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Mechanisms of CD8 T Cell-mediated Control of Mouse Polyomavirus and the Requirement for Naïve T Cell Recruitment to Maintain Immunologic Memory

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

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Abstract

Mechanisms of CD8 T Cell-mediated Control of Mouse Polyomavirus and the Requirement for Naïve T Cell Recruitment to Maintain Immunologic Memory

Human polyomaviruses (PyV) have long plagued immune-deficient patients, who may develop PyV associated pathology, due to the lack of available anti-polyomavirus agents and host determinants which identify or predict susceptibility to disease. The mouse polyomavirus (MPyV) model allows the study of the immunologic control of the virus in its natural host. $CD8^+$ T cells recognizing infected cells are required for viral control and tumor resistance. However, the effector mechanisms needed to clear intracellular pathogens (TNF-α, Fas, perforin) were dispensable for MPyV control. Work by a human PyV group identified IFN-γ as a molecule that inhibits PyV replication. Against MPyV, IFN-γ treatment of cell lines reduced MPyV gene expression and impaired viral replication in a dose-dependent manner. Mice deficient in the IFN-γ receptor ($IFN\text{-}\gamma R^{-/-}$) maintain a functional $CD8^+$ T cell compartment, yet fail to control MPyV in vivo, displaying higher viral loads and susceptibility to MPyV-tumors. $IFN\text{-}\gamma R^{-/-}$ transplanted kidneys harbor increased viral burden compared to normal wild-type (WT) kidney transplants. Finally, IFN-γ given as a therapeutic during persistent MPyV infection improved kidney resident viral control, a major reservoir of persistent mouse and human polyomaviruses. Our data demonstrate that IFN-γ is a major anti-polyomavirus effector molecule, and has therapeutic potential.

Due to the dynamics involved with antigen and inflammation during chronic viral infections, it is unknown if memory developed against PyV resembles that against latent chronic pathogens. We have found that persistently infected mice maintain a large, stable anti-viral CD8 response, yet these memory cells are short lived upon transfer to naïve mice. Interestingly, new naïve progenitors can be primed de novo, contributing to the memory T cell pool. Therefore, we developed a novel MPyV TCR transgenic system that allows the monitoring of the timing of priming and how this affects the pool of memory T cells. Late MPyV-infection recruited CD8 T cells were stably maintained, possessing improved phenotype, multi-cytokine functionality, and superior recall potential. In contrast, acute-infection recruited cells were deleted over time, and displayed characteristics of exhaustion. These findings suggest CD8 T cells recruited during persistent MPyV infection contribute significantly to the preservation of functional memory against MPyV.

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Chapter 1: Introduction

I. Adaptive immunity to viral infections

Splinters, bites, paper cuts, eating your lunch, and even in every potential breath, viruses can be anywhere and everywhere (1). Give them a route in, and they will follow it. These infectious pathogens are categorized based on nucleic acid content, viral genome organization, capsid shell structure, and presence/absence of a lipid membrane envelope. The list goes on. Viral infection can result in widespread cellular and tissue damage as these entities attempt to hijack the cells they infect in order to fulfill their replicative life cycle. The subsequent subversion of host cellular activity wreaks havoc on cellular processes as the virus and host cell battle for the machinery and nutrients required for their respective survival. Lytic viral infections are especially damaging to the cells and tissues they infect, while those that remain persistent in host cells long-term may result in persistent cell stress, chronic cellular damage, or promote tumor formation if not fully cleared. Also, the failure to clear these persistent pathogens affords them the opportunity to cause significant global tissue damage should the host breaks ever be released. As such, humans have evolved highly functional anti-viral immune responses adapted to neutralize and kill these intracellular pathogens upon infection, prevent or limit subsequent infection, as well as to keep persistent infections under long-term control. CD8 T cells play a dominant role in the clearance of these intracellular pathogens, and their effector mechanisms and ability to derive functional memory is critical to the immune system's ability to clear initial infection and respond bigger and better upon viral re-encounter.

Upon viral infection, the innate immune system is the first to detect the presence of viral particles and initiate the onset of the immune response. The initial breach in the skin or mucosal lining sets off early inflammatory factors from innate immune cells resident in these tissues, including differing classes of dendritic cells (DCs) and macrophages (2). These cells sense invading pathogens via expression of surface and intracellular pathogen-recognition receptors (3, 4). These receptors, including TLRs, RIG-I-like receptors (RLRs), and Nod-like receptors (NLRs), sense pathogen-associated molecular patterns (PAMPs) such as virion capsid structure, CpG-DNA motifs, or single-stranded RNA (5, 6). These PAMPs trigger macrophages and dendritic cell activation, resulting in the production of proinflammatory mediators like IL-1β, IL-12, and type I interferons (IFNs), as well as potent chemoattractants like CCL-2 and CCL-5, all to promote the full activation of the coming immune response (7-10). The production of type I IFNs in response to pathogens signals nearby cells of danger and activates production of several anti-viral mediators to driving and anti-viral cellular state (11, 12). Meanwhile, these inflammatory mediators also help to recruit new innate immune cells into the inflamed area.

Circulating neutrophils and monocytes migrate towards these chemoattractants and differentiate into activated phagocytes. These phagocytes help clean up debris from the initial breach, and also move in to endocytose any infected cell showing signs of distress. Neutrophil and macrophage activation in these inflamed sites generates further TNF-α, IL-1β, as well as interferon-gamma (IFN-γ), together feeding back to the ongoing inflammatory process (13, 14). Some monocytes that migrate into inflamed tissues receive the stimulus needed to differentiate into a DC rather than a macrophage (15).

DCs provide a bridge between the innate immune response and the development of an adaptive immunity by serving as an initial phagocyte that can subsequently fully activate the CD4 and CD8 T cell responses(16-18). Those DCs that phagocytose tissue and debris can then migrate away from the inflamed sites and back into the lymph.

The lymphatic fluid carries potentially infected cells, activated DCs or other phagocytes, and any tissues debris, from the site of inflammation to the lymph nodes (LNs), and later on into the spleen. These specialized tissues bring potentially infected cells or cell that engulfed pathogenic material into close contact with the adaptive immune system. Activated DCs and macrophages, having received inflammatory stimulus and engulfed potential pathogens, upregulate surface expression of MHC class I and MHC Class II (19, 20). These molecules present short digested protein epitopes (peptides) from within the phagocyte, to CD8 and CD4 T cells within the LN and spleen, earning these cells the title of antigen-presenting cell or APC (21). Normally, only self-peptides are presented on the cell surface, and as such are ignored by naïve and memory T cells in the LNs and/or spleen. However, during and infection and subsequent inflammation, pathogenic proteins can be processed and presented on these cell surface molecules to T cells. Full activation of these APCs also drives expression of costimulatory receptors and ligands required to fully activate any potential T cell response. The surface expression of CD80 and CD86 can be driven by sufficient TLR signaling as well as by ligation of CD40 with CD40L expressed on activated CD4 T cells (22, 23). The signaling of CD40 and/or TLR signals fully "licenses" DCs, improving the expression of CCL-3 and CCL-4 as well as costimulatory ligands, allowing for improved migration and subsequent activation of CD8 T cells within the lymph node (24).

Full CD8 T cell activation requires several stimuli in order to overcome the regulatory checkpoints that are in place to prevent autoimmunity during normal steady-state conditions. The interaction of the T cell receptor (TCR) with an MHC-I molecule presenting the TCR cognate peptide serves as signal l. Signal 1 is further amplified when the T cell receives costimulation from the APC via CD28/CD80 or CD40L/CD40 signaling (signal 2). Signal 1 and 2 together rapidly initiates a drive into the cell cycle, and activates the expression of needed transcription factors for expression of IL-2 (NF-κB, NFAT, and AP-1) (25). Production and release of IL-2 from the CD8 T cell, as well as potential sources from nearby activated CD4 T cells, triggers the IL-2R (CD25), further driving the cell to proliferate over the next several days, generating roughly a 4 log increase in potent $CD8^+$ cytotoxic T lymphocytes (CTLs) (26). Following this activation within the lymph nodes and spleen, these newly activated $CD8^+$ CTLs migrate out of the LNs and back to the inflamed sites. Once in the inflamed sites, having lost their necessity for costimulation, these CD8 T cells can now recognize cognate antigen expressed by infected cells and release their cytolytic potential to eliminate the infectious threat.

The environmental inflammatory milieu during the initial priming and subsequent activation of CD8 T cells also plays an integral part in the eventual developed response (27). Dubbed "Signal 3," this environment of inflammatory milieu helps provide, transmit, and imprint critical signals from the environment and APC, to the CD8 T cell (27-29). As mentioned previously, the licensing of DCs improves production of CCL-3 and CCL-4 which help CD8's better find potential antigen carrying DCs (30, 31). DCs also produce IL-12 and IFN-α and β as well as IL-1 which together optimize and alter the

activation and differentiation of developing effector cells (32), and the licensing of DCs further increases expression of these factors (27, 29, 32). Deficiency in this CD40 mediated licensing has been shown to have little effects on the initially development of the CD8 T cell response, but there is a significant reduction in CD4 levels, reducing the memory potential of the overall CD8 T cell pool (33-36). Recent evidence even suggests that DC licensing from CD4 T cell help plays a role in the increase in LN size and cellular recruitment (via increasing arteriole size) following infection of mice with HSV2 (37). Also of importance is numerous studies finding that the lack of these environmental signals can dull the expansion of responding CD8 T cells, and inhibit/limit their ability to develop into true memory cells (29, 38, 39).

Initially, IFN-γ signaling during the T cell response was thought to provide a suppressive signal to the developing response. Studies identified a role for IFN-γ signals in the elimination of the CD4 T cell response and these signals also drove the apoptosis of contracting CD8 T cells, resulting in increased T cell responses in deficient mice (40-42). Vast proliferation of CD8 T cells is also seen in IFN-γ$R^{-/-}$ mice, and these cells also display a slower rate of contraction following antigen clearance, suggesting that IFN-γ may provide an overall inhibitory signal to the differentiation of memory (42, 43). However several recent publications using acute LCMV infections have found that IFN-γ signaling stimulates the development and accumulation of the CD8 T cell response (44, 45). During these infections, early IFN-γ signaling induces increased numbers of responding T cells, and also resulted in an increased number and improved function of memory T cells. Therefore, understanding how IFN-γ and other inflammatory mediators

affect the anti-viral response during both the acute and memory phases of infections remain to be determined.

II. CD8 T cell Responses

CD8 T cells are critical agents against intracellular pathogens, because of their ability to detect pathogens capable of hiding from the innate and humoral arms of the immune response. Upon recognition of cognate antigen expression on infected cell, the CTL releases preloaded cytotoxic vesicles in a vectorial fashion towards the target cell (46). These vesicles contain effector molecules that trigger cellular death pathways, and include IFN-γ, perforin, granzymes, and TNF-α amongst others. Reception of IFN-γ signals in infected cells triggers increases in MHC I surface expression, making the infected cell and nearby cells more visible to other CD8 T cells (19, 47-49). Signaling from the IFN-γ receptor (IFN-γR) can also result in a rapid cessation of cell division, potentially halting virally induced cellular proliferation and promoting cellular apoptosis/necrosis (49, 50).

Perforin and granzyme molecules have long been appreciated for their ability to perforate target cells and initiate cell death (51, 52). These perforations arise from the perforin molecules ability to integrate into the target cell membrane creating a pore. This results in cellular leakage into the periphery, but also allows passage of the CTLs lytic granzyme proteases into the target cell. Granzymes entry through these pores, as well as entry of perforin/granzyme complexes via the mannose-6 phosphate receptor, cleaves and activates caspase 8, leading to cytochrome c release from the mitochondria and eventual

DNA cleavage via caspase associated DNAse (53). TNF-α ligation by the TNF receptor on an infected cell can also generate a cell death signal via caspase 8 activation (54, 55). Also, the infected cell's expression of Fas can be triggered by the increased expression of Fas ligand (FasL) by activated CTLs, also resulting in caspase 8 activation (54). Together, activated CD8 T cell mediated viral control includes direct increases in antigen processing and presentation, cessation of cellular proliferation, and activation of cellular death pathways.

While the humoral immune response can prevent viral spread, and can prevent reinfection by a similar pathogen, the Ab response is inefficient at detecting and targeting infected cells for clearance. How CTLs kills target cells is highly variable, with some pathogens requiring a single effector function, and others requiring poly-cytokine functionality for efficient viral clearance. The perforin/granzyme apoptotic pathway is most often associated with CTL mediated killing, and is required for control of the arenavirus LCMV and the filovirus Ebola (56-58). This direct cytolytic molecule has also been linked to viral control of HIV, West Nile, Ectromelia, and Theiller's viruses, indicating it has a broad range of pathogen control potential, regardless of acute or persistent infections (59-63). However, some viral agents show significant resistance to control by perforin and granzyme, suggesting other anti-viral mediators arm the CTL response and assist in viral clearance when this pathway is insufficient.

As previously described, TNF-α production from activated CD8 T cells can activate caspase-associated death pathways when directed at infected cells, but also serves as a potent inflammatory agent. Part of the improved viral control mediated by TNF-α may be due to its ability to improve recruitment of immune cells to the inflamed

sites (64, 65). At least part of the immunologic control of vaccinia virus is mediated by TNF-α signaling(66). Strangely though, persistent viral Hepatitis B infection within the liver has been shown to rely on TNF-α signaling from CD8 T cells, which control the virus in a non-cytolytic manner, suggesting a direct anti-viral function of TNF-α is also possibly for some infections (67-69). It is of significance that some of the TNF-α mediated viral control utilized a cyto-curative pathway (i.e. non-cytolytic), suggesting it may indeed be possible to clear viral infection without increasing cellular pathology.

Non-cytolytic effector mechanisms are very interesting viral control pathways, as they cure infected cells, thus preventing further and/or future damage of critical sites (CNS, eyes, or heart). IFN-γ is a pleiotropic cytokine that activates multiple immune processes including increased expression of MHC I and II, increased costimulatory ligand expression, the activation of caspase 1, and activation of the immunoproteosome (49, 70). Its known effector functions were further increased when it was recently found to be the cyto-curative cytokine involved in coronavirus clearance from oligodendrocytes, as well in the non-cytolytic control of HSV reactivation (71, 72). Indoleamine 2,3-Dioxygenase (IDO) may be one curative anti-viral product activated during IFN-γ signaling, as this product is known to mediate control of Measles virus, herpes simplex, influenza virus infection, and has also been associated with ongoing control of other viruses (73-77). Increased IDO expression leads to tryptophan starvation in targeted cells, as well as the production of other anti-microbial products, together which may shut off active protein synthesis pathways and limit viral replication. If IFN-γ can mediate other cyto-curative pathways to improve viral infection remain unknown, but the potential is clearly there.

III. The generation and role of CD8 T cell memory

Full viral clearance the immune response has allowed the adaptive immune response to evolve the capacity to produce a memory population. The development of memory affords the body a more rapid and larger immune response upon reinfection with the same or similar fashion (78). Following antigen-induced expansion, the CD8 T cell response contracts, with 90-95% of the responding cells contract and dying off (79). However, the small population of remaining cells that can survive the loss antigen and inflammation can develop into a true memory population (80, 81). This CD8 differentiation pathway can be triggered by a single encounter with antigen and costimulation, though there is some debate over the next steps in memory cell survival (82-84). Most research favors the linear differentiation pathway of CD8 T cell progression into a memory population, with the pool of activated cells having relieved the least antigen and/or inflammatory insult slowly deriving the eventual memory pool (85, 86). However, other means of T cell development have been proposed that relate to unequal distribution of T cell contents following initial antigen-induced cellular division (87). Regardless of the means, the survival of this small percentage of T cells provides a several fold increase in the number of antigen-specific cells. What signals allow for these cells to survive following antigen clearance?

An early finding on these memory precursor cells indicated that a small subset of developed effector CD8 T cells expressed the high-affinity IL-7 receptor (CD127) on their cell surface (86, 88). These cells preferentially survive and/or proliferate as contraction ensues, and make up more and more of the developing CD8 population (85, 89-92). This receptor expression allows this surviving population to survive independent

of infection induced IL-2 signals, to reception of the homeostatic and proliferative cytokine IL-7 present in steady-state conditions (93, 94). IL-7 signals drive increased expression of the pro-survival protein Bcl-2 and can drive some cells into cell cycle (86, 95, 96). Later, as the memory response develops, there is an increase in cells expressing the IL-2Rβ chain, CD122 (90). Together this allows for reception of multiple homeostatic and proliferative cytokine signals to aid in the development and maintenance of the developing memory response. Together, these signals, and likely others, allow these early memory precursor cells to survive the eventual clearance of antigen and loss of the IL-2 survival signals following viral control.

This survived memory CD8 T cell pool provides for a more rapid and elevated response should antigen be reencountered. The development of an L-selectin (CD62L) expressing memory population, termed T central memory (T_{CM}), allows for a population of early responding T cells that remain localized within the LNs and spleen (97, 98). Here they can more rapidly associate with other immune cells, are at increased precursor frequency, and can rapidly secrete IL-2 for proliferation upon secondary antigen encounter (99). Another memory population expresses lower levels of this LN homing marker, allowing them to better migrate into various non-lymphoid tissues, where they serve as a front-line of defense against secondary exposure (100, 101). These cells, T effector memory or T_{EM}, migrate into and out of various tissues where they respond directly upon antigen reencounter with pre-loaded cytolytic (98, 99). Together this memory response allows the body to develop immunity to potential reinfection.

More recently, several other groups have published other markers that might better describe the memory recall response to secondary infection. Hikono et al, found

that phenotyping the activation status of responding cells was superior to CD62L status in predicting recall capacity of antigen specific memory cells (102). By measuring the activation marker CD43 and the coexpression of the costimulatory marker CD27, CD8 T cells displaying a $CD27^{hi}$, $CD43^{lo}$ phenotype (associated with a low level of antigen activation) were vastly superior in recall capacity. Those cells displaying a more activated phenotype, $CD43^{hi}/CD27^{hi}$ or $CD43^{hi}$ $CD27^{lo}$ meanwhile, were highly deficient in recall potential. This study supports the hypothesis for an association with activation status and the differentiation potential of CD8 T cells; Those that have received the least amount of antigen and inflammatory insult, i.e. activation marker "lo", are those most likely to derive a true memory population with recall potential (103, 104).

Given the potential for a single cell to fully derive the effector to memory differentiation program, how antigen levels, costimulation, and/or inflammation alter the differentiation program of memory CD8 T cells is of recent focus (105). While, surface marker expression has been critical in initial studies and evaluation of memory differentiation in several models of infection, how this expression alters (directly or indirectly) the developing CD8 T cell's transcriptional program are a main focus of more recent studies. One such factor, T-bet, was originally identified for its required role in the development of T_H1 CD4 T cells, but now has been shown to play a critical role in CD8 T cell effector differentiation pathway leading to IFN-γ production and FasL expression (106-109). Targeted mutations within the T-bet locus, found that T-bet expression was not needed for acquisition of IFN-γ production and cytolytic potential in responding CD8 T cells, a finding in contradiction to the requirement of T-bet for these functions from other CD4 T cells and NK cells (108). However, antigen-specific CD8 T cell studies

found that T-bet deficiency led to decreased IFN-γ production and poor cytolytic potential, and even found expression of IL-4 and IL-10, a cytokine expression profile more associated with CD4 T cells (110, 111). While deficient in effector function, these cells maintained an ability to proliferate and expand normally, implying a potential disconnect between normal CD8 T cell effector functions and their proliferation during priming and subsequent differentiation (110).

Inflammation has recently been shown to play a large role in determining the expression level of T-bet in activated CD8 T cells. Increased levels of IL-12 can considerably enhance the level and sustained expression of T-bet in both CD8 and CD4 T cells (107, 112, 113). This may be mediated via IL-12 induced STAT4 expression, resulting in increased mTOR activity and/or associated chromatin remodeling on direct downstream genes like *gzyB* (32, 106, 112, 114). In fact, IL-12 mediated T-bet expression further promotes expression of the IL-12 receptor (IL-12Rβ2) on these signaled cells, providing a direct feedback loop to responding T cells (115). This feedback mechanism though is likely to favor short lived effector cells, which are likely needed at the onset of infection and high levels of inflammation (like IL-12), but this developmental program occurs at the expense of memory precursor generation (116). As a whole though, this development of a full effector population upon viral infection, driven in part by sustained T-bet expression, has been linked to improved viral control (117).

While T-bet may focus on the effector population at the expense of memory generation, it is clear though that some level of T-bet signaling is required for memory CD8 T cell fate decisions. Complete T-bet deficiency results in a failure to sustain and/or

increase the levels of the IL-15 receptor β (CD122) required for memory T cell homeostasis (91, 113, 116, 118). Interestingly though, this deficiency in T-bet expression by activated CD8 T cells dramatically reduces the levels of short-lived effector cells, or SLECs ($CD127^{lo}$, $KLRG1^{hi}$). This population, composed primarily of fully differentiated highly functional CTLS, is severely handicapped at antigen induced secondary expansion and homeostatic survival (88, 119). The disproportionate loss of SLECs favors the development instead of a less activated ($CD127^{hi}$, $KLRG1^{lo}$) memory-like precursor effector cell population (MPECs) (113). Together, this suggests that some basal amount of T-bet likely needs to be expressed for proper effector cell development and memory T cell differentiation, but also that is more for the development of memory CD8 T cells.

A related T-box transcription factor, eomesodermin (Eomes) has also recently become appreciated for its dynamic role in the development of CD8 T cell memory. While some CTL functions required T-bet expression, the presence of T-bet independent CD8 T cell development pathways, suggest a secondary redundant pathway for CTL differentiation (110, 120). Using dominant negative forms of, and overexpression vectors of Eomes and T-bet, Pearce et al. found that both transcription factors could function independently to drive the genes for IFN-γ production and the cytolytic molecules, perforin and granzyme. Also, due to the common T-box domain shared by these two nuclear factors, it was determined that each could partially antagonize the other, thereby competing for similar genetic targets, and suggesting a level of transcriptional redundancy (121). Deficiency of both T-bet and Eomes results in large losses of functional memory T cells, partly ascribed to the insufficient upregulation of CD122, required for homeostatic survival via IL-15 (109).

However, while these two transcription factors may have similar functions during the development of effector CD8 T cell function, recent evidence suggests that they have inverse patterns of expression during the development of memory. As described above, those cells with a T-bet^{hi} phenotype are less likely to enter the memory pool. Following the clearance of antigen from the system, Eomes expression gradually increases, and these cells show increased expression of the IL-2βR, CD122 (109). Given that Eomes expression is directly repressed by inflammation and T-bet, and given the dependence of memory T cell maintenance via IL-15 signaling following antigen clearance, Eomes-mediated maintenance and enhancement of the IL-2β chain may promote the survival of less differentiated CD8 memory precursor cells (122, 123). Together, this evidence supports the hypothesis of a direct cooperative effort between Eomes and T-bet in their roles during CD8 T-cell effector differentiation, but also that these transcription factors exhibit non-redundant roles in the development of CD8 T cell memory.

Interestingly, the transcriptional repressor Blimp-1, a molecule most often associated with terminal B cell differentiation in order to develop plasma cells, has recently been associated with numerous roles in the development and homeostasis of CD8 T cells (124). Blimp-1 expression is lowest in the MPEC population of CD8 T cells, and its expression is heightened in cells possessing a more effector-like state where it might suppress the development of true memory differentiation (125-128). While conditional deletion of Blimp-1 results in increased memory development, only haploinsufficiency (Blimp-1$^{+/-}$) improved the clearance of viral infection, implying some level of Blimp-1 expression is needed for proper differentiation pathways (127). In fact, recent studies have found that Blimp-1 deficiency impairs proper trafficking to the lung,

reduces granzyme expression, and lowers the proliferative potential of these cells to recall responses directed to this organ (128). This could be related to the decreased T-bet expression in Blimp-1 deficient cells, resulting in incomplete/improper CD8 T cell effector activation during the infection. Together it could be implied that Blimp-1 may play a critical role in how, when, and what type of CD8 T cell develops after infection, as well as affect where they go once they develop . Since studies have found that increased levels of Blimp-1 expression in CD8 T cells is associated with increased T-bet expression, while lower Blimp-1 levels is more associated with increased Eomes, Blimp-1 may serve as a master regulator of the effector to memory differentiation pathway in responding CD8 T cells (128, 129).

How and why then are various transcriptional programs triggered in the developing immune response? Several experiments have described an important interplay between the inflammatory stimulus of IL-12 and the expression of these transcription factors. The proinflammatory cytokine IL-12, highly expressed early on during infection, promotes and sustains the expression of T-bet while repressing Eomes expression (106, 130). Driven by increased IL-12, T-bet remains high in early responder CTLs, promoting the development of functional effector CD8 T cells and stalling the derivation of memory precursors (131). Cellular activation and T-bet upregulation does increase expression of IL-2Rβ chain over the course of differentiation, allowing cellular IL-15 survival in the short term, and potentially bridging the development of a memory precursor population (109). Once antigen levels clear and inflammation declines, T-bet levels decline and the repression of Eomes is relaxed allowing for the potential development of, or preferential survival of the memory precursor pool (109). Not

surprisingly then, the loss of IL-12 signaling results in a perturbed effector phase expansion/contraction response, while favoring the development of memory CD8 T cells (130). It remains to be determined if the memory pool is formed from cells expressing hi levels of T-bet that transition into Eomes^{hi} expression, or rather from a population of less antigen and inflammation insulted cells (i.e. T-bet^{int-lo}) that preferentially survive the contraction phase. Regardless, the reciprocal expression of these two transcription factors, especially in concert with different phases of inflammation, suggests that inflammatory signals act as a switch to determining effector versus memory cell differentiation.

IV: CD8 T cell memory perturbations during persistent infections

The development of true memory T cells requires the full clearance of the infectious agent, removal of inflammation from the system, and subsequent clearance of antigen, forcing those cells that survive to be fully independent from the initial stimulus. However, numerous viral infections remain a persistent and chronic burden to the immune system (132). The constant presence of viral associated Ags, or even the virus itself, results in an amalgam of CD8 T cell population having been stimulated under wildly different antigen, costimulatory, and inflammatory settings (133). These "chronic memory" T cells display varied deficiencies in these settings, and while some T cell populations can remain fully functional and survive in the system long-term, several models have shown severe deficiencies and even complete loss of these T cell populations (134-138). The study of chronic memory T cells provide invaluable

information to our understanding of T cell responses to persistent human pathogens that lead to pathologic disease(s), like HIV, hepatitis C virus (HCV), cytomegalovirus (CMV), and polyomaviruses.

Early studies using the LCMV clone-13 mouse infection model, and more recently in various other high level persistent infection models, have found that chronic memory CD8 T cells become exhausted over time in a hierarchical manner (139-142). Highly stimulated chronic memory CD8 T cells lost the ability to proliferate, co-produce IL-2, or have cytolytic capabilities upon ex vivo antigen stimulation (139). Continued antigen insult leaves responding cells unable to co-production TNF-α, and further antigen stimulus leaves cells unable to elaborate IFN-γ, thus leaving antigen specific cells without any detectable effector function (139, 143, 144). If complete incompetence of antigen specific CD8 T cells was not enough, chronic antigen stimulation in the periphery can physically delete these antigen-specific T cells from the memory pool (145, 146). CD8 T cell dysfunction also results in loss of cellular responses to the homeostatic and proliferative cytokines IL-15 and IL-7, and forces cellular survival to depend on TCR stimulation via chronic antigen (147). CTL exhaustion is also seen in HIV infections, where CD8 T cell dysfunction results in poor viral control, and also prevents protection from superinfection (148-150). Interestingly though, one study found that some cytotoxic capabilities (lytic granule release and target cell lysis) remained in otherwise cytokine exhausted cells, possibly in order to balance immunopathology with some level of viral clearance from infected cells (151).

The CD4 T cell response is critical for the development of a proper memory CD8 T cell differentiation, and yet this population can also undergo exhaustion, albeit at a

slower rate (143, 152, 153). While CD4 T cell help may not initially be required for CD8 T cell effector differentiation, deficiencies in CD4 T cell help, namely coproduction of IL-2 and a deficient DC licensing, prevents the formation of a memory pool (22, 154-157). Proper CD4 functionality is required for proper viral control of some infections as their loss results in increased viral burden, increased CD8 dysfunction, and problems in B cell responses (158-162). The exhaustion of CD4 T cells also appears hierarchical, and can create further problems in mediating viral control, but improved CD4 T cell help can improve the anti-HIV CD8 response (163-166). However, the effect of chronic infection within the CD4 T cell compartment may be more flexible than CD8 T cell exhaustion, as these cells actively switch from a classical T_H1 pathway to production of IL-21, a cytokine that is critical for the maintenance of the CD8 T cell response (167-170). Therefore the development of an amalgam of CD4 T cell phenotypes, leaves the actual effect of T cell help during varying persistent infections somewhat unclear.

Studies of T cell exhaustion, initially in mice and subsequently confirmed in humans, found that these dysfunctional cells could be marked by high expression of the inhibitory marker PD-1 (142, 171, 172). A member of the CD28 family, PD-1 signaling activates the intracellular phosphatases SHP-1 and SHP-2, leading to de-phosphorylation of activation signals downstream of the TCR, reducing the signals needed for full CTL activation (173). To further help in characterizing the exhausted CD8 T cell state, several other inhibitory receptors have recently been characterized. KLRG1 was found to be associated with CD8 T cells with poor replicative potential, identifying those cells having received higher amounts of antigenic insult and thus retaining a full effector differentiation state (88, 174, 175). Alongside senescent KLRG1 expressing cells, a

decrease in CD127 expression was noted, thus further marking the poor survival potential of these cells (86, 113). These short-term effector cells (SLECs), indeed exhibited poor survival and replicative potential compared to their $CD127^{hi}$, $KLRG1^{lo}$ counterparts (174, 176). Also, the inhibitory receptors LAG-3, 2B4, and CD160 have shown similar patterns of cellular expression on exhausted CD8 T cells during chronic infection (175, 177, 178). LAG-3 expression, for example, is increased within the antigen-specific chronic memory pool (179). This increase may serve to limit cellular accumulation and effector functions against perceived self Ags or to limit immunopathology (180).

Fitting with their association with exhaustion, Ab-mediated blockade of the PD-1 and Lag-3 pathways have been shown to improve CD8 T cells functions in both mouse and primate models of infection (171, 181-183). While individual inhibitory receptor may play distinct roles in limiting CTL potential, evidence suggests a hierarchical expression pattern between increased expression of multiple markers associated with exhaustion, and increasingly dysfunctional T cells, likely fitting with the hierarchical loss of CD8 T cell functions (162, 177, 178). And, as increased exhaustion is linked to increased levels of antigen insults, small improvements in antigen level might improve cellular functionality, further feeding back to further improvement in viral control (140, 183, 184) .

Exhaustion of the T cell compartment is not linked just to expression of inhibitory receptors, but is also associated with dysfunctional regulation of cellular transcriptional profiles (162). These transcriptional changes appear to drive exhausted T cells into a more and more effector-like state. Depending on the level of dysfunction, CD8 T cell display varied levels of T-bet, potentially related to levels inflammation and/or persistent

antigen (185). T-bet expression results in continued repression of CD127, indirectly or directly related to increased expression of Blimp-1, maintaining the effector differentiation state of and short lifespan of the cell (113). Also, SOCS-1 expression from T-bet can directly suppress IL-2 signaling and other survival cytokines, further reducing cellular survival of these exhausted cells (186). The relation of T-bet expression and IL-12 signaling, and the resultant effects on Eomes repression, and the development of memory remains a wide-open question in regards to most persistent pathogens.

The expression of Blimp-1 has also been associated as a potential marker for T cell exhaustion, with its expression levels matching the level of CD8 T cell exhaustion (125, 126, 128). Similarly, increased Blimp-1 expression was associated with increased inhibitory receptor expression and increased dysfunction of the CD8 T cell pool (126, 127). Conditional deletion of Blimp-1 restored the exhausted phenotype responding CD8 T cells, allowing them to remain functional and develop into true memory long term (128). Given Blimp-1's ability to directly block cell cycle promoter myc, and repress IL-2 signaling, high level expression may serve to maintain a highly differentiated effector state (187, 188). How Blimp-1, T-bet, Eomes, and other transcription factors interplay during chronic antigen stimulation to drive CD8 T cells away from memory and into an exhausted state requires additional studies

The study of CD8 T cell exhaustion often resides in high persistent viral load animal models, and as such, not much is known about the potential for cellular exhaustion during low level persistent viral infections. While high level persistent infections like LCMV and HIV may result in loss and complete dysfunction of the CTL response, low level latent pathogens in the herpesvirus family maintain a high level of

viral specific cells having full functional capacities (189-194). While these cells maintain most cytolytic and cytokine potential, they exhibit a highly activated cellular phenotype with poor proliferative potential, expressing high levels of KLRG1 and low levels of costimulatory ligands and cytokine receptors (133, 191, 192, 195-201). How then are these cells maintained if the population is poorly proliferative, especially given the failure to develop an antigen-independent memory phenotype when transferred into naïve animals (202, 203). A recent study found that the effector cells present in the periphery are actually derived from a small, LN resident, memory population that are continually stimulated by persistent antigen (204). Similarly, MHV-68 specific CD8 T cells are maintained via a small population of anti-viral cells that is capable of self-renewal (205).

These latent infections, resulting in persistent reactivation events that cycle between lytic and latent Ags, maintain a low level of persistent antigen and inflammation. The stability of the anti-viral CD8 T cell pool is probably maintained by a low level of proliferating cells already present, as well as late de novo recruitment of new anti-viral precursors (202, 203, 206). Interestingly, this stable CTL population did not require the homeostatic cytokine IL-15 for their survival and proliferation in vivo, and yet failed to survive or proliferate when transferred to naïve mice (205). It was speculated that this IL-15 independent maintenance required the presence of persistent antigen from reactivation events, a hypothesis supported by the failure of these cells to increase Bcl-2 levels during infection. Since antigen potentially plays a role in proliferation of effector CTLs, the possibility of new thymic recruitment of naïve progenitors into the CD8 pool cannot be ruled out as a potential mechanism to help maintain the overall pool (207, 208).

V: Polyomaviruses as a tool to study low level persistent infections

The *Polyomaviridae* family consists of several highly species specific viruses that ubiquitously infect a wide variety of host species. The serendipitous discovery of this DNA virus in 1953 from the cell free, filtered extracts, of AKR leukemic mice, served as, and confirmed the long suspicion that viruses could be etiologic agents for some neoplasms (209). These name polyomavirus (PyV) is a "meatless linguistic sandwich" (attributed to C.J. Dawe), that aptly describes the most dramatic capability of the virus itself; the ability to induce many ("poly") tumors ("oma") in numerous tissue types (210). Following the family's initial discovery in mice, PyVs were quickly discovered in a primate cell line (simian virus 40, SV40), and more recently identified in humans (211). Studies from 1971 identified 2 new human PyVs, BK and JC viruses, that were identified in a patient suffering from severe renal failure following a renal transplant, and in a separate patient suffering from progressive multi-focal leukoencephalopathy (PML) respectively (212, 213). Several new human PyVs have since followed, including the detection of Merkel cell PyV, associated with a highly aggressive cutaneous neoplasm, and a PyV associated with the development in trichodysplasia spinulosa in a heart transplant patient amongst others (214-218). These viruses have revived fears that PyV may also be tumorigenic in their human hosts, especially in the immune compromised population (219, 220).

A recent analysis of human patients in the Denver Colorado area revealed the infection pattern of these PyVs. PyV infection likely occurs early during childhood or into adolescence with over 70% of people being infected with one PyV isolate and/or another (221). While the infection is fairly ubiquitous across the human population, there

are often little to no symptoms to indicate viral infection took place. As previously implied, PyV infections are persistent life-long infections in humans. This life-long infection is believed to be controlled by the cooperative efforts of a neutralizing Ab response and the anti-viral CD8 T cell response (222-225). This hypothesis is supported by the development of severe pathological conditions when the immune system is suppressed by viral infection or immunomodulatory agents (226-228). HIV infection and subsequent development of AIDS, allow the resurgence and/or reactivation of persistent JC virus infection in some 5% of patients (229). The reactivation of JC within this site results in wide-spread, severe demyelination, potentially due to lytic viral infection, and results most often fatal case of PML. There has recently been a rash of JC virus related PML cases in people using immunomodulatory agents for regulating autoimmune disorders, like multiple sclerosis, psoriasis, and Crohn's disease (230-232). These modulatory agents, Efalizumab (anti-LFA-1) and Natalizumab (anti-VLA-4), alter T-cell trafficking and/or inhibit T-cell priming, both resulting in limitation of the immune response to any JC virus reactivation events. Likewise, the immune suppression required for renal transplants, contributes significantly to the resurgence in BK virus infection and eventual PyV-associated kidney nephropathy (PVAN), the leading cause of kidney transplant rejection (233). However, the direct mechanism of PyV pathology is poorly understood, and with the ever increasing use of immunomodulatory agents, further studies are warranted. Also, given the complete lack of any approved or even known anti-viral agents, a model allowing the evaluation of the immune response to and the study of anti-viral agents is needed.

The strict species specificity of the PyV family limits the study of any PyV to its natural host species. The original discovery of PyV in mice, affords an tractable animal model to study the viral pathogenesis and immune response to mouse PyV (MPyV). MPyV expresses 6 known genes, 3 structural and 3 non-structural, among its small ~5kb DNA genome. The non-structural genes are tumor Ags, known as LargeT (LT), MiddleT (MT), and SmallT (ST), critical for the hijacking of host cell machinery to produce excess virions. Following sufficient build-up of LargeT, the 3 structural genes (VP-1, -2, and -3), coding for the viral capsid structure, are produced in productively infected cells. The convenience of the small DNA plasmid-like genome of MPyV makes it possible to mutagenize the virus as needed, and to do so quickly. Couple the ease of MPyV genomic manipulation, with its common life-long persistence in laboratory and feral mice, and the potential for PyV study is mice is nearly boundless (134, 137, 234, 235).

The study of MPyV, as described above, utilized its ability to promote tumors in neonatal mice (234, 236, 237). Certain strains of mice were later on found to be susceptible to MPyV mediated tumorigenesis compared to others (238-240)). These tumor susceptible mice were often associated with defects in anti-viral immunity, further linking the adaptive immune response to PyV mediated control (241, 242). Mice deficient in β2m, thereby deficient any adaptive CD8 T cell response, were highly susceptible to MPyV tumors (243). However, the same was not the case for mice lacking CD4 T cells (MHC $II^{-/-}$), suggesting that it is the anti-viral CD8 response which directs viral control in mice, similar to humans PyV infections (244, 245). Genetic analysis of certain H-2^k strains of mice found that the presence of a gene encoding the mouse mammary tumor virus 7 (mtv7), severely culled a large population of CD8 T cells (246-

250). The mtv7 provirus produces a superantigen, resulting in physical deletion of CD8 T cells expressing a TCR containing Vβ6 or Vβ8.1, which conveniently for MPyV is the dominant TCR Vβ utilized by responding anti-viral T cells. $Mtv7^{+}$ mice, and not their $mtv7^{-}$ counterparts, were highly susceptible to MPyV induced tumors, and were found to bear larger viral loads, suggesting the loss of this CD8 T cell population drastically reduces viral control. Since these mice, and other tumor susceptible mice make both a T-dependent and T-independent neutralizing Ab response made against the VP-1 capsid protein, this further confirmed evidence that CD8 T cells are critical in MPyV control and also needed to curtail development of tumors (251-254).

With the advent of tetramer technology to detect antigen-specific CD8 T cells in response to various infections, multiple studies in our lab helped to define several antigen specific T cell populations in both $H\text{-}2^{k}$ and $H\text{-}2^{b}$ strains (248, 250, 255, 256). We even identified a non-classical antigen specific response against an MHC I like molecule, Q9, in mice wholly deficient in classical MHC I molecules (257). The preponderance of these CD8 T cell responses, regardless of mouse strains, target epitopes present within the viral T antigens, namely MT and LT. The developed CD8 T cell responses show variability in magnitude, immunodominant hierarchy, and functional characteristics (256, 258-260). In $H\text{-}2^{b}$ mice, the $LT_{359\text{-}368}$ (LT359) specific CD8 T cells show classical expansion/contraction/maintenance phases, and remain fully capable of IFN-γ production and TNF-α production long-term (208, 256, 259, 261, 262). While these cells appear to be maintained long-term, studies using cellular transfers from infected -> naïve mice found that these T cells failed to be maintained independent of antigen. With the use of our partial bone marrow chimera setup, we found that the overall antiviral CD4 and CD8

population is constantly bringing in newly recruited naïve precursors, and these cells appear to show increased expression of a more memory like phenotype ($CD62L^{hi}$ and/or $CD27^{hi}$) (134, 259). Further, the use of thymectomized mice, we found that the responding CD8 T cell population is lost over the course of infection if new naïve precursors cannot be primed into the response (208). So, while the antiviral response mains stable, it appears that a portion of the stable CD8 T cell maintenance arises from the late recruitment of new naïve precursors into the overall response.

While it appears that CD8 T cells specific against MPyV remain functional, the critical cytokine/cytotoxic agent controlling MPyV has yet to be discovered. As addresses above, most viral pathogens are controlled, at least in part, by one of the numerous cytotoxic effectors derived from the CTL population. However, our lab has found that cytolytic molecules like perforin and granzyme were not required for viral control and protection for MPyV associated tumors (263). Even the use of mice with a defective gene for Fas (*lpr* mice), or mice deficient in the receptor for TNF-α, found no link between these molecules and MPyV control and tumorigenesis. These studies identify that the removal of classical anti-viral molecules from the CD8 T cell compartment, fails to identify the viral control mechanism during MPyV infections. In Chapter 2, we finally ascribe how and why CD8 T cells required for viral control even when these cells may be deficient in the classical cytolytic pathways.

Meanwhile, how the CD8 T cell pool remains during MPyV infections is still unclear. Our previous findings to date supports a "conveyor belt" model of CD8 T cell recruitment, where early primed cells exhibit a more effector like profile and contribute poorly to the memory response, while those cells primed later on during infection

increasingly populate the memory pool. These late primed memory cells could arise at any time point after initial viral insult. Our bone marrow chimera model which suggests this improved phenotype during late recruitment however, does not allow us to determine the exact point(s) at which memory is more favored over effector differentiation (259). As such, it remains to be determined how the memory pool is stable, when memory development might be favored during MPyV infection, and when (or if) a true memory population develops during MPyV infection. Our development of a novel TCR transgenic system to study persistent priming of naïve cells has allowed us a window into how memory differentiation occurs during chronic MPyV infection. This improvement of late recruitment cells is confirmed and expanded in Chapter 3, allowing us to answer further questions about memory development during persistent viral infection.

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Chapter 2

IFN-γ Controls Mouse Polyomavirus Infection In Vivo

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Data presented in Figure 5 were generated in collaboration with Dr. Kenneth Newell, Dept of Surgery, Emory University, Atlanta, GA

ABSTRACT

Human polyomaviruses are associated with substantial morbidity in immune-compromised patients, including those with HIV/AIDS, recipients of bone marrow and kidney transplants, and individuals receiving immunomodulatory agents for autoimmune and inflammatory diseases. No effective anti-polyomavirus agents are currently available and no host determinants have been identified to predict susceptibility to polyomavirus-associated diseases. Using the mouse polyomavirus (MPyV) infection model, we recently demonstrated that perforin/granzyme exocytosis, TNF-α, and Fas did not contribute to control of infection or virus-induced tumors. IFN-γ was recently shown to inhibit replication by human BK polyomavirus in primary cultures of renal tubular epithelial cells. In this study, we provide evidence that IFN-γ is an important component of the host defense against MPyV infection and tumorigenesis. In immortalized and primary cells, IFN-γ reduces expression of MPyV proteins and impairs viral replication. Mice deficient for the IFN-γ receptor (IFN-$\gamma R^{-/-}$) maintain higher viral loads during MPyV infection and are susceptible to MPyV-induced tumors; this increased viral load is not associated with a defective MPyV-specific CD8 T cell response. Using an acute MPyV infection kidney transplant model, we further show that IFN-$\gamma R^{-/-}$ donor kidneys harbor higher MPyV levels than donor kidneys from wild-type mice. Finally, administration of IFN-γ to persistently infected mice significantly reduces MPyV levels in multiple organs, including the kidney, a major reservoir for persistent mouse and human polyomavirus infections. These findings demonstrate that IFN-γ is an antiviral effector molecule for MPyV infection.

INTRODUCTION

Polyomaviruses (PyV) are a family of small nonenveloped, double-stranded DNA viruses that infect a variety of avian and mammalian species, with nine human PyVs described to date (1). BK and JC PyVs are acquired during late childhood/early adolescence, and in the majority of healthy individuals, these viruses persist life-long as silent infections (2). However, in the setting of depressed immunity, these viruses are associated with life-threatening disease. Approximately 3% of HIV-1-seropositive individuals develop progressive multifocal leukoencephalopathy (PML), a usually fatal CNS demyelinating disease caused by JC virus infection (3). Recently, PML has emerged in a fraction of patients receiving VLA-4 mAb [Natalizumab (Tysabri®)] therapy for multiple sclerosis or Crohn's Disease and LFA-1 mAb [Efalizumab (Raptiva®)] therapy for severe forms of plaque psoriasis (3). Given this risk, Raptiva was withdrawn from the US market despite accumulating evidence for its clinical efficacy in inflammatory bowel disease and transplantation, and Tysabri® now carries an FDA "black box" warning. BK virus is the causative agent for PyV-associated nephropathy in kidney transplant patients and hemorrhagic cystitis in bone marrow transplant recipients (4, 5). Recently, a novel PyV was identified as the probable etiologic agent for Merkel cell carcinoma, an aggressive cutaneous malignancy seen in elderly individuals, and another PyV has been discovered in a hair follicle dysplastic disease in a heart transplant patient. These findings resurrect early suspicions that PyVs may cause human malignancies (6, 7). There are currently no effective anti-PyV therapeutic agents. Defining host immunological mechanisms and therapies that control PyV infection is therefore critical for developing strategies to interdict PyV-associated morbidity in susceptible patients.

Virus-specific $CD8^+$ T cells traffic to sites of infection where they deploy both cell contact-dependent and -independent mechanisms to quell viral replication (8). $CD8^+$ T cell-mediated cytotoxicity may entail vectorial exocytosis of perforin and granzyme loaded granules, ligation of Fas, or release of cytotoxic cytokines (e.g., TNF-α); these antiviral effectors operate by inducing apoptosis of infected cells and/or by rendering uninfected cells nonpermissive for viral infection . Host defense against different viral infections variably requires these effector pathways (8). For example, perforin-granzyme exocytosis is necessary for host immunity to infection by LCMV, murine AIDS retrovirus, and Ebola virus, whereas control of vaccinia virus infection is dependent upon TNF-α (9, 10).

Interferon (IFN)-γ constitutes another effector pathway executed by virus-specific $CD8^+$ T cells. IFN-γ receptor ligation activates the Jak/Stat-mediated signal transduction pathway to orchestrate expression of genes whose promoters contain a gamma-interferon activation site (GAS). GAS gene products modulate innate and adaptive immune responses by a variety of mechanisms, including upregulation of MHC class I and II molecules, increased expression of ligands for costimulatory and inhibitory receptors, induction of the immunoproteosome, macrophage activation, and caspase-1 production (11). In animal infection models using a variety of DNA and RNA viruses, IFN-γ has been demonstrated to inhibit viral protein synthesis, block viral genome replication, and mediate non-cytotoxic clearance of viral genomes from host cells (12-16).

Because PyVs have a narrow host range, MPyV infection provides an important model to mechanistically interrogate PyV pathogenesis and immunity in a natural host. As with the human PyVs, MPyV establishes life-long persistent infection that is

asymptomatic in immunocompetent hosts (17). Previous work from our group and others has identified $CD8^+$ T cells as central components of host immunity to MPyV infection and tumorigenesis (18-20). Further, we have shown that mice deficient in perforin, Fas, or TNF receptors control MPyV infection and retain resistance to virus-induced tumors (21). Abend et al recently reported that IFN-γ reduces BK virus gene expression and viral replication in primary human renal tubular epithelial cells (22). In this study, we show that IFN-γ also confers antiviral activity against MPyV in vitro, and, using the MPyV infection model, demonstrate that IFN-γ contributes to host antiviral defense and has therapeutic efficacy when administered during persistent infection. These findings may have implications for treating human polyomavirus infections in immunocompromised individuals.

MATERIALS AND METHODS

Mice and infections

Female C57BL/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD). IFN-γR$^{-/-}$ (B6.129S7-*IFN-γr*^{tm1Agt}/J), IFN-γ deficient (B6.129S7-*IFN-γ*^{tm1Ts}/J), and B6.PL (B6.PL-Thy.1^{a} /CyJ) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). MPyV (strain A2) was propagated and titered by plaque assay as previously described (19). Adult (8-12 wk old) and newborn mice (<24 h of birth) were inoculated s.c. in hind footpads with 1.5×10^6 PFU and $1\text{-}5 \times 10^5$ PFU of MPyV, respectively. Mice were followed up to 6 mo p.i. for development of tumors. All protocols using mice were approved by the Institutional Animal Care and Use Committee of Emory University. Mice were bred and maintained by the Division of Animal Resources of Emory University.

Assays for MPyV infection in vitro

Subconfluent monolayers of BALB.A31 (A31) cells (ATCC, Manassas VA) were infected by MPyV at an MOI of 3 unless otherwise indicated. Primary cultures of baby mouse kidney (BMK) cells from B6 and IFN-γR$^{-/-}$ mice were prepared as previously described (19). Recombinant mouse IFN-γ (PeproTech, Rocky Hill NJ) at the indicated concentration was added 24 h before infection, unless otherwise stated. Recombinant MPyV-HA virus was generated by inserting the coding sequence (TACCCATATGACGTACCTGATTACGCA) for the influenza virus hemagglutinin (HA) epitope tag (YPYDVPDYA) in-frame at the Blp1 restriction site in the Large T (LT) antigen coding sequence, as described elsewhere (23). Intracellular HA epitope tag

staining with a primary anti-HA rat monoclonal IgG antibody (clone 3F10, Roche Diagnostics, Basel, Switzerland) and an allophycocyanin-conjugated secondary goat anti-rat IgG (BD Bioscience, San Diego CA) was performed using CytoFix/CytoPerm (BD Bioscience) per manufacturer's instructions. Single infection cycle virus replication assays were performed using A31 or primary BMKs infected by MPyV at an MOI 0.1 for 60 h, with viral titer measured by plaque assay (24). Immunofluorescence detection of LT was performed on 3T3 cells plated on glass coverslips that were untreated or treated with 100 U/mL recombinant mouse IFN-γ for 24 h before and through the 48 h infection period , then fixed and stained with polyclonal rat T antigen antibody, and images were acquired and LT^+ nuclei counted as previously described (25). Western blotting was performed on similarly treated 3T3 cells using a pan-T antigen monoclonal antibody (clone F4) as described (25); the membrane was then stripped and reprobed with anti-tubulin antibody (clone DM1A).

Flow cytometric analysis

Kidney-infiltrating lymphocytes were isolated via collagenase digestion followed by percoll gradient separation as previously described (26), RBC-lysed blood, collected via submandibular incision into ACD solution A anticoagulant (BD Biosciences), and RBC-lysed splenocytes were stained with monoclonal antibodies against the following molecules: CD44, CD62L, CD8α, Thy1.1, human granzyme b, IFN-γ, IL-2, TNF-α, and Bcl-2 (BD Bioscience); CD27, CD122, CD127, IFN-γR, PD-1, LAG-3, and TIM-3 (eBioscience, San Diego CA); CD43 (Biolegend, San Diego CA); and KLRG1 (Southern Biotech, Birmingham, AL). $H\text{-}2D^b$ LT359 and $H\text{-}2K^b$ MT246 tetramers were generated

and provided as previously described by the NIH Tetramer Core Facility (Atlanta, GA) (27). Staining was performed at 4°C for 45min. Flow cytometry was performed on a FACSCalibur (BD Bioscience, San Diego, CA) and data analyzed using FlowJo software (TreeStar, Inc, Ashland, OR). Ex vivo peptide stimulation was performed for 4.5 h with the indicated peptide (1 μM) in the presence of brefeldin A. Kidney-infiltrating lymphocytes were stimulated as above with the addition of 1 x 10^6 B6.PL splenocytes as antigen-presenting cells. Cells were surface-stained as above, then fixed, permeabilized and stained intracellularly with the indicated Ab, according to manufacturer's instruction (BD Bioscience).

Quantitation of MPyV infection by PCR and plaque assays

DNA was extracted from the indicated organs and viral genome copies quantified using oligonucleotide primers and Taqman probe to the early viral genes, as previously described (27). Plaque assays on spleen and kidney samples were performed, as previously described (28).

IFN-γ therapeutic administration

B6 mice infected by MPyV for 45 days received either PBS or 2 x 10^4 U of recombinant mouse IFN-γ i.p. every 12 h for 14 days.

Kidney transplantation

Transplantation of B6 and IFN-γ$R^{-/-}$ kidneys into nephrectomized B6 recipients was performed as previously described (29). One day post-transplant, recipient mice received

1×10^6 PFU MPyV s.c. Viral genome copies in donor kidneys were quantified by PCR at 30 days p.i.

Statistical analysis

Student's *t* test with Welch's correction for variance was used for all experiments, and analysis performed using Prism statistical software (GraphPad, La Jolla CA).

RESULTS

IFN-γ inhibits MPyV protein expression and replication

To determine whether the ability of IFN-γ to inhibit gene expression and replication of BK virus (22) extends to MPyV, we infected the permissive A31 mouse fibroblast cell line with MPyV in the absence or presence of IFN-γ. IFN-γ-treated cells expressed lower amounts of MPyV Large T (LT) than untreated cells following 48 h of infection (Fig. 1A). To visualize IFN-γ's anti-MPyV effect at the single-cell level by flow cytometry and immunohistochemistry, we created a recombinant MPyV expressing the influenza hemagglutinin (HA) epitope tag embedded in-frame in LT. HA mAb western blotting of A31 cells infected by this recombinant virus, designated MPyV-HA, identified a single ~100 kDa protein, consistent with the size of LT (data not shown). Anti-HA stained cells infected by MPyV-HA are readily identified by flow cytometry, with minimal anti-HA staining of cells infected by parental MPyV infection (Fig. S1A). Also, anti-HA staining of MPyV-HA infected cells detects nuclear-localized HA-tagged LT by immunohistochemistry (IHC) (Fig. S1B). Adding IFN-γ to the culture medium resulted in a marked reduction in both the frequency and mean fluorescence intensity (MFI) of HA^+ cells, with both parameters decreasing in a dose-dependent manner (Fig. 1B & 1C, and Fig. S2A). The ability of IFN-γ to reduce HA expression was confirmed by IHC analyses, which showed a marked decrease in the percentage of cells expressing nuclear HA (data not shown). Notably, both flow cytometric and IHC assays showed an approximately 50% reduction in HA^+ cells in the presence of 100 U/ml IFN-γ. The ability of IFN-γ to inhibit MPyV protein expression also extended to primary cells. Primary cultures of baby mouse kidney cells (BMKs), which are highly permissive for

productive MPyV infection, showed a significant decrease in both frequency of HA^+ cells and MFI in anti-HA stained cells when exposed to IFN-γ (Fig. 1D and Fig. S2B). IFN-γ had little effect on HA expression by infected BMKs isolated from IFN-$\gamma R^{-/-}$ mice (Fig. 1D), excluding the possibility that IFN-γ mediates its antiviral activity through an alternate receptor. To determine whether IFN-γ inhibited production of infectious MPyV progeny, we assessed viral output by single-cycle viral replication assays in both A31 cells and primary BMK cells exposed to IFN-γ for 24 h preceding and throughout a 60 h infection period. As shown in Fig. 1E and 1F, both cell lines and primary cells treated with IFN-γ yielded approximately 50% and 70% lower viral output, respectively. In the absence of IFN-γ receptors, exogenous IFN-γ had no effect on permissivity of host cells for productive MPyV infection.

We then investigated when IFN-γ exerts its antiviral activity. MPyV genomic DNA was enumerated by quantitative PCR at various timepoints following infection in the absence or presence of IFN-γ. Without IFN-γ, viral genome numbers were relatively unchanged from input levels through the initial 24 h of infection, but then exponentially increased during the ensuing 54 h culture period (Fig. 1G). Similarly, IFN-γ treatment had no detectable effect on viral genome numbers in the first 24 h, but thereafter dampened the accumulation of viral genomes. The observed delay in IFN-γ's anti-MPyV activity fits with our findings that only modest decreases in HA^+ cells or HA MFI by MPyV-HA-infected A31 cells was seen over the first 24 h of infection, regardless of whether the cells were exposed to IFN-γ 1 d before or at the time of infection (data not shown).

We next examined whether the anti-viral activity of IFN-γ against MPyV is mediated by inducing cell death or inhibiting cell proliferation. A31 cells were stained with the vital dye 7-aminoactinomycin D (7-AAD) and the phosphatidylserine binding protein, annexin V, 48 h after infection in the presence or absence of IFN-γ. As shown in the left panel of Fig. 2A, only a small fraction of live cells were stained by annexin V (annexin V^+, 7-AAD^-) in the absence of MPyV infection, and the percentage of apoptotic cells increased marginally with infection irrespective of treatment by IFN-γ. Furthermore, MPyV infection with or without IFN-γ treatment did not result in an increased frequency of late apoptotic/necrotic cells (7-AAD^+) (Fig. 2A, right panel). Because PyV replication requires cell cycle progression, IFN-γ's antiproliferative activity for certain cell types may account for the decreased MPyV-HA gene expression in IFN-γ-treated host cells (30). By counting A31 cells that exclude trypan blue, we found that IFN-γ did not affect accumulation of viable cells over the 72 h observation period. However, when IFN-γ treated cells were infected by MPyV-A2, there was a significant reduction in cell number (Fig. 2B). Because LT overrides cell cycle checkpoints (31), these data suggest that IFN-γ handicaps T antigen-driven host cell proliferation by reducing T antigen expression and subsequent accumulation of viral genomes needed for replication. Taken together, these in vitro studies demonstrate that IFN-γ mediates anti-MPyV activity.

IFN-γ exerts anti-MPyV activity in vivo

To determine whether IFN-γ contributes to host immunity to MPyV infection and tumorigenesis, we infected B6 or IFN-γR$^{-/-}$ mice (B6 background) with MPyV and monitored viral load in the spleen and kidney. As shown in Fig. 3 (upper panel), amounts of MPyV genomic DNA in spleens of IFN-γR$^{-/-}$ mice remained similar throughout acute and persistent phases of infection; however, the kidneys of IFN-γR$^{-/-}$ mice showed significantly higher viral loads as early as the acute phase of MPyV infection (8 d p.i.), and a significant 1-2 log difference during the persistent phases of infection (Fig. 3, lower panel). Interestingly, two adult IFN-γR$^{-/-}$ mice developed gross tumors by 150 d p.i., while no wild-type mice developed tumors (Table 1). Because higher viral loads are also seen in neonatally MPyV-inoculated mice of tumor-susceptible strains (24), we investigated whether IFN-γR$^{-/-}$ mice inoculated as newborns would be predisposed to MPyV-induced tumors. Newborn-inoculated B6 mice are highly resistant to MPyV tumorigenesis (32); however, 100% of neonatally infected IFN-γR$^{-/-}$ mice developed tumors by 6 mo of age (Table 1). Interestingly, while IFN-γR$^{-/-}$ mice developed the typical constellation of MPyV-induced tumors (i.e., kidney, bone, mammary gland, salivary gland) (33), these mice also developed connective tissue tumors localized to the hind footpads, the site of virus inoculation. Thus, these data demonstrate that the absence of IFN-γR signaling impairs the ability of the host to control MPyV infection and maintain resistance to virus-induced tumors.

IFN-γR signaling reduces fitness of the MPyV-specific CD8$^+$ T cell response

Based on substantial evidence that high persistent virus levels negatively impact antiviral $CD8^+$ T cell function (34, 35), we reasoned that the higher viral loads in the IFN-$\gamma R^{-/-}$ mice would render MPyV-specific $CD8^+$ T cells dysfunctional. Further, cell-intrinsic IFN-γR signaling has also been reported to foster expansion of virus-specific $CD8^+$ T cell effectors and their differentiation into memory cells (36). We first compared recruitment and maintenance of MPyV-specific $CD8^+$ T cells in wild-type B6 and IFN-$\gamma R^{-/-}$ mice by longitudinally tracking circulating $CD8^+$ T cells that recognize the dominant D^b-restricted LT359 epitope in individual MPyV-infected mice (27). As shown in Fig. 4A, B6 mice mount a vigorous, LT359-specific $CD8^+$ T cell response that peaks around d 8 p.i., contracts through 15-21 d p.i., and is then maintained long-term. IFN-$\gamma R^{-/-}$ mice mount an equally strong LT359-specific $CD8^+$ T cell response during the acute phase of infection, but show a short delay in their rate of contraction, and then persist at frequencies similar to MPyV-infected B6 mice. This extended contraction phase fits with that previously reported for antigen-specific $CD8^+$ T cell responses in IFN-$\gamma R^{-/-}$ mice infected by *Listeria monocytogenes* (37). The splenic LT359-specific $CD8^+$ T cell population was slightly higher in acutely and persistently infected IFN-$\gamma R^{-/-}$ than B6 mice, both in terms of frequency of $CD8^+$ T cells and total numbers (Fig. 4B and not shown). This phenotype in IFN-$\gamma R^{-/-}$ mice was recapitulated at the level of cytokine effector function, where ex vivo stimulation of splenocytes with LT359 peptide elicited a higher proportion of $CD8^+$ IFN-γ^+ cells coproducing either TNF-α alone or both TNF-α and IL-2 (Fig. 4C). This numerical and functional disparity also applied to the subdominant K^b MT246-specific anti-MPyV $CD8^+$ T cell response (data not shown).

Phenotypic analysis revealed a significantly improved LT359-specific $CD8^+$ T cell response in mice lacking IFN-γ receptors (Fig. 4D). In particular, only 15-20% of D^b LT359 $tetramer^+$ $CD8^+$ T cells in either wild-type or IFN-$\gamma R^{-/-}$ mice expressed PD-1, and did so at a low MFI (Fig. 4D). No detectable staining by mAbs against LAG-3, Tim-3, or 2B4 was evident at days 8, 43, and 200 p.i. (data not shown); thus, no phenotypic or functional (Fig. 4C) signs of T cell exhaustion were apparent (38, 39). Regardless of IFN-γR status, a subset of LT359-specific $CD8^+$ T cells expressing L-selectin (CD62L) appeared over the course of infection. While MPyV-specific cells gradually acquired the anti-apoptotic molecule Bcl-2, IFN-$\gamma R^{-/-}$ mice exhibited a significantly faster acquisition and higher per cell level expression than control wild-type B6 mice. Additionally, LT359-specific $CD8^+$ T cells in IFN-$\gamma R^{-/-}$ mice showed significantly earlier acquisition and higher expression of IL-7Rα (CD127) and the costimulatory molecule CD27, with a more rapid decline of the cell senescence marker KLRG1. CD43 expression was also higher on LT359-specific $CD8^+$ T cells in IFN-$\gamma R^{-/-}$ mice, suggesting that these cells may be in a more activated state (40), possibly resulting from more frequent encounter with infected cells than those in wild type mice. Finally, both IFN-$\gamma R^{-/-}$ and B6 mice expressed equivalent levels of granzyme B over the course of MPyV infection. In aggregate, these data suggest that IFN-$\gamma R^{-/-}$ mice generate an anti-MPyV CD8 T cell response that is not only comparable in magnitude, phenotype, and function to that of wild-type mice, but also that lack of IFN-γ receptor signaling favors a qualitatively more fit antiviral $CD8^+$ T cell response.

Because IFN-γ modulates T cell trafficking to nonlymphoid organs by upregulating expression of chemokines and integrins (41, 42), we asked whether

diminished migration of antiviral $CD8^+$ T cells to IFN-$\gamma R^{-/-}$ kidneys may be associated with elevated viral loads. Using acutely MPyV-infected mice, we found similar numbers of infiltrating D^b LT359 $tetramer^+$ $CD8^+$ T cells in the kidneys of IFN-$\gamma R^{-/-}$ and wild type B6 mice (Fig. 4E). Additionally, kidney-infiltrating LT359-specific $CD8^+$ T cells in IFN-$\gamma R^{-/-}$ mice expressed a similar phenotype to that seen in the spleens of their respective hosts (Fig. 4D, and not shown). We also found no difference in the ability of kidney-infiltrating $CD8^+$ T cells to produce IFN-γ or TNF-α upon direct ex vivo stimulation, suggesting that the deficiency in IFN-γR signaling had no effect on the cytokine potential of infiltrating cells (Fig. 4E, and not shown). This data indicate that the higher MPyV levels in IFN-$\gamma R^{-/-}$ kidneys cannot be explained by impaired migration or function of kidney-infiltrating antiviral $CD8^+$ T cells.

Lack of IFN-γR signaling at the site of persistence results in significant loss of MPyV control

Because the kidney is a major site of persistence for human PyVs, and in order to circumvent potential effects of IFN-γR deficiency on T cell development and homeostasis that could influence MPyV immunity, we transplanted kidneys from IFN-$\gamma R^{-/-}$ or wild-type B6 mice into nephrectomized B6 mice, which received MPyV one day post-transplantation (Fig. 5A). We then assessed viral loads in the donor kidneys at d 30 p.i. As shown in Fig. 5B, viral genomes were approximately 50-fold higher in kidneys from IFN-$\gamma R^{-/-}$ donors. Interestingly, this difference in persistent virus levels between IFN-$\gamma R^{-/-}$ and wild-type donor kidneys mirrors the difference seen between kidneys of nontransplanted IFN-γR-deficient and -sufficient mice (Fig. 3). Despite this increased

viral burden, recipients of IFN-γR$^{-/-}$ kidneys remained healthy and the kidneys were histologically similar to wild-type kidney isografts, with neither wild type nor IFN-γR$^{-/-}$ donor kidneys in infected recipients showing histologic features of polyomavirus-associated nephropathy (i.e., interstitial fibrosis, interstitial cellular infiltrates, interstitial edema, or tubular atrophy). The increased viral burden in IFN-γR$^{-/-}$ kidney transplants could not be explained by a decrease in numbers or function of kidney-infiltrating anti-MPyV CD8^{+} T cells. IFN-γR$^{-/-}$ and WT kidney transplants contained comparable levels of MPyV-specific CD8^{+} T cells, with no differences in ex vivo LT359 peptide-stimulated IFN-γ and TNF-α production or expression of molecules shown in Fig. 4D (Fig 5C, and data not shown). Taken together, these findings support the conclusion that IFN-γ is an anti-MPyV effector molecule in vivo, and that the observed antiviral activity is largely manifested in the organ that harbors persistent PyV infection.

IFN-γ therapy reduces persistent MPyV infection

We then sought to determine if IFN-γ could exert a therapeutic effect against persistent MPyV infection. Persistently infected (30 d p.i.) B6 mice were given 2 x 10^4 U IFN-γ twice daily for 14 days, and then viral genomes were enumerated by quantitative PCR. As shown in Fig. 6A and 6B, numbers of viral genomes were significantly lower in the spleen and kidney. It is important to note that this improved viral control was not associated with significant changes in the magnitude and function of the anti-MPyV CD8^{+} T cell response (Fig. 6C, and not shown). In summary, these findings indicate that IFN-γ is an important mediator of host anti-MPyV defense and that it can operate therapeutically to reduce persistent infection.

DISCUSSION

Using the mouse-PyV infection model, we provide evidence that IFN-γ directly dampens viral replication in tissue cultured cells and contributes to the antiviral control of PyV infection in a natural host. Mice having a targeted deletion of the IFN-γ receptor were deficient in limiting MPyV replication during both the acute and persistent phases of infection, and IFN-γR$^{-/-}$ mice were highly susceptible to MPyV-induced tumors. This defect in anti-MPyV immunity was not associated with changes in the magnitude, phenotype, or function of the MPyV-specific CD8^{+} T cell response in the spleen or kidney. We further show that confining defective IFN-γ responsiveness to transplanted kidneys, a major reservoir for persistent MPyV as well as human BK and JC virus (43), results in higher viral loads, and further suggests that IFN-γ operates as a direct antiviral effector molecule in vivo. Finally, we show that IFN-γ can operate as a therapeutic agent to improve viral control against persistent MPyV infection. Taken together, these findings demonstrate that IFN-γ mediates anti-MPyV activity in vitro and in vivo.

The absence of changes in cell viability by either infected or uninfected cells argues against IFN-γ having a direct cytotoxic effect on infected cells. However, we did find a significant reduction in cellular proliferation in MPyV-infected cells exposed to IFN-γ, but this was seen only at 48 h p.i., coincident with a significant reduction in LT expression and viral genome accumulation. Similarly, IFN-γ-mediated inhibition of BK virus replication could not be ascribed to a block in cell growth, and Abend et al saw a similar 24 h delay in IFN-γ-mediated inhibition of BK virus replication (22). This delay indicates that IFN-γ sabotages the MPyV lifecycle at a point downstream of virion

binding, uptake, intracellular trafficking, uncoating, and expression of early region T antigens.

Several potential mechanisms may be invoked to account for IFN-γ control of MPyV replication. As recently reported for the MHC locus, it is conceivable that IFN-γ could trigger large-scale chromatin remodeling of the polyomavirus "minichromosome" genome and dysregulate its temporally coordinated transcription (44). By altering proteosome activity or specificity, IFN-γ may also accelerate turnover of MPyV T antigens. However, addition of proteosome inhibitors to the culture medium did not negate IFN-γ's inhibitory effect on MPyV early protein expression (data not shown). STAT molecules activated by IFN-γR signal transduction have been shown to repress gene expression (45). There are several potential GAS sequences in the MPyV genome that could serve as sites for STAT binding and thereby interfere with viral DNA replication efficiency and/or perturb viral gene expression. Host cell kinases activated by MT antigen phosphorylate key serine/threonine residues in VP1 that are required for efficient VP1 assembly into capsids (46); by reducing expression of MT antigen and/or inhibiting host serine/threonine kinases, IFN-γ may also impair capsid assembly. Finally, IFN-γ is also a potent activator of indoleamine 2,3-dioxygenase (IDO), a tryptophan-catabolizing enzyme that has been shown to inhibit replication of a number of viruses (47-49). However, preliminary studies did not reveal differences in virus levels or the magnitude, phenotype, and function of MPyV-specific $CD8^+$ T cells in persistently infected $IDO^{-/-}$ and wild type mice (data not shown). In the absence of IFN-γ-mediated upregulation of MHC molecules, virus-specific T cell recognition of infected cells may also be handicapped by lower surface epitope density. Thus, IFN-γ may operate to

control MPyV infection both directly at the level of the host cell and indirectly by improving antiviral T cell immunosurveillance.

In light of recent reports documenting the salutary effects of IFN-γ on pathogen-specific $CD8^+$ T cell responses, we were surprised to find that MPyV-specific $CD8^+$ T cell numbers and function were significantly improved in IFN-$\gamma R^{-/-}$ mice (36, 50, 51). Although higher viral loads (and presumably epitope density) in these mice are likely responsible for the heightened virus-specific T cell expansion, we found no evidence for T cell exhaustion. On the contrary, MPyV-specific $CD8^+$ T cells in IFN-$\gamma R^{-/-}$ mice were found to be phenotypically and functionally more fit than those recruited in wild-type B6 mice. These findings are in line with evidence that IFN-γ may negatively impact T cell priming, IL-2 production, and post-effector T cell contraction (37, 52). Experiments are in progress to determine whether this IFN-γ inhibitory effect operates intrinsically at the level of MPyV-specific $CD8^+$ T cells or extrinsically. It is interesting to speculate that IDO, a key immunoregulatory enzyme involved in T cell peripheral tolerance, may dually mediate IFN-γ's anti-MPyV activity (discussed above) and constrain the host virus-specific T cell response (53). Given that PyVs persist silently in healthy hosts, an IFN-γ negative feedback mechanism may constitute a host strategy to guard against antiviral T cell-mediated immunopathology while concomitantly limiting infection and preventing reactivation.

Both innate and adaptive components of host immune defense to PyV infection are likely mobilized to supply IFN-γ. Because $CD8^+$ T cells are critical for controlling MPyV infection and tumorigenesis (18-20), we favor the concept that $CD8^+$ T cells employ IFN-γ as their central anti-MPyV effector mechanism. This is in line with recent

studies suggesting that $CD8^+$ T cell-mediated control of HIV and SIV cannot be attributed to their cytolytic effector function and that $CD8^+$ T cells capable of eliciting multiple cytokines/chemokines (i.e. polyfunctional T cells) are more efficient at controlling HIV infection (54). MPyV predominantly persists in nonhematopoietic cells of epithelial and mesenchymal lineages (33) that are generally MHC class II-negative. This further supports the likelihood that virus-specific $CD8^+$ T cells, rather than $CD4^+$ T cells, provide long-term IFN-γ-mediated MPyV immunosurveillance. Additionally, antibody mediate depletion of $NK1.1^+$ cells during both the acute or persistent phases of MPyV infection have no effect on viral levels (unpublished observations). This evidence suggests that NK cells also fail to play a major role in controlling MPyV infection, consistent with the findings recently reported report by Mishra et al (55). Given the well-documented ability of IFN-γ to boost cell surface expression of peptide:MHC complexes and costimulatory molecules, our findings suggest that IFN-γ provides a three-pronged strategy to control PyV infection by promoting T cell-mediated immunity, directly inhibiting viral replication in infected host cells, and inducing a non-permissive state in uninfected neighboring cells.

Several epidemiologic studies have drawn an association between IFN-γ and susceptibility to microbial infections. Increased susceptibility to EBV-associated post-transplant lymphoproliferative disease and HPV-induced cervical carcinoma have been linked to nucleotide differences in IFN-γ promoters that affect gene expression, and a higher frequency of chronic HBV infection is seen in individuals with particular IFN-γR gene polymorphisms (56-58). A number of studies have also documented a correlation between neutralizing autoantibodies to IFN-γ and mycobacterial infections (59). The

data presented here, together with those of Abend et al (22), suggest that clinical studies may be warranted to investigate whether genetic/acquired defects which compromise IFN-γ production or IFN-γR signaling serve as host determinants that predispose recipients of kidney allografts to BK virus-associated nephropathy and/or patients receiving humoral immunotherapies to JC virus-induced PML. Finally, our findings raise the possibility that IFN-γ may offer a therapeutic option for PyV infection and its associated diseases in the immunosuppressed population.

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FIGURE LEGENDS

Figure 1. IFN-γ has anti-MPyV activity in vitro. (A) Immunoblot analysis of uninfected or MPyV-infected 3T3 cells at 48 h p.i. with or without IFN-γ (100 U/ml). (B) Representative dot plots showing the frequency of HA^+ cells at 48 h after MPyV-HA infection in A31 cells treated with the indicated concentration of IFN-γ (gates set as shown in Figure S1A based on anti-HA intracellular staining of cells infected by parental MPyV). (C) Frequency of HA^+ A31 cells, normalized to untreated cells. (D) Frequency of HA^+ cells in MPyV-HA infected B6 or IFN-γ$R^{-/-}$ BMKs in the presence (100 U/ml) or absence (Un) of IFN-γ. (E and F) Infectious virus titered by plaque assay at 60 h p.i. with MPyV (MOI = 0.1), untreated or treated with 100 U/ml IFN-γ (permissivity = virus output /virus input); (E) A31 cells and (F) B6 and IFN-γ$R^{-/-}$ BMKs. (G) Quantitative PCR assay for MPyV genome copies at the indicated times p.i. with or without IFN-γ (100 U/ml) . Data are from 3-6 independent experiments, SEM shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 2. Effect of IFN-γ on growth and death of MPyV-infected cells**.** (A) Uninfected or MPyV infected cells were left untreated (Un) or treated with IFN-γ (100 U/mL) for 48 h. Left, frequency of apoptotic (annexin V^+, 7-AAD^-) cells. Right, frequency of necrotic (7-AAD^+) cells. (B) Number of uninfected and infected cells that exclude trypan blue staining at the indicated times in the absence or presence of IFN-γ (100 U/mL). Data are from 3 independent experiments, SEM shown. * $P < 0.05$.

Figure 3. IFN-$\gamma R^{-/-}$ mice have reduced ability to control MPyV infection in the kidney. Quantitative PCR analysis for viral genomes was performed on WT B6 and IFN-$\gamma R^{-/-}$ mice at the indicated times p.i. Upper panel, Spleen; lower panel, Kidney. Data are from 3-5 independent experiments of 3-6 mice each, geometric mean indicated. * $P < 0.05$, ** $P < 0.01$.

Figure 4. IFN-$\gamma R^{-/-}$ mice maintain a functional MPyV-specific $CD8^+$ T cell response. (A) Frequency of D^b LT359 $tetramer^+$ $CD8^+$ T cells tracked longitudinally in blood of individual MPyV-infected B6 and IFN-$\gamma R^{-/-}$ mice. (B) Total splenic D^b LT359 $tetramer^+$ $CD8^+$ T cells were enumerated at the indicated time. (C) Splenocytes were assayed for intracellular IFN-γ, TNF-α, and IL-2 after ex vivo LT359 peptide stimulation at the indicated dp.i. Numbers represent % of gated $CD8^+$ cells (left plots, each pair) or % of IFN-γ^+ TNF-α^+ cells (right plots, each pair). (D) Representative histograms of D^b LT359 $tetramer^+$ $CD8^+$ T cells stained for expression of the indicated marker. Black lines, IFN-$\gamma R^{-/-}$; shaded gray, wild type. Values indicate MFIs. (E) Total number of kidney-infiltrating $CD8^+$ T cells at 8 d p.i.. Left, D^b $LT359^+$ cells; Right, IFN-γ^+ cells following direct ex vivo LT359 peptide stimulation. Data are from 2-5 independent experiments of 3-6 mice each, SEM shown.

Figure 5. Impaired control of MPyV infection in IFN-$\gamma R^{-/-}$ kidney transplants. (A) Kidneys from B6 or IFN-$\gamma R^{-/-}$ mice were transplanted into nephrectomized B6 mice that received MPyV (1×10^6 PFU s.c.) 1 d post-transplant. (B) Viral genomes were assayed

30 d p.i. by quantitative PCR (qPCR). (C) Total number of kidney infiltrating $CD8^+$ T cells in the kidney transplants at 30 d p.i.. Left, D^b $LT359^+$ cells; Right, IFN-γ^+ cells following ex vivo stimulation. Data are combined from 5-7 independent experiments with 1-2 mice each. Geometric mean indicated. * $P < 0.05$.

Figure 6. IFN-γ administration during persistent MPyV infection reduces virus levels. (A and B) Viral genomes in persistently infected mice were enumerated by quantitative PCR in the spleen (A) and kidney (B) following 14 days of IFN-γ treatment (2×10^4 U i.p., twice daily). (C) Total numbers of splenic D^bLT359 $tetramer^+$ $CD8^+$ T cells. N = 3-4 mice in each group, SEM shown. * $P< 0.05$, ** $P< 0.01$.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. MPyV-HA infection visualized by flow cytometry and immunohistochemistry. (A) Frequency of HA^+ A31 cells infected by parental MPyV (A2 strain) or MPyV-HA, stained intracellularly with anti-HA 48 h p.i. (B) Immunohistochemical staining of infected 3T3 cells by 4',6-diamidino-2-phenylindole (DAPI, Blue), HA epitope tag (red), and actin (Green) for 48 h.

Figure S2. IFN-γ reduces MPyV protein expression on a per cell basis. (A and B) MFI of intracellular HA epitope tag expression in MPyV-HA infected cells at 48 h p.i., as gated on HA^+ cells. (A) A31 cells, IFN-γ concentrations indicated. (B) B6 or IFN-$\gamma R^{-/-}$

BMKs treated with IFN-γ (100 U/ml) or untreated (Un). Data shown are from 3-6 independent experiments, SEM shown. ** $P< 0.01$, *** $P< 0.001$.

Figure 1

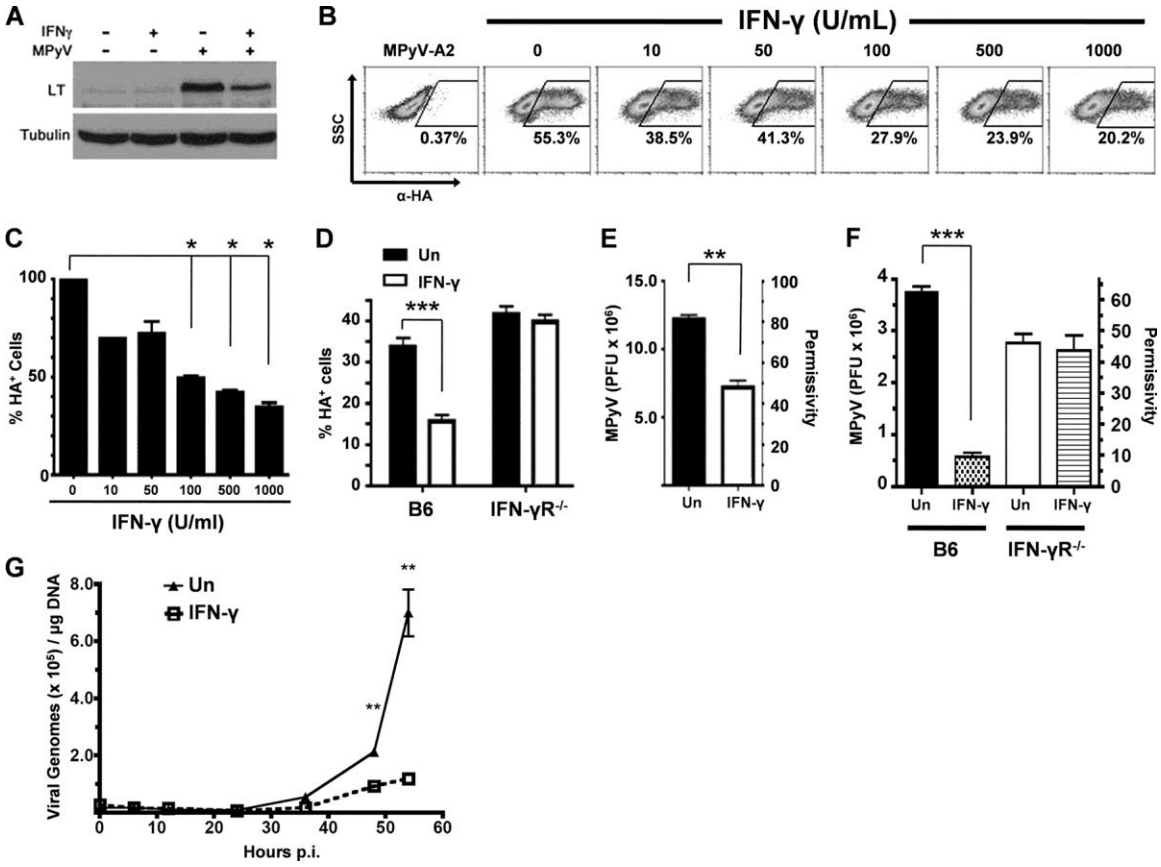

A
IFNγ
MPyV
LT
Tubulin
B
IFN-γ (U/mL)
MPyV-A2
0
10
50
100
500
1000
SSC
α-HA
0.37%
55.3%
38.5%
41.3%
27.9%
23.9%
20.2%
C
% HA+ Cells
IFN-γ (U/ml)
D
Un
IFN-γ
% HA+ cells
B6
IFN-γR-/-
E
MPyV (PFU x 10⁶)
Permissivity
F
G
Un
IFN-γ
Viral Genomes (x 10⁵) / µg DNA
Hours p.i.

Figure 2

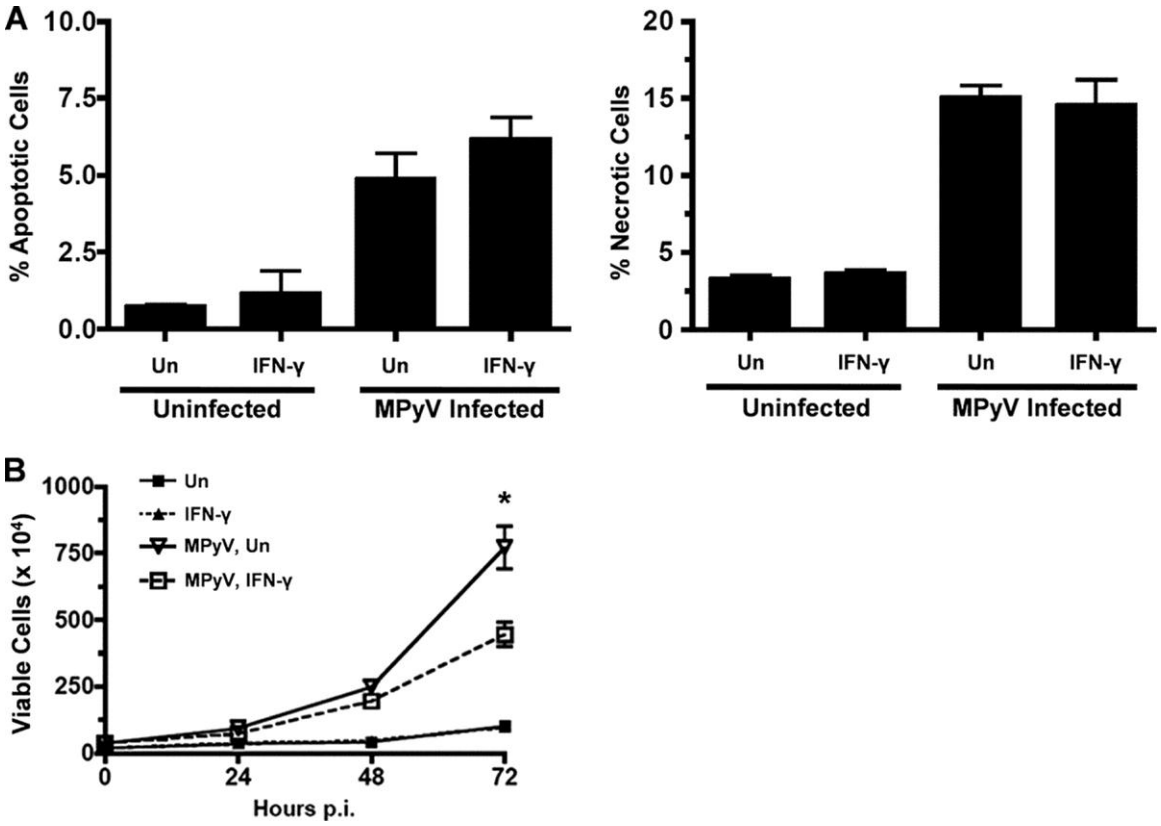

A
% Apoptotic Cells
10.0
7.5
5.0
2.5
0.0
Un
IFN-γ
Un
IFN-γ
Uninfected
MPyV Infected
% Necrotic Cells
20
15
10
5
0
Un
IFN-γ
Un
IFN-γ
Uninfected
MPyV Infected
B
Viable Cells (x 10⁴)
1000
750
500
250
0
Un
IFN-γ
MPyV, Un
MPyV, IFN-γ
*
0
24
48
72
Hours p.i.

Figure 3

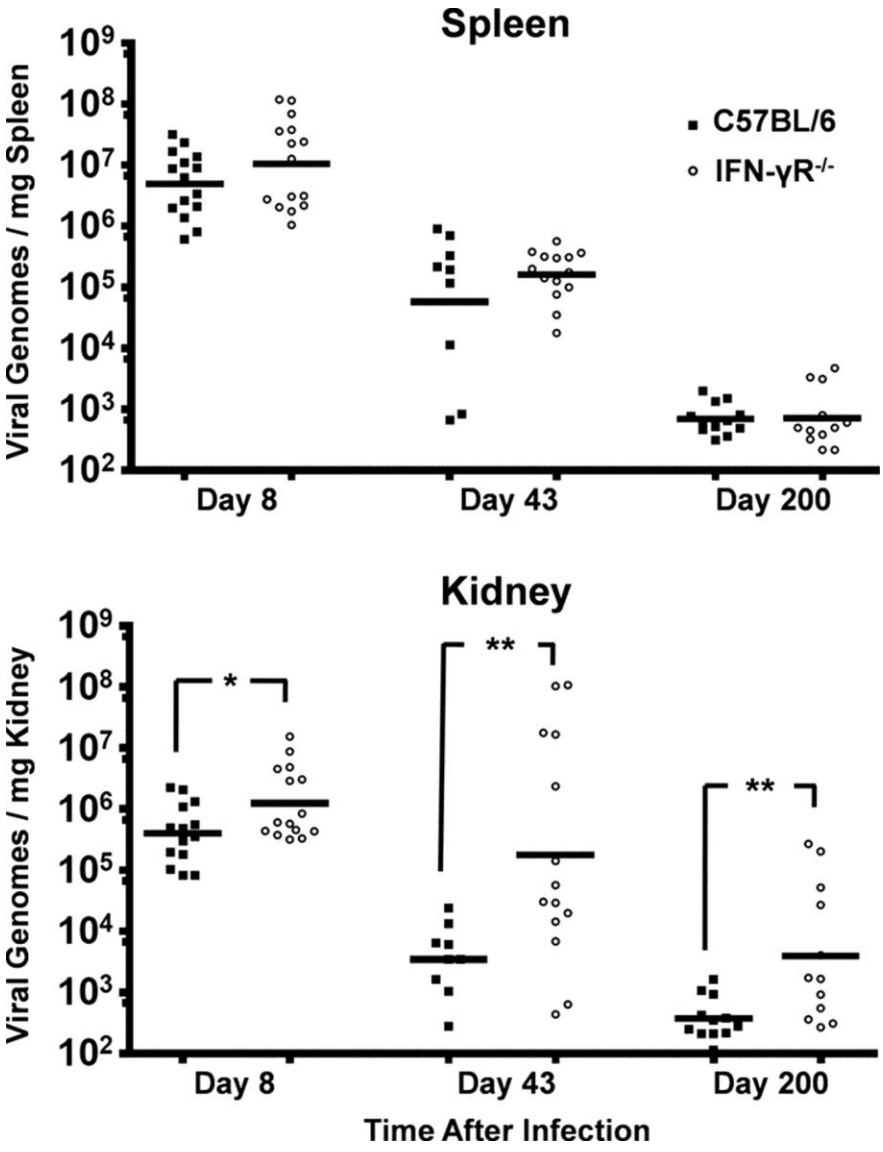

Spleen
Viral Genomes / mg Spleen
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
C57BL/6
IFN-γR$^{-/-}$
Day 8
Day 43
Day 200
Kidney
Viral Genomes / mg Kidney
*
**
**
Day 8
Day 43
Day 200
Time After Infection

Figure 4

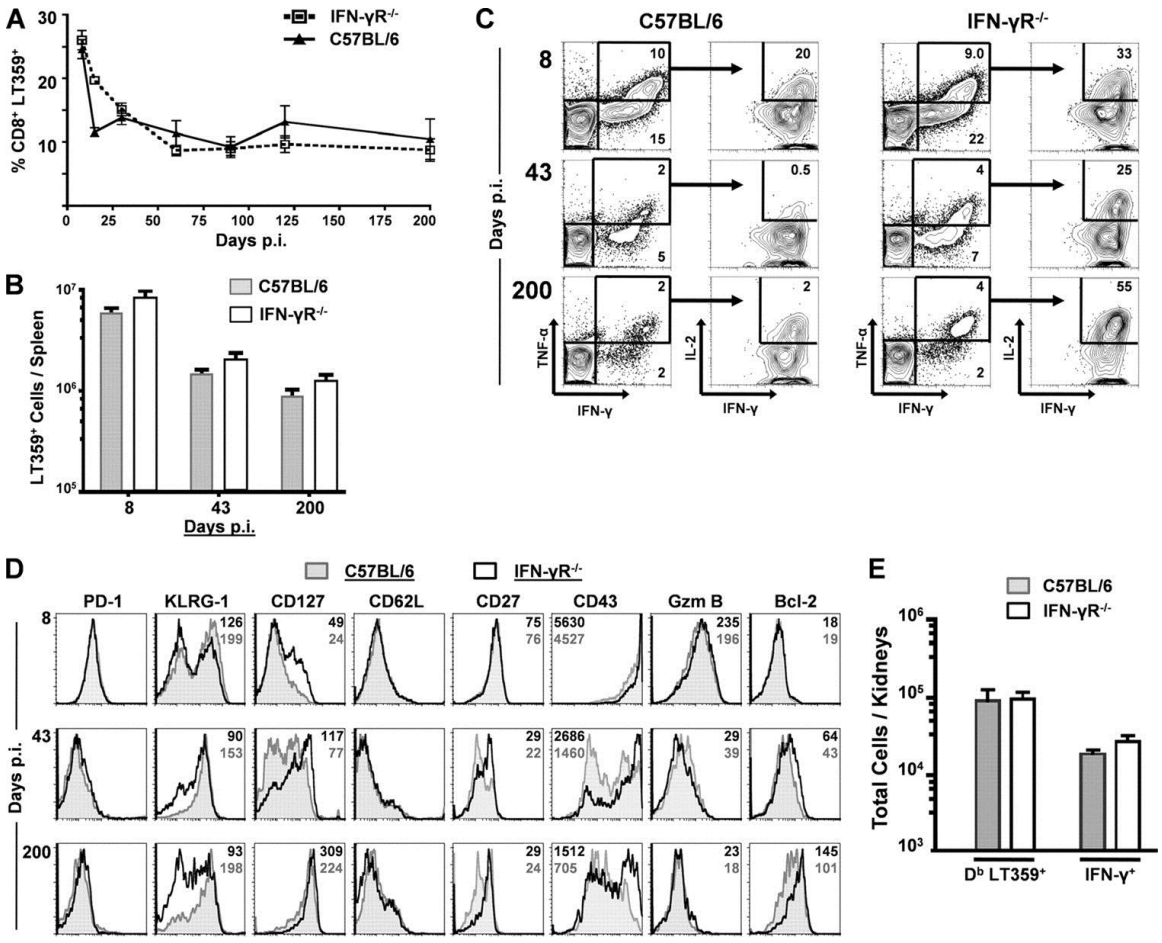

A
IFN-γR-/-
C57BL/6
% CD8+ LT359+
Days p.i.
B
C57BL/6
IFN-γR-/-
LT359+ Cells / Spleen
Days p.i.
C
C57BL/6
IFN-γR-/-
Days p.i.
TNF-α
IFN-γ
IL-2
IFN-γ
D
C57BL/6
IFN-γR-/-
PD-1
KLRG-1
CD127
CD62L
CD27
CD43
Gzm B
Bcl-2
Days p.i.
E
C57BL/6
IFN-γR-/-
Total Cells / Kidneys
Db LT359+
IFN-γ+

Figure 5

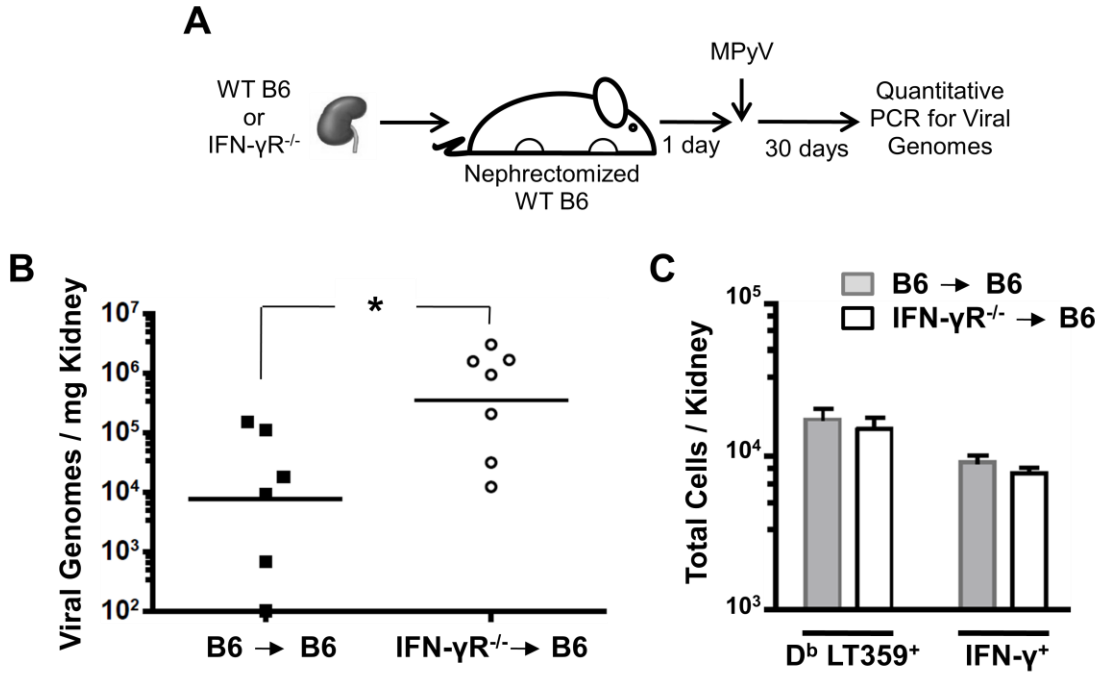
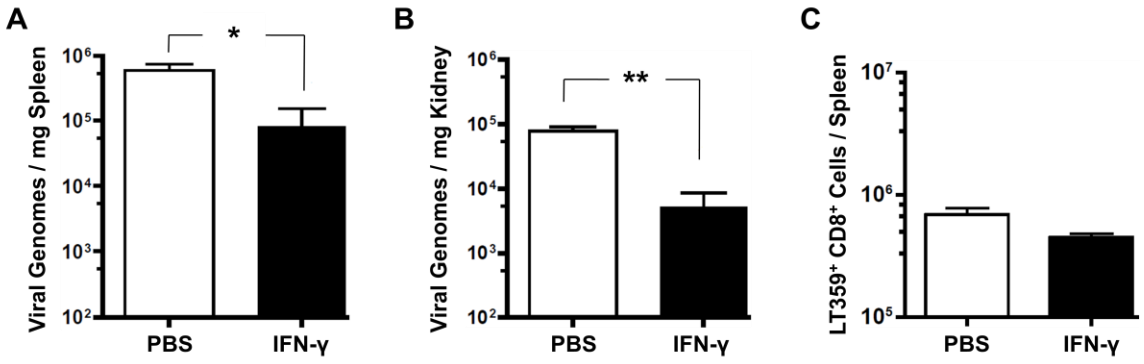
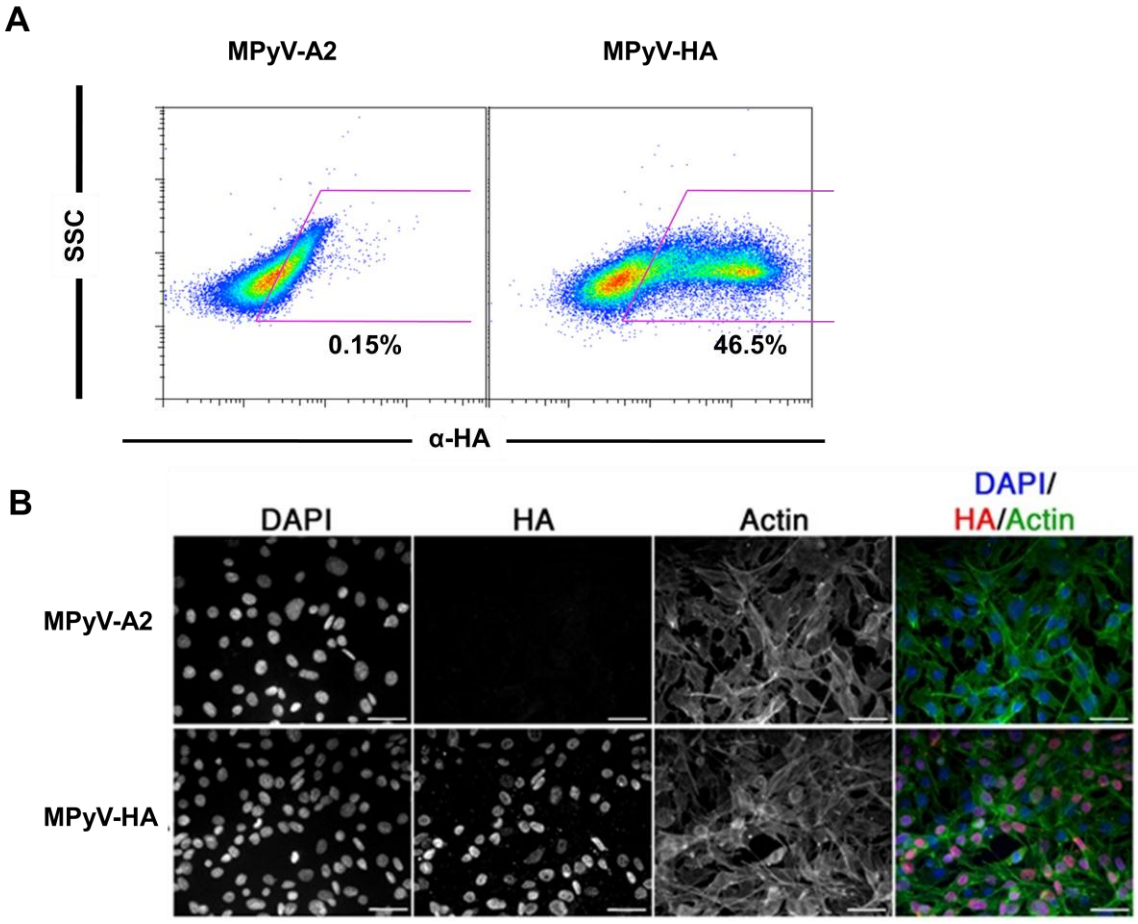

A
WT B6
or
IFN-γR-/-
Nephrectomized
WT B6
1 day
MPyV
30 days
Quantitative
PCR for Viral
Genomes
B
Viral Genomes / mg Kidney
10^7
10^6
10^5
10^4
10^3
10^2
*
B6 → B6
IFN-γR-/- → B6
C
Total Cells / Kidney
10^5
10^4
10^3
B6 → B6
IFN-γR-/- → B6
Db LT359+
IFN-γ+

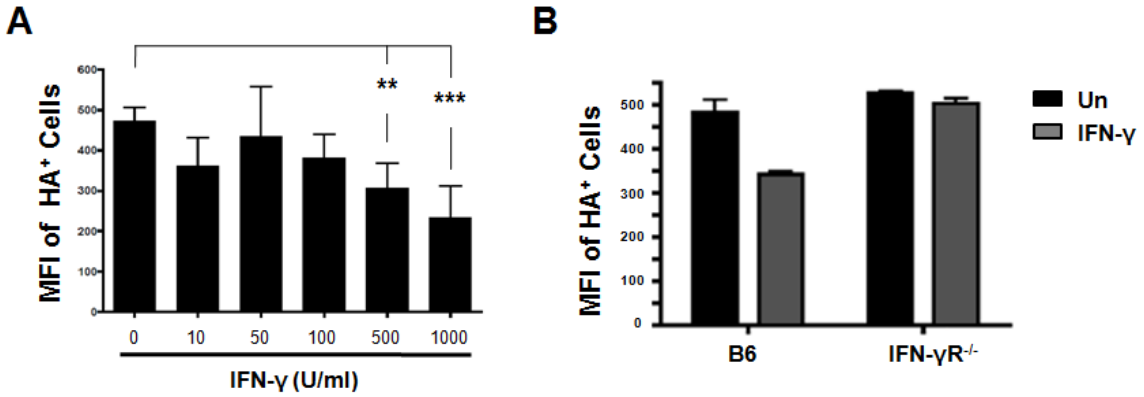
Figure 6


A
Viral Genomes / mg Spleen
10^6
10^5
10^4
10^3
10^2
*
PBS
IFN-γ
B
Viral Genomes / mg Kidney
10^6
10^5
10^4
10^3
10^2
**
PBS
IFN-γ
C
LT359⁺ CD8⁺ Cells / Spleen
10^7
10^6
10^5
PBS
IFN-γ

Supplemental Figure 1



Supplemental Figure 2



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Chapter 3

CD8 T cells recruited early in mouse polyomavirus infection undergo exhaustion

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All figures in Chapter 3 are based on data generated by the Ph.D. candidate, in cooperation with Christopher Pack, Ph.D.

Abstract

Repetitive antigen encounter, coupled with dynamic changes in antigen density and inflammation, imparts phenotypic and functional heterogeneity to memory virus-specific CD8 T cells in persistently infected hosts. For herpesvirus infections, which cycle between latency and reactivation, recent studies demonstrate that virus-specific T cell memory is predominantly derived from naïve precursors recruited during acute infection. Whether functional memory T cells to viruses that persist in a non-latent, low-level infectious state (smoldering infection) originate from acute infection-recruited naïve T cells is not known. Using mouse polyomavirus (MPyV) infection, we previously showed that virus-specific CD8 T cells in persistently infected mice are stably maintained and functionally competent; however, a sizeable fraction of these memory T cells are short-lived. Further, we found that naïve anti-MPyV CD8 T cells are primed de novo during persistent infection and contribute to maintenance of the virus-specific CD8 T cell population and its phenotypic heterogeneity. Using a new MPyV-specific TCR transgenic system, we now demonstrate that virus-specific CD8 T cells recruited during persistent infection possess multi-cytokine effector function, have strong replication potential, express a phenotype profile indicative of authentic memory capability, and are stably maintained. In contrast, CD8 T cells recruited early in MPyV infection express phenotypic and functional attributes of clonal exhaustion, including attrition from the memory pool. These findings indicate that naïve virus-specific CD8 T cells recruited during persistent infection contribute to preservation of functional memory against a smoldering viral infection.

Introduction

The inflammatory microenvironment is a central determinant that directs pathogen-specific T cell differentiation. Strong early inflammatory responses divert pathogen-specific CD8 T cells toward effector and away from memory pathways of differentiation (1, 2). Alternatively, antigen presented in low-inflammatory settings favors CD8 T cell memory differentiation (3). These microenvironment-directed shifts in T cell differentiation are associated with changes in expression of select transcription factors, including T-bet, eomesodermin (Eomes), and Blimp-1 (4, 5). Elucidation of the elements controlling CD8 T cell differentiation has been largely derived from experimental models where host immunity efficiently and completely eliminates cognate antigen.

For persistent infections, the pattern of CD8 T cell differentiation is perturbed by repetitive exposure to antigen and unresolved inflammation. In the setting of high-level persistent viremia, memory CD8 T cells express an effector phenotype (e.g., $CD62L^{lo}$ $IL\text{-}7R^{lo}$ $CCR7^{lo}$) and suffer progressive functional impairment that may culminate in deletion from the T cell pool. The severity of exhaustion experienced by these "chronic memory" T cells is dictated in large part by the level of persistent cognate viral antigen (6). However, chronic memory T cells also require cognate antigen for maintenance, but express a $T\text{-}bet^{lo}$ $Blimp\text{-}1^{hi}$ transcription factor profile and upregulate cell surface receptors that inhibit their ability to clear viral infection (7). In contrast, virus-specific CD8 T cells maintained in the setting of low-level persistent infection typically preserve most of their effector functions, and the population remains stable or gradually increases in magnitude over time (8).

Polyomaviruses establish a lifelong, low-level infection in healthy hosts of a variety of avian and mammalian species, including humans (9). The human polyomaviruses BK and JC persist as clinically silent, non-viremic infections in most individuals. With immunocompromise resulting from HIV/AIDS or humoral immunotherapeutic agents affecting T cell trafficking (e.g., Natalizumab, Efalizumab, Rituximab), JC virus may cause a life-threatening demyelinating brain disease; BK virus is a well-recognized cause of dysfunction and loss of kidney allografts (10, 11). Current evidence suggests that JC and BK virus-specific CD8 T cells control these smoldering persistent viral infections (12, 13).

During persistent infection by MPyV, virus-specific CD8 T cells express a predominantly effector phenotype, but retain cytokine and cytolytic effector functions and fail to express inhibitory markers upregulated by memory CD8 T cells that confront chronic viremia (e.g., PD-1, Lag-3, or Tim-3) (14, 15). In addition, the memory MPyV-specific CD8 T cell response is stable over the course of persistent infection (16). However, following transfer to persistently infected, congenic mice memory MPyV-specific CD8 T cells do not homeostatically proliferate and this population undergoes progressive attrition (17). Using a partial myeloablation approach to allow engraftment of congenic bone marrow in persistently infected mice, we recently demonstrated that naïve MPyV-specific CD8 T cells are primed de novo during persistent infection. Persistent infection-recruited memory antiviral T cells differ phenotypically from those recruited earlier in infection (17, 18). Whether these memory T cells differ functionally from those recruited during acute infection and to what degree they contribute to maintenance of the memory T cell compartment are not known.

Using novel mutant MPyVs and a TCR transgenic mouse model, we provide evidence that virus-specific CD8 T cells recruited during persistent MPyV infection not only favor expression of canonical markers of authentic memory T cells, but also possess superior effector functionality and secondary replicative potential over those memory cells derived from naïve precursors primed early in acute infection. Importantly, persistent infection-recruited memory CD8 T cells are maintained in all mice and do so at a higher magnitude than those recruited during acute infection, with acute infection-recruited memory cells falling below detection in a significant number of animals. Because virus-specific CD8 T cells recruited early in acute MPyV infection suffer clonal exhaustion, those recruited after acute infection likely play a central role in keeping this smoldering viral infection in check.

Materials and Methods

Mice

Female C57BL/6Ncr (B6) mice were purchased from the Frederick Cancer Research and Development Center (National Cancer Institute, Frederick, MD). B6.PL (Thy1.1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), B6.SJL-*Ptprc*^{a}/BoAiTac-H2-*Ab1*^{tm1GLM} N13 mice (I-A$^{b\ -/-}$CD45.1) were purchased from Taconic Farms (Germantown, NY) and both bred by the Emory University Division of Animal Resources. TCR-I transgenic mice bearing the TCR specific for LT206-215 from the Large T (LT) antigen of SV40 are previously described (19). TCR-I mice were crossed with B6.PL mice (Thy1.1) to create F1 mice expressing both the transgenic TCR and Thy1.1. Mice were bred and housed by the Division of Animal Resources in accordance with the guidelines of the Institutional Animal Care and Use Committee of Emory University. All mice were 6-8 wk of age at the time of infection.

Viruses and cell transfers

Mice were infected s.c in the hind footpads by 1 x 10^{6} PFU of either MPyV strain A2 (MPyV.A2) or a mutant MPyV strain expressing the D^{b}-restricted LT206-215 epitope from SV40 LT antigen (MPyV.LT206). MPyV.LT206 was generated using a QuikChange II XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) of MPyV.A2 genomic DNA to iteratively change the D^{b}-restricted LT359-368 epitope from MPyV.A2 (SAVKNYCSKL) to the D^{b}-restricted epitope LT206-215 from SV40 (SAINNYAQKL). The following primers were used: (ForLT359VI-AGGGTTTCAGCAATTAAGAATTATG, RevLT359VI-

CATAATTCTTAATTGCTGAAACCCT, ForLT359KN-GTTTCAGCAATTAATAATTATGCCTCTAAG, RevLT359KN-CTTAGAGGCATAATTATTAATTGCTGAAAC, ForLT359CA-GTTAAGAATTATGCCTCTAAGCTTTGC RevLT359CA-GCAAAGCTTAGAGGCATAATTCTTAAC, ForLT359SQ-ATTAATAATTATGCCCAGAAGCTTTGCAGC, RevLT359SQ-GCTGCAAAGCTTCTGGGCATAATTATTAAT). All primers were synthesized by Invitrogen (Grand Island, NY). Recombinant MPyV.LT206 DNA was ligated with T4 DNA ligase and baby mouse kidney cells were transfected using Lipofectamine 2000 (Invitrogen) as previously described (20). Because LT359-368 is not situated in a functional domain of LT, and does not overlap the coding sequence for the middle T oncoprotein, MPyV.LT206 is identical to MPyV.A2 in terms of replication and dissemination in vivo (Supplemental Fig. 1A), and tumor-induction when inoculated into newborn mice of an MPyV tumor-susceptible strain (unpublished data). A recombinant vaccinia virus encoding the SV40 LT206-215 epitope, designated rVV-ES-I, is previously described (21); mice received 1 x 10^6 PFU of rVV-ES-I i.v.

CD8 T cells from TCR-I transgenic mice or uninfected B6 mice were purified using a negative selection CD8 T cell isolation kit (Miltenyi Biotech, Auburn, CA) according to manufacturer's instructions. For all adoptive transfer experiments, the $CD62L^{hi}$ status of the donor TCR-I cells was > 93% (range: 93.3% to 98.1%) and the $CD44^{hi}$ status was < 8% (range: 2.4% to 8%). 100 or 1 x 10^6 TCR-I cells, or 10 x 10^6 or 20 x 10^6 polyclonal CD8 T cells were transferred. TCR-I cells were labeled with 5 μM CFSE (Invitrogen) for 10 minutes at 37°C prior to transfer of 1 x 10^6 cells (Supplemental

Fig. 1). 100 purified TCR-I cells were transferred on d -1, d 60, or d 90 after MPyV.LT206 infection, and d -1 prior to rVV-ES-I infection.

Quantification of MPyV genomes

DNA isolation and Taqman-based PCR were performed as previously described (18). The detection limit of this assay is 10 copies of genomic viral DNA.

*Synthetic peptide***s**

LT206-215 (SAINNYAQKL) and LT359-368 (SAVKNYAbuSKL, Abu = α-amino butyric acid, a cysteine structural analog) peptides were synthesized by the solid-phase method using F-moc chemistries on a Prelude peptide synthesizer (Protein Technologies, Inc.).

Cell isolation and flow cytometry

TCR-I cell numbers in the blood were determined by flow cytometric analysis using BD Trucount tubes (BD Biosciences) according to the manufacturer's directions. Single-cell suspensions of RBC-lysed spleens were prepared. Antibodies to CD8α (53-6.7), Thy1.1 (OX-7), CD62L (MEL-14), CD44 (IM7), CD45 (30-F11), Vβ7 (TR310), CD3ε (2C11), Bcl-2, human Ki-67 (B56), IFN-γ (XMG1.2), and IL-2 were purchased from BD Biosciences (San Diego, CA). CD127 (A7R34), CXCR3 (CXCR3-173), CD27 (LG.7F9), PD-1 (RMP1-30), TIM-3 (RMT3-23), and LAG-3 (eBioC9B7W) were purchased from eBioscience (San Diego, CA). Anti-KLRG-1 was purchased from SouthernBiotech (Birmingham, AL). Anti-CD43 (1B11) was purchased from BioLegend

(San Diego, CA). Anti-Blimp-1 (C-21), anti-T-bet (4B10), and anti-Eomes (N-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Annexin V staining was performed as directed using a kit from BD Biosciences. D^bLT206, D^bLT359, K^bMT246 and D^bLT638 tetramers were constructed by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Tetramer staining was performed for 45 min at room temperature. Samples were acquired on a FACSCalibur or an LSR II (BD Biosciences) and data analyzed using FlowJo software (Tree Star, Inc. Ashland, OR). For antigen recall experiments, TCR-I cells were stained with anti-Thy1.1 and anti-CD8α then sorted using a FACSAria (BD Biosciences) cell sorter.

Cells were stimulated with 1 μM to 1 pM LT206-215 peptide for 5 h in the presence of brefeldin A and intracellularly stained for IFN-γ and IL-2 as previously described (18, 22).

Gene expression analyses

Oligonucleotide primers for PCR amplification of *T-bet*, *Blimp-1*, and *L9* cDNA were previously described (23). RNA was isolated from $1 \times 10^5 - 1 \times 10^6$ FACS-sorted $Thy1.1^+$ $CD8^+$ cells using an RNeasy mini kit (Qiagen, Valencia, CA), and cDNA prepared using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK). Power SYBR green PCR master mix (Applied Biosystems) was used for quantitative PCR using an ABI PRISM 5700 sequence detection system (Applied Biosystems).

Statistical analyses

Statistical significance was determined by an unpaired, two-tailed Student's *t* test, assuming unequal variance. Experiments with 3 groups were assayed for statistical significance using a Kruskal-Wallis one-way analysis of variance test. A value for $p < 0.05$ was considered statistically significant.

Results

MPyV-specific CD8 T cells recruited during acute vs. persistent infection differ in response profiles and maintenance

Dynamic recruitment of naïve virus-specific CD8 T cells in persistently infected hosts results in an amalgam of cells having different histories of encounter with antigen and pro-inflammatory mediators. The minimal myeloablation-congenic bone marrow transfer approach we used to observe de novo priming of antiviral CD8 T cells during persistent infection is unable to resolve the phenotypic and functional impact of recruitment at defined stages of infection, because antiviral T cells are recruited throughout the engraftment interval (17). To limit the timeframe over which naïve virus-specific T cells are recruited, and to eliminate variables of precursor frequency and TCR repertoire, we developed the following MPyV-specific CD8 T cell TCR transgenic system. By mutagenizing four codons in the genome of MPyV (designated MPyV.A2), we replaced the dominant D^b-restricted LT359-368 (LT359) CD8 T cell epitope with the homologous D^b-restricted LT206-215 (LT206) epitope in SV40 LT antigen (designated MPyV.LT206), which is recognized by CD8 T cells from the "TCR-I" TCR transgenic mouse (19). TCR-I cells exhibit no cross-reactivity to syngeneic APCs presenting the LT359 peptide or infected by MPyV.A2 *in vitro* (unpublished observations), and expand in Thy1 congenic recipients acutely infected by MPyV.LT206 but not MPyV.A2 (Supplemental Fig. 1B, upper 2 panels). In addition, an LT359-specific CD8 T cell response is not generated in B6 mice infected by MPyV.LT206. Finally, the endogenous LT206-specific CD8 T cell response to MPyV.LT206 infection is similar to that of the LT359-specific CD8 T cell response to infection by MPyV.A2, and there is no change in

the frequency of the two subdominant anti-MPyV CD8 T cell responses (unpublished observations) (18).

TCR-I cells proliferate after transfer to congenic recipients infected with MPyV.LT206 for 60 d , showing that the D^bLT206 epitope is expressed long-term and is available for recognition by virus-specific CD8 T cells (Supplemental Fig. 1B, bottom panel). However, only a portion of the 1 x 10^6 donor TCR-I cells expanded within 5 d post transfer. Antigenic competition by an excessive number of naïve cells faced with the limited antigen density of persistent MPyV infection likely impaired T cell recruitment. To minimize antigenic competition and approximate physiologic numbers of antigen-specific naïve cell precursors, we transferred only 100 TCR-I cells per recipient in all subsequent experiments (24). Transfer of 100 TCR-I cells one day prior to infection allowed the generation of an endogenous LT206-specific CD8 T cell response comparable to unmanipulated infected mice (unpublished observations); virus levels showed only a modest two-fold transient decrease at d 7 p.i. following transfer, with no decrease evident by day 15 p.i. or later post infection (Supplemental Fig. 1C). These data show that the MPyV.LT206/TCR-I approach can be used to faithfully monitor the fate and function of virus-specific CD8 T cells recruited during the acute and persistent phases of infection.

Donor TCR-I cells showed strikingly different response profiles when transferred to Thy1 congenic B6 mice acutely or persistently infected by MPyV.LT206. As depicted in Fig. 1A, TCR-I cells were transferred either 1 day before infection (acute infection recruitment) or at d60 or d90 p.i. (persistent infection recruitment); acute infection-recruited TCR-I cells were assessed at d 30 and d 90 p.i. to match the time after cell

transfer and the time after infection, respectively (Fig. 1A). For acute infection recruitment, naïve TCR-I cells vigorously expanded, peaking in magnitude at 1 week p.i., then precipitously contracting to a small population (Fig. 1B). This response pattern mirrors the kinetics of antigen-specific CD8 T cell responses to acutely resolved infections. However, post-contraction, TCR-I cells fell below detection in the blood and spleen of several of these mice, with the proportion of TCR-$I^{-/low}$ chimeric mice increasing over time (Fig. 1B & 5A). In contrast, when transferred to hosts infected 90 d earlier, TCR-I cells underwent a slow progressive expansion, without an appreciable contraction phase, over a 2-mo timeframe to reach a plateau maintenance level. Notably, by d90 post transfer, the pool of circulating TCR-I cells recruited during persistent infection was 4-5-fold larger than those recruited during acute infection, and 100% of these recipients had sizeable populations of memory TCR-I cells (Fig. 1B & 5A). This response profile difference was not simply a consequence of higher virus levels in acute than persistent infection. TCR-I cells transferred to B6 mice given 1 x 10^3 PFU MPyV.LT206 underwent a delayed but similar expansion-contraction response pattern as those given 1 x 10^6 PFU inocula (unpublished observations), as we previously described for the endogenous anti-MPyV CD8 T cell response (22). The increased frequency and numbers of TCR-I cells maintained in persistently infected recipients could not be accounted for by increased expression of the Bcl-2 anti-apoptotic molecule or decreased cell death as indicated by Annexin V staining (Fig. 1C and 1D). However, a significantly higher fraction of memory TCR-I recruited in persistently infected mice expressed the nuclear factor Ki-67, indicative of recent cell division, than TCR-I cells primed at the onset of infection (Fig. 1E). This finding suggests that persistent infection-recruited TCR-

I cells retain higher replicative potential than those recruited in acutely infected recipients, which is further supported by differences in antigen recall responsiveness by these memory TCR-I populations (see Fig. 7). Together, these data indicate that the different response profiles and capacity for long-term maintenance by memory MPyV-specific CD8 T cells are instilled by recruitment during temporally distinct infection settings.

Persistent infection-recruited TCR-I cells express a phenotype associated with improved memory potential

Compared to TCR-I cells recruited in acute MPyV infection, a higher proportion of persistent infection-recruited cells expressed the IL-7 receptor-a chain (CD127). This expression level mimicked that of true memory TCR-I cells (designated "Memory") generated in response to acutely resolved infection by a recombinant vaccinia virus encoding the LT206 epitope (rVV-ES-I) (Fig. 2A). At d 30 after transfer, TCR-I cell expression of the lymphoid homing marker, CD62L, while trending higher for those recruited during persistent MPyV and resolved vaccinia virus infections, was statistically similar to acute MPyV infection-recruited TCR-I cells (Fig. 2B). Of note, a higher frequency of acute infection-recruited cells expressed CD127 at d 90 than at d 30 p.i. (Fig. 3B), which may be associated with the capacity of these cells to survive long-term. Interestingly, most TCR-I cells expressed KLRG1, a marker of T cell senescence (25), regardless of timing of recruitment (Fig. 2C and 3B), while only a fraction of cells recruited late in persistent infection expressed the CD43 marker of activation (Fig. 2D). These phenotypic differences among TCR-I cells as a function of recruitment were

recapitulated using donor polyclonal CD8 T cells from uninfected mice (Supplemental Fig. 2). These data suggest that memory MPyV-specific CD8 T cells whose progenitors were recruited during persistent MPyV infection trended toward expression of canonical markers of bona fide memory.

Low expression of CD43 with coordinate upregulated expression of CD27 and CXCR3 have been associated with memory antiviral CD8 T cells having strong potential for antigenic recall (26). As shown in Fig. 3A, acute infection-recruited memory TCR-I cells skewed toward a $CD43^{hi}$ phenotype, with only a small fraction of the $CD43^{lo}$ cells co-expressing CD27 or CXCR3, regardless of the length of time after infection. However, persistent infection-recruited memory TCR-I cells expressed less CD43 (Fig. 2D and 3A) and higher CD27 and CXCR3 molecules (Fig. 3). These $CXCR3^{hi}$ cells were also more likely to express CD127 in persistent infection-recruited memory TCR-I cells than in acute infection-recruited cells. However, the majority of acute (both d 30 and d 90 p.i.) and persistent infection-recruited memory TCR-I cells were $KLRG1^{hi}$, with few TCR-I cells in either group expressing the PD-1 or LAG-3 inhibitory receptors (Fig. 3B). Additionally, given the high KLRG1 expression by memory TCR-I cells generated under either acute or persistent infection settings, there were no phenotypically authentic $CD127^{hi}$ $KLRG1^{lo}$ memory cells (27). Taken in aggregate, these phenotyping analyses raise the possibility that memory TCR-I cells recruited during persistent infection have higher functional capabilities than those recruited during acute infection.

Persistent infection-recruited MPyV-specific CD8 T cells possess superior functionality

Although equal proportions of acute and persistent infection-recruited memory TCR-I cells produced IFN-γ upon ex vivo LT206 peptide stimulation, the latter cells were capable of multi-cytokine effector activity as shown by co-expression of IL-2 (Fig. 4A). Of note, the ability to co-produce IFN-γ and IL-2 improved with recruitment at later timepoints of persistent MPyV infection. In contrast, as shown in Fig. 4B, memory TCR-I cells recruited during acute infection failed to acquire the ability to co-produce IL-2 over time (compare d30 with d90 p.i.). LT206 peptide dose titration further showed that while the functional avidity of acute and persistent infection-recruited TCR-I cells was equivalent (i.e., similar slopes for IFN-γ production), those recruited during acute infection possessed significantly lower IFN-γ effector capability (Fig. 4C).

Because IFN-γ is an important anti-MPyV effector cytokine in vivo (14), an implication of this result is that acute infection-recruited MPyV-specific CD8 T cells are less effective in controlling this viral infection. In line with their diminished functional potential, acute infection-recruited memory TCR-I cells expressed more Blimp-1, both at the mRNA and protein level, than those recruited during persistent infection (Fig. 4D and 4E). As a transcriptional repressor of IL-2 gene expression, higher Blimp-1 levels may also underlie the lower IL-2 functional capability of acute infection-recruited memory TCR-I cells and their diminished durability in persistently infected hosts (28). By corollary, low Blimp-1 expression by memory TCR-I cells generated against acutely resolved rVV-ES-I infection is in line with their high IL-2-producing capability (Fig. 4A).

Although both acute and persistent infection-recruited memory TCR-I cells had similar T-bet transcript and protein expression levels, there was a notable increase in

Eomes expression in cells recruited during the persistent phase of infection (Fig. 4F and 4G). Therefore, while T-bet levels were similar, consistent with each memory population retaining an effector differentiation state, increased Eomes expression may explain increased survival and functional capacity of persistent infected-recruited cells (29, 30). Meanwhile, sustained high T-bet expression coupled with elevated Blimp-1 expression and low Eomes expression, may be linked to the attrition of acute infection-recruited memory TCR-I cells (29, 31).

Using D^bLT206 tetramers, we further determined that the endogenous memory anti-MPyV CD8 T cell compartment closely resembled the stability and functionality of the memory TCR-I cells recruited during persistent infection, both in the spleen and lung (Fig. 5A & 5C). However, as shown in Fig. 5B, TCR-I cells recruited during acute infection progressively lost the ability to co-produce IFN-γ and IL-2, and fell below detection in a number of recipients. This functional deficit was also evident for acute infection-recruited memory TCR-I cells isolated from the lungs (Fig. 5D). Taken together, these findings imply that MPyV-specific CD8 T cells recruited during persistent infection make a larger contribution to the memory T cell compartment than those recruited during acute infection.

CD4 T cells are required for maintenance of memory MPyV-specific CD8 T cells during persistent infection

Using the myeloablation-congenic bone marrow engraftment approach, we previously reported that recruitment of MPyV-specific CD8 T cells depended on CD4 T cell help (16). These studies used MPyV-infected I-$A^{b-/-}$ mice, which carry similar viral loads as

MPyV-infected B6 mice during acute and persistent infection. Several adaptive immune responses likely mediate MPyV control in these MHC class II-deficient mice, including a strong protective T cell-independent MPyV-specific Ab response, exaggerated expansion of anti-MPyV CD8 T cells during acute infection, and the long-term maintenance of a small functional antiviral CD8 T cell population (16, 32). Thus, differences in efficiency of antiviral CD8 T cell recruitment between CD4 T cell-sufficient and -deficient mice cannot be ascribed to differences in viral loads. However, these experiments necessitated a long engraftment period to enable detection of de novo primed antiviral CD8 T cells in persistently infected mice, and therefore did not allow us to distinguish whether CD4 T cell deficiency impaired priming, expansion, or maintenance of anti-MPyV T cells. We revisited this question using the TCR-I/MPyV.LT206 model to determine how CD4 T cell-insufficiency affected recruitment of virus-specific CD8 T cells during persistent infection. B6 and I-A^b deficient mice (I-$A^{b-/-}$) mice at d 60 p.i. received 100 TCR-I cells, and were serially bled to quantify the expansion and maintenance of the TCR-I cells. As shown in Fig 6A, TCR-I cells expanded in persistently infected, I-$A^{b-/-}$ mice, but then underwent profound contraction similar to that seen by virus-specific CD8 T cells recruited in MPyV-infected, CD4 T cell-deficient animals (16). However, post-contraction, a small fraction of these persistent infection-recruited TCR-I cells, like the endogenous polyclonal LT206-specific CD8 cells, survived and retained cytokine effector function (compare Fig. 6B with Fig. 6C and D). These data extend our previous findings by showing that the dependence on CD4 T cell help for recruiting MPyV-specific CD8 T cells during persistent infection involves mitigating the contraction and/or sustaining the memory phase.

Acute and persistent infection-recruited memory MPyV-specific CD8 T cells differ in recall potential

A cardinal property of T cell memory is rapid expansion upon re-encounter with cognate antigen. Because persistent infection-recruited TCR-I cells had an increased fraction of $CD43^{lo}$ $CD27^{hi}$ cells and higher IL-2 functionality, and possessed decreased levels of Blimp-1, we asked whether memory TCR-I cells recruited during persistent infection possessed higher replication potential than those recruited during acute infection. To test this possibility, we first compared the number of circulating TCR-I cells in acute and persistent infection, and then challenged these mice with rVV-ES-I. Five days following challenge, acute infection-recruited memory TCR-I cells showed no expansion in the blood (or spleen, unpublished observations); in marked contrast, memory TCR-I cells recruited during persistent infection expanded 50-fold (Fig. 7A).

To determine if this dramatic difference in recall potential was intrinsic to the TCR-I cells, TCR-I cells were transferred to naïve B6 recipients infected the following day or to B6 recipients infected 60 d earlier. Thirty days after transfer, $Thy1.1^{+}$ CD8 T cells were FACS-sorted and 1×10^{4} memory TCR-I cells were re-transferred to naïve B6 mice. When challenged with rVV-ES-I, donor memory TCR-I cells that were recruited during persistent infection expanded 50-fold more than those recruited during acute infection. (Fig. 7B). These data are in line with the phenotypic and functional evidence that recruitment history impacts the differentiation of MPyV-specific CD8 T cells not only with respect to cytokine effector capability, but also in potential for proliferative expansion to antigen reencounter.

Discussion

In contrast to memory virus-specific CD8 T cells that encounter aggressive chronic infections, those co-existing with low-level persistent infections are often stably maintained and retain most of their effector capabilities. Previous studies from our group and others have shown that naïve virus-specific CD8 T cells are primed de novo during persistent infection. These persistent infection-recruited T cells upregulate expression of molecules associated with a central memory T cell phenotype ($CD44^{hi}$ $CD62L^{hi}$ $CD127^{hi}$); however, their functional integrity and contribution to the memory compartment have not been previously investigated. In this study, we reexamined the question of this apparent avoidance of dysfunction and loss (exhaustion) by antiviral CD8 T cells that are confronted by low-level persistent infections. By analyzing the function and fate of naïve TCR transgenic CD8 T cells primed at different stages of MPyV infection, we show that CD8 T cell exhaustion in the environment of a low-level persistent infection is revealed when kinetics of T cell recruitment are taken into account. In addition, we provide evidence supporting the concept that naïve virus-specific CD8 T cells recruited during persistent infection are important for the integrity of a stable and functional memory antiviral T cell population.

The timeframe over which naïve CD8 T cells are recruited to acutely resolved infections has been shown to impact the balance between effector and memory differentiation. Unlike naïve virus-specific CD8 T cells recruited at the inception of an acute viral infection, those recruited during later timepoints preferentially give rise to central memory-phenotype T cells capable of antigen-induced IL-2 production and antigenic recall (33, 34). These data are line with evidence that abbreviating the duration

and reducing the peak magnitude of acute infection also favors generation of central over effector memory T cells (34, 35). Thus, even over the brief span of an acute infection, changes in antigen load, APCs, cytokines, costimulation, and availability of CD4 T cell help drive dynamic alterations in the differentiation program imprinted on viral antigen-activated naïve CD8 T cells (1). The data presented here show that even during smoldering infections such as MPyV, the direction of memory differentiation differs for virus-specific CD8 T cells recruited during acute vs. persistent infection.

In contrast to the latency-reactivation lifecycle of herpesviruses, polyomaviruses persist as smoldering infections, where viral replication may amplify in the setting of depressed immune surveillance. As a result, herpesvirus-specific CD8 T cells recognizing epitopes from lytic proteins may face a "prime-boost" scenario of antigenic encounter that guides a program of memory differentiation distinct from that imparted by non-latent persistent viral infections. This viral lifecycle difference may also underlie recent evidence that memory CD8 T cells to mouse cytomegalovirus and γ-herpesvirus infections are derived from naïve progenitors recruited during the acute phase of primary infection (8, 36). Data presented in this study indicate that naïve virus-specific CD8 T cells recruited after the acute phase of infection to MPyV are major precursors of durable functional memory. Further, we find that antiviral CD8 T cells recruited during acute infection possess diminished effector and proliferative capabilities, lack the capacity to recall upon antigen re-encounter, and are slowly lost from the memory pool – properties usually ascribed to exhausted CD8 T cells in chronic infection environments (37, 38). The precise stage of infection when differentiation is tipped toward effective memory (e.g., early after peak viremia or during persistent infection) remains to be determined.

Preliminary evidence indicates that naïve TCR-I cells transferred two weeks after infection give rise to memory cells exhibiting phenotypic and functional characteristics intermediate between those recruited at the beginning and 30 days after MPyV infection. In this connection, we previously reported that decreasing MPyV-associated inflammation during acute infection results in qualitatively superior memory antiviral CD8 T cells (22). Taken together, these data support the prediction that the proportion of naïve MPyV-specific CD8 T cells that progress toward effective memory increases over the course of infection.

During early acute viral infections, naïve T cells are exposed to high levels of antigen and inflammation, which preferentially drive differentiation toward effectors to optimize host antiviral defense. Using an acutely resolved LCMV infection model, Kaech and coworkers have identified Type I IFN and IL-12 as dominant determinants that induce expression of T-bet, Eomes, and Blimp-1, transcription factors critical for inducing CD8 T cell differentiation into cytotoxic- and cytokine-armed effectors (23). Coincident with viral clearance, T-bet and Blimp-1 transcription factors are downregulated, while Eomes expression gradually increases in concert with the emergence of self-renewing functional memory CD8 T cells (39). In contrast, during chronic LCMV infection, Blimp-1 levels remain high and closely correlate with upregulation of inhibitory receptors that mediate the dysfunction characteristic of clonal exhaustion; however, a basal Blimp-1 expression appears to be necessary for antiviral CD8 T cells to maintain effector function in the face of persistent infection. For memory TCR-I cells recruited during acute infection, we observed higher Blimp-1 expression, and lower expression of Eomes compared to persistent infection-recruited cells, suggesting a

transcriptional profile indicative of senescent effector CD8 T cells (40, 41). Of note, although Blimp-1 expression was higher on acute infection-recruited cells, we were unable to identify an association between their functional deficits and upregulation of T cell exhaustion-associated inhibitory receptors. Whether inhibitory receptors other than PD-1, Lag-3, or Tim-3 mediate functional exhaustion by memory CD8 T cells recruited during acute MPyV infection remains to be determined. The lower, but still substantial, expression of Blimp-1 by persistent infection-recruited memory TCR-I cells may underlie their higher degree of fitness.

Eomes and T-bet also direct the differentiation of effector CD8 T cells, and are required for the ability of CD8 T cells to express effector molecules (29, 42). T-bet was initially described as a master regulator driving terminal effector T cell differentiation, and several studies suggested a redundant or complementary role for Eomes. However, T-bet has recently been shown to be necessary to sustain effector function by memory antiviral CD8 T cells during chronic infection by repressing PD-1 (43). Similarly, while Eomes may play a role in the induction of an effector differentiation state, recent studies suggest that Eomes promotes preservation of the memory CD8 T cell pool. (44, 45). Both acute and persistent infection-recruited MPyV-specific CD8 T cells express T-bet, while only persistent infection-primed cells show increased expression of Eomes. This sustained T-bet expression, coupled with increased Eomes expression, may be involved in improving the capacity of persistent infection-recruited cells survive and retain antiviral effector functionality.

Using the murine CMV (MCMV) infection model, Hill and coworkers recently showed that the memory antiviral CD8 T cell compartment is a composite of naïve T

cells recruited during persistent infection and the progeny of cells primed early in infection (46). In this study, donor-derived, MCMV-specific CD8 T cells were detected months after transferring splenocytes from d7-infected mice into infection-matched, congenic recipients. Thus, the progenitors of these memory cells were primed at some point early in MCMV infection. Unlike the stable maintenance of the memory MPyV-specific CD8 T cell compartment, latent MCMV infection drives continuous expansion of CD8 T cells recognizing particular viral epitopes. Using adoptive transfer of MCMC-specific TCR transgenic CD8 T cells, Torti et al. similarly demonstrated that inflationary memory MCMV-specific CD8 T cells are recruited during acute infection, and further showed that CD8 T cell inflation results from viral antigen-driven restimulation of lymph node-resident central memory cells (47). These findings reinforce the concept that mechanisms for maintaining memory virus-specific CD8 T cells vary depending on the nature of the infectious agent.

In summary, the findings presented here lead us to propose a revised model of memory CD8 T cell maintenance to low-level persistent viral infections. We currently envision a "conveyor belt" scenario where ongoing priming of naïve virus-specific CD8 T cells is required to resupply the pool of deteriorating antiviral T cells generated at early stages of infection. As viral load and/or inflammation diminish over time, bona fide memory T cells progressively emerge and assume an increasingly larger role in maintaining effective antiviral T cell memory. Understanding this dynamic balance between persistent infection and T cell recruitment will be essential for developing interventions to bolster immunity to smoldering viral infections.

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Disclosures

The authors have no financial conflicts of interest.

Footnotes

[2]J.J.W and C.D.P contributed equally to this work.

[3]Abbreviations used: B6, C57BL/6; eomesodermin, Eomes; LCMV, lymphocytic choriomeningitis virus; LT, Large T antigen; MFI, mean fluorescence intensity; MPyV, mouse polyomavirus; p.i., postinfection; rVV, recombinant vaccinia virus; rVV-ES-I, rVV expressing SV40 LT aa 206-215.

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Figure Legends

Figure 1. Distinct response profiles for virus-specific CD8 T cells recruited during acute and persistent MPyV infection. *A,* Experimental design: 100 Thy1.1$^+$ CD8$^+$ TCR-I cells were transferred into Thy1.2$^+$ B6 mice on d -1 (acute) or d 60 or 90 (persistent) of infection by MPyV.LT206. TCR-I cells were then evaluated either 30 d or 90 d post transfer. *B,* CD45$^+$ CD8$^+$ Thy1.1$^+$ cells (TCR-I cells) in PBMCs were enumerated at the indicated timepoints after cell transfer. † and †† indicate 1 of 6 mice and 2 of 6 mice with no detectable TCR-I cells, respectively. Data is representative of 2 independent experiments of 3-6 mice. C & D, TCR-I cells were transferred either on d -1 or d 60 of infection with MPyV.LT206. At d 30 post transfer splenic TCR-I cells were analyzed for intracellular Bcl-2 and surface Annexin V binding. Left panel, representative histogram (open, acute infection-recruited; shaded, persistent infection-recruited); right panel, mean frequency ± SD. *E,* At d 30 and d 90 post transfer for acute infection recruitment, and d 30 post transfer for persistent infection recruitment (60 d p.i.), splenic TCR-I cells were analyzed for intranuclear Ki-67. Left panel, representative histograms stained for isotype control (gray) or Ki-67 (black); right panel, mean frequency ± SD. Data are representative of two independent experiments, with 3-6 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. Expression of memory markers by CD8 T cells differs with timing of recruitment during MPyV infection. *A-D,* 30 d after TCR-I cell transfer, expression of cell surface CD127, CD62L, KLRG-1, and CD43 by splenic TCR-I cells was analyzed by flow cytometry. Memory TCR-I cells generated in response to a resolved infection were

created by transferring 100 TCR-I cells to B6 mice 1 d before i.p. inoculation with rVV-ES-I (2 x 10^6 PFU). Percentages are mean frequency ± SD. *$p < 0.05$, **$p < 0.01$.

Figure 3. Minimal changes in memory phenotype of acute infection-recruited anti-MPyV CD8 T cells over long-term maintenance. Representative flow cytometry plots and histograms of splenic TCR-I cells at 30 d or 90 d after d -1 p.i. transfer (acute) or 30 d after transfer to mice at d 60 p.i. (persistent). *A*, $CD8^+$ $CD44^{hi}$ $Thy1.1^+$ splenocytes were stained for CD27, CD43, CXCR3, and CD127. *B*, Representative histograms of $CD8^+$ $CD44^{hi}$ $Thy1.1^+$ splenocytes stained for CD127, PD-1, Lag-3, KLRG1, and CXCR3. Gates are based on isotype control staining of $CD8^+$ $CD44^{hi}$ $Thy1.1^+$ cells. Percentages are mean ± SD, of 2 independent experiments of 3-6 mice each.

Figure 4. Persistent infection-recruited memory CD8 T cells express higher multi-cytokine functionality and reduced Blimp-1. *A & B*, LT206 peptide-stimulated intracellular IFN-γ and IL-2 production by splenic TCR-I cells. *A*, TCR-I cells at d 30 post transfer in acutely (d -1 p.i.) or persistently (60 or 90 d p.i.) MPyV-infected mice, and at d 30 p.i. of rVV-ES-I infection (memory). *B*, Acute infection-recruited (d -1 p.i.) TCR-I cells at d 30 or d 90 p.i., or persistent infection-recruited TCR-I cells 30 d after transfer to mice infected 60 d earlier. Values represent the mean frequency ± SD of $Thy1.1^+$ $CD8^+$ TCR-I cells either singly producing IFN-γ or co-producing IFN-γ and IL-2. Data are representative of three experiments using 3-6 mice per group. *C*, Peptide dose-response curve for acute infection-recruited (30 d or 90 d post transfer) and persistent infection-recruited (d 60 p.i.) TCR-I cells expressing IFN-γ after stimulation

with the indicated LT206 peptide concentration. Values represent the mean MFI ± SD of Thy1.1$^+$ CD8$^+$ TCR-I cells producing IFN-γ. Data are representative of 2-4 independent experiments of 3-6 mice per group. *D & F,* TCR-I cells were transferred either on d -1 (acute infection recruitment) or d 60 (persistent infection recruitment) of MPyV.LT206 inoculation, or d -1 of rVV-ES-I infection (memory) into Thy1.2$^+$ B6 recipients. d 30 post transfer, splenic Thy1.1$^+$ CD8$^+$ TCR-I cells were FACS-sorted, lysed, and cDNA was prepared. *Blimp-1* and *T-bet* transcript levels were determined by quantitative RT-PCR and normalized against *L9* ribosomal protein mRNA levels. Values ± SD were normalized to TCR-I cells purified from uninfected TCR-I mice. *E, G, and H,* Thy1.1$^+$ CD8$^+$ TCR-I cells from acute infection-recruited cells on d 30 or d 90 p.i., or persistent infection-recruited cells on d 30 post transfer were stained for intranuclear Blimp-1, T-bet, or Eomes. Values represent MFI ± SD of Thy1.1$^+$ CD8$^+$ TCR-I cells for the indicated molecule. Goat IgG isotype controls (for Blimp-1 and Eomes) stained at an MFI of 3-5 and rat IgG isotype controls (for T-bet) stained at an MFI of 2-6. Data are representative of 2-4 independent experiments of 3-6 mice per group. *$p < 0.05$, **$p < 0.01$.

Figure 5. Acute MPyV infection-recruited CD8 T cells suffer exhaustion. Splenic (*A*) and pulmonary (*C*) Thy1.1$^-$ D^b LT206 tetramer$^+$ CD8 T cells (endogenous) were enumerated in mice at d 30 or d 90 p.i. that had received TCR-I cells on d -1 p.i. (acute), or 30 d after transfer of TCR-I cells at d 60 p.i. (persistent). Thy1.1$^+$ CD8$^+$ TCR-I cells from the spleen (*B*) and lungs (*D*) were enumerated for D^b LT206 tetramer binding (top panels), LT206-stimulated intracellular IFN-γ production (middle panels) and intracellular IFN-γ and IL-2 coproduction (bottom panels). Data is compiled from 2-4

experiments of 3-6 mice each. Points on the x-axes represent individual mice with undetectable Thy1.1$^+$ CD8$^+$ cells. *$p < 0.05$, **$p < 0.01$.

Figure 6. CD4 T cells are required for maintenance of memory MPyV-specific CD8 T cells during persistent infection. B6 or I-A$^{b-/-}$ mice received 100 TCR-I cells on d 60 p.i. of MPyV.LT206 infection *A*, Enumerations of Thy1.1$^+$ CD8$^+$ PBMCs (solid line, B6 mice; dotted line, I-A$^{b-/-}$ mice) over the course of infection. *B*, Total Thy1.1$^-$ CD8$^+$ CD44^{hi} D^b LT206 tetramer$^+$ splenocytes (endogenous) or TCR-I cells (Thy1.1$^+$) were enumerated at d 30 after TCR-I cell transfer. Intracellular IFN-γ (*C*) and IFN-γ and IL-2 production (*D*) following LT206 peptide stimulation of the endogenous (Thy1.1$^-$) CD8$^+$ T cells or TCR-I cells (Thy1.1$^+$) were quantified. Values indicate mean ± SD. *$p < 0.05$, **$p < 0.01$.

Figure 7. MPyV-specific CD8 T cells recruited in persistent, but not acute, infection mount recall responses. *A*, B6 mice received 100 TCR-I CD8 T cells either 1 d before (acute infection recruitment) or at 60 d after infection (persistent infection recruitment) by MPyV.LT206. Thirty days post transfer, each mouse received 1 x10^6 PFU rVV-ES-I. The number ± SD of Thy 1.1$^+$ CD8$^+$ cells in peripheral blood was enumerated at d 0 and d 5 p.i. as described in Fig. 1. *B*, 1 x 10^4 FACS-purified TCR-I T cells that were stimulated for 30 d during acute (d 0-30 p.i.) or persistent (d 60-90 p.i.) MPyV.LT206 infection (as described in Fig. 1A), were transferred i.v. into individual naïve B6 mice; 1 day post transfer, each mouse received an i.p. infection with 1 x 10^6 PFU rVV-ES-I. Thy 1.1$^+$ CD8$^+$ cells in the spleen were enumerated at d 5 after rVV-ES-I inoculation. Values

indicated mean ± SD. Data are representative of two independent experiments using 3 mice per group. *$p < 0.05$.

Supplemental Figure 1. MPyV.LT206 infects B6 mice equivalently to wild type MPyV.A2 and specifically stimulates TCR-I CD8 T cells. (A) MPyV DNA genome copies in spleens were quantified by Taqman real-time PCR at the indicated days p.i. Each dot represents an individual mouse. (B) TCR-I CD8 T cells proliferate specifically to MPyV.LT206 in both acutely and persistently infected B6 mice. One x 10^6 CFSE-labeled TCR-I cells were transferred either 1 d before MPyV.LT206 or MPyV.A2 infection (upper two panels, respectively) or 60 d after MPyV.LT206 infection. Spleens were removed at d 5 after transfer. Plots are gated on Thy1.1$^+$ CD8$^+$ cells. (C) TCR-I CD8 T cell adoptive transfer exerts minimal impact upon long-term viral load. MPyV DNA genome copies in spleens were quantified by Taqman-based PCR at the indicated days p.i. Each dot represents an individual mouse. B6 mice were given either 100 TCR-I CD8 T cells 1 day before MPyV.LT206 infection or they received no transfer. Data are representative of two independent experiments using 3 mice per group. *$P < 0.05$.

Supplemental Figure 2. Polyclonal MPyV-specific CD8 T cells recruited during persistent infection express canonical memory markers. (A) Experimental design: naïve Thy1.1$^+$ CD8$^+$ T cells were injected i.v. into Thy1.2$^+$ B6 mice 1 d before (acute infection recruitment) or at d 50 after (persistent infection recruitment) MPyV.A2 inoculation. CD8$^+$ T cells were enriched prior to transfer (MACS CD8 T cell negative selection kit). (B) 30 d after CD8 T cell transfer, spleens were removed and expression of

cell surface CD127, CD62L, and KLRG-1 was analyzed by flow cytometry**.** Values represent the mean frequency ± SD of $Thy1.1^+$ $CD8^+$ T cells expressing the indicated surface marker. Data are representative of two independent experiments using 3 mice per group. $*P < 0.05$.

Figure 1

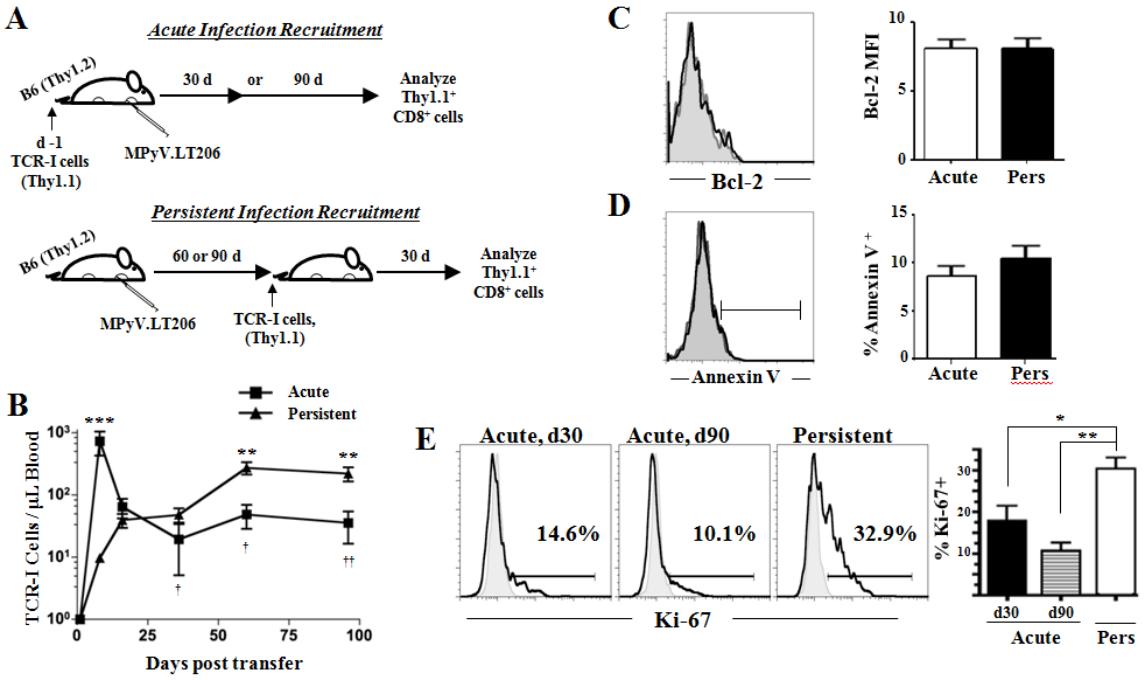

A
Acute Infection Recruitment
B6 (Thy1.2)
30 d
or
90 d
Analyze Thy1.1+ CD8+ cells
d -1 TCR-I cells (Thy1.1)
MPyV.LT206
Persistent Infection Recruitment
B6 (Thy1.2)
60 or 90 d
30 d
Analyze Thy1.1+ CD8+ cells
MPyV.LT206
TCR-I cells, (Thy1.1)
B
Acute
Persistent
TCR-I Cells / µL Blood
Days post transfer
C
Bcl-2
Bcl-2 MFI
Acute
Pers
D
Annexin V
% Annexin V+
Acute
Pers
E
Acute, d30
Acute, d90
Persistent
14.6%
10.1%
32.9%
Ki-67
% Ki-67+
d30
d90
Acute
Pers

Figure 2

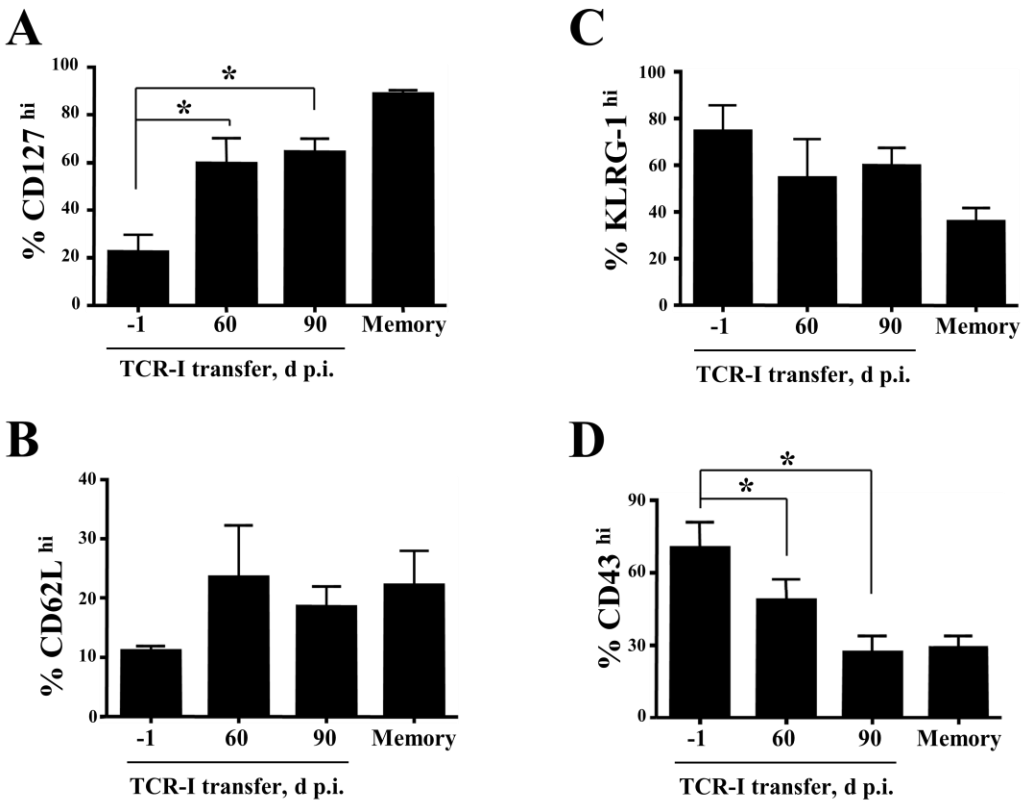

A
% CD127 hi
100
80
60
40
20
0
*
*
-1
60
90
Memory
TCR-I transfer, d p.i.
C
% KLRG-1 hi
100
80
60
40
20
0
-1
60
90
Memory
TCR-I transfer, d p.i.
B
% CD62L hi
40
30
20
10
0
-1
60
90
Memory
TCR-I transfer, d p.i.
D
% CD43 hi
90
60
30
0
*
*
-1
60
90
Memory
TCR-I transfer, d p.i.

Figure 3

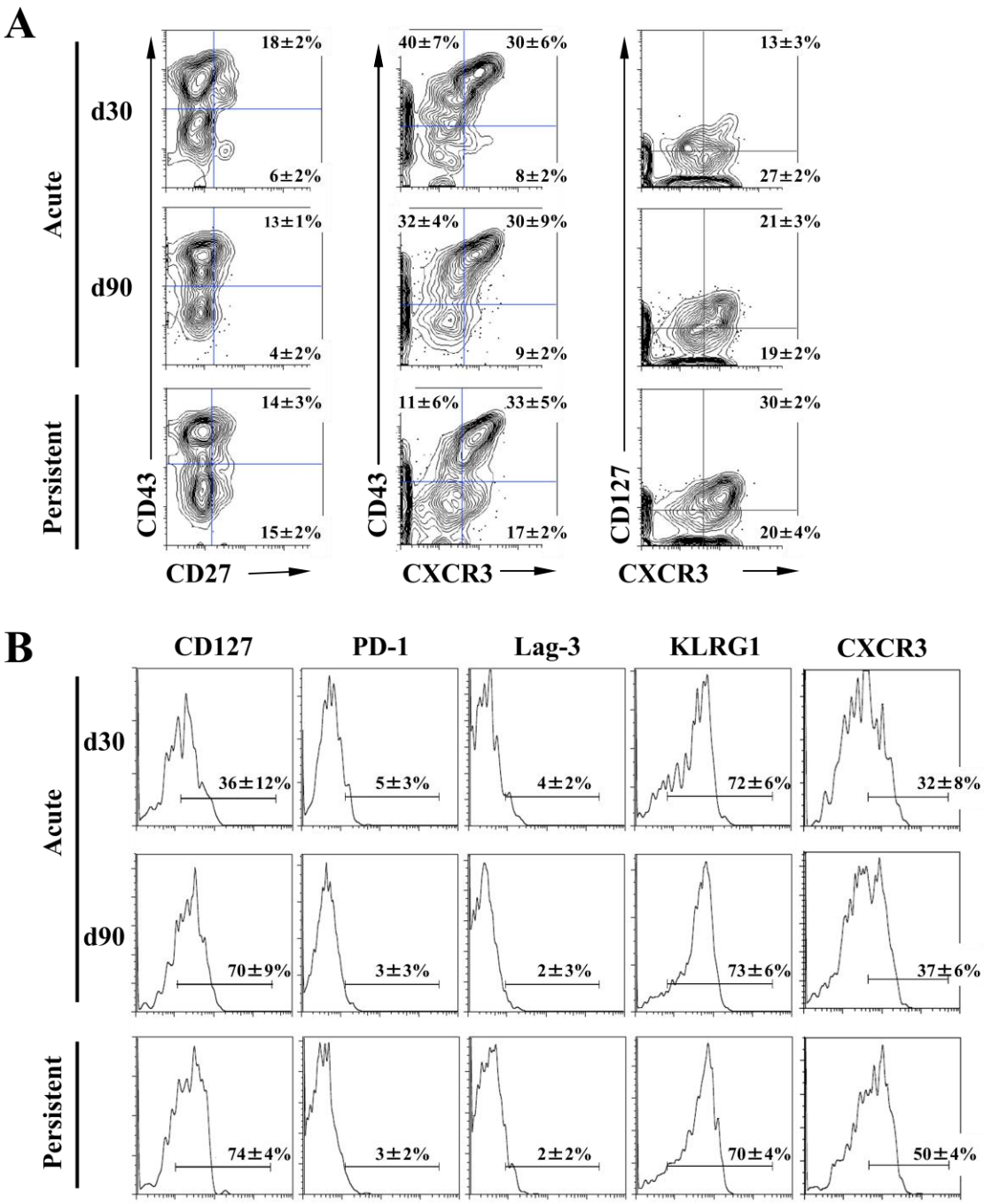

A
Acute
d30
d90
Persistent
18±2%
6±2%
13±1%
4±2%
14±3%
15±2%
CD43
CD27
40±7%
30±6%
8±2%
32±4%
30±9%
9±2%
11±6%
33±5%
17±2%
CD43
CXCR3
13±3%
27±2%
21±3%
19±2%
30±2%
20±4%
CD127
CXCR3
B
CD127
PD-1
Lag-3
KLRG1
CXCR3
Acute
d30
d90
Persistent
36±12%
5±3%
4±2%
72±6%
32±8%
70±9%
3±3%
2±3%
73±6%
37±6%
74±4%
3±2%
2±2%
70±4%
50±4%

Figure 4

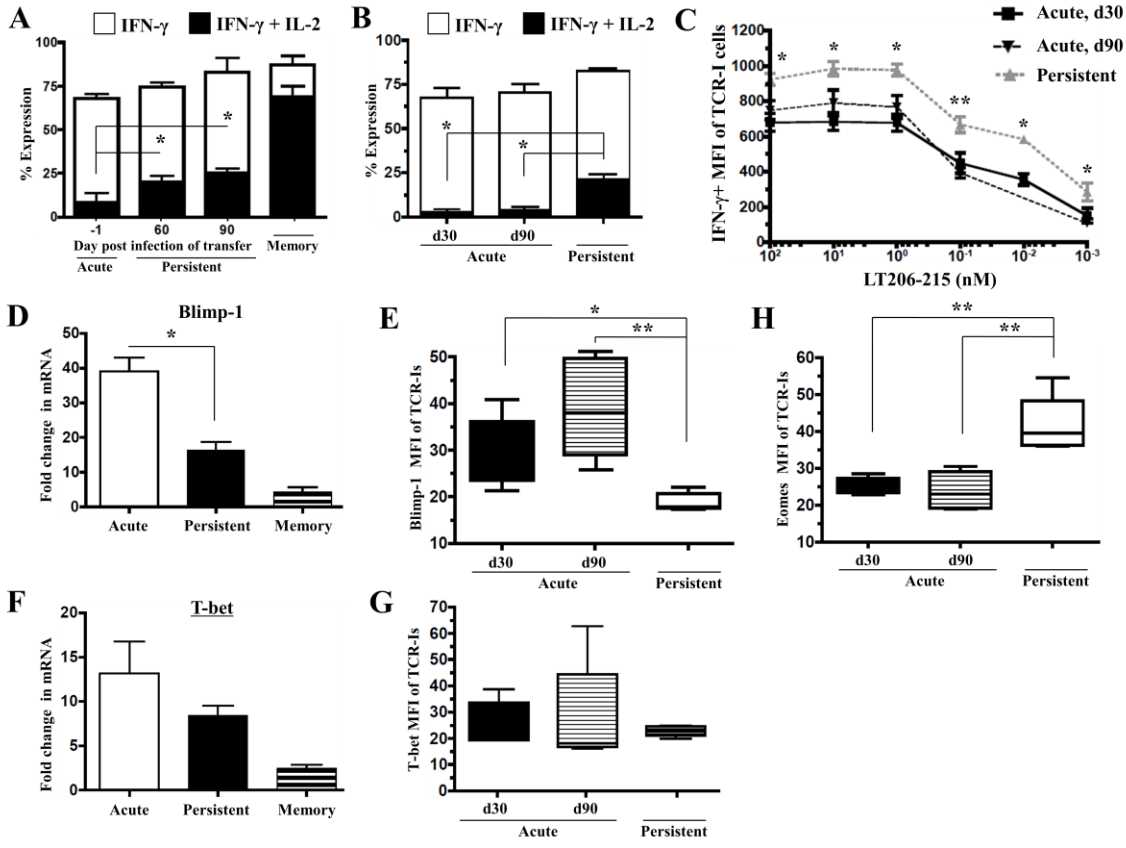

A
IFN-γ
IFN-γ + IL-2
% Expression
Day post infection of transfer
-1
60
90
Memory
Acute
Persistent
B
IFN-γ
IFN-γ + IL-2
% Expression
d30
d90
Acute
Persistent
C
Acute, d30
Acute, d90
Persistent
IFN-γ+ MFI of TCR-I cells
LT206-215 (nM)
D
Blimp-1
Fold change in mRNA
Acute
Persistent
Memory
E
Blimp-1 MFI of TCR-Is
d30
d90
Acute
Persistent
H
Eomes MFI of TCR-Is
d30
d90
Acute
Persistent
F
T-bet
Fold change in mRNA
Acute
Persistent
Memory
G
T-bet MFI of TCR-Is
d30
d90
Acute
Persistent

Figure 5

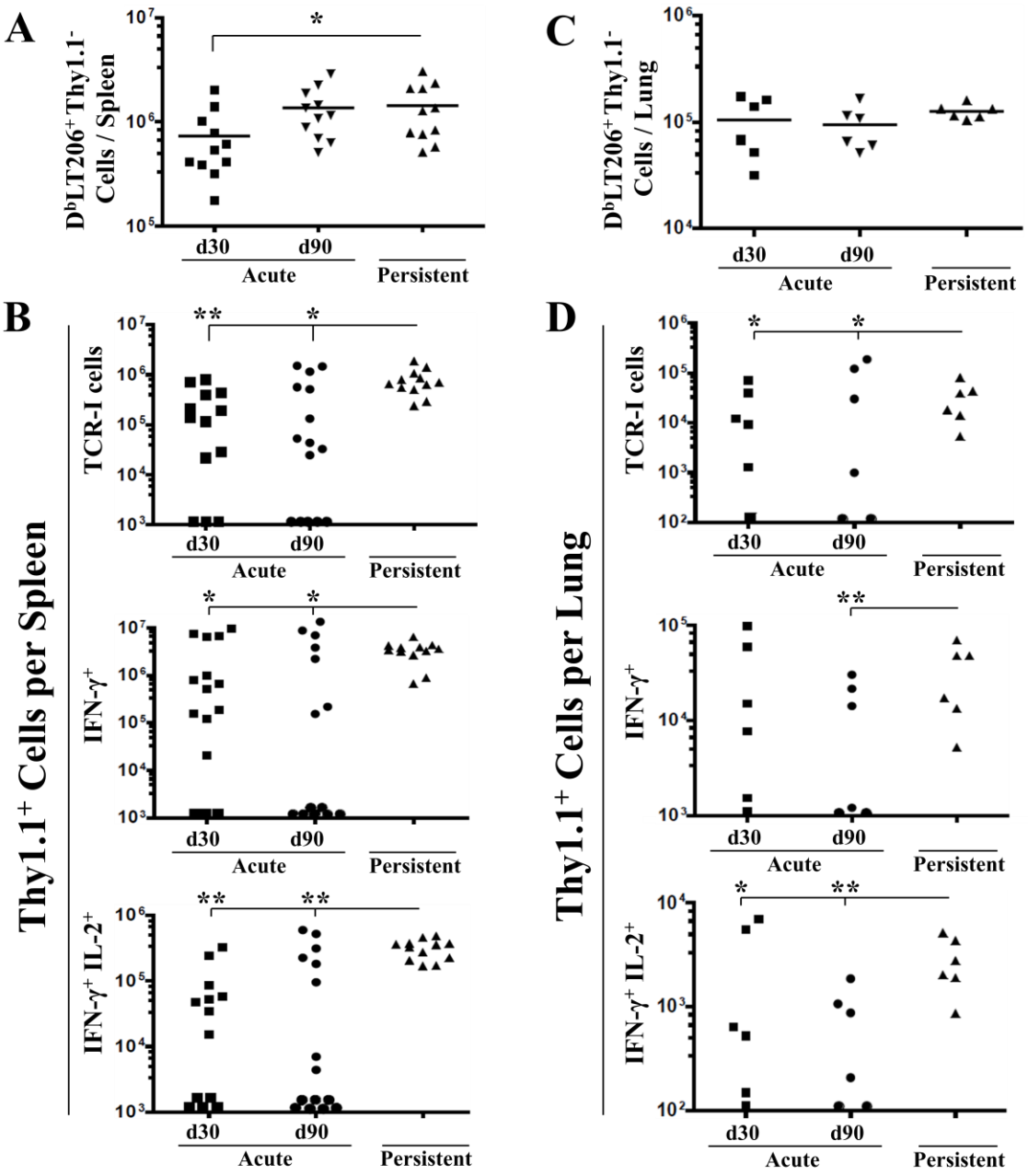

A
DbLT206+Thy1.1-
Cells / Spleen
d30
d90
Acute
Persistent
C
DbLT206+Thy1.1-
Cells / Lung
B
Thy1.1+ Cells per Spleen
TCR-I cells
IFN-γ+
IFN-γ+ IL-2+
D
Thy1.1+ Cells per Lung

Figure 6

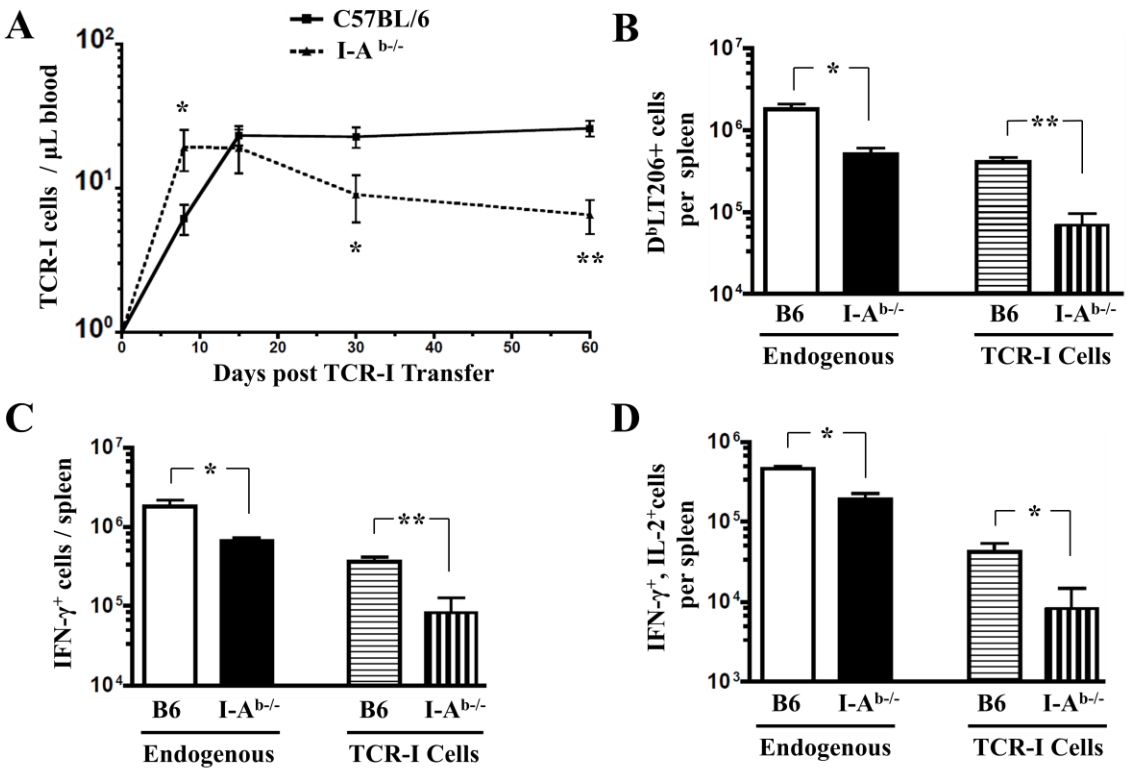
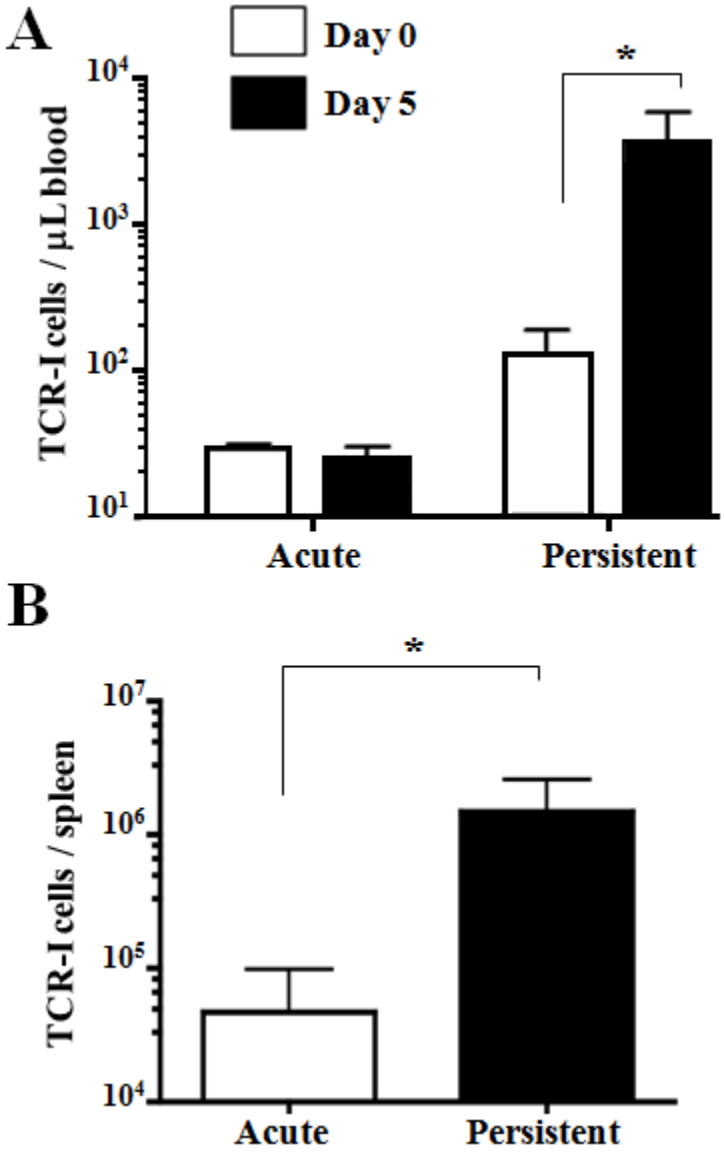
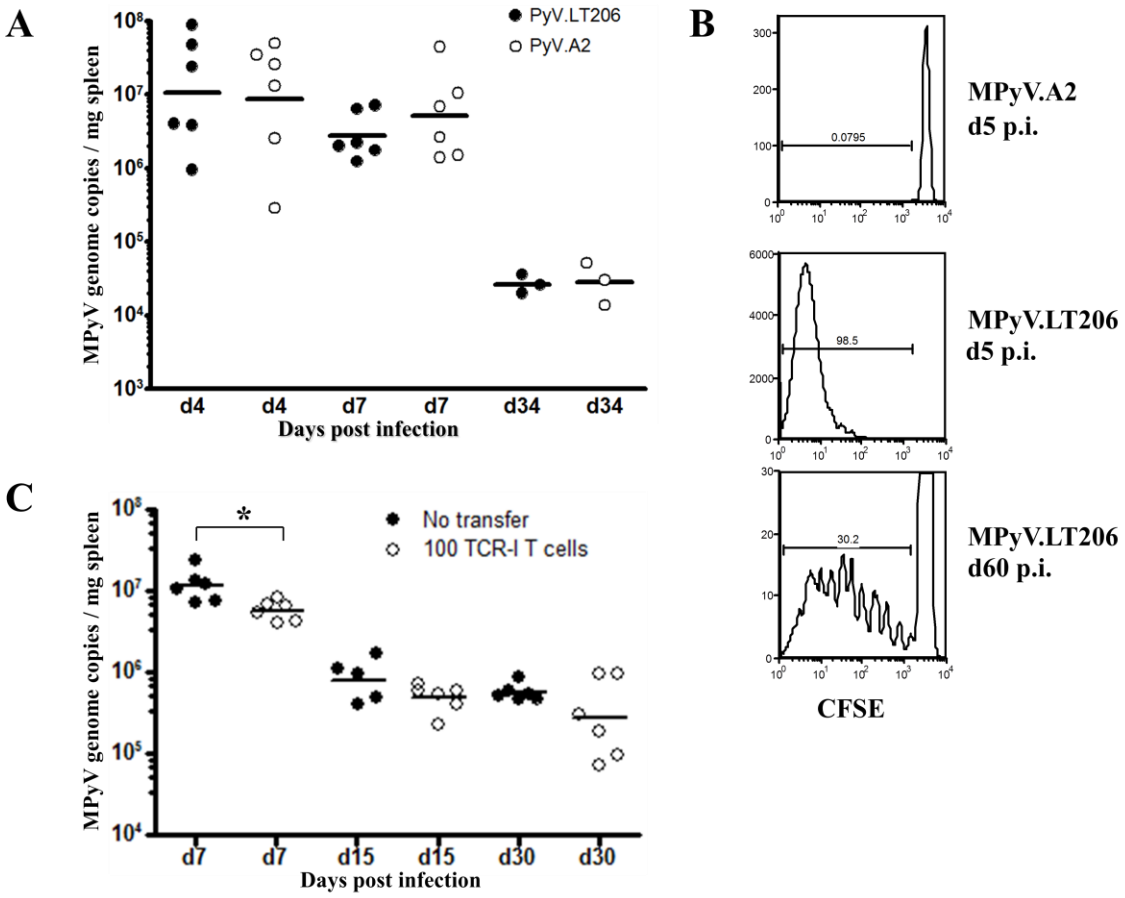

A
C57BL/6
I-A b-/-
TCR-I cells / µL blood
Days post TCR-I Transfer
B
DbLT206+ cells per spleen
B6
I-Ab-/-
Endogenous
TCR-I Cells
C
IFN-γ+ cells / spleen
D
IFN-γ+, IL-2+cells per spleen

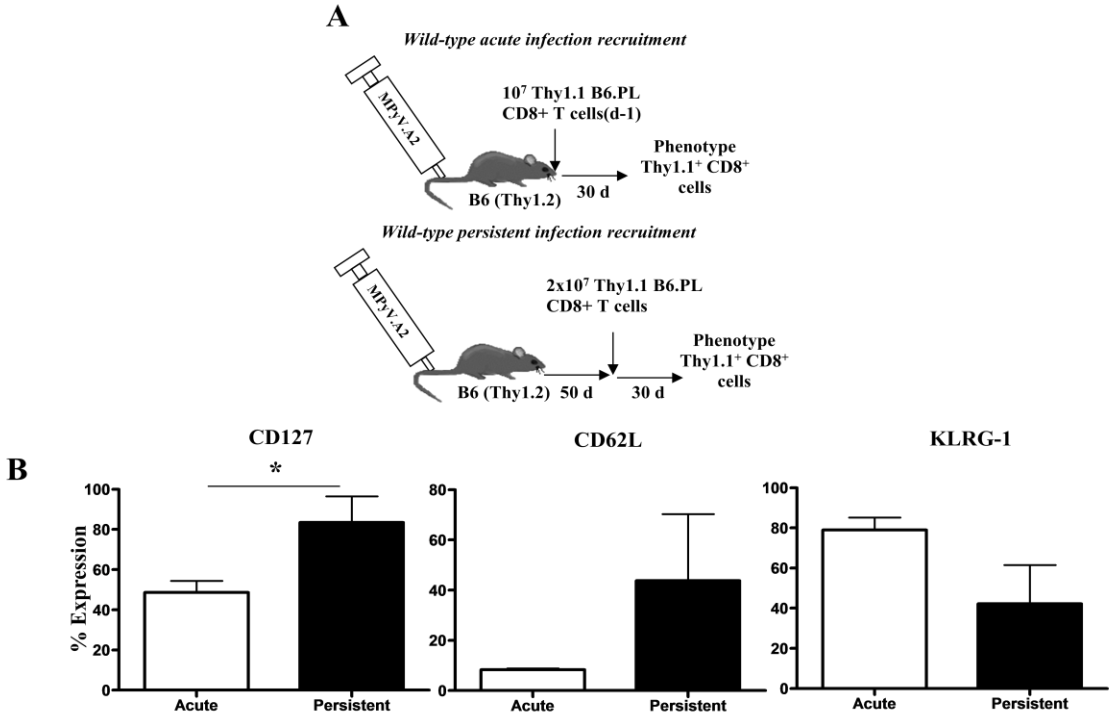
Figure 7


A
Day 0
Day 5
*
10^4
10^3
10^2
10^1
TCR-I cells / µL blood
Acute
Persistent
B
*
10^7
10^6
10^5
10^4
TCR-I cells / spleen
Acute
Persistent

Supplemental Figure 1



Supplemental Figure 2



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Chapter 4: Discussion

Numerous studies have supported the critical role that CD8 T cells play when combating various viral infections. Viruses have an uncanny ability to hide from and even alter the actions of the immune system, all in an effort to hijack, propagate, and spread to the next host. The cellular and humoral immune systems are trained during development to ignore normal, non-stressed host cells, creating a gap in immunologic control should viruses invade and hijack a cell. Within these infected cells, viruses have the ability to shut off MHC I processing, block the immune proteosome, and can increase cellular expression of inhibitory MHC I like molecules preventing the immune system from further recognizing the infections (1-4). Dysregulation of the levels of MHC I molecules on the cell surface can potentiate the activation NK cells but increased inhibitory MHC I molecules can prevent direct lysis of these infected cells (5, 6). Lowered MHC I expression, or decreased loading of viral peptides through the immunoproteosome can result in prevention and/or limitation of CTL mediated killing (7). Some viruses also directly infect "immune privileged" tissues like neurons within the CNS, where the immune system is generally limited in inflammatory and direct lytic capability (8).

Acute infections may be able to hide from or alter the immune system initially, but they eventually succumb to the adaptive arm of the immunologic response. However, persistent viral infections remain capable of hiding from or preventing their recognition by the immune system. These persistent pathogens can vary in their viral loads, from high level infections like HIV and LCMV, to low level smoldering pathogen PyV, to the latent pathogen, CMV. While persistent HIV infection is maintained due to rapid mutations allowing escape from adaptive immunologic recognition, poor CD4 T cell

help, and deficiencies in CD8 T cell functionality (i.e. cellular exhaustion), low-level persistent pathogens are thought to be under a state of constant immune surveillance (9-11). In these cases, the CTL response against these pathogens is potentially limited in order to control the level of immunopathology against the infected site (12, 13). However, the balance of the immune response with persistent infection creates a situation of chronic antigen and inflammatory stimulus, which perturbs the development of functional CTLs and their subsequent differentiation into memory. With the increased use of immunotherapies, there is a direct repression of the adaptive response, potential effects on the overall viral control, and the perturbation of the immune response that was present. Therefore, studies are needed on how to better develop and/or maintain the functional memory CTL response against these viruses, and to develop novel therapeutics that can suppress viral load and/or improve the overall CD8 response.

Our inability to initially find the effector mechanism utilized by CD8 T cells in the control of MPyV provided a problematic knowledge gap in our efforts to improve viral control in human PyV infection (14). The lack of our understanding in how the immune response mediates anti-viral control is especially glaring, since no known anti-PyV agents exist. The initial description of IFN-γ's ability to control BK virus in tissue culture spurred my interest in this pleiotropic cytokine's potential to control MPyV (15). IFN-γ resulted in significant reduction of BK virus gene expression and replication in proximal tubule cells, but the specific mechanism of IFN-γ mediated control remained. Against MPyV, we recapitulated this anti-PyV effect in Chapter 2, finding a significant and rapid inhibition of viral gene expression, with a subsequent reduction in viral replication. When taken in vivo, it was clear that IFN-γ was critical for viral control, as

IFN-γR deficient mice possessed significantly higher viral load and were highly susceptible to MPyV-mediated tumorigenesis. This defect could also be isolated to a site of viral persistence (here, the kidney) by using our novel kidney transplant model, which indicated direct IFN-γ signals are required at a site of infection for viral control. To complete my IFN-γ studies, we found that therapeutic treatment with IFN-γ improves persistent viral control. Together with the in vitro IFN-γ potential against human PyVs, our data suggests a direct link between IFN-γ expression and anti-viral control of PyV, and might provide insight a new anti-viral agent. Such an agent might directly activate downstream targets within the Jak/Stat pathway, or directly signal the IFN-γR to drive its anti-viral effects. But studies are needed to determine what signals and/or targets are critical for the anti-viral capacity of IFN-γ in order to best develop new therapeutic drugs.

How IFN-γ blocks viral gene expression and/or replication remains unknown. We found limited cellular death mediated by IFN-γ, but further evaluation of these effects are needed as we cannot rule out other means of IFN-γ induced cell death that would result in the improved control (16-19). Interestingly, the promyelocytic leukaemia protein (PML protein) associates into nuclear bodies that help recruit and harbor numerous nuclear proteins involved in varying cellular processes like apoptosis, stress responses, and viral infections (20). These structures are associated with LT during BK and SV40 genomic replication, and has been found to be in close proximity to assembled JC virus particles in the brains of patients that succumb to PML (21). Increased PML protein was recently found to block JC virus early gene expression and infection following treatment with a IFN-β (22). It was suggested that this increase in PML protein may restrict PyV proteins or the viruses needed transcription factors needed for

increasing viral gene expression. PML protein and nuclear bodies can also be induced and upregulated by IFN-γ, suggesting increased levels of PML protein or associated nuclear bodies in the anti-viral effector pathway of IFN-γ. In vitro studies of MPyV infected cells treated with IFN-γ could quickly ascribe any potential role for increased PML protein in the anti-viral effects of this cytokine.

IFN-γ could also block any of several steps within the viral life-cycle (binding, absorption, uncoating, cell-cycle initiation, to DNA encapsidation and capsid assembly), and studies are needed to best evaluate where this anti-viral block occurs. Microscopic studies (EM, deconvolution, or IHC) should be able to follow how viral entry or viral transit may be altered with IFN-γ treatment (23). Likewise, viral uncoating or viral reassembly may equally be altered by IFN-γ treatment. More studies though are needed to help focus research on novel, more specific agents that could also block the ascribed pathway, without requiring IFN-γ production from the antigen-specific CD8 response or the resulting inflammation that may occur through direct IFN-γ therapy that we utilized in our experiments.

The role of IFN-γ as a critical cytokine mediating MPyV control was intriguing as most direct anti-viral actions work through the Type-I IFNs, while IFN-γ is generally more immune stimulatory in its anti-viral functions. IFN-γ is elaborated by a wide range of immune cells. Our lab, and the human PyV lab of Igor Koralnik have suggested a critical role for CD8 T cells in mediating viral control, and our lab feels that IFN-γ's control of MPyV is likely mediated directly via CD8 T cell's vectorial secretion within the TCR/MHC synapse following recognition of infected cells (24, 25). Ongoing studies by the Lukacher lab seeks to directly link IFN-γ's anti-viral capacity with the production

from the anti-viral CD8 T cell response. While more studies are needed, our TCR-I transgenic mice have been breed to IFN-$\gamma^{-/-}$ mice to create a transgenic CD8 T cell response that is deficient in only IFN-γ. By infecting IFN-γ deficient mice with MPyV we can study the exact role of the CD8 T cell response against one epitope by injected either IFN-γ sufficient or deficient TCR-Is. This study should allow us to fully ascribe T cell target recognition and subsequent IFN-γ elaboration with direct MPyV control.

Also of interest is our finding that IFN-γ potential is generally maintained regardless of when anti-MPyV specific cells were primed, though acute-primed cells suffer from decreased overall capacity (Chapter 3). While these cells may eventually be lost and replaced by more recent recruits, the decreased IFN-γ functionality may be a means by which the immune system directly modulates the early CTL response during high level viremia, multi-tissue infection phase. However, equally possible is that other inflammatory mediators limit the IFN-γ potential of acute responding CTLs, but regardless imply a relative deficiency in viral control mediated through CD8 T cells primed during the acute phase of infection. Following the clearance of acute systemic MPyV, the virus becomes a smoldering low level infection where reduced antigen and inflammatory levels are present, resulting in an improved IFN-γ capable CTL response for those persistent primed CD8 T cells. However, this failure to clear MPyV early allows the infection to spread to the kidneys and the CNS (amongst others), both immune privileged sites of poor immunologic CTL potential, where it may remain persistent long-term.

With our finding of therapeutic potential of IFN-γ in persistently infected hosts, it may now be possible to improve persistent viral control, or to limit its resurgence during

immunomodulation. While IFN-γ is currently approved as a therapeutic against chronic granulomatous disease and osteopetrosis, it may be available for experimental approval and therapy in otherwise untreatable HIV/AIDS patients suffering from PML (26). This could serve as a CD8 independent means of controlling JCV infection in AIDS patients with PML, and might also be of use prior to or even during immunomodulatory treatment for at risk patients. This may also be possible for use in kidney transplant patients, but there is also a risk of IFN-γ treatments increasing overall inflammation (highly detrimental to renal transplant survival), and as such would require further study to evaluate the safety of such a treatment (27-30). Given our MPyV kidney transplant model, and our recent findings of how the anti-viral immune response affects kidney transplants, experiments using IFN-γ therapy during MPyV infected of allogenic kidney transplants could provide pre-clinical evidence on the effectiveness and general safety of an IFN-γ therapy regimen (27).

Since only a small subset of HIV/AIDS patients and those taking immunomodulatory agents develop PML, it is interesting to speculate that some patients suffer from genetic abnormalities within the IFN-γ or IFN-γR genes that affect his signaling pathway and promotes subsequent PyV pathology in these patients (31-34). Also, since IFN-γ is lost over the course of CD8 T cell exhaustion, these patients may also suffer from more progressive exhaustion than other patients, or be depressed in their ability to prime new anti-PyV specific CD8 T cells possessing higher IFN-γ functionality (35). Dysfunctions involving IFN-γ have been reported to play a role in certain diseases, and it would not be a stretch to envision that these deficiencies may favor the increased development of PML (36-38). Because improved viral control can serve as a positive

feedback loop on the CD8 response, which then feeds back to further viral control, even small improvements in viral control, either through improved CTL recruitment and/or function, or increasing CD4 T cell help, might vastly improve the outcome in these PyV disease patients (12, 39).

Given the capability, though decreased, IFN-γ potential of acutely primed anti-MPyV specific CTLs, we were surprised that these cells were slowly deleted from the T cell pool. The maintained functionality of a deleted cellular pool is at odds with most models of cellular exhaustion, so we were surprised that the overall CD8 T cell response requires new naive precursors to maintain the memory pool. This finding implies a direct inefficiency of acute infection priming in the development of long-lived memory CD8 responses (Chapter 3). Why then have we previously seen a stable memory pool, and yet the population is not maintained in thymectomized mice or upon transfer to naïve mice (40-42)? Our findings help fill this knowledge gap by explaining that the antigen-requirement for CD8 T cell survival resides in the require of antigen presence needed for the priming of new naïve precursors. The loss of cells in thymectomized mice, or upon transfer to naïve precursors, could be due to the inability of these mice to prime new naïve precursors needed to maintain the population.

Not only do these acute infection primed anti-MPyV specific CD8 T cells fail survive long term, but our study in Chapter 3, provides the first evidence for functional exhaustion and physical deletion of the CD8 T cell response against MPyV. While several studies from the herpesvirus field have found that an acute infection derived population continually repopulates the stable memory CD8 T cell pool against this latent virus, this does not preclude the development of more memory like cells derived from

late recruited new naïve cells (43-45). Further, it would not be surprising that the CD8 T cell response against a smoldering infection may rely differently on constant naïve precursor input, than might be required against a latent infection where there are various levels of prime/boost to lytic epitopes. Our finding provides support for the continued need of naïve precursors against this low level pathogen, in order to maintain high levels of anti-viral functionality (IFN-γ) and production of more memory-like cells.

It should be stressed that late primed anti-MPyV CD8 T cells displayed far superior levels of functionality than those CD8 T cells primed during the acute phase, and it remains unclear how this is related to antigen and/or inflammatory levels (46, 47). Obviously, acute primed cells are developed during increasing antigen levels and very high levels of inflammation, and as such may be privy to a much more effector like developmental pathway (48-50). However, our studies of low-dose infections suggest that antigen levels alone are not solely, if at all, responsible for the increased memory-like phenotype of late recruited cells (51). It is difficult to hypothesize how the inflammatory environment plays a role in the differentiation pathway of the CTL response, as there is no "acute" MPyV infection, nor has the inflammatory environment against PyVs been thoroughly studied. Utilizing mice deficient in inflammatory mediators (IFN-$\alpha R^{-/-}$, IL-$12R^{-/-}$, and even IFN-$\gamma^{-/-}$) or assaying the inflammatory levels during acute versus persistent infection would vastly improve the understanding of which cytokine(s) are involved, and then how those molecules foster a more memory-like development pathway.

Contrary to published reports of IFN-γ deficiency leading to poor CTL development, we found that the CD8 T cell response generated against MPyV in IFN-γR^{-}

$^{/-}$ mice to be of increased overall quality, than those generated in receptor-sufficient mice. Lindsay Whitton's group has directly shown that IFN-γ signaling drove increased CD8 expansion, full effector differentiation, memory development, and even was needed for full activation of anti-viral CD4 T cells (52-55). However, other groups have shown a critical role for IFN-γ in regulating CD8 T cell apoptosis and contraction following acute expansion, fitting with our data for the CTL dynamics against MPyV in IFN-γR deficient mice (50, 56-58). It is possible that IFN-γ signaling from acute infection-elicited innate immune cells (NKs and DCs) drives early inflammatory responses, leading to increased IL-12, both driving the full effector differentiation pathway of CD8 T cells (59). While we found no increased viral load in mice depleted of NK cells (JJW, unpublished observation), we did not look at the kinetic development of the CD8 T cell response, and this hypothesis remained to be studied. To separate the direct CD8 T cell control and the innate response to MPyV infection, it should be possible to place IFN-γ capable CD8 T cells into an IFN-γ KO mouse. This will allow the study of what role innate IFN-γ mediated inflammation plays in the developed memory response, while maintaining the CTL's ability to control MPyV infection through IFN-γ production. Since IFN-γ is not needed to control a DC based vaccine, the inflammatory signals driven by IFN-γ may explain why there is an improved CTL and memory response developed in IFN-γ deficient mice receiving a DC based immunization protocol (60). All together, the functional dichotomy of IFN-γ signaling and the developed immune response against various pathways remain unclear, especially given its necessity during MPyV control.

Given the direct link with increased viral load and functional exhaustion of responding CD8 T cells, we were very surprised that IFN-γR deficient mice did not show

signs of exhaustion (Chapter 2), especially given the above demonstrated role of IFN-γ in developing effector CTLs. It is unclear if IFN-γ signaling in these mice is also required for the development of CTL exhaustion during chronic viral infection, or there is merely increased recruitment of newly primed cells with increased function as described in Chapter 3. The antigen levels of MPyV and ensuing inflammation may be insufficient to drive classical T cell exhaustion during the late phases of infection. But, given our finding of decreased anti-viral functionality and survival of acute-primed cells, it cannot be ruled out that some of the improvement in the long term-response in IFN-γR$^{-/-}$ mice may be due to new naïve recruits. Given that deficiency in IFN-γ signaling lowered the KLRG1 expression in responding cells, there may be an increase of cells that have not fully divided out and therefore display an improved overall phenotype (35, 61, 62). Regardless though, it is clear this dysfunction of the acute infection-primed CD8 T cell population most likely occurs independently of viral load, and suggests that the dysfunction in this population of cells is mediated by the inflammatory environment, possibly associated with IFN-γ levels during the early phases of MPyV infection.

Recent focus on CD8 T cell dysfunction has swung from phenotypic CD8 T cell characterization to trying to understand the transcriptional pathways that are perturbed when chronic antigen and inflammation persist. This is due in part to the recent associations of inhibitory marker expression linked to changes in transcription factor expression. Studies have found that the transcriptional repressor Blimp-1 can actively regulate PD-1 expression (63). Since IL-2 drives Blimp-1 expression early during infection, PD-1 is subsequently increased on responding CD8 T cells (64). This has been hypothesized to serve as a signal to limit CTL expansion following initial activation and

prevent autoimmunity, both due to its reduction in TCR signaling to its potential downregulation of IL-2 upon antigen clearance (65, 66). Inflammation via IL-12 also directly induces and sustains T-bet expression, which is further enhanced by high levels of IL-2 that drive an effector population (67-70). Activated CD8 T cells also lose the ability to produce IL-2 following initial antigen stimulus, and only regain the ability upon full antigen clearance (71, 72). This potentially decreased amount of available IL-2, together with inflammation, may continually sustain T-bet and Blimp-1 levels (64).

We identified an interesting transcriptional program for the anti-MPyV specific CTL response over time. Acute-infection primed CD8 T cells suffered from decreased proliferative, cytokine, and survival potential, likely related to the maintenance of a Blimp-1^{hi} transcriptional level (63, 64, 73-75). Blimp-1 expression was significantly decreased in CD8 T cell primed during the persistent phase of MPyV infection, possibly due to decreased antigen and inflammation at the time of priming, a hypothesis that would also support the increased expression of Eomes (76-78). How and why T-bet levels remain high in both acute and persistent primed cells remains unclear, given the inverse relationship of T-bet and Eomes (77). Since a large proportion of the acute primed cells undergo physical deletion from the population, we may only be capturing a snapshot of just those cells that survive, while those with an even higher level expression of T-bet are preferentially lost. But, future studies are needed to fully understand the transcriptional developmental pathways in responding T cells, and how those profiles delineate an effector versus a memory CD8 response. However, it would not be surprising that a low level smoldering infection like MPyV might display a different

transcriptional program within the CD8 T cell response, as would be seen against an acute or high level chronic pathogens.

That is the problem though of persistent pathogens. Antigen and inflammation remain high, and can prevent the restoration of IL-2 responsiveness of the developing memory response. Failure to clear a pathogen can permanently imprint an effector phenotype on the developed response, preventing these cells from memory differentiation even if the environment eventually clears (79, 80). Mere re-exposure to inflammation during the late phases of antigen-driven expansion can severely blunt the ability of the CD8 T cell response to transition into memory, and even minor increases in TCR contact with cognate antigen on MHC can drive the response away from memory (81, 82).

During MPyV infections, we found an interesting amalgam of dysfunction within the responding population. While the acutely primed cells were physically deleted from a large number of mice, their inhibitory marker expression was remarkably deficient compared to that seen in other chronic infections (43, 83, 84). We find no expression of PD-1 following the initial expansion phase, and can detect no Lag-3, 2B4, or Tim-3 on MPyV specific cells, even though these cell display decreased function and eventually are lost from the memory pool. This should spur experiments to detect different phenotypic surface markers between the acute primed CTL response and those primed later during viral persistence. This might flush out new inhibitory receptors for low level pathogens, especially given our finding of cellular exhaustion with a near comlete lack of inhibitory marker expression on acute primed CD8 T cells. There could also be decreased co-stimulatory receptors in the TNF family, among others, that affect the differentiation pathway following acute infection primed CD8 T cells (85).

The lone exception of inhibitory marker expression on the anti-MPyV CD8 T cells was the continued presence of the cell senescent marker KLRG-1, both on acute-infection recruited and persistent-recruited CTLs. Given the shear disparity in survival and functionality of these two populations, this marker confounds explanation. Since there is a small KLRG-1^{lo} population in the late recruited memory compartment (as well as the acute compartment), it remains to be seen if this is a true memory population that explains the improved recall response seen in these mice (86, 87). It should also be noted that only the persistent primed population as a whole showed increased expression of Ki-67, suggesting there are cells present in this population which show signs of recent division, more so than their acute primed counterparts. Thus further studies are needed to determine if the MPyV specific response recruited late during infection is composed primarily of long-lived but mainly effector CTLs, or if, similar to that seen against CMV, there is indeed a small population of memory cells that continually repopulate the overall compartment (45, 88-91). Either way, how these memory cells develop under the condition of persistent antigen is of interest, as each may underlie different means to protect this population from immunomodulation, improve this population in potentially susceptible patients, or develop this response by means of various vaccination strategies.

On a fundamental level, how the differentiation profile of MPyV specific cells is developed over the course of infection remains of interest. While persistent primed cells develop into a more memory-like population, when or is there a critical threshold point of antigen or inflammation for improved memory development? It is possible that bona fide memory cells that would develop under conditions of acutely cleared viruses fail to develop against MPyV, especially given the poor expression of CD62L seen in MPyV

specific CD8 T cells over time. Since high level antigen presence prejudices the development of CD62L, the overall levels of MPyV Ags may be sufficient during TCR stimulation to limit L-selectin levels over time (81, 92, 93). However, since CD62L expression slowly increases over time in true memory cells, the conveyor belt of the MPyV specific response may only differentiate into $CD62L^{hi}$ cells very late during infection when antigen levels have declined to sufficient levels in various target tissues (35, 93).

While not studied in Chapter 3, it is interesting to speculate about the antigen-independent survival potential of persistent-infection recruited CD8 T cells against MPyV. Acute primed cells suffer attrition, both in the face of chronic antigen, as well as when transferred into naïve hosts. Given the cytokine potential, transcriptional, survival, and replication improvements in late recruited cells, these cells could have developed under proper conditions to favor development of true, antigen-independent memory cells. Decreased Blimp-1 may allow for sufficient IL-2 expression and signaling of the CD8 response to drive up expression of Bcl-6, and improve the maintenance of the population and favor a memory differentiation program (73, 94, 95). Also, increased CD127, CD27, and CXCR3, together with increased Eomes expression, an antigen-independent memory pool may exist, and studies should be undertaken to determine if this is true, and if so, how this population developed (77, 96, 97).

Prior to the studies described throughout this dissertation, the overall immune response against MPyV had been fairly well studied and yet important questions about CD8 T cell effector mechanisms and memory cell potential remained. How does IFN-γ signal infected cells to limit viral gene expression? Is this signaling directly through the

IFN-γR, or rather via a secondary mediator? Does IFN-γ arise directly from MPyV-specific CD8 T cells? Can IFN-γ be used as a potential therapy? Why is IFN-γ functionality decreased in acute primed cells? What inflammatory mediator (or possibly antigen) drives acute primed cells into SLECs rather than memory precursors? How can we utilize the inflammatory mediator data to better improve the acute response and develop memory?

However, we have now identified IFN-γ as a critical cytokine and linked its signaling to the perturbation of the anti-viral CD8 T cell response. While the elaboration of IFN-γ from CD8 T cells directed against infected cells remains murky, several studies demonstrating the critical role of CD8 T cells in viral control (and not other populations like NK and CD4 T cells) support this early hypothesis. Since IFN-γ is also an inflammatory cytokine produced by numerous cells, it could also play a partial role in the early exhaustion seen in acute primed cells. Based on the improved overall phenotype in the IFN-γR deficient mice, the probability of an overall drop in IFN-γ over the course of MPyV persistence might lead to an improved priming environment for late primed antigen-specific recruits. This hypothesis fits best with our findings of increased fitness during lower levels of inflammation, as well as with our new findings of CD8 T cell improvement that appears independent of overall viral burden (51). Yet further studies are warranted to better define the inflammatory events that disrupt true memory development in both acute and late primed cells, as both displayed an incomplete characteristic memory profile.

The discovery of a critical mediator of viral control, as well as our finding of inflammation-mediated dysregulation of the immune response, provides new avenues to

investigate and understand host immune mechanism that control PyV associated disease. The direct mechanism of IFN-γ remains unclear, and yet IFN-γ may still provide a potential therapeutic benefit as it is already an approved treatment in human patients. IFN-γ deficiencies (or the unknown downstream targets), might also shed light on why only a small subset of various immune suppressed patients develop PML. How to best utilize our findings as it relates to human BK virus renal pathologies remains cloudy. But, since IFN-γ can directly improve viral control of an MPyV infected kidney transplants, IFN-γ therapy could extend its efficacy into the transplant field.

The exhaustion seen in MPyV specific cells is unusual, and deciphering the mechanisms regulating it could lead to strategies that promote cellular response against persistent human PyVs and potentially other lower level persistent pathogens. Since acute-infection primed CD8 T cells can exhaust over time, it is likely that the predominant anti-PyV response in humans is from populations that are more recent recruits into the pool. Improving or maintaining the anti-PyV specific CD8 T cell response undergoing immunomodulatory treatments may be a limiting factor, but improving the response in immunodeficient patients may be possible. Also, because CD4 T cell help is required for persistent priming and maintenance of MPyV specific cells, improving help during resurgent PyV infections, possibly in concert with IFN-γ treatments, may improve the overall outcome of disease. However, studies are warranted to confirm and expand our findings in the MPyV infection model as it relates to human PyV infections and the overall CD8 T cell response.

With the human PyV field exploding in recent years (since 2008, the virus family has expanded from 5 human viruses in 2008 to 9+ today), understanding how to control

this persistent pathogen is becoming more and more important. Initially human PyVs were appreciated for their ability to cause renal failure in kidney transplant patients or PML in HIV/AIDS patients, yet the recent use of immunomodulatory agents in more and more patients has resulted in increased PML development and discovery of more PyVs. With the recent link of a new human PyV with highly aggressive skin carcinoma (Merkel cell PyV), another link of a PyV in a heart transplant patient suffering from trichodysplasia spinulosa (TSV), and the ever increasing use of immunomodulatory agents for immunologic disease, more studies are needed to improve our understating of PyV control in these and other susceptible patients (98-100). However, as this thesis describes, the control of PyV infections upon resurgence is a wide-spread problem in immunodeficient patients, and no known anti-virals exist. With the known mechanism of PyV control potentially found, and the essential role for persistent priming of PyV specific CD8 T cells now understood, this dissertation has provided the field a new set of techniques and tools by which to control this chronic infection.

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