population did not noticeably increase in Cre/Esr mice, but the frequency of $T_{EM}$ cells was reduced nearly 3-fold. Looking at just CD127 expression, we did not observe a change in the frequency of cells expressing CD127 in any population, which correlated with a lack of difference in expression of the pro-survival molecule Bcl-2 (Fig 5B and data not shown). We also did not observe variation in expression of other markers associated with the effector-memory transition such as CD27, CD43, and the senescence marker KLRG-1, which is expressed on $CD8^+$ SLECs as well virus-specific $CD8^+$ T cells in several persistent infections (data not shown). Thus, with the possible exception of $I\text{-}A^b/LT_{678}$-specific $CD4^+$ T cells, there was no appreciable shift toward a higher ratio of $T_{CM}$ to $T_{EM}$ cells despite the presumed decrease in viral antigen in Cre/Esr mice.

**Discussion**

During high load chronic infections such as with chronically infecting strains of LCMV, the function of virus-specific T cells is degraded, and memory T cells develop that appear to require antigen for their self-renewal and survival (4). Whether memory T cells to low-level persistent infections become fully dependent on antigen for their maintenance is less clear. Natural mouse polyomavirus (MPyV) provides an excellent model to study the response of virus-specific T cells to low-level persistent infection. As reported here, we have taken advantage of the virus' ease of genetic manipulation and knowledge about regulation of its replication to develop recombinant MPyVA2.Flx, in which genes critical for replication and persistence are floxed. We show that Cre-mediated deletion of the floxed region leads to a decrease in viral DNA levels in vitro, which also occurs in vivo in A2.Flx-infected Cre-expressing mice. Utilizing transgenic mice engineered to express a tamoxifen-inducible Cre, we find that targeting viral replication during persistent infection has an epitope-dependent impact on virus-specific chronic memory $CD4^+$ and $CD8^+$ T cells. Both $CD8^+$ T cells specific for nonstructural large T (LT) antigen and viral capsid (VP)-specific $CD4^+$ T cells were notably reduced. Although a decrease in LT-specific $CD4^+$ T cells was less obvious, there appeared to be a shift toward more cells bearing a central memory T cell phenotype. These results suggest that some MPyV-specific T cells are dependent on MPyV antigen for their persistence, whereas others retain the potential to differentiate into cells with phenotypes associated with bona fide memory cells capable of antigen-independent survival and robust recall responses.

Drugs have been used in several model systems to reduce or eliminate pathogen load and inflammation. Treatment with ribavirin of mice early in infection with a chronic strain of LCMV, an RNA virus, resulted in blunted viral titers and a rescue in $CD4^+$ T cell effector cytokine functionality (42). More recently, the Tarleton group used benznidazole to cure mice of persistent infection by *T. cruzi*, the causative agent of Chagas disease, and found that a stable, antigen-

independent central memory $CD8^+$ T cell population emerged, which could mount a robust protective recall response (43). Our rationale for developing the floxed MPyV A2.Flx stems from the lack of available drugs having a demonstrable direct effect against polyomaviruses. We find that Cre-mediated deletion of floxed genes essential for viral replication appears to be a viable alternative for reducing levels of MPyV both in vitro and in vivo. However, a caveat is that although we did not believe insertion in a noncoding region and a region reported to tolerate a 66 bp deletion (39) would significantly impact viral growth, we found that A2.Flx is highly attenuated compared to wildtype MPyV even when propagated on highly permissive baby mouse kidney cells. This may account for the difficulties we see in detecting viral DNA by Taqman qPCR in tissues in B6 or Cre negative control mice later in persistent infection (e.g. d60 p.i.). Our group typically measures MPyV levels using Taqman qPCR for LT, but due to the disruption of the normal LT-specific Taqman probe by the downstream loxP site near the BlpI restriction site (Figure 1A), we had to design a new LT-specific probe that hybridized upstream of the BlpI site. This probe is far less efficient at detecting even wildtype viral DNA compared with the normal probe (unpublished data) and probably compounds the problem. We have recently acquired qPCR primers specific for the major viral capsid VP1 from one of our collaborators (E. Szomolanyi-Tsuda, UMass Medical School), and it remains to be seen if these will improve our detection of A2.Flx in vivo since VP1 is untouched.

Attenuation of A2.Flx could stem from defects in the functions of LT, which is required for initiation of MPyV DNA replication and plays critical roles in controlling viral gene transcription including activating transcription of viral capsid genes (44). Replication and transcription are controlled from the noncoding region (NCR) between the coding sequences for T antigens (early genes) and viral capsid proteins (late genes). The NCR consists of a core origin of replication (ori), promoter elements, and enhancer elements (45-49). LT binds to sequences within these regulatory elements with the consensus motif of G(A/G)GGC (50) and recruits the

host cell transcriptional and DNA replication machinery (44). In addition, LT binding of the retinoblastoma protein (pRb) inactivates it and promotes the G1 → S transition (51). Based on DNA and protein sequence analysis, the BlpI site at which we inserted one loxP sequence does not encode residues critical for LT function, but it lies between the nuclear localization signal and a pRb binding site (44). By substituting one base to insert the 34 bp loxP sequence in frame with LT, we added 11 extra amino acids and changed an aspartate (D) into a tyrosine (Y). Those additions could possibly alter folding of LT and negatively impact activity of functional domains. Interestingly, although they did not observe a significant difference in viral replication based on in vitro growth curves, Bendig et al. reported that their mutant with the 66 bp deletion in the region where the BlpI site is located synthesized 60% as much viral DNA as wildtype (39). Thus, changes at this site are not as innocuous as previously thought. It is also possible that the attenuation of A2.Flx may also be due to disruptions of key cis-regulatory elements in the NCR. However, the loxP site inserted adjacent to the BglI site does not disrupt any LT binding sites, although one is in the BglI recognition sequence and another is immediately downstream (50). Even if there was a disruption of these sites, this alone would not be enough to account for the severe attenuation of A2.Flx we have observed. In addition, this site does not include any replication or transcription control elements. Therefore, it appears unlikely that this loxP insertion is detrimental to MPyV replication. Nonetheless, we did not test whether any one of these insertions alone could attenuate the virus.

The severe attenuation of A2.Flx, especially in vivo, is a glaring defect in the system that needs to be rectified. We need to redesign floxed polyomavirus so that it is far less attenuated and more detectable in normal, persistently infected mice. If the current placement of loxP at the BglI site in the NCR indeed does not significantly impair MPyV replication, that site could be left alone. To avoid disruption of protein coding sequence, the second loxP site could be inserted in the NCR but on the late gene side, where the viral capsid proteins are encoded. However, we

would need to be cautious and ensure the loxP site does not disrupt the late gene promoter sequences that lie in the enhancer (45) or the mRNA processing signals, including the mRNA leader sequence (52, 53). This leaves a stretch of 10-15 bp between the transcription start sites for the viral capsid genes and the end of the leader sequence as a potential site to insert a second loxP. Due to a lack of a nearby unique restriction enzyme site, this loxP may have to be inserted using methods other than the one we utilized to generate the original A2.Flx. We could make use of the sequence and ligation independent cloning (SLIC) method with mixed PCR inserts developed by Li and Elledge (54). If we can generate an A2.Flx v2 virus that is far less attenuated, we could splice out nearly the entire NCR following Cre recombination, which would terminate both replication and transcription.

Even if we could demonstrate that Cre activation during persistent infection ablates A2.Flx replication, the question remains as to why MPyV epitope-specific T cells did not react the same way. Our group previously observed that MPyV-specific chronic memory $CD8^+$ T cells poorly self-renew and survive despite the presence of persistent antigen, which puts an onus on de novo recruitment of antigen-specific $CD8^+$ T cells during persistent infection for maintenance (8). MPyV-specific $CD4^+$ T cells during persistent infection also appeared to display this behavior (31). Thus, during persistent MPyV infection, virus-specific T cell populations comprise a hodge-podge of cells primed under different antigenic/inflammatory conditions and having a different history of persistent stimulation. T cells primed early during high antigen and inflammation conditions will tend to be more terminally differentiated, short-lived effector/effector-memory T cells, whereas de novo primed naïve antigen-specific T cells encounter less antigen/inflammation and differentiate toward a central memory T cell phenotype (8, 9) as has been shown for latecomer T cells (27-30). However, this occurs under the shadow of repetitive antigen stimulation, which has been shown to increase T cell differentiation toward a more $CD62L^{lo}$ effector/effector-memory T cell phenotype, diminishes cytokine production especially of IL-2,

increases expression of senescent markers such as KLRG-1 (on $CD8^+$ T cells), and eventually degrades proliferative potential (15, 16). How much repetitive stimulation impacts epitope-specific T cells may depend on differences in the strength of antigenic signaling. Both $LT_{359}$-specific $CD8^+$ T cells and $VP1_{221}$-specific $CD4^+$ T cells display a more activated phenotype than $LT_{678}$-specific $CD4^+$ T cells during early to middle persistent infection with wildtype MPyV (d30-60 p.i.) as evidenced by a notable frequency of cells with low expression of CD127 and higher amounts of inhibitory receptors like PD-1 ($VP1_{221}$) or KLRG-1 ($LT_{359}$) (8, 31). As a result of less stimulation, the $LT_{678}$-specific $CD4^+$ T cells may retain some plasticity and acquire a central memory phenotype after a fall in antigen. On the other hand, more stimulated and earlier primed $VP1_{221}$- and $LT_{359}$-specific T cells die, leaving residual populations of presumably more recently primed cells for which the fate is currently unclear. These cells could continue to decline or develop into stable, antigen-independent memory T cells. Transfer of sorted cells into naïve or MPyV-infected mice could provide an answer.

Admittedly, the changes in the number of virus-specific T cells we detect are fairly modest. The $LT_{359}$-specific $CD8^+$ T cells and $VP1_{221}$-specific $CD4^+$ T cells only decreased about 2 fold in Cre/Esr mice compared to littermates, while $LT_{678}$-specific $CD4^+$ T cells appeared to decrease less obviously (Figure 3B). The residual intact A2.Flx DNA we detect in our in vitro Cre-mediated recombination assays (Figure 1B) suggests that the ratio of Cre to target DNA is critical in determining the efficiency of the Cre reaction. The stoichiometry of Cre recombination has been calculated to be 4 molecules of Cre per recombination event (34). An increase in the ratio of Ad-Cre versus A2.Flx per infected cell may lead to more Cre-mediated deletion and a larger drop in MPyV replication. Increasing Cre induction in Cre/Esr mice by administering more tamoxifen may also result in effects that are more visible (33). An experiment is currently underway in which Cre/Esr and littermate mice persistently infected with A2.Flx (d35 p.i.) have been given 10 rather than the 5 consecutive injections of tamoxifen initially used (Figure 3A).

Even if a more dramatic effect on chronic memory MPyV-specific T cells is detected, we are still left with the question of whether the loss in cells was due to an inability to de novo prime naïve antigen-specific cells or the death of  T cells primed earlier that required antigen for their self-renewal and survival. In addition, residual T cells would themselves still consist of cells with varying antigen stimulation experiences. To better control the timing of T cell priming, we have recently constructed an MPyV in which the $D^b$-restricted $LT_{359}$ epitope was mutated by single base substitutions to the highly homologous $D^b$-restricted $LT_{206-214}$ epitope from SV40 LT antigen, for which TCR-1 TCR transgenic $CD8^+$ T cells are specific (55). We are currently in the process of constructing an MPyV that would combine features of this MPyV.LT206 virus with A2.Flx. This MPyV.Flx.LT206 virus will enable us to adoptively transfer TCR-1 cells at different time points post infection into Cre/Esr and littermates then administer tamoxifen at differing times post transfer to reduce viral replication. We can thereby better test the hypothesis that less stimulated MPyV-specific T cells can develop into bona-fide memory T cells imbued with the ability to persist independently of antigen and mount a robust, protective recall response.

**Figure Legends**

**Figure 1.** Cre-mediated deletion of A2.Flx impacts viral replication in vitro. (A) Schematic depicting genome of A2.Flx compared to wildtype MPyV strain A2. Result of Cre-mediated recombination of A2.Flx is also depicted. Black dot indicates origin of replication. Arrows above the DNA indicate PCR primers flanking the floxed region, which were used to determine the extent of deletion. (B) PCR of DNA from nMuMG cells following infection with wildtype (WT) MPyV or A2.Flx with or without AdCre co-infection. NMuMG cells were first infected with AdCre for 24 hrs prior to infection with MPyV. PCR was run on DNA isolated from cells 48 h p.i. with A2.Flx or WT MPyV. (C) Copies of MPyV genome in A2.Flx infected compared with A2.Flx/Ad-Cre co-infected nMuMG as measured by qPCR. Infections done as discussed in (B). Data are from 2 independent experiments.

**Figure 2.** MPyV-specific $CD4^+$ and $CD8^+$ T cell responses to A2.Flx are mounted in B6 mice and mice constitutively expressing Cre (CMV-Cre). (A) QPCR assay for copies of MPyV genome in spleen and kidney at d20 p.i. (B) Frequency of $LT_{359}$-specific $CD8^+$ T cells in spleen at d20 p.i. as measured by $D^b/LT_{359}$ tetramer (*left*) or intracellular IFN-γ staining assay following direct ex vivo $LT_{359-368}$ peptide stimulation (*right*). (C) Splenic $LT_{678}$-specific (*left graph*) and $VP1_{221}$-specific (*right graph*) $CD4^+$ T cells at d20 p.i. Frequencies are of $CD4^+$ T cells that are $I\text{-}A^b$ $tetramer^+CD44^{hi}$ (*left side of graphs*) or IFN-$\gamma^+CD154^+$ following direct ex vivo stimulation with indicated peptides (*right side of graphs*).

**Figure 3.** Loss of virus-specific chronic memory T cells following ablation of A2.Flx during persistent infection. (A) Experimental set-up. Cre/Esr or transgene negative littermate controls were infected with A2.Flx. Tamoxifen was administered starting at d35 p.i. and given once daily

for 5 days. 30 days after the last tamoxifen injection, mice were sacrificed and MPyV-specific T cell responses assessed. (B) Comparison of the numbers of MPyV-specific $CD8^+$ and $CD4^+$ T cells in Cre/Esr versus Cre negative controls. Number of $LT_{359}$-specific $CD8^+$ T cells as assessed by $D^b/LT_{359}$ tetramer staining (*left*). Number of $CD4^+$ T cells specific for $LT_{678\text{-}690}$ and $VP1_{221\text{-}235}$ as assessed by I-$A^b$ tetramer staining (*right*). (C) qPCR assay for MPyV genomic DNA in spleen and kidney. Data in all graphs represent mean of 5-6 mice per group in two independent experiments. P values calculated using the nonparametric Mann-Whitney U test.

**Figure 4.** Functional competence of MPyV-specific T cells is unaltered by reduction in A2.Flx replication during persistent infection. (A) Comparison of the frequency of tetramer versus intracellular IFN-γ staining of (from left to right) $LT_{359}$-specific $CD8^+$ T cells, $LT_{678}$-specific $CD4^+$ T cells, and $VP1_{221}$-specific $CD4^+$ T cells in Cre/Esr mice versus littermates. Splenocytes were stained directly ex vivo with $D^b/LT_{359}$, I-$A^b/LT_{678}$, or I-$A^b/VP1_{221}$ tetramers. Alternatively, they were stimulated ex vivo with the respective peptides and stained intracellularly for IFN-γ, CD154 ($LT_{678}$- or $VP1_{221}$-stimulated only), and IL-2. (B) IL-2 co-production with IFN-γ. Data shown is from the same mice depicted in Figure 3.

**Figure 5.** Epitope-specific impact of persistent antigen on phenotype of MPyV-specific chronic memory $CD4^+$ and $CD8^+$ T cells. (A) Frequency of tetramer$^+$$CD44^{hi}$ $CD4^+$ and $CD8^+$ T cells that are $CD62L^{hi}CD127^{hi}$ central memory ($T_{CM}$), $CD62L^{lo}CD127^{hi}$ effector memory ($T_{EM}$), or $CD62L^{lo}CD127^{lo}$ effector ($T_{eff}$) cells. Splenocytes were stained directly ex vivo with the indicated tetramers and for CD4, CD8, CD44, CD62L, and CD127. (B) Level of Bcl-2 expressed by MPyV-specific $CD4^+$ and $CD8^+$ T cells from spleen as measured by geometric mean fluorescence

intensity (gMFI) of staining by antibody for Bcl-2. Data are from the same mice shown in Figure 3. P values calculated using the nonparametric Mann-Whitney U test.

**Figure 1**

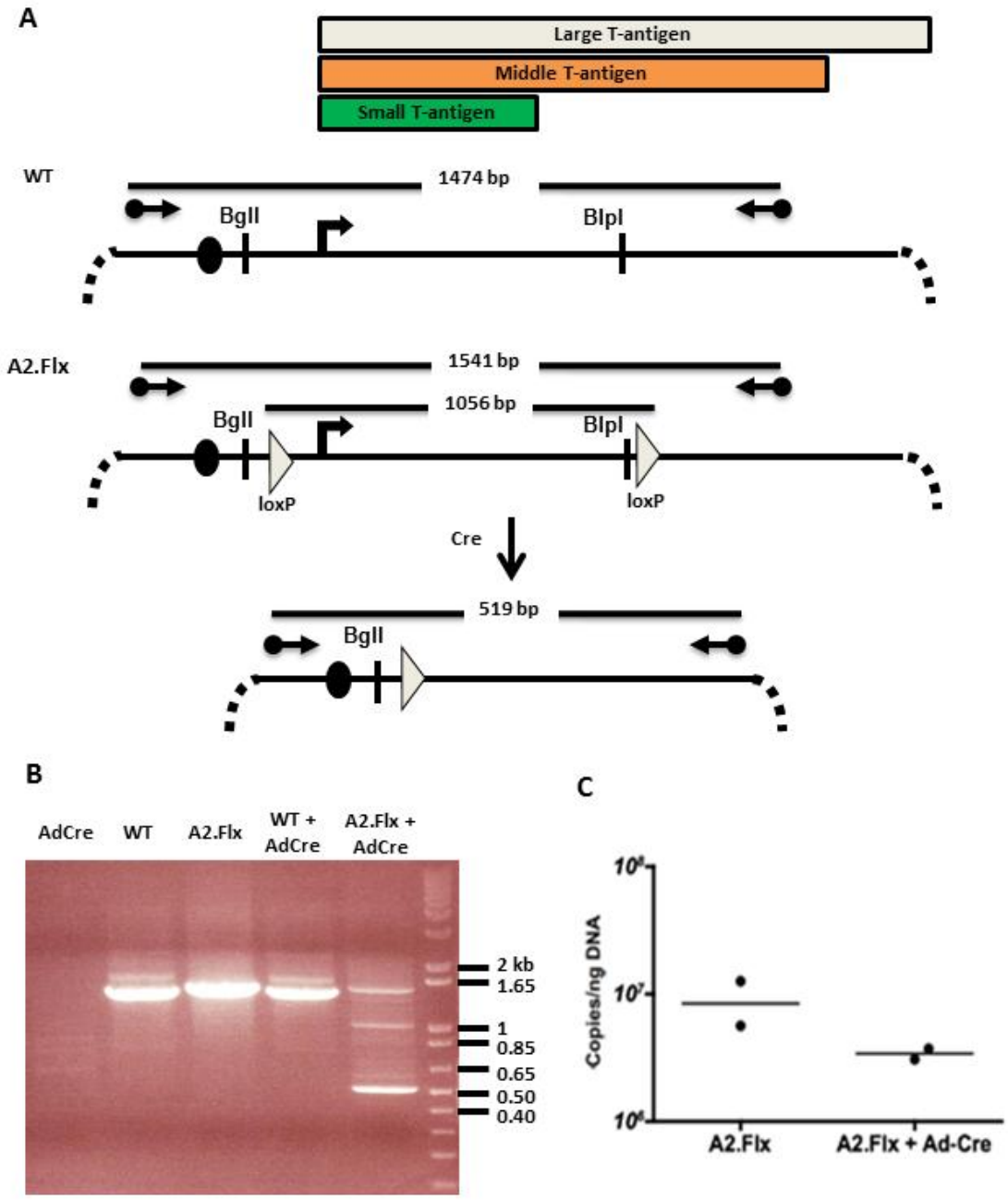

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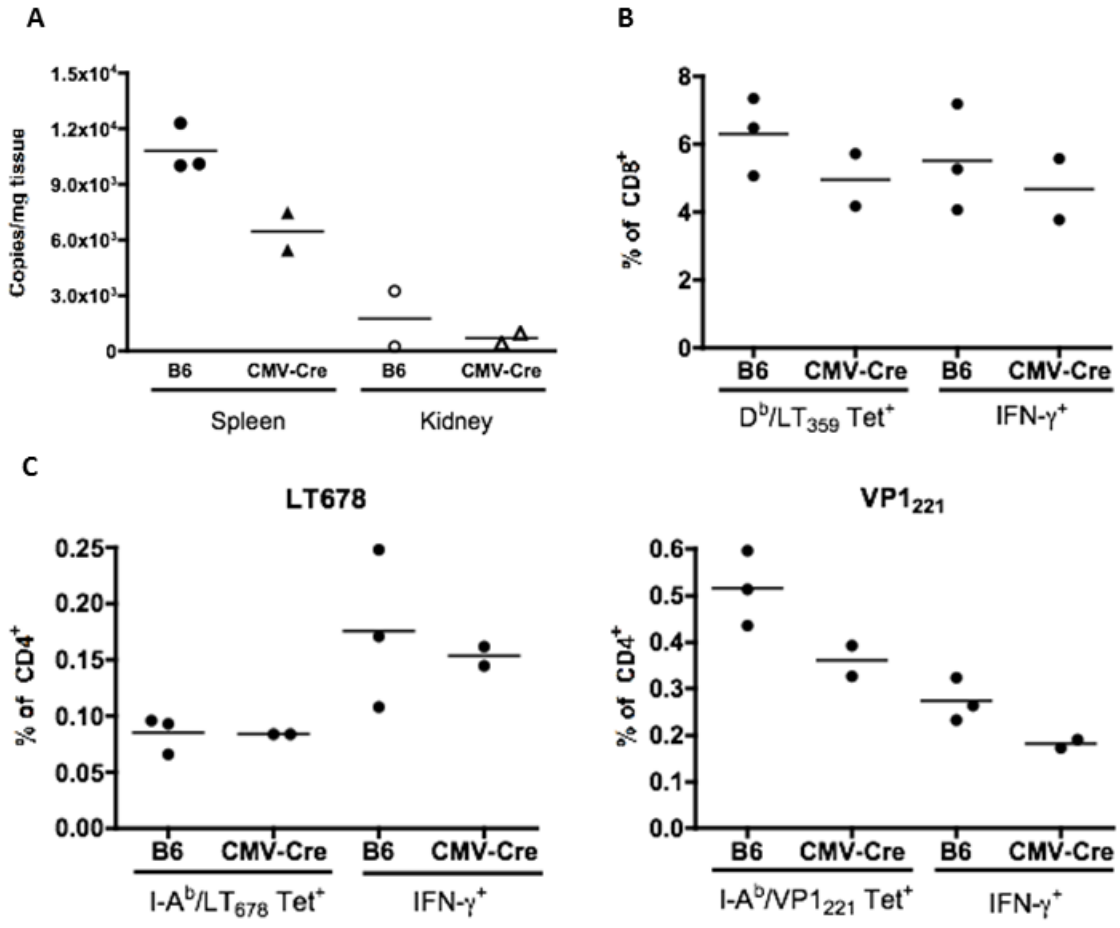
**Figure 2**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IFN-γ⁺

**Figure 3**

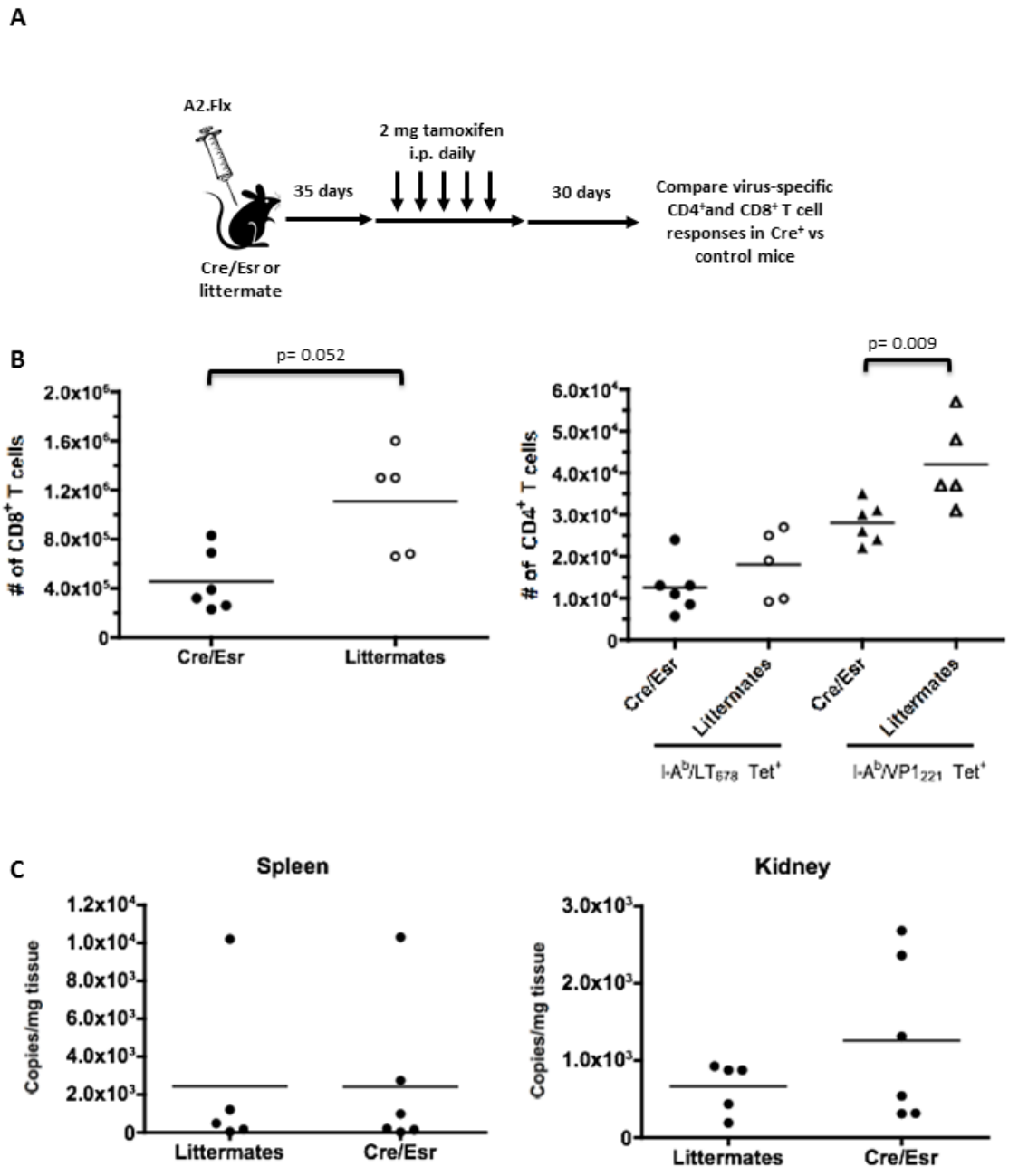

A
A2.Flx
Cre/Esr or littermate
35 days
2 mg tamoxifen i.p. daily
30 days
Compare virus-specific CD4+and CD8+ T cell responses in Cre+ vs control mice
B
p= 0.052
# of CD8+ T cells
2.0x10^6
1.6x10^6
1.2x10^6
8.0x10^5
4.0x10^5
0
Cre/Esr
Littermates
p= 0.009
# of CD4+ T cells
6.0x10^4
5.0x10^4
4.0x10^4
3.0x10^4
2.0x10^4
1.0x10^4
0
Cre/Esr
Littermates
Cre/Esr
Littermates
I-Ab/LT678 Tet+
I-Ab/VP1221 Tet+
C
Spleen
Copies/mg tissue
1.2x10^4
1.0x10^4
8.0x10^3
6.0x10^3
4.0x10^3
2.0x10^3
0
Littermates
Cre/Esr
Kidney
Copies/mg tissue
3.0x10^3
2.0x10^3
1.0x10^3
0
Littermates
Cre/Esr

**Figure 4**

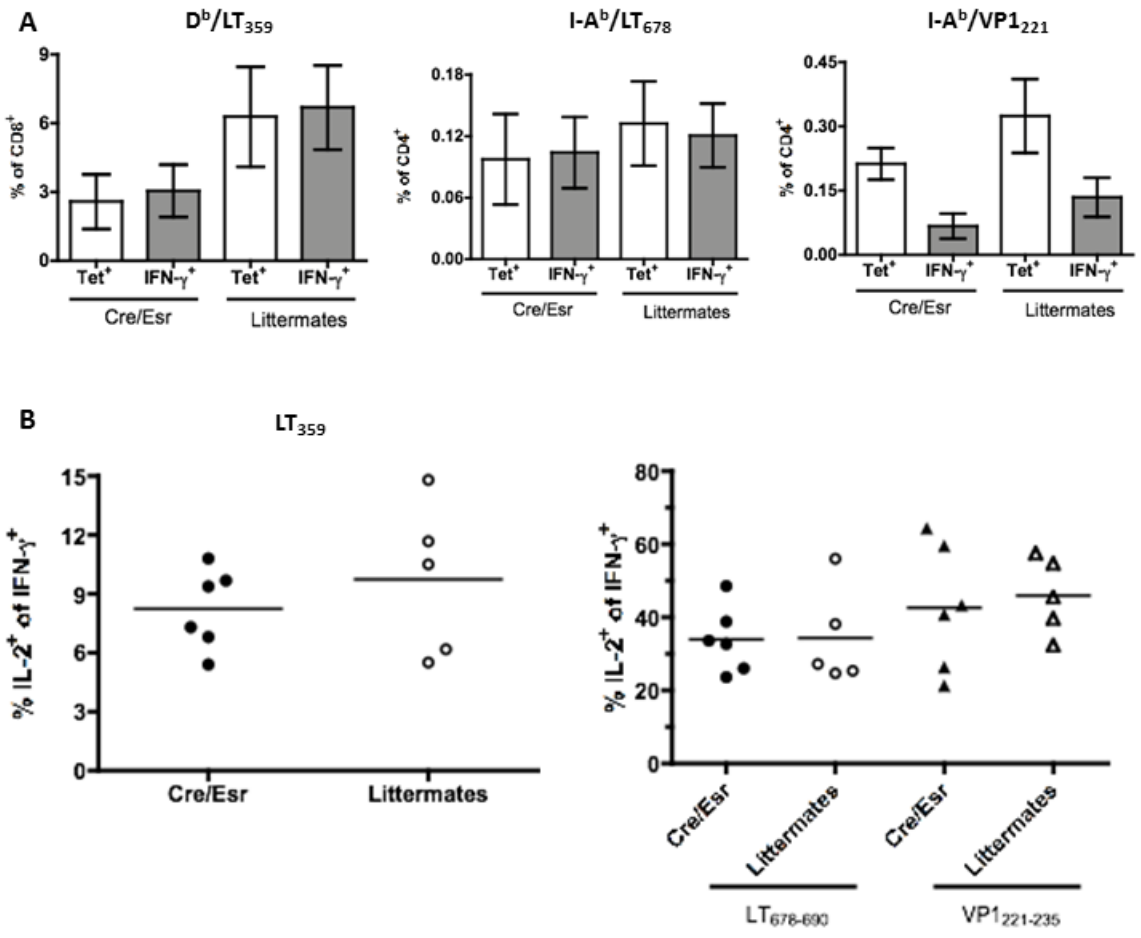
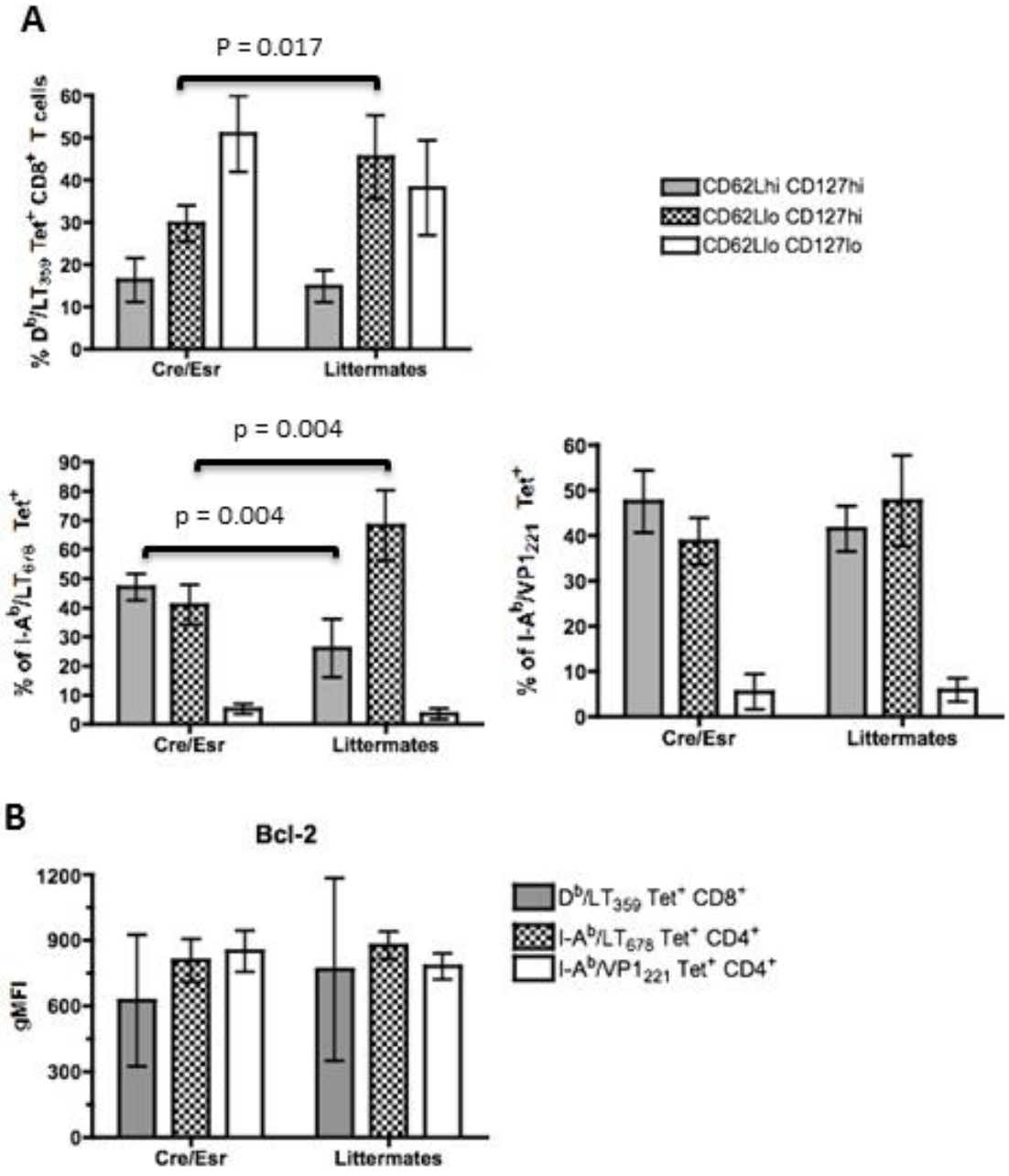

A
D^b/LT359
I-A^b/LT678
I-A^b/VP1221
% of CD8+
% of CD4+
Tet+
IFN-γ+
Cre/Esr
Littermates
B
LT359
% IL-2+ of IFN-γ+
LT678-690
VP1221-235

**Figure 5**


A
P = 0.017
% D^b/LT359 Tet+ CD8+ T cells
CD62Lhi CD127hi
CD62Llo CD127hi
CD62Llo CD127lo
Cre/Esr
Littermates
p = 0.004
p = 0.004
% of I-A^b/LT678 Tet+
% of I-A^b/VP1221 Tet+
B
Bcl-2
gMFI
D^b/LT359 Tet+ CD8+
I-A^b/LT678 Tet+ CD4+
I-A^b/VP1221 Tet+ CD4+

**References**

1. Kaech, S. M., and E. J. Wherry. 2007. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity 27:393-405.
2. van Leeuwen, E. M., J. Sprent, and C. D. Surh. 2009. Generation and maintenance of memory CD4(+) T Cells. Curr. Opin. Immunol. 21:167-72.
3. Surh, C. D., and J. Sprent. 2008. Homeostasis of naive and memory T cells. Immunity 29:848-62.
4. Wherry, E. J. 2011. T cell exhaustion. Nat. Immunol. 12:492-9.
5. Wherry, E. J., D. L. Barber, S. M. Kaech, J. N. Blattman, and R. Ahmed. 2004. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. Proc. Natl. Acad. Sci. USA 101:16004-9.
6. Shin, H., S. D. Blackburn, J. N. Blattman, and E. J. Wherry. 2007. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. J. Exp. Med. 204:941-9.
7. Bannard, O., M. Kraman, and D. T. Fearon. 2010. Cutting edge: Virus-specific CD8+ T cell clones and the maintenance of replicative function during a persistent viral infection. J. Immunol. 185:7141-5.
8. 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. 203:2263-9.
9. 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. 174:7950-60.

10. Snyder, C. M., K. S. Cho, E. L. Bonnett, S. van Dommelen, G. R. Shellam, and A. B. Hill. 2008. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. Immunity 29:650-9.

11. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401:708-12.

12. Stubbe, M., N. Vanderheyde, M. Goldman, and A. Marchant. 2006. Antigen-specific central memory CD4+ T lymphocytes produce multiple cytokines and proliferate in vivo in humans. J. Immunol. 177:8185-90.

13. Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat. Immunol. 4:225-34.

14. Wherry, E. J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. J. Virol. 78:5535-45.

15. Jelley-Gibbs, D. M., J. P. Dibble, S. Filipson, L. Haynes, R. A. Kemp, and S. L. Swain. 2005. Repeated stimulation of CD4 effector T cells can limit their protective function. J. Exp. Med. 201:1101-12.

16. Masopust, D., S. J. Ha, V. Vezys, and R. Ahmed. 2006. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. J. Immunol. 177:831-9.

17. Cush, S. S., K. M. Anderson, D. H. Ravneberg, J. L. Weslow-Schmidt, and E. Flano. 2007. Memory generation and maintenance of CD8+ T cell function during viral persistence. J. Immunol. 179:141-53.

18. Obst, R., H. M. van Santen, D. Mathis, and C. Benoist. 2005. Antigen persistence is required throughout the expansion phase of a CD4(+) T cell response. J. Exp. Med. 201:1555-65.

19. Blair, D. A., and L. Lefrancois. 2007. Increased competition for antigen during priming negatively impacts the generation of memory CD4 T cells. Proc. Natl. Acad. Sci. USA 104:15045-50.

20. Hataye, J., J. J. Moon, A. Khoruts, C. Reilly, and M. K. Jenkins. 2006. Naive and memory CD4+ T cell survival controlled by clonal abundance. Science 312:114-6.

21. Whitmire, J. K., N. Benning, B. Eam, and J. L. Whitton. 2008. Increasing the CD4+ T cell precursor frequency leads to competition for IFN-gamma thereby degrading memory cell quantity and quality. J. Immunol. 180:6777-85.

22. Williams, M. A., E. V. Ravkov, and M. J. Bevan. 2008. Rapid culling of the CD4+ T cell repertoire in the transition from effector to memory. Immunity 28:533-45.

23. 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 26:827-41.

24. Prlic, M., G. Hernandez-Hoyos, and M. J. Bevan. 2006. Duration of the initial TCR stimulus controls the magnitude but not functionality of the CD8+ T cell response. J. Exp. Med. 203:2135-43.

25. Harty, J. T., and V. P. Badovinac. 2008. Shaping and reshaping CD8+ T-cell memory. Nat. Rev. Immunol. 8:107-19.

26. Joshi, N. S., W. Cui, A. Chandele, H. K. Lee, D. R. Urso, J. Hagman, L. Gapin, and S. M. Kaech. 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. Immunity 27:281-95.

27. Catron, D. M., L. K. Rusch, J. Hataye, A. A. Itano, and M. K. Jenkins. 2006. CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. J. Exp. Med. 203:1045-54.

28. D'Souza, W. N., and S. M. Hedrick. 2006. Cutting edge: latecomer CD8 T cells are imprinted with a unique differentiation program. J. Immunol. 177:777-81.

29. Jelley-Gibbs, D. M., D. M. Brown, J. P. Dibble, L. Haynes, S. M. Eaton, and S. L. Swain. 2005. Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. J. Exp. Med. 202:697-706.

30. Fousteri, G., A. Dave, A. Juedes, T. Juntti, B. Morin, L. Togher, D. L. Farber, and M. von Herrath. 2011. Increased memory conversion of naive CD8 T cells activated during late phases of acute virus infection due to decreased cumulative antigen exposure. PLoS One 6:e14502.

31. 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. 185:1692-700.

32. 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. 160:1724-34.

33. Hayashi, S., and A. P. McMahon. 2002. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev. Biol. 244:305-18.

34. Sauer, B. 1998. Inducible gene targeting in mice using the Cre/lox system. Methods 14:381-92.

35. Soeda, E., J. R. Arrand, and B. E. Griffin. 1979. Polyoma virus. The early region and its T-antigens. Nucleic Acids Res. 7:839-57.

36. Soeda, E., J. R. Arrand, and B. E. Griffin. 1980. Polyoma virus DNA: complete nucleotide sequence of the gene which codes for polyoma virus capsid protein VP1 and overlaps the VP2/VP3 genes. J. Virol. 33:619-30.

37. 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 283:445-53.

38. Benjamin, T. L. 2006. Polyoma Viruses, p. 105-139, The Mouse in Biomedical Research, 2nd ed. Elsevier, San Diego, CA.

39. Bendig, M. M., T. Thomas, and W. R. Folk. 1980. Viable deletion mutant in the medium and large T-antigen-coding sequences of the polyoma virus genome. J. Virol. 33:1215-20.

40. Schwenk, F., U. Baron, and K. Rajewsky. 1995. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic Acids Res. 23:5080-1.

41. Frentsch, M., O. Arbach, D. Kirchhoff, B. Moewes, M. Worm, M. Rothe, A. Scheffold, and A. Thiel. 2005. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. Nat. Med. 11:1118-24.

42. Brooks, D. G., D. B. McGavern, and M. B. Oldstone. 2006. Reprogramming of antiviral T cells prevents inactivation and restores T cell activity during persistent viral infection. J. Clin. Invest. 116:1675-85.

43. Bustamante, J. M., L. M. Bixby, and R. L. Tarleton. 2008. Drug-induced cure drives conversion to a stable and protective CD8+ T central memory response in chronic Chagas disease. Nat. Med. 14:542-50.

44. Pipas, J. M. 1992. Common and unique features of T antigens encoded by the polyomavirus group. J. Virol. 66:3979-85.

45. Cahill, K. B., and G. G. Carmichael. 1989. Deletion analysis of the polyomavirus late promoter: evidence for both positive and negative elements in the absence of early proteins. J. Virol. 63:3634-42.

46. Luthman, H., M. G. Nilsson, and G. Magnusson. 1982. Non-contiguous segments of the polyoma genome required in cis for DNA replication. J. Mol. Biol. 161:533-50.

47. Mueller, C. R., W. J. Muller, and J. A. Hassell. 1988. The polyomavirus enhancer comprises multiple functional elements. J. Virol. 62:1667-78.

48. Muller, W. J., D. Dufort, and J. A. Hassell. 1988. Multiple subelements within the polyomavirus enhancer function synergistically to activate DNA replication. Mol. Cell Biol. 8:5000-15.

49. Triezenberg, S. J., and W. R. Folk. 1984. Essential nucleotides in the polyomavirus origin region. J. Virol. 51:437-44.

50. Cowie, A., and R. Kamen. 1984. Multiple binding sites for polyomavirus large T antigen within regulatory sequences of polyomavirus DNA. J. Virol. 52:750-60.

51. Benjamin, T. L. 2001. Polyoma virus: old findings and new challenges. Virology 289:167-73.

52. Cowie, A., C. Tyndall, and R. Kamen. 1981. Sequences at the capped 5'-ends of polyoma virus late region mRNAs: an example of extreme terminal heterogeneity. Nucleic Acids Res. 9:6305-22.

53. Treisman, R. 1980. Characterisation of polyoma late mRNA leader sequences by molecular cloning and DNA sequence analysis. Nucleic Acids Res. 8:4867-88.

54. Li, M. Z., and S. J. Elledge. 2007. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat. Methods 4:251-6.

55. Staveley-O'Carroll, K., T. D. Schell, M. Jimenez, L. M. Mylin, M. J. Tevethia, S. P. Schoenberger, and S. S. Tevethia. 2003. In vivo ligation of CD40 enhances priming against the endogenous tumor antigen and promotes CD8+ T cell effector function in SV40 T antigen transgenic mice. J. Immunol. 171:697-707.

**Chapter 4: Discussion**

$CD4^+$ T cells are an integral component of the immunosurveillance against viral infection. Much of the literature demonstrates a critical impact of $CD4^+$ T cell "help" on the generation of optimal antibody responses and optimal virus-specific $CD8^+$ T cell responses in models of both acutely resolved and persistent infections (1-4). Effector $CD4^+$ T cells express a wide array of cytokines and surface molecules that have been the focus of studies into the molecular mechanisms of $CD4^+$ T cell help. However, $CD4^+$ T cells differentiate into subsets of T helper ($T_H$) cells that express different sets of effector molecules and perform different functions. $T_H$ differentiation is mercurial, and changes in the surrounding conditions could alter the particular properties of an effector $CD4^+$ T cell (5). This issue could be particularly problematic during persistent viral infections, where the environment is dynamic. Thus, there is a need to study virus-specific $CD4^+$ T cell responses as they develop over the course of persistent infection.

Studies of acutely resolved infections showed that virus-specific $CD4^+$ T cells could develop into functional and heterogeneous memory T cells that are maintained by antigen-independent self-renewal (6). However, during chronic high load viral infections such as with HIV/SIV (7) and chronically infecting strains of LCMV (e.g., clone 13 (8)), virus-specific $CD4^+$ T cells become functionally exhausted and increase expression of inhibitory receptors (9-11). Although more recent studies with LCMV chronic infection models suggest that virus-specific $CD4^+$ T cells are not actually exhausted but driven into an altered differentiation state by the high antigen load (12), less is known regarding how $CD4^+$ T cells cope with low-level persistent infections. We and others working with low-level persistent viruses such as mouse polyomavirus, mouse and human cytomegalovirus (CMV) (13, 14), and mouse γ-herpesvirus 68 (MHV68) (15, 16) have begun to address this knowledge gap. In addition, we could not find studies addressing the issue of $CD4^+$ memory T cell development during persistent infection (i.e., "chronic memory $CD4^+$ T cells"). This is in marked contrast to chronic memory $CD8^+$ T cells for which studies

using multiple persistent viral infection models show that maintenance requires antigen for self-renewal and/or de novo priming of virus-specific $CD8^+$ T cells (17-21).

Mouse polyomavirus (MPyV) has proven to be a valuable model for studying T cell responses in the setting of low-level persistent infection. Taking advantage of its rather small size genome, which encodes only 6 genes, Kemball et al. identified several $CD8^+$ T cell epitopes in nonstructural T antigen proteins in B6 ($H\text{-}2^b$) mice by screens of overlapping peptides covering the viral proteins (17). Studies of immunodominant $D^b$-restricted $LT_{359}$-specific $CD8^+$ T cell populations showed that MPyV-specific $CD8^+$ T cells remain polyfunctional despite persistent infection (17, 22). More importantly, maintenance of MPyV-specific chronic memory $CD8^+$ T cell populations was found to require continual recruitment of new naïve antigen-specific $CD8^+$ T cells during persistent infection (17, 20). This process depends on $CD4^+$ T cell help (23). However, a detailed analysis of the functional profile and fate of MPyV-specific $CD4^+$ T cells had to wait until we identified $I\text{-}A^b$-restricted epitopes. The identification of two $CD4^+$ T cell epitopes and our ability to track the responding populations over the course of infection through newly developed peptide:major histocompatibility complex (pMHC) II tetramers and intracellular cytokine assays mark major steps forward. These tools enabled us to determine if MPyV-specific $CD4^+$ T cells become dysfunctional over time and whether chronic memory $CD4^+$ T cell populations decline or are maintained.

Tracking both the large T antigen (LT)- and major capsid protein VP1- specific $CD4^+$ T cells from acute to persistent phases of MPyV infection unveiled unexpected heterogeneity in function and phenotype (Chapter 2). Much of this diversity may well have remained hidden without access to pMHC II tetramers for both epitopes. In particular, we were struck by the diminished functionality displayed by VP1-specific $CD4^+$ T cells starting early in acute infection and sustained through the persistent phase, which was not seen with LT-specific $CD4^+$ T cells. This was not expected since MHC class Ia-restricted MPyV-specific $CD8^+$ T cells were fully

functional. The difference in function between the two epitope-specific $CD4^+$ T cell populations extended to the pattern of effector cytokine polyfunction (IFN-γ, TNF-α, IL-2). Heterogeneity in effector cytokine polyfunctionality amongst epitope-specific $CD4^+$ T cells to low-level persistent infection was also recently observed by Arens et al in MCMV infection (13). However, unlike us, they did not utilize pMHC II tetramers, and it remains unclear whether MCMV-specific $CD4^+$ T cells too can lose function. PMHC II tetramers also allowed us to detect the heterogeneity in CD127 and PD-1 expression within the VP1-specific but not LT-specific $CD4^+$ T cell population. Thus, reliance only upon cytokine assays may not provide a full picture of what is going on with $CD4^+$ T cells in a low-level persistent infection.

The phenotypic and functional heterogeneity between both MPyV-specific $CD4^+$ T cell populations could be the result of differences during priming in the strength and duration of antigenic signaling, which are critical determinants that drive differentiation of $CD4^+$ T cells (24). The pattern of viral protein expression could impact access to the MHC class II presentation pathway and subsequent antigen availability. MPyV capsid proteins like VP1 are expressed in productively infected cells, whereas nonstructural proteins like LT are produced in all infected cells regardless of virion production. As LT is produced in the cytoplasm of infected cells, it is likely inefficiently shunted into the MHC class II presentation pathway unless taken up by phagocytic APCs from the debris of lysed cells. VP1 could be acquired via the same route, but as MPyV virions bind to sialylated glycoproteins and glycolipids expressed on the surface of cells (25), receptor mediated endocytosis could provide an additional source of VP1 for processing by the MHC class II pathway. As a result, the chances for antigen encounter by VP1-specific $CD4^+$ T cells would be much greater than for LT-specific $CD4^+$ T cells. Alternatively, VP1-specific $CD4^+$ T cells may express higher avidity TCRs. Others have shown a direct correlation between mean fluorescence intensity (MFI) of tetramer binding and TCR affinity for pMHC (26, 27). The higher I-$A^b$/$VP1_{221}$ tetramer gMFI compared to that of I-$A^b$/$LT_{678}$ (data not shown) would seem to

support this. Additional evidence for high avidity TCR comes from the narrow TCR Vβ repertoire for VP1-specific $CD4^+$ T cells. The work of Baumgartner and colleagues has shown that the efficiency of T cell activation by antigen impacts clonotypic diversity (28). Less stable pMHC II complexes and lower antigen doses focus antigen-specific $CD4^+$ T cell responses toward a less diverse TCR repertoire composed of higher affinity clonotypes. Since dendritic cells (DCs) can be infected by MPyV (29) and production of viral capsid protein presages cell death, only high affinity VP1-specific $CD4^+$ T cell clones would be expected to be primed if antigenic signal were to suddenly dissipate. Overall, VP1-specific $CD4^+$ T cells appear to be more strongly stimulated with a visible proportion of cells being $CD127^{lo}$ and PD-$1^{hi}$. In future, it may be of interest to generate antigen-specific T cell clones to test the impact of antigen dose on activation and differentiation. Recently a novel 2D assay that measures TCR-pMHC binding affinity by T cell:pMHC- coated red blood cell contact was used to compare affinities of viral antigen-specific and autoantigen-specific $CD4^+$ T cells (30). This could be used to test the TCR affinity of sorted VP1- versus LT-specific $CD4^+$ T cells.

Despite differences, both LT- and VP1-specific $CD4^+$ T cell populations appear to be maintained by the same mechanism. Both underwent minimal contraction and were stably maintained through the persistent phase of infection, which contrasts with some reports that pathogen-specific $CD4^+$ memory T cells emerging after resolution of an acute infection steadily decline over time (31-33). This would seem to reinforce assertions by some that memory $CD4^+$ T cells are dependent on some antigen for optimal survival (34). However, we found that MPyV-specific $CD4^+$ T cells in persistently infected mice minimally proliferate and are highly apoptotic. This largely rules out antigen-driven proliferation, which is exhibited by LCMV chronic memory $CD8^+$ T cells (18), as the mechanism of maintenance of MPyV-specific chronic memory $CD4^+$ T cell populations. Instead, the striking similarity to the lack of proliferation and poor survival of MPyV-specific $CD8^+$ T cells (20) led us to investigate the possibility that naive virus-specific

$CD4^+$ T cells are still being primed during persistent infection. The partial bone marrow (BM) chimera protocol used to demonstrate de novo $CD8^+$ T cell priming (17, 20) proved highly useful as it allowed us to demonstrate de novo priming of both MPyV antigen-specific $CD4^+$ T cells using our pMHC II tetramers. The finding that naive $CD4^+$ T cells specific for both LT and VP1 are de novo recruited in persistently infected mice affirms our long-held contention that MPyV productively replicates continually at low levels. It is interesting that both virus-specific $CD4^+$ and $CD8^+$ T cell populations are actively maintained by de novo recruited antigen-specific cells. This reflects on the close cooperation between these two critical cell types. $CD4^+$ T cells may be needed to license APCs to drive de novo priming of naïve virus-specific $CD8^+$ T cells as a result of reduced inflammation during persistent infection. Although we are not the first to clearly demonstrate de novo priming of $CD4^+$ T cells specific for persisting viruses, the study showing de novo generation of $CD4^+$ T cells against persistent viral infections in baboons merely placed this in the context of immune reconstitution (35). We, however, are showing it as a mechanism to maintain $CD4^+$ T cell immunosurveillance. As both virus-specific $CD8^+$ T cell and anti-MPyV antibody responses are drastically diminished in persistently infected mice lacking $CD4^+$ T cells (23), we feel that continual de novo recruitment of new MPyV-specific $CD4^+$ T cells is critical for maintaining support for B cells and $CD8^+$ T cells to keep persistent virus in check. Since $CD4^+$ T cells are critical for control of other persistent infections as well, others are starting to look at the possibility of continued recruitment of virus-specific $CD4^+$ T cells in their systems. Most recently, Freeman et al demonstrated that adoptively transferred TCR transgenic (Tg) $CD4^+$ T cells specific for an epitope in MHV68 were activated during both acute and latent phases of infection (15).

Continual de novo generation of MPyV-specific $CD4^+$ T cells throughout the course of persistent infection has potential implications regarding $CD4^+$ T cell heterogeneity in vivo. The literature is replete with studies demonstrating that differentiation of $CD4^+$ T cells into distinct $T_H$ subsets is regulated by the antigenic, inflammatory, and costimulatory environment during

priming of naïve $CD4^+$ T cells (36, 37). There is increasing evidence, however, that $CD4^+$ T cells may be far more plastic than originally thought. For instance, Hegazy et al. recently showed that they could convert in vitro generated LCMV-specific memory $T_H2$ cells into dual personality $T_H1+2$ cells, which simultaneously expressed characteristics of both $T_H1$ and $T_{H2}$ cells, when rechallenged in vivo with LCMV (38). As persistent infection is a state of continuous rechallenge, shifts in the overall cytokine and antigen environment could reprogram chronic memory antigen-specific $CD4^+$ T cells. This has been shown to occur during chronic LCMV infection where virus-specific $CD4^+$ T cells lose IL-2 production but ramp up production of IL-21 and are redirected from a $T_H1$ into a T follicular helper ($T_{FH}$) cell program (8, 12, 39). Although we did not observe a loss in IL-2 production, we too see increased IL-21 production by both VP1- and LT-specific $CD4^+$ T cells during persistent MPyV infection (Chapter 2, Figure 3). We would argue, however, that this is unlikely to be because of just reprogramming of antigen-experienced virus-specific $CD4^+$ T cells but could reflect differential programming of de novo recruited cells primed under conditions different from those primed earlier in acute or persistent infection.

Different antigen and inflammation conditions can also influence the development of memory T cells into either central memory T ($T_{CM}$) cells or effector memory T ($T_{EM}$) cells. So-called "latecomer" T cells primed after acute infection or immunization receive lower antigenic and inflammatory cues and develop into $CD62L^{hi}$ $T_{CM}$ cells, which are considered intermediately differentiated (40-43). Repetitive and/or overly robust antigen stimulation, on the other hand, pushes T cells into more terminally differentiated $CD62L^{lo}$ effector T ($T_{Eff}$)/$T_{EM}$ cells as seen in chronic LCMV(44). We thus expected to find that de novo primed MPyV-specific $CD4^+$ T cells would also be $CD62L^{hi}$. However, we were unable to satisfactorily phenotype the donor-derived $I\text{-}A^b$ $tetramer^+$ $CD4^+$ T cells in our chimeras due to mouse-to-mouse variability and low frequencies. De novo primed MPyV-specific $CD8^+$ T cells, on the other hand, have been shown to have a $CD62L^{hi}$ $CD27^{hi}$ $T_{CM}$ cell phenotype as compared to the predominant $CD62L^{lo}$ $T_{eff}$/$T_{EM}$

cell phenotype of the bulk population (17, 20). Furthermore, the frequency of $T_{CM}$ phenotype virus-specific $CD8^+$ T cells in normal infected mice gradually increases over time. This suggested that de novo recruited virus-specific $CD8^+$ T cells are actively contributing to the overall heterogeneity of the virus-specific T cell pool (20). Indirect evidence that de novo primed virus-specific $CD4^+$ T cells also contribute to chronic memory T cell heterogeneity can be seen in the biphasic staining for PD-1 and CD127 on VP1-specific $CD4^+$ T cells during persistent infection at d60 p.i. (Chapter 2, Figure 5). It is possible that the $CD127^{hi}$ and PD-$1^{lo}$ VP1-specific $CD4^+$ T cells represent recently recruited cells while $CD127^{lo}$ and PD-$1^{hi}$ cells are older, more stimulated cells. Besides the low detectability of donor-derived $CD4^+$ T cell priming, another key issue of the BM chimera approach is the length of time (about 90 days for the $CD4^+$ T cell experiments) between bone marrow transfer and analysis of virus-specific T cells. This was given to allow for engraftment and thymic selection to take place. As a result, the donor population itself is an amalgam of cells primed at different times and enduring varying degrees of persistent stimulation. A legitimate question is how we would know that the changes in phenotype over time are due to newly emerging virus-specific T cells rather than cells primed earlier that retained plasticity and were reprogrammed.

To control the timing of priming, we need a TCR Tg cell adoptive transfer system. This approach is being used to compare acute versus persistent phase infection primed $CD8^+$ TCR Tg cells specific for an epitope in SV40 LT antigen, which was engineered into MPyV by site-directed mutagenesis of the homologous immunodominant $D^b$-restricted LT epitope. Like the de novo primed $CD8^+$ T cells, TCR-I Tg cells primed during the persistent phase of MPyV.LT206 infection adopt more $T_{CM}$-like properties including higher CD127 expression, increased cytokine production per cell (e.g., IL-2), improved survival, and superior recall capacity over acute primed cells (JJ Wilson, manuscript submitted). Hoping to make similar comparisons with antigen-specific $CD4^+$ T cells, we created two new MPyVs carrying the epitopes recognized by OT-II and

SMARTA TCR Tg $CD4^+$ T cells. In several experiments, we found that although the OT-II and SMARTA TCR Tg cells are primed during acute infection with MPyV.OVA-II and MPyV.GP66, respectively, only 1 out of every 3 recipient mice still retains detectable cells past d30 p.i. In contrast, after transfer into mice persistently infected with MPyV.GP66 (d35 p.i.), we have been able to detect priming of about 40-50% of adoptively transferred SMARTA cells at 60d post-transfer. These cells are mainly $CD62L^{lo}$ and $CD127^{hi}$ (unpublished data). The $CD62L^{lo}$ phenotype may be a result of continued stimulation after initial priming. Nonetheless, the results confirm the $CD8^+$ TCR Tg data that acute primed T cells may not survive as well as those primed during the persistent phase of infection. Future studies using the SMARTA TCR Tg system in conjunction with MPyV.GP66 could be done to address the requirements for persistent phase priming as well as the mechanisms of $CD4^+$ T cell help during persistent infection. A major issue with the recombinant MPyVs, however, is that the epitopes are expressed in frame with T antigens. The TCR Tg cells would probably give us insights into regulation and function of the LT-specific $CD4^+$ T cells, but these potential findings may not necessarily apply to capsid-specific $CD4^+$ T cells. Ideally, it would be useful to have both VP1-specific and LT-specific $CD4^+$ TCR Tg cells so that we would not have to modify MPyV and risk attenuating the virus.

Although continual de novo recruitment of virus-specific T cells may be a way to maintain a functional anti-viral T cell pool during persistent infection, thymic involution associated with aging would be expected to diminish the available pool of naïve antigen-specific T cell precursors (45). Thus, a key question is whether MPyV-specific T cells are capable of developing into antigen-independent memory T cells. Preliminary experiments using the A2.Flx/ tamoxifen-inducible Cre system (Chapter 3) showed an impact on MPyV-specific T cell survival when Cre was induced during persistent infection that was epitope-dependent. The significant decline in both the LT-specific $CD8^+$ and the VP1-specific $CD4^+$ T cells but not the LT-specific $CD4^+$ T cells in $Cre^+$ mice may be related to the strength and duration of their stimulation by

antigen. Stronger repetitive antigen stimulation may have led to terminal differentiation, while weaker and/or less stimulation led to an intermediate state that permitted some plasticity when antigen conditions changed. This hypothesis could be tested by adoptive transfer of virus-specific TCR Tg cells at various time points prior to Cre induction and observe their fate as viral replication/antigen diminishes.

As costimulatory molecules play a key role in T cell priming (46), it would be interesting to determine the costimulatory requirements for de novo MPyV-specific $CD4^+$ T cell priming. The CD40:CD154 (CD40L) interaction has been shown to be critical for priming of $CD4^+$ T cells during the primary response as well as programing of effector function in memory cells in a peptide immunization model (47). CD27, which is highly expressed by both MPyV-specific $CD4^+$ T cell populations during persistent infection (Chapter 2, Figure 5), is also reported to play a role in $CD4^+$ T cell activation (48-50). In addition, we found high expression of inducible costimulator (ICOS) on both LT- and VP1-specific $CD4^+$ T cells during persistent MPyV infection (unpublished data). ICOS expression is upregulated following activation on $CD4^+$ T cells and is a critical regulator of $CD4^+$ T cell proliferation, survival, effector function, and memory development (51). In particular, ICOS is critical for the development of the $T_{FH}$ subset that is directly involved in helping B cells (36). The effect of blocking any of these pathways on MPyV-specific $CD4^+$ T cells should be investigated.

As previously mentioned, there was enhanced peptide-stimulated production of the cytokine IL-21, a member of the IL-2 family, by both LT- and VP1-specific $CD4^+$ T cells during persistent infection. Activated $CD4^+$ T cells are the predominant source of IL-21, which is a pleiotropic cytokine with effects on various immune cells including $CD8^+$ T cells and B cells. IL-21 appears to enhance the generation of memory $CD8^+$ T cells and inhibits T cell senescence (52). Recently, three groups showed that IL-21 is critical for maintenance of functional virus-specific $CD8^+$ T cells in persistent high load infection by chronically infecting strains of LCMV (39, 53,

54). The importance of IL-21 to $CD8^+$ T cells during low-level persistent infections is thus far unclear, but a recent paper studying memory inflation of MCMV-specific $CD8^+$ T cells reported IL-21 not to be critical for that process (55). IL-21 is highly expressed by $T_{FH}$ cells, the $CD4^+$ T cell subset responsible for B cell help, and has critical roles in driving plasma cell differentiation and class switching depending on the additional signals (1). Intriguingly, some VP1-specific $CD4^+$ T cells during persistent MPyV infection might be $T_{FH}$ cells, which express high levels of ICOS and PD-1 in addition to IL-21 (1). It remains to be seen what exactly IL-21 is doing during persistent MPyV infection, but given the multiple cells affected, we should proceed with caution.

Prior to work covered in this dissertation, we knew very little about how virus-specific $CD4^+$ T cells cope with low-level persistent infections. Although we have found that the impact of persistent MPyV infection on the differentiation and function of virus-specific $CD4^+$ T cells to be more complex than previously thought, our work has furthered understanding of how highly critical virus-specific $CD4^+$ T cell populations can be sustained in the face of continuous bombardment by antigen. Despite differences between low-level persistent viruses, there may also be commonalities in the way $CD4^+$ T cells respond to them. Clearly, further studies are needed to better understand the mechanisms that drive and control $CD4^+$ T cell responses to persistent viral infection. Findings regarding MPyV-specific $CD4^+$ T cells could have applicability for $CD4^+$ T cell responses to infection by human polyomaviruses (HuPyV), which though normally clinically silent, can cause severe disease in the immunosuppressed. Like MPyV, $CD4^+$ T cell responses to BK virus (BKV), which is associated with kidney transplant rejection, have been identified directed toward LT and VP1 (56). Recent work has also identified $CD4^+$ T cells responding to the newly discovered HuPyV Merkel cell polyomavirus (57), a potential cause of aggressive Merkel cell carcinoma. Our host immune defenses must be kept on constant alert for not only incoming viral threats but also those that have managed to evade them and establish a niche within us. Continual support by $CD4^+$ T cells other immune cells is essential to maximizing

the effectiveness of immunosurveillance against the ever-present threats posed by even normally clinically silent pathogens.

**References**

1. Crotty, S. 2011. Follicular helper CD4 T cells (TFH). Annu. Rev. Immunol. 29:621-63.
2. Williams, M. A., B. J. Holmes, J. C. Sun, and M. J. Bevan. 2006. Developing and maintaining protective CD8+ memory T cells. Immunol. Rev. 211:146-53.
3. Altfeld, M., and E. S. Rosenberg. 2000. The role of CD4(+) T helper cells in the cytotoxic T lymphocyte response to HIV-1. Curr. Opin. Immunol. 12:375-80.
4. Wherry, E. J. 2011. T cell exhaustion. Nat. Immunol. 12:492-9.
5. O'Shea, J. J., and W. E. Paul. 2010. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. Science 327:1098-102.
6. van Leeuwen, E. M., J. Sprent, and C. D. Surh. 2009. Generation and maintenance of memory CD4(+) T Cells. Curr. Opin. Immunol. 21:167-72.
7. Younes, S. A., B. Yassine-Diab, A. R. Dumont, M. R. Boulassel, Z. Grossman, J. P. Routy, and R. P. Sekaly. 2003. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. J. Exp. Med. 198:1909-22.
8. Brooks, D. G., L. Teyton, M. B. Oldstone, and D. B. McGavern. 2005. Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. J. Virol. 79:10514-27.
9. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature 443:350-4.
10. D'Souza, M., A. P. Fontenot, D. G. Mack, C. Lozupone, S. Dillon, A. Meditz, C. C. Wilson, E. Connick, and B. E. Palmer. 2007. Programmed death 1 expression on HIV-

specific CD4+ T cells is driven by viral replication and associated with T cell dysfunction. J. Immunol. 179:1979-87.

11. Kaufmann, D. E., D. G. Kavanagh, F. Pereyra, J. J. Zaunders, E. W. Mackey, T. Miura, S. Palmer, M. Brockman, A. Rathod, A. Piechocka-Trocha, B. Baker, B. Zhu, S. Le Gall, M. T. Waring, R. Ahern, K. Moss, A. D. Kelleher, J. M. Coffin, G. J. Freeman, E. S. Rosenberg, and B. D. Walker. 2007. Upregulation of CTLA-4 by HIV-specific CD4(+) T cells correlates with disease progression and defines a reversible immune dysfunction. Nat. Immunol. 8:1246-54.

12. Fahey, L. M., E. B. Wilson, H. Elsaesser, C. D. Fistonich, D. B. McGavern, and D. G. Brooks. 2011. Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells. J. Exp. Med. 208:987-99.

13. Arens, R., P. Wang, J. Sidney, A. Loewendorf, A. Sette, S. P. Schoenberger, B. Peters, and C. A. Benedict. 2008. Cutting edge: murine cytomegalovirus induces a polyfunctional CD4 T cell response. J. Immunol. 180:6472-6.

14. Casazza, J. P., M. R. Betts, D. A. Price, M. L. Precopio, L. E. Ruff, J. M. Brenchley, B. J. Hill, M. Roederer, D. C. Douek, and R. A. Koup. 2006. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. J. Exp. Med. 203:2865-77.

15. Freeman, M. L., C. E. Burkum, K. G. Lanzer, M. K. Jensen, M. Ahmed, E. J. Yager, E. Flano, G. M. Winslow, D. L. Woodland, and M. A. Blackman. 2011. Cutting Edge: Activation of Virus-Specific CD4 T Cells throughout gamma-Herpesvirus Latency. J. Immunol.

16. Stuller, K. A., S. S. Cush, and E. Flano. 2010. Persistent gamma-herpesvirus infection induces a CD4 T cell response containing functionally distinct effector populations. J. Immunol. 184:3850-6.

17. 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. 174:7950-60.

18. Shin, H., S. D. Blackburn, J. N. Blattman, and E. J. Wherry. 2007. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. J. Exp. Med. 204:941-9.

19. Snyder, C. M., K. S. Cho, E. L. Bonnett, S. van Dommelen, G. R. Shellam, and A. B. Hill. 2008. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. Immunity 29:650-9.

20. 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. 203:2263-9.

21. Wherry, E. J., D. L. Barber, S. M. Kaech, J. N. Blattman, and R. Ahmed. 2004. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. Proc. Natl. Acad. Sci. USA 101:16004-9.

22. 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. 178:7267-75.

23. 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. 179:1113-21.

24. Kim, C., and M. A. Williams. 2010. Nature and nurture: T-cell receptor-dependent and T-cell receptor-independent differentiation cues in the selection of the memory T-cell pool. Immunology 131:310-7.

25. 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 384:389-99.

26. Fazilleau, N., L. J. McHeyzer-Williams, H. Rosen, and M. G. McHeyzer-Williams. 2009. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nat. Immunol. 10:375-84.

27. Reichstetter, S., R. A. Ettinger, A. W. Liu, J. A. Gebe, G. T. Nepom, and W. W. Kwok. 2000. Distinct T cell interactions with HLA class II tetramers characterize a spectrum of TCR affinities in the human antigen-specific T cell response. J. Immunol. 165:6994-8.

28. Baumgartner, C. K., A. Ferrante, M. Nagaoka, J. Gorski, and L. P. Malherbe. 2010. Peptide-MHC class II complex stability governs CD4 T cell clonal selection. J. Immunol. 184:573-81.

29. 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. 74:4093-101.

30. Sabatino, J. J., Jr., J. Huang, C. Zhu, and B. D. Evavold. 2011. High prevalence of low affinity peptide-MHC II tetramer-negative effectors during polyclonal CD4+ T cell responses. J. Exp. Med. 208:81-90.

31. Homann, D., L. Teyton, and M. B. Oldstone. 2001. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. Nat. Med. 7:913-9.

32. Pepper, M., J. L. Linehan, A. J. Pagan, T. Zell, T. Dileepan, P. P. Cleary, and M. K. Jenkins. 2009. Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. Nat. Immunol. 11:83-9.

33. Schiemann, M., V. Busch, K. Linkemann, K. M. Huster, and D. H. Busch. 2003. Differences in maintenance of CD8+ and CD4+ bacteria-specific effector-memory T cell populations. Eur. J. Immunol. 33:2875-85.

34. MacLeod, M. K., E. T. Clambey, J. W. Kappler, and P. Marrack. 2009. CD4 memory T cells: what are they and what can they do? Semin. Immunol. 21:53-61.

35. Kalina, T., H. Lu, Z. Zhao, E. Blewett, D. P. Dittmer, J. Randolph-Habecker, D. G. Maloney, R. G. Andrews, H. P. Kiem, and J. Storek. 2005. De novo generation of CD4 T cells against viruses present in the host during immune reconstitution. Blood 105:2410-4.

36. Choi, Y. S., R. Kageyama, D. Eto, T. C. Escobar, R. J. Johnston, L. Monticelli, C. Lao, and S. Crotty. 2011. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity 34:932-46.

37. Zhu, J., and W. E. Paul. 2010. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. Immunol. Rev. 238:247-62.

38. Hegazy, A. N., M. Peine, C. Helmstetter, I. Panse, A. Frohlich, A. Bergthaler, L. Flatz, D. D. Pinschewer, A. Radbruch, and M. Lohning. 2010. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. Immunity 32:116-28.

39. Elsaesser, H., K. Sauer, and D. G. Brooks. 2009. IL-21 is required to control chronic viral infection. Science 324:1569-72.

40. Catron, D. M., L. K. Rusch, J. Hataye, A. A. Itano, and M. K. Jenkins. 2006. CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. J. Exp. Med. 203:1045-54.

41. D'Souza, W. N., and S. M. Hedrick. 2006. Cutting edge: latecomer CD8 T cells are imprinted with a unique differentiation program. J. Immunol. 177:777-81.

42. Fousteri, G., A. Dave, A. Juedes, T. Juntti, B. Morin, L. Togher, D. L. Farber, and M. von Herrath. 2011. Increased memory conversion of naive CD8 T cells activated during late

phases of acute virus infection due to decreased cumulative antigen exposure. PLoS One 6:e14502.

43. Jelley-Gibbs, D. M., D. M. Brown, J. P. Dibble, L. Haynes, S. M. Eaton, and S. L. Swain. 2005. Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. J. Exp. Med. 202:697-706.

44. Kaech, S. M., and E. J. Wherry. 2007. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity 27:393-405.

45. Lynch, H. E., G. L. Goldberg, A. Chidgey, M. R. Van den Brink, R. Boyd, and G. D. Sempowski. 2009. Thymic involution and immune reconstitution. Trends Immunol. 30:366-73.

46. Sharpe, A. H. 2009. Mechanisms of costimulation. Immunol. Rev. 229:5-11.

47. MacLeod, M., M. J. Kwakkenbos, A. Crawford, S. Brown, B. Stockinger, K. Schepers, T. Schumacher, and D. Gray. 2006. CD4 memory T cells survive and proliferate but fail to differentiate in the absence of CD40. J. Exp. Med. 203:897-906.

48. Hendriks, J., L. A. Gravestein, K. Tesselaar, R. A. van Lier, T. N. Schumacher, and J. Borst. 2000. CD27 is required for generation and long-term maintenance of T cell immunity. Nat. Immunol. 1:433-40.

49. Kurche, J. S., M. A. Burchill, P. J. Sanchez, C. Haluszczak, and R. M. Kedl. 2010. Comparison of OX40 ligand and CD70 in the promotion of CD4+ T cell responses. J. Immunol. 185:2106-15.

50. Soares, H., H. Waechter, N. Glaichenhaus, E. Mougneau, H. Yagita, O. Mizenina, D. Dudziak, M. C. Nussenzweig, and R. M. Steinman. 2007. A subset of dendritic cells induces CD4+ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo. J. Exp. Med. 204:1095-106.

51. Simpson, T. R., S. A. Quezada, and J. P. Allison. 2010. Regulation of CD4 T cell activation and effector function by inducible costimulator (ICOS). Curr. Opin. Immunol. 22:326-32.

52. Yi, J. S., M. A. Cox, and A. J. Zajac. 2010. Interleukin-21: a multifunctional regulator of immunity to infections. Microbes Infect. 12:1111-9.

53. 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 324:1576-80.

54. 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 324:1572-6.

55. Walton, S. M., N. Torti, S. Mandaric, and A. Oxenius. 2011. T-cell help permits memory CD8(+) T-cell inflation during cytomegalovirus latency. Eur. J. Immunol. 41:2248-59.

56. Zhou, W., M. Sharma, J. Martinez, T. Srivastava, D. J. Diamond, W. Knowles, and S. F. Lacey. 2007. Functional characterization of BK virus-specific CD4+ T cells with cytotoxic potential in seropositive adults. Viral. Immunol. 20:379-88.

57. Iyer, J. G., O. K. Afanasiev, C. McClurkan, K. Paulson, K. Nagase, L. Jing, J. O. Marshak, L. Dong, J. Carter, I. Lai, E. Farrar, D. Byrd, D. Galloway, C. Yee, D. M. Koelle, and P. Nghiem. 2011. Merkel Cell Polyomavirus-Specific CD8+ and CD4+ T-cell Responses Identified in Merkel Cell Carcinomas and Blood. Clin Cancer Res 17:6671-80.